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EVIDENCE THAT PROSTAGLANDINS DO NOT HAVE A ROLE IN THE CYTOSTATIC ACTION OF ANTI-INFLAMMATORY DRUGS

MARIA C. F. DE MELLO*, BARBARA M. BAYER and MICHAEL A. BEAVEN

Laboratory of Cellular Metabolism, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.

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Abstract—The following evidence suggests that the cytostatic action of nonsteroidal anti-inflammatory drugs is not due to inhibition of prostaglandin synthesis. First, measured cytostatic activity was not related to the reported ability of the drug to inhibit prostaglandin synthetase. Indomethacin, for example, inhibited growth of rat hepatoma (HTC) cell cultures only at concentrations in excess of those reported to be required to inhibit prostoglandin (PG) synthetases. Compounds reported to have no or weak prostoglandin synthetase-inhibitory activity, such as salicylic acid and phenacetin, were cytostatic, whereas congeners of phenylbutazone, antipyrine and aminopyrine, which have been reported to retain some synthetase inhibitory activity, were not cytostatic. Second, the prostaglandins and arachidonic acid did not reverse the effects of indomethacin and, in high concentrations, also inhibited growth. The actions differed in that the inhibition by indomethacin was slow in onset (24 hr) and reversible, whereas that by PGA_1 and PGA_2 was apparent within 60 min and was irreversible. Third, no detectable prostaglandin synthesis was observed with the HTC cell cultures. Indomethacin did interfere with the incorporation of labeled arachidonic acid into the phospholipid fraction of the cells, but this action was not shared by the other anti-inflammatory drugs.

In previous papers, we showed that anti-inflammatory drugs arrested the growth of rat hepatoma (HTC) and human fibroblast cultures in the G_1 phase of the cell cycle [1, 2]. The effect was reversible, and upon removal of drug the cultures resumed growth in synchrony to produce a surge and decay in DNA synthesis and, after 24 hr, a sharp increase in mitotic index and cell number [2]. Of particular interest was the fact that the order of potency of these drugs in producing these effects paralleled their reported ability to inhibit prostaglandin (PG) synthesis [3].

In this paper, the possible role of the prostaglandins in this phenomenon was examined by several experimental approaches. Drugs were selected and tested for their cytostatic activity, either because they were reported to be weak inhibitors of the prostaglandin synthetases or because they were closely related to the drugs tested previously. The effects of the prostaglandins on culture growth in the presence and absence of indomethacin and the effects of indomethacin on prostaglandin synthesis were also investigated.

MATERIALS AND METHODS

Materials. L-Leucine[4,5-^{3}H(N)], 5 Ci/mmole, was obtained from the New England Nuclear Corp., Boston, MA, and arachidonic acid[1-^{14}C], 50 mCi/mmole, from the Amersham/Searle Corp., Arlington Heights, IL.

The prostaglandins were a gift from Dr. John Pike, the Upjohn Co., Kalamazoo, MI, and arachidonic acid was purchased from the Sigma Chemical Co., St. Louis, MO. Antipyrine, aminopyrine and acetaminophen were purchased from the Sigma Chemical Co., the Aldrich Chemical Co., Inc., Milwaukee, WI, and the Eastman Kodak Corp., Rochester, NY, respectively. Other drugs were obtained from the sources described earlier [1]. The purity of the prostaglandins was checked by thin-layer chromatography (t.l.c.) by procedures described later.

Drugs were dissolved in Eagle's No. 2 medium supplemented with 10% fetal calf serum and neutralized by titration with 1 N NaOH. The prostaglandins were dissolved in absolute ethanol and added in volumes of up to 10 μl. The same volume of ethanol was added to control cultures. Media were prepared by the Media Unit, Division of Research Services, NIH. All solutions were sterilized by filtration.

Cell cultures. Rat hepatoma cell cultures were maintained as described earlier [2, 3]. Suspensions of confluent cultures were prepared and diluted 1:20 in Eagle's No. 2 medium [1]. The diluted culture was distributed into individual wells (1000 μl, ~30,000 cells/well) of Costar tissue culture cluster plates (16 mm diameter wells, Cat. No. 3524, Cambridge, MA). The plates were incubated in a National incubator (Heinicke Instr. Co., Hollywood, FL) at 37° under an atmosphere of 95% air and 5% CO_2 which was saturated with water. Under these conditions, the cultures entered exponential growth on day 2 and became confluent (1–1.2 × 10^6 cells/well) on day 5. The compounds or vehicle were added at the start or at later stages of culture growth as indicated in the text.

