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Comparative Studies of Normal, 'Spontaneously' Transformed and Malignant Human Urothelium Cells *in Vitro**

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Abstract—*In numerous* in vitro *studies of cell-mediated cytotoxicity directed against transitional cell carcinomas (TCC) urothelial cells of normal origin have served as control material. In the present work, which includes 13 human bladder cell lines of non-malignant or malignant origin, a comparison was made of morphology, chromosomal mode, growth fraction and tumorigenicity in nude mice. Signs of apparently spontaneous transformation were seen in two cell lines of non-malignant origin. The possibilities for cross-contamination as an explanation of these signs of 'spontaneous' transformation are discussed. During these studies radiation-dependent cytotoxicity of disposable microtissue culture plates was revealed as another important source of error.*

INTRODUCTION

THE urothelium-derived cell lines described in the following report were established for use in a series of immunological investigations [1–5]. Similar lines have been propagated in other laboratories [6–15].

However, bladder cell lines propagated *in vitro* vary markedly in morphology, chromosomal number, growth pattern, differentiation and sensitivity to cell-mediated cytotoxicity. Using such cells in *in vitro* immunological tests therefore necessitates a detailed analysis and classification [5, 16, 17]. We have previously reported studies of the sensitivity of a number of our cell lines to natural cytotoxicity, their HLA antigen expression [5] and ability to invade and destroy normal tissue *in vitro* [18]. In the present investigation our cell lines were allocated to four groups based on (1) origin of the cultured material, (2) growth fraction, (3) morphological characteristics and (4) chromosomal mode. The tumorigenicity of nine lines was tested in nude mice and the apparently spontaneous transformation of two lines of normal origin is described and discussed. Also important for future immunological studies is the finding that microplates may be toxic to human bladder cells. A possible correlation between clinical and pathological malignancy of donor tissue and the *in vitro* growth potential was also looked for, even where such a correlation has not been found by others, either in conventional monolayer cell cultures [7, 8, 14, 19–21] or in more complex culture systems [22].

A detailed comparison of the growth pattern of the present cell lines and a series of human bladder cell lines of normal origin will be published later.

MATERIALS AND METHODS

Cell cultures

Tissue was minced into small fragments by scissors in a dry disposable Petri dish and placed on the dry substratum of a 25-cm² flask (Falcon 3031 or NUNC) to which 5 ml of medium were added while the flask was kept upside down. In order to facilitate the attachment of the explant

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the flask was left in this position for 2 hr at 37°C in 5% CO_2-enriched humidified air. After this preincubation the tissue was floated with medium by slowly turning the flask right side up.

The flask was then left untouched for 2 days. The medium was changed 2–3 times per week, depending on the cell density. When a confluent monolayer of cells was observed the cells were transferred by treatment with 0.25% trypsin in phosphate-buffered saline (PBS) minus calcium and magnesium. Mycoplasma tests were carried out by the method described by Hayflick [23]. FIB 41B culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) was used throughout the experiments [2]. Two series of experiments were carried out. In pilot experiments with HU 456, HU 549 and HU 609 the medium was supplemented with penicillin (250,000 IU/l) and streptomycin (25 mg/l). In a second series of consecutive explantations the medium in primary cultures was further supplemented with gentamycin (50 mg/l), but after the first 2–3 days all cultures were left without antibiotics. The normal urothelium cell line HCV 29 [24] and the TCC-derived T 24 cell line [6], both obtained from J. Fogh, were included in this study for comparison. They were propagated in the same medium as the other cell lines with penicillin and streptomycin. Lines not in use were stored in 10% dimethylsulfoxide (DMSO) in liquid nitrogen.

Growth fraction

The cells were seeded in disposable Petri dishes from NUNC for continuous [^{3}H]-thymidine labeling. The medium was changed 3 days later and on the following day the cells were incubated with tritium-labeled thymidine ([^{3}H]-TdR; 1 μCi/ml, Amersham) at 37°C with 5% CO_2 in air. When cells were incubated for more than 24 hr with [^{3}H]-TdR the medium was changed daily, with new medium containing the same concentration of [^{3}H]-TdR. After incubation times varying from 1 to 72 hr the labeling was terminated by incubation with 2 mM 'cold' thymidine for 0.5 hr. The cultures were then rinsed twice with PBS, fixed in 1 part acetic acid plus 3 parts of 96% ethanol for 5 min and finally air-dried. Autoradiography was performed with Kodak nuclear track emulsion type NTB2 (exposure time 6–8 days). After developing by Kodak D-19 and fixation in Kodafix solution the cultures were again air-dried and finally faintly stained with a 10% Giemsa stain for 1–2 min. The percentage of labeled nuclei (labeling index: LI) was based on counting a total of 500 cells. Growth fraction (maximal labeling index) was estimated according to Smets [25].

Growth in microtissue culture plates

HU 456 and T 24 cells were seeded in disposable microtissue culture plates No. 3034 from Falcon or the corresponding plates from NUNC with 60 wells, each containing 20 μl of cell suspension in tissue culture medium supplemented with antibiotics and 10% FBS. Approximately 100 cells per well were inoculated at time 0. After incubation for 3 days cultures were rinsed in PBS, fixed in methanol, stained by Giemsa stain and counted semiautomatically in a Leitz classimate [2]. Growth was investigated on Falcon plates from different lots and on NUNC plates pretreated with varying doses of ^{60}Co radiation.

Light microscopy

Living cells were investigated in an inverted phase contrast microscope. For the final morphological investigation of cell cultures the monolayers were washed twice in phosphate-buffered saline, pH 7.4 (PBS), fixed for 10 min or more in methanol, stained in 15% Giemsa stain in water for 15–20 min, washed and destained for 2–5 min in water and finally air-dried.

Electron microscopy

Specimens for electron microscopy of cell lines HU 609, HU 456 and T 24 were obtained by trypsinizing the cells and then centrifuging the material at 400 *g* for 20 min at 4°C. The pellets were fixed in 0.2 M Karnowsky fixative, pH 7.2, at 4°C for 3–10 hr, washed in 0.2 M cacodylate buffer, pH 7.2, at room temperature, post-fixed in 1% osmium in *S*-collidine buffer, dehydrated in ethanol solutions and embedded in Epon 821. Sections were cut on a Reichert Ultra Microtome OM-U3, stained with 2% uranyl acetate for 20 min and counterstained with a solution of lead citrate. Sections were examined under a Philips electron microscope 301 operating at 80 kV.

Chromosome analysis

Chromosomes were counted after 4 hr of treatment with 4 mg/l colcemid in well spread metaphases. After 15–30 min of pretreatment with Tyrode's solution diluted 1/10 the cells were fixed in modified Carnoy's fluid, consisting of glacial acetic acid and 99% ethanol(1/3) and stained with Giemsa stain.

