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Actions of a Progestogen on Human Breast Cancer Cells: Mechanisms of Growth Stimulation and Inhibition

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Abstract—*Medroxyprogesterone acetate (MPA) reduced the proliferation of MCF-7 cells with a maximal response at 100 nM. A subline ('M' cells) resistant to this action remained responsive to antioestrogen but showed a poor response to dexamethasone (DEX). Experiments with mixtures of MPA and DEX showed that MPA acts on wild-type MCF-7 cells through a glucocorticoid as well as a progestogen mechanism. The growth of 'M' cells was stimulated by MPA at 100 nM or greater and the drug increased polyamine concentrations in 'wild-type' as well as 'M' cells. It is suggested that both progestogen and glucocorticoid receptors may mediate the effects of high-dose MPA therapy in breast cancer. The possible stimulation of the growth of some cell types by MPA requires further investigation.*

INTRODUCTION

ADDITIVE hormonal therapy has largely replaced endocrine ablation in the treatment of breast cancer and is far better tolerated than the chemotherapeutic agents available at present. Unfortunately favourable responses are invariably followed by 'escape' from control and the events leading to this phenomenon are not fully understood. Hormonal agents used therapeutically may enhance, as well as inhibit, tumour growth. Adverse effects of the antioestrogen, tamoxifen, have been observed clinically [1–5] and growth stimulation has been demonstrated in cultured human breast cancer cells [6]. It is thus possible that prolonged treatment with such agents can speed tumour progression even before 'escape' is clinically observable.

No growth-promoting effects appear to have been detected clinically for progestogen therapy, which is effective in a considerable proportion of patients [7]. The growth-inhibitory actions are thought to be mediated by progestogen receptors [8, 9] but these compounds interact with other intracellular hormone receptors [10–14] and can simulate actions of a variety of hormones, including those of glucocorticoids [15] and oestrogens [16].

Using a human breast cancer cell line and the progestogen medroxyprogesterone acetate (MPA) we have found that this drug can act on its target cells through several mechanisms and that, apart from its growth-inhibitory actions, it can increase intracellular polyamine content and cell proliferation. Our results may have implications with regard to the selection of patients for progestogen therapy and the long-term use of high doses.

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MATERIALS AND METHODS

Chemicals

Inorganic chemicals, ether and benzoyl chloride were from Fisons, Loughborough, Leicestershire; *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES) from British Drug Houses, Poole, Dorset, or Sigma Chemical Company, Poole, Dorset; glutamine, insulin (from porcine pancreas), charcoal, diaminohexane, putrescine hydrochloride, spermidine dihydrochloride, spermine trihydrochloride, oestradiol and dexamethasone from Sigma; dextran (T70) from Pharmacia, Uppsala, Sweden; dithiothreitol from Boehringer, Mannheim, Germany; glycerol (AR) from May and Baker, Manchester; ethylenediaminetetraacetic acid (EDTA) and scintillator chemicals from Koch-Light, Slough, Berkshire; and methanol (HPLC grade) from Rathburn, Walkerburn, Scotland. Diaminohexane was converted to its hydrochloride and recrystallized before use.

Tritium labelled oestradiol (92 Ci/mmol), ORG 2058 (45 Ci/mmol) and triamcinolone (30 Ci/mmol) were from Amersham International, Amersham, Buckinghamshire.

Eagle's minimum essential medium (EMEM), foetal calf serum (FCS) non-essential amino acid concentrate (NEAA), trypsin-EDTA and Earle's balanced salt solution, calcium- and magnesium-free (EBSS) were from Gibco, Paisley, Scotland.

Cell culture

Human breast cancer cells (MCF-7) were maintained in flasks with 25 cm^2 growth area (Sterilin or Corning, Fisons, Loughborough, Leicestershire) with growth medium consisting of Eagle's minimum essential medium (EMEM) containing HEPES and supplemented with FCS (4.7% v/v), NEAA concentrate (0.93% v/v), glutamine (2mM) and insulin (0.6 μg/ml). Infections were not a problem and no antibiotics were needed. Cells were passaged once a week.

