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Mitogens for Murine Embryo Cell Lines

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The growth-promoting activities of fetal bovine serum, cortisol, phorbol myristate acetate, prostaglandin F2α, insulin, epidermal growth factor, and fibroblast growth factor were evaluated on four murine embryo cell lines (Swiss 3T3, Balb 3T3, M2, and C3H10T½). Each cell had an unique response spectrum to this collection of reported mitogens. Phorbol myristate acetate and prostaglandin F2α were active only on selected cell lines; cortisol was inactive on all four lines. Serum, epidermal growth factor, and fibroblast growth factor were able to stimulate cell division in all four lines, albeit to varying degrees for the different target cells.

Key words: insulin, mitogenesis, epidermal growth factor, fibroblast growth factor, prostaglandin F2α, phorbol myristate acetate

Continuous cell lines derived from murine embryo tissue often exhibit density-dependent inhibition of growth in culture. Because of their convenience for culture experiments and their capacity for clonal isolation, murine embryo-derived cell lines have been used as model systems for studying growth control and the initiation of cellular proliferation [1].

Clonal murine embryo cell lines have also been used in the study of viral [2] and chemical transformation [3, 4], and more recently in studies of chemical cocarcinogenesis [5, 6]. The Balb 3T3 [7] and Swiss 3T3 (clone 42) [8] lines have been used extensively for growth control studies [1]; the C3H-derived lines 10T½ [9, 10] and M2 [11] have been primarily utilized for chemical carcinogenesis studies.

A number of agents of diverse chemical structures have been reported to be mitogenic for one or another of these cell lines. These include polypeptides such as epidermal growth factor (EGF) [12], fibroblast growth factor (FGF) [13], and insulin [14]; prostaglandin F2α [15]; steroids such as cortisol [16]; and the potent cocarcinogen phorbol myristate acetate (PMA) [17]. Our laboratory has been interested in the mechanism of the initiation of cellular proliferation by such mitogens [12, 18, 19]. In this report we have tested a number of mitogens, representing a range of chemical structures, on four clonal murine embryo cell lines. Our objectives have been fivefold: i) to determine the ubiquity of

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individual mitogens on various cell lines; ii) to determine whether these murine embryo cell lines are responsive to a similar spectrum of mitogens; iii) to extend growth control studies to those cell lines (M2 and 10T½) which have been used primarily for carcinogenesis studies; iv) to define mitogen-cell systems to which we can extend selection for nonproliferative variants [19], and v) to determine whether agents which are cocarcinogens in culture [5, 6] are mitogenic in culture.

MATERIALS AND METHODS

Mitogens

EGF was prepared in our laboratory by the method of Savage and Cohen [20]. Insulin was purchased from Sigma (St Louis, Missouri), PGF2α was the gift of Dr John Pike of Upjohn Co (Kalamazoo, Michigan). PMA was purchased from Consolidated Midland (Brewster, New York), cortisol was from Calbiochem (San Diego, California). FGF was purchased from Collaborative Research (Waltham, Massachusetts).

Cell Lines

Swiss 3T3 was obtained from Dr C. Fred Fox (UCLA). We have previously used this cell line in a variety of studies concerned with the mitogenic activity of EGF [12, 18, 19, 21]. Balb 3T3 (clone 31) was also obtained from Dr Fox. The M2 cell line was provided by Dr Hans Marquart (Sloan Kettering); C3H10T½ was from Dr William Benedict (University of Southern California).

Cell Culture

All cells were grown at 37° in 10% CO_2 in Dulbecco's modified Eagle's medium (DME, Gibco), supplemented with 5% fetal calf serum (FCS, Reheis), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Growth curves and mitogen-stimulation studies were generally done in 60-mm dishes (Falcon), in 5 ml of medium. Cells were plated at densities of $1-3 \times 10^5$ cells per dish. Initial mitogenic studies for FGF were done in Linbro 17-mm multiwell trays, in 2.0 ml of medium, in order to conserve FGF. Cells were plated at a proportionally reduced number. Subculture of cells and removal of cells from the culture plates for counting were as described previously [12]. Cell counts were carried out on duplicate plates, using an Electrozone Celloscope Model 112TH (Particle Data Inc, Elmhurst, Illinois).