Heterotransplantation

Nude mice were inoculated with the cell lines indicated in Table 4. The experiments were made with 4- to 5-week-old nude mice of both sexes, the progeny of 10–12 backcross cycles in a gene transfer to BALB/c mice (BALB/c/A/BOM/Fib, $1-(\frac{1}{2})^{10-12}$. The animals were kept under minimal disease conditions and given sterile food and

drinking water. Cells were grown in large numbers in roller bottles or conventional plastic tissue culture flasks. Removed from the bottles by trypsinization, the cells were washed 3 times in PBS and resuspended in a minimal volume (0.1–0.2 ml) of PBS. Cell suspensions were injected subcutaneously into the right flank of nude mice. The number of cells and animals used in each experiment is shown in Table 4. Tumors derived from the T 24 cell line in nude mice were serially transplanted into new nude recipients. These animals were inoculated with finely minced tumor tissue. Serial transplantation with tumors derived from other cell lines was not attempted. Following inoculation mice were observed once a week for tumor development at the inoculation site. The animals were examined for possible metastases to lymph nodes and organs. Tumor specimens from nude mice were fixed in formalin, embedded in paraffin and sections of 7 μm were stained with hematoxylin and eosin, periodic acid Schiff (PAS) and alcian blue stain. Sections from mouse-grown tumors were compared with histological slides of biopsy material from tumors in patients from whom the cell lines were derived.

Secondary culture

Tumors from nude mice were re-explanted *in vitro* using the same technique as described for primary cultures. Chromosome analysis was also carried out in re-explanted cells.

RESULTS

Establishment of cell lines

The material is displayed in Table 1. HU 456, HU 549 and HU 609 cell lines were established in pilot experiments carried out to evaluate different media. Cultures showing rapid primary outgrowth were selected for further cultivation. HU 697–HU 1325 represents the material of a consecutive series of explantations where all cultures were kept under the standardized conditions already described. Cultures showing no primary cell outgrowth after 2 weeks were discarded. Primary outgrowth was obtained from 19 of 28 TCC in the series and from a biopsy of a squamous cell carcinoma of the bladder. Growth could usually be seen within 48 hr of cultivation. The resulting islands of cells surrounding the original explants were composed of epithelial cells and sometimes a very few fibroblasts (Fig. 1). Fibroblast overgrowth did not occur. The primary outgrowth phase usually lasted 1–2 weeks, followed by a phase of sluggish growth lasting several weeks. In the group of cultures selected from previous experiments HU 456 did not enter into sluggish growth until its first passage. The material derived from a squamous cell carcinoma (HU 1305) showed no sluggish growth at any time. Following the phase of sluggish growth, cells in most of the cultures slowly died off. However, in cultures from 6 TCC patients in the consecutive series the sluggish growth phase was followed by a secondary growth phase during which five cell lines were established. These lines have been propagated for 15–24 passages so far. One line (HU 1207) stopped growing in the 7th passage. All lines were stored in liquid nitrogen. None were contaminated by mycoplasmas. In Table 1 it can be seen that there is no correlation between the clinical or histological tumor classification and the ability of the explants to produce primary or secondary growth. Similarly, no correlation could be established between the course of disease and the *in vitro* growth potential (Table 2).

Classification of cell lines

The established cell lines could be allocated into four groups according to origin, chromosome number, morphology and growth fraction, as shown in Table 3. The fast-proliferating tumorigenic lines (group B, Table 3) included two 'spontaneously' transformed sublines derived from the two normal lines in group A.

Toxicity of microtissue culture plates

When using the present cell lines for microcytotoxicity assays of cell-mediated immunity it was found that the microplates sometimes exerted a toxic effect. Figure 2 shows the results of growth studies of HU 456 in Falcon microculture plates from two different lots. Two different culture media were used: freshly prepared medium and medium which had been stored at room temperature for 1 month before use. Clearly, growth was severely impeded by plates from one lot, whereas no significant difference due to the medium could be seen. The curves represent the best and the poorest lots of plates from Falcon. Growth results with some other lots were in between.

Similar results were obtained with T 24 cells. The significance of radiation sterilization by ^{60}Co was demonstrated by the growth of HU 456 in microculture plates from NUNC exposed to 0.25–0.75 Mrad before use (Fig. 3). At time 0 100 cells per well were seeded. After 3 days of incubation in nonradiated plates the cell number had increased to about 500 cells per well, while a marked and dose-dependent cytotoxicity was noted on plates after radiation. Other lines, for instance of melanoma or osteosarcoma cells, were less sensitive to the toxic effect of microplates.

Table 1. Growth results in relation to clinical and pathological classifications of tissue donor patients

Patient No.	Age (yr)	Sex	T	Gr.	Inv.	Epithelial growth Prim.	Epithelial growth Sec.	Max. pass. No.
HU 456	72	M	2	1	*	+	+	100
HU 549	66	M	2	2	0	+	+	20
HU 609	49	F	normal†			+	+	67
HU 697	79	M	1	2	0	+	0	
HU 812	62	M	1	1	0	+	0	
HU 826	67	M	2	3	+	0	0	
HU 863	68	M	1	3	0	0	0	
HU 891	58	M	1	2	0	0	0	
HU 961	65	M	1	2	0	+	+	24
HU 1125	63	M	1	1	0	+	+	17
HU 1197	65	M	1	2	+	+	0	
HU 1206	67	M	1	1	0	+	0	
HU 1207	75	F	1	1	0	+	+	7
HU 1209	74	F	3	3	+	+	0	
HU 1210	85	F	3	3	+	+	+	15
HU 1212	69	F	1	3	?	0	0	
HU 1213	67	F	2	3	+	+	0	
HU 1233	75	M	1	2	0	+	0	
HU 1244	66	F	1	2	0	+	+	15
HU 1251	66	M	1	2	0	+	0	
HU 1257	63	M	‡	3	?	+	0	
HU 1264	75	M	1	3	0	+	+	16
HU 1266	60	M	2	3	+	0	0	
HU 1275	67	M	1	1	0	+	0	
HU 1277	70	M	2	3	+	0	0	
HU 1292	68	F	1	1	0	+	0	
HU 1305	48	M	squamous			+	+	23
HU 1308	73	F	1	1	0	+	0	
HU 1309	85	M	1	1	0	0	0	
HU 1310	46	F	1	3	+	+	0	
HU 1324	83	F	1	2	0	0	0	
HU 1326	48	M	1	1	0	0	0	

T = T1–T4: the TNM-classification of malignant tumors, Geneva 1974; Gr. = Gr.1–Gr.4: grade classification [26]; Inv. = + or 0 indicates presence or absence of microscopical invasiveness; Prim. = primary growth *in vitro*; Sec. = growth through several *in vitro* cell passages; Max. pass. No. = highest *in vitro* passage number obtained. HU 1207 stopped growing in passage 7. The remaining cultures are still proliferating.