To develop a strain resistant to the growth-inhibitory actions of MPA, cells were grown and passaged for 6 months with growth medium containing the drug at a concentration of 1 μM. Treatment was commenced when cells approached confluence, and after the first 2 passsages the cells grew in the presence of MPA at a similar rate to 'wild-type' cells in the absence of the drug. Prior to experiments, the MPA-treated subline was passaged and grown in the absence of the drug for the number of passages stated.

Steroids and other small molecules were removed from FCS as described by Butler *et al.* [17] but using half the concentrations of charcoal and dextran. The resulting stripped foetal calf serum (SFCS) was sterilized by filtration in succession through 0.65 (Millipore, Molsheim, France), 0.45 (cellulose nitrate, Whatman, Maidstone, Kent) and 0.2 (Acrodisc, Gelman, Ann Arbor, Michigan, USA) μm filters.

Experimental media consisted of EMEM containing SFCS, glutamine and NEAA concentrate at the same concentrations as growth medium, but no insulin. Hormones were added in ethanol to give the concentrations stated for each experiment, the final ethanol concentrations in the media not exceeding 0.2%. Control media contained vehicle only.

For each treatment, 5×10^4 to 1×10^5 cells were seeded into 3 or 4 replicate 25 cm^2 flasks and maintained in growth medium for 24 hr prior to replacement by experimental media. At the end of each experiment, monolayers were washed once with Earle's balanced salt solution (5 ml) and nuclei released [17] prior to counting in a Coulter Industrial counter and polyamine assay (see below).

Steroid receptors. Oestrogen, progestogen and glucocorticoid receptors (ER, PR and GR) were assayed just prior to confluence. Most of the medium was decanted, the upper surface of the flask cut out using a heated scalpel blade and the cells carefully scraped off the growth surface into the small volume of medium remaining.

The cell sheets were centrifuged at 500 ***g*** for 5 min and the supernatant (medium) rejected. The pellet was resuspended (using a Kontes Duell glass/glass homogenizer) in 1.5 ml HED buffer (HEPES, 20mM, EDTA, 1.5mM, dithiothreitol 250 μM, pH 7.4, with glycerol, 10% v/v). Careful homogenization dispersed the cells without causing appreciable disruption. The suspension was centrifuged at 500 ***g*** for 5 min and the pellet resuspended in 1.5 ml HED buffer using a vortex mixer. An aliquot of the cell suspension was removed for determination of DNA [18].

Receptor site concentrations were estimated either in cytosol and nuclear preparations [19] or by means of a whole cell assay. Fifty μl of a 12 nM solution of ^3H-E_2 or of 20 nM solutions of ^3H-ORG 2058 or ^3H-triamcinolone was added to 150 μl of cell suspension to give final concentrations of 3 and 5 nM, respectively. Non-saturable binding was estimated in parallel tubes by addition of a 250-fold excess of DES or 100-fold excess of ORG 2058 or dexamethasone. The mixtures were incubated at 8°C for 18 hr, the cell suspensions filtered through Whatman GF/C filter discs and the cells washed 3 times with 5 ml 0.9% saline. The discs with their cells were dried overnight at 60° C and counted in a toluene-PPO (5 g/l) scintillator in a Sorvall Mark 3 counter at 56% efficiency.

Results were expressed as fmol steroid bound per mg DNA.

Polyamines

Polyamines were estimated in aliquots equivalent to 5×10^5 or more cells, using the method of Redmond and Tseng [20]. Diaminohexane (4 nmol/10^6 nuclei) was used as internal standard and extracts were analysed using a Model U6K Universal Liquid Chromatography Injector and Model 6000A Solvent Delivery System (Waters Associates Inc., Hartford, Northwich, Cheshire) and a dimethyloxylsilyl-Hypersil column (100 × 5 mm) with guard column containing the same packing material (Shandon, Runcorn, Cheshire).

Statistical calculations

Student's *t*-test was used for significance testing of growth experiments.