*. Present address: Instituto de Biofisica, UFRJ, Centro de Ciencias da Saude, Bloco G, Ilha do Fundao, Rio de Janeiro, R.J., Brazil.

Determination of cell counts. The cultures were washed twice with 1.0 ml of a Ca^{2+}- and Mg^{2+}-free Dulbecco's solution before the addition (0.2–1.0 ml) of a 0.025% (w/v) trypsin solution in Ca^{2+}- and Mg^{2+}-free Dulbecco's solution. The plates were incubated at room temperature, and when the cells had completely separated, soy bean trypsin inhibitor (0.15%, w/v) was added. The cell count was determined by use of a Neubauer counting chamber.

Measurement of [^{3}H]leucine incorporation. [^{3}H]Leucine (20 μCi/20 μl aliquot) was added to each well. The culture plates were incubated at 37° for 3 hr (unless stated otherwise), after which time the medium was removed and the cultures were rinsed twice with 0.5 ml of Locke's solution, once with 0.5 ml of ice-cold 10% trichloroacetic acid (TCA) solution, and finally with 1.0 ml ethanol. The TCA precipitate was dissolved in 0.2 ml Hyamine hydroxide (Packard Instrument Corp., Downers Grove, IL) and transferred to a glass scintillation vial. Each well was rinsed with 0.2 ml methanol. Radioactivity was assayed by liquid scintillation spectrometry (efficiency for ^{3}H, 23–25 per cent, and ^{14}C, 85 per cent).

Studies with arachidonic acid. Arachidonic acid[1-^{14}C] (50 nCi/20 μl aliquot) was added to each well on day 3 and the cultures were incubated for a further 20 hr. Aliquots (20 μl) of the medium were removed for assay of radioactivity and for chromatography. The medium from 72 wells (58×10^6 cells) was pooled and mixed with 40 μl of a mixture of unlabeled arachidonic acid and prostaglandins (100 μg of each) in ethanol. The medium was evaporated to dryness by lyophilization, and the residue was extracted three times with 10 ml of a mixture of ethyl acetate–isopropanol–0.2 N HCl (3:3:1). The extracts were pooled and evaporated just to dryness under a stream of N_2. This residue was dissolved in 100 μl ethanol for chromatography.

After removal of the medium, the cells were washed and treated with TCA, as described in the previous section. The TCA precipitate was washed twice with water (0.5 ml) and extracted with 1.0 ml ethanol. Aliquots (50 μl) of the ethanol extract were assayed for radioactivity, and the remainder was evaporated to dryness under a stream of N_2. The residue and medium were each mixed with 20 μl of an ethanolic solution of unlabeled prostaglandins and arachidonic acid (50 μg of each). Ten microliters of this mixture were spotted onto t.l.c. silica gel plates (Quanta gram precoated 5 × 20 cm plates, Quantum Industries, Fairfield, NJ), and the prostaglandins were separated by chromatography in a benzene–*p*-dioxane–glacial acetic acid (200:200:10 parts by volume) mixture [4]. The chromatograms were exposed to iodine to locate the prostaglandins and cut into segments, 2.5 mm wide, for the assay of radioactivity.

Identification of the arachidonic acid metabolite. One major metabolite of [^{14}C]-arachidonic acid was observed on the chromatogram. This compound was assumed to be a phospholipid on the following basis. The labeled metabolite was present exclusively in the particulate fraction of the cells and was precipitated by 0.4 N perchloric acid. It was readily extracted from the perchloric acid precipitate into ethanol. When chromatographed in the solvent system described above, the metabolite remained at the origin. Mild alkaline hydrolysis (0.5 M NaOH, in 75% methanol at 37° for 45 min), according to the procedure of Lands and Samuelsson [5], resulted in the dissappearance of the labeled metabolite and the reappearance of a material which migrated as arachidonic acid.

