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Failure of 5-Fluorouracil and Methotrexate to Destroy the Reproductive Integrity of a Human Breast Cancer Cell Line (MCF-7) Growing *In Vitro**

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Abstract—*Human breast cancer cell (MCF-7) in continuous culture were exposed to clinically achievable extra-cellular concentrations of methotrexate and 5-fluorouracil. A* 24-hr *treatment with 5-fluorouracil (up to* 7.7×10^{-6} M*) or methotrexate (up to* 10^{-5} M*) failed to reduce the colony-forming ability of MCF-7 cells, although marked dose-dependent effects on the incorporation of* [^{3}H]*-deoxyuridine into DNA were observed immediately after treatment. These effects correlated with a dose-dependent delay in cell population growth rate. However, following treatment with concentrations of the anti-metabolites which failed to reduce colony-forming ability, control cell population growth rate ultimately resumed. Increasing the dose of methotrexate to* 10^{-4} M *resulted in a reduction in MCF-7 cell colony-forming ability, inhibition of* [^{3}H]*-thymidine incorporation into DNA and a fall in cell number within three days of treatment. Thymidine failed to modulate the growth inhibitory effects of the two antimetabolites. It is concluded that methotrexate and 5-fluorouracil appear to exert a cytostatic rather than a cytotoxic effect on MCF-7 cells.*

INTRODUCTION

CHEMOTHERAPY and hormone manipulative therapy are of proven value in the management of metastatic breast cancer. However, the results of clinical trials involving combined hormone and cytotoxic drug therapy have been equivocal [1, 2]. The availability of human breast cancer cell lines which synthesise appropriate hormone receptors and respond to physiological and pharmacological concentrations of hormones provides a model for the study of the interactions of hormones and cytotoxic drugs and their effects in tumour cell survival. The human breast tumour cell line MCF-7 synthesises functional oestrogen, prosterone, glucocorticoid and insulin receptors [3, 4], and has been well characterised as malignant and epithelial in origin [5]. Prior to beginning a study of possible interactions between hormones and cytotoxic drugs we have investigated the response of MCF-7 cells to anticancer drugs alone. Cells were exposed to clinically achievable concentrations of 5-fluorouracil and methotrexate. Since it has been shown that not all end points used to assess cytotoxic drug effects provide a measure of reproductive cell death [6], we have studied the effect of antimetabolites on (a) incorporation of radioactively labelled precursors into nucleic acids, (b) colony-forming ability and (c) cell population growth rate. In view of the conflicting reports concerning the modulating effect of thymidine on 5-fluorouracil-induced cytotoxicity [7, 8] we have also investigated the effect of this nucleoside on the biological response of MCF-7 cells to 5-fluorouracil.

MATERIALS AND METHODS

Cell culture conditions

MCF-7 cells, obtained from Dr. Marvin Rich, Michigan Cancer Foundation, were routinely cultured in Eagles Minimal Essential Medium with Earles Salts, supplemented with 10% foetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml insulin. Cells were grown in a 95% air/5% CO_2 atmosphere at 37°C and had a population doubling time of 36 hr. For some experiments cells were

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grown in medium containing serum which had been exhaustively dialysed against six changes of physiological saline in order to remove endogenous nucloetides. Dialysed and non-dialysed serum contained $<10^{-11}$ M oestrogen.

Colony survival tests

Appropriate numbers of MCF-7 cells were plated into 5-cm petri dishes (Sterilin, Middlesex, England) and allowed to attach for 24 hr. Medium was then replaced with medium containing 5-fluorouracil (Sigma, Dorset, England) or methotrexate (Lederle Laboratories, Hampshire, England). Following a 24-hr drug exposure period, cells were incubated in drug-free medium for 3–4 weeks, with a medium change every 3–4 days. Surviving colonies of fifty or more cells were then counted and expressed as a percentage of control. Control plating efficiency was 5–10%.

