Mitogenic hormones and tumor promoters greatly increase the incidence of colony-forming cells bearing amplified dihydrofolate reductase genes

(methotrexate resistance/phorbol esters/mezerein/mitogenic hormones/disproportionate DNA replication)

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ABSTRACT   Previous work has shown that the presence of a phorbol ester tumor promoter, phorbol 12-myristate 13-acetate (PMA), during a single-step selection for methotrexate (MTX)-resistant mouse 3T6 cells results in an up to 100-fold increase in the incidence of MTX-resistant, colony-forming cells. MTX resistance of most of these cells is due to amplification of the gene for dihydrofolate reductase (DHFR), the target enzyme for MTX. We show here that other active, noncytotoxic phorbol ester tumor promoters, such as phorbol 12, 13-didecanoate and 20-phorbol 12,13-butyrate, at their optimal concentrations ($\approx 0.1\,\mu$M) are approximately equal to PMA in increasing the incidence of MTX-resistant 3T6 colonies. Mezerein, a potent second-stage tumor promoter, but a weak complete promoter, increases the incidence of MTX resistance up to 350-fold, the strongest effect for any of the agents so far tested. PMA analogs that are inactive as tumor promoters, such as phorbol or phorbol 12,13,20-triacetate, have no effect on the incidence of MTX-resistant 3T6 colonies. Anthralin, a nonphorbol tumor promoter, is $\approx 40\%$ as active as PMA in the MTX selection assay. Remarkably, the hormones insulin, arginine vasopressin, and epidermal growth factor, all of which are mitogenic for 3T6 cells, also exert a strong PMA-like effect on the incidence of MTX-resistant 3T6 colonies under conditions of MTX selection. The effect of insulin at its optimal concentration (≈ 1 μg/ml) is $\approx 70\%$ that of PMA. Although the effect of PMA on the incidence of MTX-resistant 3T6 colonies does not significantly depend on the initial density of seeded cells or volume of the medium added, the analogous effect of insulin is strongly influenced by these parameters. Mevalonic acid, arachidonic acid, thymidine, caffeine, and nicotine, all of which are known to influence patterns of DNA synthesis in mammalian cells, were tested at their highest noncytotoxic concentrations and failed to produce any significant effect on the incidence of MTX-resistant 3T6 colonies. We discuss possible mechanisms of hormone- and tumor promoter-facilitated gene amplification in mammalian cells, relationship of mitogenic hormones to tumor promoters, and also implications of our findings for the problem of drug resistance in cancer chemotherapy.

We have recently reported that phorbol 12-myristate 13-acetate (PMA), a noncytotoxic, nonmutagenic phorbol ester tumor promoter, greatly increases the incidence of mouse cells bearing amplified dihydrofolate reductase (DHFR) genes under conditions of cytotoxic selection for resistance to a DHFR enzyme inhibitor, methotrexate (MTX) (1). Subsequent work by Tlsty *et al.* (2) has confirmed this result and, in addition, showed that *cytotoxic* treatments of mammalian cells (with hydroxyurea or UV light) *before* selection for MTX resistance also lead to an increased incidence of colony-forming cells bearing amplified

DHFR genes. These findings (1, 2) provided an indirect support for an earlier hypothesis that one pathway of carcinogenesis and tumor progression may operate through a generalized *increase in the frequency* of disproportionate DNA replication within a single cell cycle, resulting in *accelerated* gene amplification (3). Targets for such "facilitated" gene amplification may include genes responsible for the initial stages of carcinogenesis or other genetic loci—for example, those influencing metastatic potential or immunosuppressive properties of evolving tumor cells (3–12). Facilitated gene amplification could greatly speed up evolution of cellular systems under selection pressure, a developing tumor being one example of such a system. Indeed, double-minute chromosomes (DMs) and homogeneously staining chromosomal regions (HSRs), the two cytological indicators of gene amplification (5, 13–15), have been consistently detected in cells from a wide variety of naturally occurring tumors but were not reported to occur in cells from normal tissues (9). A different aspect of gene amplification that is also clinically important is the emergence of drug-resistant tumor cells during cancer chemotherapy. In many cases such cells are drug-resistant due to amplification of specific genes whose products are involved with drug's action, transport, or metabolism (5, 9, 14–16).