RESULTS

Lack of correlation between cytostatic activity and reported ability to inhibit prostaglandin synthetase activity. Phenylbutazone inhibited the growth of HTC cell cultures in a dose-dependent manner (ED_{50}, 0.37 mM), whereas two other pyrazolone drugs, antipyrine and aminopyrine, had little effect on culture growth in concentrations up to 4 mM (Fig. 1). Acetaminophen, like the parent compound phenacetin [3], inhibited growth completely in concentrations of 2.5–5 mM. As in previous studies [3], cell counts

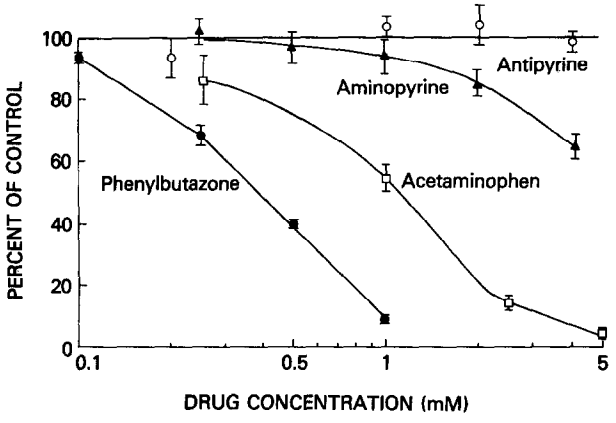

Fig. 1. Effects of phenylbutazone (●), acetaminophen (□), aminopyrine (▲) and antipyrine (○) on HTC culture growth. Cultures were grown in the presence of various concentrations of drugs added on day 2, and cell counts were determined on day 5. Values are means ± S.E.M. for six cultures and are expressed as a percent of the mean cell count (932,800 ± 61,000) of control cultures (no drug).

and [^{3}H]leucine incorporation into protein declined in parallel with increasing concentrations of drugs.

When the cytostatic activity and the reported ability to inhibit prostaglandin synthetases are compared (Table 1), it is evident that meclofenamic acid and indomethacin inhibit growth only at concentrations far exceeding those required to inhibit prostaglandin synthesis. Phenacetin and salicylic acid, which have little or no synthetase inhibitory activity, are cytostatic, whereas antipyrine and aminopyrine, which are weak inhibitors, are not cytostatic. Gentistic acid, the metabolite of salicylate, was reported by Flower *et al.* [6] to be inactive on dog spleen synthetase but to be more active than aspirin as an inhibitor of rabbit kidney prostaglandin synthetase [9]. This compound, however, has no cytostatic or anti-inflammatory activity (Table 1). There does appear to be a general correlation between the cytostatic and the anti-inflammatory activities of these drugs (Table 1).

Failure of the prostaglandins to reverse the effects of indomethacin. Culture growth, as measured by [^{3}H]leucine incorporation, was reduced when cultures were grown in the presence of indomethacin and was reduced further by the addition of PGE_1 (Fig. 2). PGE_1, PGE_2 and arachidonic acid were tested over a wide range of concentrations (0.01–100 μg/ml) and were found not to antagonize the effects of indomethacin and, in fact, inhibited culture growth (Fig. 3). All of the prostaglandins available to us inhibited growth to a varying extent, with those of the A and E series being the most potent in this respect (solid lines, Fig. 4). There was little difference in the curves for cultures containing prostaglandins and those containing indomethacin and prostaglandin when correction was made for the inhibition produced by indomethacin alone (dotted lines, Fig. 4).

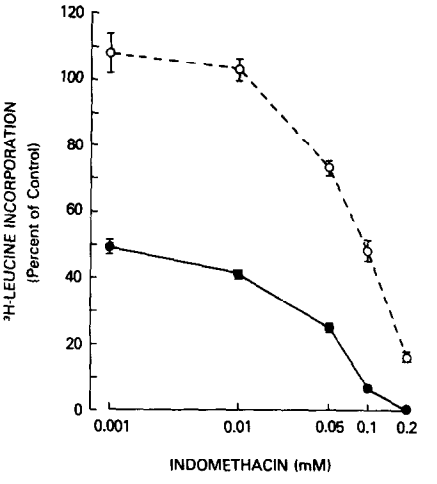

Fig. 2. Effects of various concentrations of indomethacin, alone (○——○) or in the presence of 12.5 μg/ml prostaglandin E_1 (●——●), on culture growth as measured by the incorporation of [^{3}H]leucine into protein. The drugs were added on day 0, and [^{3}H]leucine incorporation was determined on day 3. The values are calculated as a percent of the incorporation for control cultures (23,187 ± 782 cpm) and are the means ± S.E.M. for six cultures.

