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Doxorubicin Interactions at the Membrane: Evidence for a Biphasic Modulation of Inositol Lipid Metabolism

Michael G. Thompson and John A. Hickman

Doxorubicin, when incubated for 30 minutes with [^{32}P]-labelled human erythrocyte membrane vesicles, produced an elevation of [^{32}P]inositol-1,4,5-trisphosphate levels. The maximum rise was obtained with 10^{-8} mol/l doxorubicin [132 (S.E. 13%) of control, $n = 6$, $P = 0.001$]. However, when the inositol lipids were examined, there was no evidence that doxorubicin stimulated the breakdown of [^{32}P]phosphatidylinositol-4,5-bisphosphate under resting conditions, suggesting that the elevated levels of [^{32}P]inositol 1,4,5-trisphosphate were not the result of the stimulation of phospholipase C. Instead, it was found that the dephosphorylation of inositol 1,4,5-trisphosphate by a 5′-phosphomonoesterase was partially inhibited by 10^{-8} mol/l doxorubicin so that the rise in [^{32}P]inositol 1,4,5-trisphosphate resulted from the inhibition of the breakdown of constitutively released [^{32}P] inositol 1,4,5-trisphosphate. Similar data was also obtained with another aminoglycoside antibiotic, neomycin. The release of [^{32}P] inositol 1,4,-bisphosphate and [^{32}P] inositol 1,4,5-trisphosphate and the breakdown of the inositol lipids in response to calcium (2.5×10^{-4} and 10^{-3} mol/l) stimulation was enhanced by doxorubicin (10^{-6} to 10^{-12} mol/l). These effects on resting and stimulated inositol lipid metabolism are discussed with reference to the paradoxical effects of doxorubicin to both stimulate and inhibit proliferation, according to concentration.
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INTRODUCTION

THE AMINOGLYCOSIDE antibiotic doxorubicin is a potent antitumour drug in humans yet its mechanism of action remains controversial. As an amphipath it is capable of physical interactions with DNA, the nuclear enzyme topoisomerase II and membranes [1–3]. Evidence that its cytotoxicity is expressed through a membrane-mediated effect comes from both biophysical and biochemical studies [4–6] but few reports have attempted to address the question of what consequences may ensue from a doxorubicin–membrane interaction which could account for cytotoxicity. Recently, Poseda *et al.* have suggested an attractive but as yet unsubstantiated hypothesis that a doxorubicin-mediated elevation of diacylglycerol, brought about by membrane receptor activation, could activate protein kinase C [7]. This idea implies that doxorubicin becomes enmeshed in growth factor-mediated events in the cell and it might therefore be expected that this would modulate the ability of the cells to traverse the G_1–S transition of the cell cycle. Most studies do

not support this expectation but instead show that doxorubicin brings about a cytostasis, prior to cell death, in the G_2–M phase [8, 9]. Indeed, it has been suggested recently that the drug inhibits a critical event in the steps leading to mitosis [10]. As a model appropriate for studies of the effects of doxorubicin on both membrane and cytoskeletal function we, and others, have studied its effects on the human erythrocyte membrane [11–15]. In this system, doxorubicin in the 1–10 μmol/l range rapidly inhibited calcium-induced morphological transitions [13] which are considered to be mediated by co-ordinated changes in the membrane and cytoskeleton [16]. Modulation of cell shape may be a mechanism whereby the drug interferes with events prior to cytokinesis [17]. Doxorubicin has a particular avidity for negatively-charged molecules [18] and we have previously reported that at concentrations which inhibited morphological transitions of human erythrocytes, the drug inhibited the metabolism of a singular pool of the highly charged polyphosphoinositol lipids [14]. This pooling of inositol lipids in erythrocytes has been described in detail elsewhere [19–22]. In the erythrocyte these lipids play an important role in membrane cytoskeletal interactions and in the control of cell shape [23, 24]. In proliferating cells the role of the lipid in modulating membrane–cytoskeletal interactions is not so well defined, and the metabolism of the inositol lipid phosphatidylinositol-4,5-bisphosphate ($PtdIns4,5P_2$) is largely understood for its role in generating a signal for the initiation of proliferation [25]. However, recent work has suggested $PtdIns4,5P_2$ plays a pivotal role in modulating the interaction of profilin, cofilin and destrin with actin [26, 27] and profilin with phospholipase C [28].