We report below that tumor-promoting phorbol esters other than PMA, nonphorbol tumor promoters, and most significantly, mitogenic hormones, such as insulin, vasopressin, and epidermal growth factor (EGF), also greatly increase the incidence of MTX resistance in 3T6 cells under conditions of MTX selection.

MATERIALS AND METHODS

Single-Step MTX Selection Assay. The procedure was described in detail previously (1). MTX [(+)amethopterin], bovine insulin, and arginine vasopressin were obtained from Sigma. Phorbol derivatives were obtained from Consolidated Midland (Brewster, NJ). Mezerein was obtained from CCR (Eden Prairie, MN). EGF was obtained from Bethesda Research Laboratories.

Cloning of MTX-Resistant 3T6 Cells and DNA Isolation. The protocol used was essentially the one described previously (1, 17), except that a dot blot manifold (Schleicher & Schuell) was used, and quantitation of dot hybridization patterns was carried out by cutting out and counting individual dots of ^{32}P-

Abbreviations: DHFR, dihydrofolate reductase; PMA, phorbol 12-myristate 13-acetate; PDD, phorbol 12,13-didecanoate; PDBu, 20-phorbol 12,13-butyrate; PTA, phorbol 12,13,20-triacetate; MTX, methotrexate; EGF, epidermal growth factor; MVA, mevalonic acid; Me$_2$SO, dimethyl sulfoxide; DMs, double-minute chromosomes; HSR, homogeneously staining region.

labeled DNA (this procedure yields more accurate results than visual comparisons of dot intensities; data not shown). The cloned mouse DHFR cDNA probe (pDHFR11; ref. 18) was a gift from R. Schimke. The cloned mouse β^{maj}-globin cDNA probe (pCR1-M9; ref. 19) was donated by V. Volloch.

RESULTS

Incidence of MTX-Resistant 3T6 Colonies Is Greatly Increased in the Presence of Either Phorbol or Nonphorbol Tumor Promoters. Fig. 1A shows that the presence of PMA during MTX selection results in up to a 100-fold increase in the incidence of MTX-resistant mouse 3T6 cell colonies, as described in detail previously (1). The enhancing effect of PMA varied from one independent experiment to another from ≈50-fold to ≈110-fold, whereas duplicate plates within one and the same experiment showed <20% variation (ref. 1 and data not

shown). Whenever a comparison was made between the effect of PMA and of another compound on the incidence of MTX resistance (see below), measurements of the PMA effect were carried out in a parallel experiment with the same population of 3T6 cells.