For polyamine assays, within-batch between-flask coefficients of variation (c.v.) were estimated from analyses in duplicate flasks [21]. Means of duplicate assays were considered to be significantly

different ($P < 0.05$) if they differed by 4.3 × c.v.% where 4.3 is the appropriate value of t at $(n_1 + n_2 - 2)$, i.e. 2 degrees of freedom.

RESULTS

Effects of MPA on cell proliferation

Under the conditions of our experiments 'wild-type' MCF-7 cells responded to physiological levels of E_2 with increased, and to MPA with decreased, proliferation (Fig. 1). The growth-inhibitory effect of MPA was statistically significant at 1 nM ($P < 0.001$) and the effect at 100 nM was significantly greater than that at 50 nM ($P < 0.01$). Further increasing the concentration to 500 nM did not appear to have any additional growth-inhibitory effect.

The proliferation rate of 'M' cells was not reduced by MPA during their first, second, third or fourth passage in the absence of the drug, as illustrated in Fig. 2. At concentrations of 100 and 500 nM MPA significantly increased the proliferation of these cells ($P < 0.05$ and $P < 0.001$, respectively). This growth-stimulatory effect of MPA was demonstrated in 5 separate experiments (statistically significant in 4 experiments) spread over more than one year.

Effects of DEX on cell proliferation

That 'M' cells appeared to be resistant to the growth-inhibitory effects of DEX as well as MPA was suggested by the results shown in Fig. 3(A). Cell numbers of 'M' cells were not reduced at concentrations of DEX which significantly decreased the proliferation of 'wild-type' cells ($P < 0.01$, $P < 0.001$ and $P < 0.001$ at 5, 50 and 500 nM, respectively, Fig. 3B). However, in another experiment it could be shown that 'M' cells were not completely resistant to DEX. Small but significant decreases in cell numbers were detected at concentrations of 500 nM ($P < 0.01$) or greater (Fig. 3C). Unlike MPA, DEX did not increase the proliferation of 'M' cells at high concentrations.

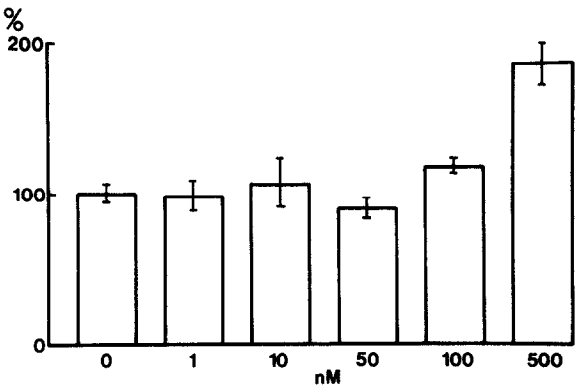

Fig. 2. Effect of medroxyprogesterone acetate on proliferation of 'M' cells. Cells during the third passage in the absence of the drug were exposed for 7 days to media containing SFCS and MPA at the concentrations shown. Results expressed as in Fig. 1. Mean control: 0.75 × 10^6 cells/flask.

Presence of steroid receptors

Hormone receptor binding site concentrations of 'M' and 'wild-type' cells were compared on three occasions. On the first, 'M' cells harvested after the 6th passage in the absence of MPA and grown with 5% FCS and insulin (0.6 μg/ml) were found to contain appreciable levels of total ER and cytoplasmic PR binding sites (Table 1). In both cell types practically all the ER was associated with the nuclear fraction. It could be argued that, by the 6th passage without MPA, the 'M' cells could have

