in their potential for collagen synthesis has been demonstrated by several authors[19-21]. Our hypothesis is in agreement with previous histological observations[22] and the findings published by Wegrowsky et al.[23] showing an increase in collagen content and synthesis in fibroblast cultures established from samples of irradiated fibrotic tissue. Another hypothesis would be the release from irradiated DE of some activator(s) of collagen synthesis.

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Phenytoin-induced DNA synthesis and inositol 1,4,5-trisphosphate formation in L-929 fibroblasts

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Summary. Culture of L-929 fibroblasts in the presence of phenytoin (2.5–5.0 μg/ml) increased DNA synthesis, as indicated by increased [^{3}H]thymidine uptake, while a higher dose (20 μg/ml) inhibited DNA synthesis. In like manner, a low dose of phenytoin (5.0 μg/ml) was effective in increasing inositol 1,4,5-trisphosphate formation while a higher dose (10 μg/ml) tended to inhibit this activity. These data suggest that the formation of inositol phosphate second messengers may play a role in phenytoin-induced fibroblast proliferation and connective tissue growth.
Key words. Phenytoin; fibroblasts; inositol phosphates; DNA synthesis.

Phenytoin (5,5-diphenylhydantoin) has been used as an effective drug for the control of seizures since its clinical introduction in 1938[1]. Since that time phenytoin therapy has been associated with overgrowth of gingiva in about half of those receiving the drug[2] and less frequently, excessive connective tissue growth in other areas[3-5]. Further, phenytoin has been shown to be mitogenic for fibroblasts in vitro[6-8]. However, the mechanism by which phenytoin induces connective tissue growth or mitosis of fibroblasts has not been determined. The relationship of some mitogens to the receptor-coupled hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to form the intracellular 'second messengers', inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol[9-11] led us to compare the mitogenic properties of phenytoin and its possible activation of the phosphoinositide transmembrane signal mechanism in cultured fibroblasts. We report here that a critical in vitro concentration of phenytoin (within the range of therapeutic blood levels) increased both the rate of DNA synthesis and cellular levels of IP_3 and its metabolites, inositol 1,4-bisphosphate (IP_2) and inositol 1-phosphate (IP_1), in L-929 fibroblasts.

Materials and methods

Cell culture and assessment of mitogenic properties of phenytoin. The mitogenic activity of phenytoin in vitro was assessed by determination of [^{3}H]thymidine incor-

porated by the test cells according to the methods of Martin et al.[12]. Briefly, L-929 fibroblasts, 10,000/well, were seeded in 18-mm dia wells in Dulbecco's modified Eagle medium (DMEM) plus 2% fetal bovine serum (FBS) and antibiotics (100 units penicillin + 700 µg streptomycin + 25 µg fungizone/ml medium) and incubated at 37 °C in 5% CO2 and 95% air for 3 days. The cells were then fed with fresh DMEM plus 2% FBS, antibiotics and 10 µCi [^{3}H]thymidine/well (spec. act. = 88 Ci/mM). Groups of 5 wells each were treated with: phenytoin, 1.25, 2.5, 5, 10 or 20 µg/ml; or an equal volume (10 µl) of drug solvent (1:1:1 absolute ethanol: propylene glycol: 2.5 mM NaOH). After 3 days, the medium was removed, the dishes placed on ice, the cells washed once with 2 ml cold phosphate-buffered saline, and fixed for 10 min in 2 ml absolute methanol. The cells were then washed with distilled water and extracted twice (10 min each) with 1 ml cold 10% TCA. The residue was washed with 2 ml water and digested with 0.5 ml of 0.3 M NaOH for 20 min at 37 °C. A 0.2-ml aliquot of the digest was neutralized with an equal volume of 0.3 M HCl and placed in 10 ml of scintillation cocktail. The quantity of isotope in each assay was measured with a Beckman scintillation counter, the data being expressed as disintegrations per minute (DPM).

