Pergamon

European Journal of Cancer Vol. 31A, No. 2, pp. 222–229, 1995

0959-8049/95 $9.50+0.00

0959-8049(94)00434-X

Clonal Dominance Between Subpopulations of Mixed Small Cell Lung Cancer Xenografts Implanted Ectopically in Nude Mice

K. Aabo, L.L. Vindeløv and M. Spang-Thomsen

Clonal evolution of neoplastic cells during solid tumour growth leads to the emergence of new tumour cell subpopulations with diverging phenotypic characteristics which may alter the behaviour of a malignant disease. Cellular interaction was studied in mixed xenografts in nude mice and during *in vitro* growth of two sets of small cell lung cancer (SCLC) subpopulations (54A, 54B and NYH, NYH2). The tumour cell lines differed in cellular DNA content enabling flow cytometric DNA analysis (FCM) to be used to monitor changes in the fractional composition of the mixed cell populations. The progeny clone 54B was found to dominate the parent 54A clone when grown as mixed subcutaneous xenografts in nude mice, whereas no dominance was exerted during *in vitro* growth. The *in vivo* dominance could not be explained by differences in growth kinetics between the two tumour cell lines, and the interaction was not dependent on 54B being in excess in mixed tumours. The dominance was dependent on close *in vivo* contact as no remote effect on the growth of 54A was found when the dominating 54B cells were growing in the opposite flank of tumour-bearing mice. Irradiation inactivated 54B cells were unable to exert the dominating effect, indicating that the interaction required viable and proliferating cells. Clonal dominance was not found in mixed NYH-NYH2 tumours indicating that the dominance mechanism(s) may not always be operational between subpopulations in heterogeneous tumours. Recognition of interaction between tumour cell populations may result in a better understanding of the behaviour of heterogeneous human malignancies.

Key words: cell interactions, subpopulations, heterogeneity, clonal dominance, clonal interaction, small cell lung cancer, nude mice

Eur J Cancer, Vol. 31A, No. 2, pp. 222–229, 1995

INTRODUCTION

CLONAL EVOLUTION of genetically unstable neoplastic cells has been suggested to cause emergence of new tumour cell clones resulting in tumour cell heterogeneity (subpopulations) in malignant tumours [1]. Despite considerable interest during the past few years in the cellular heterogeneity of malignant tumours, expressed as phenotypic diversity among tumour cells [2–7], information is limited about cell–cell interaction among human tumour cell subpopulations in solid tumours.

Using phase microscopy to identify individual clones after disaggregation of mixed tumours followed by *in vitro* tumour cell growth, Leith and associates [8] have shown that equal mixtures of two subpopulations of a human colon tumour grown in nude mice were unstable until a stable equilibrium of a 1/9 composition was reached. Whether this change in cell composition in the mixed solid tumours was due to clonal interaction or simple growth kinetic differences between the two cell lines cannot be evaluated as no growth kinetic parameters were indicated in this study. Similarly, in a human breast carcinoma (MDA-MB-435), one single, genetically tagged clone was found to dominate a mixed tumour [9]. However, in this case the most likely explanation was growth kinetic differences between the clones as the dominating clone grew faster than the other clones. Using genetic tagging of a human renal cell carcinoma grown in nude mice, Staroselsky and coworkers [10] demonstrated that distinct clones dominated metastases of different organs, and concluded that clonal dominance was influenced by organ environment. Cellular interaction indicating clonal dominance has also been demonstrated in murine tumour systems [11–18].

Small cell lung cancer (SCLC) has been shown to be a heterogeneous tumour with respect to several phenotypical as well as genotypical characteristics [19], including cellular DNA content [20]. In order to evaluate the effect of cellular heterogeneity on tumour evolution in SCLC, we studied the changes in cellular composition in artificially mixed tumour xenografts of two sets of SCLC subpopulations (54A, 54B and NYH, NYH2) in nude mice and *in vitro*. Since the cell lines were distinguishable by differences in cellular DNA content, the changes in the proportions of the cell lines were monitored by flow cytometric

Correspondence to K. Aabo.
K. Aabo and M. Spang-Thomsen are at the University Institute of Pathological Anatomy, University of Copenhagen, Frederik V's Vej 11, DK-2100 Copenhagen, Denmark; and L.L. Vindeløv is at the Department of Haematology L, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.
Revised 3 Aug. 1994; accepted 5 Sep. 1994.

DNA analysis (FCM). We found that in one pair of subpopulations (54A, 54B), the progeny population (54B) was able to dominate the parent population (54A) when the cells were growing together in mixed solid tumours. The interaction required viable and proliferating cells. The interaction could not be demonstrated *in vitro*. No such interaction was demonstrated between another pair of SCLC subpopulations (NYH, NYH2).

MATERIALS AND METHODS

Cell lines

The tumour cell lines 54A and 54B were obtained as described elsewhere [21] by *in vitro* cloning of a cell line (CPH SCCL 054) established from an untreated SCLC. The cell lines differed in DNA content with DNA indices determined by FCM of 2.30 for 54A and 1.30 for 54B. The proliferation kinetics were similar for the two cell lines [22]. The cells were maintained in Eagle's minimal essential medium supplemented with 20% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

OC-NYH (NYH) was originally obtained from a previously untreated patient with SCLC as described elsewhere [23]. The DNA index was 1.30. During *in vitro* culture, a new population NYH2 with a DNA index of 1.15 emerged. The cell lines were maintained at 37°C in RPMI 1640 with 10% fetal calf serum in a humidified atmosphere with 7.5% CO_2 [24]. The cell lines were free of mycoplasma contamination.

Mice

Six-week-old specific pathogen free athymic nude mice of BALB/c background (Bomholtgaard Breeding and Research Centre, Ry, Denmark) were used as tumour-bearing hosts. The mice were kept under sterile conditions in laminar air flow benches with a room temperature of 25 ± 2°C and a relative humidity of 55 ± 5%. Sterile food and water were given *ad libitum*. For tumour maintenance, serial transplantation was performed under general anaesthesia with propanidid (Epontol®) by inserting tumour blocks of about 2 mm^3 subcutaneously (s.c.).

The 54A–54B experiments

In vivo *experiments*. Tumours weighing 1.5–2.0 g were excised and disaggregated into single cell suspensions using a combined mechanical–enzymatic procedure [25]. The fraction of viable cells was determined by the nigrosin dye exclusion test. Cell mixtures of 54A/54B were prepared in proportions of 1000/1, 100/1, 10/1, 1/1, 1/10, 1/100 and 1/1000. Samples of 10^6 cells from each mixture and controls were prepared for FCM. Aliquots of 0.1 ml (= 5×10^6 cells) were inoculated s.c. in the right flank of male nude mice, 2–4 mice per mixture. Six control animals were inoculated in both flanks with either 5×10^6 54A or 5×10^6 54B cells. After 3 weeks of tumour growth, fine-needle aspirations from the individual tumours were prepared for FCM. In order to ensure that the aspirate was representative for the entire tumour, the aspiration needle was moved in many directions under continuous aspiration. Aspirations were repeated 6 weeks after inoculation.