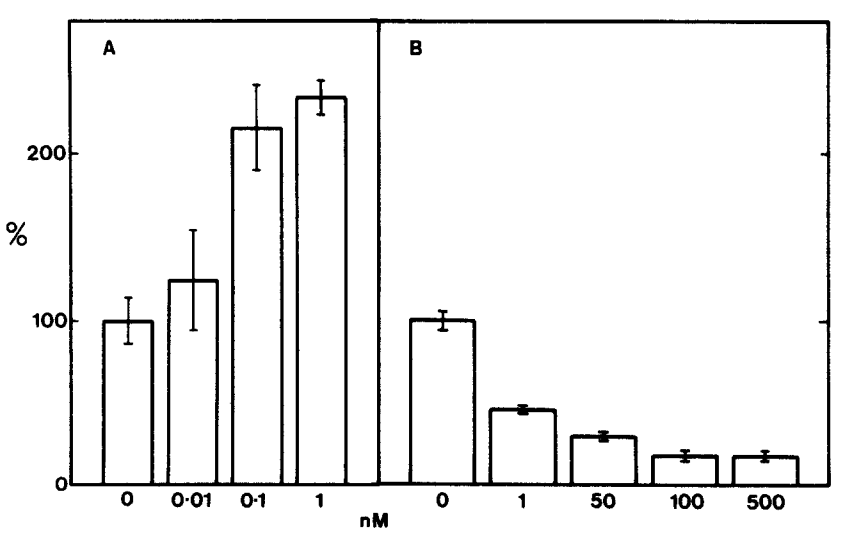

Fig. 1. Effects of oestradiol and medroxyprogesterone acetate on proliferation of 'wild-type' cells. Cells were exposed for 7 days to media containing SFCS and the concentrations shown of E_2 (A) or MPA (B). Cell counts expressed as % of mean control (A-1.3 × 10^6, B-2.4 × 10^6 cells/flask), vertical bars indicate ± S.D.

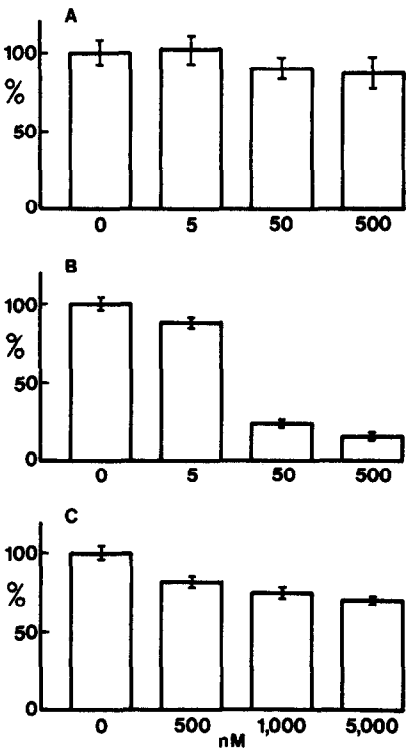

Fig. 3. Effects of dexamethasone on proliferation of 'wild-type' and 'M' cells. 'M' cells during the third passage in the absence of MPA were exposed for 8 days (A, C) and 'wild-type' cells were exposed for 6 days (B) to media containing SFCS and DEX at the concentrations shown. Results expressed as in Fig. 1. Mean controls: (A) 2.0 × 10⁶, (B) 3.3 × 10⁶, (C) 3.8 × 10⁶ cells/flask.

reverted to 'wild-type' receptor characteristics. Therefore, on a second occasion, 'M' cells were studied after 1 passage in the absence of the drug, using whole cell receptor assays. Concentrations of ER, PR and GR binding sites were 328, 877 and 103 fmol/mg DNA (Table 1), while those for cells grown in the presence of MPA (1 μM) gave estimates of 195, 97 and 0 fmol/mg DNA for ER, PR and GR, suggesting reduced binding to, or 'down regulation' of, all 3 receptors. Levels of PR and GR but not ER, were lower in 'M' than in 'wild-type' cells. On a third occasion, 'M' cells contained lower levels of all 3 receptors than 'wild-type'.

Interactions of MPA and DEX

Because 'M' cells contained very low levels of GR and appeared to be not only completely resistant to the growth-inhibitory actions of MPA but were also relatively unresponsive to DEX, it seemed likely that the action of MPA was, at least partly, mediated by GR. To investigate this possibility, the effects of combinations of the 2 drugs on 'wild-type' cells were examined in detail. If MPA and DEX act through different mechanisms, then the effect of one should be additive at optimal concentrations of the other. If, on the other hand, they have a common mode of action, a maximal effect of one will prevent an additional action of the other.

