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Genetic Instability of Cell Lines Derived from a Single Human Small Cell Carcinoma of the Lung*

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Abstract—*Specimens from a human small cell carcinoma of the lung were established as a cell line* in vitro. *Flow cytometric DNA analysis demonstrated only one tumor cell population in the parent tumor as well as in the early passages* in vitro. *After six passages* in vitro, *two new subpopulations with different DNA content appeared. By cloning, permanent cell lines were established from the new subpopulations, whereas the original population stopped growing. The cloned cell lines were characterized by morphology, chromosomes analysis, electron microscopy and plating efficiency; the stability of the DNA content was examined regularly by flow cytometric DNA analysis and instability was found in one of the cloned cell lines. Chromosome analysis showed that the cloned cell lines consisted of more than one population after* 17 in vitro *passages. Both cloned cell lines produced tumors in nude mice. Genetic instability was demonstrated in these mouse-grown tumors as well. Development of resistance to antineoplastic treatment may be due to heterogeneity in sensitivity among subpopulations in a tumor. Isolation of populations with different DNA contents allows the study of interaction between subpopulations and the observations provide evidence in support of the hypothesis of clonal evolution.*

INTRODUCTION

SMALL cell carcinoma of the lung (SCCL) is shown to be heterogeneous with respect to various biological features including morphology, hormone production and DNA content [1-3]. According to the theory of clonal evolution this heterogeneity is a result of genetic instability [4, 5]. Although SCCL responds dramatically to chemotherapy, a relapse of the tumor occurs in most patients and only a few of them are long-term survivors [6]. The development of resistance to antineoplastic treatment may be due to heterogeneity in the sensitivity of different subpopulations in a tumor.

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Biological diversity among cloned subpopulations in murine tumors is well documented [7, 8]. Cell lines established from different metastatic sites from a single human colonic carcinoma have previously been described as differing in DNA content [9]. Cloning of cell lines derived from a human bladder carcinoma and from a human colonic carcinoma at low *in vitro* passage resulted in subpopulations with different chromosome content and diversity in a number of biological properties [10, 11]. Recently, a study demonstrated that cellular heterogeneity for chemotherapy in murine cancer metastasis can develop rapidly even in tumors of unicellular origin [12].

SCCL grown *in vitro* has previously been described as stable with regard to DNA content [13]. In this study, heterogeneity and genetic instability in cloned as well as uncloned cell lines established from a single SCCL metastatis are described. The cell lines were characterized by flow cytometric DNA analysis, karyotype, morphology, electron microscopy and hormone

analysis. The tumorigenicity of the cells grown *in vitro* was examined by the formation of tumors in nude mice. Flow cytometric DNA analysis demonstrated, even within tumors of clonal origin, instability in the DNA content. These observations are considered substantial evidence in support of the hypothesis of clonal evolution.

MATERIAL AND METHODS

Tumor

Tumor tissue was obtained from a lymph node metastasis from a 49-yr-old male patient with disseminated untreated SCCL, subclassified as the intermediate type [2].

Tissue culture

The tumor sample was transferred to the laboratory in phosphate-buffered saline, and 1 to 2-mm^3 tumor blocks were explanted in liquid culture (passage 1). Four weeks after plating, the growing cells were passaged to agar culture (passage 2) to obtain fibroblast-free cell populations [14]. After the agar passage, the tumor cells were grown serially in liquid culture (from passage 3).

The explants and liquid cultures were grown in 200-ml culture flaks (Nunclon®) in Eagle's minimal essential medium (MEM), containing Earl's salt supplemented with 20% fetal bovine serum, MEM amino acid, MEM vitamins, L-glutamine, glucose 10% (0.5 ml/100 ml), gentamycin (2 μg/ml) and mycostatin (10 units/ml). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air.

Cells from the explant cultures were plated in 35-mm plastic Petri dishes in liquid medium as described above, on top of a layer of hardened 0.25% agarose (Difco) containing 26% of the culture medium described above supplemented with 13% fetal calf serum, 2.5% sheep red blood cells and mercaptoethanol (50 μM) [14].

Cloning procedure: 10^2 cells were plated on agar as described above. The dishes were checked for reaggregation in a dissecting microscope on days 1 and 2 after plating. Dishes with reaggregation were excluded. Three weeks later single colonies of more than 50 cells were picked up with a 6-μl pipette, transferred to liquid culture medium in 50-ml culture flasks (Nunclon®) and incubated until a cell number of about 10^5 cells was reached. The procedure was repeated twice.