Incorporation of radioactively labelled precursors into DNA

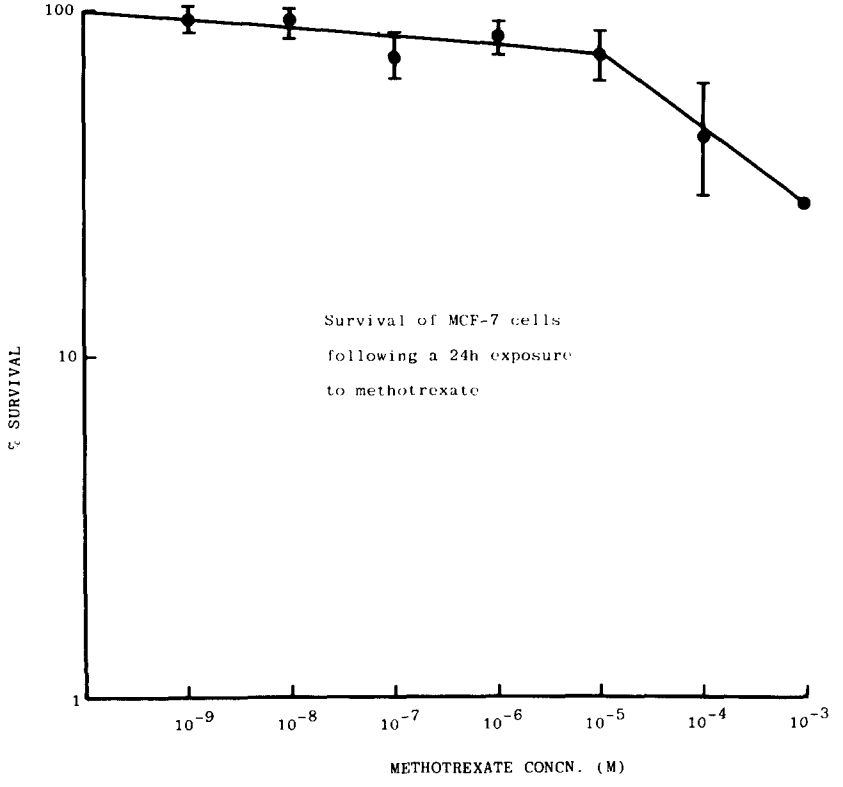
Approximately 10^5 MCF-7 cells were plated into each of a number of wells of a multi-well culture dish (Flow Laboratories, Irvine, Scotland). Cells were treated as previously described and immediately after treatment the medium was replaced with medium containing 1 μCi/ml [^{3}H]-thymidine (specific activity 28 Ci/mmole, Radiochemical Centre, Amersham) or 1 μ Ci/ml [^{3}H]-deoxyuridine (specific activity 25 Ci/mmole). After 1 hr incorporation was stopped by adding ice-cold 0.85% saline, cells were removed from the wells by trypsin/EDTA treatment and the degree of precursor incorporation into nucleic acids assessed following filtration through glass fibre discs, as previously described [9]. All assays were performed in quadruplicate.

Cell population growth studies

Approximately 10^4 cells were plated into the multi-well dishes and treated with the drugs, as described. At three-day intervals the mean number of cells in four wells was determined using an electronic particle counter (Coulter Electronics Model ZB1, Harpenden, England), following removal of the cells from the wells by trypsin/EDTA treatment.