In a parallel experiment, 54B cells were implanted into the one flank (15 mice) and subsequently, when the 54B tumours had reached a size of approximately 5 mm, the 54A cells were implanted into the opposite flank in order to evaluate a possible remote effect of 54B on 54A. Animals implanted with only 54A tumour cells served as controls.

In order to evaluate the effect of radiation killed 54B cells on 54A cells in mixed solid tumours, a cell suspension of 54B was irradiated with a single X-ray dose of 180 Gy using a Stabilipan (Siemens) (dose rate: 5.32 Gy/min. ±2% at 300 kV and 8 mA). After irradiation, a 1/1 mixture of 54A and irradiated 54B was prepared (FCM: 49/51%). Of each cell line, 2×10^6 cells in 0.2 ml of culture medium were inoculated in both flanks of 10 animals. Non-irradiated 54B cells mixed with 54A cells served as controls (FCM: 49/51%). Approximately 2×10^6 irradiated 54B cells inoculated in both flanks of 10 animals served as documentation for the killing of the 54B cells by the irradiation. After 4 weeks of growth, fine needle aspirates were taken from the tumours for FCM.

In vitro *experiment*. 54A and 54B tumours were disaggregated, and triplets of 1/1 mixtures and unmixed 54A and 54B control cells were cultured as described. The initial total number of cells per culture was 2×10^6 in the mixtures and 10^6 in unmixed controls, respectively; 5 ml of medium were added to each culture twice weekly.

After 1 week of incubation, the cells were harvested by trypsinisation with Versene/trypure (1/1 solution). The cells were washed once in PBS (230 *g* for 5 min) and resuspended in culture medium. The total cell number per culture flask was determined using a haemocytometer and samples for FCM were prepared.

The NYH–NYH2 experiment

In vivo *experiment*. From *in vitro* cell cultures, NYH and NYH2 were harvested and a 1/1 mixture containing 5×10^7 cells per ml of each cell line was prepared. Aliquots of 0.2 ml (10^7 cells) were inoculated in both flanks of 15 animals. As controls, 5×10^6 cells of each cell line were inoculated in both flanks of 10 animals. After 4 weeks of tumour growth, fine-needle aspirations from the tumours were performed for FCM.

Measurement of tumour cell growth

After tumour take, the tumours were measured bidimensionally with callipers twice weekly. The products of the measurements (mm^2) were used to construct the growth curves. Using a computer program [26], the median tumour volume doubling times at a preselected tumour volume of 350 mm^3 were calculated.

In the 54A–54B *in vitro* experiment, the total number of harvested cells after 7 days of incubation served as a measure of the growth rate.

Flow cytometric DNA analysis

Samples from single cell suspensions (inoculates and *in vitro* cultures) and from fine-needle tumour aspirates were analysed by FCM using a FACS III (Becton Dickinson, Sunnyvale, California, U.S.A.) [27]. The proportion of each cell line was estimated by a maximum likelihood method [27]. The DNA index of the tumour cells was determined as the ratio of the DNA content of the tumour G_0+G_1 cells to that of human diploid cells by use of chicken and trout red blood cells as internal standards [28].

RESULTS

Tumour growth

The median tumour growth curves of the four cell lines are illustrated in Figure 1. No difference in growth between the 54A and the 54B tumours could be demonstrated. In the NYH system, the NYH tumours grew faster than the NYH2 tumours.

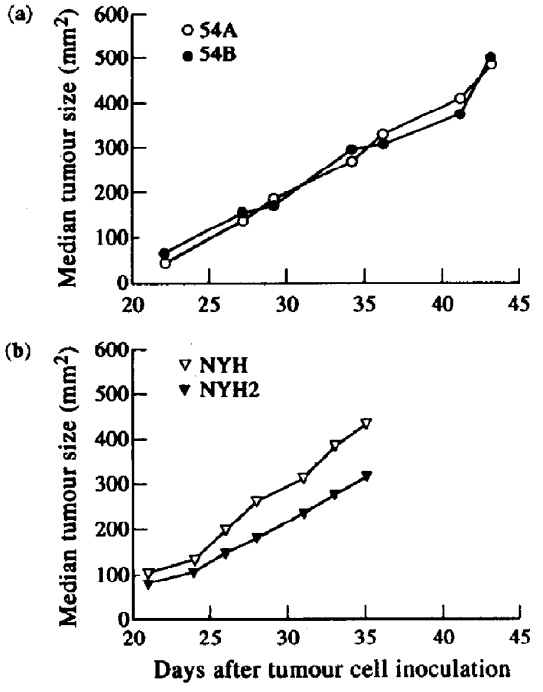

Figure 1. Median growth curves of 54A (n = 9) and 54B (n = 9) human SCLC tumours (a) and human SCLC NYH (n = 18) and NYH2 (n = 19) tumours (b) after inoculation of 5 × 10^6 cells s.c. in nude mice. The tumour size is expressed as the product of the measurements of two perpendicular diameters (mm²).

The calculated median tumour volume doubling times were 3.5 days for 54A, 3.8 days for 54B, 3.1 days for NYH and 4.0 days for NYH2.

Cell cycle distributions

From Table 1, it appears that 54A and 54B had nearly identical cell cycle distributions whereas a higher percentage of NYH2 cells were in G_0+G_1 phase and less in S and G_2+M phases compared with NYH.

The 54A–54B experiments

In vivo *experiment.* All but one mouse (54A/54B: 100/1) had tumour take. As illustrated in Figure 2, the fraction of 54B cells progressively increased, and the fraction of 54A cells decreased in all tumours. FCM of the intended 1000/1 mixture of 54A/54B showed only 54A cells as expected in view of the FCM detection limit of 2–5%. However, after 3 weeks of tumour growth, 54B cells appeared in the FCM histograms of the tumours, and after 6 weeks 54B cells were the predominant cell type in the tumours.