The periodic testing of cultures by fluorescent Hoechst 33258 stain, in mycoplasma agar medium and in electron micrographs was performed to detect any mycoplasma contamination.

Heterotransplantation

A suspension of 5×10^6 cultured tumor cells in 0.1 ml medium was injected s.c. into the flanks of 6-week-old female SPF nude mice (BALB/c nu/nu, BOM) kept under sterile conditions in laminar air flow clean benches [15]. Established tumors were transplanted serially by the s.c. inoculation of tissue blocks of about 2 mm into the flanks of the recipient mice [15].

From 10 days after transplantation the tumors were measured three times a week. Mathematical analysis of the growth data was based on a transformed Gompertz function [15], which depicts the growth rectilinearly when the tumor size $\ln(\ln A(\max) - \ln A(t))$ is plotted as a function of time; $A(\max)$ = theoretical maximum area, $A(t)$ area at time (t). The growth of the tumors were thus characterized by the slope (α) of the growth curves and by the value $A(\max)$.

Immunohistochemical procedure

The tumors were examined for production of ACTH, gastrin, calcitonin and methionine enkephalin. Tumor-bearing nude mice were lightly anesthetized with diethylether and intracardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Thin slices were cut from well-perfused, non-necrotic (peripheral) parts of the tumor and postfixed overnight at 4°C in the formaldehyde solution. Following soaking in 20% sucrose in 0.1 M sodium phosphate buffer, pH 7.4, the slices were frozen on cryostat chucks in melting Freon-22. The specimens were processed as previously described [16–18]. Specificity checks included the use of antisera pre-incubated with varying amounts (5–100 μg/ml diluted serum) of appropriate antigens and potentially cross-reacting peptides.

Electron microscopy

Cell cultures for transmission electron microscopy were fixed by mixing the cell suspension (10^6 cells) with Karnovsky's fixative. After centrifugation (500 **g**/5 min) the cells were postfixed for 30 min in Karnovsky's fixative diluted (1:2) with distilled water. The cells were enrolled in 2% Noble agar at 40°C followed by fixation for 2 hr in 1% osmium tetroxide. The cells were dehydrated in ethanol (99%) and 3,3-epoxypropane and embedded in Vestopal W. Ultrathin sections were post-stained with magnesium uranyl acetate and lead citrate.

Cells for scanning electron microscopy were fixed as above. The pellet of free cells and cells adhering to the culture vessel were dehydrated in ethanol, critical point dried in CO_2 (Polaron E

3000) and sputter-coated with gold palladium (Polaron E 5100).

Flow cytometric DNA analysis

Samples for flow cytometry from the original tumor and the mouse-grown tumors were obtained by fine-needle aspirations. Tumor cells grown *in vitro* were harvested by gentle scraping with a 'rubber policeman', without the use of enzymes. The tumor cells were suspended in citrate buffer, frozen in liquid N_2, and stored at -80°C until the analysis [19]. Before analysis the samples were stained with propidium iodide as described previously [20]. The flow cytometer used was a FACS III cell sorter (Becton Dickinson, Sunnyvale, CA). The DNA content was expressed by the DNA index (DI), defined as the ratio of the DNA content of the G_1 tumor cells to that of diploid human cells. Chicken and rainbow trout red blood cells were used as internal standards [21]. The percentage of cells in the cell cycle phases were determined by the statistical analysis of the DNA distributions [22].

Chromosome analysis

Cell cultures of the two cloned subpopulations were analysed in *in vitro* passages 17-19. Two hours prior to harvest ethidium bromide (5 μg/ml) was added, to obtain elongated chromosomes [23], along with colcemid (0.04 μg/ml). The cells were detached by trypsin-EDTA, hypotonically treated (0.075 M KCl, 20 min), fixed three times in methanol:glacial acetic acid (3:1), dropped onto ice cold, wet slides and air-dried. Slides were stained by quinacrine mustard [24], examined and photographed using a Leitz Ortholux II microscope equipped with epifluorescence. Some slides were destained and restained by silver to detect nucleolar organizer regions (AgNOR-technique) [25] or by the C banding technique to detect constitutive heterochromatin [26]. Staining with distamycin A/DAPI followed the description of Schweizer *et al.* [27]. All analysis were performed using photographic prints.

