TRANSITIONAL CELL CARCINOMA IN SOFT AGAR CULTURE

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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CONTENTS

VOORWOORD:

Dit onderzoek maakte deel uit van het onderzoeksprogramma "kwantitatieve morfologie, celbiologie en epidemiologie van (pre-)maligne afwijkingen". Het betrof een samenwerkingsproject van de afdeling Urologie (hoofd Prof. Dr. F.M.J. Debruyne) en de afdeling Pathologische Anatomie (hoofd Prof. Dr. G.P. Vooys).

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CHAPTER 1.

GENERAL INTRODUCTION.

INTRODUCTION:

Most common tumors of the bladder are the epithelial type tumors. Histologically, the vast majority of the epithelial tumors are the transitional cell type tumors.(1)

The histological classification separates transitional cell tumors from squamous, glandular and undifferentiated forms. (Table 1.)

Transitional cell papilloma has a highly restrictive definition i.e. "a papillary tumor with a delicate fibrovascular stroma covered by regular transitional epithelium indistinguishable from that of the normal bladder and not more than six layers thick". There should be no evidence of anaplasia. Because of this high threshold the proportion of papillomas is very limited in most series; less than three percent of all epithelial tumors of the bladder.

Transitional cell carcinoma is classified, considering histological grade and growth pattern. In bladder cancer histological and cytological grading is of considerable clinical importance. The grading is based on anaplasia i.e. cellular abnormalities in size, shape, staining and mitotic activity. By the W.H.O. a classification is proposed in which three grades are employed. (2,3)

Grade 1 applies to tumors that have the least degree of cellular anaplasia, compatible with the diagnosis of malignancy; Grade 3 applies to tumors with the most severe degree of anaplasia; Grade 2 lies in between. The growth patters are described using four groups as follows:

1) Papillary: the tumor is growing in the lumen of the bladder.
2) Infiltrating: the tumor is growing in the wall of the bladder.
3) Papillary and infiltrating: the tumor is doing both.
4) Non-papillary and non-infiltrating: the tumor is confined to the surface.

Table 1. Histological classification of urinary bladder tumors (1)

I. Epithelial tumors

A. Transitional cell papilloma
B. Transitional cell papilloma, inverted type
C. Squamous cell papilloma
D. Transitional cell carcinoma
E. Variants of transitional cell carcinoma
   1. with squamous metaplasia
   2. with glandular metaplasia
   3. with squamous and glandular metaplasia
F. Squamous cell carcinoma
G. Adenocarcinoma
H. Undifferentiated carcinoma

II. Non-epithelial tumors

A. Benign
B. Malignant
   1. Rhabdomyosarcoma
   2. Others

III. Miscellaneous tumors

A. Phaeochromocytoma
B. Lymphomas
C. Carcinosarcoma
D. Malignant melanoma
E. Others

IV. Metastatic tumors and secondary extensions

V. Unclassified tumors

VI. Epithelial abnormalities

A. Papillary (polypoid) "cystitis"
B. Von Brunn's nests
C. "Cystitis" cystica
D. Glandular metaplasia
E. "Nephrogenic adenoma"
F. Squamous metaplasia

VII. Tumor-like lesions

A. Follicular cystitis
B. Malakoplakia
C. Amyloidosis
D. Fibrous (fibroepithelial) polyp
E. Endometriosis
F. Hamartomas
G. Cysts

Transitional cell carcinoma is not confined to the epithelium of the bladder. In the modern concept of the collecting system, the urinary tract is a single functional unit.(4) It is this concept of a total urothelial disease that explains the distribution and behavior of many tumors.(5) In 1952 Melicow presented the results of a "Histological study of the vesical urothelium intervening between gross neoplasms in total cystectomy" (6) in which 10 bladder specimens were studied, all bearing gross invasive cancer. Apart from sections of the tumors, pieces of smooth, normal appearing bladder tissue were made. These sections showed foci of inflammation and urothelial hyperplasia and metaplasia, but also occasional localized areas of pronounced cellular atypism. These areas were labeled carcinoma in situ or intra-urothelial cancer. Carcinoma in situ was described as an equivalent of Bowen's disease, or intra-epithelial cancer of the epidermis. (7) It was stated that carcinoma in situ of the urothelium were all early focal cancers limited to their respective surface linings, but possesing a potential for invasiveness.

Because of the multicentric character of the disease in a number of patients, an evaluation has to be made of the neoplastic potential of the tumor. For this purpose the urologist must obtain additional material. This material can be tissue samples taken from selected mucosal sites or cellular samples obtained from urine or bladder washings. (8) The mucosal biopsies from selected sites are preferably taken using a "cold" biopsy instrument and should be submitted to the pathologist in a fixative in separate containers, each clearly marked by the site of origin. In the standard procedure used by the Clinical Collaborative Group A of the National Bladder Cancer Project biopsies are taken of apparently normal mucosa adjacent to the tumor and three other biopsies of apparently normal mucosa, one lateral to each ureteral orifice and one from the upper posterior wall. (9)

Besides the classification of the histological type and the grade of the tumor the clinical and histologic stage of the tumor have to be determined.

Table 2. Clinical versus microscopical staging.

CLINICALLY:	MICROSCOPICALLY:
	pTis: pre-invasive carcinoma, carcinoma in situ.
	pTA : papillary non-invasive carcinoma.
T0 : no evidence of primary tumor.	pT0 : no evidence of tumor found on histological examination of the specimen.
T1 : on bimanual examination no tumor or a freely mobile mass may be felt: this should not be felt after complete transurethral resection of the lesion.	pT1 : tumor not extending beyond the lamina propria.
T2 : on bimanual examination there is induration of the bladder wall which is mobile; there is no residual induration after complete transurethral resection.	pT2 : tumor with invasion of superficial muscle (not more than halfway through muscle coat).
T3 : on bimanual examination induration or a mobile nodular mass is palpable in the bladder wall which persists after transurethral resection of the exophytic portion of the lesion.	pT3 : tumor with invasion of deep muscle (more than halfway through muscle coat) T3a; or with invasion of perivesical tissue, T3b.
T4 : tumor fixed or extending to neighbouring structures: T4a: tumor infiltrating prostate, uterus or vagina. T4b: tumor fixed to pelvic wall and/or abdominal wall.	pT4 : tumor with invasion of prostate or other extravesical structures.

Clinically the tumor is staged according to the T.N.M.-classification (U.I.C.C.).(10) There are differences between the clinical classification and the histopathological classification. (Table 2)

The N-classification is used to describe involvement of regional (N0-N3) and juxta-regional lymphodes (N4).
The M-classification describes the presence of distant metastases; M0, no evidence of distant metastases and M1, positive evidence of distant metastases.

Microscopically it is not possible for the pathologist to differentiate between pT2 and pT3a on endoresection specimens. For this differentiation a cystectomy specimen is needed to evaluate depth of infiltration in the muscle coat of the bladder.

For prognosis the lamina propria is an important border. The recurrence rate is higher after transurethral resection in T1 tumors than in T0 tumors.(11)

Other prognostic factors for likelihood of recurrence were multiplicity, size equal to or greater than 3 cm and abnormal selected mucosal biopsies.

Positive urinary cytology and higher grade correlated with prognosis though less strongly.(11) According to the study of the EORTC Genito-Urinary Tract Cancer Cooperative Group the number of tumors at presentation is the most important prognostic factor followed by, in order of importance, the recurrence rate at entry of the study and size of the largest tumor.(12) However Loening et al (13) found no predictive value for tumor recurrence of initial grade, stage, number and size of bladder tumors. None of the observed parameters alone or in combination could predict tumor recurrence.

The recurrence rate of urothelial tumors is high, 50-80%.(14,15) A fourth of the tumors will recur as higher grade lesions (15)

and depending on the histologic grade of the original tumor 8 to 57% of the patients will have subsequent invasive tumors. (16) This suggests a progression with the time from low-grade low-stage tumors to high-grade infiltrating tumors. This observation could not be confirmed by Kaye and Lange (17), who found that of 166 patients with invasive bladder cancer only 16% had prior non-invasive bladder tumors. They reported that death from bladder cancer occurs primarily among patients who have never had a recognized, early bladder tumor.

According to grade and most important stage of the bladder cancer, different treatment modalities are applied.

Superficial bladder tumors are treated with transurethral electroresection or fulgeration.This therapy is based on experiments with animal model systems (carcinoma-induced tumors in syngeneic mice) (18) and prospective randomized clinical studies as performed by the EORTC Genito-Urinary Tract Cancer Cooperative Group comparing agents as Thiotepa, Epipodophyllotoxin (VM 26) and TUR alone.(19) Thiotepa significantly reduced the recurrence rate as compared with the control group or VM 26. Intravesically administered doxorubicin (Adriamycin) showed effect on the recurrence rate after TUR.(20) Also the effects on the recurrence rate were studied using high dose oral Bacillus Calmette-Guerin (BCG) (21) showing effect on the recurrence rate. Also the combination intravenous cyclophosphamide plus orally or intravesically administered BCG was reported to have an effect on the recurrence rate.(22) In animal tumor model systems these effects of BCG on superficial bladder tumors could be confirmed.(23,24)

Also the effect of intravesical administered BCG was evaluated on CIS of the bladder. Fifty-five percent of the patients treated with BCG obtained complete regression for a median duration of 18 months as determined by negative urine cytology and negative bladder biopsies.(25) Some effect of Adriamycin intravesically was showed against CIS (26) and against superficial bladder tumors without prior resection.(27)

Intravesically administered Cisplatin showed little effect on CIS and pT1 tumors.(28) Also intravenous Cisplatin induced no complete remissions of superficial bladder tumors.(29) The current treatment of superficial transitional cell carcinoma of the bladder consists of transurethral resection of the exophytic tumor. It is advisable to take separate biopsies of the bottom of the resected area. Furthermore random cold cup biopsies are taken from the normal appearing bladder mucosa at selected sites. After resection, intravesically chemotherapeutic drugs (Adriamycin, Thiotepa) are administered for several months. Radiotherapy for low-stage bladder cancer is not the optimal treatment for these patients.(30)

At the moment follow-up measures consist of repeated cystoscopy and urine cytology. The patients who had a TA-T1 transitional cell carcinoma, accompanied by CIS or patients with CIS alone need very careful follow-up. As soon as progression occurs to infiltrating tumor more definite measures are needed.

Treatment for infiltrating bladder tumors (T2,T3) without metastases is in discussion. Radiation therapy is still controversial as a treatment for bladder cancer given pre-operatively and as radical treatment.(31,32) For highly selected cases (small, solitary tumors with limited infiltration) interstitial irradiation is proposed as an effective measure. (33,34) In the treatment of T3NXNO carcinoma of the bladder the combination of radium inplant and external irradiation was proposed for tumors smaller than 5 cm.(35) The results were excellent in low grade tumors without vascular invasion, prognosis was poor if a high grade tumor with vascular invasion was treated. Most common practice for deeply infiltrating bladder tumors is the combination of pre-operative radiation followed by radical cystectomy.(36,37,38,39) Radiation doses varied from 2000-6000 rad. Early death of the patients was related to the post-operative complications due to the combined approach. Later deaths were due to tumor recurrences and late complications.