The results of 2 such experiments are shown in Fig. 4. The effects of MPA and DEX were significantly additive at optimal as well as sub-optimal levels of DEX ($P < 0.001$ in both cases). On the other hand DEX could produce a significant ($P < 0.001$) additive effect only when a sub-optimal, growth-inhibitory concentration of MPA was present. Further support for a glucocorticoid mechanism for the growth inhibitory effect of MPA is provided by recent experiments with a subline of MCF-7 cells grown in the presence of dexamethasone and partially resistant to its action. These cells also exhibit reduced responsiveness to MPA (data not shown).

Effect of MPA on intracellular polyamine content

To assess the reliability of estimates of polyamines a pooled preparation from MCF-cells was analysed with each of 7 batches of assays. Mean values were: putrescine 0.58 ± 0.054 (S.D.), spermidine 2.2 ± 0.24 and spermine 1.6 ± 0.14 fmol/cell and estimates of between-batch coefficients of variation were 9, 11 and 9%, respectively. Within-batch

Table 1. Estimates of receptor sites in 'wild-type' (W) and 'M' cells

Experiment	Assay	Cells	Receptor binding sites (fmol/mg DNA) ER	PR	GR
I	Fractionated	W	732	799	—
		M*	294	556	—
II	Whole cell	W	348	3282	353
		M†	328	877	103
III	Whole cell	W	636	3363	454
		M‡	286	644	143
			295	853	164

*Sixth passage in absence of MPA.
†Second passage in absence of MPA.
‡Third passage in absence of MPA.

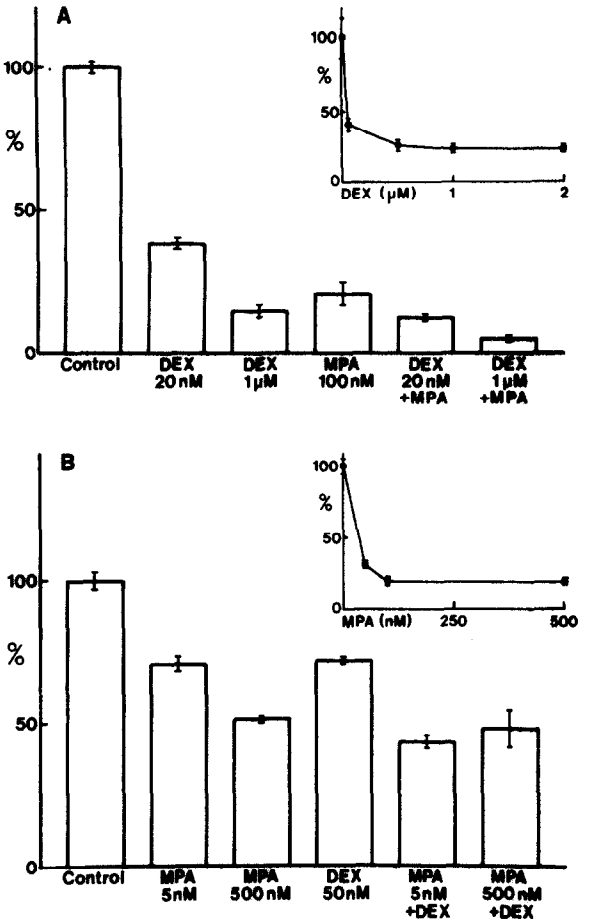

Fig. 4. Effects of medroxyprogesterone acetate, dexamethasone and combinations of these drugs on proliferation of 'wild-type' cells. Cells were exposed for 6 (A) and 5 (B) days to media containing SFCS and suboptimal or optimal concentrations of DEX (A) or MPA (B) without and with addition of MPA (A) or DEX (B) at the concentrations shown. Insets show dose–response curves for DEX (A) and MPA (B). Results expressed as in Fig. 1. Mean controls: (A) 3.1 × 10^6, (B) 2.9 × 10^6 cells/flask.

assays of preparations from 31 duplicate flasks gave estimates of between-flask coefficients of variation of 12, 9 and 12% for putrescine, sperimidine and spermine, respectively. These latter figures indicate the true experimental errors, since samples from any one experiment were analysed in the same batch. Addition of polyamines (4nmol) to duplicate samples containing 2.2, 10.6 and 8.0 nmol endogenous putrescine, spermidine and spermine, respectively, gave mean recoveries of 108, 103 and 103%. Estimates of cellular polyamine content ranged from 0.1 to 3, 1 to 15 and 1 to 9 fmol/cell of putrescine, spermidine and spermine and 0.02, 0.03 and 0.05 fmol/cell, respectively, could be detected, but not estimated accurately.