Assay for inositide phosphate formation. The ability of phenytoin to initiate hydrolysis of membrane PIP_2 was assessed by measuring the formation of IP_3, and its metabolic products, IP_2 and IP_1 according to the methods of Downes and Michell[13] as modified by Jamieson and Villereal[14]. L-929 fibroblasts were cultured in 100-mm dia plates in DMEM + 5% FBS and antibiotics as described above. Before confluence was reached, the FBS level was reduced to 2%, [^{3}H]myo-inositol (2 µCi/ml final concentration, specific activity 22.8 Ci/mM) added to each plate and cultured 48 h to label the membrane PIP_2. The cultures were then washed 3 times with sodium bicarbonate: HEPES buffered HBSS to remove the unbound tritiated inositol, incubated one additional hour at 37 °C in air, and given a final wash with HBSS.

The final wash was replaced by the assay incubation medium, fresh HBSS (pH 7.2) containing 10 mM LiCl and 1 µg/ml insulin (added 10 min prior to assay). Lithium chloride is utilized to inhibit IP phosphatase, and thus increase the assay products[10]. Duplicate (occasionally triplicate) plates received either varying concentrations of phenytoin, an equal volume of its solvent, or 25 nM bombesin. The plates were then incubated for 20 min at 37 °C, the medium removed and cellular activity halted by the addition of 4 ml of cold 10% TCA. The cells were extracted in the TCA for 15 min on ice, the extracts transferred to individual centrifuge tubes, the plates rinsed with an additional 2 ml of TCA and the combined extract washed 5 times with equal volumes of anhydrous diethyl ether. The aqueous layer was removed and neutralized with 26 mM sodium tetraborate and applied to AG1-8X anion exchange columns (2 ml bed volume). The columns were washed with 15 ml water, 10 ml 5 mM sodium tetraborate/60 mM ammonium formate and eluted sequentially with 15 ml 0.2 M, 0.4 M, and 1.0 M ammonium formate each in 0.1 M formic acid. In some cases all three inositol phosphates are eluted with 1.0 M formate buffer. The ability to separate the three inositol phosphates or to elute them simultaneously with the 1.0 M buffer was verified by use of [^{3}H]IP_3,[^{3}H]IP_2,and [^{3}H]IP_1 standards (spec. act. 1 Ci/mM each). Aliquots of the eluates, 0.7 ml, were added to 10 ml of scintillation cocktail and the radioactive label present measured in a scintillation counter, the data recorded as DPM. Each assay was performed 2 or more times. Due to variations in cell numbers and other conditions between assays, test data are expressed as the difference (D) between each value and the corresponding solvent control. Bombesin served as a positive control to verify the response of each assay.

Results and discussion

The increased uptake of [^{3}H]thymidine by phenytoin-treated L-929 fibroblasts indicate that phenytoin is capable of increasing the rate of DNA synthesis and thus, mitotic activity of these cells (fig. 1). The optimum dose level was 2.5–5 µg/ml of medium, while higher levels tended to suppress DNA synthesis. The peak effective dose range of 2.5 to 5.0 µg/ml confirms the observations of other workers[6–8]. The higher dose levels seemed to produce a cytotoxic effect since the 20 µg/ml level produced a significant lowering of [^{3}H]thymidine incorporation relative to the solvent-treated controls. Benveniste and Bitar[7] also reported a decreased incorporation of tritiated thymidine in cultured phenytoin-treated gingival fibroblasts at 10 µg/ml, although this level was not significantly below control, it was markedly lower than the peak value found with 5 µg/ml. Moy et al.[8] also noted a peak incorporation of tritiated thymidine in cultured hu-

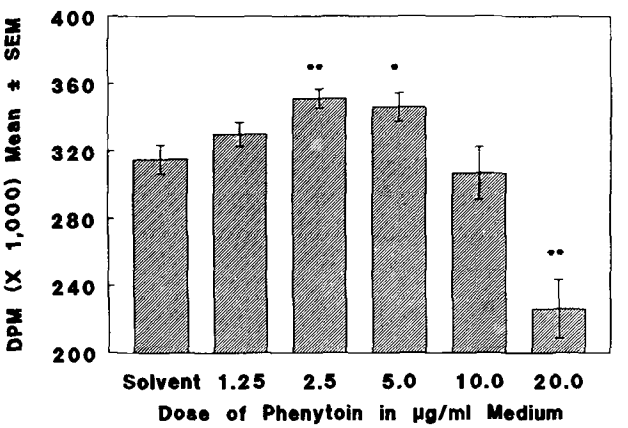

Figure 1. [^{3}H]Thymidine uptake in phenytoin-treated L-929 fibroblasts. For each group, N = 5. Compared with drug solvent control group, * = $p < 0.05$; ** = $p < 0.01$.