Several adjuvant chemotherapy protocols have been reported (40, 41,42) because of the high probability of subclinical metastatic disease in patients with deeply infiltrating bladder tumors. The results are still questionable because no highly effective drug has been identified against transitional cell carcinoma.

To improve survival of patients with metastasized transitional cell carcinoma many cytotoxic drugs, single and in combination have been evaluated. Much attention was given to Methotrexate (43,44), Adriamycin (45), Bleomycin (46) and most recently Cis-Diamine-Dichloride Platinum II (47) as single agents. Many drug combination regimens were evaluated. (48,49,50) Reviews on systemic chemotherapy used against metastasized transitional cell carcinoma are given by Carter (51,52), Pavone-Macaluso (53), DeKernion (54), Bush (55), Smith (56), Baumgartner (57) and Paulson (58). It can be concluded that no highly effective drug or drug combination exists against transitional cell carcinoma. Effectivity of CDDP (cisplatin) is most consistent as is expressed by response rates between 33-57% in several studies. (58) It is questionable if the higher toxicity of combination drug regimens is in balance with better results. The overall duration of remissions is in the range of 5-6 months practically unrelated to the drug regimen used.(58)

At the time the present study was started (1981), patients with metastasized transitional cell carcinoma were treated with the combination: cisplatinum, adriamycin and 5-fluorouracil.(59) Because of the moderate effect this combination seemed to have on transitional cell carcinoma and the serious side effects accompanying this drug combination, need was felt for an in vitro test for sensitivity testing of tumors against cytotoxic drugs. At that time first reports became available about the Hamburger and Salmon double layer soft agar method.(60,61,62,63,64,65) Favorable results were presented about correlations between in vitro and in vivo drug sensitivity and resistance of a variety of solid tumors.(62,63,65,66)

From fresh unfixed tumor material single cell suspensions are

prepared mechanically (crossed scalpels or scissors) eventually followed by enzymatic disaggregation (67) using 0.8% collagenase type II and 0.002% DNase. After washing the cells and disposal of cell clumps in the suspension (by means of filtering and aspirating the cell suspension through fine needles) the cells are counted in a hemocytometer and viability is determined with a dye exclusion test (Trypan blue).
The cell suspension is diluted to $3x10^6$ cells per ml. For drug sensitivity testing 0.5 ml aliquots of the cell suspension are exposed to the various drugs to be tested. Drug concentrations are 10% of the peak plasma concentration in vivo achievable. Exposure to the drug lasts one hour. After washing the cells, the cells are plated in triplicate in a 0.3% agar layer of double enriched CMRL1066 over a bottom layer of 0.5% agar in double enriched McCoy's 5A medium in 35 mm petri dishes. The originally described conditioned medium was omitted in the present study. In an incubator at 37°C and 6% CO_2 in the atmosphere tumor cell colonies were allowed to develop from the presumed stem cells present in the cultures. The colonies formed are counted. The difference between the control cultures (not drug pre-treated cells) and the drug pre-treated cells in culture indicates drug sensitivity or resistance. A minimum of 30 colonies in the control dishes were demanded to accept a culture as evaluable; for sensitivity 70% inhibition of colony growth was needed.(66) Colony counting is performed using an inverted microscope or an automated colony counter, Omnicon Feature Analysis System II (Bausch and Lomb, Rochester NY,USA).(68)

This culturing system was not yet systematically used on transitional cell carcinoma. Some reports were available based on limited numbers of tumors (69,70) which gave us an opportunity to investigate the possibilities of this system to test cytotoxic drugs on transitional cell carcinoma. It would be possible to compare our results with other laboratories with a similar experience with other solid tumors.

As described by Hamburger (71) an ideal in vitro test should

be simple, statistically acceptable, easily standardized, cheap, rapid, flexible and capable of taking into account various methods of drug action and should offer reasonable correlations with in vivo effects. The double layer soft agar culturing system is based upon the theoretical existence of stem cells in a tumor as was first described by McCulloch and Till in 1961.(71) These stem cells are held responsible for the cell renewal in a tumor, the growth of a tumor, and eventually for outgrowth of tumors at distant localisations c.q. metastases. Less than 1% of the total number of cells are believed to have stem cell properties. In the double layer soft agar culturing system the stem cells, better called clonogenic cells, of malignant tumors will form colonies with time (3-4 weeks). These colonies can be counted. The first question to be answered by the present study was whether it is possible to culture transitional cell carcinoma in a routine set up, and eventually to test cytotoxic drugs with this system comparing the results with those reported for other solid tumors in the first retrospective studies published.

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CHAPTER 2.

SOFT AGAR CULTURE OF HUMAN TRANSITIONAL CELL CARCINOMA COLONIES FROM URINE.

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ABSTRACT:

Soft agar culture of transitional cell carcinoma colonies from urine, bladder irrigation fluid, and transurethral resection solid tumor specimens demonstrate generally better growth of tumor cell colonies from urine than from irrigation fluid. Growth of tumor colonies from urine was adequate to evaluate presence of tumor and to study growth parameters of the tumor colonies in vitro, although the number of colonies produced from urine was inadequate to evaluate sensitivity or resistance to chemotherapeutic agents. Growth in soft agar of transitional cell carcinoma colonies from urine may offer a simple, noninvasive method of evaluating the effectiveness of therapy and the prognosis for tumor progression.
(Key words: Tumor stem cells; Soft agar culture; Clonogenic cell culture; Transitional cell carcinoma; Bladder cancer)
Am J Clin Pathol 1982; 78: 690-694.

INTRODUCTION:

Since the report of Hamburger and Salmon (3) describing the culture technics of tumor "stem" cell colonies from humans in a double layer soft-agar system, this cloning system (HTCS) of tumors from humans has become a popular investigational tool to determine resistance or sensitivity of individual patients' tumors to chemotherapeutic agents in vitro (2,9,10). Preliminary data suggest that this method may prove very helpful in predicting patients' response in vivo to chemotherapy regimens (2).

In addition to determining chemotherapy sensitivity and resistance at least two other potential applications of this tumor cell culture method have been suggested (Herman and colleagues: Arch Pathol Lab Med, in press). Growth of tumor colonies in soft agar may provide a means of assessing the effectiveness of chemo- or radiation therapy by determining the presence of "viable", i.e., clonogenic, tumor cells in post-therapy biopsy specimens. In addition, the parameters of growth in soft agar may be a predictor of tumor behavior, i.e., prognosis, especially in those tumors, such as ovarian epithelial and papillary transitional cell, where the borderline between "benign" and malignant is not sharply discernible by routine pathologic examination.

Further investigation and potential routine clinical application of the cloning system of tumors from humans in these and other problems in clinical oncology will be facilitated considerably by the development of technics for obtaining quality tumor cells for culture with minimal trauma to the patient. So far, most reports dealing with culture of tumors from humans have used tumor biopsies as a source of tumor cells. Increasing use has been reported of effusions and irrigation fluids (1,6, 11). The present work reports the culture of transitional cell colonies from urine specimens of humans and compares the results of culture of bladder biopsy and irrigation specimens.

MATERIALS AND METHODS:

Twenty patients admitted to the Radboud Hospital for transurethral resection of a transitional cell bladder tumor (newly diagnosed or recurrent) formed the population of the present series. From these 20 patients, 18 solid tumors, 12 urine samples and 12 bladder irrigation fluid samples were obtained for HTCS. The urine was collected in a sterile urine bag before the resection was started, through the cystoscope sheath. After the urine collection the bladder was filled with 50-100 ml 5% glucose in water. Then the bladder was gently lavaged with a Thoomy syringe. This irrigation fluid was also collected in a sterile urine bag.

Following urine and irrigation fluid collection, the transurethral resection was started. Approximately 1-2 gr tissue was taken for HTCS after ensuring that adequate, representative material was taken for routine histopathologic study. For HTCS the exophytic portion of the tumor was taken in most cases.

After collection, the urine and irrigation fluid were brought to the pathology laboratory and transferred to sterile 50 ml centrifuge tubes. The samples were spun for 10 min at 1,500 rpm, the supernatant discarded, and 10-20 ml McCoy's wash was added to the cell pellet. After thorough vortexing, the cell suspensions were combined into one tube, urine and irrigation fluid separately. After another washing of the cells, McCoy's wash was added to the cell pellet, 2-5 ml according to the estimated number of cells obtained and the specimens were processed further as described below for the cell suspensions obtained from solid tumor.

The solid tumor was brought to the pathology laboratory in sterile 0.9% NaCl solution and further processed as recently described in detail (9). In brief, the tissue was minced with scissors for 10 min in McCoy's wash through a metal sieve with 60 holes per inch. The suspension of cells was then syringed

once manually through a 25 g 1 in needle and washed twice in McCoy's wash. Trypan-blue-excluding cells were determined using 0.1 ml cell suspension and 0.1 ml trypan blue. The cell concentration was determined in a Burker-Turk hemocytometer. The concentration of large nucleated cells was then adjusted to $3x10^6$ cells per ml McCoy's wash. Culture dishes (35mm) were seeded with $5x10^5$ large nucleated cells per dish in double layer soft agar exactly as described elsewhere (3,10), except that conditioned medium was not used. Culture dishes were examined within 24 hours after plating to evaluate presence of clumps seeded into culture and rule out infection of the culture. Colonies of tumor cells were first examined after one week after plating.

Part of the cell suspension not used for plating was air-dried and stained by the Papanicolaou technic to assess cell morphology and the types of cells brought into culture. Detailed morphologic studies of the colonies and other structures were performed on agar layers mounted on standard glass slides (8) and stained with the Periodic Acid Schiff reaction. From selected cultures, parrafin sections were made of colonies, stained with hematoxylin and eosin (7), and the tumor histology in the original biopsy specimens.

RESULTS:

Table 1 summarizes the patient and culture data in the present series. Of the total 42 samples cultured, 25 showed growth in the soft-agar system, growth being defined as five or more colonies composed of 30 or more tumor cells per plate. An additional 7 cultures could not be evaluated because of bacterial or fungal infection of the cultures or a markedly bloody background. Of the 12 urines cultured, 8 showed growth, which compares favorably with the 12 of 18 tumor samples which showed growth and is notably better than the 5 of 12 specimens from irrigation fluid which showed evaluable growth.