RESULTS

Serum Concentrations and Growth of Murine Embryo Cell Lines

Several of these cell lines have been shown to grow to specified saturation densities that are a function of serum concentration. Mitogenic stimulation by "growth factors" mimics increased serum concentration in this experimental paradigm [19]. In order to standardize our experimental comparisons of all lines and mitogens, we first compared the effect of serum concentration on growth rate and saturation density for the four lines, under identical conditions. Multiple dishes of each cell line at a density of approximately 1×10^5 cells per dish were plated in DME + 5% FCS. On the second day appropriate plates were switched to medium containing 5, 10, or 15% FCS. Duplicate plates were counted daily, and growth curves were constructed. In this experiment the plates, medium,

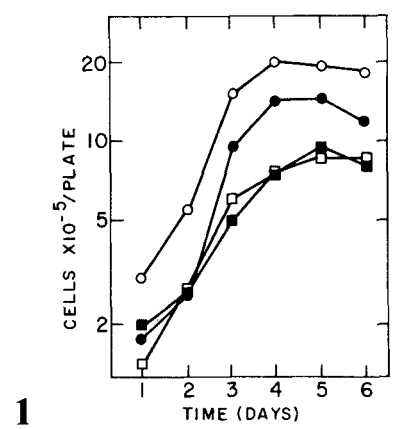
trypsin, etc were identical. The single variable was the cell line under examination. Saturation densities for the four cell lines were within two doublings of one another (Fig 1). All cell lines showed an increased saturation density when grown in progressively higher serum concentrations (Fig 2). The M2 cell line demonstrated a somewhat reduced response to serum in comparison with the other three lines.

Criteria for Mitogenic Activity

We have used two criteria for measuring mitogenic stimulation. In the first, cells grown continuously in the presence of a mitogen grow to a higher saturation density [1, 19]. Examples of two positive responses (Swiss 3T3 cells) and a positive and a negative response (M2 cells) are illustrated in Figure 3. All mitogen-cell line combinations positive in this first test were verified by the addition of the mitogen to confluent nondividing cells. Daily cell counts were then performed to evaluate mitogenic response. Examples of several positive mitogenic responses by this criteria are shown in Figure 4. FGF was tested only by the first (continuous growth) assay.

Mitogenic Responses of Swiss 3T3, Balb 3T3, M2, and C3H10T½ to EGF, Insulin, Cortisol, PGF2α, and PMA

Each cell line was plated in 60-mm dishes. One day after plating, mitogens were added at concentrations indicated in Figure 5. Mitogen concentrations were chosen to bracket those reported in the literature to be effective for one of these cell lines. Cell counts were then carried out on successive days until a plateau value (Fig 1, Fig 3) was achieved. Saturation density values for each of the cell lines exposed to this group of mitogens are shown in Figure 5.



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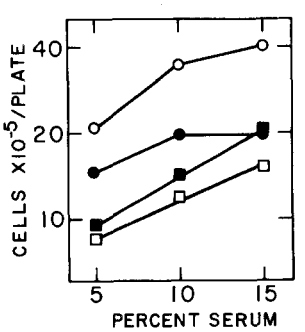

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Fig 1. Growth of murine embryo cell lines in DME + 5% FCS. Cells were plated at approximately 1×10^5 cells per dish. Cell counts were performed daily. Data are the average of duplicate plates. ○), Swiss 3T3; ●), M2; □), 10T½; ■), Balb 3T3.

Fig 2. Saturation density of murine embryo cell lines as a function of serum concentration. Cells were plated at 1×10^5 cells per 60-mm dish in DME + 5% serum. Twenty-four hours later appropriate plates were switched to fresh medium containing 5%, 10%, or 15% serum. Cell counts were performed daily. ○), Swiss 3T3; ●), M2; □), 10T½; ■), Balb 3T3.