RESULTS

Figure 1 shows that exposing MCF-7 cells to methotrexate for 24 hr had a significant effect on subsequent colony-forming ability only at concentrations of 10^{-4} M and above. A 24-hr exposure of cells to 5-fluorouracil (7.7×10^{-9}–7.7×10^{-6} M) had no significant effect on colony-forming ability (Fig. 2). However, concentrations of methotrexate and 5-fluorouracil



Fgi. 1. *Colony-forming ability of MCF-7 cells following a* 24-hr *exposure to methotrexate. (Mean and S.E. of four determinations).*

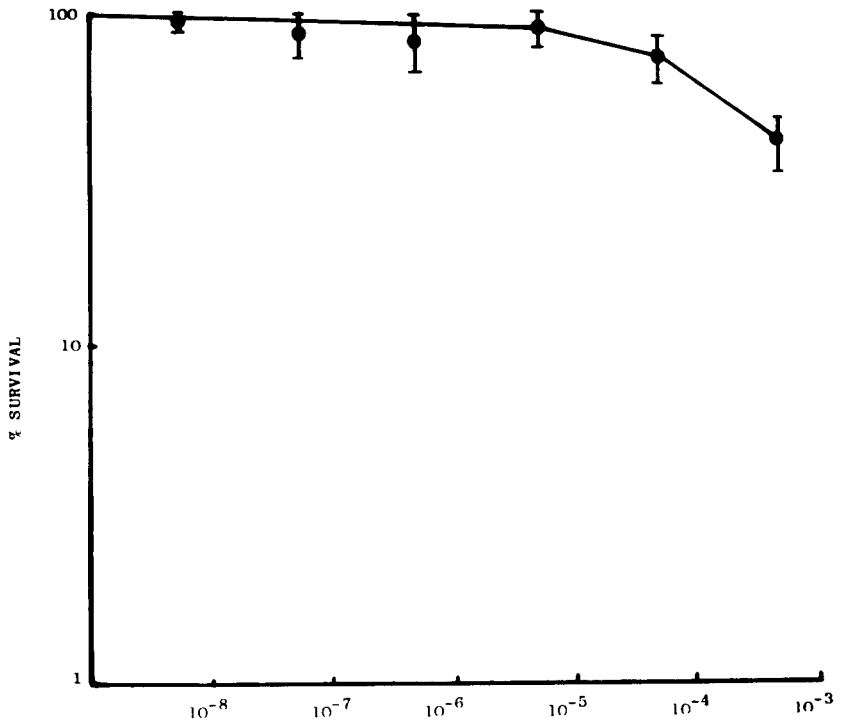


Fig. 2. Colony-forming ability of MCF-7 cells following a 24-hr *exposure to 5-fluorouracil. (Mean and S.E. of four determinations).*

which were not ultimately cytotoxic had a marked effect on [^{3}H]-deoxyuridine incorporation into DNA immediately following treatment. Thus, [^{3}H]-deoxyuridine incorporation was reduced by 90% following a 24-hr exposure of MCF-7 cells to 10^{-6} M methotrexate (Fig. 3) and by 70% following treatment with 3.85×10^{-6} 5-fluorouracil (Fig. 4). Figure 3 also shows that concentrations of methotrexate which reduced the colony-forming ability of MCF-7 cells (10^{-4} and 10^{-3} M) also inhibited the incorporation of [^{3}H]-thymidine into DNA.

Concentrations of methotrexate and 5-fluorouracil which inhibited [^{3}H]-deoxyuridine incorporation but not colony-forming ability nevertheless had a marked effect on MCF-7 cell population growth rate. A dose-dependent effect on growth rate was observed following treatment with methotrexate (Fig. 5), but a reduction in starting cell number was only seen following treatment with 10^{-4} M methotrexate. In all cases control growth rate eventually resumed. (Under these experimental conditions control cell population increased exponentially until there was approximately 3×10^5 cells per well.) The effect of a 24-hr exposure of MCF-7 cells to 5-fluorouracil on population growth rate is shown in Fig. 6. A transitory delay was observed following treatment with 7.7×10^{-6} M 5-fluorouracil, a dose which reduces [^{3}H]-deoxyuridine incorporation by 85% (Fig. 3). In order to mimic the higher extracellular concentrations of 5-fluorouracil obtainable *in vivo* for a short time following intravenous bolus injection, we also investigated the effect of a 1-hr exposure to the drug on MCF-7 cell population growth and [^{3}H]-deoxyuridine incorporation into DNA. The results shown in Fig. 4 and Fig. 6 could be reproduced following a 1-hr exposure to 5-fluorouracil if the concentrations used were ten times that used for the 24-hr exposure.