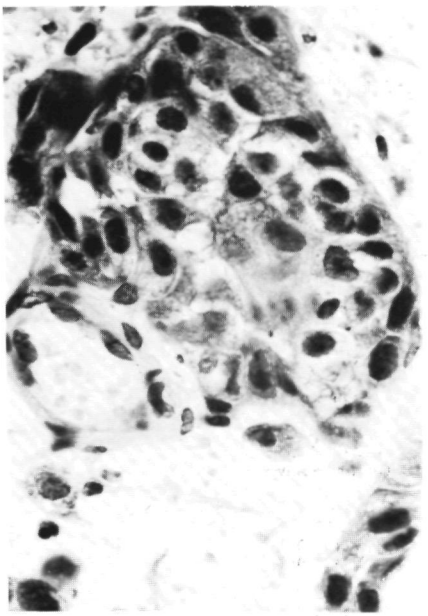
Table 1 Human Transitional Cell Carcinomas Cultured in HTCS Clinical and Pathologic Data and Culture Results

Patient Number	Age/Sex Comments	Pathologic Grade	Pathologic Stage	Culture Plates*		Colony Count per Plate Mean (Range)†
1	31/m TUR	II–III	T1b-T2N0M0	Urine Irrigation fluid Tumor	1/1 17/3 137/6	Infection Infection 85 (75–95)
	Cystectomy (after 2000 rad radiation)			Tumor	4/3	No growth
2	68/m recurrence	0	T0	Urine Irrigation fluid	less than 1/0 3/3	 2?
3	65/m	II–III	T2NXM+?	Urine Irrigation Fluid Tumor	15/6 0 78/6	Growth positive‡ Growth positive‡
4	53/m	III	pT2	Urine Irrigation fluid Tumor	15/6 10/6 78/6	28 (23–40) 9 (6–13) 77 (61–105)
5	70/m recurrence	II–III	T1NXM0	Urine Irrigation fluid	1/1 5/3	Growth positive‡ 10 (4–16)
6	70/f	I–II	TANXM0	Urine Irrigation fluid Tumor	9/5 43/4 12/6	19 (13–29) Growth negative 26 (12–45)
7	56/m recurrence (after adriamycin therapy)	I–II	TA-T1NXM0	Urine Irrigation fluid	1/1 5/3	Growth negative Growth negative
8	69/m	I	TANXM0	Urine Irrigation fluid	1/1 1/1	Not counted bloody background Not counted bloody background
9	64/m	II	T1NXM0	Urine Irrigation fluid Tumor	1/1 2/2 1/1	24 4 (0–8) 1
10	76/m	II–III	T2NXM0	Urine Irrigation fluid Tumor	6/6 8/6 124/6	25 (13–33) More than 100 More than 100
11	53/m same patient as number 4, completion of resection	III	pT2	Urine Irrigation fluid Tumor	36/3 94/3 149/3	32 (30–34) 1 (0–3) 5 (2–6)
12	74/f	II–III	pT1	Urine Irrigation fluid Tumor	32/6 23/6 153/3	15 (6–24) 52 (11–104) 17 (15–20)
13	62/f	I–II	TANXM0	Urine Irrigation fluid Tumor	6/6 6/3 27/6	Infection Infection 70 (52–93)
14	76/m	II	T1-T2NXM0	Tumor	72/3	75 (71–78)
15	48/f	I–II	TANXM0	Tumor	3/3	2?
16	46/m	I	TANXM0	Tumor	206/9	Growth negative
17	47/m	II	T1-T2NXM0	Tumor	184/9	17 (5–39)
18	65/f	II–III	T3NXM+	Tumor	34/9	77 (50–89)
19	76/f	I	T1NXM0	Tumor	134/9	Growth negative
20	70/m	II	T2NXM0	Tumor	33/3	10 (9–12)
21	35/m	II	T2NXM0	Tumor	59/3	Infection
	Recurrence during adriamycin therapy					

* 5×10^5 large nucleated cells per culture plate. Number of possible cultures from the specimen/Number of culture dishes actually set up.

† Mean and range of colony counts.

‡ Growth clearly recognizable colonies could not be counted because of bacterial or fungal infection.



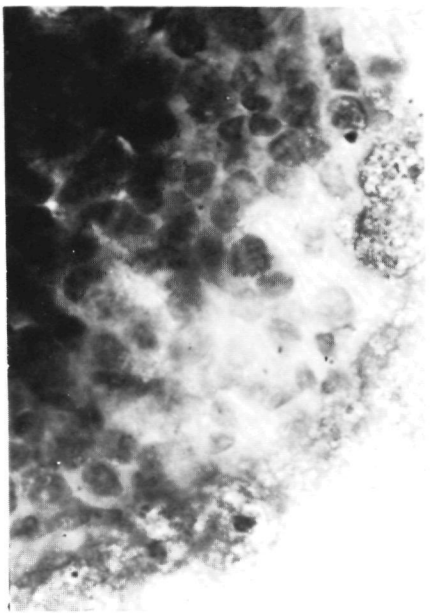


Fig. 1 (left). Nest of transitional cell carcinoma (Grade II, Stage pT1) in bladder lamina propria of 52-year old man. Hematoxylin and eosin, (x40).

Fig. 2 (right). Colony of transitional cell carcinoma from the same tumor illustrated in Figure 1 after 2.5 weeks of growth in soft-agar culture. The cytologic and "histologic" features of the colony reproduce those of the tumor cell nest in the biopsy material of Figure 1. PAS (x40).

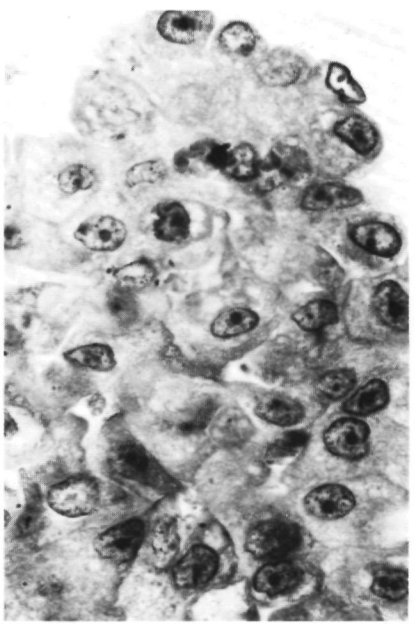


Fig. 3. 4u section of paraffin-embedded colony of transitional cell carcinoma, similar to the intact colony shown in Figure 2. Hematoxylin and eosin, (x50).

Table 2 Correlation of Tumor Grade with Number of Colonies Produced in Soft Agar (Mean, Range)

Grade	Urine	Irrigation Fluid	Tumor
0	——	2 (?)	——
I	*	*	——
	——	——	Growth negative
	——	——	Growth negative
I–II	19 (13–29)	Growth negative	26 (12–45)
	Growth negative	Growth negative	——
	†	†	70 (52–93)
	——	— —	2 (?)
II	24	4 (8–0)	1
	——	——	75 (71–78)
	——	——	17 (5–39)
	——	——	10 (9–12)
	——	——	†
II–III	†	†	85 (75–95)
	——	——	Growth negative§
	‡	——	‡
	‡	10 (4–16)	— ——
	25 (13–33)	>100	>100
	15 (6–24)	52 (1–104)	17 (15–20)
	——	——	77 (50–89)
III	28 (23–40)	9 (6–13)	77 (61–105)
	32 (30–40)	1 (0–3)	5 (2–6)

* Not counted because of markedly bloody background in culture

† Not evaluable because of bacterial or fungal infection in culture

‡ More than five tumor cell colonies clearly recognizable but reliable counting was not possible because of bacterial or fungal infection

§ No growth after 2 000 rad radiation to the bladder (same patient as the first line of the II–III group)

Table 3 Correlation of Tumor Stage with Number of Colonies Produced in Soft Agar (Mean, Range)

Stage	Urine	Irrigation Fluid	Tumor
pT0	——	2 (?)	——
pTA	19 (13–29)	Growth negative	26 (12–45)
	*	*	——
	†	†	70 (52–93)
	——	——	2 (?)
	— —	——	Growth negative
pTA–T1	Growth negative	Growth negative	——
pT1	‡	10 (4–16)	——
	24	4 (0–8)	——
	15 (6–24)	52 (11–104)	17 (15–20)
	Growth negative		——
pT1–T2	†	†	85 (75–95)
	——	——	§
	——	——	75 (71–78)
	— —	——	17 (5–39)
	——		‡
pT2	‡	——	‡
	28 (23–40)	9 (6–13)	77 (61–105)
	25 (13–33)	>100	>100
	32 (30–34)	1 (0–3)	5 (2–6)
	— —	-	10 (9–12)
	——	——	†
	——	——	77 (50–89)

* Not counted because of markedly bloody background in culture

† Not evaluable because of bacterial or fungal infection in culture

‡ More than five tumor cell colonies clearly recognizable but reliable counting was not possible because of bacterial or fungal infection

§ No growth after 2 000 rad radiation to the bladder (same patient as the first line of the pT1–T2 group)

At least one culture plate from each sample was evaluated after termination of the culture by examination of a PAS-stained agar layer containing colonies. In all cases the morphologic features of the cells comprising the tumor colonies in agar were clearly recognizable as being those of transitional cell carcinoma. In most but not all cases, the histo- and cytopathologic grading of the tumor as grown in agar agreed with the grade of tumor as seen in the histologic material. Figure 1 shows an invasive transitional cell carcinoma in hematoxylin-and-eosin-stained, parrafin block material. Figure 2 is a colony of tumor cells from the same tumor after 2.5 weeks in agar culture. Figure 3 is a hematoxylin-and-eosin-stained, parrafin section of a colony similar to that shown in Figure 2. The cytologic and histologic features of the cell nests are comparable.

Table 2 shows the correlation of colony growth in agar with histopathologic grading of the tumor. Table 3 shows the correlation of colony growth with tumor stage (pT) according to the UICC criteria (4). These data demonstrate that, in general, more colonies are produced in agar from tumor samples than from either urine or irrigation fluid. However, in general, urine seems to produce evaluable growth more reliably than irrigation fluid. Although the number of samples tested is small, the number of colonies per culture plate produced from urine samples shows a trend toward correlation with grade and stage of tumor, a trend not identifiable in cultures of irrigation fluid or solid tumor specimens.

DISCUSSION:

The present data demonstrate that evaluable growth of human transitional cell carcinoma colonies in double-layer soft-agar culture can be obtained from urine samples. The number of colonies per sample and the percentage of samples producing colonies compares favorably with soft-agar culture of bladder irrigation fluid, though these parameters of growth are infe-

rior to culture of a solid tumor obtained by transurethral resection. The number of colonies produced from urine samples in soft-agar culture tends to correlate with the pathologic grade and stage of tumor.