We wished to pursue studies on the effects of doxorubicin on inositol lipid metabolism, and we were intrigued by a recent report that low concentrations of doxorubicin (10^{-9} mol/l) stimulated the growth of a variety of cell types [29] and augmented the growth stimulus provided by platelet-derived growth factor and epidermal growth factor [7]. This suggests that at low concentrations, doxorubicin may stimulate inositol-lipid metabolism and we have again used the human erythrocyte membrane to examine any such effects since they relate to the modulation of changes in morphology. We report here that concentrations of doxorubicin (10^{-6}–10^{-8} mol/l) produce elevated levels of inositol-1,4,5-trisphosphate ($InsP_3$) in native human erythrocyte vesicles via a mechanism which does not involve $PtdIns4,5P_2$ breakdown, whereas when the membranes are treated with calcium, to stimulate phospholipase C action, these concentrations of doxorubicin augment $PtdIns4,5P_2$ breakdown. The results are discussed in the context of doxorubicin's known interactions with the membrane and cytoskeleton, and in the context of the effects of the drug on cell growth.

MATERIALS AND METHODS

Materials

Human blood was donated by healthy colleagues and used immediately. Doxorubicin and neomycin were purchased from Sigma. [^{32}P]-phosphate was purchased from Amersham (UK). All other chemicals and reagents were of analytical grade.

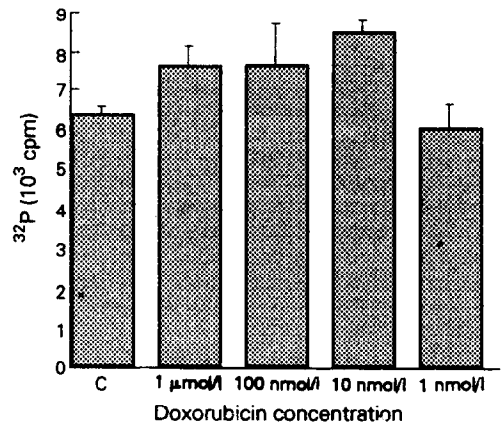

Fig. 1. Concentration-dependent elevation of [^{32}P]-inositol 1,4,5-trisphosphate in human erythrocyte vesicles by various concentrations of doxorubicin. Representative experiment, counts/minute of [^{32}P] inositol 1,4,5-trisphosphate, mean (S.D.) of duplicate determinations. C = controls.

[^{32}P] phosphate labelling of erythrocyte inositol-lipids

Whole blood (20 ml) was drawn from a normal healthy volunteer into a heparinised tube and centrifuged at 3000 *g* for 5 min. The supernatant and buffy coat were removed and the pelleted erythrocytes were washed once with 154 mmol/l NaCl in 1.5 mmol/l Hepes pH 7.2 and once in a citrate buffer comprising 5 mmol/l sodium pyruvate, 5 mmol/l inosine, 1 mmol/l adenine, 10 mmol/l glucose, 57.7 mmol/l trisodium citrate and 50 mmol/l Hepes, pH 7.2. 10 ml packed erythrocytes were then incubated with 10 ml citrate buffer containing 259 MBq [^{32}P]-phosphate at 37°C for 2 h as described previously [14].