Since it is known that nucleosides present in serum can modulate the anti-metabolic effects of 5-fluorouracil and methotrexate [7, 8, 10], cell population growth experiments were repeated with cultures grown in medium supplemented with dialysed serum or in medium containing dialysed serum and supplemented with 10^{-6} or 10^{-4} M thymidine. Control cell population doubling time was unaffected under these conditions and the effects of 5-fluorouracil and methotrexate on MCF-7 cell population growth shown in Fig. 5 and Fig. 6 were also unaffected by the presence or absence of thymidine in the culture medium.

DISCUSSION

In studies designed to determine the effect of cytotoxic drugs on tumour cells *in vitro*, inhibition of incorporation of radioactively labelled precursors into DNA, colony survival

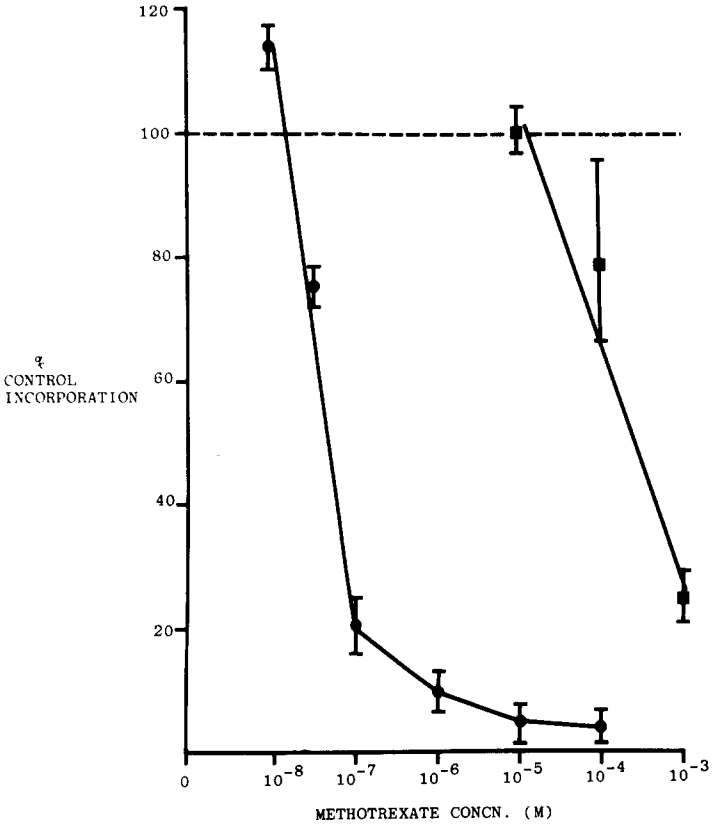


Fig. 3. The effect of a 24-hr *exposure to methotrexate on the incorporation of* [^{3}H]*-deoxyuridine* (●–●), *or* [*s*[^{3}*H*]*-thymidine,* (■–■) *into DNA of MCF-7 cells. (Mean and S.E. of four determinations).*