*Microscopy of the biopsy revealed no invasiveness despite large masses of tumor tissue in the bladder. Only two months later widespread TCC metastases were found.

†Donor nephrectomized for a clear cell-type renal carcinoma.

‡TCC in the renal pelvis.

Table 2. Tumor growth in TCC tissue donor patients for 2 yr following explanation in relation to in vitro *growth*

	Patients with non-progressive tumor disease	Patients with progressive tumor disease	
Primary growth from explant	14	5	
			($2P > 0.05$)
No growth from explant	6	3	
Secondary growth from explant	6	0	
			($2P > 0.05$)
No secondary growth from explant	14	8	
Primary but no secondary growth	8	5	
			($2P > 0.05$)
Primary and secondary growth	6	0	

$2P$ = level of significance, estimated by Fischer's exact test.

Table 3. Allocation of cell lines from normal or malignant urothelium into 4 groups, A–D, with common characteristics

	Cell line	Growth fraction %	Cell size	Cell shape	Polarization in dense cultures	Modal chromosome number	Tumorigenicity in nude mice	Invasiveness *in vitro* [18]	HLA antigen expression [5]	Sensitivity to natural cytotoxicity [5]	Origin
A	Hu 609	98	Medium	Heterogenous	Present	45–46	0	0	+	High	NU
	HCV 29	86	—	Less heterogenous	—	46	0	0	NI	High	NU
B	HU 456	93	Medium	Uniform	Absent	82	+	+	0	High	T2, Gr1, Oi
	HU 549	98	—	—	—	NI	+	NI	0	—	T2, Gr2, Oi
	T 24	84	—	—	—	91	+	+	0	—	Gr3
	HU 609 T	95	—	—	—	44–60	+	+	0	—	HU 609
	HCV 29 T	89	—	—	—	70	+	+	NI	—	HCV 29
C	Hu 961	19	Large	Heterogenous	Absent	45	NI	NI	+	Low	T1, Gr1–2, Oi
	HU 1125	14	—	—	—	43	0	NI	+	—	T1, Gr1, Oi
	HU 1210	16	—	—	—	45	NI	NI	+	—	T3, Gr2, +i
	HU 1244	5	—	—	—	NI	NI	NI	+	—	T1, Gr2, Oi
	HU 1264	13	—	—	—	NI	NI	NI	NI	—	T1, Gr3, Oi
D	HU 1305	88	Small	Uniform	Absent	Approx. 70	+	NI	0	NI	SQ

(A) Fast-proliferating cell lines from normal urothelium; (B) fast-proliferating cell lines from TCC or *in vitro* transformed 'normal' urothelium cells; (C) slowly proliferating cell lines from TCC; (D) cell line from squamous cell carcinoma of the urinary bladder.
NU = normal urothelium; T = T-classification of tumor; Gr = Gr-classification of tumor [26]; Oi = non-invasive; +i = invasive; NI = not investigated.
All data refer to cultures containing 10% fetal bovine serum.

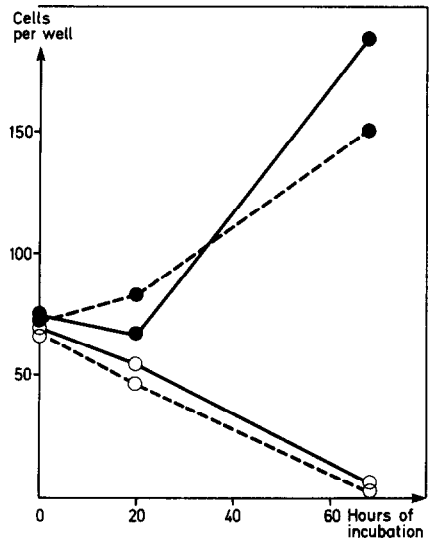

Fig. 2. Pronounced depression of the growth of HU 456 in one lot of disposable microculture plates. • = in Falcon plates, lot I; ○ = in Falcon plates, lot II; ——— = in freshly prepared culture medium; – – – – – = in medium stored at room temperature for 1 month.

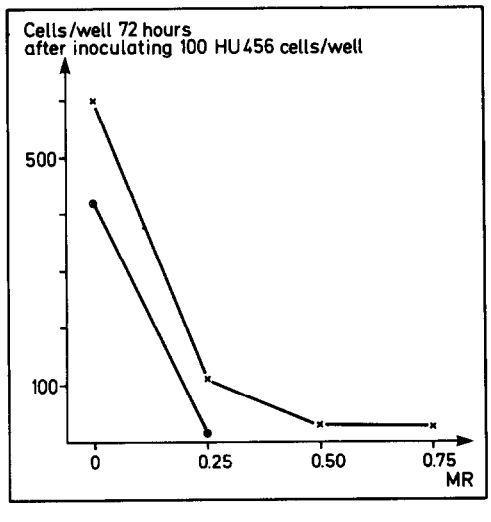

Fig. 3. Radiation-dependent cytotoxicity of disposable microtissue culture plates on cells growing in monolayer culture. + and ⊙ = two different lots of microplates (NUNC); MR = *megarad.*

Light microscopy

Cell lines within each of the groups A–D in Table 3 revealed common characteristics, but there were obvious differences between the groups. HU 609 cells from group A (Fig. 4:5) were of medium size, yet varied considerably in shape and size. There were a few scattered giant cells, each containing from 1 to 10 nuclei. The cells were sharply outlined polygonal or elongated in one or more directions. Real spindle-like cells were not seen. In densely growing cultures 'piling up' and some degree of polarization was seen, including the frequent appearance of elongated cells tending to grow in parallel bands. Generally, cultures of HCV 29 resembled HU 609. However, certain differences were noted: HCV 29 was more homogenous, with a mixture of rounded or elongated cells with a rather pronounced tendency to polarize in parallel bands in dense cultures but without piling up. There was no overgrowth of elongated cells during subcultivation.

The TCC-derived lines and the morphologically transformed normal lines of group B could not be distinguished from one another by light microscopy (Fig. 4:1–3, 6 and 8). Microscopic examination revealed uniform monolayers of rounded or polygonal medium-sized cells with uniform nuclei and some characteristic semilunate cells with the nucleus at the concave border of the cytoplasm. Proliferation could take place from single scattered cells, and when confluence was achieved the cells were densely packed with little tendency to multilayer growth and no piling up. The only sign of organization was some tendency of semilunate cells to form curved bands or rings, containing 4–6 cells in cultures with low cell density. Giant cells were not present.