The evaluation of sensitivity or resistance of tumors to chemotherapeutic agents in vitro is the most technically demanding application of the cloning system of tumors from humans. In addition to demanding low between-plste variability of number of colonies and low infection rates, this application requires growth of at least 30-40 colonies per control (non-drug-incubated) culture plate to reproducibly recognize significant tumor sensitivity to a chemotherapeutic agent in vitro. For this application of HTCS, the use of urine samples as reported here is still inadequate.

However a second application of tumor cell culture, assessing the effectiveness of therapy by determining the presence of "viable" or clonogenic tumor cells after therapy is clearly feasible using urine samples. A positive soft-agar culture following therapy of a transitional cell carcinoma clearly indicates that tumor cells with demonstrable proliferative capacity remain. How this information can be used to improve clinical management of patients with transitional cell carcinoma must be explored further by parallel and prospective clinical trials. The interpretation of a negative post-therapy soft-agar culture is made difficult by the relatively low growth rates of tumors in soft agar (9). Further improvement of the technics of HTCS may provide a partial solution to this problem. In addition, the fact that collection of urine samples is non-invasive makes frequent culturing possible and may in part negate some of the problems of low growth rate in culture (comparable to blood cultures in SBE).

The third application of soft-agar culture of transitional cell carcinoma, evaluation of prognosis, clearly deserves further attention. The present data suggest a correlation of number of colonies produced in culture with the pathologic grade and

stage of tumor. If in fact further data support this correlation, the culturing of repeated urine samples over time may provide a relatively easy, non-invasive technic for monitoring patients, especially those with low-grade papillary transitional cell tumors, for signs of progression, especially early invasion. For this purpose, the availability of an automated colony counter (5) offers the potential of a more sophisticated evaluation of tumor colony growth in culture. The availability, from the automated counter, of other parameters of colony growth, such as size distribution of colonies, rate of change of colony size distribution with time, and early rate of colony development, will provide objective quantitative measures of biologic behavior of tumors in vitro. These measures should provide a basis for comparison of in vitro growth with in vivo behavior that is both more varied and more reliable than manual observation and counting (5).

The present study shows that the HTCS technic is easily implemented in, and integrated into, the routine work flow of a pathology department, requiring little more in personnel and space than a very modest microbiology laboratory. The development of non-invasive technics for obtaining tumor samples , as well as the routine soft-agar culturing of tumor samples obtained for other purposes, will allow this method to be evaluated further for potential contributions to solving a variety of patient management problems in clinical oncology.

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CHAPTER 3.

SOFT AGAR CULTURES OF TRANSITIONAL CELL CARCINOMA COLONIES FROM URINE, IRRIGATION FLUIDS AND TUMOR SAMPLES.

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ABSTRACT:

Soft agar cultures of transitional cell carcinoma from urine, irrigation fluid and transurethral resection solid tumor specimens show good colony growth. Growth of tumor colonies produced from urine was adequate to evaluate the presence of a viable tumor, since 12 of 18 noninfected cultures showed growth. The number of colonies produced was adequate to evaluate the sensitivity or resistance to chemotherapy agents in only 3 of 18 cultures. For irrigation fluid, similar results were obtained: 9 of 17 noninfected cultures showed growth, while only 3 of 17 were adequate for drug-sensitivity evaluation. For the evaluation of sensitivity or resistance, tumor cell suspensions were most appropriate, 7 of 25 noninfected cultures showed 30 or more colonies/dish, whereas 17 of 25 showed growth. With the possibility of obtaining growth of tumor cells derived from urine, a prospective study is proposed to define the value of repeated urine cultures in monitoring the status of the urothelium of patients treated for transitional cell carcinoma.

Key words: Tumor cells - Stem cells - Soft agar culture - Transitional cell carcinoma - Bladder cancer.

INTRODUCTION:

Noninvasive papillary transitional cell carcinoma is often multifocal and has a recurrence rate after surgical excision of 40-70% (Soloway, 1980). Approximately 10% of these tumors recur with an increase in grade and/or stage (Gilbert et al., 1978).

Chemotherapy of the noninvasive tumors attempts to prolong the recurrence-free interval after transurethral resection (TUR). A variety of agents have been tried as intravesical therapy (e.g. adriamycin, mitomycin C, epodyl, thiotepa, cisplatin, VM 26). For advanced disease a truly effective chemotherapeutic regimen has not yet been developed. Cisplatin, as a single agent or in combination, is under investigation as are other drugs and combinations (Merrin et al., 1975; Cross et al.,1976; Yagoda et al., 1977, 1978; Turner et al., 1979). These regimens are not without risks and toxic side effects are usually dose-limiting.

To prevent morbidity induced by ineffective treatment, many techniques have been investigated to develop a predictive laboratory method to determine individual tumor sensitivity to the proposed drugs. Much attention has been given to the human tumor clonogenic cell culture, HTC3, developed by Hamburger and Salmon (1977). This cloning system, in which the human tumor clonogenic or "stem" cells produce colonies in a two-layer soft agar system, has become a popular investigational tool to determine the resistance or sensitivity of individual patients' tumors to chemotherapeutic agents in vitro. Retrospective studies indicate that, in the tumor types investigated, this method predicts tumor sensitivity in vivo with 60-70% accuracy (Hamburger, 1981).

In addition to determining chemotherapy sensitivity and resistance, at least two other potential applications of the tumor cell culture system have been suggested (Herman et al., 1983a, b; Kirkels et al., 1982). Growth of tumor cell colonies in

soft agar may provide a means of monitoring the effectiveness of all forms of therapy by determining the presence of clonogenic and thus viable cells in post-therapy biopsies. Furthermore, in vitro growth parameters may provide a predictive means of determining tumor behavior, especially in those tumors where the borderline between benign and malignant is not sharply discernible by routine pathologic examination; e.g. papillary transitional cell carcinoma.

For further investigation of these and other questions, it is very important to obtain good quality tumor cells for culture with a minimum of trauma to the patient. Most published reports deal with cultures of tumor biopsies and more recently of effusions and irrigation fluids (Buick et al., 1979; Stanisic and Buick, 1980; Niell et al., 1982). The present paper reports the culture of human transitional cell carcinoma colonies from urine, as well as tumor biopsies and bladder irrigation fluid, and compares the results of tumor cell culture from these three specimen sources.

MATERIALS AND METHODS:

From patients admitted to the Urology Department of Radboud Hospital for TUR of a transitional cell bladder tumor (newly diagnosed or recurrent), 32 tumor samples, 29 urine samples and 29 bladder irrigation fluid samples were obtained for HTCS3.

The urine, collected before resection, was obtained in a sterile urine bag through the cystoscope sheath. After the urine collection, 50-100 ml 5% glucose in water was instilled into the bladder. Then the bladder was lavaged with a Thoomy syringe. This irrigation fluid was also collected in a sterile urine bag. After fluid collection, tumor resection was started. Approximately 3-5 g tumor tissue was taken for HTC3 after ensuring that adequate representative material was submitted for

routine histologic examination. In most cases the exophytic part of the tumor was taken for HTC3

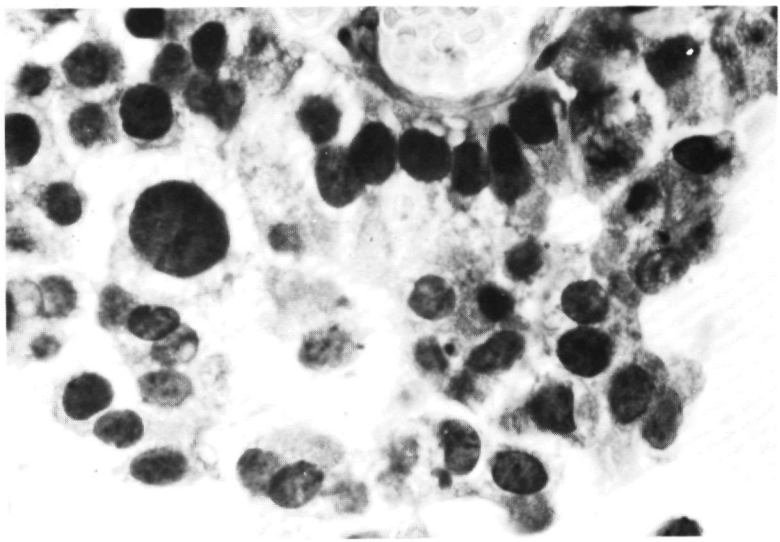
After collection,all samples were transported to the pathology laboratory where the urine and irrigation fluid were transferred to sterile 50 ml centrifuge tubes. The cells were centrifuged twice (400 g for 10 min), resuspended in 2-5 ml McCoy's wash according to the estimated number of cells obtained, and processed further as described below for the cell suspensions obtained from solid tumor.

The solid tumor was brought to the laboratory in sterile 0.9 NaCl solution and further processed as described elsewhere in detail (Salmon, 1980).

In brief, the tumor tissue is minced with scissors for 10 min in McCoy's wash through a sterile metal sieve. The resultant cell suspension and the pieces of tumor remaining in the sieve are incubated with collagenase and DNase for 2 h at 37° C in a 6% CO_2 atmosphere (Slocum et al., 1981), syringed once manually through a 25-gauge needle and washed twice with McCoy's wash. Trypan-blue-excluding cells are determined using 0.1 ml of the cell suspension, and the number of large nucleated cells is determined in a Bürker Turk hemocytometer. The cell concentration is then adjusted to $3x10^6$ large nucleated cells/ml McCoy's wash.

35-mm culture dishes are seeded with $5x10^5$ large nucleated cells per dish in double-enriched CMRL 1066 with 0.3% agar on a bottom layer of 0.5% agar exactly as described by Salmon et al. (1978), except that conditioned medium is not used. Culture dishes are examined within 24 h after plating to rule out infection of the culture and to evaluate the presence of clumps seeded into culture.

Since the patients did not receive antibiotics as routine, we tried to suppress possible infection with a double amount of penicillin/streptomycin added to the McCoy's wash during prepara-



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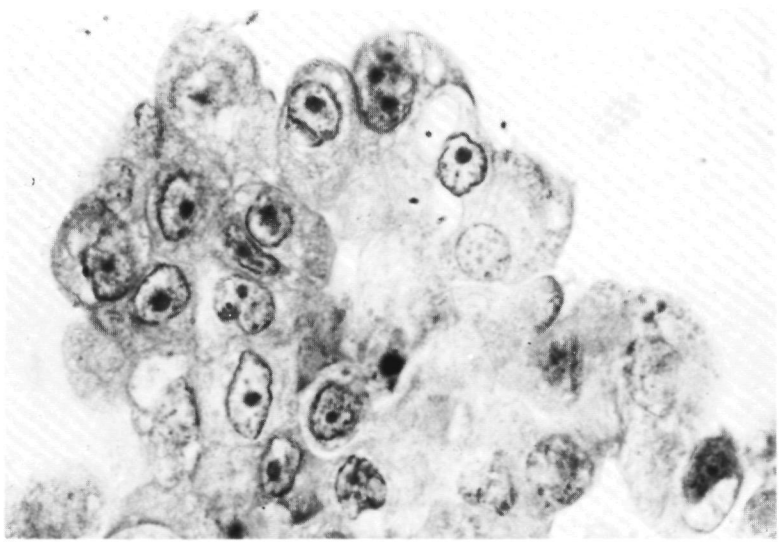

2.