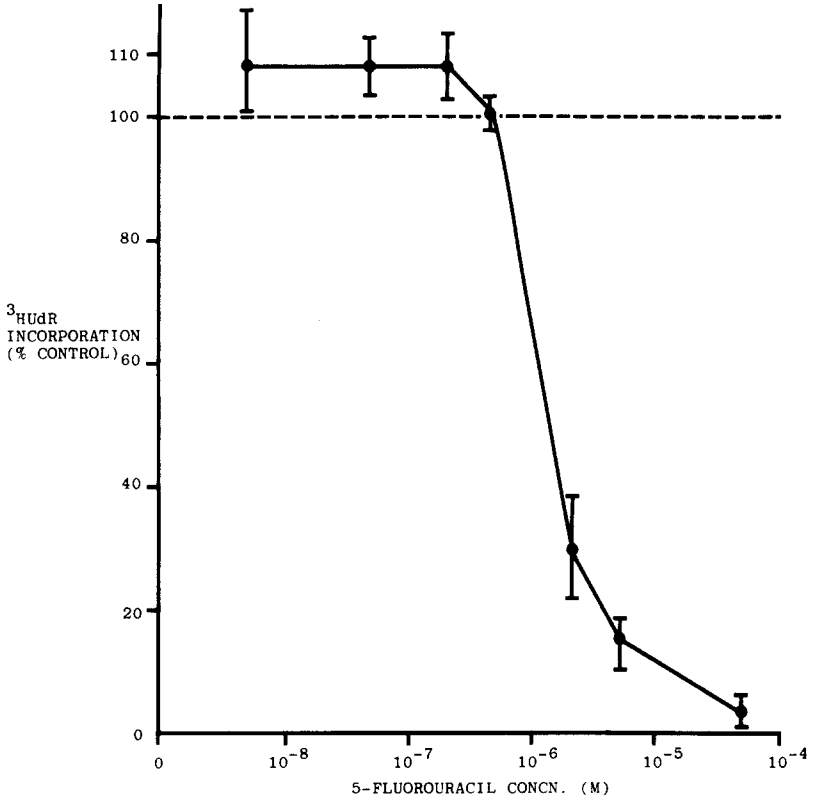


Fig. 4. The effect of a 24-hr *exposure to 5-fluorouracil on the incorporation of* [^{3}*H*]*-deoxyuridine into DNA of MCF-7 cells. (Mean and S.E. of four determinations).*

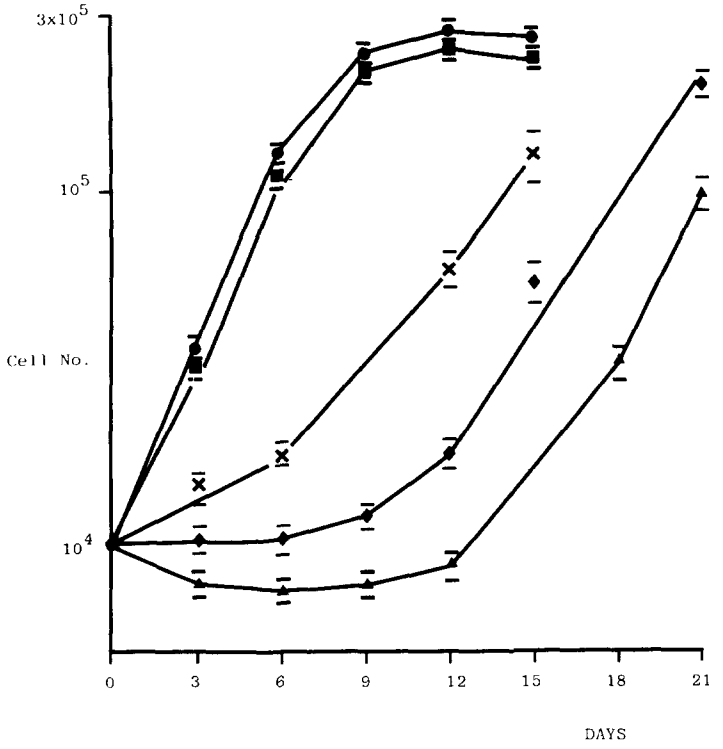


Fig. 5. The effect of a 24-hr *exposure of MCF-7 cells to methotrexate on cell population growth rate.* ●–●, *controls;* ■–■, 7.7×10^{-7} M; ×–×, 10^{-7} M; ◆–◆, 10^{-6} M; ▲–▲, 10^{-4} M. (*Mean and S.E. of four determinations*).