Table 1. Cell cycle distributions of two pairs of human small lung cancer cell lines (54A, 54B and NYH, NYH2) measured by flow cytometric DNA analysis of the inoculated tumour cells

Cell line	DNA index	Cell cycle distributions in %		
		G_0+G_1	S	G_2+M
54A	2.30	82	14	3
54B	1.30	83	13	4
NYH	1.30	57	38	5
NYH2	1.15	79	20	1

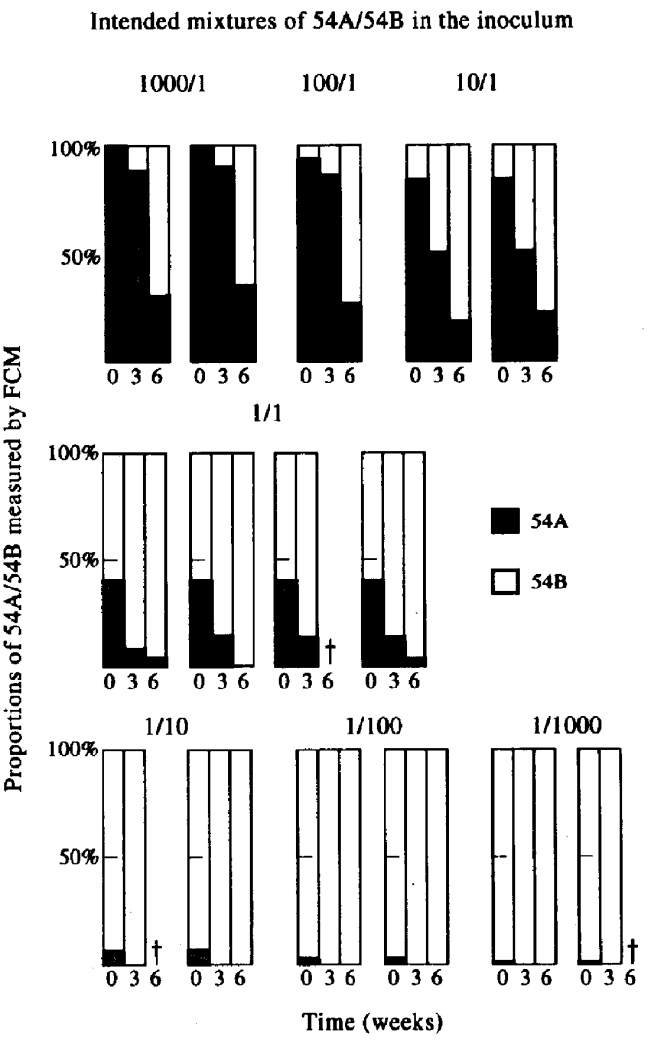

Figure 2. Relative proportions of the human SCLC cell lines 54A and 54B measured by flow cytometric DNA analysis in various inoculated cell mixtures and in fine-needle tumour aspirates after 3 and 6 weeks of subcutaneous tumour growth in nude mice. Progressive dominance of the 54B population was found in all tumours. Each set of columns represents an individual tumour. † death of host animal.

In one of the intended 1/1 mixed tumours (FCM determined proportions of 54A and 54B: 40% and 60%), 54A could not be detected after 6 weeks of tumour growth. Only 54B cells were detectable in tumours originating from inoculated mixtures with excess of 54B. Figure 3 shows the progressive dominance of 54B cells 3 and 6 weeks after subcutaneous growth of mixed tumours (54A/54B: 10/1) in a selected series of FCM histograms.

In the experiment in which 54A and 54B grew in opposite flanks of the animals, the growth of 54A was not affected by the 54B tumours compared with 54A controls (data not shown).

Irradiation experiment. After 4 weeks of observation, all tumours (n = 15) in the 54A/irradiated 54B group contained only 54A cells (Figure 4). In the 54A/54B mixed control group, all tumours (n = 14) were totally dominated by 54B cells (Figure 4), substantiating the results in the initial experiment. There was no tumour growth in the 54B irradiated group indicating that the irradiation dose was sufficient to kill all the 54B tumour cells.

In vitro *experiment.* From the intended 1/1 mixture of 54A and 54B, a relative median increase in 54A and a corresponding

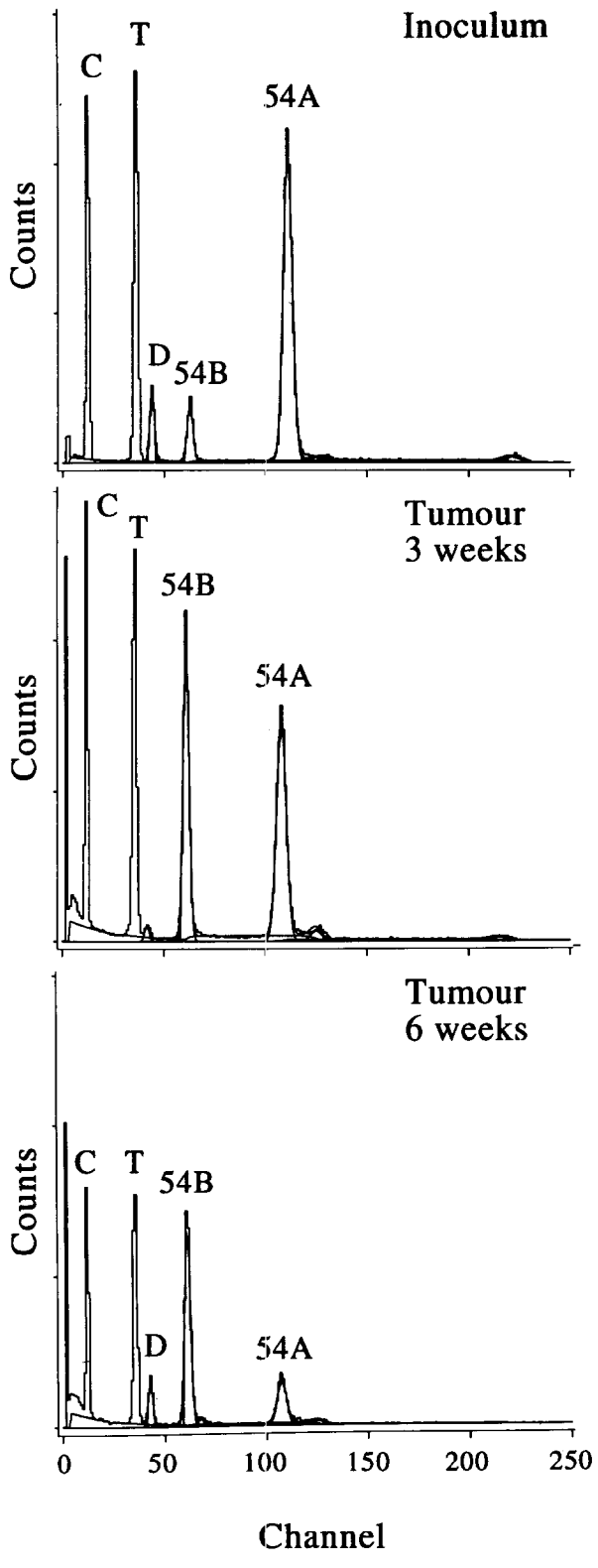

Figure 3. Selected series of DNA histograms from FCM analysis of an intended 10/1 inoculated 54A/54B cell mixture and of fine-needle tumour aspirates after 3 and 6 weeks of tumour growth. The relative proportions of 54A and 54B in the inoculum were 84 and 16%, respectively. After 3 and 6 weeks, 54B had increased to 50 and 77%, respectively, and the percentage of 54A had decreased correspondingly. C: chicken red blood cells, T: trout red blood cells, D: diploid host cells.