RESULTS

DNA histograms from the original tumor and from early passages *in vitro* are shown in Fig. 1. In the original tumor (Fig. 1A) only one tumor cell population, with DI = 1.57, could be identified. In passages 3-6 the DI of the tumor cell population did not differ significantly from that of the original tumor (Fig. 1B). In passage 7 (Fig. 1C) three different populations were demonstrated. The DI of the main population was identical to that of the patient tumor, but two new populations had appeared, with a DI = 1.26 and 2.51, respectively. During passages 8-10 of the uncloned cultures one of the subpopulations (DI = 1.26) totally overgrew the other two, and after passage 10 only tumor cells with DI = 1.26 were detectable in the cultures (Fig. 1D).

Cells from passage 7, in which the new subpopulations appeared, were cloned and permanent cell lines representing the two new subpopulations with DI = 1.26 and DI = 2.51 were established (Fig. 1E and F), whereas the cloning of the original tumor cell population was unsuccessful. One of the cloned cell lines (DI = 2.51) has at present been followed for 52 passages and no significant changes in the DNA content have been detected. In the other cloned cell line (DI = 1.26) tumor cells with doubled DNA content appeared in *in vitro* passage 19 (Fig. 2), but in the subsequent 33 passages only one population (DI = 1.26) was present in the histograms.

During passages 3-6 the tumor cells were growing as colonies slightly attached to the

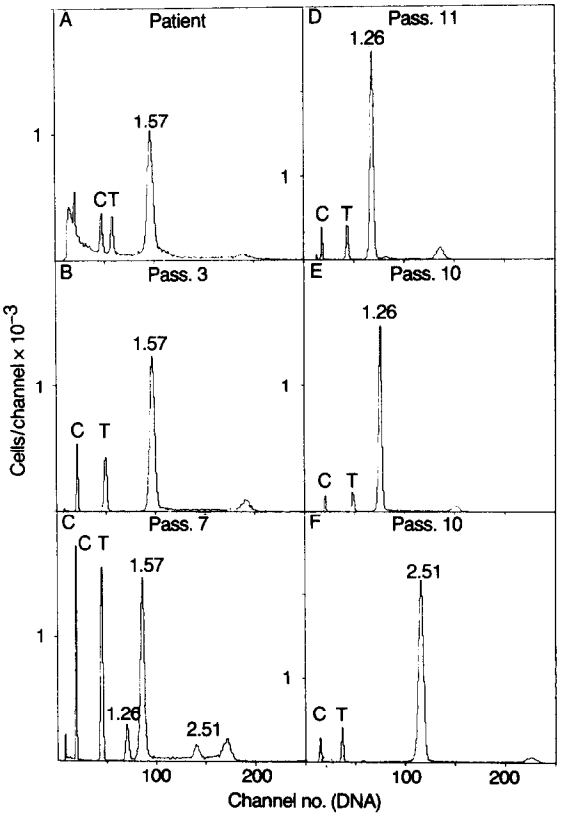

Fig. 1. DNA distributions of the patient tumor and tumor cells grown in vitro. *The DI is indicated by the number on top of the G_1 peak and the peaks marked C and T represent internal standards to calculate the DI. (A) DNA distribution of the patient tumor. (B) DNA distribution of the tumor cells in passage 3. (C) DNA distribution of the tumor cells in passage 7. Three different subpopulations are present. (D) DNA distribution from the uncloned cell line in passage 11. (E and F) DNA distribution of the two different cell line cloned in passage 7.*

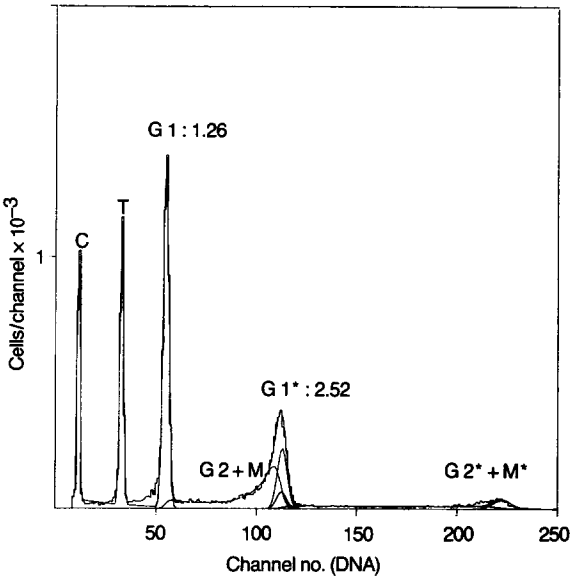

Fig. 2. DNA distribution of a cloned cell line in passage 19 in vitro. *The DI is indicated by the number on top of the G_1 peak. The peaks marked C and T represent internal standards to calculate the DI. Two tumor cell populations are seen. The parts of histograms produced by G_1 and G_2 + M cells are indicated.*

bottom of the flasks. In passage 7, where the new populations were detected, the tumor cells grew mostly as a monolayer culture. The cloned cell lines (DI = 1.26 and DI = 2.51) grew as monolayer cultures in the first passage after cloning, but thereafter the growth morphology alternated between floating aggregates and monolayer cultures.