Fig. 1. Transitional cell carcinoma grade II-III in a bladder tumor biopsy. HE. x50.

Fig. 2. Thin section of a paraffin-block colony of transitional cell carcinoma grown from a urine sample of the same patient illustrated in figure 1, after 3 weeks in soft-agar culture. The "histologic" and cytologic features of the colony reproduce those of the tumor cell nest in the biopsy material of figure 1. PAS. x50.

tion of the single cell suspension, with no improvement of the results. To prevent introduction of an air-borne infection in the incubator during incubation, we put the individual culture dishes in a larger (90 mm) Petri dish with good results. In the cultures described in this report, the number of infected cultures is significantly lowered.

Colonies of tumor cells are examined with an inverted microscope beginning after 1 week in culture. A colony of tumor cells is defined as a three-dimensional cellular aggregate with smooth borders containing 30 or more cells (Herman et al., 1983a, b).

Part of the cell suspension used to prepare the culture is air dried on a standard glass microscope slide and Papanicolaou stained to assess cell morphology and the types of cells brought into culture.

Detailed morphologic studies of the colonies and other structures in culture are performed on agar layers mounted on standard glass slides (Salmon, 1979) and stained with the periodic acid-Schiff reaction. From selected cultures, formalin-fixed paraffin blocks are made and 4- to 6-um sections are prepared, similar to routine histologic preparations (Pavelic et al., 1981) and stained with hematoxylin and eosin. The tumor colony morphology is compared with the tumor histology and/or cytology in the original biopsy specimens.

RESULTS:

In table I a summary is given of the numbers of cultures performed from the different sources of tumor cells. Only the results from cultures performed on urine, irrigation fluids and tumor samples obtained from patients with histologically proven transitional cell carcinoma are included. From an initial 90 cultures, 60 were evaluable. The remaining 30 cultures were not

evaluable due to fungal and/or bacterial infections.

TABLE I.SOURCES OF TUMOR CELLS AND GROWTH IN CULTURE.

	Number of cultures	Infected	Number of evaluable cultures	Number of growth positive cultures	
				> 5 colonies/ dish	> 30 colonies/ dish
Urine	29	11	18	12	3
Irrigation fluid	29	12	17	9	3
Tumor cell suspension	32	7	25	17	7

Table I also shows the number of cultures with 5 or more colonies and 30 or more colonies/culture plate. 5 Colonies/culture plate is accepted as an arbitrary number of colonies in a culture to conclude that it is growth positive. 38 of 60 cultures showed 5 or more colonies/culture plate. 30 or more colonies/culture plate are needed for in vitro drug testing. For evaluation of drug sensitivity or resistance, tumor cell suspension cultures can best be used, since 7 of 25 cell suspension cultures showed a mean of 30 or more colonies/culture plate.

For evaluation of growth potential, urine and tumor cell suspensions are equally useful as a source of tumor cells. 12 of 19 urine cultures showed positive growth, compared with 17 of 25 tumor cell cultures. Cultures of irrigation fluid showed slightly worse results: 9 of 17 cultures were growth positive.

TABLE II. CORRELATION OF TUMOR GRADE AND MEAN NUMBER OF COLONIES PRODUCED IN SOFT AGAR.

Tumor grade	Urine	Irrigation fluid	Tumor cell suspensions
I	-	-	0
	-	-	22 (14-27)
I-II	24 (13-40)	0	27 (12-45)
	0	0	-
	14 (8-24)	33 (27-40)	8 (0-26)
	-	-	69 (52-93)
	5 (1-10)	7 (6-9)	14 (10-19)
II	22	8	1
	-	-	85 (75-95)
	5 (2-8)	12 (9-22)	16 (5-39)
	-	-	9 (5-12)
	-	-	11 (4-16)
	-	-	4 (2-6)
	-	-	119 (88-145)
II-III	25 (24-40)	9 (3-13)	77 (61-105)
	32 (30-34)	-	-
	3 (0-6)	2 (1-3)	5 (2-6)
	-	0 (0-1)	3 (0-7)
	-	10 (4-16)	-
	25 (13-33)	100	100
	-	-	70 (50-89)
	15 (6-24)	52 (11-104)	17 (15-22)
	0	0	15 (9-25)
	44 (25-63)	0	0
	0	-	-
	0	0	0
III	0 (0-2)	10 (6-15)	-
	-	-	75 (71-78)
	54 (24-96)	-	0
	0	0	0

The ranges are given in parentheses.

TABLE II shows the correlation of tumor grade and the number of colonies produced in soft agar culture. The available data suggest that the results are consistent with the assumption that with increasing tumor grade the plating efficiency increases also. But additional experience is needed to document this possibility.

After termination of the culture, at least one culture plate from all samples showing growth was evaluated by examining a PAS-stained agar layer containing colonies. In all cases the morphology of the cells comprising the tumor colonies in agar was clearly recognizable as originating from transitional cell carcinoma. Cytopathologic grading of the tumor colonies grown in agar agreed in most cases with the grade of tumor as found in the histologic material. In HE-stained paraffin block material from a biopsy of a grade II-III transitional cell carcinoma, the cell nest shown in figure 1 is very similar histologically and cytologically to the tumor cell colony shown in figure 2, which was grown in soft agar from cells obtained from a urine sample of the same patient (3 weeks in culture).

DISCUSSION:

The present data demonstrate that evaluable growth of human transitional cell carcinoma colonies in double-layer soft agar culture can be obtained from urine samples, bladder irrigation fluid and tumor biopsies. Compared with bladder irrigation fluid cultures, the number of colonies produced from urine is in the same range, but both are inferior to the number of colonies produced in tumor cell suspension cultures prepared from solid tumors obtained by TUR. A major problem with the use of tumor biopsy material is to obtain a representative sample of tumor. It is possible that the exophytic part, used for culture in the present series, shows a different behavior in culture than the deep, sometimes infiltrating, part of the tumor.

The technically most demanding application of the HTC3 is the in vitro evaluation of tumor sensitivity and resistance to chemotherapeutic agents. This application requires a low between plate variability of the number of colonies and a low infection rate. Infection can be introduced in culture by using in vivo infected tumor material, or during the course of sample preparation and incubation of the culture plates. Occult bacterial colonization of bladder tumors has been described (Appell et al., 1980) which may be responsible in part for the high infection rate in our material.

Since growth of at least 30-40 colonies/control plate (non-drug incubated) is necessary to recognize reproducibly significant sensitivity or resistance to chemotherapeutic agents, cultures from urine samples are still inadequate for this application of HTC3.

However, a second application of the HTC3, determining "viable" or clonogenic tumor cells to assess the effectiveness of therapy (Herman et al., 1983a), is clearly feasible with urine cultures. If growth of transitional cell carcinoma colonies in cultures is found after therapy, tumor cells with proliferative potential clearly remain. With cytological examination of the cells in urine, if therapy effects are found it is not clear whether the remaining tumor cell still have a potential to replicate in vivo. With repeated cell cultures from urine, we try to investigate the biological behavior of these remaining tumor cells during and after therapy. It is not yet clear how this information can help to improve clinical management of the patient with transitional cell carcinoma. Prospective trials have to be initiated for further evaluation. Evaluation of a negative post-therapy soft agar culture is difficult because of the relatively low growth rates of tumors in soft agar in general. Further improvement of the HTC3 technique may provide a partial solution to this problem. In addition, urine sample collection is noninvasive which makes frequent repeated culturing possible.

Low stage transitional cell carcinoma of the bladder, where

treatment regimens with intravesical chemotherapeutic agents are proposed to prolong the recurrence-free interval (Soloway, 1980; Schulman et al., 1981), can be studied with the HTC3 technique in a randomized prospective trial. In this form of prospective trial all tumors are cultured and sensitivity and/or resistance assessed if possible.

All patients treated are followed with repeated urine cytology, cystoscopy and culturing of urine in HTC3 to assess tumor activity. Comparing these methods, the value of repeated culturing of urine in HTC3 as a means to search for viable tumor cells can be seen.

Further evaluation of patient prognosis can be made by studying the growth pattern of tumor colonies in HTC3. Evaluable growth parameters include the number of colonies and size distribution of colonies with time, all of which can be investigated in repeated cultures. For this purpose manual counting of colonies with an inverted microscope is not adequate (Kressner et al., 1980; Herman et al., 1983b). The availability of an automated colony counter, which gives not only a total number of colonies but also size distributions, makes more sophisticated growth studies possible.

In the management of transitional cell carcinoma it is of great value to identify those aggressive tumors which will readily metastasize and treat them more aggressively than the tumors that have a more "benign" prognosis. Thus, the relative ease of culturing transitional cell tumor colonies from urine, bladder irrigation fluid and tumor biopsies may allow HTC3 evaluation to make significant contributions to patient management throughout the entire course of the disease.

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CHAPTER 4.

"VIABLE" TUMOR CELLS IN POSTTHERAPY BIOPSY SPECIMENS.

A potential application of human tumor clonogenic cell culture.

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ABSTRACT:

Human carcinoma tissues were grown in culture for two to four weeks using the two-layer soft agar technique. All cultures that showed growth of tumor cell colonies also showed well-preserved, apparently healthy tumor cells lying singly. These cells showed neither proliferative capacity nor necrosis or morphologic degeneration during the time in soft agar. Thus, morphologic criteria seem to be poor indicators of tumor cell proliferative potential, at least in the short term. However, the method of soft agar tumor clonogenic cell culture itself provided a direct measure of tumor cell proliferative capacity, ie, the formation of colonies from single tumor cells. This may be valuable in directly assessing the presence of "viable" tumor cells in biopsy specimens taken after therapy, and thus guide further patient therapy. (Arch Pathol Lab Med 1983; 107: 81-83)

INTRODUCTION:

It is common practice in oncology to take biopsy specimens from areas of tumor localization to assess the effectiveness of therapy. In these situations, the pathologist is often asked if "viable" tumor cells appear in the specimen. To respond to this question, the pathologist may attempt to evaluate the viability of tumor cells by morphologic characteristics, especially nuclear and chromatin preservation. Both the pathologist and the oncologist usually are well aware of the severe limitations of this method. However, because the reliable diagnosis of persisting viable tumor cells can potentially be a major contribution to choice of further therapy, better insight into the criteria for evaluation of posttherapy biopsy specimens would be of value to both the pathologist and the clinician.