decrease in 54B of 14% was found after 7 days of *in vitro* growth (Figure 5). The number of 54A control cells had increased from 10^6 cells per culture flask to a median of 6.3×10^6 cells per culture flask (range $5.5–7.2 \times 10^6$). Similarly, 54B had increased from 10^6 cells per culture flask to a median of 3.9×10^6 cells per culture flask (range: $3.6–4.6 \times 10^6$).

The NYH-NYH2 experiment

In the NYH experiment, 17 of 26 tumour takes in the NYH/NYH2 mixed tumours were evaluable, i.e. the fine-needle tumour aspirations contained enough cells for determination of the cell proportions. In the inoculated mixture, the proportions of NYH and NYH2 were 56 and 44%, respectively. The median proportions of NYH and NYH2 after 4 weeks of tumour growth were 70 and 30%, respectively (range: 52/48%–100/0%; Figure 6).

DISCUSSION

We have described clonal interaction leading to clonal dominance by the progeny subpopulation of a human SCLC cell line (54B) over the parent subpopulation (54A) when grown in close contact in tumour xenografts in nude mice. The interaction required viable and proliferating cells since irradiation inactivated 54B cells were unable to dominate (growth inhibit) the 54A cells. The dominance was not dependent on the 54B cells being in excess, and was not due to differences in growth kinetics between the two cell lines. This was probably the case in the other studied pair of human SCLC subpopulations NYH and NYH2, a finding also reported by Price and colleagues [9].

The presented data indicate that subpopulations may dominate other subpopulations in heterogeneous tumours leading to an apparently more homogeneous tumour than is in fact the case. The dominated subpopulation may be undetectable by the method used, and after extinction of the dominating population by chemotherapy or radiation treatment, the dominated population may acquire sufficient growth conditions to become detectable. The dominance phenomenon is apparently not always operational between subpopulations of heterogeneous tumours, as clonal dominance was only found in one of the two examined sets of subpopulations.

The observed clonal dominance required *in vivo* growth conditions as the phenomenon was not found when 54A/54B mixtures were grown *in vitro*. A possible explanation is that stromal components are necessary participants in the process of dominance as was suggested by Staroselsky and colleagues [10]. This is supported by the observation that no remote effect on the growth of 54A was seen when the dominating 54B cell population was growing apart from the dominated population. Using multicellular spheroids, in which subpopulations presumably also have intimate contact, Rak and Kerbel [29] demonstrated clonal dominance *in vitro*.

Using repeated DNA flow cytometry on fine needle-aspirations from the evolving tumours, we have, in more detail than in previous studies, been able to detect changes in the fractional composition of heterogeneous tumours. Previous studies on human and murine tumour systems have used several techniques. Only in one study was FACS analysis of cellular DNA content performed on disaggregated tumours, as a supplement to identification of the cell composition end-point of disaggregated tumours by growth of tumour cells in selective media [11]. In that study, agreement was found between the two assays and, the colony forming assay was preferred for future studies. In murine systems, others have also used a clonogenic assay after