Scanning electron microscopy showed that the surface of the tumor cells was covered by a great number of microvilli and blebs (Fig. 3). No differences in the surface structure of the two cell lines were detected whether the cells grew as floating aggregates or as monolayer cultures.

No evidence of mycoplasma contamination was encountered, as revealed by negative cultures and the failure to detect these organisms by electron microscopy.

The characteristics of the cell lines are summarized in Table 1.

In the subpopulation with DI = 1.26, two hyperdiploid clones with modal numbers of 56 (three cells) and 57 (six cells) were identified, the karyotypes being: 56, X, -Y, +X, +2, +7, +8, +8, +20, +22, +M1, +M2, +M3, t(9;14)(p11;q24)/57, X, -Y, +X, +2, +7, +8, +8, +19, +20, +22, +M1, +M2, +M3, t(9;14)(p11;q24) (Fig. 4). The clones with modal numbers of 56 and 57 thus had an identical karyotype except for an additional chromosome 19 in the latter.

The analysis of the cell line with DI = 2.51 showed a hypertetraploid karyotype with a modal number in the range 109–115. Although a random loss of single chromosomes was found in this population, probably due to the selection of slightly overspread metaphases for analysis due to the large number of chromosomes, it was possible to construct a karyotype by analysing several cells. This cell line contained the same marker chromosomes (M1, M2, M3), the same reciprocal translocation and numerical aberrations affecting the same chromosomes (X, Y, 2, 7, 8, 19, 20, 22) as was found in the hyperdiploid cell line. Thus the presence of two copies of each of the marker chromosomes and the translocation chromosomes, four copies of X, six copies each of chromosomes 2, 7, 19, 20 and 22, and eight copies of chromosome 8 were found. In addition, a clone containing five copies of chromosome 5 was identified in the hypertetraploid population.

Many endoreduplicated metaphases were seen in both the hyperdiploid and hypertetraploid cultures (Fig. 4F). Also, hypertetraploid metaphases without evidence of endoreduplication were seen in the hyperdiploid population, and metaphases with over 200 chromosomes (hyperoctaploid) were seen in the hypertetraploid population.

Two normal chromosomes 3 were found in the hyperdiploid population (and four in the hypertetraploid population). Both homologues contained brightly fluorescent centromeric regions (Fig. 4E) and there was no evidence of chromosome 3 material participating in the formation of marker chromosomes. A number of chromosomal rearrangements which must have taken place during tumor evolution were identified. A reciprocal translocation t(9;14)(p11;q24) (Fig. 2A) was observed. Two long arms of chromosome 1 formed a marker chromosome (M1) (Fig. 4C). This marker was not just an isochromosome for the long arm of chromosome 1. Three large heterochromatic C-band-positive, DA/DAPI-positive blocks were distributed unevenly around the centromere. Each block was considerably larger than the heterochromatic blocks seen on the normal chromosome 1, indicating that amplification of the heterochromatic region(s) of chromosome(s) 1 must have occurred. One acrocentric marker chromosome (M2) contained an active NOR region on the short arm, and it mostly resembled a deleted chromosome 14 (Fig. 4B). The third marker chromosome (M3) was subtelocentric, of the size of chromosome 17, did not contain active NOR regions, was DA/DAPI-negative and contained a very small centromeric C band (Fig. 4E). It could not be characterized further. By AgNOR staining, all three chromosomes 22 showed different-sized NOR regions (Fig. 4B), indicating that either

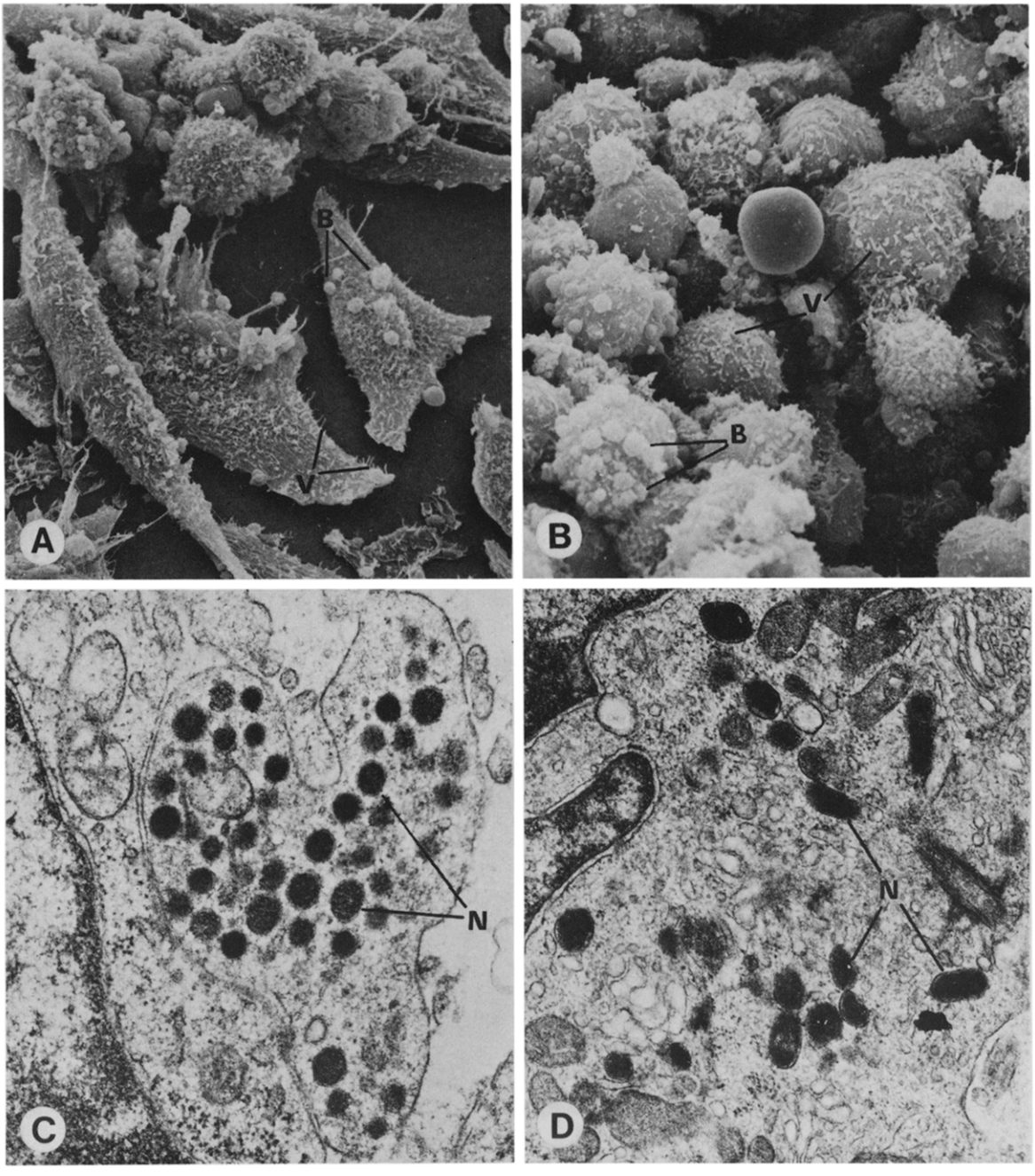

Fig. 3. *(A) and (B) are scanning electron micrographs of SCCL cell cultures, (A) mostly with flattened cells attached to the culture vessel and only few spherical cells, (B) from cultures of floating, mostly spherical cells. Blebs (B) and microcilli (V) are present in both cultures. (C) and (D) are transmission electron micrographs of SCCL cells, (C) from a patient tumor, (D) from a cell culture. Neurosecretory type granules (N) with a dense core and a translucent periphery are present in both. The granules are larger and more irregularly shaped in cultured cells. Magnifications:* (A) × 900; (B) × 1500; (C) × 40.000; (D) × 28,000.

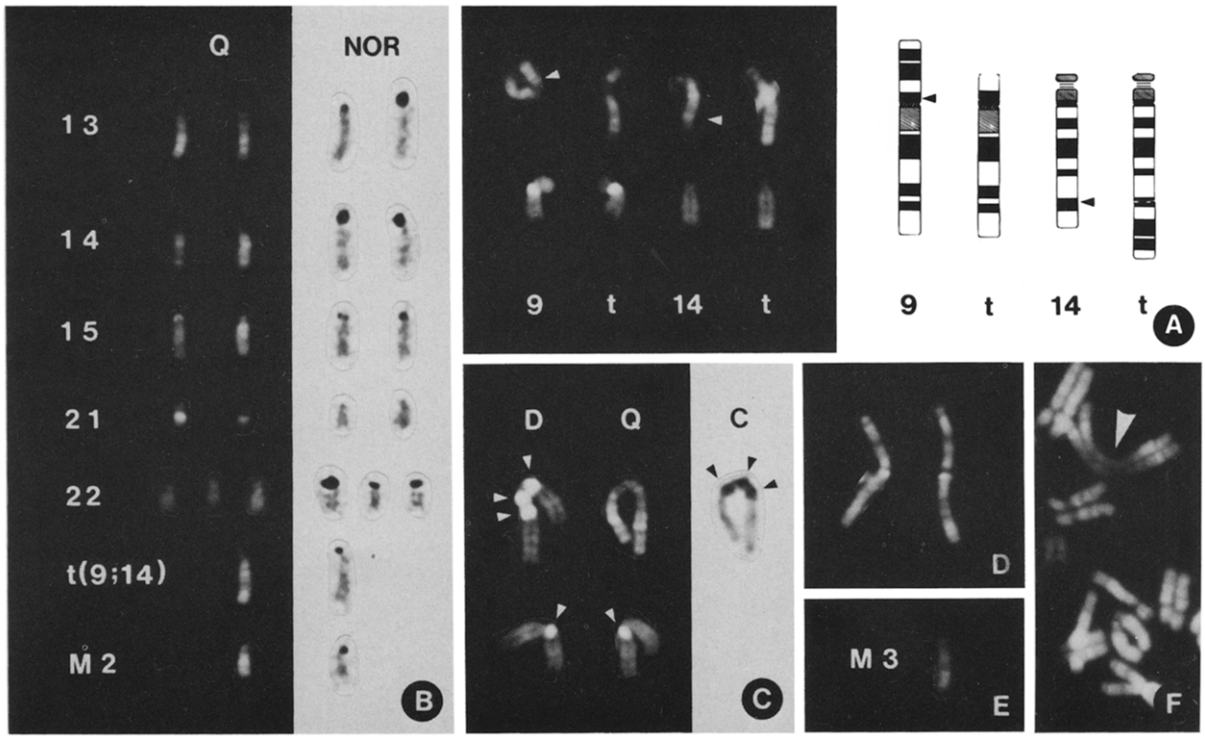

Fig. 4. Chromosomal findings in both cloned subpopulations. Staining with quinacrine mustard except where otherwise indicated. (A) Reciprocal translocation t(9;14) (p11; q24). Lower row: DA/DAPI staining. Diagram from ISCN [41]. Arrows on breakpoints. t: translocation chromosomes. (B) Acrocentric chromosomes from a single hyperdiploid metaphase consecutively stained with quinacrine mustard and silver to reveal Q-band polymorphisms and NOR. (C) Upper row: marker chromosome (M1) with two long arms of chromosome 1 and three amplified heterochromatic regions (arrows). (D) DA/DAPI staining; C: C banding. Lower row: DA/DAPI-stained normal chromosomes 1 from the hyperdiploid subline with arrows on heterochromatic regions. (D) Two normal chromosomes 3 from the hyperdiploid cell line. (E) Unidentified marker chromosome (M). (F) Part of endoreduplicated cell; arrow on M1.

Table 1.

DNA index ± S.D.	Plating efficiency	S-phase ± S.D.
1.57*	—	0.28 ± 0.03
1.57†	3.4	0.20 ± 0.03
1.26 ± 0.03‡	3.3	0.18 ± 0.03
2.51 ± 0.06§	2.9	0.20 ± 0.09

Characteristics of the patient tumor and tumor cells grown *in vitro*.
*Patient tumor.
†*In vitro* passage three.
‡Mean values from 23 samples in 18 different passages.
§Mean values from 25 samples in 21 different passages.

deletion, amplification or translocation of ribosomal genes must have occurred.

The cloned cell lines produced transplantable tumors in nude mice. Several hundred tumors in different experiments were examined with flow cytometric DNA analysis. The DNA content of the heterotransplanted tumors was identical to that of the corresponding *in vitro* grown cells, and the DI of the tumors was stable during passages 1-7 in nude mice.

A new subpopulation with DI = 2.73 appeared in passage 8 of the hypertetraploid tumor (Fig. 5A). The new subpopulation was present in all 28 tumors. The ratio between the two clones varied from 1:95 to 1:1 in the individual tumors. The tumor cell population with DI = 2.73 disappeared in passage 10 and could not be demonstrated in the subsequent passages. In the tumor with DI = 1.26 a population with DI = 1.39 was present in 2/12 tumors in passage 9 (Fig. 5B). Serial transplantations were performed on tumors containing the population with DI = 1.26 only, and in the subsequent passages no changes were found.

The histological structure and cellular morphology of the two subpopulations grown in nude mice were identical to those of the original patient tumor and dense core bodies were demonstrated by electron microscopy in both tumors.

Tests for production of ACTH, gastrin, calcitonin and methionine-enkephalin were found to be negative in both tumors.

Growth data of the two tumors was described according to a transformed Gompertz function and the growth curves were identical in both tumors; $A(\max) = 1096\ mm^2$, α hyperdiploid = -0.0338 and α hypertraploid = -0.0346.

The temporary presence of new tumor cell populations in the tumors did not cause any significant changes in the growth curves.

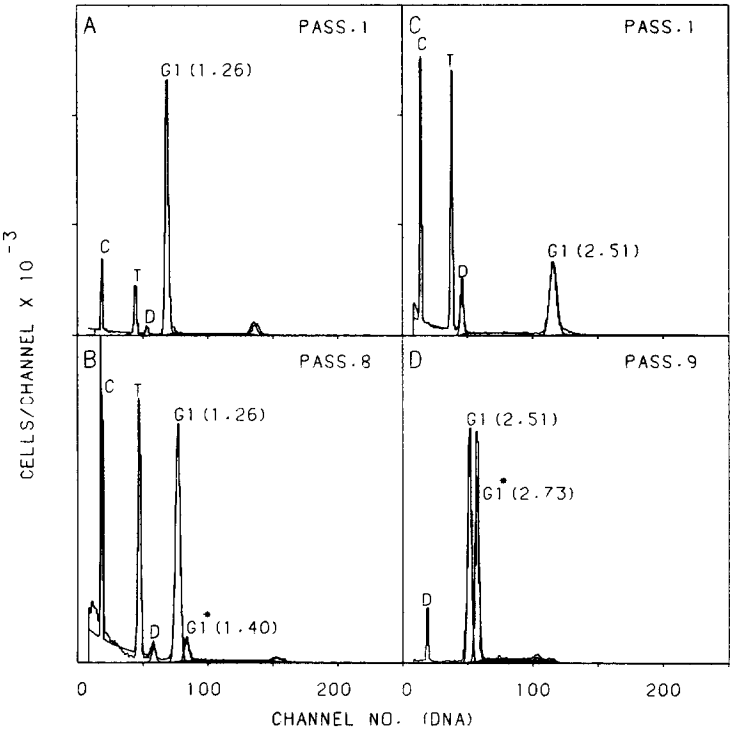

Fig. 5. DNA distribution of tumors established in nude mice from the two cloned cell lines. C and T represents internal standards to calculate the DI, the peak marked D represents normal mouse stroma cells and the G_1 fractions are indicated. The DI is indicated by the number in parentheses. (A) DNA distribution of mouse-grown tumor in passage 1, established from the cell line shown in Fig. 1(E). (B) DNA distribution of the same tumor after eight passages in nude mice. A new tumor cell population marked G is seen. (C) DNA distribution of mouse-grown tumor in passage 1, established from the cell line shown in Fig. 1(F). (D) DNA distribution of the same tumor after nine passages in nude mice. A new tumor cell population marked G* is seen.*

DISCUSSION

This study has demonstrated genetic instability of an SCCL grown *in vitro* and after transplantation into nude mice. The data presented provide no information on whether the subpopulations with DI = 1.26 and DI = 2.51 pre-existed in small fractions in the original tumor or arose from a mutation in one of the initial passages *in vitro*. However, the new subpopulations that appeared in the cloned tumors both *in vitro* and *in vivo* must be a result of genetic instability.

The chromosome analysis of the two cloned subpopulations in passages 17–19 identified several types of genetic instability which must have occurred both prior to and after cloning in passage 7. Since the same structural and numerical aberrations found in the hyperdiploid population were observed *in duplo* in the hypertetraploid population the two populations must have a common origin. The complete duplication of the genome must have taken place either prior to or after the *in vitro* culturing of the tumor. The occurrence of endoreduplicated mitosis in both subpopulations at passage 19 indicate that this type of instability still occurred *in vitro*.

Evidence of clonal evolution in the isolated subpopulations is represented by the aberrations that are different in the two subpopulations. Since the hyperdiploid line invariably contained only two copies of chromosome 5, this indicates that an extra copy of chromosome 5 must have been added to a hypertetraploid cell after duplication and after cloning. Similarly, the presence of two clones in the hyperdiploid population, differing only in the number of chromosome 19 (2 vs 3), indicates further clonal evolution.

Small differences in chromosome numbers can not be measured by flow cytometry [21], clearly demonstrated in the hyperdiploid cell line. Although a single peak with DI = 1.26 was observed by flow cytometry, chromosome analysis indicated that this population consisted of at least two clones, one with 56 and one with 57 chromosomes. In contrast to flow cytometry, only few cells are examined by chromosome analysis. Tumors may therefore contain an appreciable number of subpopulations not detectable by flow cytometry or by chromosome analysis.

It has been reported that the deletion of part of the short arm of chromosome 3 can be seen in cell lines from small cell lung cancer [28]. Other investigators have not been able to confirm this finding [29, 30]. In the present cell lines two normal chromosomes 3 were seen even by high-resolution analysis (Fig. 4D). By using quinacrine fluorescence, which highlights centromeric heterochromatic regions on most chromosomes 3, including the present ones, we were able to exclude the presence of centromeric regions derived from chromosome 3 on any of the markers. Thus we have found no evidence of a specific defect of chromosome 3 in these cell lines.

The presence of a translocation t(9;14) is interesting since specific reciprocal translocations have been identified in other malignant cell types, the prime examples being t(8;14) (q24;q32) in Burkitt's lymphoma [31] and t(9;22) (q24:q11) in CML [32]. Cell-transforming genes (oncogenes) have been mapped to the regions involved in these tumor-specific translocations [33], and it is believed that one of the carcinogenic steps may be alteration/activation of such oncogenes by the translocation [34]. Each of the translocation breakpoints found in the present cell lines (9p11;14q24) have been involved in structural rearrangements in some individual cases of malignancy [35], but whether specific genes associated with the malignant state may be present at 9p11 and/or 14q24 are unknown.

In a recent study SCCL was divided into pure SCCL (SCCL-P) and its morphological/biochemical variants (SCCL-V) [36]. The SCCL-V fail to produce peptide hormones, have no neurosecretory granules, have a higher plating efficiency *in vitro*, show a higher growth rate in nude mice compared to that of SCCL-P, and are characterized by the finding of a greatly amplified c-myco gene. The relative high plating efficiency and growth rate, and the negative finding on ACTH, gastrin, calcitonin and methionine-enkephalin in the here-described cell lines correspond well to the SCCL-V, except for the finding of dense core bodies in all the subpopulations.

It is obscure why one subpopulation (DI = 1.26) overgrew the other two populations from passage 7 to passage 10 of the uncloned cell line *in vitro*, since all the subpopulations detected demonstrated the ability to grow. It has been shown that near-tetraploid tumors often appear to contain an appreciable number of near-diploid cells, but no growth advantages have been demonstrated [37, 38]. Cell kinetics provide no explanation of the overgrowth, since no differences in S-phases were found (Table 1). Furthermore, the macroscopic growth curves of the hyperdiploid and hypertetraploid tumors were identical.

This disappearance of new subpopulations in the heterotransplanted tumors may be explained by the hypothesis of the creation of sterile subpopulations which stopped proliferating after a few number of divisions [38]. Another possible explanation could be selection during the transplantation procedure [39].

Tumor heterogeneity in model systems was previously described for experimental as well as for human tumors and the detection of genetic instability in experimental tumors of unicellular origin was described recently [7-12]. According to the theory of clonal evolution the pronounced heterogeneity in biological properties in SCCL are due to genetic instability. Therefore, it is not surprising to detect instability in the DNA content of these tumors *in vitro* and *in vivo* although the tumors were established from cloned cell lines. The results indicate that the inherited genetic instability is preserved in the model systems. Development of resistance to antineoplastic treatment may be due to heterogeneity in the sensitivity of different subpopulations. Isolation of subpopulations with different DNA contents from a single tumor makes it possible to study interaction between subpopulations in treated and untreated tumors.

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