Using the same single-step MTX selection assay (1), we have subsequently tested other phorbol esters and found that phorbol 12,13-didecanoate (PDD) and 20-phorbol 12,13-butyrate (PDBu), both of which are comparable to PMA in their tumor promoting activity on mouse skin (20–24), are also approximately equal to PMA in increasing the incidence of MTX-resistant 3T6 colonies (Fig. 2). Optimal concentrations of PDD and PDBu in the MTX selection assay were determined as described for PMA (1) (data not shown) and were found to be indistinguishable from the optimal PMA concentration (≈0.1 μM; ref. 1). On the other hand, the addition of either a weak phorbol ester tumor promoter phorbol 12,13,20-triacetate (PTA) or
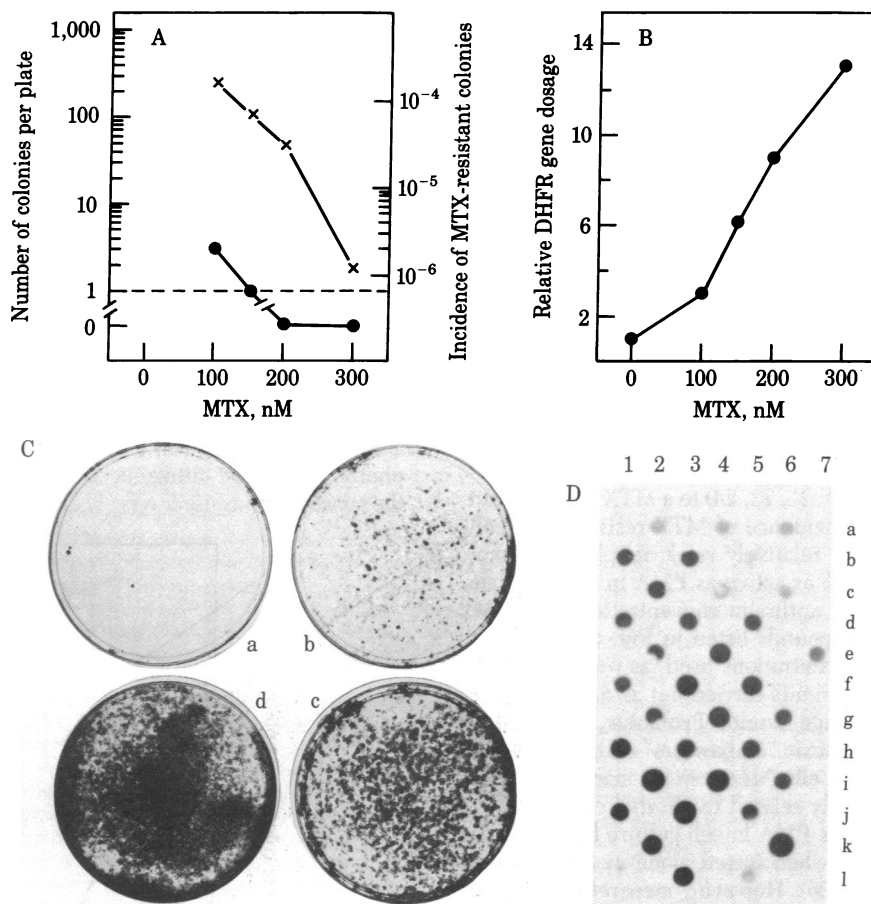

