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Characterisation of a Cell Line (LCC-18) from a Cultured Human Neuroendocrine-differentiated Colonic Carcinoma

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A cell line (LCC-18) from a neuroendocrine colonic tumour was established. The tumour cells retained their endocrine characteristics through more than 100 passages and showed positive immunocytochemistry for synaptophysin, vasoactive intestinal polypeptide (VIP) and glucagon. The culture medium also contained VIP and glucagon, which indicates that mechanisms for release of some of the active peptides were preserved. Transplantation of LCC-18 tumour cells into nude rats resulted in tumour formation with similar endocrine characteristics. The c-*myc* gene was amplified which might have been a prerequisite for establishment of the cell line. The chromosomes in LCC-18 were studied by G-banding and C-banding. The cell line had a distinctive mode in the hypotriploid region, at S=61. The double minute (Dms) positive stemline karyotype showed numerical and structural aberrations more similar to findings in ordinary colonic adenocarcinomas than to observations in previously studied, pure intestinal neuroendocrine tumours. The Dms may be correlated with amplification of c-*myc*. LCC-18 may become valuable for studies of neuroendocrine differentiation, regulation of growth and production and release of hormones and for studies of drug effect.
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INTRODUCTION

NEUROENDOCRINE GUT tumours, i.e. carcinoids, are clinically and pathologically well characterised neoplasms with varying and only partly defined hormonal characteristics [1]. Depending on localisation and hormone production of the primary tumour and its metastases, the clinical symptoms include hypoglycaemia, recurrent gastric ulcers or the carcinoid syndrome [2]. Neuroendocrine tumours are classified according to the regulatory peptides or amines they produce or store. The gastrointestinal endocrine tumours are usually highly differentiated neoplasms, with a low rate of proliferation in *vivo*. Therefore, these tumours are difficult to culture and only a few cell lines have been reported [3, 4].

Here we describe a hindgut neuroendocrine-differentiated tumour cell line investigated by immunocytochemistry, light microscopy, cytogenetics, *in situ* hybridisation and blotting. These techniques allow characterisation for expression of endocrine features and oncogenes and for chromosomal aberrations.

PATIENTS AND METHODS

Case Report

The patient was a 27-year-old Australian man with a history of sarcoidosis. In December 1985 he was referred to the Royal Melbourne Hospital because of a 3-week history of fever, diarrhoea and vomiting. Examination revealed an upper abdominal mass. At laparatomy, a hard, irregular, 10 cm tumour infiltrating around the transverse colon was seen, and regional lymph nodes were enlarged. Histological examination showed an ulcerated, poorly differentiated intestinal epithelial tumour growing in solid sheets. In February 1986, the patient was reoperated because of recurrent tumour growth. Subsequent chemotherapy was unsuccessful. The patient died 4 months later in April 1986.

In vitro culture

Material from the untreated primary tumour was used to establish the cell line, which has been designated LCC-18. After disaggregation, the tumour cells were cultured in RPMI 1640 (Flow) supplemented with 10^{-8} mol/l hydrocortisone, 5 μg/ml bovine insulin, 10 μg/ml human transferin, 10^{-8} mol/l 17 β-oestradiol and 3×10^{-8} mol/l selenium (Hites medium) supplemented with 10% fetal calf serum (FCS) [5]. The cells were frozen after approximately 3 months in culture and sent to the Ludwig Institute for Cancer Research, Uppsala. The cells were further subcultured by either of the above culture technique or in flasks coated with epithelial cell matrix (ECM) (Kebo Lab, Spånga, Sweden) [6] and containing Hites medium supplemented with 10% FCS.

Transplantation experiments

10^6 cells per animal were transplanted subcutaneously in nude *RNu/rnu* rats and the animals were followed up for tumour growth for up to 4 months. Observed tumours were studied by conventional histopathology and immunocytochemistry. The transplantation experiments were approved by the local ethical committee.

Immunocytochemistry and silver impregnation

Immunocytochemistry and silver impregnation were done on paraffin-embedded tissue sections and on *in vitro* cultured

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tumour cells. The cells were harvested and resuspended in culture media at 1–2 × 10^5/ml, centrigued on glass slides in a Shandon cytospin 2 (at 280 g for 5 min) and fixed in 4% neutral buffered formalin for 10 min.