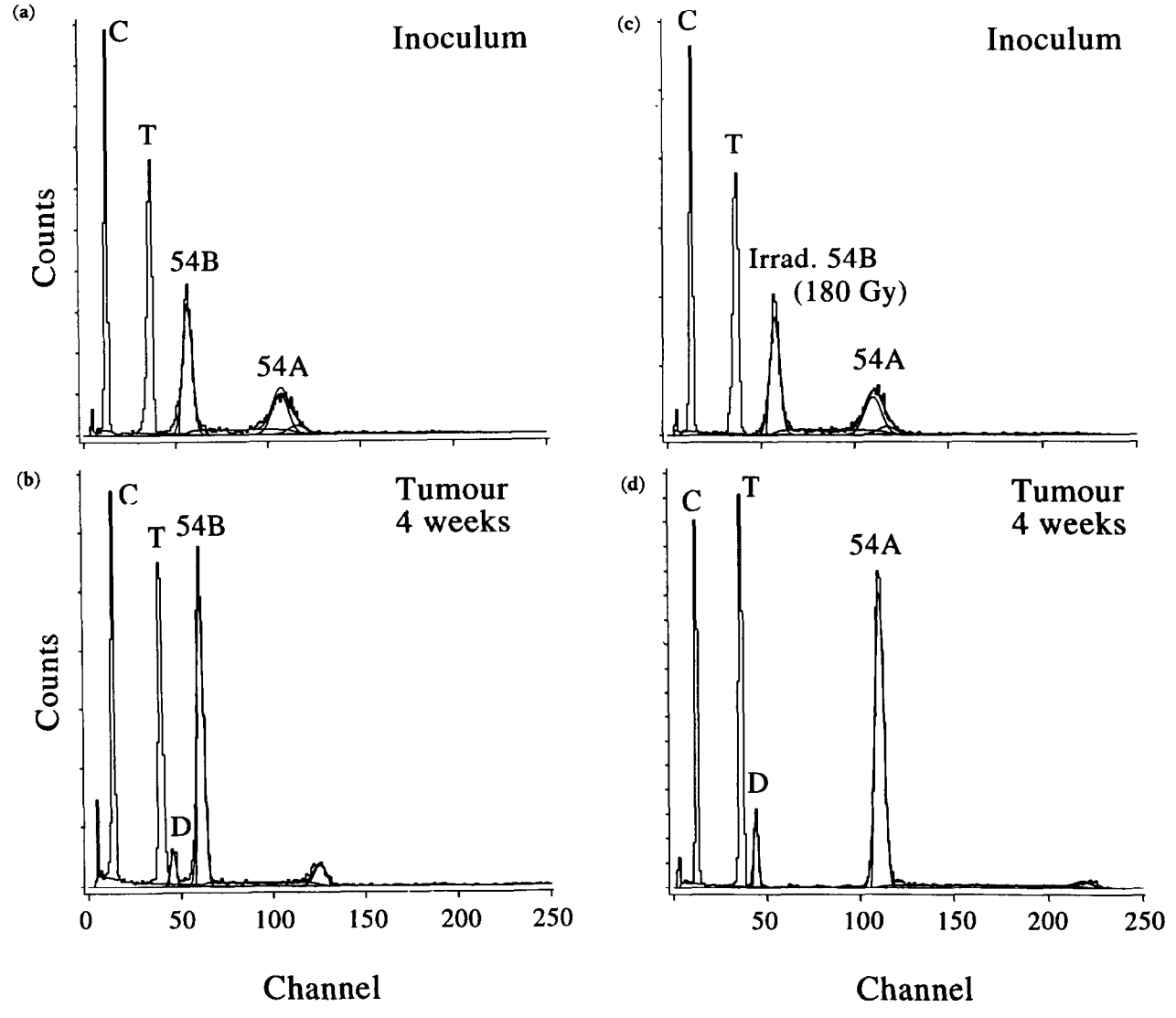

Figure 4. DNA histograms from FCM analysis of an intended 1/1 mixture of 54A and 54B (a) and of a fine-needle tumour aspirate respresentative of the median of 14 tumours, 4 weeks after inoculation of the tumour cell mixture s.c. into nude mice (b). The relative proportions of 54A and 54B in the inoculum were 49 and 51%, respectively. After four weeks of tumour growth, only the 54B population could be detected. The (c) DNA histogram shows the cell distribution of an intended 1/1 mixture of 54A and preirradiated 54B cells (180 Gy). The relative proportions of 54A cells and of irradiated 54B cells in the inoculum were 49 and 51%, respectively. The (d) histogram shows the cell distribution in a fine-needle tumour aspirate after four weeks of tumour growth. All 15 tumours were totally dominated by 54A cells. C: chicken red blood cells, T: trout red blood cells, D: diploid host cells.

tumour disaggregation to identify subpopulations [14, 16, 30]. In these systems, the cells were not grown in selective media, but differed in colony morphology [30] or in karyotype [14, 16]. One of the problems with the colony forming assay is that only selected cells are examined, i.e. colony forming cells that survive the disaggregation procedure. The drawback of chromosome analysis is that only the small fraction of cells in mitosis can be analysed, however, metaphase cells are not likely to be representative of the entire cell population of a tumour. FCM on fine-needle tumour aspirates using computer analysis of the DNA histograms allows analysis of hundreds to thousands of cells directly and repeatedly from the tumours with a high sensitivity (a few percent depending on the amount of cellular debris in the samples) as long as the cell populations have a distinguishable difference in DNA content. The use of fine-needle biopsy from solid tumours may lead to a sampling error if the subpopulations are not evenly distributed within the tumours. In order to overcome this obstacle, we moved the aspiration needle in many directions under continuous aspiration to ensure that the aspirate was representative for the entire tumour. Others have used genetic tagging of cells in order to detect the individual subpopulations [9, 10, 12, 15, 17]. Some of the problems with this method is the low sensitivity [17], or loss of the marker DNA sequence during DNA replication as a consequence of genetic instability or through repair mechanisms. In addition, the behaviour of the transfected cells may be altered [31]. Thus, the stability of the marker used to discriminate the clones under analysis is essential for the interpretation of the results. The cellular DNA content was stable following *in vivo* growth, but other potential markers may be quite heterogeneous in these individual cell populations.

Only a few studies have been performed on compound human tumour systems *in vivo*. In a human breast carcinoma, one genetically tagged clone was found to dominate in mixed tumours [9]. However, this seemed to be due to a faster growth of this clone. In a human renal cell carcinoma xenograft, distinct

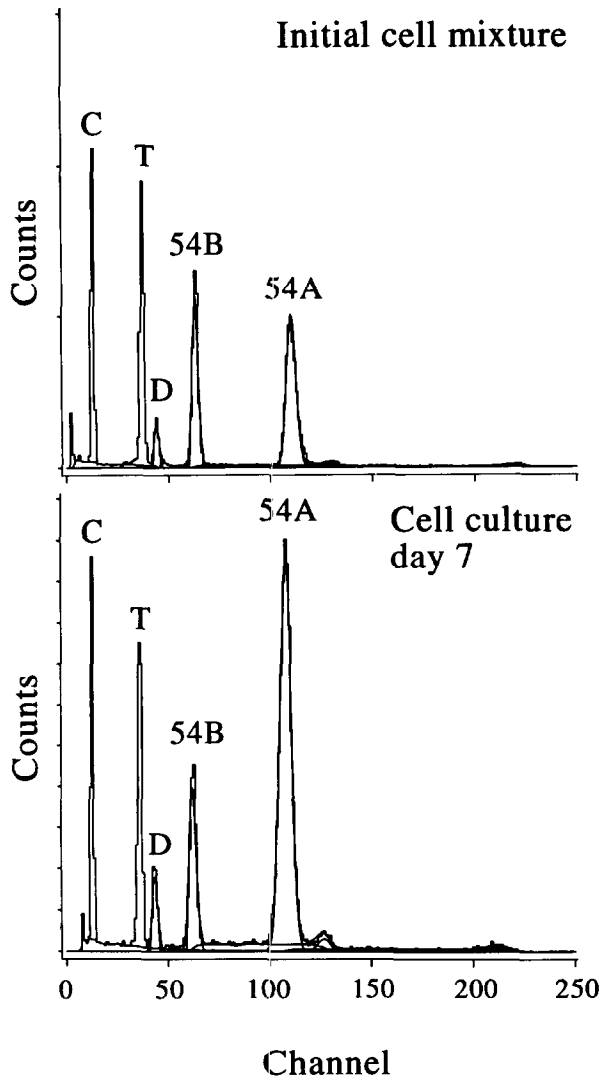

Figure 5. DNA histograms from FCM analysis of an intended 1/1 mixture of 54A and 54B and of the median of 3 harvested cell cultures 7 days after incubation. The relative proportions of 54A and 54B in the initial mixture were 56 and 44%, respectively. After 7 days of cell culture, 54A had increased to 70%, and correspondingly 54B had decreased to 30%. C: chicken red blood cells, T: trout red blood cells, D: diploid host cells.