FIG. 1. Effects of PMA and mezerein on incidence of 3T6 cells bearing amplified DHFR genes; determination of DHFR gene dosage. (A) Confluent 3T6 monolayers in medium A were reseeded in 10-cm plates (1.5×10^6 cells per plate) containing MTX in the same medium (1). ●, 3T6 cells grown, reseeded, and selected in the absence of PMA; ×, same, but PMA was added at 162 nM (0.1 μg/ml) at the start of MTX selection. (B) Relative DHFR gene dosage in MTX-resistant 3T6 cells as a function of selecting MTX concentration. Experimental points in this graph were derived directly from the dot hybridization data (shown in D). (C) Stained colonies of MTX-resistant 3T6 cells (1.5×10^6 cells initially seeded per plate in the presence of 150 nM MTX) selected in the absence of PMA and mezerein (plate a), in the presence of 162 nM PMA (plate b), or in the presence of 162 nM mezerein (plate c). Plate d is the same as plate c, but 1.85×10^6 3T6 cells were initially seeded. (D) Determination of relative DHFR gene dosages in MTX-resistant 3T6 cells by a DNA·DNA dot hybridization assay. Equal amounts of the total nuclear DNA (1 μg) purified from different sublines were denatured, applied onto a nitrocellulose filter, and hybridized with the cloned ^{32}P-labeled DHFR cDNA (pDHFR11; see *Materials and Methods* and ref. 18). An identical parallel set of DNA dots was hybridized with cloned ^{32}P-labeled β^{maj}-globin cDNA probe (pCRI-M9; data not shown; see *Materials and Methods* and refs. 1 and 19). All dots represent independently selected MTX-resistant 3T6 sublines. DNA from control, MTX-sensitive 3T6 cells (dots 2a, 4a, 5l, and 6a). DNA from 3T6 cells selected for resistance to 100 nM MTX in the presence of either 162 nM mezerein (dot 1d) or 162 nM PMA (dots 2g, 6c, and 7e) or insulin at 1 μg/ml (dots 1f and 5j) or no additions (dot 2e). DNA from 3T6 cells selected for resistance to 150 nM MTX in the presence of either 162 nM mezerein (dots 1b and 5h) or 162 nM PMA (dots 3b, 3h, 3l, and 6g) or insulin at 1 μg/ml (dots 1j, 5d, and 6i) or no additions (dots 2c, 2k, and 3d). DNA from 3T6 cells selected for resistance to 200 nM MTX in the presence of either 162 nM mezerein (dot 3f) or 162 nM PMA (dots 4i and 5f) or insulin at 1 μg/ml (dot 4e) or no additions (dots 1h and 4g). DNA from 3T6 cells selected for resistance to 300 nM MTX in the presence of either 162 nM PMA (dot 6k) or insulin at 1 μg/ml (dot 3j) or no additions (dot 2i).
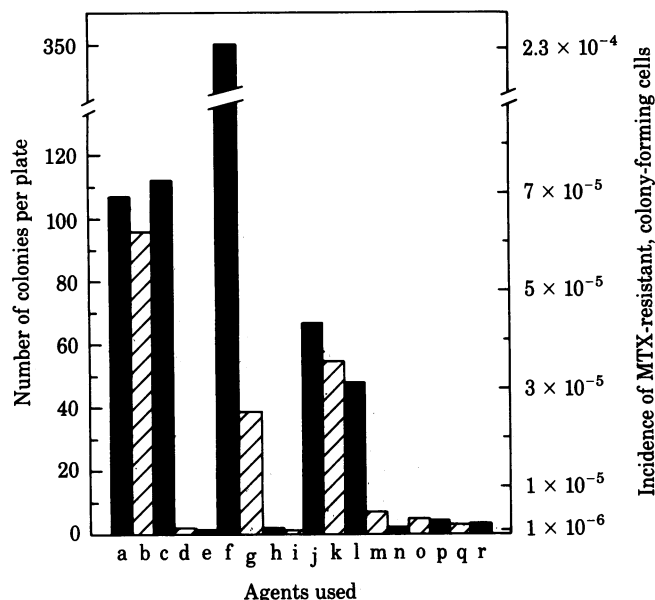
FIG. 2. Influence of various noncytotoxic agents on incidence of MTX resistance in 3T6 cells. Confluent 3T6 monolayers were reseeded at 1.5 × 10⁶ cells per 10-cm plate in the presence of 150 nM MTX and one of the following agents: a, PMA; b, PDD; c, PDBu; d, PTA; e, phorbol; or f, mezerein (a–f at 162 nM); g, anthralin (10 nM); h, 0.01% dimethyl sulfoxide (Me₂SO); i, 0.1% Me₂SO; j, insulin (1 μg/ml); k, arginine vasopressin (10 ng/ml); l, EGF (10 ng/ml); m, mevalonic acid (MVA) (1 mM); n, arachidonic acid (100 nM); o, thymidine (20 μM); p, caffeine (50 μM); q, nicotine (60 μM); r, no addition (control). For the tumor promoters and hormones the concentration listed is that which resulted in the greatest incidence of MTX-resistant 3T6 colonies. Nicotine and caffeine were present at their highest noncytotoxic levels as determined by a plating efficiency assay (1).

a nonpromoting phorbol (20, 21, 23, 24) to a MTX assay did not significantly increase the incidence of MTX-resistant 3T6 colonies (Fig. 2). Anthralin, a relatively weak nonphorbol tumor promoter (23, 25), is ≈40% as active as PMA in the MTX selection assay at an optimal anthralin concentration (≈10 nM) (Fig. 2). None of the compounds listed in Fig. 2 is itself cytotoxic for 3T6 cells at concentrations used, as was verified by plating efficiency measurements carried out as described (1).