Cytospun cells and the paraffin sections, from the primary tumour and the transplanted cells were stained by the Grimelius silver nitrate method [7]. They were also stained immuncytochemically [8] with different antisera, neurofilament, cytokeratin, vaso-active intestinal polypeptide (VIP), somatostatin, pancreatic polypeptide (PP), polypeptide YY (PYY), insulin, gastrin and serotonin. The tissue sections were then exposed to the primary antiserum in optimal dilution overnight at room temperature followed by the ABC technique (Vector Laboratories, Burmingame, California). The reaction was visualised with 3-amino-9-ethyl-carbazol. Negative controls were obtained by omission of the primary antiserum and by use of A431 cells.

The concentration of neuron specific enolase (NSE) in cells was determined by radioimmunoassay (RIA) [9]. The levels of VIP and glucagon in culture medium were also analysed [10, 11].

Cytogenetic analysis

The cytogenetic studies were done in three identical preparations. Since the observations agreed, the findings are summarised below. The methods for G-banding and C-banding have reported [16]. The chromosomal nomenclature follows that of ISCN [17].

RNA extraction

Total RNA was extracted by the method of Auffray and Rougeon [12]. Cells were homogenised in buffer containing a 3 mol/l lithium chloride, 6 mol/l urea, 0.2% sodium dodecyl sulphate (SDS) and 1 μl/ml Antifoam A (Sigma), and kept on ice overnight. The following day the precipitate was centrifuged at 16 000 *g* for 20 min. The resulting pellet was dissolved in TES solution (10 mmol/l triethanolamine, pH 7.5, 1 mmol/l EDTA and 0.5% SDS) and extracted once with phenol followed by an extraction with chloroform and isoamylalcohol (24:1). The RNA was preeipitated with 0.1 volumes of 3 mol/l sodium acetate, pH 6 and 2.2 volumes of ethanol. Before electrophoresis the RNA was poly-$(A)^+$ enriched on a oligo-dT cellulose column (Pharmacia).

Northern blot analysis

RNA samples (10 μg per sample) were electrophoresed on an agarose gel containing formaldehyde and blotted onto a Hybond-C extra filter (Amersham). The filter was prehybridised in a buffer consisting of 20% formamide, 5 × sodium saline citrate (SSC) (1 × SSC:0.15 mol/l sodium chloride and 0.0125 mol/l trisodium citrate, pH 7.0), 5 × Denhardt's solution, 5 mmol/l Na_2HOP_4, 5 mmol/l NaH_2PO_4 0.1% SDS, and 200 μg/ml salmon sperm DNA, for 4 h at 42°C, followed by hybridisation for 8 h (in the same buffer as used in prehybridisation). During hybridisation, about 2 × 10^6 cpm of a ^{32}P labelled c-*myc* probe (a 1.2 kb *Pst*I fragment of RYC 7.4, obtained from Dr Carlo Croce) was added per ml of buffer. The probe was labelled with a multiprime labelling kit (Amersham). After hybridisation, the filters were washed in 2 × SSC and 0.5% SDS at 65°C, and if necessary also in 0.1 × SSC and 0.1% until a low background activity was obtained. Filters were put on film and kept at −70°C in the presence of intensifying screens (DuPont).

To quantify the amount of mRNA loaded per lane, the filter was hybridised to a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (pHcGAP3, obtained from Dr RayWu, Cornell University) [13] and to 1.9 kb *Bam*Hi fragment of the cDNA for human β-actin [14].

DNA extraction and Southern blot analysis

Cells were incubated at 37°C for 20 h in TE buffer (10 mmol/l Tris–HCl and 1 mmol/l EDTA, pH 7.5) containing proteinase K and SDS at a final concentration of 100 μg/ml and 1%, respectively. After incubation, the samples were extracted once with an equal volume of phenol, followed by two extractions with isobutanol and isopropanol (7:3). The DNA was precipitated with 2.2 volumes of ethanol. Samples of LCC-18 DNA and normal human macrophages (15 μl per sample) were digested with *Eco*RI, and size-fractionated on a 0.8% agarose gel. The Southern blotting and hybridisation procedure was the same as for the northern blot analysis.