Thus, while indomethacin and the prostaglandins appear to inhibit growth in an additive fashion, their actions differed in several ways. The effects of

Table 1. Effectiveness of anti-inflammatory drugs as inhibitors of HTC cell growth, prostaglandin synthetase activity and carrageenan-induced edema of the rat paw*

	Cell growth (ED_{50}, μM)	Prostaglandin synthetase (ID_{50}, μM)					Rat paw edema (ED_{50}, μmoles/kg)	
Drug	Ref. 3	Ref. 6	Ref. 7	Ref. 8	Ref. 9	Ref. 10	Ref. 6	Ref. 8
Strongly cytostatic								
Meclofenamic acid	30	0.1			20	1.4	50	
Indomethacin	~140	0.2	0.5	2	30	4	18	6
Phenylbutazone	370†	7.2	12	56	1200	19	324	39
Cytostatic								
Phenacetin	1700			NA (100)				‡
Acetaminophen	1000†	660		NA (100)	3900	640	NA (1000)	‡
Salicylic acid	1100				>10,000	70,000		
Acetylsalicylic acid	1800	37	83	4	9000		830	420
Non cytostatic								
Aminopyrine	>4000†		91					
Antipyrine	NA†				4900§			
m-Hydroxybenzoic acid	9000	NA (700)					NA (1000)	
Gentisic acid	NA	NA (650)			Active ″		NA (970)	

* Drugs are compared in terms of ED_{50} (cell growth or edema inhibition) or ID_{50} (prostaglandin synthetase inhibition). The prostaglandin synthetase systems used were from dog spleen [6], sheep seminal vesicle [7], rat platelets [8], bovine seminal vesicle [9] and rabbit kidney [9]. When tested for ability to inhibit carrageenan-induced edema of the rat paw, drugs were administered p.o. NA, not active; highest concentration tested is in parentheses.

† Present work.

‡ Equal in potency to aspirin.

§ Ref. 10.

″ Reported [9] to be 32 times more active than salicylic acid and 50 per cent more active than aspirin.

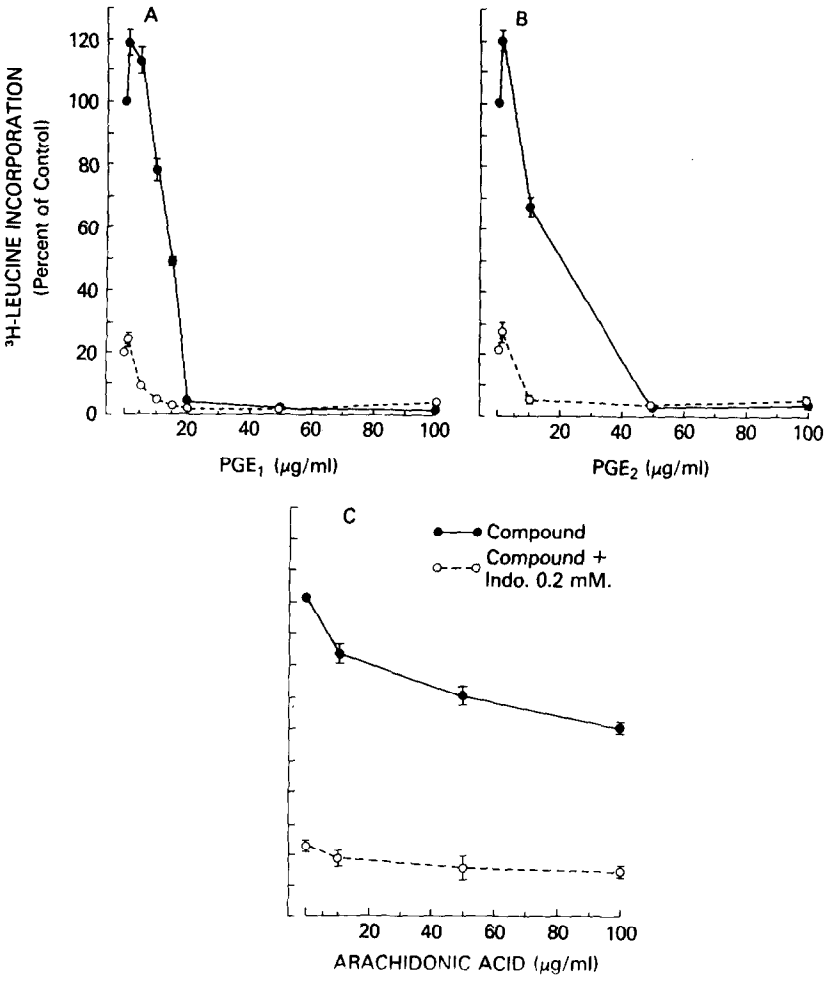

Fig. 3. Effects of different concentrations of prostaglandin E_1 and E_2 and arachidonic acid on growth of HTC cell cultures grown in the absence (●——●) or the presence (○- - -○) of 0.2 mM indomethacin. Drugs were added on day 0, and [^{3}H]leucine incorporation was determined on day 3. Values are expressed as a percent of that of control cultures (no drug) and are the means ± S.E.M. of six cultures. Additional experiments (data not shown) with prostaglandin E_1 and E_2 in concentrations of 0.01–1 μg/ml showed no significant difference from controls.