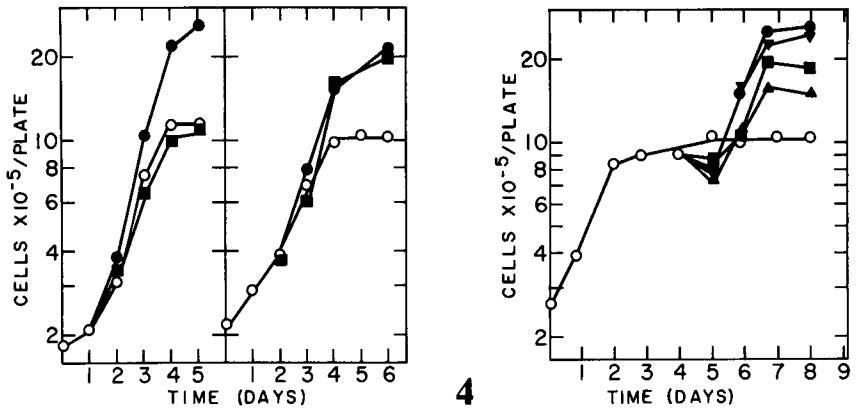

Fig 3. Increase in saturation density of M2 (left) and Swiss 3T3 (right) cells in response to continuous exposure to EGF and PGF2α. Cells were plated as described in Figure 1. After 24 h EGF (10 ng/ml) or PGF2α (100/ ng/ml) was added to appropriate plates. Data are the average of duplicate cell counts. •), EGF; ▪), PGF2α; ○), control.

Fig 4. Stimulation of cell division in quiesent Swiss 3T3 cells by various mitogens. 3T3 cells were plated as described in Figure 1. At day 4 EGF (10 ng/ml), PMA (100 ng/ml), PGF2α (100 ng/ml), or insulin (10,000 ng/ml) was added to appropriate plates. Data are the average of duplicate cell counts. •), EGF;▾), PMA; ▪), PGF2α; ▲), insulin, ○), control.

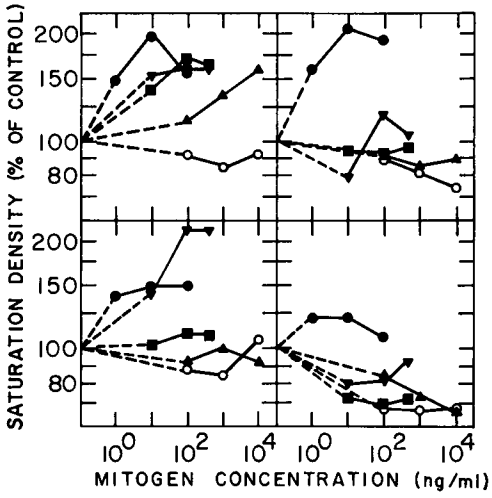

Fig 5. Saturation densities of Swiss 3T3 (top left), Balb 3T3 (top right), M2 (bottom left), and 10T½ cells (bottom right), exposed to various mitogens. Cells were plated at approximately 1 × 10^5 cells per 60-mm dish. After 24–72 h mitogens were added as indicated. Duplicate plates were used for daily cell counts. •), EGF; ○), cortisol; ▲), insulin; ▪), PGF2α;▾), PMA.