Mezerein, a Second-Stage Tumor Promoter, Is the Most Potent of Known Noncytotoxic "Enhancers" of the Incidence of MTX Resistance in 3T6 Cells. Mezerein is a macrocyclic plant diterpene that is structurally related to phorbol esters and has many of the same effects as PMA in cell culture but is considerably weaker than PMA when tested alone as a tumor promoter on mouse skin (20, 26). However, mezerein was shown to be quite potent when used during the second stage of tumor promotion as defined by using the mouse skin model (20–22, 26). We found mezerein to increase the incidence of MTX-resistant 3T6 colonies ≈350-fold as compared with an ≈110-fold increase elicited by PMA under the same conditions in a parallel MTX selection experiment (Figs. 2 and 3).

Hormones Insulin, Arginine Vasopressin, and EGF All Increase the Incidence of MTX-Resistant 3T6 Colonies Under Conditions of MTX Selection. As shown in Figs. 2–4, addition of optimal concentrations of either insulin, arginine vasopressin, or EGF to the MTX selection assay results in an ≈70-fold, ≈60-fold, or ≈50-fold increase, respectively, of the incidence of MTX-resistant 3T6 colonies, as compared with an ≈110-fold increase elicited by PMA under the same conditions in a parallel series of single-step MTX selections. A significant (≈3-fold) increase in the incidence of MTX-resistant 3T6 colonies is caused

by insulin even at 10 ng/ml, which is 1/100th of the optimal insulin concentration (Fig. 2 and data not shown). All of the above hormones as well as PMA and other tumor-promoting phorbol esters are mitogenic and noncytotoxic for 3T6 cells at the concentrations used (refs. 1, 27, and 28 and data not shown).
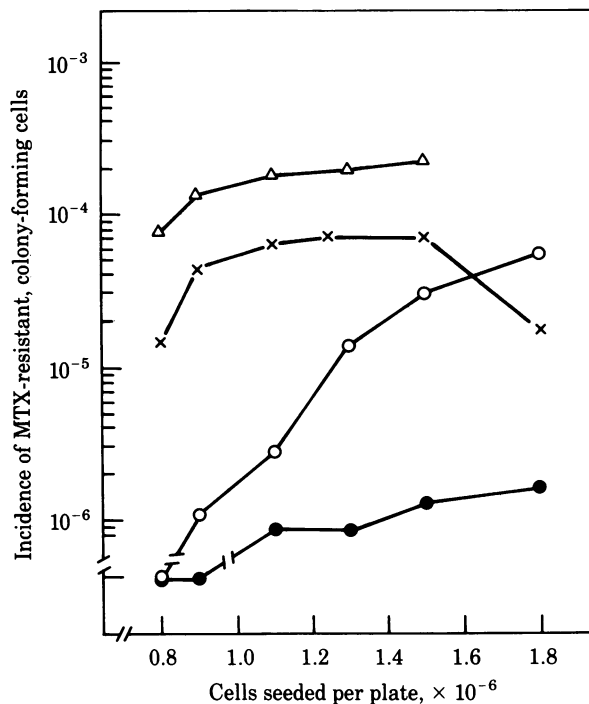

FIG. 3. Incidence of MTX-resistant 3T6 colonies as a function of initial cell density in the presence of either insulin, PMA, or mezerein. Varying amounts of 3T6 cells were seeded in 10-cm plates in medium A containing 150 nM MTX and either no additions (●), insulin (1 μg/ml; ○), PMA (162 nM; ×), or mezerein (162 nM; △).
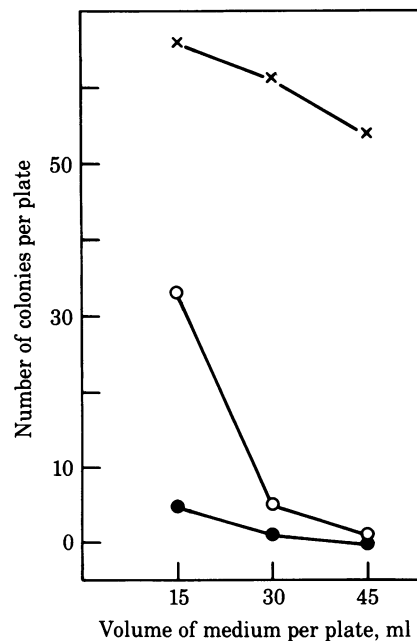