indomethacin and phenylbutazone, but not those of PGA_1 and PGE_2, were reversed by washing the cultures (Table 2). Complete repression of [^{3}H]leucine incorporation was noted within 3 hr upon addition of PGA_1 and PGA_2 but not of indomethacin or the other prostaglandins (Table 3). More detailed examination of the effects of PGA_1 and PGA_2 showed that the inhibition of [^{3}H]leucine incorporation was apparent within 45 min after the addition of the PG (Fig. 5) and that at this point washing of the cultures reversed the inhibition (Fig. 5, insert). These results indicate that the initial effect of PGA_1 and PGA_2 on [^{3}H]leucine incorporation was reversible but that prolonged exposure to the prostaglandins led to irreversible changes and cessation of culture growth.

Effects of the anti-inflammatory drugs on prostaglandin synthesis and arachidonic acid metabolism. Prostaglandins A, E and F were not detected in the culture medium by radioimmunoassay during days 1–5 of HTC growth*, and no labeled prostaglandins were detected in cultures incubated with [^{14}C]arachidonic acid (Fig. 6). By comparison, the presence of labeled prostaglandin E was apparent in cultures of rabbit renal interstitial cells (Fig. 6). The [^{14}C]arachidonic acid was slowly incorporated (62–70 per cent by 24 hr and 96 per cent by 48 hr) into a phospholipid fraction of the HTC cell, and free arachidonic acid could be recovered from this fraction by mild hydrolysis (Fig. 6). The incorporation of [^{14}C]arachidonic acid into the phospholipid was inhibited by indomethacin but not by phenylbutazone or sodium salicylate (Fig. 7).

* The assays were performed for us by R. Zussman, NHLBI.

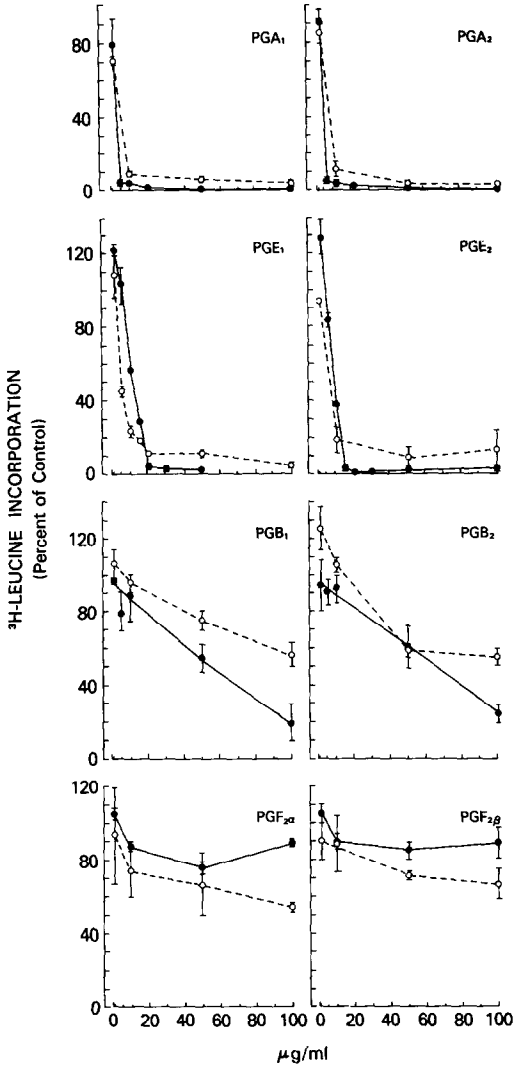

Fig. 4. Effects of the various prostaglandins on HTC cell cultures grown in the absence (●——●) or the presence of 0.2 mM indomethacin (○— — —○). The experimental design and graphical representation of data are identical to that shown in Fig. 2 except that the values for the indomethacin-containing cultures (○— — —○) are expressed as a percent of the incorporation observed in cultures containing indomethacin alone (5800 ± 520 cpm). Values are means ± S.E.M. of six cultures.