Measurement of inositol phosphates and phosphoinositides from erythrocyte vesicles

Erythrocyte vesicles were produced by the lysis of ^{32}P-labelled erythrocytes, prepared as described above, in 200 ml 1 mmol/l EDTA in 20 mmol/l Tris, pH 7.4. The lysate was centrifuged at 15 000 *g* for 10 min and the pellet washed four times in this lysing solution until it was white, then given a final wash in 20 mmol/l Tris (pH 7.4) with no EDTA. The erythrocyte vesicles (0.2 ml, equivalent to approximately 3 mg protein/ml) were incubated at 37°C with or without doxorubicin or neomycin for up to 30 min. The preparation was then added to 8 ml ice-cold distilled water and centrifuged at 15 000 *g* for 10 min. Aliquots (7 ml) of the supernatant were removed and loaded on to anion-exchange columns of Dowex-1 (1 ml × 10, 200–400 mesh, formate form). The inositol-phosphates were separated by the method of Downes and Michell [30] and measured by Cerenkov counting. The phosphoinositides were extracted by treatment of the pellet obtained by centrifugation at 15 000 *g* for 10 min with 3.75 ml chloroform: methanol: 12 mol/l HCl (50:100:1) for 20 min; 1.25 ml chloroform and 1.25 ml 1 mol/l HCl were then added to separate the phases. The mixture was vortexed and centrifuged at 3000 *g* for 5 min, the upper phase was discarded and to the lower phase added the upper phase from chloroform: methanol: 1 mol/l HCl (1:1:0.9). After mixing and centrifugation as before, the upper phase was discarded and 1 ml portions of the lower phase, which contained the polyphosphoinositides, were subjected to mild alkaline hydroly-

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Table 1. The effect of doxorubicin on inositol lipids in human erythrocyte vesicles

Treatment	PtdInsP (cpm)	PtdInsP2 (cpm)
Control	8316 (461)	20 286 (842)
Doxorubicin (nmol/l)		
100	9019 (757)	20 764 (1177)
10	7214 (553)	22 437 (150)
1	7180 (617)	20 954 (1654)

Mean (S.D.).

sis by the method of Ellis *et al.* [31]. Analysis of the deacylated inositol-lipids was performed by the method of Downes and Michell [30].

RESULTS

Incubation of stringently washed plasma membranes with concentrations of doxorubicin (10^{-6}–10^{-8} mol/l) for 30 min produced an elevation of [^{32}P]-InsP$_3$ levels, whilst at 10^{-9} mol/l, levels reverted to those of the controls. The maximum effect was observed with 10^{-8} mol/l doxorubicin and was 132 (S.D. 13%) of control ($n = 6, P = 0.001$). Figure 1 shows a representative experiment with counts per minute; the mean increases from six experiments were significantly different from the controls at the $P = 0.05$ level except for 10^{-9} mol/l doxorubicin which was not significantly different from the controls.