In situ hybridisation

The cultured cells were resuspended in culture media at concentration of 10^6/ml spun on object slides in a Shandon Cytospin 2 at 280 *g* for 5 min and fixed in 4% paraformaldehyde for 1 min. *In situ* hybridisation was done as described [15]. ^{35}S-labelled RNA probes were transcribed *in vitro* from plasmid SP65 (Promega Biotec, Madison), containing the 1.5 kb *Cla*I and *Eco*RI fragment of cDNA for c-*myc* in antisense direction. The 1.9 kb *Bam*Hi fragment of the cDNA for human β-actin [14] was also subcloned in SP65 vector (Promega), and labelled in the same way. Labelled probe (10^6 cpm per section) was mixed with hybridisation buffer and added to the tissue sections on slides. Hybridised preparations were autoradiographed with NTB 2 nuclear track emulsion (Eastman Kodak) diluted 1:1 with distilled water. After exposure for 5 days at 4°C slides were developed in Dektol (Kodak) [15], and counter stained with Mayer's haematoxylin and eosin. Control slides included the following: (a) positive and negative cultured control cells for the probe to check the specificity: SCLC cell line U 2020 known to have amplified c-*myc* [15] and unstimulated human peripheral lymphocytes as negative controls; (b) an RNA probe transcribed with the plasmid (SP64 with the same insert ligated in sense direction), used to estimate the background; and (c) cell preparations checked for the presence of cellular mRNA by hybridising with the RNA probe for human β-actin.

RESULTS

Histopathology

In routine fixed and paraffin-embedded material, the primary tumour was originally diagnosed as a poorly differentiated, neuroendocrine colon carcinoma with neuroendocrine cells diffusely spread over the entire tumour. There were no criteria for a diagnosis of an adenocarcinoma with glandular or papillary structures containing neuroendocrine cells. The tumour had fairly uniform cells growing in sheets and nests (Fig. 1a). The Grimelius stain showed a weak positive reaction but immunoperoxidase staining was positive for VIP. The tumour was therefore classified as a neuroendocrine differentiated colon carcinoma.

The tumour cells transplanted in nude rat showed a subcutaneous palpable tumour after 8–10 weeks. The transplanted tumours had the same histopathological characteristics as the primary tumour and displayed uniform cells growing in small or larger nests mixed with necrotic tumour cells. No metastases were observed.

The transplanted tumours showed a positive reaction with

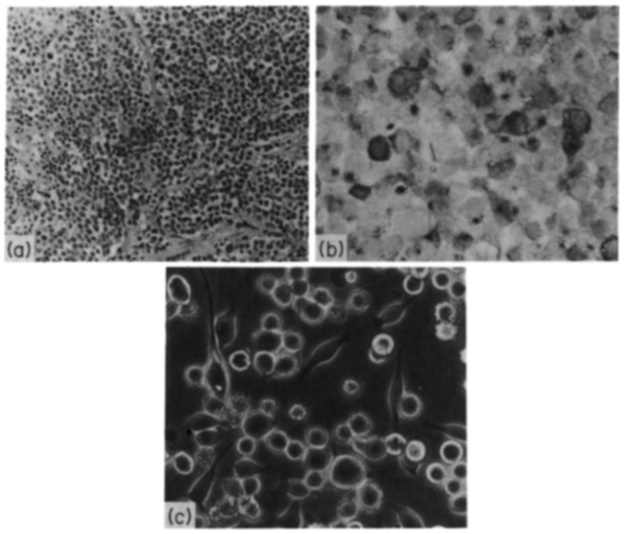

Fig. 1. Primary tumour, showing a poorly differentiated neuroendocrine colonic carcinoma. (a) Haematoxylin–eosin, × 200; (b) transplanted tumour cells positively stained with the Grimelius argyrophil silver technique, × 600; (c) cultured tumour cells growing singly cells or slightly aggregated, × 400.

