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COMMUNICATIONS

A new method for studying cell growth in suspension, and its use to show that indomethacin enhances cell killing by methotrexate

JOHN D. GAFFEN*, ALAN BENNETT, MICHAEL R. BARER†, *Department of Surgery, The Rayne Institute, King's College School of Medicine and Dentistry, London SE5 9NU, and †Department of Microbiology, School of Hygiene and Tropical Medicine, London WC1E 7HT, UK*

Microturbidimetry has been used to measure the growth of cells in suspension. Disaggregated mouse NC carcinoma cells in culture medium were added to the wells of a microtitre test plate and incubated. Absorbance of 600 nm light was measured daily for 4 days using a microplate reader. As the cells grew, light absorbance increased. Methotrexate 2–40 ng ml^{-1} reduced cell growth; this effect was increased by indomethacin 1 μg ml^{-1}, possibly by displacing methotrexate from its binding by serum protein or by enhancing cell uptake of methotrexate. Similar results were obtained by conventional clonogenic assays. The new technique offers simplicity, better reproducibility, and substantial savings in time and cost.

Colony formation in semisolid medium is used to study the growth of normal or malignant cells (Park et al 1971; Courtenay 1976). Since this technique is laborious, and the results often difficult to interpret (Meyskens et al 1983), we have used a microplate reader to study the effect of drugs on the growth of malignant cells in suspension. This technique is novel, although other micro-methods have been reported (Keusch et al 1972; Gentry & Dalrymple 1980). The results are similar to those obtained using colony formation in agarose gel. They also demonstrate that indomethacin increases the cytotoxicity of methotrexate.

Materials and methods

NC carcinoma cells (Hewitt et al 1976) were obtained from a WHT/Ht mouse with peritoneal metastases following subcutaneous injection. Cells in a sample of peritoneal fluid were maintained in MEM + 10% newborn bovine serum, 50 units ml^{-1} of penicillin and streptomycin, and 1% non-essential amino acids. All the cells in these experiments were from the same primary culture which was subcultured over 4 months. Throughout this period they remained tumourigenic as shown by s.c. injection of 10^6 NC cells/100 μl Tyrode solution into WHT/Ht mice (Bennett et al 1979). In each case palpable tumours developed within 21 days, and 5/6 mice who had their tumours excised subsequently developed metastases.

The medium was prepared from powder (Flow Laboratories), methotrexate 5–20 μg ml^{-1} was made up in 150 mM NaCl adjusted to pH 8·4 with 0·1M NaOH, and indomethacin 0·5 mg ml^{-1} was dissolved in water containing $NaHCO_3$ (final pH 7·8). All water for preparing the medium and the drugs was double-distilled in glass, and sterilization was by filtration. The volumes of added drug solutions did not exceed 2.7 μl ml^{-1} medium.

Cloning in agarose gels

NC cells grown in suspension were pelleted, resuspended in 1 ml trypsin/EDTA solution (0·05%/0·02%, w/v, Flow Laboratories) and incubated for 30 s at 37 °C. Medium, 9 ml was rapidly added and the cells gently disaggregated by repeated passage into and out of a pipette. The cell numbers were determined using a Coulter counter and diluted so that 100 μl contained 4 × 10^4 cells that were viable as shown by trypan blue exclusion. 10 ml 0·5% agarose maintained at 42 °C were mixed with 10 ml double-strength MEM + 20% newborn bovine serum at 37 °C containing drugs or vehicle, and 4 × 10^4 cells were added. After mixing, 5 ml aliquots were rapidly dispensed into dishes and incubated for two weeks (37 °C, 5% CO_2 in air, 100% humidity). Colonies were counted after staining with neutral red (Norman et al 1978). Final drug concentrations were: methotrexate (Lederle) 2–8 ng ml^{-1}, indomethacin (Merck, Sharp and Dohme) 1 μg ml^{-1}, both drugs together or vehicle only. There were 4 experiments giving a total of 38 replicates for vehicle controls, and 12–16 replicates at each drug concentration.

In 6 other experiments survival curves were constructed for methotrexate 2–12 ng ml^{-1} or indomethacin 0·01–50 μg ml^{-1}. There were 24 replicates for vehicle controls and 12 replicates at each drug concentration.

Microturbidimetric assays

To each well of a 96 well micro test plate were added 150 μl of MEM + 10% NBS containing drugs or vehicle at 1·3 times the desired final concentration. Disaggregated NC cells (2·5 × 10^4 in 50 μl) or medium only for blanks

* Correspondence.

were then added, and the plates incubated (37 °C, 5% CO_2 in air, 100% humidity). Absorbance of 600 nm light in each well was determined daily for 4 days using a Dynatech microplate reader.

