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Growth and Ploidy of Human Osteosarcoma Xenografts in Serial Passage in Nude Mice*

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Abstract—*Six human osteosarcoma tumors have been transplanted to and maintained as xenografts in serial passage in nude mice. The xenografts, characterized by growth rate, histologic appearance, and DNA flow cytometric data, are now in the* 6–17 *passages in mice. Histologic appearances and growth rates of the xenografts were unchanged through all passages except for one xenograft in which the growth rate doubled in passage* 3. *All six tumors had hyperploid DNA contents. The ploidy levels were unchanged, compared to their original tumors, in all passages of five xenografts. One xenograft, concomitantly with the increase in growth rate, also doubled the ploidy from* 2.8 *to* 5.6C. *There was a good agreement between the proportion of S-phase cells and mitotic indices of the six xenografts. The growth rate, depending also on cell loss, was less related to the proliferative activity of the xenografts. It was concluded that osteosarcomas have a stable aneuploid DNA content in nude mice, but polyploidization can occur after a number of passages.*

INTRODUCTION

TRANSPLANTATION of human tumors to nude (athymic) mice has become an important tool in cancer research. The rationale for employing this model is the supposition that the human xenografts grow and respond to treatment in a similar fashion as the original tumor in the patient [1]. It is therefore essential that the xenografts retain the characteristics of the original tumors, even after several passages in mice. The stability of transplanted tumors have been evaluated by histologic appearance and growth rates [2,3], circulating human tumor markers [4], and chromosome analysis [5]. Additional means of characterizing xenografts are valuable, particularly if the nude mouse model is used for studying antineoplastic agents in human tumors [1].

Malignant tumors often exhibit chromosomal aberrations leading to increased (hyperploid) amounts of DNA in the cell nuclei, that can be quantified by DNA cytometry [6]. Since most osteosarcomas have hyperploid DNA contents [7,8], determination of ploidy may be used to characterize osteosarcoma xenografts and compare them with their original tumors. Determination of *S*-phase proportion of the cell cycle by DNA flow cytometry can be used to measure the proliferative activity of the xenografts.

Histologic appearances, growth rates, and DNA cytometric data of six human osteosarcomas, maintained in serial passage in nude mice for more than 1 yr, are presented. The purpose of the study was to determine if xenogenic osteosarcomas retain the ploidy of their original tumors, and to investigate the relation between growth rate and proliferative activity of the tumors.

MATERIALS AND METHODS

Animals

Six to 12 weeks old, female, nu/nu, BALB/c, mice (Harlan Sprague-Dawley Inc., Indianapolis, Indiana, U.S.A.) were kept in sterilized cages and fed a standard laboratory diet.

Tumors

Human tumor tissue, for transplantation to nude mice, was obtained either at biopsy or resection/amputation. No chemo-, immuno-, or radiotherapy

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had been given prior to surgery. The osteosarcoma tumors were numbered from T1 to T6. T1 was a Grade IV osteoblastic lesion of the distal femur of an 11 year old male. The original tumor was characterized by high cellularity with only negligible mineral and no mature bone. T2 was a Grade III osteoblastic lesion of the proximal femur of an 11 year old male. This tumor contained large amounts of osteoid, with smaller areas of chondroid differentiation, but no mature bone. The tumor was poorly vascularized but only minute areas of necrosis were seen. T3 was a Grade III fibroblastic lesion of the distal femur of a 62 year old female. The tumor was highly pleomorphic with large amounts of mineralized osteoid. T4 was a Grade IV osteoblastic lesion of the proximal humerus of an 18 year old male. Apart from larger areas of necrosis, the tumor had a similar appearance as T1. T5 was a Grade IV osteoblastic lesion of the distal femur of a 53 year old female. The tumor contained relatively large amounts of osteoid in some areas with chondroid differentiation, but no mature bone. Furthermore, the tumor displayed a variable mostly moderate cellularity and a large number of small vessels. T6 was a Grade IV chondroblastic lesion of the proximal tibia of a 16 year old male. The tumor was fairly well vascularized and contained moderate amounts of poorly mineralized osteoid. Although variable, some areas were cellular with high degree of pleomorphism.

Transplantation

Tumor tissue was minced, and one or two, 1–2 mm^3 pieces were transplanted subcutaneously over the flanks through a 5 mm incision. Four to ten mice in each passage were inoculated with tumor tissue under ether anaesthesia. The mice were killed when the tumors reached a diameter of approximately 15 mm. At autopsy tumor tissue was removed for histologic preparation, DNA flow cytometry, and inoculation in new mice. Since some tumors contained central necrosis, peripheral tumor tissue was chosen for transplantation. There was no selection between fast and slow growing tumors for transplantation between mice.