A relation between growth rate and cellular polyamine content can be demonstrated for MCF-7 cells. Concentrations were low during the lag phase following passage, increased once logarithmic growth began (Fig. 5) and decreased again as confluence was reached (data not shown). Physiological doses of oestradiol increased polyamine content by a factor of 2 or more [22]. Addition of MPA to media containing 5% SFCS significantly increased the polyamine content of 'M' cells after 24 hr exposure and by 48 hr levels had fallen to those of untreated controls (Fig. 6A and B). In 'wild-type' cells only putrescine content had increased at 24 hr (Fig. 6C) in contrast to 'M' cells in which levels of all 3 polyamines had increased. There was no significant difference between the effects of 10 and 500 nM MPA on 'wild-type' cells.

DISCUSSION

The MCF-7 cell line reflects practically all the responses to hormones of human breast tumours *in vivo*. The mitogenic effects of oestrogens [23] and antioestrogens [6] at low concentrations and growth-inhibitory actions of these hormones at high concentrations [6, 23] are consistent with clinical observations [1–5, 24, 25]. Similarly, the beneficial clinical effects of glucocorticoid [26] and progestogen [7] therapy are manifested in MCF-7 cell cultures as growth inhibition. There is evidence that human malignant tumours, including those of the

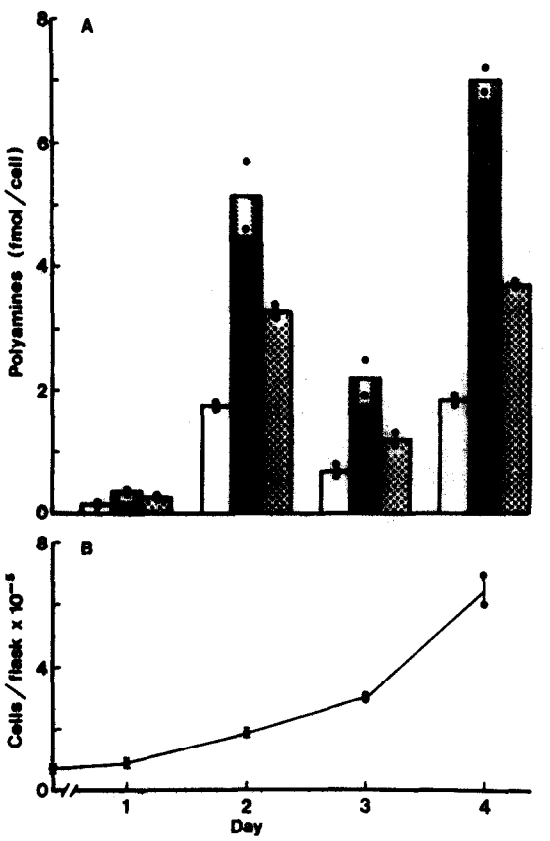

Fig. 5. Estimate of cellular polyamine content in relation to proliferation rates. Cells were seeded on day 0 and harvested after 1, 2, 3 and 4 days in growth medium. (A) content of putrescine (open bars), spermidine (hatched bars) and spermine (dotted bars), (B) cell numbers.