The growth of human solid tumors in soft agar (1) recently provided a unique opportunity to study the biology of human tumor cells of proven proliferative capacity. The clinical applications of this technique have primarily been directed at in vitro evaluation of chemotherapeutic responsiveness or resistance of tumors as a potential guide for individualizing the choice of a chemotherapy regimen in vivo (2,3). We studied tumor cell morphologic structure in soft agar culture, and its relevance to the problem of posttherapy evaluation of tumor biopsy specimens.

MATERIALS AND METHODS:

One hunderd thirty-six human tumors, primarily carcinomas, were cultured, using the two-layer soft agar method (1), in the department of pathology, Radboud Hospital, Nijmegen, the Netherlands. Routine surgical specimens of solid tumors were brought sterile and unfixed to the department of pathology.
Using sterile technique, a pathologist examined the material and chose tissue for soft agar culture (approximately 1 to 2 g)

and routine histopathologic study.

Tissue selected for soft agar culture was further processed according to the detailed description recently published (1). Briefly, the tissue was minced with scissors for five minutes through a metal sieve of approximately ten holes per square centimeters into McCoy's wash medium. The suspension of cells was then syringed once manually through a 25-gauge, 2.5-cm needle and washed twice in McCoy's medium. The percent of trypan blue-excluding cells was determined using 0.1 mL of the cell suspension ($<5\%$ to $>95\%$ in the present material) and the concentration of nucleated cells determined in a hemocytometer. The concentration of cells was then adjusted to $3x10^6$ nucleated cells per milliliter of McCoy's wash medium. Culture dishes, 35 mm, were seeded with $5x10^5$ nucleated cells per dish in double-layer soft agar, exactly as described elsewhere (4), except that conditioned medium was not used. Culture dishes were examined within 24 hours after plating to evaluate presence of clumps seeded into culture, and to rule out infection of the culture. Colonies of tumor cells were examined beginning one week after plating.

Air-dried, Papanicolaou-stained smears of the cell suspension used for plating the cultures allowed morphologic assessment of the cell composition and morphologic structure of the cell types brought into culture. After the growth of colonies, detailled morphologic assessment of the colonies as well as of other cells or particles in the agar was carried out on agar layers removed from the culture dishes, mounted on standard glass slides, and stained with PAS.

RESULTS:

Of the 136 tumors cultured, 54 grew in the culture system, growth being defined as five or more colonies of 30 or more cells per dish. Examination of the cultures 24 hours after pla-

ting showed distributions of tumor cells, interstitial cells, and inflammatory cells that exactly reflected those seen on the Papanicolaou-stained smears of single-cell suspensions. Further growth of colonies showed development of three-dimensional cell groups with smooth borders (Figs 1 and 2).

The most striking observations on these cultures resulted from examination of the PAS-stained agar layers after termination of the cultures. In all cultures that showed colony growth, examination of the colonies after two to four weeks showed that they consisted of tumor cells with the same characteristics as the tumor cells observed in the preculture, single-cell suspension. The colonies displayed morphologic features comparable with the histologic slides of the tumors. Figure 1, left, shows a mucinous adenocarcinoma found in the tunica albuginea of a 67-year-old man. Figure 1, center, shows a tumor cell colony from this same tumor after three weeks' growth in soft agar. The smooth border of tumor cell colonies developed in agar is apparent. The morphologic characteristics of the tumor cells in soft agar culture reproduced exactly those seen in the original histologic slides (Fig 1, left and center). Figure 2, left, shows a well-differentiated squamous cell carcinoma of the sublingual region metastatic to a cervical lymph node in a 76-year-old woman. Figure 2, center, shows a colony of cells from this tumor after four weeks' growth in soft agar culture. The similarity of cell features, as well as the formation in culture of a squamous nest similar to those seen in the original histologic structure, are apparent. Large colonies (more than 100 cells) often showed pyknotic tumor cells in the middle of the colony after three to four weeks in culture. We have not observed growth of fibroblast or hematopoietic colonies, in agreement with the observations of others (1).

Of special importance is the observation of single tumor cells (not located within colonies) in the agar layers after two to four weeks in culture. These tumor cells are well preserved and show the same nuclear and cytoplasmic features as tumor cells in colonies. Particularly noteworthy are the well-preserved chromatin structure and nuclear and cytoplasmic boundaries

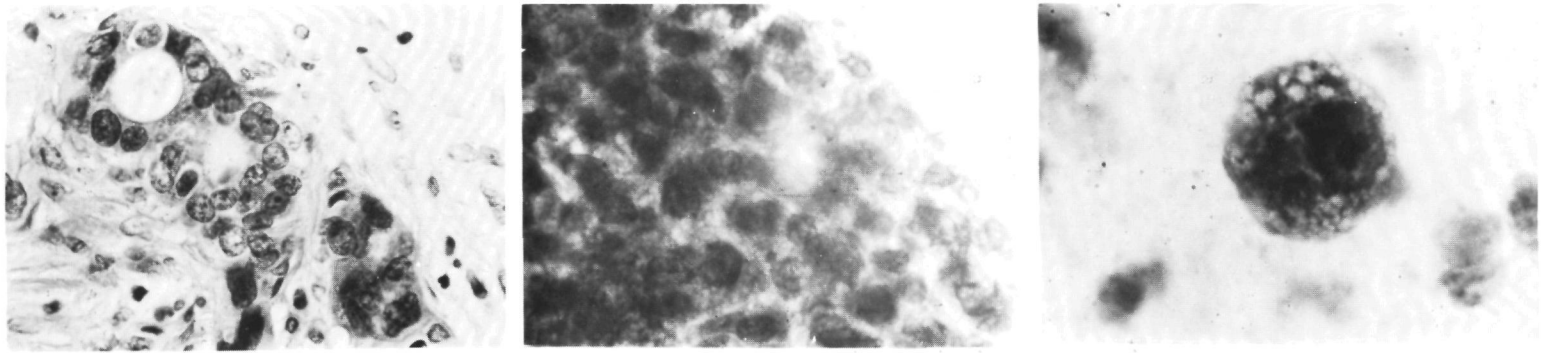

Fig. 1.- Mucinous adenocarcinoma metastatic to tunica albuginea of testis of 67-year old man. Primary was unknown. Left, Initial specimen (hematoxylin-eosin, original magnification x40). Center, Colony of tumor cells grown in soft agar from tumor shown at left, after three weeks in culture (PAS, original magnification x50). Right, Single well-preserved adenocarcinoma cell in same soft agar culture as colony shown at center (PAS, original magnification x100).

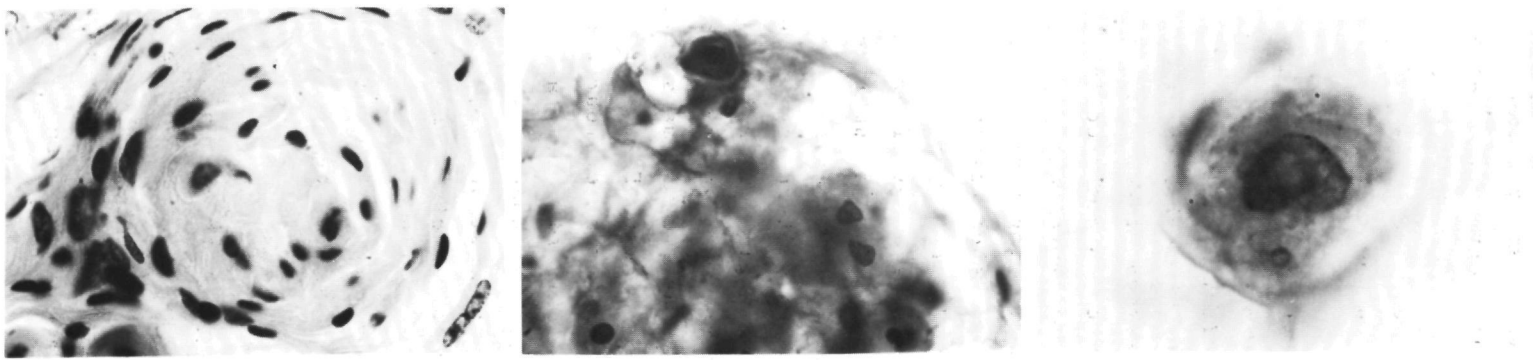

Fig. 2.- Cervical lymph node biopsy specimen with metastatic squamous cell carcinoma of sublingual origin in 76-year old woman. Left, Initial specimen (hematoxylin-eosin, original magnification x50). Center, Soft agar culture of squamous cell carcinoma shown at left, after four weeks in culture. Cytologic and histologic features of in vitro tumor cell colony reproduce those seen in biopsy material (PAS, original magnification x50). Right, Single well-preserved squamous carcinoma cell in same soft agar culture plate as tumor cell colony shown at center. Excellent morphologic preservation of cell after four weeks in agar is apparent (PAS, original magnification x100).

(Figs 1, right, and 2, right). Figure 1, right, shows an adenocarcinoma cell in the same agar layer as the tumor cell colony in Fig 1, center. The cell lies singly, clearly is well preserved, and shows cytologic features that make it readily identifiable as an adenocarcinoma cell. This cell is morphologically identical to the cells in the tumor cell colony (Fig 1, center). However, this and similar cells demonstrated no proliferative potential during the three weeks in soft agar culture. Similary, Fig 2, right, shows a single, well-preserved, squamous carcinoma cell from the same soft agar culture as the colony in Fig 2, center. In both cultures (Figs 1, right, and 2, right), the well-preserved single tumor cells are surrounded by necrotic debris and ghosts of tumor, inflammatory and stromal cells originally seeded into the agar culture.

COMMENT:

The present study demonstrated that cells may remain morphologically well preserved for up to a month in soft agar culture without demonstrating any proliferative capacity. Because proliferative capacity is the most important definition of viability in terms of tumor therapy, morphologic preservation or even ability to incorporate metabolites, exclude dye, or other commonly used measures of cell viability may in fact be misleading in evaluation of clinical tumor material.

The conditions of cell growth in soft agar culture clearly are highly artifactual when compared with those of in vivo tumor growth. Cells that remain well preserved by morphologic criteria but do not proliferate in soft agar culture may well retain the potential for growth in vivo. As dormant tumor cells, they may in fact be responsible for eventual regrowth of tumor after therapy. However, because of the serious consequences for the patient, the present observations emphasize that the morphologic characteristics of cells should be regarded as an unreliable indication of their proliferative potential. Thus, basing an assessment of viable tumor cells on the examination of rou-

tine cytologic or histologic preparations is clearly beyond the capabilities of routine pathologic techniques. Most pathologists and oncologists are aware of this limitation of morphologic methods. The frequency with which the question of viable tumor cells in posttherapy biopsy specimens is posed, however, indicates the importance of finding a more reliable technique with which to provide an answer.