Growth rates

The diameters of the tumors were measured regularly with a caliper, tumor volumes were calculated for an elliptical sphere with the formula $\pi \times a \times b \times c/6$, where a, b, and c, are the diameters in three planes, respectively. The mean tumor volume was calculated from the log volumes of all tumors in each passage, and plotted on a semilogarithmic graph. Growth rate was expressed as the volume doubling time (VDT), calculated by the formula $\log 2 \times (t1-t0)/\log(v1-v0)$ where $v0$ was the volume at day $t0$, when a measurable tumor was first observed, and $v1$ was the volume on day $t1$, when the mice were killed.

Histologic examination

Original and transplanted tumor tissue were cut into 5 mm thick slices. The tumors were fixed by immersion in 2% paraformaldehyde — 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.4 [9] for at least 24 hr at room temperature, except for the original specimen of T2 which was fixed in 5% formalin and primarily embedded in paraffin. Some specimens were subsequently demineralized in 22% formic acid, 10% sodium citrate in water pH 1.9 for 1–3 days. The specimens were then dehydrated in several washings graded 70–95% ethanol and embedded in glycolmethacrylate (JB-4, Polysciences Inc., England). Approximately 1.5 μm thick sections were cut with a Sorvall JB 4A microtome from one to three blocks covering both central and peripheral parts of the tumor and stained with hematoxylin and eosin. The sections were examined by qualitative and semiquantitative light microscopy. The tumors were classified according to Dahlin [10] and graded according to Broders *et al.* [11]. Mitotic index was counted as the number of mitotic figures per 20 high power fields of vision (corresponding to approximately 1500–2000 cells) in the most cellular areas of the tumor [12].

DNA flow cytometry

The original tumor specimen and tumor tissue from at least two mice in each passage were analyzed with DNA flow cytometry by methods described in detail previously [13,14]. The specimens were squeezed through a nylon gauze with isotonic Tris–EDTA-buffer. After centrifugation the cells were fixed in ice cold 96% ethanol. The fixed cells were washed in Tris–EDTA-buffer with 1 μg/ml RNase. Suspensions of single cell nuclei were obtained by pepsin treatment and stained using 2.5×10^{-5}M ethidium bromide in Tris–EDTA-buffer with a molarity of 395 mOsm. The DNA content of the cell nuclei was determined in a flow cytometer ICP 11 (Phyme, West Germany, now Ortho Instruments, U.S.A.) equipped with a xenon lamp (Osram × BO 75 W). The excitation and emission wavelengths were 455–490 nm and 590–630 nm, respectively. The output was sorted with a 256 multichannel analyzer.

The flow cytometer was standardized with human lymphocytes, isolated in a Ficole gradient, and subsequently prepared at the same time as the tumor material. The amplitude of the light pulses from the lymphocytes was adjusted to channel number 70. If necessary, the illumination was adjusted so that the coefficient of variation was less than 3%. The ploidy level of the tumor cell popula-

tion was defined as the DNA value of the G1/G0 cells in the tumor cell population in relation to the DNA value of normal diploid cells (lymphocytes = 2C). The proportion of cells in various phases of the cell cycle was determined according to the simplified method of Baisch *et al.* [15] after correction for background. For further details see Tribukait [16].

RESULTS

All six xenografts grew as lobulated tumors with a surrounding pseudocapsule. The tumors did not invade underlying structures and no metastases were encountered. No host morbidity, that could be attributed to the tumors, was observed. The take rate varied considerably between different passages of all xenografts, but was usually more than 50%.

Even though the histological appearance of each tumor varied substantially from one field of vision to another the xenografts retained the basic characteristics of their original tumors, both as regards type and grade. For example T3, a Grade III tumor with abundant osteoid formation, also exhibited similar amounts of extracellular matrix with the same degree of mineralization in mice (Fig. 1). T4 was originally, as well as in the mouse, highly anaplastic with large areas of necrosis and only scanty areas of osteoid without mineralization (Fig. 2). The mitotic indices of the original tumors were generally lower than their xenografts, especially T2 in which the original tumor was fixed in formalin and paraffin embedded.

The range in growth rate of the first five xenografts, expressed as the mean VDT, was 5.8 days (T1) to 14.9 days (T3) (Table 1). There was no sign of altered growth rate with increasing number of passages of these five xenografts (Fig. 3). The growth rate showed a tendency to diminish with increasing tumor volumes. Although the VDT was nearly constant, the tumor volume at a certain time after transplantation showed considerable variability. This is demonstrated in Table 2 where the VDT of nine tumors of T1 in passage 5, together with the tumor volumes 40 days after transplantation, are presented.