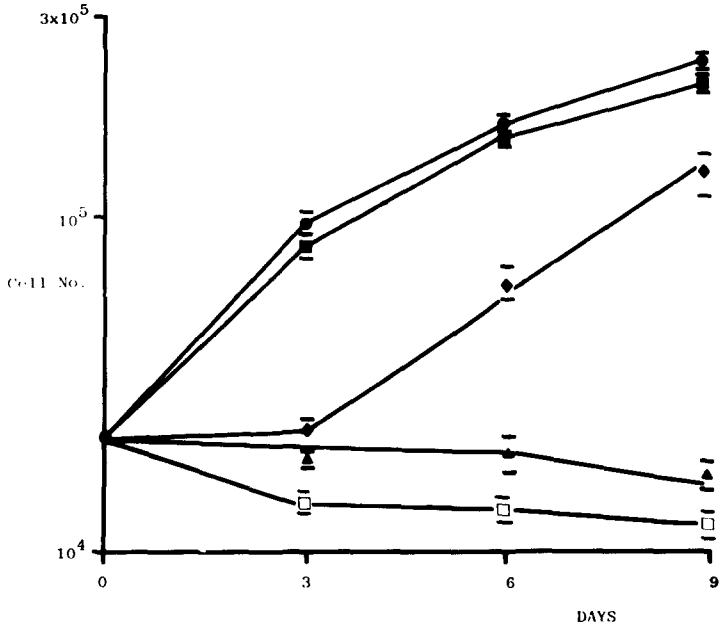


Fig. 6. The effect of a 24-hr *exposure of MCF-7 cells to 5-fluorouracil on cell population growth rate.* ●–●, control; ■–■, 7.7×10^{-7} M; ◆–◆, 7.7×10^{-6} M; ▲–▲, 7.7×10^{-5} M; □–□, 7.7×10^{-4} M. (*Mean and S.E. of four determinations*).

tests and cell population growth delay have all been used as experimental end-points [6, 11, 12]. In a comparative study Roper and Drewinko [6] concluded that only colony survival tests provided a valid index of tumour cell kill. The concentration and exposure times used for 5-fluorouracil and methotrexate treatment in this study would have been deemed effective against MCF-7 cells if inhibition of incorporation of nucleic acid precursors or initial population growth delay had been used as end-point determinants. However, eventual colony-forming ability was unaffected and extending the period of population growth rate analysis up to three weeks after treatment showed that the treated cells ultimately resumed control

growth rate (Figs. 5 and 6). Both methotrexate and 5-fluorouracil are believed to owe their cytotoxic action to their ability to inhibit the synthesis of deoxythymidine monophosphate from deoxyuridine monophosphate; methotrexate, by inhibition of dihydrofolate reductase, reduces intracellular reduced folate levels and 5-fluorouracil, though its active metabolite, 5-fluorodeoxyuridine monophosphate, inhibits thymidylate synthetase. Hence, inhibition of incorporation of [^{3}H]-deoxyuridine into DNA following treatment with these anti-metabolites cannot discriminate between inhibition of precursor synthesis and inhibition of DNA synthesis *per se*. Inhibition of incorporation of [^{3}H]-deoxyuridine into DNA may, therefore, overestimate cell sensitivity if thymidine triphosphate pools were maintained in the presence of 5-fluorouracil and methotrexate via the "salvage" pathway through the action of thymidine kinase. Nevertheless, inhibition of DNA synthesis as determined by reduction of incorporation of [^{3}H]-deoxyuridine into DNA did correlate with biological response as measured by cell population growth inhibition. Immediately following treatment with 10^{-7} M or 10^{-6} M methotrexate, [^{3}H]-thymidine incorporation was not inhibited (Fig. 3), although population growth delay did subsequently occur (Fig. 5). Presumably this reflects the fact that incorporation of thymidine into DNA does not utilise the biochemical pathway blocked by methotrexate. However, at higher doses of methotrexate (10^{-4} M and 10^{-3} M) [^{3}H]-thymidine incorporation is inhibited (Fig. 3) and colony-forming ability is impaired (Fig. 1). At such high doses methotrexate may inhibit *de novo* purine synthesis, although the relationship between rates of purine biosynthesis and [^{3}H]-thymidine incorporation into DNA is not clear.