The TCC-derived lines of group C differed markedly from the aforementioned. The cells were larger, with one or more nuclei in abundant cytoplasm, and the cultures were rather inhomogenous. Multilayering did not occur and growth ceased before a really dense monolayer arose (Fig. 4:9–13). Line HU 1305 (Fig. 4:4), from a squamous carcinoma, consisted of small, uniform, very densely packed round or polygonal cells, always growing in clusters or islands. They could hardly be trypsinized into real monocellular suspension and growth seemed only to take place from clusters rather than from single cells. In the densely packed areas numerous small, round cyst-like structures were seen, containing debris of often strongly eosinophilic cellular material.

Electron microscopy

HU 609, HU 456 (Fig. 5) and T 24 (not shown) were investigated in *in vitro* passages 34, 31 and an unknown number. The ultrastructures of the tissue cultures were very similar, all with features supporting the epithelial origin of the cultured cell lines. In all the lines derived from normal as well as neoplastic bladder epithelium, cellular junctions similar to those described as hemidesmosomes and desmosomes by others [27, 28] were present, thus supporting an epithelial origin of the cultured cells. Neither virus-like particles nor tonofilaments could be detected in any of the cultured cell lines. Cultured line T 24 established

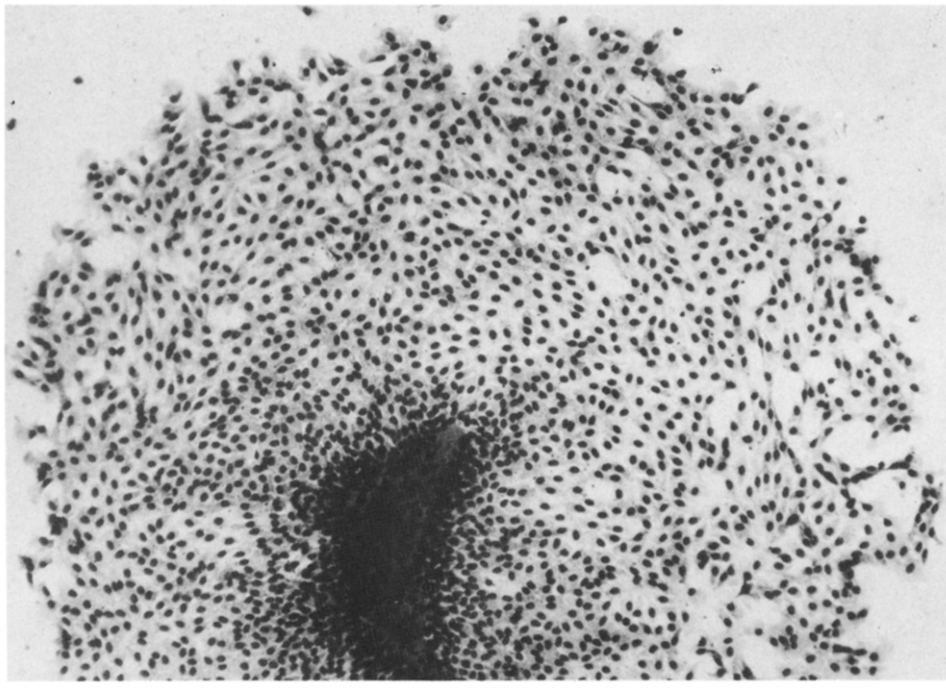

Fig. 1. *Primary growth from TCC, Gr* 2, *at day* 3 *after explantation. The central dark area is the biopsy fragment from which cells move out as a monolayer. Giemsa stain.*

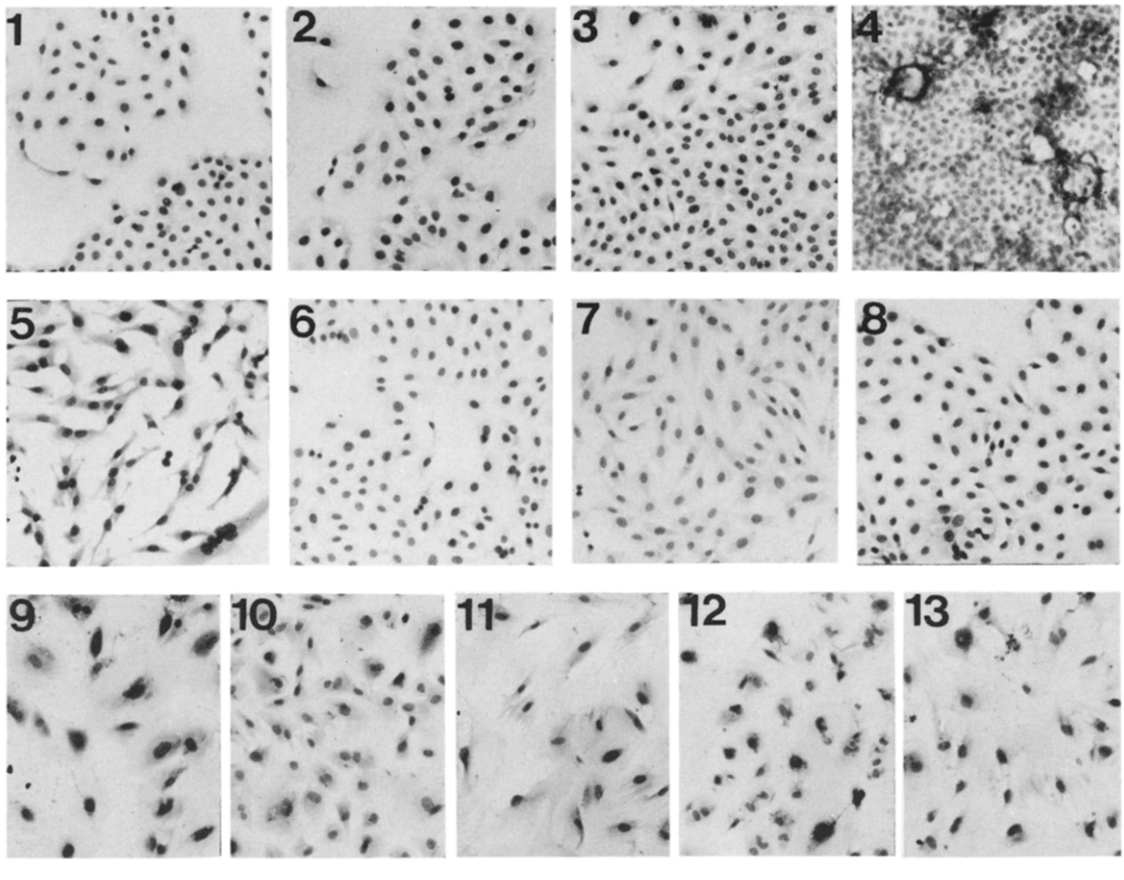

Fig. 4. *Light microscopy of cell lines from TCC or squamous cell carcinoma of the urinary bladder or from normal urothelium.* 1 = *HU* 456; 2 = *HU* 549; 3 = *T* 24; 4 = *HU* 1305; 5 = *HU* 609; 6 = *HU* 609 *T*; 7 = *HCV* 29; 8 = *HCV* 29 *T*; 9 = *HU* 961; 10 = *HU* 1125; 11 = 1210; 12 = *HU* 1244; 13 = *HU* 1264. *The monolayers have been rinsed in PBS, fixed in methanol* (10 min) *and stained by a* 20% *Giemsa stain in water* (15 min). *Magnification* = × 70.