man skin fibroblasts treated with 5 µg phenytoin/ml medium, with below control values at 25 and 50 µg. The latter appeared to be a significant decrease, although statistical treatment was not provided. These data provide further evidence that the mitogenic effects of phenytoin are not limited to gingival fibroblasts.

Culture conditions markedly affect the mitogenic properties of phenytoin. Benveniste and Bitar[7] reported that phenytoin increased DNA synthesis in human gingival fibroblasts only when in the log growth phase. Recently Modeer et al.[15] reported increased cell numbers and DNA synthesis in phenytoin-treated gingival fibroblasts in culture at a dose of 20 µg/ml medium (serum free). The cause of the mitogenic effects of the higher dose of phenytoin seen by these authors is not clear.

The effect of bombesin (our positive control for the phosphoinositide turnover study) on tritiated thymidine uptake was also tested on L-929 fibroblasts under the same conditions as used for the mitogenic test of phenytoin. Six wells containing 25 nM bombesin were compared with six wells of untreated cells. Bombesin produced a 14% increase when compared with the controls, but this was not significant (498,861 ± 24,462 vs 431,220 ± 35,227 DPM, Means ± SE respectively). This weak response correlates with the small increase in IP formation produced by bombesin in the L-929 cells (fig. 2).

Interestingly, the level of phenytoin which was optimal for the stimulation of IP_3 formation (fig. 2) was within the same range as that required for increasing DNA synthesis in this fibroblast cell line. The 2.5 µg/ml dose level was effective in stimulating DNA synthesis, but ineffective for stimulation of IP_3, while the 5.0 µg level was effective in stimulating both processes. Higher dose levels tended to inhibit the formation of IP_3, IP_2 and IP_1 as was true for DNA synthesis. The reason for the lack of correlation of the two processes at the lowest dose level is not clear. The two cellular processes may not be related or there may be a difference in the sensitivity of the two assay procedures as used in our laboratory.

While hydrolysis of membrane PIP_2 has been shown to be associated with the mitogenic properties of some growth factors such as platelet-derived growth factor and other mitogens such as bombesin[16–19], these data do not indicate whether or not there is a cause and effect relationship between the two events seen in this study. Although this study does not provide definitive answers to such questions, it does open a new avenue of investigation for the study of mechanisms by which phenytoin stimulates connective tissue growth and potentially some of the other diverse effects this drug has on various cell types[20].

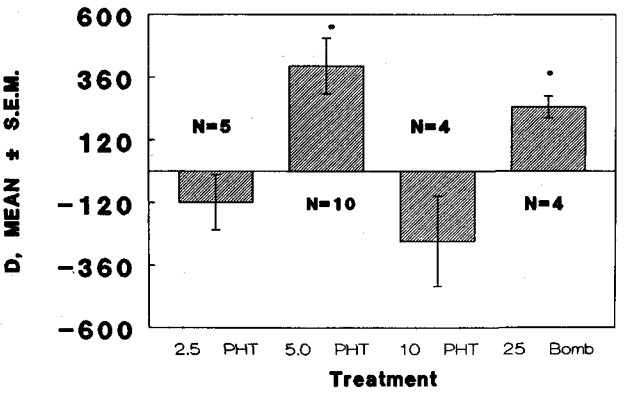

Figure 2. Formation of intracellular [^{3}H]inositol phosphate second messengers in L-929 fibroblasts treated with phenytoin (µg/ml) or bombesin (nM). D = individual difference between drug-treated cells and solvent-treated cells. Significance of those differences: * = $p < 0.05$.

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