T6 grew slowly in the first two passages with VDT of 19 and 13 days, respectively (Table 3). In passage 3, the VDT was reduced to only 6 days, and in the subsequent 2 passages retained this fast rate of growth (Fig. 3). The increase in growth rate was not accompanied by changes in histologic appearance, but the xenograft underwent polyploidization as described below.

Five tumor were aneuploid (3.7C, 3.9C, 2.7C, 2.9C, 2.8C), while one (T1) was diploid–tetraploid (Table 1). The ploidy levels were the same as the original tumors in all passages in mice (Fig. 4), except for T6. The original tumor of T6 had one aneuploid peak at 2.8C and at the double value, 5.6C, a rather high additional peak was seen (Fig. 5). There was an additional small peak at 11.2C, indicating that some of the cells at 5.6C were proliferating. In the first two passages of T6 the DNA histograms were unchanged, but in passage 3 the xenograft had almost completely lost the 2.8C peak in all four tumors of this passage (Fig. 5). This doubling of the ploidy level occurred concomitantly with a doubling of the growth rate of this xenograft (Table 3). In the following two passages the xenograft retained this higher ploidy level and the 2.8C peak completely disappeared. The proportion of cells in *S*-phase of T6 was however approximately the same, when calculated from the 2.8C peak in passages 1–2 and the 5.6C peak in passages 3–4. In passage 5 the proportion of *S*-phase cells was lower than in previous passages but the growth rate was unchanged. In T3, which originally contained a rather low proportion of aneuploid cells at 3.9C, the proportion of this aneuploid cell population increased considerably in passage 8, which may also indicate further development towards aneuploidy (Fig. 4).

For the purpose of evaluating the correlation of the different parameters of proliferation, the mean proportion of *S*-phase cells of each of the 6 xenografts was plotted against the mean VDT (Fig. 6a), and against the mean mitotic indices (Fig. 6b). There was a poor correlation between the proportions of *S*-phase cells and VDT, whereas the mitotic indices and the *S*-phase proportions were linearly correlated.

DISCUSSION

The six human osteosarcoma xenografts presented here were characterized by histologic appearance and ploidy level. In all initial xenografts these parameters were in good agreement with their original tumors. With the exception of T6, the growth rates were quite stable through all passages, in concurrence with reports of different tumors transplanted from children to nude mice [17]. Other investigators have noted increased growth rate after the first passages [18–20].

The growth rate of individual tumors in the same passage was also constant, which contrasts to a previous report of neuroblastoma xenografts in nude mice, where large differences in growth rate were observed [21]. This apparent disagreement may actually be due to differences in defining the growth rate. In the referred study the growth rate was defined as the tumor area 3 weeks after inoculation, instead of the VDT as in the present study. This difference in defining the growth rate was illustrated by T1 where the VDT of nine tumors in passage 5 varied considerably less than the final volumes, and there was no correlation

Table 1. Characteristics of the five human osteosarcomas T1–T5 and their xenografts in nude mice

Tumor	Passages in mice	VDT (days)*	Mitotic index*†	Cells in S-phase (%)*	Ploidy level‡
T1					
Original			28	15	2.0 + 4.0C
Xenografts	14	5.8 (2.2)	29 (7.1)	22 (3.3)	2.0 + 4.0C
T2					
Original			1 §	16	3.7C
Xenografts	11	7.0 (1.6)	16 (4.1)	14 (2.7)	3.6C (3.6–3.8)
T3					
Original			4	4	3.9C
Xenografts	9	14.9 (3.2)	14 (5.7)	7 (2.9)	3.9C (3.8–4.1)
T4					
Original			24	44	2.7C
Xenografts	10	8.7 (1.9)	42 (9.6)	25 (4.5)	2.7C (2.6–2.8)
T5					
Original			24	21	2.9C
Xenografts	6	10.2 (1.3)	19 (4.4)	20 (2.9)	3.0C (2.9–3.0)

* Values are means with standard deviations in parentheses of all passages from untreated mice. There were 2–10 tumors in each passage.
† Number of mitoses/20 high power fields.
‡ Diploid cell populations have DNA relative values of 2C. Range in parentheses.
§ Original tumor was fixed in formalin and paraffin embedded.

between VDT and tumor size. We suggest that the VDT is the most adequate expression of growth rate, since only observed growth is included in the calculation, whereas other expressions of growth rate include the time from tumor inoculation until a tumor is first observed, the so called lag-time. In the previously mentioned study of neuroblastoma, flow DNA cytometry was also performed with calculation of the size of different cell cycle proportions. The percentage of cells in *S*-phase showed a small variability and was constant and independent of tumor size at 3 weeks, which strengthens the view that tumor volume at a specified period after transplantation is not a relevant parameter of tumor growth. The VDT has however other limitations as an expression of the proliferative activity of tumors, which will be discussed below.

Table 2. VDT, calculated from volumes at 15 *and* 40 days *after transplantation, and tumor volumes at day* 40 *of nine tumors of T1 in passage* 5. *There was one tumor per mouse*

Tumor	VDT (days)	Volume (mm^3)
a	4.8	1469
b	5.9	75
c	4.4	880
d	4.3	3024
e	4.1	1762
f	5.5	904
g	3.8	367
h	4.0	2835
i	4.0	1232
Mean (SD)	4.5 (.7)	1394 (1012)

In agreement with previous studies of human tumor lines in nude mice the histologic appearance of the transplanted tumors was similar to the original tumors [17], and also produced the type of extracellular matrix that is characteristic of osteosarcoma of different types. We did not observe any mouse derived new bone formation surrounding the tumors, as has been described after implantation of lyophylized osteosarcoma tissue to nude mice [22]. Although variation occurred, the mitotic indices were of the same magnitude from passage to passage in each of the six tumors. The very low values obtained in the original tumor of T2 may be explained by the fact that this tumor specimen was fixed in formalin and primarily embedded in paraffin. It is well known that such fixation and embedding procedure gives rise to considerable artefacts compared to paraformaldehyde-glutaraldehyde fixation [9] and subsequent plastic embedding [23]. Original tumors were fixed 2–4 hr after surgery, whereas xenografts were fixed immediately upon removal from the mice. This may explain the lower mitotic indices in original tumors since the recorded mitotic activity is also influenced by the mode and rate of fixation [24]. Other investigators have also reported increased mitotic activity in xenografts as compared to original tumors [25]. Therefore the higher mitotic indices in xenografts may be due to tumor heterogeneity, differences in cell loss, and tumor nutrition.

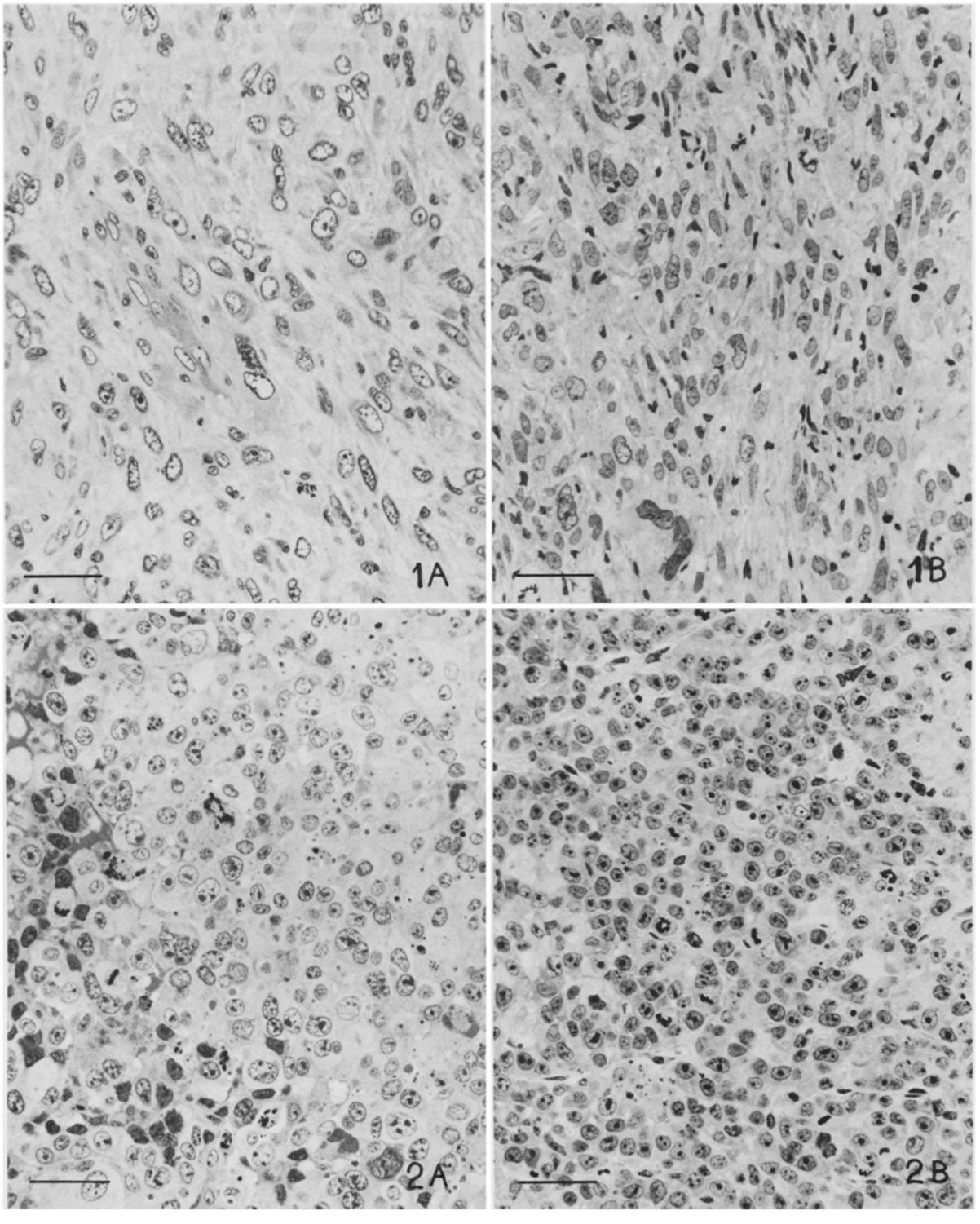

Fig. 1. *T3 a Grade III fibroblastic osteosarcoma.* (*A*) *Original tumor; and* (*B*) *after* 6 *passages in nude mice. Hematoxylin and eosin stain. Bar* = 30 μm.

Fig. 2. *T4 a Grade IV osteoblastic osteosarcoma.* (*A*) *Original tumor; and* (*B*) *after* 5 *passages in mice. Hematoxylin and eosin stain. Bar* = 30 μm.

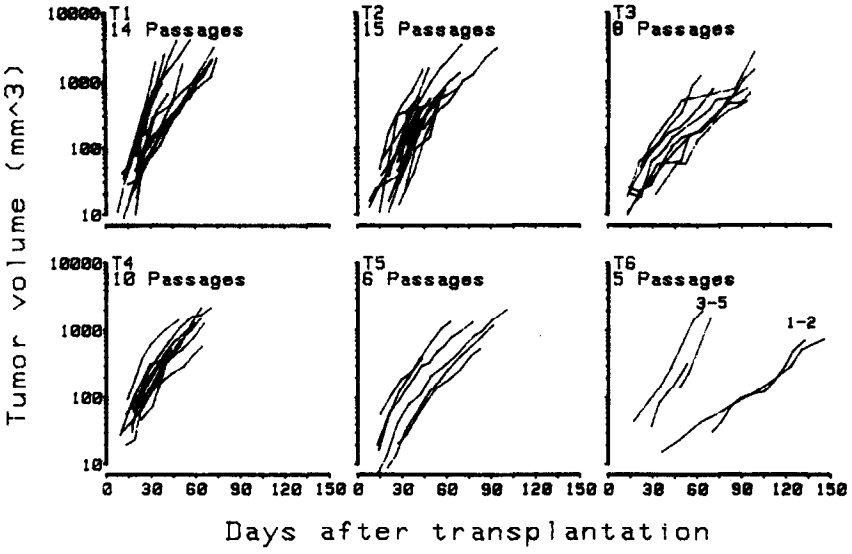

Fig. 3. Growth curves of human osteosarcoma xenografts T1–T6. Each line represents the mean of 2 or more tumors in each passage. The figures in the chart of T6 refers to passage number. Logarithmic volume scale.

Chromosomal abnormalities of malignant tumors are detected by flow DNA cytometry, due to increased amounts of DNA [6]. All six osteosarcoms were hyperploid, confirming previously reported clinical DNA cytometric studies of osteosarcoma [7,8]. In five of these, the ploidy levels were unchanged even after more than 2 yr passage in mice. Thus no subsequent major chromosomal changes occurred in these tumors that could be detected cytochemically. The sole exception was T6 in which the 5.6C peak became predominant over the 2.8C peak in passage 3. There was thus no change to a different aneuploid DNA content, but only selection of the 5.6C cells. Similarly a colonic carcinoma xenograft in nude mice, studied with flow DNA cytometry, underwent a selection of aneuploid polyploid clones [26]. However the change in ploidy from 2.6C to 5.2C of this tumor occurred in passage 56, 4 yr after transplantation to mice, and not in the first few passages as in T6 in this study. It thus appears that aneuploid DNA content is a relatively stable alteration in the chromosomal content of these tumors, but a selection of polyploid clones can occur, which was also

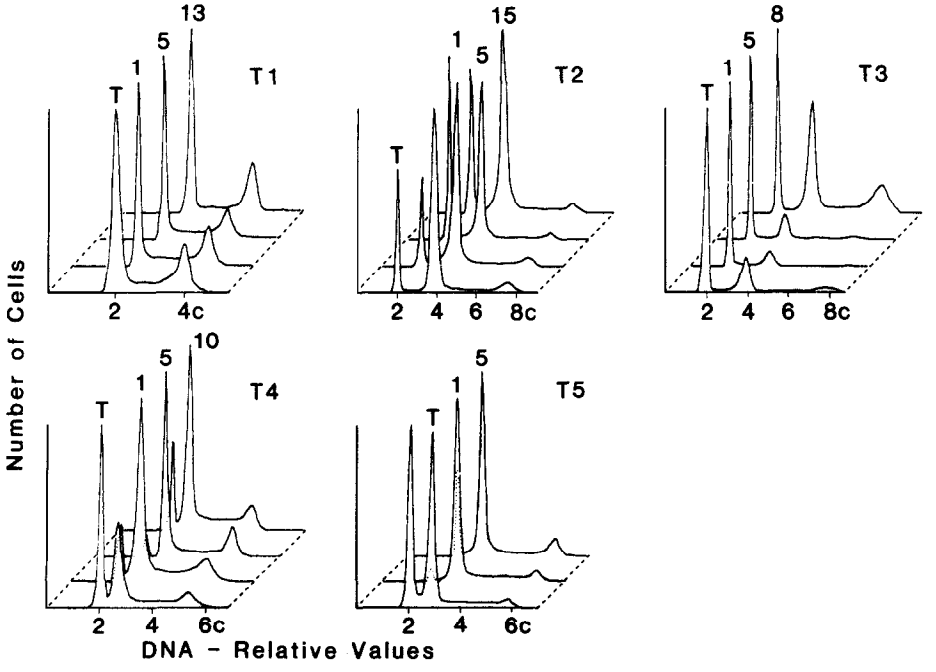

Fig. 4. Representative DNA histograms of osteosarcomas T1–T5. T refers to the original tumors and the figures to passage number. Note the higher peaks at 4C and 8C of T3 in passage 8.

Table 3. Characteristics of the original tumor T6 and tumors of the 5 first passages in nude mice

	VDT (days)	Mitotic index	Cells in *S*-phase (%)	Ploidy level
Original passage		7	23	2.8C
1	19	34	30	2.8C
2	13	21	30	2.8C
3	6	16	28	5.6C
4	8	19	26	5.6C
5	8	21	17	5.6C

suggested in passage 8 of T3. Even if the ploidy level of the xenografts were stable during the observation period of more than one year, continuous monitoring of tumors of subsequent passages are necessary to ensure that no clonal evolutions occur. This is especially important since clonal subpopulations may have different sensitivity to antineoplastic drugs [27].

S-phase determinations of bone tumors may entail difficulties because of debris and necrosis in these mineralized tumors. The proportion of *S*-phase cells were however fairly well reproducible. The means of the *S*-phase proportions of all passages of each tumor were linearly correlated with the means of mitotic indices. These two methods quantify independently the proportion of proliferating cells, which strengthens the assertion that they are relevant for judging the proliferative activity. We wish to emphasize that individual tumors showed considerable variation in these parameters. When relating the proportion of *S*-phase cells to the growth of the tumor it should be noticed that the

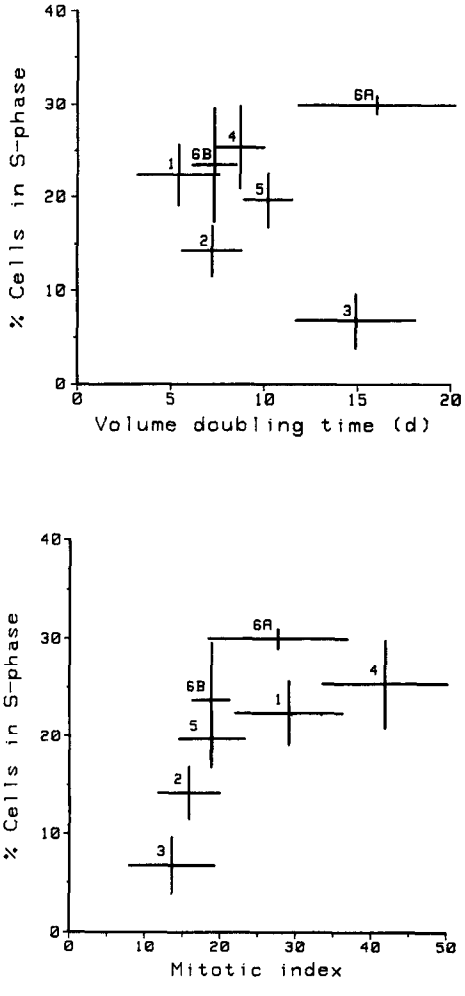

Fig. 6 (a) Relation between the S-phase proportion and volume doubling time; and (b) relation between the S-phase proportion and mitotic index of the 6 osteosarcoma xenografts. The intersections represent the means and the lines 2 SD. The numbers refer to tumor numbers. 6A refers to passages 1–2 and 6B to passages 3–5 of T6.

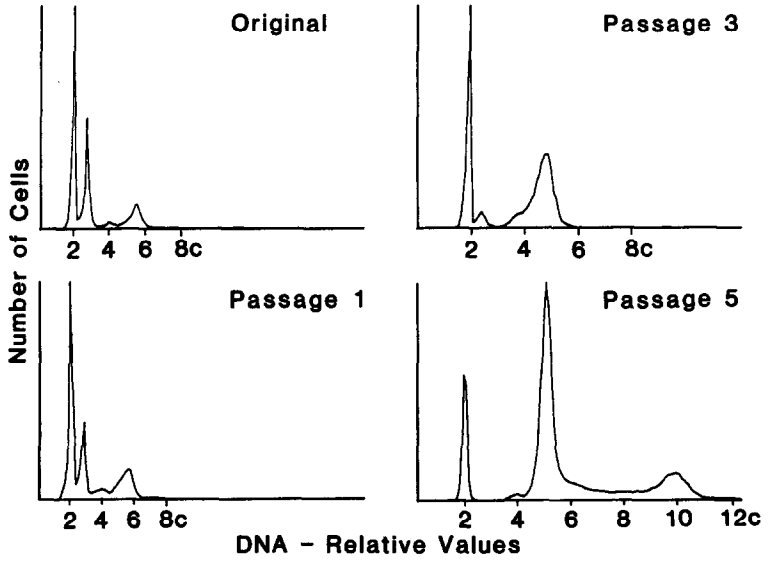

Fig. 5. Representative DNA histograms of T6, illustrating the change from 2.8C to 5.6C in passage 3. The original tumor had a small 11.2C peak which could be seen with magnification.

growth rate is the net of cell proliferation and cell loss, and is further dependent on bleeding and extracellular matrix formation. These factors may explain the poor correlation between the *S*-phase proportions and VDT. A doubling of cell volume, and protein and RNA contents is normally found in G2 cells, compared to G1 cells [28]. An additional factor influencing growth rate might be the volume of the individual cells. The observation in T6 of a doubling of the growth rate simultaneously with doubling of the nuclear DNA content, without increase in *S*-phase proportion or mitotic index, supports this suggestion.

The present study has shown that DNA cytometric analysis of osteosarcoma tumors, and their xenografts, may be a valuable method of characterizing tumors maintained in nude mice. DNA cytometry provides important information, not only in analyzing the effect of chemotherapy, but also when investigating the relation between growth, cell proliferation, and ploidy level of human tumors in nude mice.