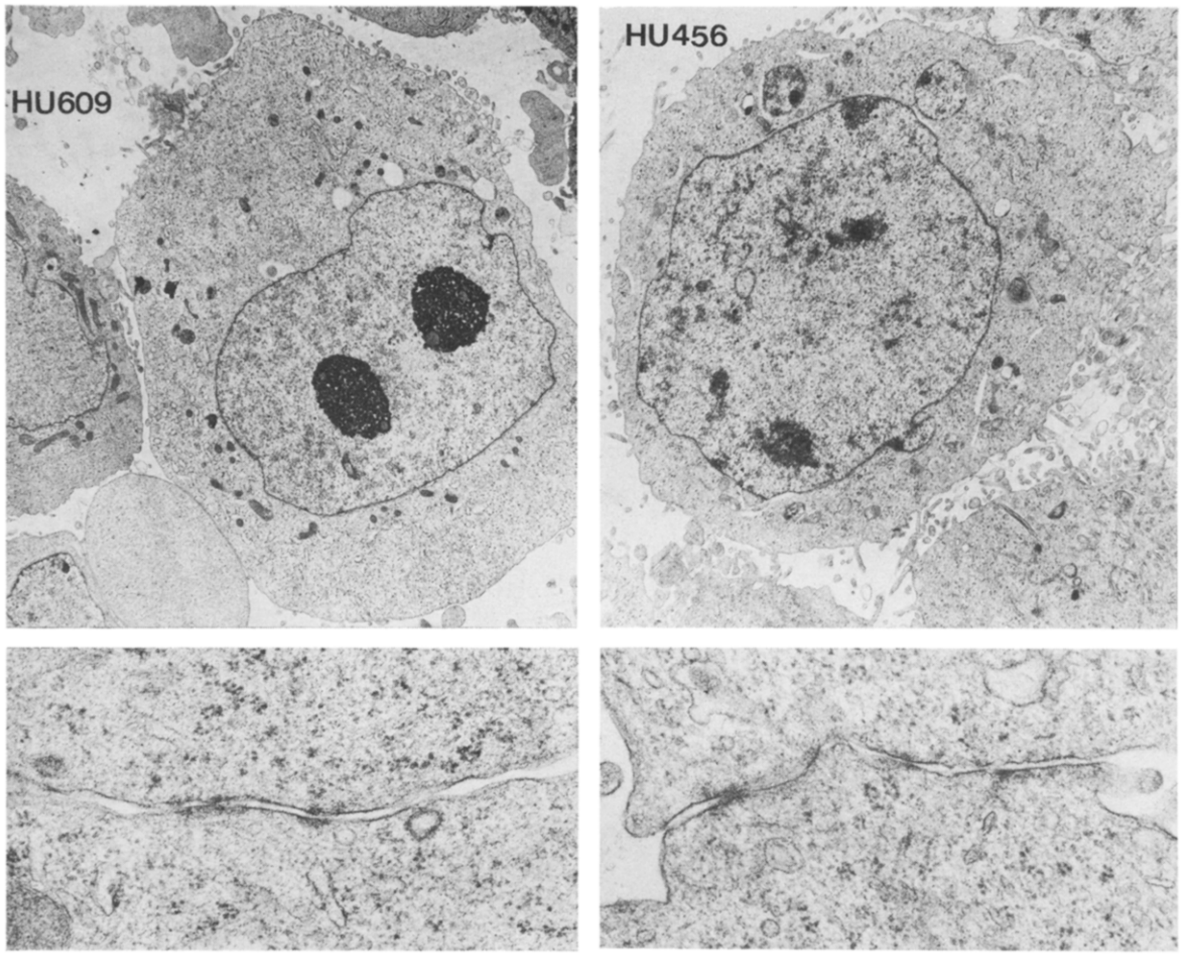

Fig. 5. Electron microscopy appearance of cell line HU 609 from normal urothelium and cell line HU 456 from TCC of the bladder. HU 609: note the prominent nucleoli and microvilli (×2040). Inset shows desmosome like structures (×23,800); HU 456: the outline of the nucleus is more irregular than that of HU 609 (×3128). Inset demonstrates desmosomes similar to those present in HU 609 (×23,800).