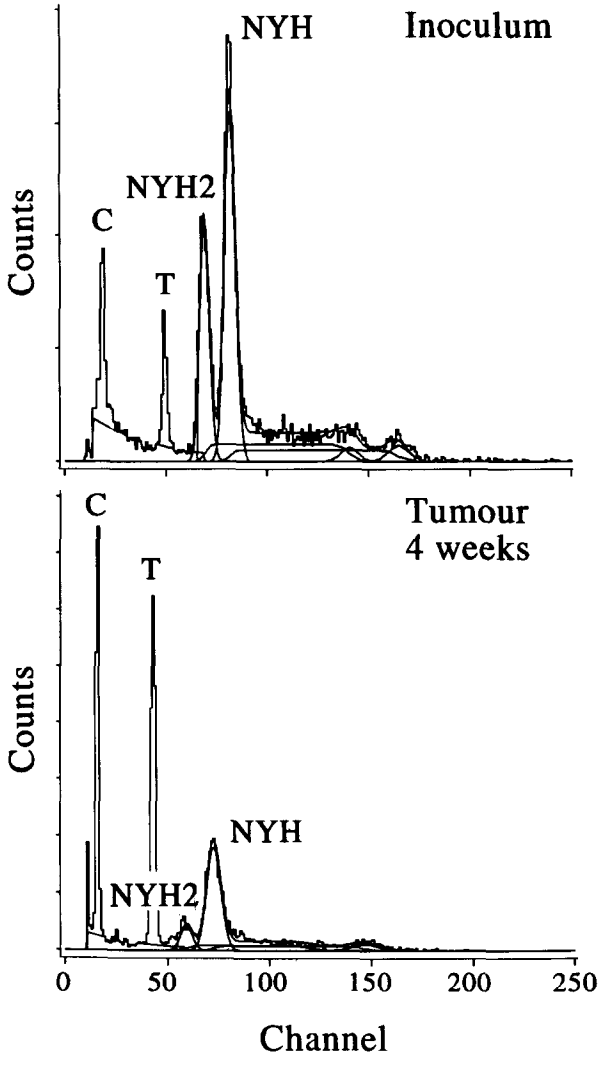

Figure 6. DNA histograms from FCM analysis of an intended 1/1 mixture of NYH/NYH2 and of fine-needle tumour aspirate representing median changes in 17 tumours 4 weeks after tumour cell inoculation. The relative proportions of NYH and NYH2 in the inoculum were 56% and 44%, respectively. After 4 weeks, NYH had increased to 70%, and the percentage of NYH2 had decreased correspondingly. C: chicken red blood cells, T: trout red blood cells.

clones were found to dominate metastases of different organs, indicating that the different clones may be influenced differently by organ environments [10]. We used ectopic (subcutaneous) implantation of the pulmonary tumour cells, but if there is a difference in local environmental influence on the individual tumour cell clones, implantation in the orthotopic site (lung) or another site may lead to different results.

Clonal interaction has also been demonstrated in murine systems. Miller and colleagues found non-immunological contact dominance among two murine mammary carcinoma sublines grown together in immune competent mice [13]. The same phenomenon was described in a mouse fibrosarcoma [15] and in the mouse Shionogi carcinoma system [14]. In a mouse mammary carcinoma [12, 17] and a mouse melanoma [16], metastatic clones were found to dominate non-metastatic clones. These studies indicate that, during tumour progression, emergence of 'more' malignant subpopulations with metastatic properties may lead to dominance of 'less' malignant parent populations, thus changing the phenotype of the primary tumour.

The mechanism of clonal dominance has yet to be elucidated. Probably the dominated cells are forced into a quiescent G_0 state, equivalent to what is seen when hormone dependent tumour cells are deprived of their hormonal drive [32, 33]. Thus, a short range paracrine or juxtacrine mechanism involving either a growth inhibitor secreted by the dominating clone or a growth promoter secreted by the dominated clone and specifically stimulating the dominating clone could be an attractive explanation. A growth inhibitor is probably the most likely, as no enhancement of tumour growth rate was seen in the present or in other studies. As radiation killed inhibitor cells had no effect, such a factor may have a short-lived effect on the dominated cells thus requiring continuous secretion to be effective. No growth inhibition could be demonstrated *in vitro*, a finding also reported by others [14, 15].

Transforming growth factor beta [TGF-β] has been shown to inhibit the growth of various cell lines *in vitro*, whereas other cell

lines have lost the inhibitory response [34, 35]. In addition, TGF-β may be involved in the control of proliferation and differentiation of several lung-derived cell types [36, 37]. The effect of TGF-β on cell lines is dependent on the presence of cell surface receptors [34, 35]. Both 54A and 54B express TGF-β receptors and TGF-β messenger RNA [38], and both cell lines are inhibited by TGF-β_1 *in vitro* [39]. Whether 54B cells are producing TGF-β has yet to be investigated. If they do, it may, as no domination was found *in vitro*, be secreted in an inactive form which requires activation *in vivo* in order to down-regulate the growth of 54A. In contrast to our findings, Theodorescu and associates [40] found that TGF-β_1 production by a dominated subpopulation which had been irradiated had a growth stimulating effect on the dominating clone when co-inoculated *in vitro*, using an indirect ^{3}H-thymidine incorporation assay as end-point of growth. Using a tumour lag time assay, they claimed that a similar growth stimulating effect *in vivo* (Révész effect [41]) by the irradiated subpopulation was due to TGF-β. They also found that the dominating clone had four times higher TGF-β receptor levels compared with the dominated clone. In contrast to other studies, which found that TGF-β is a growth inhibitor, the conclusion of this study was that TGF-β_1 is a growth stimulator and may be responsible for the clonal dominance phenomenon. Growth stimulators, such as epidermal growth factor, TGF-α, platelet derived growth factor, nerve growth factor and insulin-like growth factor 1 and 2, had no growth stimulatory effect in their system. Thus, it seems unresolved whether TGF-β or other negative growth regulators [42] alone or in combination are responsible for clonal dominance. Another explanation for the missing interaction *in vitro* may be that the cells require intimate cell contact in order to release or activate the growth regulators, or that an interaction with stroma cells or intercellular matrix is necessary [43, 44].

Adhesion molecules, such as NCAM and E-cadhedrin, have been shown to be involved in cellular interactions during embryonic development [45–47]. A decrease in adhesion molecules on the surface of malignant cells may be contributing to the metastatic phenotype [47, 48]. In the nude mouse system, the human SCLC tumours did not metastasise, however, the explanation for this may be a yet unknown but lacking interaction between the tumour cells and the host stroma. Whether the expression of adhesion molecules on the tumour cells was involved in the cellular interaction described in the present study cannot be evaluated with certainty. Both 54A and 54B cells have weak expression of E-cadhedrin in xenografts, but strong expression *in vitro*. 54B cells showed a slightly higher expression of NCAM than 54A both *in vitro* and in xenografts [49]. These differences may have contributed to the 54B dominance on 54A.