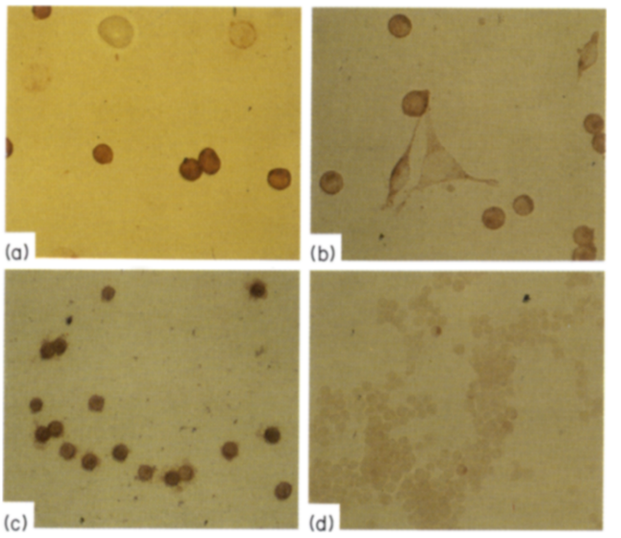

Fig. 2. Cultured cytospun cells demonstrating positive immunocytochemical staining against (a) tyrosine hydroxylase, (× 300); (b) synaptophysin (× 600); (c) L-dopa decarboxylase (× 600); (d) chromogranin A, with few cells positively stained (× 300).

Grimelius silver impregnation in about 15% of cells (Fig. 1b). Immunocytochemistry with different antisera revealed a positive reaction for neuroendocrine markers such as chromogranin A, L-dopa decarboxylase and synaptophysin. Positive immunoreactivity was also demonstrated for VIP and substance P in 10–20% of the tumour cells, with varying intensity of the staining. The other antisera were negative, and antisera tested on A431 cells showed no immunoreaction.

In vitro culture

After more than 150 passages *in vitro*, the tumour cells grow as round uniform single cells, floating free in the medium or loosely anchored to the bottom of the flasks (Fig. 1c). The cell dividing time was approximately 24 h, irrespective of culture method.

The Grimelius silver nitrate reaction revealed positive reaction on cytospun cells and immunocytochemical staining for neuroendocrine "markers" was positive for tyrosine hydroxylase (Fig. 2a), synaptophysin (Fig. 2b) and L-dopa decarboxylase (Fig. 2c). A weaker staining was noted for chromogranin A (Fig. 2d) [18–20] and NSE, but measurements of NSE from the cell pellet showed 0.998 μg NSE per mg protein, a concentration similar to that found in neuroblastoma [21].

VIP, substance P and glucagon were identified immunocytochemically with polyclonal antisera but the staining was not homogeneous, some cells demonstrating a strong reaction while others were only weakly positive or remained unstained. Glucagon was also detectable in culture medium from the cell line at a concentration of 30 pmol/l and extraction from the cell pellet revealed a high concentration of glucagon (260 pmol/l) and VIP (more than 100 pmol/l).

Cytogenetic findings

Figure 3 shows the stemline karyotype and the morphological characteristics of some recurrent marker types. The cell line had distinct mode in the hypotriploid region, at S=61. The spread

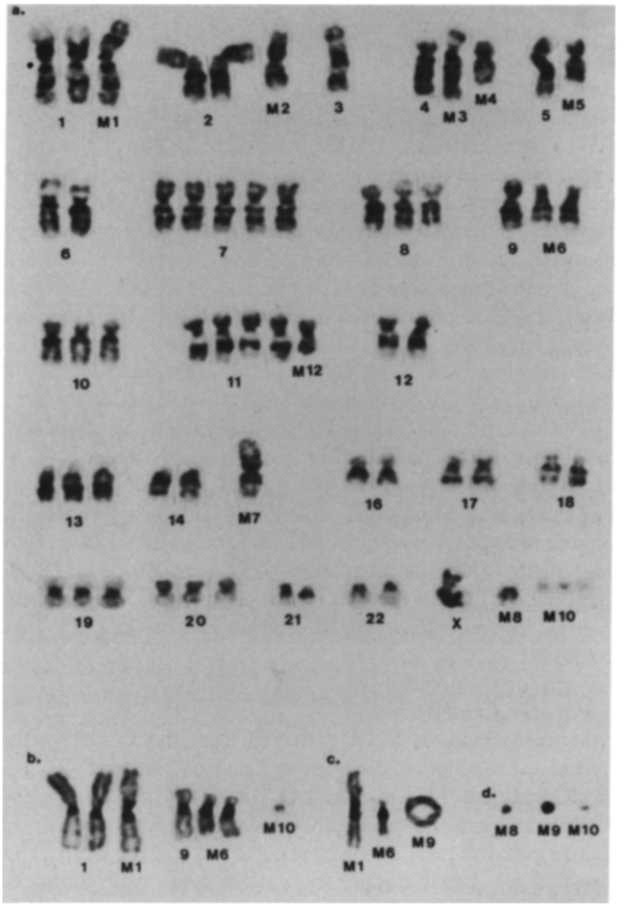

Fig. 3. (a) G-banded karyotype of modal cell in LCC-18; (b–d) C-banded partial karyotypes showing different marker types in modal-near-modal of LCC-18, × 3200.