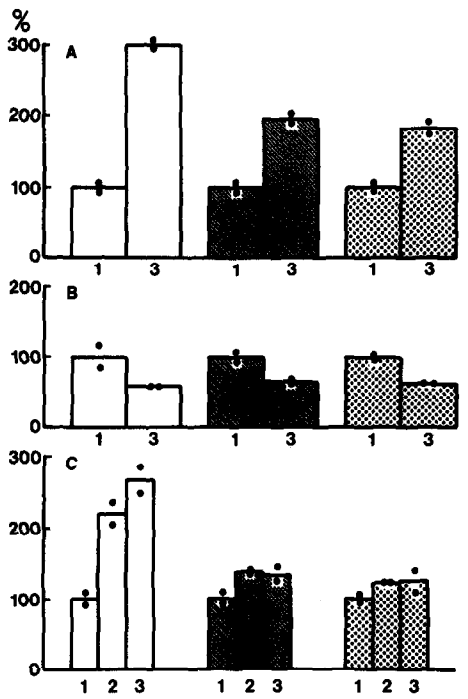

Fig. 6. Effects of medroxyprogesterone acetate on polyamine content of 'M' (A, B) and 'wild-type' (C) cells. Monolayers grown with medium containing FCS followed by medium containing SFCS for 2 days were exposed to control medium (1) or media containing 10 nM (2) or 500 nM (3) MPA for 24 (A, C) or 48 (B) hr prior to harvesting.

breast, contain heterogeneous cell populations [27–29]. Similarly, the MCF-7 cell line contains cells differing in their responses to oestrogen and antioestrogen [30, 31], and we show here that cells varying in responses to MPA are also present. Since this cell line displays so many of the properties of tumours *in vivo* it seems reasonable to use it as a model and, albeit cautiously, extrapolate results obtained with it to the clinical situation.

In our experiments MPA consistently reduced the proliferation of MCF-7 cells in the absence of E_2. This contrasts with the results of Vignon and coworkers [32] who could only reproducibly demonstrate an effect of another progestogen (R5020) on T47D cells in the presence of E_2 or on a cloned subline of MCF-7 in the presence of tamoxifen. We cannot explain this discrepancy at present.

The development of 'M' cells has made it possible to examine all the effects of a progestogen and gain an insight into its modes of action. The concentration of PR binding sites in 'M' cells, though apparently lower than those in 'wild-type' cells, was not negligible. Failure to respond to the growth-inhibitory action of MPA suggests that the PR may be defective or that the effect of the drug is not mediated by PR. Since 'M' cells contained very low levels of GR and responded poorly to dexamethasone, it seemed possible that the growth-inhibitory effects of MPA are, at least partly, mediated by GR. Our experiments on the interactions of MPA and DEX with respect to cell proliferation indicate a progestogen action not shared with that of a glucocorticoid and an additional growth-inhibitory effect by a glucocorticoid action (Fig. 4). DEX had no effect on 'wild-type' cells whose growth was maximally inhibited by MPA. These results are consistent with studies by Lippman and coworkers [33] who demonstrated the direct effect of DEX on MCF-7 cells and showed that progestogens bind to both progesterone and glucocorticoid binding sites. Hormone action appears to be receptor- rather than hormone-specific since cross-reaction of one hormone with the receptor of another can evoke a response untypical of the hormone. This was first suggested for an androgen/oestrogen receptor interaction by Ruh and Ruh [34] and was confirmed by the growth-stimulatory effect of 5α-dihydrotestosterone through interaction with ER in MCF-7 cells [35].

The glucocorticoid actions of progestogens have been demonstrated by several investigators [15] and the involvement of such a mechanism in the growth-inhibitory action of MPA is not surprising. Since progestogens bind to GR sites with lower affinity than to PR sites, it is possible that tumours containing cells with functional PR respond to low doses of progestogens while those with functional GR but no PR require higher dosage. Distinct actions of progestogens at low and high receptor affinities could resolve the controversy regarding dosage [7].

The claim that the growth-inhibitory action of progestogens may rest solely on their interaction with PR binding sites [32] and the proposed strategy for hormone therapy based on this assertion [8, 9] require re-examination. Vignon and coworkers [32] demonstrated no effect of DEX (50 nM) in the presence of E_2 (1 nM) while R5020 (100 nM) in the presence of E_2 and DEX significantly reduced proliferation, as judged by DNA determinations, of T47D cells. In further experiments, these workers showed that a rat mammary tumour cell line containing GR but no PR binding sites was resistant to 100 nM R5020. No assays of GR binding site concentration or affinity under the specific experimental conditions were presented, nor was an effect of DEX demonstrated in either case. It is possible, therefore, that no glucocorticoid effects of R5020 were observed either because there was no demonstrable glucocorticoid action or because the concentration was insufficient at the prevailing affinity. Our data do not contradict the therapeutic implications of PR-mediated effects of progestogens [8, 9] but raise the possibility that they are based on an over-simplification and that other modes of action should be taken into account.