Direct cellular communication via gap junctions [50, 51] between the tightly packed cells in solid tumours but not in tissue culture, may represent another putative mechanism. Miller and associates [52] have shown that resistance against cytotoxic drugs can be transferred from one subpopulation to another when the tumour cells are growing together in solid tumours.

The phenomenon of clonal interaction resulting in cellular dominance between tumour cell subpopulations may explain important tumour biological characteristics. According to the clonal evolution theory [1], genetic instability of neoplastic cells results in the emergence of a number of new subpopulations. However, in many clinical tumours, only one or a few subpopulations are detected, depending on the methods used. The explanation could be that pre-existing subpopulations are extinct or suppressed below the detection limit of the method used by cellular dominance imposed by other subpopulations. Using FCM, Vindeløv and colleagues [53] detected a new clone in a human colon carcinoma xenograft in nude mice in the 56th transplant generation. After three transplant passages, the parent population could no longer be detected despite no differences in growth kinetics between the two populations. The most likely explanation is that the new population inhibited the parent population by clonal dominance.

The mechanism by which the leukaemic stem cells are able to inhibit normal haematopoiesis leading to pancytopenia has been insufficiently explained. One explanation could be that the proliferation of normal haematopoietic stem cells are contact dominated by the leukaemic stem cells either directly or via the stroma cells of the bone marrow. The leukaemic stem cells may induce the stroma cells to overproduce a growth inhibitor causing growth inhibition of the normal haematopoietic stem cells [54]. The phenomenon of cellular dominance may also be involved in tumour stem cell to normal cell interaction during invasive tumour growth, probably resulting in apoptosis of surrounding normal tissue cells. Finally, development of relapse after induction of remission by cytotoxic drugs, which is a common event during the treatment of malignant disease, may in part be explained by facilitation of growth of suppressed and drug resistant subpopulations after eradication of a dominating population which was sensitive to the treatment.

1. Nowell PC. The clonal evolution of tumor cell populations. *Science* 1976, **194**, 23–28.
2. Dexter DL, Calabresi P. Intraneoplastic diversity. *Biochem Biophys Acta* 1982, **695**, 97–112.
3. Fidler IJ, Hart IR. Biological diversity in metastatic neoplasms: origins and implications. *Science* 1982, **217**, 998–1003.
4. Poste G, Greig R. The experimental and clinical implications of cellular heterogeneity in malignant tumors. *J Cancer Res Clin Oncol* 1983, **106**, 159–170.
5. Woodruff MFA. Cellular heterogeneity in tumours. *Br J Cancer* 1983, **47**, 589–594.
6. Heppner GH. Tumor heterogeneity. *Cancer Res* 1984, **44**, 2259–2265.
7. Dexter DL, Leith JT. Tumor heterogeneity and drug resistance. *J Clin Oncol* 1986, **4**, 244–257.
8. Leith JT, Faulkner LE, Bliven SF, Lee ES, Glicksman AS, Dexter DL. Disaggregation studies of xenograft solid tumors grown from pure or admixed clonal subpopulations from a heterogeneous human colon adenocarcinoma. *Invasion Metastasis* 1985, **5**, 317–335.
9. Price JE, Bell C, Frost P. The use of genotypic marker to demonstrate clonal dominance during the growth and metastasis of a human breast carcinoma in nude mice. *Int J Cancer* 1990, **45**, 968–971.
10. Staroselsky AN, Radinsky R, Fidler IJ, Pathak S, Chernajovsky Y, Forst P. The use of molecular genetic markers to demonstrate the effect of organ environment on clonal dominance in a human renal-cell carcinoma grown in nude mice. *Int J Cancer* 1992, **51**, 130–138.
11. Miller BE, Miller FR, Wilburn DJ, Heppner GH. Analysis of tumor cell composition in tumours composed of paired mixtures of mammary tumour cell lines. *Br J Cancer* 1987, **56**, 561–569.
12. Waghorne C, Thomas M, Lagarde A, Kerbel RS, Breitman ML. Genetic evidence for progressive selection and overgrowth of primary tumors by metastatic cell subpopulations. *Cancer Res* 1988, **48**, 6109–6114.
13. Miller BE, Miller FR, Wilburn D, Heppner GH. Dominance of a tumor subpopulation line in mixed heterogeneous mouse mammary tumors. *Cancer Res* 1988, **48**, 5747–5753.
14. Ichikawa T, Akimoto S, Hayata I, Shimazaki J. Progression and selection in heterogeneous tumor composed of androgen-responsive Shionogi carcinoma 115 and its autonomous subline (Chiba Subline 2). *Cancer Res* 1989, **49**, 367–371.
15. Enoki Y, Niwa O, Yokoro K, Toge T. Analysis of clonal evolution