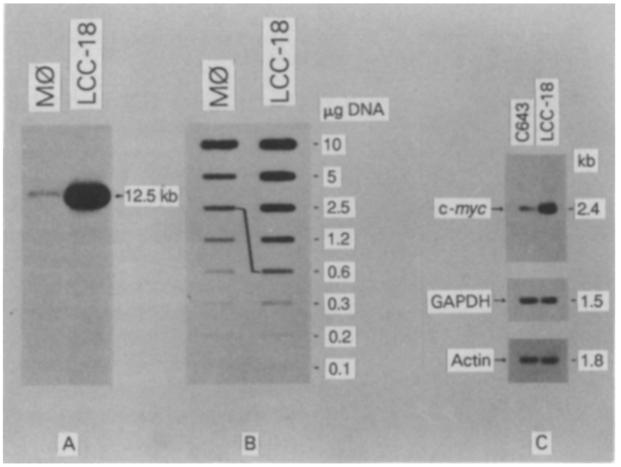

Fig. 4. Southern and northern blots of c-*myc* in LCC-18 cells. (a) Southern blot with a radiololabeled c-*myc* cDNA probe, (b) Gene copy number calculated in serial dilution of DNA from LCC-18 cells and normal macrophages. Bands of equal intensity are indicated. (c) Northern blot of c-*myc* mRNA expression with a ^{32}P-labelled c-*myc* cDNA probe. To assure that same amount of RNA was loaded in the two lanes, the filter was also hybridised to a GAPDH and an actin cDNA probe.

was restricted and mainly found on the negative side of the mode. Very few doubling products of modal cells were observed.

The stemline can be written as follows: 61, XO, −Y, +i(1q) (M1), +t(2p9q) (M2), t(4q17q) (M3), t(4p?15q) M4), del(5) (q22-23) (M5), +3×C7, +8, −9+2×del (9) (p13-21) (M6), +10, +2×C11, +13, i(15q) (M7), −15, +19, +20, +1-2 minute marker (M8), +0-2 ring chromosomes of varying sizes (M9), +1-5 double-minutes (DMS) (M10). The origin of the small minute markers of varying sizes as well as the derivation of the monocentric ring chromosomes could not be determined. However, it seemed likely that the minutes were products of breakage–fusion cycles affecting the ring chromosomes. 86% of the 50 cells studied contained markers of the specific type Dms. In most of the positive cells, there were only 1-3 Dms and usually only 1. As shown in Figure 3, these Dms were large. In addition there were a few cells with 10–30 Dms, but most of these were usually extremely small and at the detection limit.

The variant cells differed from the S-cells by numerical deviations, mainly losses, affecting the chromosomes 3, 4, 5, 11, 14 and 17. A few variant cells showed gains of chromosomes 2, 7 and 8. In addition some variant cells contained new, usually unique marker types: del(3) (p11) (one cell); t(3pDorGp) (one cell); del(5) (q12) (one cell); del(6) (p13) (one cell); del (11) (q22) (six cells) (M11); del (12) (p11) (one cell); t(12q15q) (one cell); t(16;?) (16pter > 16q24::?) (four cells; and del(X) (q25) (one cell).

Southern and northern blots

Southern blots of DNA extracted from the LCC-18 cells, hybridised to a human c-*myc* probe, showed a normal 12.5 Kb *Eco*RI fragment (Fig. 4a). The LCC-18 cells, however, appeared to have an increased gene copy number, which was confirmed in a quantitative comparison by serial dilution of the DNA. This revealed a 3–4-fold amplification of the c-*myc* gene compared with the copy number in normal human macrophages (Fig. 4b). To determine if the increase in gene number was reflected by an increased c-*myc* mRNA level, poly (A)$^+$ enriched RNA from LCC-18 cells were northern blotted. The LCC-18 cells showed a 3–4 times increased c-*myc* transcript level compared with an anaplastic thyroid carcinoma cell line known to have normal level of c-*myc* transcripts (N.E. Heldin, personal communication) (Fig. 4c).