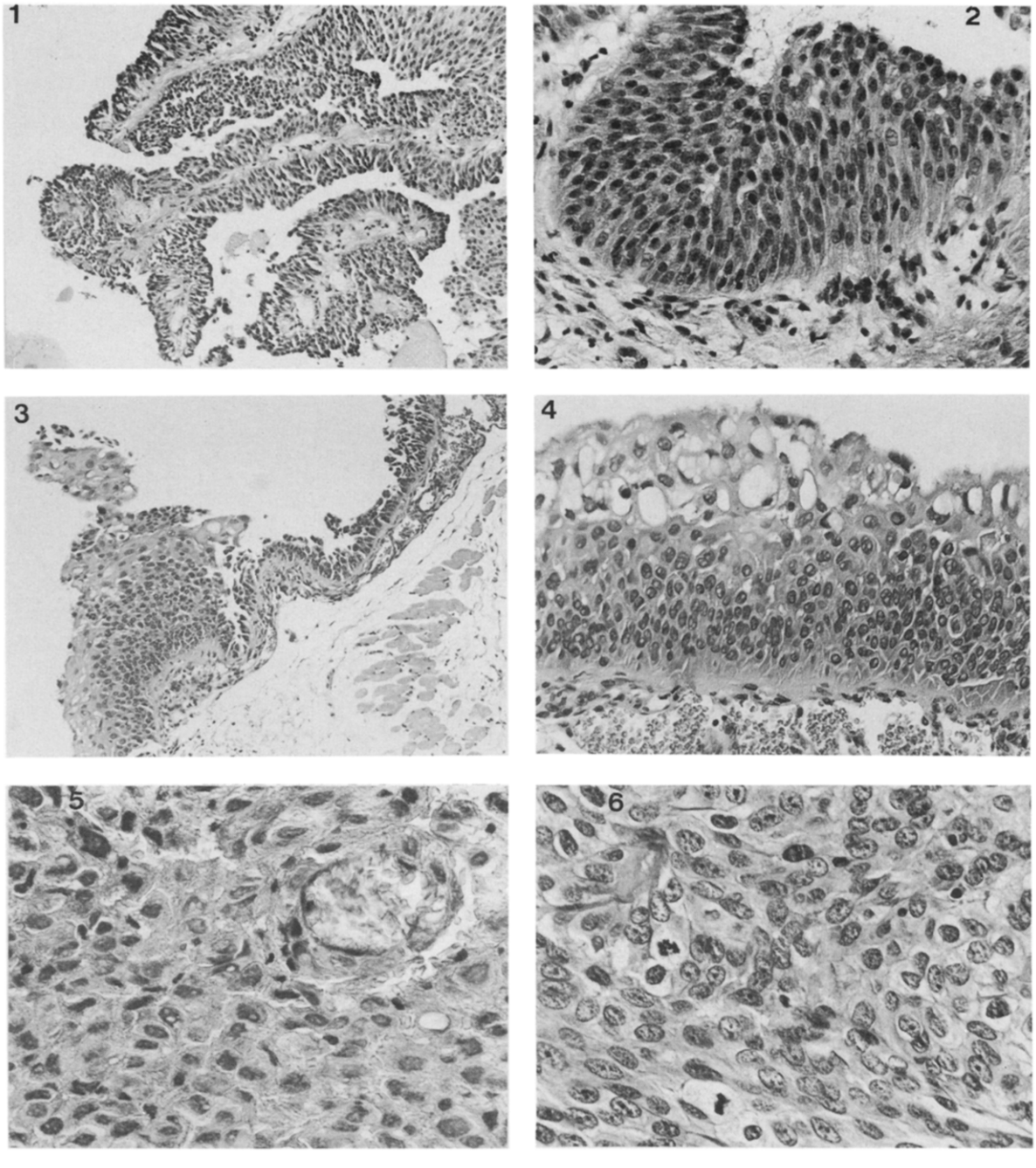

Fig. 6. Reproduction of the histological characteristics of transitional cell carcinoma, comparable with the original patient tumors, in mouse-grown tumors originating from cell lines HU 456 and T 24 subcutaneously inoculated in nude mice. 1 = *HU 456, patient tumor* (×27); 2 = *HU 456, patient tumor* (×170); 3 = *HU 456, mouse-grown tumor* (×27); 4 = *HU 456, mouse-grown tumor* (×170); 5 = *T 24, mouse-grown tumor* (×272); 6 = *T 24, patient tumor* (×272).

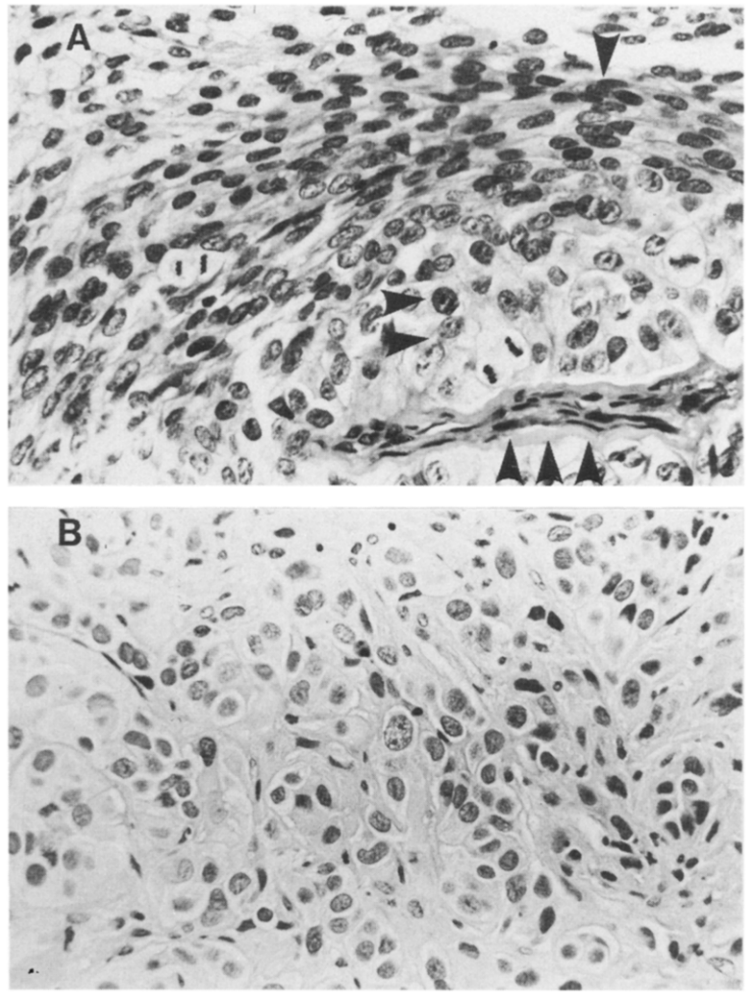

Fig. 7. Histology of tumors induced in nude mice by inoculation of tissue cultured cells from TCC (A) or in vitro *transformed normal urothelium cells (B). (A) Microscopical examination of T 24-induced tumor shows a whirl pattern with imitation of papillomatous growth. Numerous stalks of connective tissue are penetrating the tumor, covered with epithelial cells of varying appearance, i.e. polygonal basal cells close to the stalks and cells of increasing spindle like the shape above; (B) examination of HCV 29 T-induced tumor shows a uniform pattern of epithelial growth with very sparse stroma. The tumor grows in alveolar formations consisting of polygonal epithelial cells with moderate nuclear polymorphism. ⟹ = connective tissue stalk; ⟹ = polygonal cells; ⟶ = spindle-like cells.*