DISCUSSION

A variety of pharmacological actions of the anti-inflammatory drugs has been attributed to inhibition of prostaglandin synthesis [6, 9, 11, 12]. Our studies were designed specifically to see whether such inhibition might be responsible for the cytostatic properties of these drugs. The findings suggest otherwise, since we found that (1) the abilities of the drugs to inhibit the cell growth and the reported inhibition of prostaglandin synthetase are poorly correlated; (2) prostaglandin synthesis was not detected at any stage of growth of the HTC cell cultures; and (3)

Table 2. Reversibility of the effects of indomethacin and prostaglandins on HTC cell culture growth*

Addition	[³H]Leucine incorporated (% of control) Unwashed	Washed
Control (no drug)	100 ± 2	100 ± 3
Phenylbutazone (0.5 mM)	2 ± 0.2	83 ± 5
Indomethacin (0.2 mM)	11 ± 0.2	64 ± 5
PGA_1 (10 μg/ml)	0.1 ± 0.1	0.4 ± 0.4
PGE_2 (10 μg/ml)	14 ± 5	21 ± 6

* Values are means ± S.E.M. (N = 6). The amount of [³H]leucine incorporated in control cultures was for unwashed 82,800 ± 1800 and for washed 112,900 ± 3000 dis./min/well. Drugs were added at the start of culture growth. On day 3 of growth, the cultures were left unwashed or were washed twice with fresh medium. The cultures were reincubated for an additional 4 days before measurement of [³H]leucine incorporation.

exogenous prostaglandins did not reverse the effects of indomethacin. There is wide variability in the sensitivity of synthetase preparations, and since exogenous prostaglandins may not reproduce the effects of endogenous prostaglandins or of the unstable prostacyclin and endoperoxides, the lack of prostaglandin synthesis is perhaps the most compelling evidence against the involvement of prostaglandins.

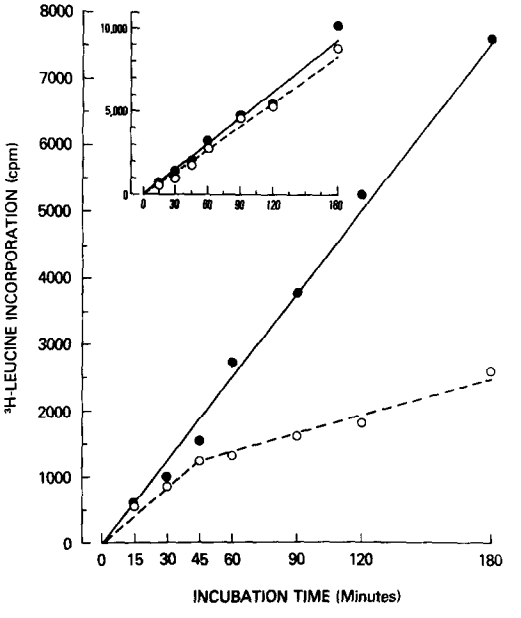

Fig. 5. Inhibition of [³H]leucine incorporation by prostaglandin A_1. Prostaglandin A_1, 10 μg/ml, and [³H]leucine (○— — —○) or [³H]leucine alone(●——●) were added to 3-day-old HTC cell cultures. An additional set of cultures was incubated for 45 min with 10 μg/μl PGA_1 and washed twice with fresh medium before the addition of [³H]leucine (insert). Cultures were removed at the indicated times and the label incorporated into protein was determined. Values are means ± S.E.M. for six cultures.

Table 3. Inhibition of [^{3}H]leucine incorporation by indomethacin and prostaglandins: short-term effects*

Prostaglandin	[^{3}H]Leucine incorporation	
	Without indomethacin (% of control)	With indomethacin (0.2 mM)
Control (no prostaglandin)	100 ± 2	68 ± 3
A_1	6 ± 1	2 ± 0.2
A_2	7 ± 1	5 ± 1
B_1	82 ± 2	52 ± 4
B_2	83 ± 2	36 ± 9
E_1	83 ± 2	68 ± 3
E_2	94 ± 3	58 ± 4
$F_{2\alpha}$	108 ± 8	60 ± 2

* Values are means ± S.E.M. (N = 6). Prostaglandins (50 μg/ml) alone or with indomethacin (0.02 mM) and [^{3}H]leucine were added simultaneously to 3-day-old cultures. The cultures were reincubated for 3 hr, and leucine incorporation was measured. The incorporation for control cultures was 9732 ± 203 cpm/culture.