Mitogenic Response of Swiss 3T3, Balb, 3T3, M2, and C3H10T½ to FGF

Cells were plated in 17-mm Multiwell trays on day 1. On day 2 various FGF concentrations were added to appropriate wells. Cell counts were done for three successive days when cells were confluent, to determine saturation densities. Data are summarized in Table I. In a second experiment, also summarized in Table I, the mitogenic efficiency of

TABLE I. Mitogenic Activity of EGF and EGF

	5% serum	EGF (100 ng/ml)	FGF (10 ng/ml)	FGF (100 ng/ml)
Experiment 1				
Swiss 3T3	100	172	144	217
M2	100	284	121	199
10T½	100	127	85	122
Balb 3T3	100	312	122	171
Experiment 2				
Swiss 3T3	100	203	–	230
M2	100	174	–	166
10T½	100	142	–	154

All data are expressed as percent of cell number at saturation in DME + 5% FCS. Experimental values are the average of duplicate plates counted at saturation after continuous growth in the presence of mitogens. Experiment 1 was carried out in 17-mm Multiwell trays. Experiment 2 was performed in 60-mm culture dishes.

EGF and FGF were compared, at a single concentration, for cells grown in 60-mm plates as described above.

DISCUSSION

All four cell lines grow to characteristic saturation densities, dependent on serum concentration, then cease dividing, and enter a "stationary" state. Increased serum can initiate cell division in confluent nondividing cells of each cell line (data not shown). All these cell lines exhibit the characteristic form of growth control reviewed previously [1].

Results in these mitogen studies are somewhat variable; positive results for most of the mitogens ranged from 50% to 100% increases in cell numbers. Nevertheless, the distinction between a positive and a negative response is fairly clear-cut. There are clear differences in the response spectrum of these cell lines to various mitogens. The Swiss 3T3 cell line appears to have the broadest response spectrum (Fig 5); we will probably use this cell line most extensively in extending our variant selection approach [19] to the study of mitogenesis.

Cortisol had no mitogenic effect on any of the cell lines tested. Since a partial mitogenic response to cortisol has been reported in one of these cell lines [16], the possibility exists that subtle experimental variables or clonal variations within populations in different laboratories are responsible for this difference.

Insulin is stimulatory only for Swiss 3T3 cells, and then only at very high concentrations when compared to the other polypeptide mitogens. Thus, in an experiment in which the added polypeptides were the only variables, a 50% increase in saturation density occurred in response to 1 ng/ml of EGF; a similar growth response in Swiss 3T3 cells required 1,000–10,000 ng/ml of insulin (Fig 5).

The cocarcinogen PMA was mitogenic for both Swiss 3T3 and M2, but not for 10T½. Our data for 10T½ confirm the recent results of Peterson et al [22]. These results are of interest for several reasons. The M2 and 10T½ cell lines are both derived from C3H embryo tissue. That they respond differently to PMA consequently suggests that variation(s) in mitogenic response is not simply a function of strain differences. The 10T½ cell line has been used for PMA-induced cocarcinogenesis studies [5, 6]. The lack of a mitogenic

response suggests that these cells can be used to probe the puzzling and controversial relationship between mitogenic activity and "promotion" [23]. It is possible that a very low level of enhanced cell division in response to PMA occurs in 10T½ cells, commensurate with the frequency of induced transformation. This hypothesis would require that the transformation of a cell stimulated by the mitogen be a frequent event. To investigate this question one would need to carefully characterize entry of 10T½ cells into the S phase of the cell cycle in response to PMA.

PGF2α was mitogenic only for Swiss 3T3 cells. It is, however, a potent mitogen for these cells (see Jimenez de Asua, Clingan, and Rudland [15], and Fig 5). This observation should make this cell line and companion cell lines valuable tools for characterizing the biology and chemistry of the PGF2α receptor.

The two polypeptides EGF and FGF have mitogenic potential for all murine embryo-derived lines of this type. The degree of stimulation does differ, however. For example, while Swiss 3T3 and Balb 3T3 responded to optimal EGF exposure with a complete doubling, the two C3H-derived lines M2 and 10T½ had only partial responses. Observation of the percentage of labeled nuclei (ie, the number of cells entering S) and measurement of the density of EGF receptors should be particularly interesting in these cases of partial response. The difficulty in obtaining FGF [13] in comparison to EGF [20], and the rapidly accumulating knowledge of the chemistry and biology of EGF, suggest that EGF will be an easier system to analyze in molecular terms.

ACKNOWLEDGMENTS

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