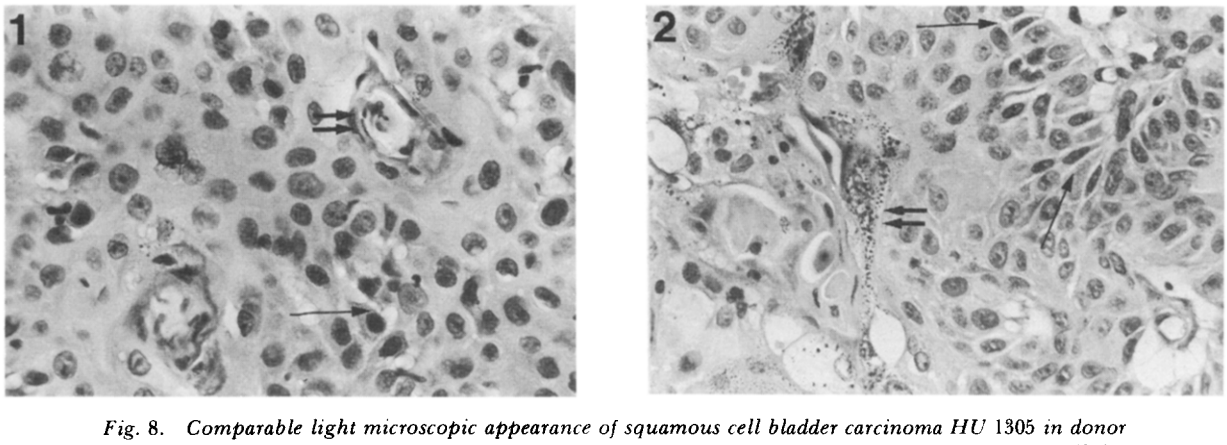

Fig. 8. Comparable light microscopic appearance of squamous cell bladder carcinoma HU 1305 in donor patient (1) and nude mouse (2) inoculated with cell line HU 1305 (×248). ⟶ = intercellular bridges; ⟹ = keratinization.

from an urinary bladder carcinoma appeared as previously described by others [6]; however, in our cultures several desmosome-like cell junctions were also present.

Morphological transformation

Signs of 'spontaneous' morphological transformation occurred in HCV 29 and HU 609 lines of non-malignant origin.

The HCV 29 cell line, which was obtained from J. Fogh, was derived from the histologically normal bladder mucosa from a patient who had previously been irradiated because of bladder cancer. During propagation of this cell line in our laboratory changes from an elongated cell shape into more polygonal or round cells were seen in some of the cultures. These morphological changes were accompanied by the development of aneuploidy and tumorigenicity in nude mice. From these transformed cultures a subline HCV 29 T was established. Similar changes were later seen in the HU 609 cell line.

Even though HU 609 has been cultured to passage No. 67 without morphological changes, signs of 'spontaneous' irreversible morphological transformation occurred in several subcultures from the frozen stock (*in vitro* passages 14–20) of this cell line, always between passages 30 and 35. Just before altered cell types appeared in HU 609 the cultures showed increased granulation with some tendency to detach. In the next few days rounded or semilunate cells like those in TCC cultures of group B proliferated from multiple centers in the flask, and within 1–2 weeks the culture was totally transformed into such cells. The morphological transformation was followed by chromosomal alterations (Table 3), i.e. a shift from euploid to aneuploid modal chromosome number, and epithelial tumors could be established in nude mice with cells from the transformed subline but not with cells from the nontransformed line. From one of these transformed cultures subline HU 609 T was established. As an additional sign of malignancy both HCV 29 T and HU 609 T invade embryonic chick heart tissue when aggregates of transformed cells are associated with heart fragments *in vitro* [18]. Studies of HU 609–HU 609 T further demonstrated loss of HLA antigen expression after transformation [5].

Chromosome analysis

Chromosome numbers in lines HU 609, HU 456, HU 1125, HU 961, HU 1210 and subline HU 609 T were counted in *in vitro* passages 23, 41, 14, 10 and 33 respectively. Chromosome counts were made on HCV 29, HCV 29 T and T 24 in an unknown number of passages. Modal chromosome numbers are given in Table 3.

Growth patterns of tumors in nude mice

Results of inoculation of different cell doses of 9 human cell lines are given in Table 4. Animals inoculated with the normal lines (HU 609 or HCV 29) at doses up to 8.4×10^7 cells did not develop tumors within an observation period of 3–8 weeks. Also, the slow-growing HU 1125 cell line of TCC origin was found to be nontumorigenic in nude mice at cell doses varying from 10^5 to 2×10^6 cells. After a latency period of less than 1 week progressively growing tumors were observed at the inoculation site in nude mice inoculated with 10^7–10^8 cells of TCC lines HU 456 and HU 549 and the transformed normal-derived lines HU 609 T and HCV 29 T. Tumor development with a similar short latency period was seen after inoculation of 10^6 HU 1305 cells from a squamous

Table 4. Results of inoculation of urothelium-derived cell lines in nude mice

Cell line	No. of cells per mouse	No. of mice with tumors	Total No. of mice
HU 456	8.4×10^7	1	5
	7.0×10^7	2	2
	5.0×10^7	0	2
	1.7×10^7	1	5
	10^7	1	2
	10^5–3.4×10^6	0	15
HU 549	10^8	1	2
	5.0×10^7	0	2
	10^5–10^7	0	27
T 24	5.0×10^7	2	2
	10^7	0	2
HU 1125	2.0×10^6	0	4
	10^5–10^6	0	15
HU 1305	3.3×10^7	3	5
	10^7	5	5
	10^6	2	5
	10^2–10^5	0	20
HU 609	8.4×10^7	0	5
	2.0×10^7	0	2
	1.7×10^7	0	7
	1.3×10^7	0	3
	4.0×10^6–10^7	0	5
HU 609 T	8.4×10^7	2	5
	1.7×10^7	1	5
	10^7	4	5
	10^5–3.4×10^6	0	15
HCV 29	10^7	0	8
	0.9×10^7	0	3
HCV 29 T	10^7	5	5

cell carcinoma of the bladder. T 24 cells grow very slowly in nude mice: after the inoculation of 5×10^7 cells progressive tumor growth was not seen until after 4–8 weeks. However, on further transplantation of these tumors a latency period of less than 1 week was observed. No spontaneous regressions were recorded.

Microscopic examination of tumors in nude mice inoculated with line HU 456 disclosed a well-defined cyst outlined by multilayered epithelium resembling urothelium. Only a few papillary structures protruded into the lumen, which otherwise appeared empty. PAS and alcian blue stainings were negative. The degree of cellular atypia was comparable in mouse-grown and human tumors (Fig. 6:1–4).

Tumors in nude mice inoculated with line T 24 were also well circumscribed, consisting of solid islands of epithelial cells separated by a connective tissue stroma. No papillary structures were seen. The microscopic appearance of the mouse-grown tumors corresponded to the most dedifferentiated area in human biopsy material (Fig. 6:5–6). Serial transplantation of T 24-induced tumors was successful in 2 passages with preservance of morphology. However, infection made further serial transplantation impossible. The HU 609 T and HCV 29 T sublines produced solid carcinomas in nude mice (Fig. 7). The histological pictures of the mouse-grown HU 609 T and HCV 29 T tumors were identical but differed from that of T 24, as also demonstrated in Fig. 7. On re-explantation *in vitro* HU 456-, T24-, HCV 29- and HU 609 T-induced tumors from nude mice, showed the same growth pattern as the established lines after only 1 week. Chromosome analysis of re-explanted cells showed the human karyotype. The mouse-grown tumor from HU 549 was not saved for microscopy.