Despite a wide range in the sensitivities of different tissue synthetases (Table 1), the concentration at which indomethacin inhibited growth was still 30–500 times that required to inhibit prostaglandin synthesis, and other synthetase inhibitors, such as aminopyrine [7] and gentisic acid [9], were not cytostatic even at high (4 mM) concentrations. Salicylic acid, on the other hand, which does not inhibit prostaglandin synthetase at levels up to 10–70 mM [9], partially inhibited culture growth in concentrations of 0.5–1.0 mM [3]. A striking example of the distinction between cytostatic and prostaglandin syn-

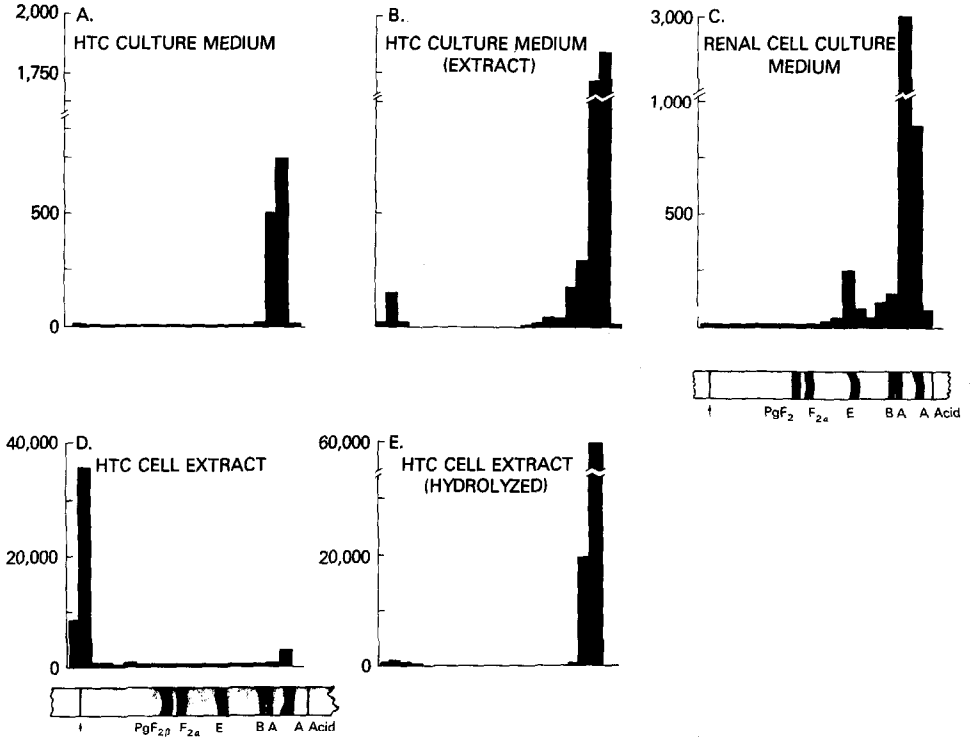

Fig. 6. Thin-layer chromatograms of culture medium (A) and extracts of medium from 72 cultures (58×10^6 cells) (B) after incubation of HTC cultures with [^{14}C]arachidonic acid. For comparison, chromatograms of culture medium from renal cell cultures treated similarly are shown (C). Also shown are chromatograms of extracts of the washed hepatoma cells before (D) and after (E) hydrolysis with alkali. Three-day cultures were incubated for 24 hr with [^{14}C]arachidonic acid. The medium and extracts were mixed with unlabeled standards and chromatographed on silica gel plates as described in Materials and Methods. Prostaglandin $F_{2\alpha}$ and $F_{2\beta}$ were separated in this system but not those of the A, B and E series. A acid = arachidonic acid.