FIG. 4. Incidence of MTX-resistant 3T6 colonies as a function of the volume of medium per plate during MTX selection: comparison of the effects of insulin and PMA. Confluent 3T6 cells in medium A were reseeded at 0.9 × 10⁶ cells per 10-cm plate in the same medium containing 150 nM MTX and either no additions (●), insulin (1 μg/ml; ○), or PMA (162 nM; ×) in volumes of the medium varying from 15 to 45 ml per plate.

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Most of MTX-Resistant 3T6 Clones Have Increased Dosages of DHFR Genes. Mouse cells were shown to acquire resistance to MTX either through an amplification of the DHFR gene, which leads to an overproduction of the target DHFR enzyme (5, 9–12, 18), through mutations rendering the DHFR enzyme less sensitive to inhibition by MTX, or through mutations decreasing MTX uptake by cells (5). Of these causes of MTX resistance, the DHFR gene amplification appears to be the most frequent one under a variety of conditions (5, 9–12). This is in agreement with the results shown in Figs. 1 *B* and *D* for 3T6 sublines that were single-step selected for resistance to different MTX concentrations either in the presence or absence of PMA, mezerein, or insulin. Of 38 independently derived 3T6 sublines selected for resistance to different concentrations of MTX in the presence of added PMA, insulin, or mezerein, only 4 sublines (3 resistant to 100 nM MTX and 1 resistant to 200 nM MTX) did not have an increased DHFR gene dosage (Fig. 1D, dot 6c and data not shown). Significantly, it is the selecting MTX concentration but not the presence of tumor promoters or hormones that determines the DHFR gene dosage in MTX-resistant 3T6 clones as has been found previously for the PMA-MTX system (1) and is shown in Fig. 1D for the mezerein-MTX and insulin-MTX systems.

Insulin Effect on Incidence of MTX-Resistance in 3T6 Cells Strongly Depends on Initial Cell Density and Volume of Added Medium During MTX Selection. When the amount of 3T6 cells seeded per a 10-cm plate at the start of selection for resistance to 150 nM MTX in the presence of insulin is increased less than 50% from 0.9×10^6 to 1.5×10^6 cells per plate, the incidence of MTX resistant 3T6 colonies is increased $\approx$25-fold, from $\approx$1 $\times 10^{-6}$ to $\approx$2.5 $\times 10^{-5}$ (Fig. 3). Analogous effects of arginine vasopressin and EGF display a strikingly similar dependence on the initial cell density (data not shown). In contrast, in a parallel single-step MTX selection in the presence of PMA, the increase in the number of initially seeded 3T6 cells from 0.9×10^6 to 1.5×10^6 cells per plate resulted in a relatively small increase in the incidence of MTX-resistant 3T6 colonies, from $\approx$4 $\times 10^{-5}$ to $\approx$6 $\times 10^{-5}$ (Fig. 3). Selection experiments carried out in the presence of mezerein, PDD, PDBu, or anthralin also did not show a significant dependence of the incidence of MTX resistance on the initial cell density, in striking contrast to the results with the insulin-MTX system (Fig. 3 and data not shown).

The fact that insulin added to the 3T6 growth medium is much less metabolically stable than PMA (29) apparently does not contribute to the results shown in Fig. 3 because experiments in which insulin was added to 3T6 cultures at either 4- or 12-hr intervals from the start of MTX selection produced similar results (data not shown). It should be also noted that a decrease in the initial cell density significantly *below* 0.9×10^6 cells per a 10-cm plate does lead to a significant decrease in the incidence of MTX-resistant 3T6 cells selected in the presence of either PMA (1) or mezerein (data not shown).