Thus, a more optimistic second conclusion may be drawn from the present observations. Colonies of tumor cells are easily recognizable in soft agar culture and their presence indicates that tumor cells of relatively robust proliferative ability, a direct and compelling measure of viability, were present in the original material from the patient. A reliable positive answer to the oncologist's question of whether viable tumor cells were present in a biopsy specimen can be given if tumor cell colonies are produced from the biopsy material in soft agar culture. This demonstration of robustly proliferating tumor cells in posttherapy biopsy specimens is, to our knowledge, new information. The further questions of how this information can be used in patient management and wether this information can in fact have a significant effect on reducing patient morbidity and mortality must be answered in parallel and prospective clinical trials.

Similary, the meaning of no growth in posttreatment soft agar culture, (negative results), especially in cases where cytologic or histologic material shows evidence of tumor cells, remain to be defined. The prospects for obtaining reliable negative data from the soft agar technique, given the relatively low growth rates of tumors in this system (1), heavily depend on further development of the system through better understanding of the growth conditions of various tumor types. The presence after two to four weeks in soft agar of tumor cells that appear healthy yet do not proliferate may be ambiguous. Either these cells are dormant and retain the capacity to reenter the cell cycle and proliferate, or they may be in fact end stage, possibly retaining metabolic capacity, but without further proliferative capacity. This and similar questions that relate in

vitro and in vivo behavior are presently subjects of continuing investigation.

Finally, the present series demonstrated that the human tumor clonogenic cell culture technique was relatively easy to implement in, and integrate into, the routine work of a pathology laboratory. In addition to determining sensitivity or resistance of tumors to chemotherapeutic agents, this system is potentially applicable to other problems in routine treatment of tumor patients.

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CHAPTER 5.

IN-USE EVALUATION OF THE OMNICON AUTOMATED TUMOR COLONY COUNTER.

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ABSTRACT:

The reproducibility and accuracy of the Omnicon (Bausch and Lomb Inc., Rochester, NY) automated tumor colony counter for counting tumor colonies growing in double layer soft agar is evaluated and the reproducibility is compared with manual tumor colony counting. Replicate within day run-to-run colony counts of the Omnicon show a median correlation coefficient (r) $>$ 0.985, and day-to-day median r of $>$ 0.980. In contrast, for manual colony counting, the best intra-observer reproducibility achieved is a r of 0.943 and the best inter-observer reproducibility is a r of 0.831. Analysis of results from individual culture plates counted by the Omnicon on 5 separate days shows a median coefficient of variation of colony counts over 5 days of less than 20%. Counting of culture plates during incubation shows that the Omnicon is counting tumor colonies developing after plating of a single cell suspension.

Key terms: Clonogenic culture, tumor colonies, tumor cloning, soft agar culture.

INTRODUCTION:

The recent development of short-term culture of human primary tumors has offered the clinical laboratory the possibility of monitoring dynamic rather than static properties of human tumor cells. Specifically, the human tumor cloning system (HTCS) applied to solid tumors by Salmon et al., allows the growth in a two-layer soft agar system of tumor colonies from clonogenic, presumptive "stem" cells.(5,6) The first proposed application of this system to clinical oncology was the in vitro determination of chemotherapeutic sensitivity and resistance of tumors to allow individualizing of chemotherapeutic regimens.(5) Further clinical applications have been proposed, including posttherapy evaluation of persistent or recurrent tumor (1) and evaluation of grade and stage of tumor based on in vitro growth characteristics.(2)

Soon after the potential clinical usefulness of the HTCS was recognized, an automated tumor colony counter was developed (3) to speed up the process of counting colonies and determining chemotherapeutic drug resistance or sensitivity by computation of the inhibition of colony growth produced by preincubation of cell samples with cytostatic agents. Since the automated colony counter also gives information on the size distribution of tumor colonies, additional data bearing on tumor colony growth parameters in vitro have become available and are potentially useful new data which may contribute to all of the proposed clinical applications of HTCS cited here. However, before the full potential of the automated colony counter can be realized, the reproducibility and accuracy of its basic function of counting tumor colonies in soft agar culture must be established. The present report deals with initial in-use experience with the automated colony counter.

MATERIALS AND METHODS:

In the Pathology Department of Radboud Hospital, all urologic and gynecologic tumors received are cultured by the two layer soft agar method. Routine surgical specimens of solid tumors are brought sterile and unfixed to the Department of Pathology. A pathologist using sterile technique examines the material and chooses tissue for soft agar culture (approximately 1-2 g) and routine histopathology. Tissue selected for soft agar culture is further processed according to the detailed description recently published.(6)

Briefly, the tissue is minced with scissors for 5 min through a metal sieve of 60 holes/inch into McCoy's wash medium. The suspension of cells is then treated with collagenase and DNase for 2 hr (7), syringed manually once through a 25 g 1 inch needle and washed twice in McCoy's medium. Trypan blue-excluding cells are determined using 0.1 ml of the cell suspension, and the concentration of nucleated cells is determined in a Burker-Turk haemocytometer. The concentration of cells is then adjusted to $3x10^6$ nucleated cells/ml McCoy's wash. Culture dishes (35 mm) are seeded with $5x10^5$ nucleated cells per dish in double layer soft agar exactly as described elsewhere (5) except that conditioned medium is not used. Culture dishes are examined within 24 hr after plating to evaluate presence of clumps seeded into culture and to rule out infection of the culture. Colonies of tumor cells are examined beginning 1 week after plating. A colony of tumor cells is defined as a spherical three-dimensional cellular aggregate with smooth borders containing 30 or more cells.(1)

Air-dried Papanicolaou stained smears of the cell suspension used for plating the cultures are made from all specimens. These smears allow visual assessment of the cell composition and morphology of the cell types brought into culture. Following the growth of colonies, detailed morphologic assessment of the colonies as well as other cells and particles in the agar is carried out on agar layers removed from the culture dishes,

mounted on standard glass slides, and stained with the periodic acid Schiff (PAS) reaction. From selected cultures, formalin-fixed paraffin blocks are made and 4-6 um sections are prepared, similar to routine histologic preparations.(4) These sections are stained with haematoxylin and eosin as well as other routine histologic stains as appropriate for the type of tumor.

Automated colony counting:

The Omnicon (Bausch and Lomb Inc., Rochester, NY) was used for the present evaluation of automated colony counting. A detailed description of the theory and operation of this instrument has been published.(3) The instrument used in the present work was similar to that previously described except that it was fitted with an inverted microscope and a fully automatic 36-dish microscope stage. This allowed walk-away operation with counting of 36 culture dishes taking an average of 40-45 min.

For the present study, culture dishes from the routine workload of the laboratory including a spectrum of both gynecologic and urologic tumors were fixed in 4% glutaraldehyde. These cultures were unselected except that an attempt was made to include cultures showing the full range of colony counts: <30 to >1500 colonies/plate. For automated analysis, 108 culture dishes were counted by the Omnicon as three 36-dish sets. For determination of within day run-to-run reproducibility, the sets were counted twice in successsion with no changes in position of the dishes, settings of the instrument controls, etc. A total of 13 different within day comparisons were made on different days. For determination of day-to-day reproducibility, the same 108 culture dishes as 36-dish sets were counted five times on 5 or 6 different days over a period of 8 days with demounting and remounting of the cultures as well as readjustment of the illuminator source and scanner sensitivity each day. This provided a total of 35 different day-to-day comparisons. Care was taken however, to orient the culture dishes identically each day using an indelible ink mark on the side of the culture dish. Because of drying and cracking of the agar layers with ageing, (in spite

of glutaraldehyde fixation, hydration and refrigeration of the culture dishes) 29 of the culture dishes became unusable during the course of the study leaving a total of 79 culture dishes from which evaluable data were obtained.

Manual colony counting:

For comparison with automated colony counting, 155 culture dishes were counted by three different observers working in the culture laboratory and responsible for routine counting of colonies. For technical reasons, primarily deterioration and drying of the agar, these culture dishes were not the same dishes used for Omnicon counting but were from the same types of material with a similar distribution of numbers of tumor cell colonies. All colony counting was done with the culture plates coded so that the observer did not know whether or not he had seen the culture plate previously, nor did the observer know the results of previous counts, if any.

RESULTS:

Omnicon reproducibility:

Figure 1A shows the distribution of r values of duplicate Omnicon counts on the same day, each point representing one 36-dish set. The modal r value is > 0.990 and median r value is > 0.985.

The day-to-day r values of automated colony counting are presented in Figure 1B. Each point represents the r value of colony counts from one of the three 36-dish sets on 1 day with counts obtained from the same set on 1 of the other days. Thus, each day's colony count is compared with every other day's count for each of the sets of 36-dishes. The modal day-to-day r value is > 0.990 and the median > 0.980.

Since the distribution of both run-to-run and day-to-day r values showed considerable skewness with several day-to-day

r values less than 0.750, the variability of Omnicon counting was evaluated individually for each culture plate. Figure 2 presents the distribution of coefficients of variation (cv) for each culture dish computed from the colony counts on 5 different days. The modal cv is 6%, the median cv 10%, and 61 of the 79 evaluable culture dishes show a cv of automated counting less than 20%. When the mean number of colonies per dish is compared with the cv, a tendency to lower cv with higher colony counts is seen in all three test sets (r=-0.115, -0.217, and -0.380).

Reproducibility of manual counting:

For comparison with automated colony counting, 155 culture dishes were counted by at least two observers and/or by the same observer twice to assess intra- and inter-observer variability. Table I presents the results of these studies. The best reproducibility is achieved by observer A counting the same plates twice. Observer C also shows good reproducibility with duplicate counting of the same culture dishes (consistency), but clearly produces very different results from observers A and B. Observer A had, at the time of the present study, 1.5 yr experience with the soft agar culture technique after training in one of the most active and experienced HTCS laboratories. Observers B and C were trained by observer A and all three observers count culture plates routinely in our laboratory. It is clear that the best reproducibility achieved by manual counting falls short of that achieved by automated counting. In addition, the inter-observer variability is such as to call into question the use of data in a laboratory where more than one observer counts culture dishes.