We initially considered the possibility that [^{32}P] InsP$_3$ production was the result of phospholipase C-stimulated [^{32}P] PtdIns4,5P$_2$ breakdown. Recent work has shown that short chain alcohols stimulated phospholipase C through an interaction with a guanine nucleotide binding protein (G-protein) [32]. We have found that guanine nucleotides and aluminium fluoride produced a small elevation of [^{32}P]InsP$_3$ levels in human erythrocyte membranes (M.G.T. and J.A.H., unpublished) and we considered the possibility that doxorubicin might work in a similar manner to short chain alcohols, perhaps exerted via a physical perturbation of coupling (discussed below). However, when [^{32}P]PtdIns4,5P$_2$ and [^{32}P]InsP$_3$ were assayed in the same experiment, elevated levels of [^{32}P]InsP$_3$ were observed without a corresponding decrease in [^{32}P]PtdIns4,5P$_2$ (Table 1) suggesting that doxorubicin did not stimulate [^{32}P]PtdIns4,5P$_2$ breakdown under what might be termed "resting" conditions where phospholipase C was not activated above the level of constitutive breakdown. In addition, a time course of [^{32}P]InsP$_3$ levels over a 30 minute incubation period showed a continual fall that was partially inhibited by 10^{-8} mol/l doxorubicin (Fig. 2a). Similar data was also obtained with another aminoglycoside antibiotic, neomycin (Fig. 2b). The percentage fall after 10 minutes was 88.8 (S.D. 5.7%) for doxorubicin compared to 71.5 (5.1%) in the controls ($P < 0.05, n = 3$). The disappearance of [^{32}P]InsP$_3$ could have resulted from its phosphorylation to inositol-1,3,4,5-tetrakisphosphate (InsP$_4$) by a 3′-kinase or its dephosphorylation by a 5′-phosphomonoesterase to [^{32}P] inositol-1,4-bisphosphate (InsP$_2$) [30, 33]. There is as yet no evidence for a 3′-kinase in human erythrocyte vesicles [34] and the data suggest that the fall in [^{32}P]InsP$_3$, observed in the controls, was the result of its dephosphorylation by a membrane-bound 5′-phosphomonoesterase, as has been concluded by others [30, 33]. This conclusion was also supported by an observation of a corresponding rise in [^{32}P]InsP$_2$ and [^{32}P] inorganic phosphate (Pi) (data not shown).

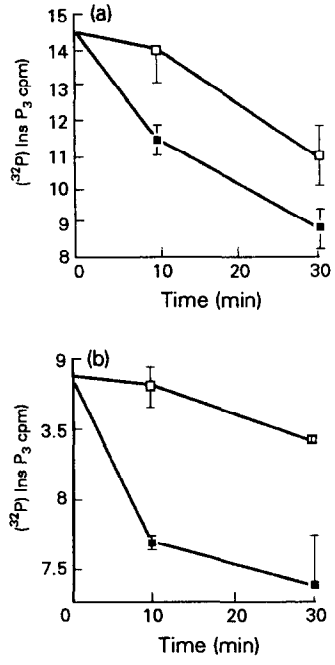

Fig. 2. Time course of [^{32}P] inositol 1,4,5-trisphosphate levels over a 30 min incubation period in human erythrocyte vesicles in the absence and presence of either (a) 10^{-8} mol/l doxorubicin (□) or (b) 10^{-8} mol/l neomycin (□). Control values (■).

We have previously shown that concentrations of doxorubicin in the millimolar range, which inhibited the morphological transition of very high cell densities of human erythrocytes (10^9/ml) also inhibited the breakdown of the inositol lipids [14]. These experiments had to be performed at a high cell density for the analysis of lipid levels, conditions which demanded a proportional increase in drug concentration in order to modulate changes in cell shape. Additionally, we showed, and confirm here, that this concentration of doxorubicin inhibited the calcium-induced release of inositol phosphates from membrane vesicles (Fig. 3). However, when the vesicles were incubated with lower concentrations of doxorubicin (10^{-6} to 10^{-12} mol/l),

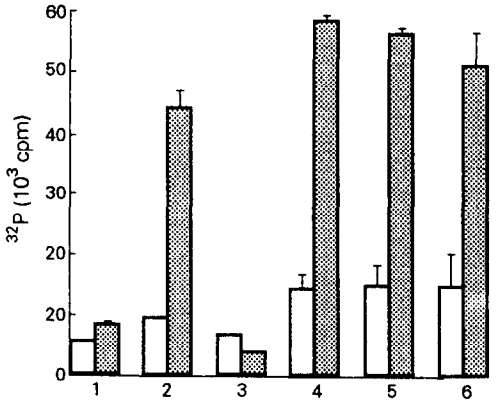

Fig. 3. Concentration-dependent effects of doxorubicin on 2.5×10^{-4} mol/l calcium-stimulated release of InsP$_2$ (□) or InsP$_3$ (▨) in human erythrocyte membrane vesicles over a 30 min period. Doxorubicin was added 10 min prior to the addition of calcium. 1 = controls, 2 = 2.5×10^{-4} mol/l Ca^{2+}, 3 = 1 mmol/l doxorubicin + 2.5×10^{-4} mol/l Ca^{2+}; 4 = 1 μmol/l doxorubicin + 2.5×10^{-4} mol/l Ca^{2+}; 5 = 1 nmmol/l doxorubicin + 2.5×10^{-4} mol/l Ca^{2+}; 6 = 1 pmol/l doxorubicin + 2.5×10^{-4} mol/l Ca^{2+}.