The growth-promoting effects of MPA, uncovered by the use of 'M' cells but not dem-

onstrable in 'wild-type' cells (Fig. 2), occurred at concentrations found in the plasma of patients treated with the drug [36, 37]. Such action is consistent with a recent report of a similar action demonstrated *in vitro* and suspected *in vivo* [38]. To our knowledge, no 'withdrawal responses' similar to those observed with tamoxifen [5] have been reported so far, but if other therapy is instituted immediately on discovery of recurrence such effects could be missed. We hope that awareness of the possibility of a growth-promoting action will prompt a careful search for it in the clinical situation, so that its incidence can be assessed. In our experiments, growth stimulation was detected at MPA concentrations 100 nM or greater. So far no such effect has been reported *in vivo*. Prolonged treatment with MPA should perhaps be restricted to situations in which the effect of the drug on tumour growth is evaluable, particularly in patients whose tumours lack GR binding sites.

Our estimates of putrescine concentrations in MCF-7 cell were always lower than those for spermidine and spermine, in agreement with results for tumour tissue [39]. However, our results for spermine tended to be lower than those for spermidine while the reverse has been reported for tumour tissue [39]. The rapid increases in polyamine levels following exposure to MPA (Fig. 6) are consistent with a growth-promoting effect and may be due to increased synthesis or decreased catabolism of polyamines. The increases were not sustained and by 48 hr polyamine levels of MPA-treated cells had fallen below those of controls (Fig. 6B). It is thus possible that polyamine catabolic enzymes are involved in these changes. The increases in putrescine content of 'wild-type' cells (Fig. 6C) require further investigation. While increases in cellular polyamine content tend to be associated with cell proliferation, it does not follow that decreased growth rates involve decreased polyamine concentrations. Cells which, like 'M' cells, show increases in polyamines and may show increased proliferation in response to MPA may be undetectable in a population in which the majority of cells respond by decrease in growth rates. Studies of polyamine content of tumour samples in response to progestogens *in vitro* may reveal the presence of cells on which the drug may have adverse effects. It would thus be possible to identify patients in whom high doses or prolonged exposure to the drug or both should be avoided.

The mechanism(s) by which progestogens may stimulate growth and increase intracellular polyamine content require investigation. They can evoke oestrogenic effects [16] and bind to ER, though with an affinity which may be too low to be of pharmacological importance [10, 11]. Whether MPA at high concentrations may evoke an oestrogenic effect, rather than an antioestrogenic one, as has been suggested [10], requires further investigation. If the growth-promoting effect of MPA proved to be mediated by ER, the growth of tumour components containing ER could be stimulated by high dosage of the drug counteracting any GR-mediated inhibitory effects. Simultaneous administration of MPA and an antioestrogen may reduce or abolish possible oestrogenic effects of the progestogen. High response rates have been observed with such combined hormone treatment schedules [9], although other results [25] do not support this.

More work is needed, before the mechanisms of growth-inhibitory and growth-stimulatory effects of MPA, and possibly other progestogens, are fully understood. Our findings support the possibility [13] that hormone receptors other than PR may be involved in the actions of these drugs and should be examined more widely in relation to clinical response. The possible growth-stimulatory effects should not be ignored and great caution exercised with regard to high dosage. Further progress can still be made in the selection of patients for treatment, delay of tumour progression and use of optimum dosage and drug combinations for each patient. The questions raised in this paper require further study with these ends in view.

Note added in proof. Regressions following withdrawal of megestrol acetate therapy have been reported recently (Nowakowski V, Bonomi P, Anderson KM, Straus A, Economou SG. *Breast Cancer Res Trest* 1986, **8**, 82). We have not tested this progestogen, but it is possible that, like MPA, it may promote proliferation of some breast cancer cells.

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