Results

In the cloning assays indomethacin 1 μg ml^{-1} had little or no effect on colony formation ($P<0·3$, Student's *t*-test for unpaired data), but indomethacin 10–50 μg ml^{-1} or methotrexate 4–12 ng ml^{-1} concentration-dependently inhibited cell survival (Fig. 1). However, cell survival was less when indomethacin 1 μg ml^{-1} was combined with methotrexate (overall difference $P<0·01$, Student's *t*-test for unpaired data), the effect of indomethacin being similar at each methotrexate concentration ($P = 0·75$, one way analysis of variance) (Fig. 2).

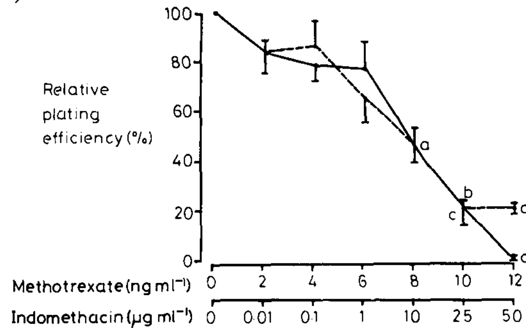

FIG. 1. Clonogenic concentration-response curves for the survival of NC carcinoma cells treated with indomethacin 0·01–50 μg ml^{-1} (solid line), or methotrexate 2–12 ng ml^{-1} (broken line). Each point is the mean ± s.e.m. of 12 dishes from 3 experiments (24 dishes for controls). a, $P < 0·1$; b, $P < 0·05$; c, $P < 0·005$; d, $P < 0·001$, Student's unpaired *t*-test on the raw data.

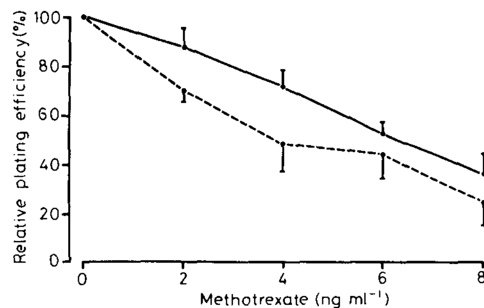

FIG. 2. Clonogenic concentration-response curves for the survival of NC carcinoma cells treated with methotrexate 2–8 ng ml^{-1} alone (solid line) or with indomethacin 1 μg ml^{-1} (broken line). Each point is the mean ± s.e.m. of the means of 4 experiments with 4 replicates/experiment (8 replicates/experiment for vehicle controls). Indomethacin increased the effect of the methotrexate ($P<0·01$, Student's *t*-test for paired data on overall difference).

In the turbidimetric method it was not possible to make valid readings soon after setting up the assays because of air bubbles. As the cells grew and multiplied, light absorbance in the microplate wells increased. Vehicle and drug controls gave low absorbance readings (0·003 ± 0·0005 and 0·0005 ± 0·0004 respectively, mean ± s.e.m. of 4 experiments, each with 8 replicates). The mean reading from the initial inoculum of 2·5 × 10^4 cells/well after approximately 15 h was 0·013 ± 0·001 s.e.m. absorbance units which increased to 0·031 ± 0·002 on day 4 (4 experiments each with 8 replicates). In the presence of drugs the mean absorbance readings at day 4 (0·013 ± 0·001) approximates to zero growth (cytostatis); readings below this suggest that cell death and lysis may have occurred, and this seemed to be so on microscopic examination. Methotrexate 4–40 ng ml^{-1} inhibited the reduction of light transmission. Up to 10 ng ml^{-1} cell growth was less, and 15–40 ng ml^{-1} may have been cytolytic since light transmission was more than through the 15 h cultures. Indomethacin 1 μg ml^{-1} alone had little or no effect, but it increased the effect of most concentrations of methotrexate (Fig. 3).

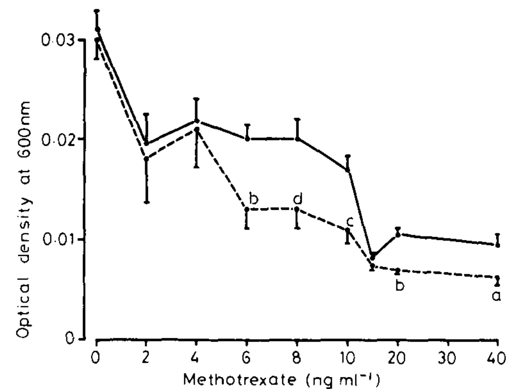

FIG. 3. The effect of methotrexate 2–40 ng ml^{-1} alone (solid line) or with indomethacin 1 μg ml^{-1} (broken line) on the growth of NC carcinoma cells measured by turbidimetry. Each point is the mean ± s.e.m. of the means of 4–7 experiments with 8 replicates/experiment (16 replicates/experiment for vehicle controls). a, $P < 0·1$; b, $P < 0·01$; c, $P < 0·005$; d, $P < 0·001$, Student's *t*-test for paired data.