Comparison of automated and manual counting:

All culture plates counted by the Omnicon were also counted by observer A. The r value for the three sets of culture dishes comparing automated and manual counting were 0.513, 0.630 and

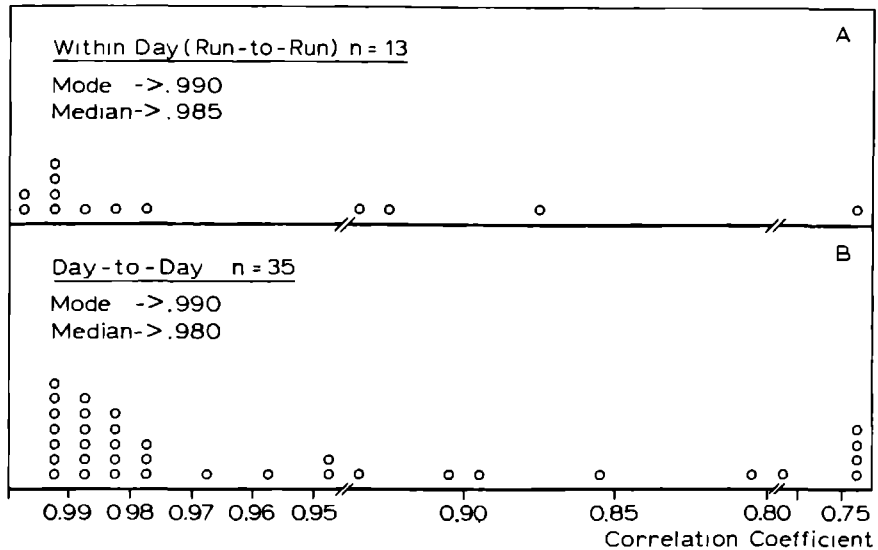

Fig. 1. Distribution of correlation coefficients of within-day run-to-run (A) and day-to-day (B) replicate Omnicon tumor colony counts. Each point represents one 36-plate set counted twice on the same day (A) or on two different days (B). Ordinate: number of observations; abscissa: correlation coefficient.

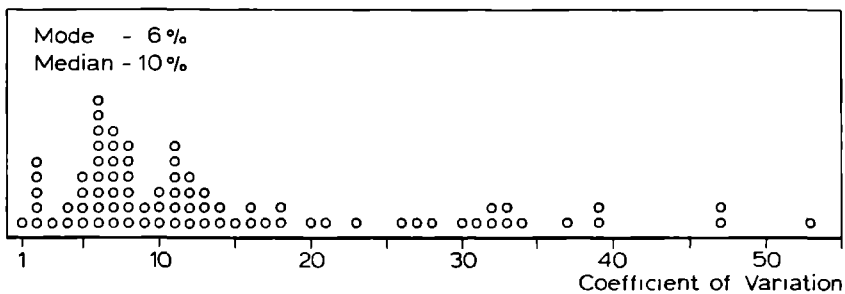

Fig. 2. Distribution of coefficients of variation of tumor colony counts for 79 evaluable culture dishes. Each point represents one culture dish counted with the Omnicon automated tumor colony counter on 5 different days.

0.891, respectively, similar to the inter-observer reproducibility shown by manual counting (Table I). In general, the manual counts were lower by factors of 2 to 5 than the Omnicon counts (Discussion).

Accuracy of automated colony counts:

Because of the poor correlation of manual colony counts by different human observers, the accuracy of the Omnicon was assessed independently of manual counting. The essential question to determine the accuracy of automated colony counting is whether the automated counts reflect development of tumor colonies in culture over time, dependent only on the presence of living cells in the original cell suspension. Table II shows an example of such a study. MatLyLu rat prostate carcinoma cells, maintained in our laboratory, were planted in soft agar culture. Because of the high plating efficiency and rapid growth of this undifferentiated tumor, $5x10^3$ cells were used per culture plate in place of the $5x10^5$ cells used for human primary tumors. As controls, MatLyLu tumor cells were prefixed in neutral buffered formalin for 2 hr, washed twice with PBS and planted in culture. During incubation, pairs of culture plates were removed from culture and counted (Table II). Because of high likelihood of infection of the culture plates during the time they were being counted by the Omnicon in an open laboratory and the subsequent risk of infection of other cultures in the incubator, the plates were not reincubated after counting. Thus, the data points in Table II represent different pairs of culture plates from the same tumor cell suspensions, processed and set in culture at the same time

As shown in Table II, the Omnicon counted no colony development in the culture plates planted with formalin-fixed cells, while the unfixed cells formed microscopically verified colonies over 9 days in culture. The development during 3 weeks incubation of Omnicon-counted tumor colonies from a human breast carcinoma is also shown in Table II. Colony growth is slower and the plating efficiency is lower in primary human tumor cultures.

TABLE I. INTER-OBSERVER REPRODUCIBILITY OF MANUAL TUMOR COLONY COUNTS[a].

	Observer		
	A	B	C
A	0.943 (25)[b]	0.831 (150)	0.478 (114)
B		0.733 (18)[b]	0.643 (110)
C			0.929 (15)[b]

[a] Correlation coefficient (number of culture plates).
[b] Pooled data for 3 observers, each observer counting the same culture plate twice: total N=58; r=0.877.

TABLE II. DEVELOPMENT OF TUMOR COLONIES IN SOFT AGAR CULTURE[a]

Days in culture	MatLyLu Rat Carcinoma[b]		Human Breast Carcinoma
	Formalin fixed	Unfixed	Pleural Fluid[c]
1			4±1
2	3±0	0±0	
3			12±3
6	2±1	419±40	22±2
9	2±2	521±136	
10			45±10
13			45±3
16			43±1
19			60±7
21			70±35

[a] Mean ±SE; two culture plates per data point. Omnicon counts
[b] $5x10^3$ Cells plated per culture dish.
[c] $5x10^5$ Cells plated per culture dish.

DISCUSSION:

The present study indicates that the Omnicon automated tumor colony counter functions with a high reproducibility. The median within day run-to-run r of $\geq$ 0.985 and median day-to-day r of $\geq$ 0.980 are significantly better than the reproducibility achievable by manual colony counting (Table I).

In order to achieve this reproducibility, instrumental variables must be carefully controlled. The most sensitive hardware component of the Omnicon is the light source. It is important that the light source be adjusted each day such that the maximum illumination, judged by level of TV-scanner saturation, is as high as possible and the same from day-to-day. It was noted in the present study that increased variability in colony counts was recorded just before the light bulb burned out. The addition of a feedback circuit for monitoring light intensity at the field of view might obviate this problem.

The cultures which were subject to high day-to-day cv were examined manually and compared with cultures producing low variability. The single most important cause of lack of day-to-day reproducibility of colony counts of individual culture dishes is the presence of tumor colonies at different levels in the agar layers. The most common levels of colony growth seem to be the upper and lower agar layers. Two or more layers of tumor cell colonies in a single culture dish seem to cause difficulties with the automatic focussing of the Omnicon such that different planes of focus are chosen from run to run and day to day, with inevitable variability in colony counts resulting. Preliminary manual inspection of culture dishes to ascertain that the level of colony growth within the culture dish is reasonably uniform should eliminate many of the culture dishes which give high cv with automated counting.

The observation that day-to-day cv tend to be inversely related to colony count in individual culture dishes may also be

related to problems with the automated focussing of the Omnicon. If the number of tumor cell colonies in a culture dish is so low that the instrument does not have a tumor cell colony to use for focussing in the first field examined, variable planes of focus will be used from run-to-run. A minimum threshold number of colonies per culture dish as a prerequisite for accepting Omnicon counts may help in eliminating from automated counting those culture dishes which show higher variability.

The Omnicon rejects fields of view from counting if the total optical density of the field is too high. In the present study, data from a particular culture dish were not included in the statistics if 10 or more fields (of a total of 35 scanned per culture plate) were rejected because of high optical density. This was an arbitrary choice based on counting statistics; further refinement of this threshold for rejecting Omnicon counts may be possible.

The most difficult problem in evaluating Omnicon results and comparing them with manual counting of tumor cell colonies is the question of accuracy. The most widely accepted present definition of a tumor cell colony in the double layer soft agar system is a reasonably regular round structure composed of 30 to 50 or more cells. Since identification by inverted phase microscopy of individual cells in colonies is difficult, it seems that in practice the most important criterion used by laboratory personnel is colony size. Since the size of a tumor colony composed of 30 cells can vary significantly depending on the tumor type, the number of cells actually required to meet the definition of tumor cell colony may vary both among human observers and for the Omnicon. This may explain in large part the poor inter- and intra-observer reproducibility of manual counting as well as the poor correlation between the Omnicon and manual counting.(3) During the initial phases of the present study, comparison of Omnicon and manual counts and further examination of fixed, PAS stained agar layers demonstrated that our laboratory's criteria for tumor colonies had become too stringent, requiring in practice 100 or more cells

for a structure to be accepted as a tumor colony.

The tumors used in the present study to evaluate Omnicon performance included a range of gynecologic and urologic tumors. Since the individual tumor cells of different tumor types vary widely in size, a tumor colony in agar composed of 30 cells will also vary in size. Thus, for optimal tumor colony counting, the size threshold accepted by the Omnicon may need to be varied depending on the specific tumor type.

The fact that growth of tumor colonies in agar during the period of incubation is clearly reflected in automated counts from a rapidly growing animal tumor and from a more slowly growing primary human tumor (Table II) supports the interpretation that the Omnicon is counting tumor colonies from two very different tumor types. Since colonies are not recognized by the Omnicon during incubation of formalin-fixed cells in the soft agar system, artefacts such as drying or cracking of the agar do not seem to contribute to the colony count produced by the Omnicon.

In general the reproducibility of the Omnicon is adequate for routine use in counting of tumor colonies in soft agar, and is clearly better than the reproducibility achieved by manual counting. Furhter evaluation of this instrument will allow criteria to be formulated to identify the types of cultures and properties of specific culture dishes which cause poor reproducibility of automated counting of specific cultures. The present study suggests that these may constitute approximately 20% of the total culture dishes in a routine tumor cloning laboratory. The remaining 80% of cultures, evaluable by automated colony counting, will also provide additional data of potential value such as colony size distribution during growth in culture and changes in size distribution caused by chemotherapeutic agents. This additional data is unavailable from manual counting from manual counting techniques.

ACKNOWLEDGEMENTS:
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CHAPTER 6.

PATTERNS OF TUMOR COLONY DEVELOPMENT OVER TIME IN SOFT AGAR CULTURE.

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ABSTRACT:

Human tumors were cultured by the two-layer soft-agar technique and the time course of tumor colony development was evaluated during periods of up to 6 weeks in culture. All colony counting was performed with an automated tumor colony counter (Omnicon: Bausch and Lomb, Inc, Rochester, NY, USA). This instrument provided colony counts per culture plate in six size categories from > 60 um diameter colonies to > 149 um diameter colonies. Six to 24 culture plates were used for each "growth curve", generally 24. Control (non-drug-treated) cultures were obtained from 117 tumors, of which 25 also provided enough cells to allow evaluation of the time course of colony development after exposure to cytotoxic agents. The development of colonies in non-drug-treated plates usually demonstrated a lag phase, a logarithmic growth phase to maximum colony development and a subsequent deterioration of colonies. In spite of clumps seeded into the agar, real colony growth could be recognized by frequent colony counting of culture dishes, although the temporal patterns of growth were compared with suspensions containing clumps from the same tumor. Drug pre-incubation caused changes in the temporal pattern of colony growth as well as in the total number of colonies. