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Growth Characteristics of Head and Neck Squamous Cell Carcinoma in Nude Mice

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Abstract—*Growth behaviour of human squamous cell carcinoma from the head and neck region was studied in nude mice. Tumour growth was observed in* 10 *out of* 13 *tumours transplanted with a primary take rate of* 77 *and* 100% *for serial passaging. The take rate percentage related to the number of tumour inocula used, varied from* 10 *to* 90% *in the first passage and from* 70 *to* 100% *in the subsequent passages. No significant difference could be established in the growth character, except from the first to the second passage. Histological studies demonstrated preservation of the original histopathological features, micro- and macroinvasion of two tumour lines into the fibrous capsule and cyst formation. Comparison of growth curves with histological features demonstrated that growth curves are not a reliable measure for the number of vital tumour cells present.*

INTRODUCTION

SINCE the first successful transplantaion of a human colonic carcinoma in athymic nude mice [1], many studies dealing with xenotransplantation of a large variety of malignant tumours in this animal model have been reported [2–4].

These tumours present during repeated passages in this animal a histopathological picture, which is very similar to that of the original tumour [3, 5, 6], although the degree of differentiation may change [3, 4, 6, 7]. In addition, the human origin of the growing tumour cells has repeatedly been demonstrated by chromosome analysis [8]. The degree of success in establishing a tumour line varies considerably between various tumour types and appears to be largely dependent on the degree of cellular differentiation and whether tumours are used from the primary site, from metastases or from recurrent tumours [2, 4, 5, 9–12]. At present, only limited data are available on the transplantability and growth behaviour of squamous cell carcinoma of the head and neck region in the nude mouse [10, 13–18]. These studies reveal a rather low take rate, varying between 26 and 40% for primary takes and of about 50% for serial passaging. Although the factors responsible for this invariably low take rate are not fully understood, it is discured that the degree of differentiation [10, 15] and the possibility of bacterial contamination of the primary grafts [10] play an important role. However, Wennerberg *et al.* [15] failed to find a significant difference in take rate between specimens taken as non-sterile biopsies and as sterile excisions. Growth characteristics of these tumour xenografts have only been determined from measurements of tumours grafted subcutaneously and hardly any data are available which compare growth curves and histological features. This report deals with transplantation of a series of head and neck squamous cell carcinoma with special reference to primary take rate and serial passaging as well as to the interrelationship between histological features and growth curves.

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METHODS

Tumour tissue was obtained from fresh surgical specimens of previously untreated squamous cell carcinomas from different primary sites in the head and neck region. A solid piece of tumour measuring about 1 × 1 cm was dissected from the surgical specimen, under sterile conditions, immediately after removal from the patient. Since the surface of the tumour is always contaminated, the specimens were dissected from the deeper parts of the tumour and the necrotic tissue was removed. Further cleansing was achieved by rising the tumour tissue three times in cold balanced Hanks solution (4° C). The tissue was subsequently stored in this solution until use.

Transplantation was performed within 1 hr of

tumour excision. For inoculation of the tumour into the nude mouse, the tissue block was cut into pieces of about 1–2 mm^3 and these were implanted subcutaneously in the flanks of ten nude mice (female Balb/c, nu/nu, age 7–12 weeks, obtained from TNO-breeding unit, Rijswijk, The Netherlands). The mice were kept in cages with filter caps and provided with sterile bedding, food and drinking water containing Terramycin (250 mg/l). The ambient room temperature was 26° C and the air humidity 70%. For serial transplantation, the tumour graft was removed under ether anaesthesia and the same procedure was followed as for the primary transplants. The number of mice used for each tumour varied between 10 and 20.

Tumour growth was measured with the use of a Vernier caliper at weekly intervals, and biweekly in the case of rapidly growing tumours. Tumour volume was calculated as 0.5 × the product of the three dimensions and was plotted on a linear scale [19]. For histological studies, fragments from the original tumour and the entire tumours grown in the mouse, were fixed in buffered formaldehyde (4%; pH 7.4). After dehydration in graded alcohols and paraffin embedding, sections (7 mu) were stained either with haematoxylin-eosin, methyl-green pyronin or Masson–Goldner for keratin.

RESULTS

A total number of 11 primary squamous cell carcinomas and two lymph node metastasis were transplanted. The sites of origin, the TNM- and the histopathological classification are shown in Table 1. Primary takes were observed in 10 out of 13 tumours corresponding to 77%. Tumour lines were established from all these tumours (100%). The passage levels are indicated in Table 1. Only five of the tumours are presently carried in continuous passage.

The take-rates, i.e. the number of takes in relation to the number of mice transplanted for the first and subsequent passages, are summarized in Table 2. These data show a very low primary take for seven tumours, considerably increasing, sometimes to 100%, during serial passaging.

Growth curves

Growth curves of transplants from the same tumour revealed a large variation. This was true not only for the primary transplants, but also for the subsequent passages as shown in Fig. 1 for tumour 5. The variation in the growth curves, increasing with time after transplantation could not be related to the size of the inoculum, since it appeared that transplantation of tumour fragments of different size failed to show any clear correlation

Table 1. *Head and neck squamous cell carcinoma transplanted in nude mice*

Localisation	Histology	Passage
1. Supraglottic $T_1N_2M_0$	Poorly diff., keratinizing	No growth
2. Epiglottic $T_2N_1M_0$	Mod. diff., keratinzing	
– primary tumor		10th
– Lymph node metastasis		10th
3. Epiglottic $T_4N_1M_0$	Poorly to mod. diff., keratinising	No growth
4. Piriform sinus $T_2N_1M_0$	Mod. diff., keratinizing	10th
5. Tongue $T_2N_0M_0$	Mod. diff., keratinizing	5th
6. Tongue $T_2N_0M_0$	Mod. to well diff., keratinizing	6th
7. Epiglottic $T_1N_0M_0$	Well to mod. diff., Focally keratinizing	3rd*
8. Tongue $T_1N_0M_0$	Mod. diff., non keratinizing	3rd*
9. Supraglottic $T_3N_0M_0$	Mod. to well diff., keratinizing	4th
10. Piriform sinus $T_1N_1M_0$	Mod. to well diff., keratinizing	No growth
– lymph node metastasis		
11. Tongue $T_2N_1M_0$	Mod. diff., keratinizing	3rd*
12. Tongue $T_2N_0M_0$	Poorly to mod. diff., non keratinizing	2nd*
Total number 13	Prim. takes 10/13 (77%) Line establishment 10 (100%)	

*Terminated at indicated passage

Table 2. Percentual take rate (number of takes in relation to the number of mice transplanted). For the first passage 10 mice were used. For serial passaging the number of mice varied between 10 and 20

Tumour	Passages									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
1.	0									
2. Prim. tumour	30(3/10)	80(8/10)	100(10/10)	100(20/20)	85(17/20)	85(17/20)	100(20/20)	100(2/20)	100(20/20)	100(20/20
2. L. node metast.	10(1/10)	33(3/10)	86(12/14)	100(18/18)	100(20/20)	90(18/20)	100(17/17)	100(10/10)	100(10/10)	90(18/20)
3.	0									
4.	10(1/10)	80(8/10)	92(12/13)	93(14/15)	80(16/20)	80(12/15)	85(17/20)	70(14/20)	100(10/10)	75(15/20)
5.	100(10/10)	100(15/15)	93(14/15)	100(8/8)						
6.	30(3/10)	100(10/10)	87(13/15)	100(20/20)	80(16/20)	80(12/15)				
7.	10(1/10)	90(9/10)	100(10/10)							
8.	20(2/10)	90(9/10)	90(9/10)							
9.	20(2/10)	20(2/10)	100(10/10)	100(10/10)						
10. L. node metast.	0(0/10)									
11.	90(9/10)	60(6/10)	80(8/10)							
12.	80(8/10)	90(9/10)								

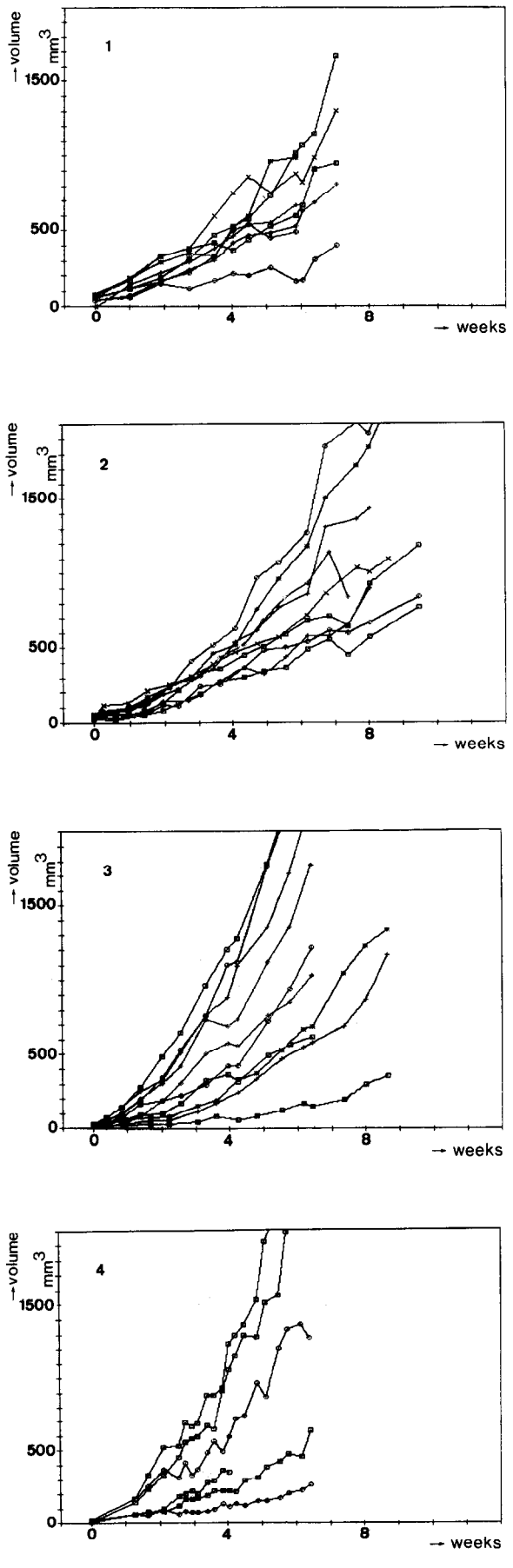


Fig. 1. Growth curves of tumour 5 during the first four passages. Growth curves of tumours which were used for histological studies, during the first 5 weeks are not indicated.

between size and the type of growth curve. In order to study the possibility as to whether selection of fast and slow growing populations of tumour cells might be the underlying cause of the observed variation of the growth curves, fragments of both tumours with a flat and a steep growth curve were transplanted. Both resulted in a comparable variation of the growth curve pattern. Because of this large individual variation of growth curves obtained from one tumour, these curves could not be used as a reliable parameter for calculating the growth rate in the various passages. Therefore the duration of the initial lag-phase, i.e. the time needed for the tumour to obtain a general mean diameter of about 4 mm, was chosen as the parameter for growth.

The initial lag-phase appeared to be the largest for the first passage and to decrease in the higher passages to a rather stable value, as determined for six tumours (Table 3). The initial lag-phase for the first passage appeared to vary between 3.6 and 9.8 weeks, with the exception of tumour 7 which showed the extra-ordinarily high value of 33 weeks. In further passages, the initial lag-phase varied between 1.1 and 5.1 weeks. No significant difference was found for this value between the primary tumour 2 and its lymph node metastasis.

Histology

Histological examination of the transplanted tumours demonstrated preservation of the original histopathological characteristics up to the highest passage level studied. Only tumour 7 showed a slight dedifferentiation. Metastasis to lymph nodes or other organs was not observed in any of these lines. Throughout the observation period all tumours were surrounded by a fibrous capsule with many capillaries. Because of the very low number of tumours available from the primary passages, histology of tumour growth was studied during serial passaging

After 1 week, the implantation site showed scattered islands of proliferating tumour cells concentrated in the periphery, accumulations of extravasated erythrocytes, fibroblasts and capillaries (Fig. 2 A, B). This area was separated from the surrounding host tissue by a layer of fibroblasts with numerous granulocytes and capillaries. After 2 weeks, the islands of tumour cells had grown together (tumour size about 2 mm^3), remaining separated from each other by connective tissue septa of different sizes (Fig. 3A). This resulted in a tumour mass composed of various compartments (Fig. 3 B, C). The connective tissue septa were covered by a layer of cells containing numerous mitotic figures, as was the periphery of the tumour.

During further enlargement of the tumour, necrosis and/or desquamation became visible in

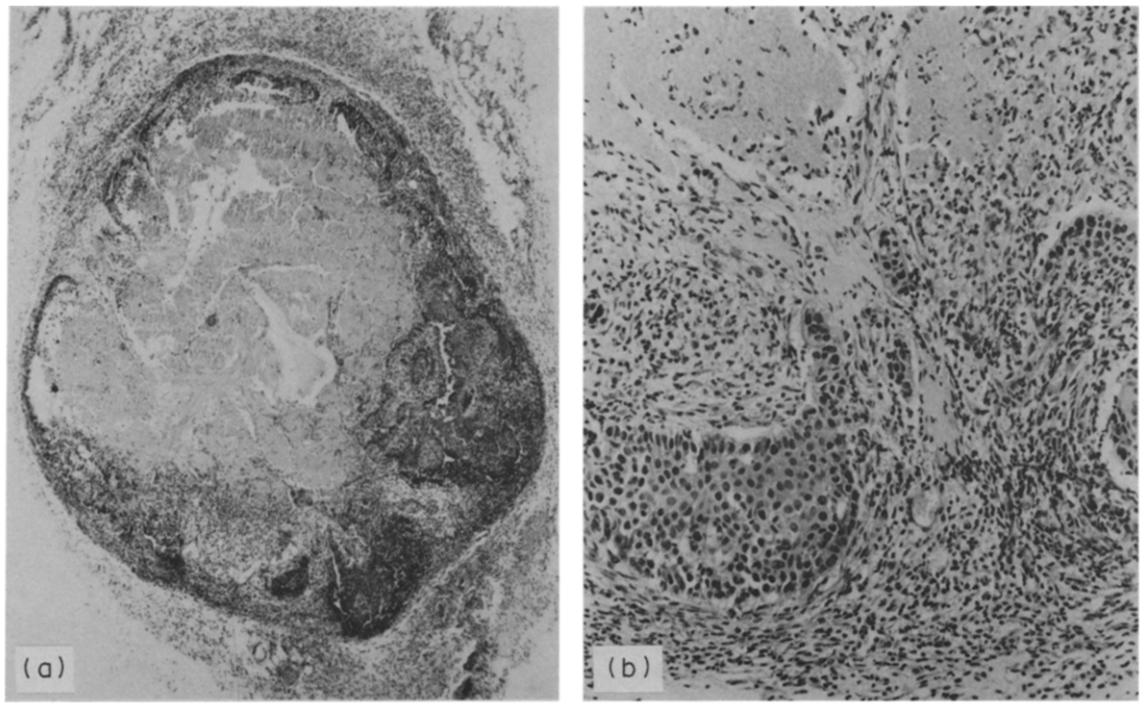


Fig. 2. *Micrographs* (*survey* A *and detail* B) *of growth characteristics of tumour* 4 (4th *passage*), *after* 1 week. *The site of implantation is surrounded by a fibrous capsule. Clusters of growing tumour cells are present in the peripheral part. The central part contains extravasal erythrocytes and fibrocytes* (*Haematoxylin–eosin*, A × 40; B × 125).

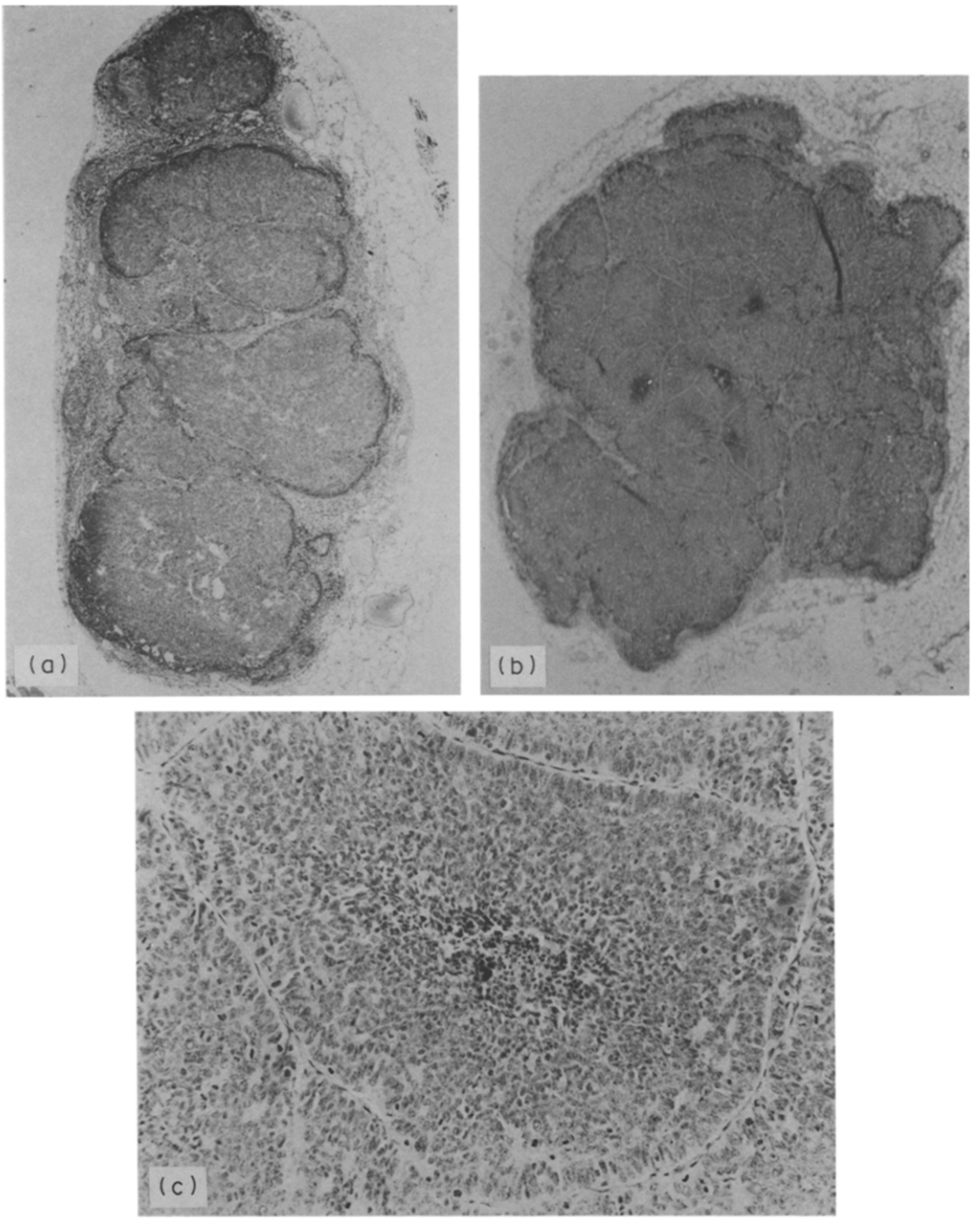


Fig. 3. *Micrographs of tumour* 4 (4th *passage*) *after two* (A; *tumour size* 2.0 mm^3) *and* 3 weeks (B, C; *tumour size* 12.0 mm^3). *Fig.* A *shows encapsulated tumour mass with connective tissue septa of varying size in between. Fig.* B *shows one massive tumour, composed of various compartments. First signs of cellular necrosis, clearly visible at higher magnification in* C(× 125) (*Methylgreen pyronin.*)

Table 3. Initial tumour growth of six established head and neck squamous cell carcinoma lines. Initial time (in weeks ±S.D.; n: *number of tumors measured) is defined as the time needed to obtain a general mean diameter of about* 4 mm

Tumor	Passages									
	1	2	3	4	5	6	7	8	9	10
2(prim	7 0.50 (n=5)		3.48 ±0.63 (n=10)	3.52 ±1.67 (n=20)	3.17 ±1.24 (n=17)	3.24 ±2.15 (n=17)	2.60 ±0.08 (n=20)	3.02 ±1.20 (n=20)	2.86 ±0.53 (n=20)	3.77 ±1.74 (n=20)
2(metast)	7.4 (n=1)		5.10 ±0.20 (n=12)	4.53 ±1.63 (n=18)	3.45 ±1.57 (n=20)	4.35 ±1.70 (n=19)	3.01 ±1.11 (n=17)	4.05 ±2.54 (n=10)	3.80 ±0.01 (n=10)	3.20 ±0.92 (n=18)
4	9.8 (n=1)	4.30 ±0.80 (n=8)	3.65 ±2.00 (n=12)	2.46 ±0.70 (n=13)	2.67 ±1.16 (n=16)	2.05 ±0.04 (n=10)	1.55 ±0.81 (n=17)	1.63 ±0.35 (n=14)	2.06 ±1.01 (n=20)	2.47 ±0.96 (n=15)
5	3.6 ±0.01 (n=10)	1.19 ±0.06 (n=14)	1.80 ±0.08 (n=15)	2.10 ±0.10 (n=12)	1.97 ±0.18 (n=8)					
6	9.10 ±0.02 (n=3)	1.08 ±0.30 (n=10)	2.68 ±0.71 (n=13)	3.81 ±1.28 (n=20)						
7	33 (n=1)	3.13 ±0.38 (n=9)	3.76 ±1.85 (n=10)							

the central parts of the tumour. This was often already observed at a tumour size of 30–50 mm^3 (Figs. 4A, 5A). The presence of cellular necrosis and/or keratin appeared to differ from line to line and was related to the degree of keratinization and the size of the tumours (Figs. 4A, B; 5A, B). In all tumours larger than 100 mm^3, the major part of the tumours consisted of keratin and debris, (Figs. 5B; 6A) sometimes showing a typical onion skin appearance (Fig. 5B). A layer of vital tumour cells, often showing local discontinuities and of varying thickness was present in the periphery and along the connective tissue septa or remnants of these structures which contained capillaries (Fig. 6A, B). Throughout the observation periods, micro- and macroinvasion of tumour cells into the fibrous capsule was frequently found in two tumour lines (2 and 4) (Fig. 7A, B). This gave rise to the formation of satellite tumours, sometimes resulting in a lobular appearance. The earliest capsular invasion was already demonstrable after two weeks at a tumour size of 2 mm^3.

At a tumour size of more than 500 mm^3, an increasing number of tumours started to fluctuate on palpation and appeared to be filled with a viscous fluid. In the non-keratinizing tumours such cystic development could already be found at a much smaller size.

Apart from differences in degree, this course of events was noticed in all tumour lines studied during various passages. Tumours which from their growth curves were classified as slow growing, revealed the same histologic features as fast growing tumours after comparable survival times.

DISCUSSION

The present study demonstrated that tumour lines of squamous cell carcinoma from the head and neck region can be established in nude athymic mice, retaining the original histopathological features of the donor tumours. No metastasis to lymph nodes or other organs was observed. These observations generally agree with those made by others [10, 15, 16, 20], although both increased and decreased cellular differentiation have been described by Sharkey *et al.* [4] and Braakhuis *et al.* [10]. Kyriasis *et al.* reported a lymph node metastasis of a human epidermoid carcinoma of the larynx in nude mice [21, 22].

The transplants grew as circumscribed tumours without infiltration into the surrounding tissue, except for the invasion in the fibrous capsule. A comparable observation has been described with a laryngeal epidermoid carcinoma by Kyriasis *et al.* [21].

In comparison with other studies on these tumours, a higher take rate in primary and serial passaging has been obtained in this study. This discrepancy is difficult to explain, but the high take-rate obtained (10/13 primary graft takes) demonstrates that head and neck squamous cell carcinoma has been transplanted more successfully than in previous experiments [10, 15]. Moreover, it indicated that they do not belong to the category

of tumours which are difficult to grow as gastric, mammary and genital tract tumours.

Since it has been shown that poorly differentiated tumours had a better primary take rate than moderately and well differentiated head and neck squamous cell carcinoma [10, 15], the degree of differentiation of the transplanted tumours might be held responsible for this difference in take rate. However, the present findings give no support to this, although the number of tested tumours is rather small. In this study a primary take rate of 77% was obtained chiefly with moderately to well differentiated tumours, while two out of the three tumours which failed to take were poorly to moderately differentiated.

Also tumour site, [10, 15, 23] tumour size, [15, 23] malignancy grading [23] and the clinical course [24, 25] have been shown to influence tumour growth in nude mice. According to Wennerberg *et al.* [23] tumour size appeared to be more important than the degree of differentiation. However, the small number of tumours of various categories tested in the present study and the absence of data on the clinical course do not permit to conclude on such a relationship.

A more acceptable explanation for the difference in take rate between the present study and those of others is likely to be found in the number of inocula transplanted. The data given in Table 1 indicate that the number of primary takes with respect to the number of inocula used is often very low (sometimes only 1 out of 10).

In the further passages the take rate considerably increases, sometimes reaching 100%. This is a confirmation of previous studies [10, 26].

Determination of the growth of squamous cell carcinoma xenografts appeared to be difficult, owing to the large divergency between growth curves of the same tumour. This variation could not be attributed to the presence of fast and slow curving population of tumour cells or to the size of the inocula, since initially the nutrition of the transplanted tumour is merely dependent on diffusion and therefore only the cells in the peripheral part will survive (Fig. 2A, B) [27]. With a larger inoculum only the initial lag-phase will change.

Although host factors relating to the mitigated immune response and to the vascular supply of the tumours cannot be entirely excluded as possible explanations for the variation in growth curves, the histological observations suggest that accumulation of keratin and/or cellular debris within the tumour contributes to a large extent to this. This study demonstrates that increase in the size of tumours beyond 50 mm^3 is passively influenced by cellular debris and desquamation products. This influence increases with the size of the tumour and shows individual variation. Calculation of growth rate of larger squamous cell carcinoma xenografts, from tumour doubling time is therefore an unreliable measure, since it is not related to the fraction of vital tumour cells, but to the amount of accumulated dead material. In view of these observations, the time needed for the tumour to obtain a general mean diameter of 4 mm proved to be a more reliable parameter in determining tumour growth characteristics.

This approach revealed a rather long initial lag-phase for primary transplants, decreasing in the subsequent passages to a limiting maximum value, and varying between different tumour lines. A comparable difference between primary transplant time and passage time has also been established by other authors for various other human tumour lines [8, 11, 28].

These data together with the difference in the percentage take-rate for primary transplants and serial passaging, are suggestive of an adaption of the tumour to the changed environmental conditions in the host which is assumed to result in an increase in the growth fraction as well as a reduction in the cell loss factor in subsequent passages [29–31]. The decrease in passage time observed between first and subsequent passages corresponds with the increased mitosis from first to second passage reported by Fu and Steel for rat mammary tumours [31] and with the increase in *S*-phase percentage cells in human lung tumours [32]. An increased rate of ^{3}H-thymidine incorporatic in tumour DNA in the second passage when compared with the first passage of head and neck squamous cell carcinoma, was recently reported by Wennerberg [15]. He suggested that this might be explained by a recruitment of G_0 cells or by stem cell selection of clones of rapidly proliferating cells. This latter suggestion seems to be in conflict with the observation of Lindenberger [3] who, using flow cytofluorometry, failed to find any change in DNA-distribution in xenografts of squamous cell carcinoma from first to third passage.

Summarizing, this study demonstrates that moderately to well differentiated squamous cell carcinoma from the head and neck region can be successfully established as lines in athymic nude mice. Combined studies of both growth curves and histology are indispensable when investigating growth rate and growth behaviour, owing to the absence of a distinct relationship between tumour size and the amount of vital tumour tissue. This is of special importance when this model is used for evaluating the effect of various therapies.

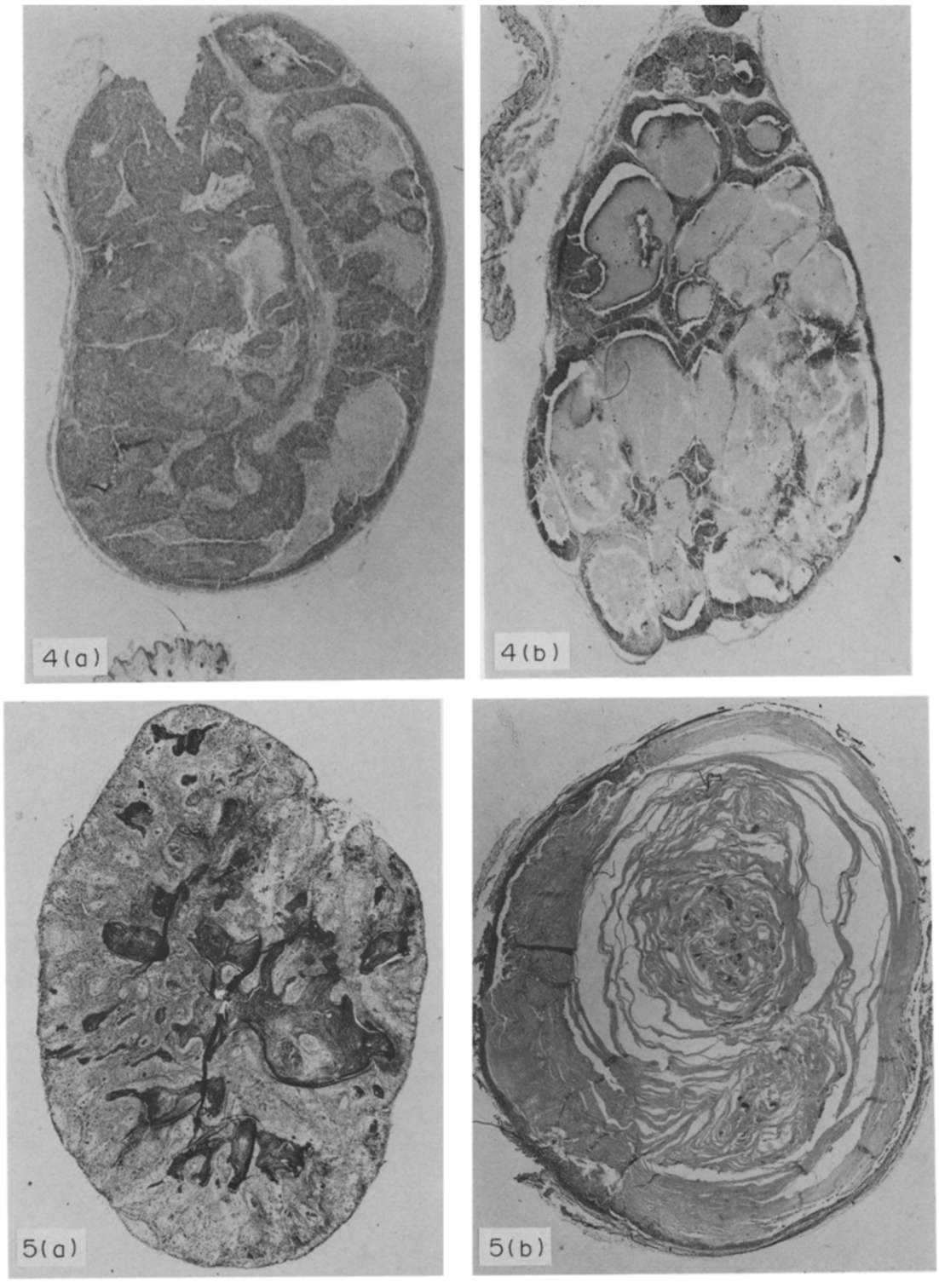
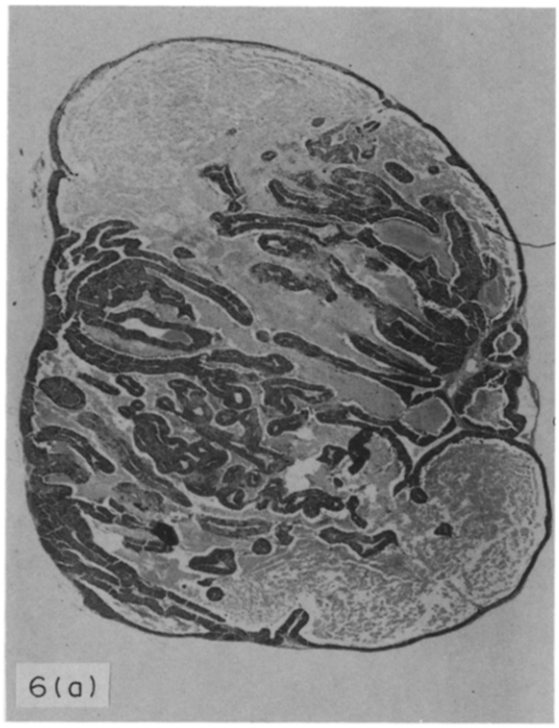
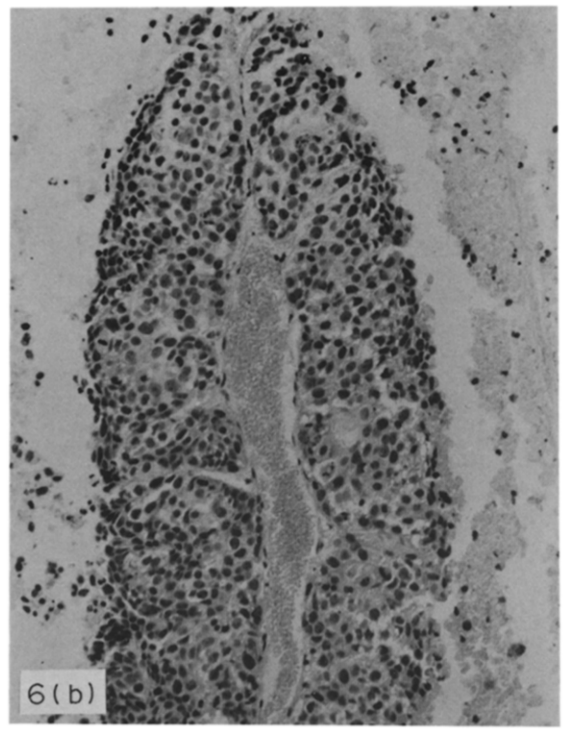
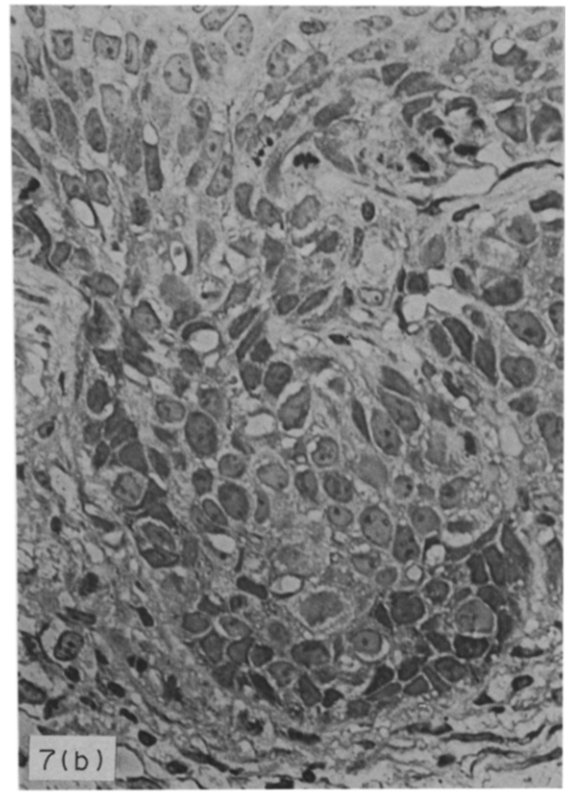


Fig. 4. *Micrographs of tumour* 4 *after* 4 weeks (A; *tumour size* 40 mm^3) *showing severe necrosis and after* 5 weeks (B; *tumour size* 80 mm^3). *By far the largest part of the tumour consists of dead material. This tumor is only slightly keratinizing* (*Haematoxylin–eosin*).

Fig. 5. A. *Micrograph of tumour* 2 (*metastasis*, 4th *passage*) *after* 4 weeks (*tumor size* 50 mm^3). *Note starting keratinization in the central parts. This tumour is moderately keratinizing* (*Haematoxylin–eosin*). B. *Onion peel appearance of the strongly keratinizing tumour* 9 (*tumour size* 200 mm^3) *after* 6 weeks. *This tumor consists of a large mass of keratin, surrounded by a thin layer of vital tumour cells* (*Haematoxylin–eosin*).







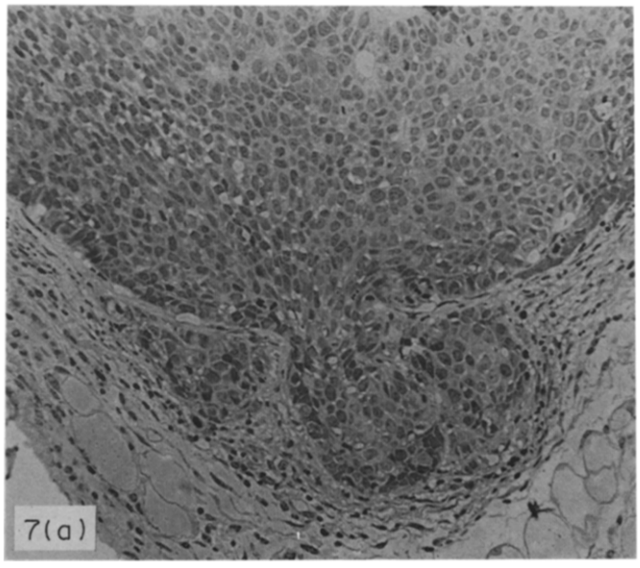


Fig. 6. *Micrograph of tumour* 4 (4th *passage*) *after* 6 weeks (*tumour size* 300 mm^3). *Vital cells are only present in the periphery and along the vascularized connective tissue septa.* B. *Higher magnification of septum with vital tumour cells and central capillary* (*Haematoxylin–eosin*, B × 125).

Fig. 7.A,B *Invasion of tumour cells into the fibrous capsule. Tumour* 4, 2 weeks *after transplantation* (4th *passage*)

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