In situ hybridisation

In situ RNA–RNA hybridisation with a probe complementary to c-*myc* on unstimulated LCC-18 cells showed that most of the cells expressed high mRNA level for c-*myc*, as judged by the number of grains precipitated per nucleus. Unstimulated control peripheral blood lymphocytes contained less than 1–2 grains per cell, which was considered to be the background level. LCC-18 mRNA did not hybridise with sense RNA probe, indicating that mRNA hybridised with antisense RNA probe was specific for c-*myc*. Hybridisation with β-actin probe showed that most of the cells expressed a high mRNA level, except for a few cells that contained a significantly lower level and which were probably damaged during preparation of the slides. LCC-18 cells hybridised with c-*myc* probe also contained such cells with a fewer number of grains.

DISCUSSION

Tumours of the colon and rectum include different types of malignancies: true adenocarcinomas, adenocarcinomas with some dispersed neuroendocrine cells, so called "small cell" undifferentiated carcinoma with neuroendocrine features and true hindgut carcinoids [22, 23]. The definitive diagnosis depends on special techniques such as electron microscopy, silver impregnation and immunocytochemistry. We used silver staining and immunocytochemistry to characterise the established cell line LCC-18. Ths, argyrophil silver impregnation was positive and immunocytochemical studies revealed tyrosine hydroxylase, synaptophysin, L-dopa decarboxylase and chromogranin A. Tumour cells transplanted to nude rats showed essentially the same neuroendocrine properties.

There are few reports about cell lines from neuroendocrine gut tumours [3, 4]. There are also few reports about human colorectal carcinoma cell lines with neuroendocrine features; however, most of these have not been fully characterised for neuroendocrine markers [22, 23]. Since there might be an overlap between colorectal adenocarcinoma, and low differentiated colorectal carcinoids, it is important to elucidate the true neuroendocrine potential of each cell line. Hindgut tumours, such as LCC-18, might contain peptides such as substance-P and glucagon. Furthermore, chromogranins, a family of glycoproteins found in most neuroendocrine cells [18] and also found in neuroendocrine tumours, were identified in our cell line. Immunocytochemical staining of sections from the transplanted tumours of LCC-18 demonstrated clones of well-differentiated endocrine cells with a positive reaction for all endocrine markers and other cells that stained negatively or weakly. It is thus possible that LCC-18 is derived from an intermediate type of tumour cells with the ability to differentiate both in an exocrine and endocrine manner (the endocrine cells with a capacity to synthesise and release peptides). Another possibility might be that some of the tumour cells lost their endocrine properties and storage capacity for peptides or amines during subculture. This has been reported for the cell lines RIN-r and RIN-m, which gradually decreased hormone production during culture [26].

There are few reports about chromosomal aberrations in gastrointestinal endocrine or neuroendocrine tumours. One

undifferentiated small cell carcinoma of the small intestine, probably a carcinoid showed in its pseudodiploid stemline, as the sole deviation, a reciprocal translocation: T(11;22) (q23q11) [27]. This specific structural change, which is characteristic for Ewing sarcomas, peripheral neuroepitheliomas and Askin tumours [27], was not found in any LCC-18 cell. Nor was this translocation observed in any cell of the other neuroendocrine tumour cell lines previously studied (COLO 320 and COLO 321) [30].

The most distinguishing karyotypic features of COLO 320 and COLO 321 were, particularly in early passages, the occurrence of numerous Dms; some were as large as the Dms seen in LCC-18. During subculture, homogeneously staining regions (such markers were not seen in LCC-18) developed and 14 to more than 20 different ordinary marker types occurred. None of these, however, was identical to any of the markers seen in S-cells or variant cells in the LCC-18. The most noteworthy features in LCC-18 were absolute or proportional losses of 1, 5 and D-group chromosomes and gains of E-group and F-group chromosomes. Only losses in group D and gains in group F were found in LCC-18.

The following similarities were found in our cell line and colorectal carcinoids [27, 29]: absolute or proportional losses of 13, 18 and of 1p and 17p; over-representation of 7, 13 and 20; common involvement of 1 and 17 in unbalanced structural rearrangements; and the occurrence of some colorectal carcinoid tumours with one or only a few Dms. Further studies are necessary to establish whether neuroendocrine differentiated carcinoma, at least of the colonic type, are really aberrate compared with ordinary adenocarcinomas.