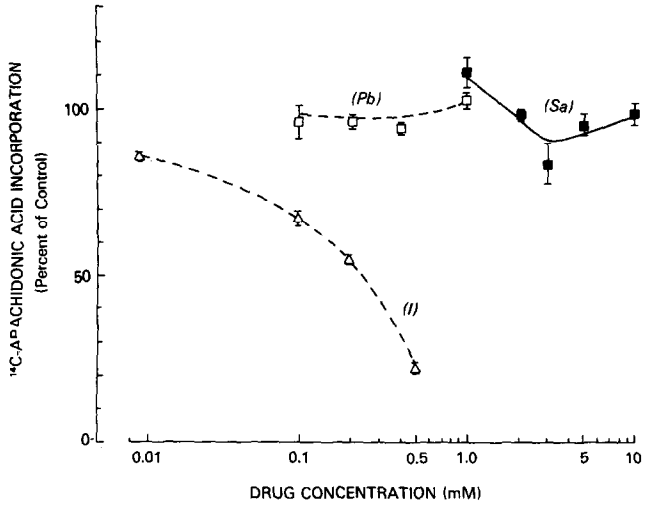

Fig. 7. Effects of indomethacin (△), phenylbutazone (□) and sodium salicylate (■) on the incorporation of [^{14}C]arachidonic acid into the phospholipid fraction of HTC cell cultures. The drugs and [^{14}C]arachidonic acid were added to 3-day-old cultures and the incorporation was measured after 24 hr. Values are means ± S.E.M. of six cultures.

thetase inhibitory activities was the difference between acetaminophen and antipyrine. Both drugs are equipotent inhibitors of bovine seminal vesicle prostaglandin synthetase [8], but only one, acetaminophen, inhibited culture growth. In contrast to the wide variation in the sensitivity of synthetase preparations, the cytostatic activity of the anti-inflammatory drugs shows no such variation; both transformed and non-transformed cell lines appeared to be equally sensitive to inhibition by these drugs (unpublished data).

There is no evidence that the prostaglandins have a general or essential role in the regulation of cell growth, but they do have a variety of effects on cell growth and differentiation. Those of the E series inhibit growth of several cell lines [13–18], although stimulation of cell proliferation by $PGE_{2\alpha}$ [19, 20], and in two cell lines by PGE_1 and PGE_2 [21, 22], has been reported. Some of these effects may be mediated through activation of a prostaglandin-sensitive adenylate cyclase system. Prostaglandin E_1, for example, suppresses transformation of blood lymphocytes [23] but induces transformation of thymic lymphocytes [24]. In neuroblastomas [12], activation of adenylate cyclase by prostaglandin E_1 precedes morphological differentiation. In another cell line, 3T3 mouse embryo fibroblast, prostaglandin E_1 potentiates the insulin-induced differentiation of this cell line into adipocytes but, at later stages, inhibits this reaction [25]. In high concentrations, however, the prostaglandins appear to be cytotoxic. Others have noted that inhibition of a mouse leukemic lymphoblast culture by prostaglandins E_1, E_2 and $F_{2\alpha}$ in concentrations of 25–100 μg/ml was accompanied by a significant reduction in numbers of viable cells within 1 hr [15]. In our studies, all of the prostaglandins, to a greater or lesser extent, inhibited culture growth, and inhibition was evident only with relatively high levels of the prostaglandins. The inhibition was irreversible and differed in several respects from that induced by the anti-inflammatory drugs. In the case of prostaglandins A_1 and A_2, an immediate effect on [^{3}H]leucine incorporation was observed, whereas the effects of indomethacin were apparent only after 1–2 days of culture growth. The inhibition by phenylbutazone, indomethacin (this paper) and salicylates [3] was reversed completely by washing the cultures, whereas growth did not resume upon removal of prostaglandin.

The arrest of cell growth is not in itself a toxic action of the anti-inflammatory drugs, and these drugs may be useful tools to study the cell cycle. Both transformed and non-transformed cells can be arrested for prolonged periods (8 days) of time without detriment to their viability or ability to resume replication once drug is removed [1]. After removal of drug, 98 per cent or more of the cells resume growth with a considerable degree of synchrony [2].

An unaccountable factor in our comparison of cytostatic action and reported inhibition of prostaglandin sythetase is that in tissue culture the anti-inflammatory drugs are partially bound to protein in the medium (53 per cent in the case of indomethacin). With human diploid fibroblast, a cell line that, unlike the HTC culture, is capable of proliferation in the presence of low serum concentrations, a 2-fold enhancement of the cytostatic activity of indomethacin was observed in the presence of the 2% fetal calf serum instead of the 10% fetal calf serum (unpublished data).

The anti-inflammatory drugs have, at high concentrations, a variety of biochemical actions, but because inhibition of prostaglandin synthesis is the one consistent finding with therapeutic concentrations of these drugs [26] the effects on prostaglandin synthesis are the most likely explanation for their anti-inflammatory properties. However, alternative mechanisms have been proposed [27, 28], and the present studies suggest that the cytostatic action of these drugs, which might be one component of their therapeutic action, is not attributable to inhibition of prostaglandin synthesis.

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