Table 2. The effect of doxorubicin on the breakdown of [^{32}P]-labelled inositol lipids stimulated by calcium and the release of inositol phosphates

	PtdIns4P	InsP2	PtdIns4, 5P2	InsP3	Total
Control	100	100	100	100	100
2.5 × 10^{-4} Ca^{2+}	69 (9.8)	82 (14)	70 (1)	458 (87)	99 (2)
1 mmol/l dox. + 2.5 × 10^{-4} Ca^{2+}	105 (18)	32 (14)	125 (16)	54 (8)	107 (2)
1 μmol/l dox. + 2.5 × 10^{-4} Ca^{2+}	63 (10)	164 (13)	64 (4)*	663 (118)†	108

Percentage changes (S.D.) from the controls. Student's *t* test for data obtained with Ca^{2+} alone compared to that with Ca^{2+} added after a 10 min incubation with 1 μmol/l doxorubicin: * $P < 0.05$, † $P < 0.01$ ($n = 5$).
dox. = doxorubicin.

the drug enhanced inositol phosphate release in response to calcium, suggesting that under conditions of the stimulation of phospholipase C, the activity of the enzyme was augmented (Fig. 3). This finding is supported by the observation that 10^{-6} mol/l doxorubicin enhanced inositol lipid breakdown in response to calcium (Table 2). Interestingly, at 10^{-3} mol/l doxorubicin not only was the breakdown of the lipids inhibited, in agreement with our previous findings [14], but the drug also appeared to increase the recovery of these lipids (Table 2), which suggests to us that the drug may be perturbing the distribution of the "pools" of the lipid. The ability of 10^{-6} mol/l doxorubicin to enhance inositol phosphate release, in response to 2.5 × 10^{-4} mol/l calcium, occurred in a time-dependent manner (Fig. 4) and was also observed when vesicles were stimulated by 10^{-3} mol/l calcium (data not shown).

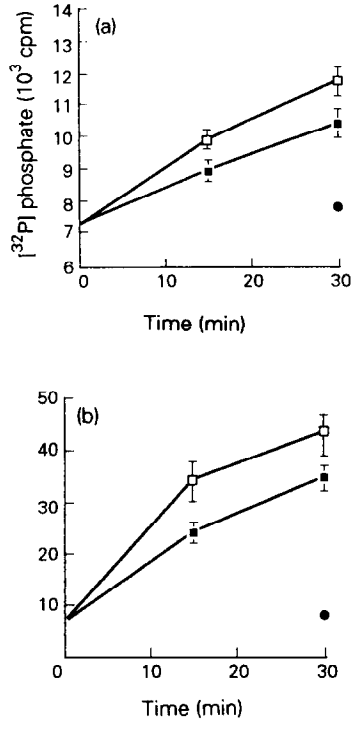

Fig. 4. Time course of effects of 10^{-6} mol/l doxorubicin on 2.5 × 10^{-4} mol/l calcium-stimulated release of (a) $InsP_2$ or (b) $InsP_3$ in human erythrocyte membrane vesicles over a 30 min period. □ = Doxorubicin + calcium, ■ = calcium alone, ● = control.