To explain the much stronger effect of the initial cell density on the incidence of MTX-resistant colonies selected in the presence of insulin *versus* PMA, it is sufficient to assume that although both compounds are mitogenic for partially MTX-resistant 3T6 cells that emerge during MTX selection, PMA is either a more potent mitogen or requires lower concentrations of MTX block-bypassing compounds (released from dying, MTX-sensitive cells) in the medium to induce growth of "nascent" MTX-resistant cells. The finding that the insulin-mediated increase in the incidence of MTX resistance has a far greater dependence on the volume of medium added per plate as compared with the PMA-mediated effect (Fig. 4) is also consistent with the above explanation.

Effects of PMA and Insulin Present Together During MTX Selections Are Approximately Additive. At all of the cell densities tested, from 1×10^6 to 1.75×10^6 3T6 cells per a 10-cm plate, the effect of simultaneous presence of optimal concentrations of insulin and PMA is approximately equal to the sum of the effects of insulin and PMA alone (Fig. 5). In contrast, the effect of simultaneous presence of optimal concentrations of PMA and another phorbol ester promoter, PDD (Fig. 2), on the incidence of MTX resistance is indistinguishable from the effects of each of these compounds present alone in the MTX selection assay (data not shown). One interpretation of these results is that there may be little overlap between the sets of cell targets for growth-promoting activities of PMA and insulin in the nascent population of MTX-resistant 3T6 cells.

MVA, Arachidonic Acid, Thymidine, Caffeine, and Nicotine Do Not Significantly Change the Incidence of MTX Resistance in 3T6 Cells. All of the above compounds are known to influence patterns of DNA synthesis in mammalian cells. Arachidonic acid (a prostaglandin precursor) and MVA, the rate-limiting intermediate in cholesterol biosynthesis, have both been shown to increase in concentration after PMA treatment (30). Addition of MVA or arachidonic acid to cell cultures stimulates DNA synthesis (31). Plant alkaloids caffeine and nicotine also stimulate DNA synthesis by subconfluent cell cultures (32, 33). Presence of a nucleotide precursor thymidine in the growth medium has been shown to increase the plating efficiency of 3T6 cells (1). As shown in Fig. 2, none of the above compounds, when tested at its highest noncytotoxic concentrations in the single-step MTX selection assay, significantly increases the incidence of MTX-resistant 3T6 colonies.
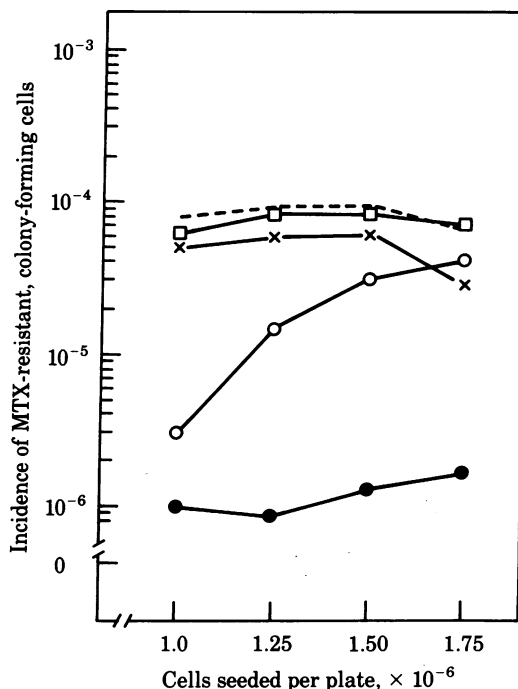

FIG. 5. Effect of simultaneous presence of PMA and insulin during MTX selection of 3T6 cells is approximately additive as compared with effects of PMA and insulin alone. Varying amounts of confluent 3T6 cells in medium A were reseeded in 10-cm plates in the same medium containing 150 nM MTX and either no additions (●), insulin (1 μg/ml; ○), PMA (162 nM; ×), or *both* PMA and insulin at the above concentrations (□). The dashed curve shows the calculated "precise additivity" pattern produced by summing up the effects of PMA and insulin when present separately in the single-step MTX selection assay.