in a tumor consisting of pSV2neo-transfected mouse fibrosarcoma clones. *Jpn J Cancer Res* 1990, **81**, 141–147.
16. Staroselsky A, Pathak S, Fidler IJ. Changes in clonal composition during *in vivo* growth of mixed subpopulations derived from the murine K-1735 melanoma. *Anticancer Res* 1990, **10**, 291–296.
17. Samiei M, Waghorne CG. Clonal selection within metastatic SP1 mouse mammary tumors is independent of metastatic potential. *Int J Cancer* 1991, **47**, 771–775.
18. Aabo K, Vindeløv LL, Spang-Thomsen M. Interaction between three subpopulations of Ehrlich carcinoma in mixed solid tumours in nude mice: evidence of contact domination. *Br J Cancer* 1994, **70**, 91–96.
19. Carney DN, Gazdar AF, Nau M, Minna JD. Biological heterogeneity of small cell lung cancer. *Semin Oncol* 1985, **12**, 289–303.
20. Vindeløv LL, Hansen HH, Christensen IJ, *et al.* Clonal heterogeneity of small-cell anaplastic carcinoma of the lung demonstrated by flow-cytometric DNA analysis. *Cancer Res* 1980, **40**, 4295–4300.
21. Engelholm SA, Vindeløv LL, Spang-Thomsen M, *et al.* Genetic instability of cell lines derived from a single human small cell carcinoma of the lung. *Eur J Cancer Clin Oncol* 1985, **21**, 815–824.
22. Spang-Thomsen M, Clerici M, Engelholm SA, Vindeløv LL. Growth kinetics and *in vivo* radiosensitivity in nude mice of two subpopulations derived from a single human small cell carcinoma of the lung. *Eur J Cancer Clin Oncol* 1986, **22**, 549–556.
23. de Leij L, Postmus PE, Buys CHCM, *et al.* Characterization of three new variant type cell lines derived from small cell carcinoma of the lung. *Cancer Res* 1985, **45**, 6024–6033.
24. Roed H, Christensen IJ, Vindeløv LL, Spang-Thomsen M, Hansen HH. Interexperiment variation and dependence on culture conditions in assaying the chemosensitivity of human small cell lung cancer cell lines. *Eur J Cancer Clin Oncol* 1987, **23**, 177–186.
25. Engelholm SA, Spang-Thomsen M, Brünner N, Nøhr I, Vindeløv LL. Disaggregation of human solid tumours by combined mechanical and enzymatic methods. *Br J Cancer* 1985, **51**, 93–98.
26. Rygaard K, Spang-Thomsen, M. 'Growth'—a computer program for determination of mean growth curves and calculation of response to therapy of solid tumor xenografts. In Wu B-q, Zheng J, eds. *Immune-deficient Animals in Experimental Medicine*. Basel, Karger, 1989, 301–306.
27. Vindeløv LL, Christensen IJ. A review of techniques and results obtained in one laboratory by an integrated system of methods designed for routine clinical flow cytometric DNA analysis. *Cytometry* 1990, **11**, 753–770.
28. Vindeløv LL, Christensen IJ, Nissen NL. Standardization of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. *Cytometry* 1983, **3**, 328–331.
29. Rak JW, Kerbel RS. Growth advantage ('clonal dominance') of metastatically competent tumor cell variants expressed under selective two- or three-dimensional tissue culture conditions. *In Vitro Cell Dev Biol* 1993, **29A**, 742–748.
30. Leith JT, Michelson S, Faulkner LE, Bliven SF. Growth properties of artificial heterogeneous human colon tumors. *Cancer Res* 1987, **47**, 1045–1051.
31. Kerbel RS, Waghorne C, Man MS, Elliott B, Breitman ML. Alteration of the tumorigenic and metastatic properties of neoplastic cells is associated with the process of calcium phosphate-mediated DNA transfection. *Proc Natl Acad Sci USA* 1987, **84**, 1263–1267.
32. Sutherland RL, Green MD, Hall RE, Reddel RR, Taylor IW. Tamoxifen induces accumulation of MCF 7 human mammary carcinoma cells in the G_0/G_1 phase of the cell cycle. *Eur J Cancer Clin Oncol* 1983, **19**, 615–621.
33. Osborne CK, Boldt DH, Clark GM, Trent, JM. Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G_1 phase. *Cancer Res* 1983, **43**, 3583–3585.
34. Barnard JA, Lyons RM, Moses HL. The cell biology of transforming growth factor β. *Biochim Biophys Acta* 1990, **1032**, 79–87.
35. Shipley GD, Pittelkow MR, Wille JJ, Scott RE, Moses HL. Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. *Cancer Res* 1986, **46**, 2068–2071.
36. Jetten AM, Shirley JE, Stoner G. Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGFβ. *Exp Cell Res* 1986, **167**, 539–549.
37. Masui T, Wakefield LM, Lechner JF, La Veck MA, Sporn MB, Harris CC. Type β transforming growth factor is the primary differentiation-inducing serum factor for normal bronchial epithelial cells. *Proc Natl Acad Sci USA* 1986, **83**, 2438–2442.
38. Damstrup L, Rygaard K, Spang-Thomsen M, Poulsen HS. Expression of transforming growth factor β (TGFβ) receptors and expression of $TGF\beta_1$, $TGF\beta_2$ and $TGF\beta_3$ in human small cell lung cancer cell lines. *Br J Cancer* 1993, **67**, 1015–1021.
39. Nørgaard P, Damstrup L, Rygaard K, Spang-Thomsen M, Poulsen HS. Growth suppression by transforming growth factor-β_1 of human small cell lung cancer cell lines is associated to expression of the type II receptor. *Br J Cancer* 1994, **69**, 802–808.
40. Theodorescu D, Caltabiano M, Greig R, Rieman D, Kerbel RS. Reduction of TGF-beta activity abrogates growth promoting tumor cell–cell interactions *in vivo*. *J Cell Physiol* 1991, **148**, 380–390.
41. Révész L. Effect of lethally damaged tumor cells upon the development of admixed viable cells. *J Natl Cancer Inst* 1958, **20**, 1157–1186.
42. Massagué J, Weinberg RA. Negative regulators of growth. *Curr Opin Genet Dev* 1992, **2**, 28–32.
43. Wu K-f, Pope JH, Ellem KAO. Inhibition of growth of certain human tumour cell lines by a factor derived from human fibroblast-like cell lines. I. Demonstration by mixed culture and by use of cell washings. *Int J Cancer* 1985, **35**, 477–482.
44. Ruoslahti E. Control of cell motility and tumour invasion by extracellular matrix interactions. *Br J Cancer* 1992, **66**, 239–242.
45. Levi G, Duband J-L, Thiery JP. Modes of cell migration in the vertebrate embryo. *Int Rev Cytol* 1990, **123**, 201–252.
46. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991, **251**, 1451–1455.
47. Thiery JP, Boyer B, Tucker G, Gavrilovic J, Valles A-M. Adhesion mechanisms in embryogenesis and in cancer invasion and metastasis. *Ciba Found Symp* 1988, **141**, 48–69.
48. Johnson JP. Cell adhesion molecules in neoplastic disease. *Int J Clin Lab Res* 1992, **22**, 69–72.
49. Rygaard K, Møller C, Bock E, Spang-Thomsen M. Expression of cadherin and NCAM in human small cell lung cancer cell lines and xenografts. *Br J Cancer* 1992, **65**, 573–577.
50. Mehta PP, Bertram JS, Loewenstein WR. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell* 1986, **44**, 187–196.
51. Yamasaki H. Role of cell–cell communication in tumor suppression. *Immunol Ser* 1990, **51**, 245–266.
52. Miller BE, Machemer T, Lehotan M, Heppner GH. Tumor subpopulation interactions affecting melphalan sensitivity in palpable mouse mammary tumors. *Cancer Res* 1991, **51**, 4378–4387.
53. Vindeløv LL, Spang-Thomsen M, Visfeldt J, Povlsen CO, Jensen G, Nissen NI. Clonal evolution demonstrated by flow cytometric DNA analysis of a human colonic carcinoma grown in nude mice. *Exp Cell Biol* 1982, **50**, 216–221.
54. Olsson I, Olofsson T. Tumor associated myelopoiesis inhibiting factors. *Leuk Res* 1990, **14**, 715–716.

Acknowledgement—We thank Dr Henrik Roed, Department of Oncology, Rigshospitalet, Copenhagen for supplying the NYH and the NYH2 tumor cells. This study was supported by grants from the Danish Cancer Society and the Danish Medical Research Council.