DISCUSSION

The plasma membrane is the site where many events controlling cell proliferation and differentiation, many of which are subverted in malignancy, are expressed [35]. Doxorubicin has been shown to both stimulate and inhibit cell growth and we have presented data in this paper which may provide an explanation for these paradoxical activities: the disruption of inositol lipid metabolism. The elevation of $InsP_3$ levels under both resting and activating conditions for phospholipase C provide a potential mechanism by which doxorubicin could induce cell growth (Figs 1 and 3), although, as pointed out earlier, the drug normally inhibits growth at the G_2/M phase of the cell cycle. PtdIns4,5P_2 modulates cytoskeletal interactions (see Introduction) and must therefore play an important role in the modulation of cell shape; the accumulated literature suggests that the presence of PtdIns4,5P_2 prevents the depolymerisation of actin. In the erythrocyte, there is strong evidence that PtdIns4,5P_2 controls cell morphology, making it an excellent model for studies of changes in cell shape. Changes in morphology have been suggested to play a pivotal role in the control of gene expression and proliferation in nucleated cells [17].

It remains to be determined what consequences our results have for the signalling role of the inositol phosphates in replicating cells. The magnitude of the changes observed in erythrocytes was small but may relate to the similarly modest effects observed when doxorubicin was used as a mitogen [29]. We have previously presented evidence that calcium levels did not change immediately after doxorubicin treatment of leukaemic cells at cytotoxic concentrations, but these experiments were not able to measure small, transient changes in intracellular calcium concentrations using fluorescent probes for calcium, because of the inherent fluorescence of doxorubicin [43]; nor did we measure the effects of very low concentrations of the drug. It was recently shown that treatment of mouse sarcoma cells with 5 × 10^{-6} mol/l doxorubicin led to a rapid elevation of diacylglycerol (DG) levels [7]. It was argued that its source might be either inositol lipids or phosphatidylcholine; the magnitude of the rise suggests that it was possibly elevated under conditions where there was an inhibition of DG kinase. Nevertheless, our experiments show that although doxorubicin had no effect on the inositol lipids *per se* in unstimulated erythrocyte membrane vesicles, in vesicles treated with calcium so as to activate phospholipase C we found low concentrations of the drug to enhance inositol lipid breakdown (Fig. 3). This would be expected to transiently elevate DG levels in addition to those of the inositol phosphates and, if it occurred in quiescent cells capable of growth, might be of a magnitude sufficient to stimulate mitosis.

Our data show that in the resting state doxorubicin elevated erythrocyte membrane vesicle $InsP_3$ levels (Fig. 1). There was no suggestion that PtdIns4,5P_2 breakdown was involved and since the human erythrocyte does not appear to be able to resynthesise PtdIns4,5P_2 after it has been cleaved by phospholipase C [36], the levels of the lipid could not have been replenished. Instead, doxorubicin appeared to produce elevated $InsP_3$ levels through inhibition of $InsP_3$ breakdown by a 5′-phosphomonoesterase (Fig. 2), the only known metabolic route for $InsP_3$ in human erythrocyte vesicles [30, 33].

In a previous study of the effect of doxorubicin on inositol

lipid metabolism, we reported that concentrations of the drug (10^{-4} to 10^{-3} mol/l) inhibited the shape change of dense populations of erythrocytes by preventing the breakdown of a pool of PtdIns4,5P_2 [14]. Doxorubicin, at similar concentrations, also inhibited the labelling of phosphatidylinositol- 4-phosphate (PtdIns4P) and PtdIns4,5P_2 with [^{32}P] phosphate (M.G.T. and J.A.H., unpublished). Another aminoglycoside antibiotic, neomycin, has also been suggested to bind to PtdIns4,5P_2 so as to prevent its labelling and breakdown [37–42]. The similarity in the effects of this aminoglycoside and doxorubicin suggests that the latter drug may also act in a similar manner, complexing with the inositol phosphates preventing InsP_3 degradation by the 5′-phosphomonoesterase, although as an amphipathic compound the actions of doxorubicin on cell membranes are likely to be more complex.