A comparison of the effects of methotrexate and 5-fluorouracil on MCF-7 cell colony-forming ability and population growth rate emphasises the difficulties inherent in interpreting such data. Thus, a 24-hr treatment with 10^{-4} M and 10^{-6} M methotrexate results in a growth delay of approximately 8 and 6 doubling times respectively (Fig. 5). From these data a maximum cell kill of 99.5% could be calculated ($2^{-8} = 5 \times 10^{-3}$ surviving fraction). However, the results from the colony survival tests indicated that 10^{-4} M methotrexate treatment resulted in only a 40% cell kill (Fig. 1). A possible explanation of this discrepancy could be that cells treated with methotrexate prior to assessing colony-forming ability died reproductively, releasing nucleotides and nucleosides into the medium and thus reversing the action of the drug. However, one would predict that this effect, if it occurred, would be more marked in the population growth rate experiments since the cells were plated at a higher density than in the colony survival experiments (see Materials and Methods). It is known that the antimetabolic effects of methotrexate and 5-fluorouracil may be markedly affected by exogenous nucleosides. Thus chicken fibroblasts were found to be resistant to methotrexate when grown in medium containing serum, and thus resistance was attributed to the presence of thymidine [10]. A recent report has also suggested that thymidine, by increasing the incorporation of 5-fluorouracil into RNA, enhances the antitumour effect of this antimetabolite [8]. In contrast, other studies have shown that thymidine has no effect on the antiproliferative effects of 5-fluorouracil or, by providing the end product of the inhibited reaction, protects cells against the drug [7, 13]. The antiproliferative effects of methotrexate and 5-fluorouracil on MCF-7 cells shown in Figs. 5 and 6 were unaffected when the experiments were repeated using medium containing dialysed serum or medium to which exogenous thymidine had been added to a final concentration of 10^{-6} M or 10^{-4} M.

An alternative to selective nucleoside rescue as an explanation of the apparent discrepancy between the colony survival data and the data from the population growth curves would be that methotrexate and 5-fluorouracil exert a cytostatic rather than a cytotoxic action in MCF-7 cells, with actual cell kill being reflected—in the case of methotrexate—by a reduction in colony-forming ability (Fig. 1), inhibition of [^{3}H]-thymidine incorporation (Fig. 3) and a fall in cell number within three days of treatment (Fig. 5). Further biochemical and kinetic evaluation of MCF-7 cells following treatment with antimetabolites will be necessary to resolve these questions.

Depending upon the treatment regime, extracellular concentrations of methotrexate up to 10^{-4} M may be achieved *in vivo* for 24 hr [14]. However, in most clinical situations relevant to the treatment of breast cancer a 24-hr plasma concentration of 10^{-6} M is rarely exceeded [15, 16]. Extracellular concentrations of 5-fluorouracil up to 7.7×10^{-6} M can be reached during continuous infusion [17], and transitory levels ten times this value occur following i.v. bolus injection [18]. It is concluded, therefore, that clinically achievable extracellular concentrations of methotrexate and 5-fluorouracil do not appear to destroy the reproductive in-

tegrity of a human breast cancer cell line growing *in vitro*, although antimetabolic effects and population growth delay do occur as a result of treatment. The mechanism of cellular recovery is not yet clear, and the effect of combination drug treatment and the influence of hormones to which MCF-7 cells are responsive remains to be established.

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