C-*myc* encodes a DNA-binding protein and is expressed in several proliferating normal and neoplastic tissues. Our analysis of LCC-18 revealed a 3-4 fold amplification of c-*myc*, which was also reflected in similarly increased mRNA levels. We also observed Dms in the LCC-18 cells, and amplification of the c-*myc* gene has often been observed in tumour cell lines with Dms [25, 28]. Several studies of colorectal carcinomas have reported increased expression of c-*myc* [28–30]. However, this could not be ascribed to gene amplification but was proposed to be due to either an increased transcription rate or an increased mRNA stability. Our discrepant results are most likely related to the occurrence of Dms in most of the LCC-18 cells. This very specific chromosomal aberration and its equivalent homogeneously staining regions and abnormally banding regions are all known to correlate with DNA amplification, especially oncogene amplification [31]. Accordingly, the cytogenetic abnormality of Dms in LCC-18 might have been important for the establishment of this cell line.

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Neoadjuvant Chemotherapy in Operable Breast Cancer

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Primary chemotherapy in localised breast cancer may prevent tumour spread during surgical treatment and reduce proliferation of micrometastases. A randomised clinical trial, in 196 premenopausal and postmenopausal patients with operable (T2–3, N0–1b) breast cancer, was started in November 1983 at the Institut Curie to compare neoadjuvant and adjuvant regimens of chemotherapy with radiotherapy with or without surgery. The patients have been followed up for 35–70 months (median 54). A neoadjuvant group received two monthly cycles of intravenous doxorubicin/cyclophosphamide/5-fluorouracil before locoregional therapy and four cycles subsequently. Six monthly cycles following locoregional therapy were administered to the adjuvant group. Because of inclusion of postmenopausal and/or node-negative patients, compliance was less than optimal in 39 patients who were analysed separately according to actual dose received. Tumour response, evaluated after two cycles of neoadjuvant chemotherapy, was significantly associated with dose ($P = 0.003$). Survival showed a slight non-significant advantage for the neoadjuvant group. Survival plotted by actual dose was also similar. Neoadjuvant chemotherapy was safe and at least as effective as the adjuvant regimen. Patients have been accrued to a subsequent larger trial of chemotherapy as first-line treatment.
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INTRODUCTION

CHEMOTHERAPY AS a primary treatment in localised breast cancer has been advocated for a variety of theoretical reasons, based on both biological and clinical premises. Experimental systems suggest that non-curative reduction of tumour cell burden results in an increase in the proliferation of residual tumour cells, possibly due to the release of serum growth factors [1]. Clinical studies [2] have shown that cyclophosphamide, tamoxifen and radiotherapy, given prior to surgery, prevent an increase in metastatic tumour growth and prolong survival. According to the Goldie–Coldman hypothesis [3], the number of drug-resistant phenotypes generated by spontaneous mutation will increase concomittantly with tumour growth. The risk of generation and multiplication of resistant cells can therefore be minimised by initiating chemotherapy as soon as possible, thus preventing further cell proliferation.

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A randomised clinical trial involving 196 patients was initiated in November 1983 at the Institut Curie with the aim to compare neoadjuvant and adjuvant regimens in operable breast cancer. These patients have been followed up for 35–70 months (median 54) and the treatment efficacy as well as percentages of breast conservation have been evaluated.

PATIENTS AND METHODS

Patient presentation

Between November 1983 and March 1986, 196 patients were accrued in this study and randomised to receive either neoadjuvant ($n = 100$) or adjuvant (96) therapy. The criteria for inclusion were as follows: tumour size T2–T3, axillary nodes not involved clinically or involved but not adherent (N0, N1b) no prior cancer, no serious concomittant illness, and below 65 years of age. 15 patients were excluded due to errors of randomisation, poor patients' or physicians' complicance, or because treatment was at outside institutions. At the time this trial was conducted, the premise that surgically node-negative patients would benefit from adjuvant chemotherapy was not generally accepted and accordingly incomplete treatments (neoadjuvant) or treatment omissions (adjuvant) occurred not infrequently. For the purpose of this communication, patients were divided into four groups (Table 1) according to whether