DISCUSSION

Possible Mechanisms of Hormone- and Tumor Promoter-Increased Incidence of Cells Bearing Amplified Genes. There are two classes of interpretations formally consistent with our results. In the first one, the presence of either tumor promoters or hormones in the single-step MTX selection assay increases (either directly or indirectly) the probability of gene amplification through disproportionate DNA replication (1, 3, 5) in many different chromosomal domains, including the one containing the DHFR gene. In the second class of interpretations, tumor promoters and hormones do not increase the probability of gene amplification *per se*, but rather the probability of *colony formation* by either preexisting or newly formed cells bearing amplified DHFR genes. A qualitative correlation between the ability of a substance to act as a mitogen for 3T6 cells and its ability to increase the incidence of MTX-resistant 3T6 colonies (see *Results*) is consistent with the second class of interpretations but also with one interpretation from the first class—namely, that while cytotoxic conditions of selection may "prime" cells for an increased probability of disproportionate DNA replication, the action of a mitogenic substance, such as PMA or insulin, is also required for a cell to enter a "replicative state" (analogous but not necessarily identical to a normal S-phase state) where the disproportionate DNA replication would actually occur (see also refs. 34 and 35). Taken together, our data favor both of these two related pathways in which *mitogenic* hormones and tumor promoters act by increasing both the probability of disproportionate DNA replication (via increased numbers of cells entering a replicative state in the presence of a cytotoxic drug) and the probability of survival and colony formation by nascent mutant cells bearing amplified genes.

Another means by which the incidence of MTX-resistant cell colonies can be increased is by briefly treating the cells with a cytotoxic agent other than MTX (such as hydroxyurea or UV light) shortly before subjecting them to MTX selection (ref. 2; see also refs. 23 and 35). The effect of these transient *cytotoxic* treatments on the yield of cells bearing amplified genes is likely to be due to a *direct* influence of the above cytotoxic agents on the probability of disproportionate DNA replication (2, 3, 34, 35), in contrast to the proposed mechanism by which the analogous effect is produced *indirectly* through the use of *noncytotoxic, mitogenic* hormones and tumor promoters.

One interpretation of the striking similarity of hormones and tumor promoters in the gene amplification assay (see *Results*) is that noncytotoxic tumor promoters such as phorbol esters act in their tumor-promoting mode as nothing more than powerful, cell type-specific mitogens. Both the fact that a number of mammalian hormones have been shown to possess tumor-promoting properties (36–38) and the recent identification of a phorbol ester receptor as a specific protein kinase (39, 40) are consistent with the "mitogenic" interpretation of the phenomenon of tumor promotion (see, however, refs. 41 and 42).

Significance of Hormone-Facilitated Gene Amplification for the Problem of Drug Resistance in Cancer Chemotherapy. Extensive evidence on drug resistance in both animal and human tumors strongly suggests that resistance of tumor cells to either a single cytotoxic drug, such as MTX (5, 9–12), or to a number of different drugs (16) simultaneously is often due to amplification of specific genes or sets of genes during cytotoxic selection for such drug-resistant cells in cancer chemotherapy. Regardless of which of the suggested mechanisms of the hormone-facilitated gene amplification (see above) will prove to be the correct one, it is clear that the incidence of colony-forming drug-resistant cells can vary dramatically *in vitro*, depending on the conditions of selection (see *Results* and refs. 1 and 2). One implication of this discovery is that by manipulating the conditions of cytotoxic chemotherapy of tumor cells it may be possible to reduce the incidence of drug-resistant cells.

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