Discussion

These experiments form part of a study into the improved response of mice with NC tumours to cytotoxic chemotherapy when a prostaglandin synthesis inhibitor is also given (Bennett et al 1979, 1982). The conventional clonogenic assay was used initially, but was found to be time-consuming and variable. Because the plating efficiency of the NC cells varied so greatly (0·5–14%) some experiments produced >1000 colonies/plate. This, in conjunction with the difficulty of deciding which clones contained >50 cells, made scoring difficult and tedious, and contributed to the wide variations within and between experiments. The microturbidimetry technique has many advantages. It gives results similar to the clonogenic method, but in contrast is

relatively quick, reproducible and inexpensive. However, the interpretation of changes in light absorbance may need to take into account alterations in cell size or cell disintegration in addition to proliferation.

The ability of indomethacin 1 $\mu g\ ml^{-1}$ to potentiate methotrexate cytotoxicity may merely reflect the ability of organic acids to displace methotrexate from binding sites on the proteins in the newborn bovine serum (Dixon et al 1969), but another possibility is increased transport into the malignant cells. Prostaglandin E_2 inhibited methotrexate uptake by L1210 cells (Henderson et al 1978), so that indomethacin might increase uptake by inhibiting prostaglandin synthesis.

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Cardiovascular effects in the Sprague-Dawley rat of 8-hydroxy-2(di-*N*-propylamino) tetralin, a selective 5-hydroxytryptamine receptor agonist

K. Gradin*, A. Pettersson, S. Hjorth, T. Hedner, L. E. Arvidsson†, B. Persson, *Department of Pharmacology, University of Göteborg, Box 33031 Göteborg and †Department of Organic Pharmaceutic Chemistry, University of Uppsala, Uppsala, Sweden*

The intravenous administration of 8-hydroxy-2(di-*N*-propylamino) tetralin, a selective 5-HT receptor agonist, caused a biphasic blood pressure response and bradycardia in Sprague-Dawley rats. The initial pressor response involved peripheral α_1-adrenoceptors since it was present in pithed rats and was antagonized by prazosin. Though the intracerebroventricular route of administration was not more effective the hypotension and bradycardia were probably of central origin. The bradycardia was prevented by pretreatment with atropine and propranolol suggesting an involvement of vagal as well as sympathetic activity. These results support the view that central 5-HT receptor activation reduces the blood pressure and heart rate.

There is accumulating histochemical (Bobillier et al 1976) and pharmacological (Kuhn et al 1980) evidence to suggest that central 5-hydroxytryptaminergic (5-HT) mechanisms are involved in blood pressure (BP) regulation. However, the nature of that involvement is not well understood since apart from unusually large interspecies variations (Kuhn et al 1980) the various 5-HT receptor subtypes have not been physiologically defined. Thus, central 5-HT neurons have been ascribed both inhibitory (Baum & Shropshire 1975) and facilatory (McCall & Humphrey 1982) effects on sympathetic outflow.

* Correspondence.

8-Hydroxy-2(di-*N*-propylamino) tetralin (8-OH-DPAT) is a new 5-HT receptor agonist that biochemically (Hjorth et al 1982) and electrophysiologically (Fallon et al 1983) has a pharmacological profile that is characteristic for compounds active at 5-HT_1 receptors (Martin & Sanders-Bush 1982). In radioligand studies it has recently been further defined to bind specifically to 5-HT_{1A} receptors (Middlemiss & Fozard 1983). Unlike other putative 5-HT receptor agonists, such as lisuride, 8-OH-DPAT lacks appreciable effects on central adrenergic or dopaminergic receptors (Hjorth et al 1982).

In view of the conflicting data on BP in the rat following central 5-HT receptor activation (Kuhn et al 1980) we have investigated the cardiovascular effects of 8-OH-DPAT in the rat.

Methods

Male Sprague-Dawley rats (Anticimex, 220–250 g) were used. Mean arterial BP and heart rate (HR) were recorded in conscious or pithed rats through indwelling catheters (carotid artery, Trolin 1975). Intravenous catheters (jugular vein, Trolin 1975) and intracerebroventricular catheters (i.c.v., lat. ventricles, Garcia-