REFERENCES

1. Golden A, Johnson RK, Venditti JM. Usefulness and limitations of murine tumor models for the identification of new antitumor agents. *Antibiotics Chemother* 1980, **28**, 1–7.
2. Fogh J, Fogh JM, Orfeo T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 1977, **59**, 221–225.
3. Fogh J, Tiso J, Orfeo T, Sharkey FE, Daniels WP, Fogh JM. Thirty-four lines of six human tumor categories established in nude mice. *J Natl Cancer Inst* 1980, **64**, 745–751.
4. DiPersio L, Kyriazis AP, Michael JG, Pesce AJ. Monitoring the therapy of human tumor xenografts in nude mice by the use of lactate dehydrogenase. *J Natl Cancer Inst* 1979, **62**, 375–379.
5. Fogh JM, Fogh J. Comparison of a human tumor cell line before and after growth, Chromosome analysis. In: Fogh J, Giovanella BC, eds. *The Nude Mice in Experimental and Clinical Research*. New York, Academic Press 1978, 225–226.
6. Barlogie B, Raber MN, Schumann J, Johnson TS, Drevinko B, Swartzenburger DE, Göhde W, Andreff M, Freireich EJ. Flow cytometry in clinical cancer research. *Cancer Res* 1983, **43**, 3982–3997.
7. Kreicbergs A, Broström L-Å, Cewrien G, Einhorn S. Cellular DNA content in human osteosarcoma. Aspects on diagnosis and prognosis. *Cancer* 1982, **50**, 2476–2481.
8. Kreicbergs A, Silvferswärd C, Tribukait B. Flow DNA analysis of primary bone tumors, relationship between cellular DNA content and histopathologic classification. *Cancer* 1984, **53**, 129–136.
9. Karnovsky MJ. A formaldehyde-glutaraldehyde fixative of high osmolarity for the use in electron microscopy. *J Cell Biol* 1965, **27**, 137–138.
10. Dahlin DC. *Bone Tumors: General Aspects and Data on* 3987 *Cases* (2nd edn). Springfield, Illinois, Charles C. Thomas, 1967, 156–157.
11. Broders AC, Hargrave R, Meyerding HW. Pathological features of soft tissue fibrosarcoma with special reference to the grading of its malignancy. *Surg Gynecol Obstet* 1939, **69**, 267–280.
12. Price C. Osteogenic sarcoma: an analysis of survival and its relationship to histologic grading and structure. *J Bone Joint Surg* 1961, **43B**, 300–313.
13. Tribukait B, Moberger G, Zetterberg A. Methodological aspects on rapid-flow cytofluorometry for DNA analysis of human urinary bladder cells, In: *First Int Symp on Pulse Cytophotometry* (Nijmegen) 1974. Ghent, European Press Medikon 1975, 50–60.
14. Tribukait B, Esposti P. Comparative cytofluorometric and cytomorphological studies in non-neoplastic and neoplastic human urothelium. *Pulse-Cytophotometry*. Ghent, European Press 1976, 176–178.
15. Baisch H, Göhde W, Linden WA. Analysis of PCP-data to determine the fraction of cells in the various phases of the cell cycle. *Rad Environ Biophys* 1975, **12**, 31–39.
16. Tribukait B. Flow cytometry in surgical pathology and cytology of tumors of the genito-urinary tract, In: Koss LG; Coleman DV, eds. *Advances in Clinical Cytology*. New York, Masson 1984, Vol II, 163–189.
17. Neely JE, Ballard ET, Britt AL, Workman L. Characteristics of 85 pediatric tumors heterotransplanted into nude mice. *Expl Cell Biol* 1983, **51**, 217–227.
18. Mattern J, Wayss K, Haag D, Toomes H, Volm M. Different growth rates of lung tumours in man and their xenografts in nude mice. *Europ J Cancer* 1980, **16**, 289–291.
19. Nilsson OS, Bauer HCF, Broström L-Å. Growth characteristics of human malignant tumours and cell lines in serial passages in nude mice. *Acta Orthop Scand* 1983, **54**, 75–79.
20. Wennerberg J. Changes in growth pattern of human squamous cell carcinomas of the head and neck during serial passages in nude mice. *Int J Cancer* 1984, **33**, 245–250.
21. Sordillo P, Hansen H, Helson L, Helson C. Cytofluorometric analysis of tumours in nude mice, In: Sparrow S, ed. *Immunodeficient Animals in Cancer Research*. London, Macmillan Press 1980, 191–206.

22. Urist MR, Grant TT, Lindholm TS, Mirra JM, Hirano H, Finerman GAM. Induction of new-bone formation in the host bed by human bone-tumor transplants in athymic nude mice. *J Bone Joint Surg* 1979, **61-A**, 1207–1216.
23. Weibel ER, Knight BW. A morphometric study on the thickness of the pulmonary air-blood barrier. *J Cell Biol* 1964, **21**, 367–384.
24. Graem N, Helweg-Larsen K. Mitotic activity and delay in fixation of tumour tissue. *Acta Pathol Microbiol Scand* 1979, **87**, Sect A, 375–378.
25. Sharkey FE, Bains-Grebner M. Increased mitotic activity in human tumors transplanted to athymic nude mice. In: Reed ND, ed. *Proc Third Int Workshop on Nude Mice*. New York, Gustav Fischer 1982, 457–462.
26. Vindelöv 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. *Expl Cell Biol* 1982, **50**, 216–221.
27. Heppner GH, Dexter DL, DeNucci T, Miller FR, Calabresi P. Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. *Cancer Res* 1978, **38**, 3758–3763.
28. Skog S, Tribukait B. Discontinuous RNA and protein syntheses and accumulation during cell cycle of Erlich ascites tumor cells. *Exp Cell Res* 1985, **159**, 510–518.