Tumors in nude mice inoculated with HU 1305 were circumscribed, consisting of solid sheets of squamous cells with keratinization and formation of horn pearls. Intercellular bridges were present (Fig. 8:2). The microscopic picture corresponded well to the moderately differentiated squamous cell carcinoma in the patient (Fig. 8:1).

DISCUSSION

The lack of any correlation between clinical and pathological tumor classifications of donor tissue or further growth during follow-up investigations of donor patients and the *in vitro* growth potential of TCC cells in the present investigation (Tables 1 and 2) agrees with the findings of others [7, 8, 14, 19, 20, 22]. Thus *in vitro* growth potential cannot be used as a prognostic tool. A comparative study of 8 TCC-, 1 SQ- and 2 NU-derived cell lines from human urothelium revealed that the lines could be allocated to 4 subgroups, each with common characteristics, as shown in Table 3, which also includes immunological parameters and investigation of invasiveness *in vitro* as described in previous publications [5, 18]. The epithelial nature of group B TCC-derived cell lines was indicated by the morphology in light- and electron microscopy and finally confirmed by the formation of epithelial tumors after inoculation in nude mice, which further documented that the lines were tumorigenic and thus seem to be malignant. Furthermore, the degree and type of differentiation in mouse grown-tumors derived from HU 456, T 24, and HU 1305 cell lines corresponded exactly to the respective human tumors, indicating that these cell lines seem to represent fundamental properties of the donor tissue. Likewise, the epithelial nature of the NU-derived HU 609 and HCV 29 lines was indicated by the light- and electron microscopic findings and further confirmed by the formation of epithelial tumors from the transformed sublines. However, in contrast to the tumor-derived lines, cultures derived from normal tissue contained a number of morphological variants in the monolayer, including elongated cells forming parallel bands. It cannot be excluded that some of these cells were fibroblasts, but fibroblast overgrowth did not occur and only one cell type was seen using the electron microscope.

It is possible that the differences between non-transformed NU-derived and group B TCC-derived cell lines with regard to tumorigenicity and chromosomal aberrations shown in this investigation and tissue type antigen expression and invasiveness in xenogeneic normal tissue described elsewhere [5, 18] represent *in vitro* parameters of importance in distinguishing between benign and malignant human epithelium cells. Further studies are needed before definite criteria of malignancy can be established [29, 30]. The morphological alterations occurring spontaneously during *in vitro* cultivation of the NU-derived cell lines HU 609 and HCV 29 were irreversible; characteristically, the cell culture acquired a morphological appearance indistinguishable from the malignant TCC-derived group B lines. The morphological alterations were followed by chromosomal aberrations, acquisition of tumorigenicity as described in this investigation and further by loss of surface antigens (not investigated in HCV 29) and acquisition of invasiveness as described elsewhere [5, 18], changes which according to others may be associated with neoplasia [31–34]. According to

Pontén [29] and Smets [30] infinite growth has invariably been associated with malignant alteration. This claim was not supported by Kieler *et al.*'s [35] studies of spontaneous malignant alteration of ST/a mouse lung fibroblasts. In this investigation subcultures of HU 609 showed signs of morphological alteration, but the maternal cell line had remained unaltered for 1½ yr and 67 passages at the time this report was written.

As pointed out by Pontén [29], the criteria of malignant transformation have been based on experiments with unstable rodent cells prone to spontaneous aneuploidy, genetic imbalance and secondary alterations, changes which are not seen in human cells. In fact, 'spontaneous' malignant transformation of human cells has only rarely been described [36–39].

Cross-contamination between cell lines grown at the same time in one laboratory may be a source of serious errors which should be considered when the development of the HU 609 T and HCV 29 T sublines is described as the result of 'spontaneous' transformation of the maternal cell lines of non-malignant origin. Cell lines HU 456 and T 24 from TCC, HU 609 and HCV 29 from NU and SAOS 2 from osteosarcoma were cultured at the time transformation was observed. However, only HU 456 and T 24 need further comments as the growth pattern of SAOS 2 *in vitro* was clearly different from the remaining lines. The morphological appearance of the cultured HU 609 T, HCV 29 T, HU 456 and T 24 did not differ sufficiently to exclude cross-contamination, but the differences between the histological pictures of tumors produced in nude mice by the TCC-derived cell lines and the 'spontaneously' transformed cell lines represent a strong argument against contamination of the latter by the former. The degree and type of differentiation of cell line-induced tumors in nude mice seem to represent rather stable cellular characteristics according to the present findings with HU 456, T 24 and HU 1305 cell lines and experiments with other cell lines [34]. Furthermore, the development of T 24 tumors showed a latency period which was much longer than that of HU 609 T and HCV 29 T tumors.

On the other hand, the present data do not exclude cross-contamination between HU 609 and HCV 29 and their respective sublines. If cross-contamination has taken place the HCV 29 T subline is probably the most suspect. This cell line originated from a histologically normal bladder mucosa which had been irradiated for transitional cell carcinoma. It cannot be excluded that the original explant contained a few pre-neoplastic cells or cells which had been initiated by irradiation. However, 'spontaneous' transformation of HU 609 has also been seen by M. Troye at the Wennergreen Institute in Stockholm (personal communication).

At the time when 'spontaneous' transformation was observed no chemical carcinogens were used in our tissue culture laboratory. However, during storage in liquid nitrogen the cell lines had been exposed to DMSO, which has been shown to be a weak carcinogen [40].

The TCC-derived cell lines could be allocated to either of two groups, B or C (Table 3), each with common characteristics. There were great differences between the groups with respect to the growth fraction, modal chromosome number and morphology. And characteristic differences in sensitivity to spontaneous lymphocyte-mediated cytotoxicity and tissue-type antigen expression have been reported [5]. The biological significance of these differences is not clear. The large, slowly growing HLA antigen expressing group C cells may represent a more differentiated variant of malignant cells, but it may also be the progeny of non-malignant cells possibly present in the donor tumor biopsy. Sufficient numbers of these cells have not been available for quantitative comparisons of tumorigenecity in nude mice, but so far tumors have not been grown.

The pronounced influence on cell growth of different microtissue culture plates is of extraordinary importance for immunological *in vitro* studies and other experiments where cells are grown as monolayers on such plates. In investigations of the cytotoxicity of lymphocytes from TCC patients in our laboratory [2–5] all new lots of plates were tested for cytotoxicity before use and nonirradiated plates have been used in most of these experiments since such plates revealed optimal growth conditions without significant bacterial contamination.

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