The mechanism whereby doxorubicin enhances calcium-stimulated phospholipase C activity, its function, and which pool of inositol lipid becomes its substrate are not known. Presumably, in erythrocytes it controls shape, and our data may explain why, in a previous study [13], we found low concentrations of the drug to augment the shape change stimulated by calcium. We have observed that the guanine nucleotide analogue guanosine 5′-O-(3′-thiotri-γ phosphate) (GTPγS) enhanced inositol phosphate release in erythrocyte vesicles (unpublished), and we consider it possible that the effect of doxorubicin may be mediated through a G-protein. Interestingly, neomycin has been shown to modulate GTPase activity in platelet membranes in a biphasic manner [44]. One of the central questions regarding the mechanism of receptor coupling to second messenger generators concerns the vectorial interplay between the components, including the G-proteins [45]. It has been shown that the tubulin of the cytoskeleton may modulate G-protein function [46]. The profound biophysical effects of doxorubicin, such as changes in membrane fluidity [5], and the interaction of anthracyclines with cytoskeletal components [11], including tubulin [47] may modulate these interactions so that, according to concentration, it may both stimulate or inhibit inositol lipid metabolism. We would argue that, at cytotoxic concentrations, the potential for effects on modulation of the cytoskeleton, because of changes in inositol lipid metabolism, may be more compatible with the imposition of a G_2/M block, as suggested by others [10].

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In vitro Influence of Gastrin, Oestradiol and Gonadotropin-releasing Hormone on HCT-15 and LoVo Human Colorectal Neoplastic Cell Proliferation

Robert Kiss, Isabelle Salmon, Olivier Pauwels, Stephan Gras, Andre Danguy, Chantal Etievant, Jean-Lambert Pasteels and Jean Martinez

We set up *in vitro* several human colorectal neoplastic cell lines that we labelled "hormone-sensitive" (HS) in comparison to the original cell lines which appeared to be rather "hormone-insensitive" (HI). We used LoVo and HCT-15 human colorectal neoplastic cell lines and studied the influence of 17β-oestradiol (E2), gastrin and two gonadotropin-releasing hormone (GnRH) analogues, HRF and buserelin, on the proliferation of the HS and HI variants of the LoVo and HCT-15 cell lines. Cell proliferation was evaluated by a colorimetric assay, the MTT test. Our results show that E2, gastrin, HRF and buserelin did not induce a significant stimulatory influence on the HI variants of the LoVo and HCT-15 cells, i.e. the cells that were cultured in a hormone-free 10% FCS-supplemented medium. In sharp contrast, the colorectal cells cultured for 30 passages in an E2 and/or gastrin + 1% FCS-supplemented medium showed a marked tropic response to E2, gastrin, HRF and buserelin. However, the HS variants of the HCT-15 cells appeared less sensitive to the two GnRH analogues than did the HS variants of the LoVo cells.
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INTRODUCTION

IT IS becoming more and more evident that the gastrointestinal tract is a target for various hormones from at least three different origins, i.e. gastrointestinal, gonadal and hypothalamo-pituitary tissues. Indeed, many gastrointestinal peptides including gastrin, secretin, cholecystokinin, glucagon, somatostatin and bombesin have been reported to affect the *in vivo* growth of the digestive mucosa [1]. Of these, the effect of gastrin has been widely reported as stimulating the growth of normal digestive mucosa [1–5]. Hoosein and colleagues [2] stated that such a gastrin-induced tropic effect included stimulation of RNA, protein and the DNA synthesis occurring in mammalian gastric and duodenal as well as colonic mucosa. Many experiments have also demonstrated that gastrin stimulates the growth of gastrointestinal tumours *in vitro* [1, 2, 6–9] as well as *in vivo* [7, 10–12].

Peters and coworkers [13] reported several lines of evidence indicating that reproductive factors may also play a role in the aetiology of colon cancer. For example, nulliparity has been associated with an increased risk of colon cancer, as have