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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.
Eur J Cancer, Vol. 27, No. 10, pp. 1268–1274, 1991.

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

hysterectomy and early menopause [13]. Harrison *et al.* [14] showed that physiological concentrations of oestradiol significantly stimulated the proliferation of one gastric and two colorectal neoplastic cell lines.

Bastie and colleagues [15] demonstrated that hypophysectomy affected the small intestine as well as gastric mucosa, with the hypophysectomy-induced cell diferentiation not being similarily affected at all levels of the digestive tract. Finally, several works proved that gonadoliberin (GnRH) influences *in vitro* the proliferation of human breast cancer cells, which are in general oestrogen-sensitive [16–19]. For this reason, we investigated whether not only gastrin and oestradiol but also GnRH might influence the proliferation of gastrointestinal neoplastic cells.

We describe the influence of gastrin, 17β-oestradiol (E2) and two GnRH analogues, HRF and buserelin, on the proliferation of two gastrointestinal cell lines: the HCT-15 colorectal adenocarcinoma [20] and the LoVo metastatic colorectal adenocarcinoma [21]. We set up hormone-sensitive (HS) and hormone-insensitive (HI) variants of both the cell lines. The proliferation level of gastrin, E2-, HRF- and buserelin-treated HI and HS variants of HCT-15 and LoVo cells was assessed by means of the MTT test, which is a simple colorimetric assay based on the ability of living cells to reduce a tetrazolium-based compound (MTT) to a blue formazan insoluble product [22, 23].

MATERIALS AND METHODS

Chemicals, cells and medium

Gastrin and 17β-oestradiol were purchased from Sigma (St Louis, Missouri). The two GnRH analogues HRF (Pro-His-Trp-Ser-Tyr-Leu-Arg-Pro-Gly-NH2) and buserelin (Gly-His-Trp-Tyr-Ser-D--Ser(tertButyl)-Leu-Arg-Pro-ethylamide) were kindly provided by Wyeth-Ayerst Belgium and Hoechst Belgium. The LoVo (CCL229) and HCT-15 (CCL225) cells were obtained from the American Type Culture Collection.

The cells were maintained as monolayers cultured at 37°C in closed Falcon plastic dishes (Becton Dickinson) containing Eagle's minimal essential medium (MEM) (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 0.6 mg/ml glutamine (Gibco) and a mixture of 200 IU/ml penicillin, 200 μg/ml streptomycin and 0.1 mg/ml gentamycin (Gibco). In our experiments FCS was heat-inactivated for 1 h at 56°C.

HS and HI variants of LoVo and HCT-15 cell lines

We set up eight variants of each of the two cell lines. For this purpose, the HCT-15 and LoVo cells were cultured in closed Falcon plastic dishes containing MEM plus either 10% (variant cells labelled HCT-15/10/CT, LoVo/10/CT) or 1% (HCT/1/CT, LoVo/1/CT) fetal calf serum without hormones. These four variants were further cultured without hormones or in MEM, to which either 10 nmol/l oestradiol (HCT-15/10/E2, HCT-15/1/E2, LoVo/10/E2, LoVo/1/E2), or 10 nmol/l gastrin (HCT-15/10/G, HCT-15/1/G, LoVo/10/G, LoVo/1/G), or both hormones, i.e. 10 nmol/l E2 + 10 nM gastrin (HCT-15/10/EG, HCT-15/1/EG, LoVo/10/EG, LoVo/1/EG) had been added. We thus set up 16 variant cell lines from the two original HCT-15 and LoVo cell lines. With respect to these 16 variant cell lines, the influence of the gastrin, E2, HRF and buserelin pulse on proliferation and differentation was assessed at the 30th passage (T30), i.e. after 30 weeks of long-term culture in MEM + 10% or 1% FCS, with or without E2, gastrin and E2 + gastrin.

Experimental schedule for cell proliferation assessments

Cell proliferation was assessed by means of the MTT assay, which was performed according to Carmichael [24], but with modifications. Briefly, after incubation of the cells for 72 h, the culture medium was removed and replaced with 100 μl MTT (Sigma) at 1 mg/ml RPMI medium (Seromed, Germany). The plates were incubated for 3 h at 37°C and then centrifuged for 7 min at 400 *g*. The medium was replaced with 100 μl dimethylsulphoxide (DMSO). The multiwells were shaken on a plate-shaker for 10 min; they were then read on a Bio-Tek Instrument Microplate Reader (EL 308), using a test wavelength of 570 nm and a reference wavelength of 630 nm (23).

The 16 variant cell lines were incubated in a hormone-free medium for 24 h to ensure good plating conditions. The control cells were incubated in a hormone-free medium during the whole experiment, while the hormone-treated cells were incubated in

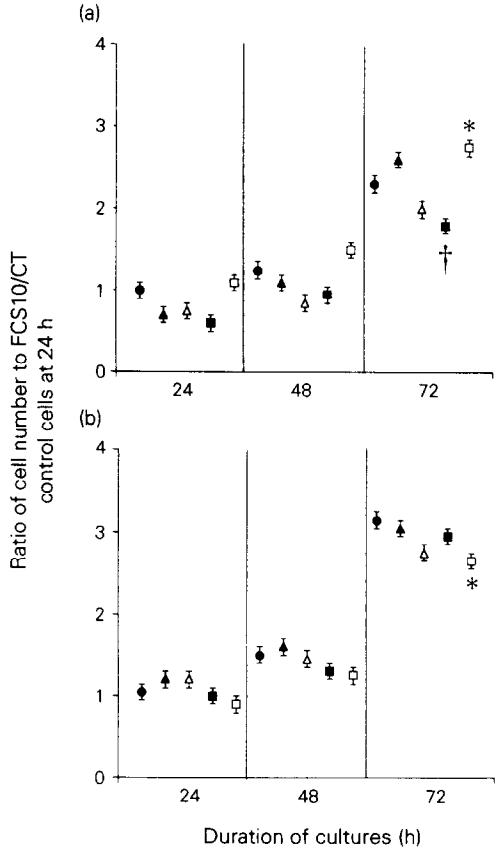

Fig. 1. Effect of 1 nmol/l (△) and 0.1 μmol/l (▲) 17β-oestradiol (E2) and 1 nmol/l (□) and 0.1 μmol/l (■) gastrin on the proliferation of human LoVo colorectal cancer cells. The control cells were cultured in a medium to which no pulses of E2 or gastrin were added (●). FCS10/CT cells were cultured for 30 passages in a medium supplemented with 10% FCS (a) while the FCS10/E2 + G cells were cultured for 30 passages in a 10% FCS medium supplemented with 10 nmol/l E2 + 10 nmol/l gastrin (b). * $P < 0.05$; † = $P < 0.01$; (Fisher *F* test).

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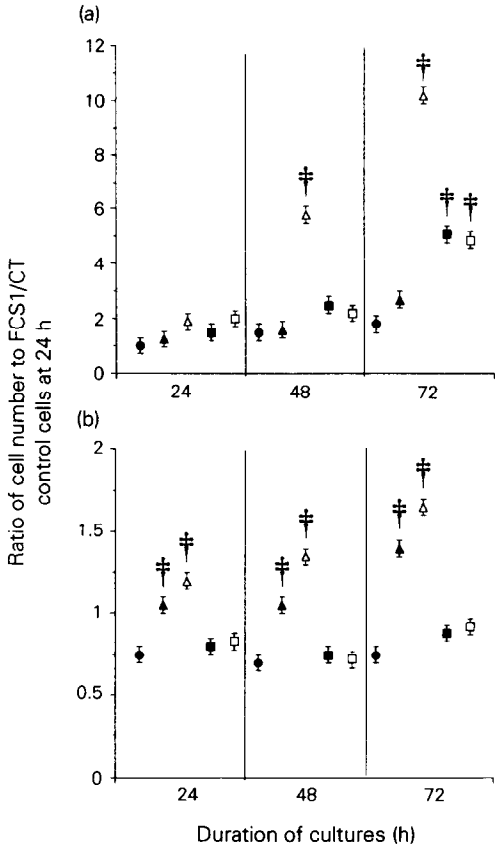

Fig. 2. FCS1/CT (a) and FCS1/E2 + G (b) LoVo cells cultured for 30 passages in a 1% FCS medium not supplemented with E2 and/or gastrin or supplemented with 10 nmol/l gastrin and 10 nmol/l E2. Symbols as Fig. 1. ‡= $P < 0.001$.

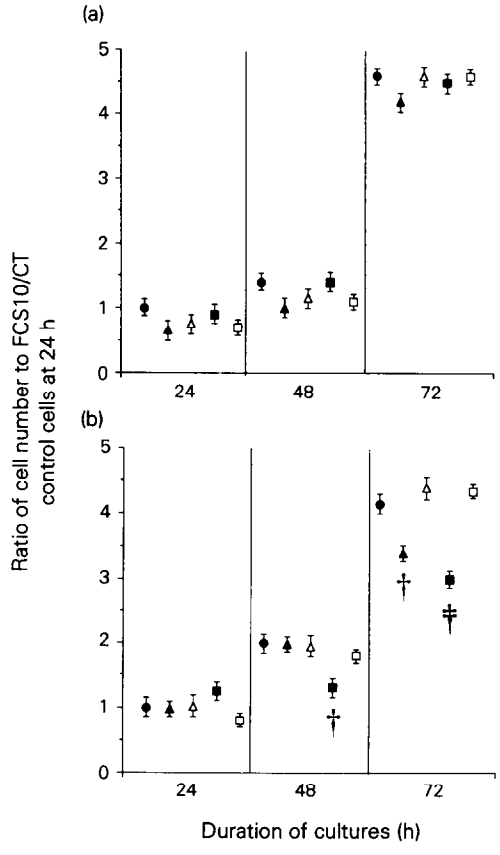

Fig. 3. Effect of 17β-oestradiol (E2) and gastrin on the proliferation of (a) FCS10/CT and (b) FCS10/E2+G HCT-15 colorectal cancer cells. Symbols as Fig. 1. * = $P < 0.05$; † = $P < 0.01$; ‡ = $P < 0.001$.

a medium containing either 1 nmol/l or 0.1 μmol/l of gastrin, or E2, or HRF or buserelin. All the assays were performed in sextuplicate after 72 h of culture in control or hormone-treated conditions.

Statistical analyses

Cell proliferation assessments were reported as mean (S.E.) statistically compared using the Fisher F test (one-way variance analysis).

RESULTS

Cell proliferation assessments relative to E2 and gastrin stimulation

Figures 1–4 illustrate the influence of a pulse of 17β-oestradiol (E2) and gastrin on four HCT-15 and four LoVo variant cell lines. Since the results obtained on HCT-15/EG and LoVo/EG variant cell lines were identical to those obtained on HCT-15/E, HCT-15/G, LoVo/G, and LoVo/E cells, we did not represent these latter in Figs 1–4 for the sake of clarity. We arbitrarily defined as 1 the quantity (number) of HCT-15 and LoVo cells living at 24 h in the control medium supplemented with 10% FCS (HCT-15/10/CT and LoVo/10/CT cells) or 1% FCS (HCT-15/1/CT and LoVo/1/CT). All optical density values assessed by the MTT test were expressed as a ratio compared to the reference value equal to 1 in the case of (1) the HCT-15/10/CT and the LoVo/10/CT cells and (2) the HCT-15/1/CT and the LoVo/1/CT cells living at 24 h: this made an easy comparison possible between all the experimental conditions.

The gastrin and E2-induced influences on the four LoVo cell variants from long-term cultures with the presence of 10% FCS, i.e. LoVo/10/CT (Fig. 1a), LoVo/10/G (data not shown), LoVo/10/E (data not shown), and LoVo/10/EG (Fig. 1b), had only a borderline statistical significance: no clear-cut hormone-induced response was observed. In sharp contrast, gastrin and 17β-oestradiol dramatically increased the proliferation of the LoVo cell variants from long-term 1% FCS cultures, i.e. LoVo/1/CT (Fig. 2a), LoVo/1/G (data not shown), LoVo/1/E (data not shown), and LoVo/1/EG (Fig. 2b). Furthermore, the LoVo cell variants from long-term cultures whose medium contained 1% FCS + hormone, i.e. gastrin (LoVo/1/G, data not shown), or oestradiol (LoVo/1/E, data not shown), or both hormones (LoVo/1/EG, Fig. 2b) responded differently to the gastrin or E2 pulse as compared to the LoVo cell variants from the long-term cultures whose medium contained only 1% FCS, e.g. not supplemented by hormone(s) (LoVo/1/CT, Fig. 2a). Whereas 0.1 μmol/l E2 did not influence the proliferation of the LoVo/1/CT cells (Fig. 2a) it markedly increased that of the LoVo/1/gastrin (data not shown) and the LoVo/1/EG cells (Fig. 2b), as did 1 nmol/l E2. Gastrin significantly stimulated the cell proliferation of both the LoVo/1/CT (Fig. 2a) and the LoVo/1/G (data not shown) cells, while it seemed to be without apparent effect on the LoVo/1/EG cells, whatever the dose used (Fig. 2b). The gastrin and E2-induced increase of the LoVo cell proliferation appeared earlier, i.e. at the 24th hour, in the LoVo/1/G (data not shown), the LoVo/1/E (data not shown), and the LoVo/1/EG (Fig. 2b) cells as compared to what occurred in the LoVo/1/CT cells (Fig. 2a). The present results indicate that the four LoVo

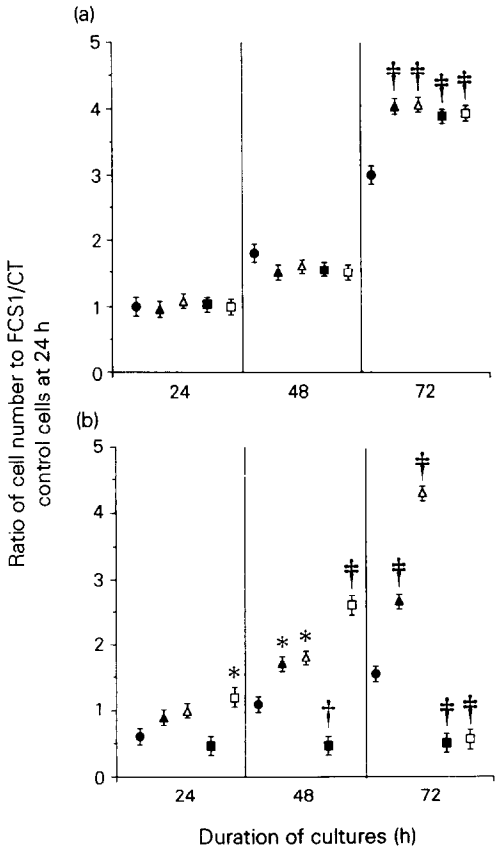

Fig. 4. Effect of 17β-oestradiol (E2) and gastrin on the proliferation of (a) FCS1/CT and (b) FCS1/E2+G HCT-15 cells. Symbols as Fig. 1.

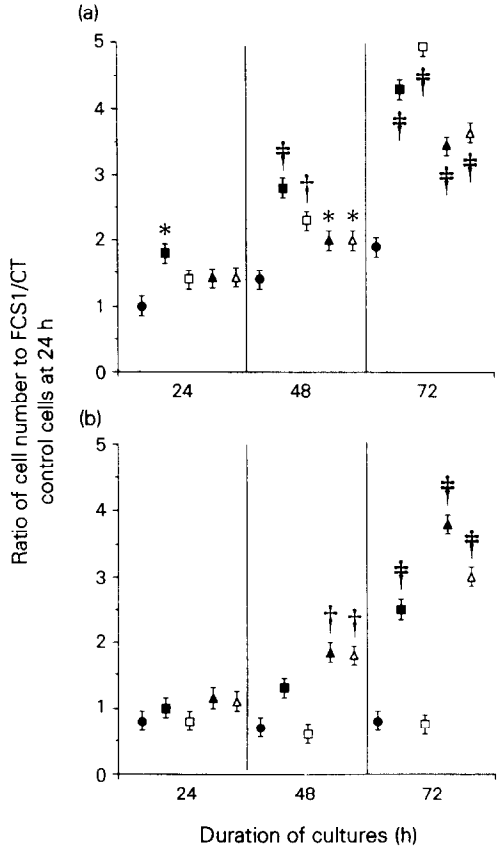

Fig. 5. The effect of two GnRH analogues, HRF at 1 nmol/l (□) and 0.1 μmol/l (■), and buserelin 1 nmol/l (△) and 0.1 μmol/l (▲), on the proliferation of human LoVo colorectal cancer cells. Control cells were cultured in an exogeneous GnRH-free medium (●). "FCS 1%/CT" cells were cultured for 30 passages in a medium supplemented with 1% FCS (a); "FCS 1%/E2 + G" variants for 30 passages in a medium supplemented with 1% FCS 10 nmol/l oestradiol and 10 nmol/l gastrin (b).

cell variants from long-term cultures with the presence of 10% FCS appeared significantly less hormone sensitive than those from the long term cultures with the presence of 1% FCS. We therefore labelled "hormone-insensitive" (HI) the 4 LoVo cell variants from the 10% FCS long-term cultures and "hormone-sensitive" (HS) the 4 LoVo cell variants from the 1% FCS long-term cultures.

Comparing the results observable in Figs 3a–4b to those reported in Figs 1a–2b, it appears that the E2 and gastrin-induced proliferative effects on the HCT-15 cells from the 1% FCS long-term cultures are distinct from those reported with respect to the LoVo cells. Both the 0.1 μmol/l E2 and the 0.1 μmol/l gastrin significantly inhibited HCT-15/1/G cell proliferation, while 1 nmol/l gastrin significantly stimulated it, with the 1 nmol/l E2 being without any apparent effect (data not shown). Whatever the dose used, i.e. 1 nmol/l and 0.1 μmol/l, oestradiol stimulated the HCT-15/1/EG cell proliferation in contrast to gastrin, which inhibited such proliferation (Fig. 4b). The four gastrin and E2-supplemented media significantly stimulated the HCT/1/CT cell proliferation (Fig. 4b). The results obtained on the HCT-15 cells from the 10% FCS long-term cultures (Figs 3a, b) were not far different from those obtained on the LoVo cells cultured in the same conditions (Figs 1a, b). In other words, we also labelled HI the HCT-15 cells from the 10% FCS long-term cultures and HS those from the 1% FCS long-term cultures.

The results reported in Figs 1–4 thus indicate that several factors seemed to modulate the gastrin and E2-induced proliferative effect exerted on HCT-15 and LoVo colorectal cancer cells. Firstly, the fact that the cells were cultured for many passages with the presence of 10% or 1% FCS renders them either "hormone-sensitive" or "hormone-insensitive". The control HCT-15 and the LoVo cells cultured for 30 passages in a 1% FCS-supplemented medium grew significantly more slowly as compared to the same control cells that were cultured in a 10% FCS-supplemented medium (Figs 1–4).

Secondly, the fact that the HCT-15 and LoVo cells were cultured for several passages in hormone-free or hormone-supplemented media also significantly influenced their response phenotype with respect to the E2- and gastrin-induced proliferative influence. Finally, the origin of the cells, i.e. HCT-15 and LoVo, also modulated the E2- and gastrin-induced response (Figs 1–4).

Cell proliferation assessments relative to GnRH analogue stimulation

The two GnRH analogues, HRF and buserelin, significantly stimulated the LoVo/1/CT cell proliferation at both 1 nmol/l and 0.1 μmol/l dosage (Fig. 5a), as was also the case with 0.1 μmol/l of both HRF and buserelin on the LoVo/1/EG cells (Fig. 5b). In contrast, only HRF stimulated the LoVo/1/EG cell proliferation at the 1 nmol/l dose (Fig. 6b). Comparing Figs 6a, b to Figs 5a, b, the HCT-15 cells appeared relatively insensitive to both HRF and buserelin according to their cell proliferation

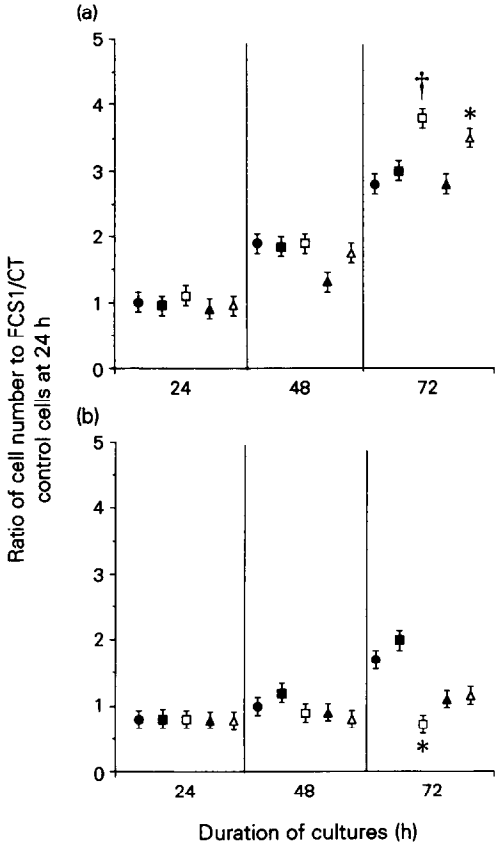

Fig. 6. The effect of HRF and buserelin on HCT-15 cells. Symbols as Fig. 5.

level, as opposed to the LoVo cells. The behaviour of the LoVo/1/G and the LoVo/1/E cells in HRF and buserelin-supplemented media was identical to that of the LoVo/1/EG cells (data not shown). The same features as reported here on the HCT-15/1/CT cells were observed with respect to the HRF and buserelin-induced effect on the HCT-15/1/G and the HCT-15/1/E cells (data not shown). We observed no statistically significant HRF- and buserelin-induced stimulation on the four LoVo and the four HCT-15 variant cell lines cultured for 30 passages in a 10% FCS-supplemented medium (data not shown).

DISCUSSION

The majority of patients with colorectal cancers have serosal penetration and nodal involvement at the time of operation. However, as Upp and colleagues claimed [12], the treatment of colon cancer relies heavily upon surgical resection and no widely effective adjuvant therapy is presently available. Lamers and Jansen [5], Watson *et al.* [8] and Singh *et al.* [25] suggested that since gastrointestinal hormones are known to influence the growth of gastrointestinal cancer, the hormonotherapy of gastrointestinal tumours might represent a new treatment in the near future. A comparison must be therefore made with breast cancers which are successfully treated by endocrine manipulation when specific receptors for steroid hormones are present in the cancer [5].

Upp and colleagues [12] showed that stage C and D patients with gastric receptors (GR) of less than 10 fmol/mg protein died during the early period of follow-up, whereas all the stage C and D patients that they studied and who showed GR > 10 fmol/mg protein were alive during this period; in the same way, Dukes' A and B colon cancers showed a significantly greater proportion of high gastrin receptor content than did more advanced cancers [12]. These authors suggested that the GR content of colon cancers may have a prognostic significance and may identify a group of patients with colon cancer that may benefit from hormonal therapy with antigastrin drugs [12]. Since this may be an epiphenomenon, it should be emphasised that these are preliminary observations of uncertain significance. However, the understanding of the influence of hormones on colorectal cancers seems to be important with respect to such a potential treatment.

At the physiological level, Singh *et al.* [26] showed that the tropic effects of gastrin on MC-26 mouse colon cancers are mediated by the regulation and maintenance of gastrin receptors in the cancer cells. Accordingly, we set up several variant cell lines of the HCT-15 and the LoVo cells that we arbitrarily labelled "hormone-sensitive" (HS) and "hormone insensitive" (HI) according to whether they showed a significant tropic response to oestradiol and/or gastrin (HS cells) or no response (HI cells) at all to these hormones. It is clear that the HCT-15 and LoVo cells that we labelled HI might show sensitivity to other hormones not tested here, in the same way as various growth factors which have been demonstrated to regulate colorectal neoplastic cell growth [6]. Be that as it may, the HCT-15 and LoVo cells that we labelled HI showed no or only a very weak topic response to oestradiol, gastrin and GnRH analogues, whereas the cells labelled HS showed a marked tropic response to these hormones. Tropicity was assessed by means of the MTT assay which is a colorimetric assay indirectly assessing the number of live cells in the culture medium. Therefore, it does not make it possible to give numerical values to the changes in cell number with added hormones. However, it should be emphasised that we obtained a high correlation between the indirect tropicity assessment performed by means of the MTT assay as opposed to direct cell counting performed by means of digital Feulgen-stained nuclei analysis [27].

We cultured the LoVo and HCT-15 cells during 30 passages in E2 and/or gastrin supplemented media because we think that gastrin and/or oestradiol might induce the up-regulation of gastrin receptors on colorectal neoplastic cells. We are now performing binding experiments to investigate the level of GR on the HI and HS cell variants. Using long-term treatment with prolactin, we had previously applied an experimental schedule *in vivo* such to develop hormone-sensitive variants from hormone-insensitive mouse MXT mammary tumour [28].

Our results must be compared to certain work reported elsewhere that showed an absence of tropicity in colorectal cells with respect to gastrin. Indeed, although gastrin has been widely shown to stimulate the *in vitro* growth of colorectal cancers [1, 2, 6–9], Weinstock and Baldwin were unable to demonstrate any increase in cell numbers or ^{3}H-thymidine uptake in response to gastrin; this was true for several gastrointestinal neoplastic cell lines [29, 30]. We obtained the same results using the variants of the LoVo and HCT-15 cells that labelled HI. In the same way, Watson and coworkers [8] reported that two newly established adenocarcinoma cell lines showed a response to pentagastrin at passage 2 but not at passage 6; they concluded that long-established cell lines did not respond to pentagastrin and suggested that tropic responses to gastrin may be underestimated by using long-established tumour cell lines [8]. Our results clearly demonstrate that it is possible to restore significant tropic response to gastrin and/or oestradiol with respect to colorectal neoplastic cells that have apparently lost such a

physiological property. It must be remembered that this was done by culturing HI cells for 30 passages in E2 and/or gastrin-supplemented media, and that a significant hormone sensitivity appeared as early as the 15th passage (data not shown). Furthermore, the results showed that lowering the level of FCS in the culture medium from 10% to 1% also endowed the cells with a "hormone sensitive" phenotype.

We observed that whereas oestradiol significantly stimulated the growth of the HCT-15 and LoVo cell HS variants, it remained without any apparent clear-cut effect on HI variants. The level of the E2-induced tropic effect was relatively similar to that induced by gastrin.

Some gastrointestinal cancers had already been shown to be oestrogen receptor-positive in 10% to 50% of the cases, depending on the method of detection used [31].

Considering the results obtained with the GnRH analogues, this is the first time to our knowledge that a significant tropic effect of this hormone has been demonstrated on human colorectal neoplastic cells. We observed that the HS variants of the LoVo cells were significantly more sensitive in terms of tropicity to the two GnRH analogues under study than were the HS variants of the HCT-15 cells. We obtained no significant-RF or buserelin-induced effect on the proliferation of the HI variants of both the LoVo and the HCT-15 cells (data not shown).

With respect to breast cancers, Miller *et al.* [17], Blankenstein *et al.* [16] and Scambia *et al.* [19] demonstrated a tropic GnRH-induced response in breast cancer cells, although Eidne *et al.* [32] failed to demonstrate such a feature, arguing that the absence of a GnRH-induced effect might be related to "culture conditions". Our findings corroborate such observations. Furthermore, Giaccheti and colleagues [33] also showed that human breast cancer cells possess gastrin-releasing peptide-like receptors. We are now pursuing experiments to characterise the influence of GnRH analogues and antagonists on the HI and HS variants of the LoVo and the HCT-15 cells.

In conclusion, our results show that 17β-oestradiol, gastrin and GnRH analogues did not induce significant tropic response on the LoVo and HCT-15 human colorectal cancer cells cultured in a conventional medium supplemented with 10% fetal calf serum. We labelled such cells hormone-insensitive (HI). We set up hormone-sensitive (HS) variants of the HCT-15 and LoVo cells by culturing HI cells for 30 passages in a medium supplemented with 1% FCS only, and/or gastrin plus oestradiol. These HS cells showed a highly significant tropic response to oestradiol, gastrin and GnRH analogues. The basal proliferation rate of such HS cells was significantly lower than that of the HI cells, suggesting dedifferentiation of the former as compared to the latter.

The quantitation of cytodifferentiation is of fundamental importance to any study addressing the question of growth in transformed cell phenotype. We are therefore performing assays to study whether changes in proliferation are maintained in the cytodifferentiated status of the target cell. This would make it possible to obtain in a near future a meaningful interpretation of endocrine responsiveness of colon cancer cells to gonadal hormones such as oestradiol and gonadotropin-releasing hormone.

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Mechanisms of Acquired Resistance to Methotrexate in a Human Squamous Carcinoma Cell Line of the Head and Neck, Exposed to Different Treatment Schedules

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Mechanisms of acquired resistance to methotrexate (MTX) were evaluated in HNSCC-11B cells which were made resistant to methotrexate either by continuous (11B-MTX-C) or by pulse exposure (11B-MTX-P) to the drug. 11B-MTX-C cells were 91-fold resistant to methotrexate and 30-fold or 49-fold crossresistant to trimetrexate and 10-EdAM, respectively. Dihydrofolate reductase (DHFR) activity was increased 63-fold in 11B-MTX-C cells together with a decrease in [^{3}H]-methotrexate transport and folylpolyglutamate synthase (FPGS) activity (2.5-fold and 3.8-fold, respectively). Against two novel antifolates targetting enzymes other than DHFR, minor crossresistance was observed for ICI-198, 583, but full sensitivity was retained for DDATHF. 11B-MTX-P cells were 46-fold resistant to methotrexate and 47-fold crossresistant to ICI-198,583 in short-term drug exposure, but showed only minor changes in methotrexate sensitivity following prolonged drug exposure. The resistant phenotype in 11B-MTX-P cells were characterised by a 5.6-fold decrease in FPGS activity. These results suggest that different mechanisms of methotrexate resistance in HNSCC cells *in vitro* can be obtained dependent on the schedule of exposure to the drug.
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INTRODUCTION

SURGERY AND RADIOTHERAPY are the primary modalities of treatment in patients with squamous cell carcinomas of the head and neck (HNSCC) [1]. Chemotherapy is most commonly reserved for end-stage disease. Active drugs for the treatment of head and neck carcinomas include methotrexate, cisplatin, bleomycin and 5-fluorouracil (5-FU). The overall response rate to each of these four agents has ranged from 15% to 30% [2].

The antimetabolites remain among the most effective drugs in HNSCC, with methotrexate as its most used agent [3]. Although methotrexate is considered to be an "active" drug, only one third of patients will have an objective but transient response. This may be due to either inherent or acquired cellular resistance to methotrexate. The mechanisms by which neoplastic cells become resistant have been the subject of intense research efforts and a variety of such mechanisms have now been identified [4]. Four mechanisms of resistance to methotrexate have been extensively studied *in vitro* [5], and are of significant importance for resistance in HNSCC cell lines [6–9]. These mechanisms include: (a) increase of the intracellular level of dihydrofolate reductase (DHFR), the target enzyme of methotrexate, usually as a result of DHFR gene amplification; (b) alteration of DHFR, with decreased affinity for methotrexate; (c) decrease of methotrexate transport into the cell; and (d) decrease of intracellular polyglutamylation of methotrexate.

In all of these studies, however, the development of methotrexate resistance *in vitro* was induced by stepwise increasing concentrations of methotrexate to the cell culture. Recently Pizzorno *et al.* [10] have shown for leukaemia cells *in vitro* that pulse doses of methotrexate, in an attempt to mimic clinical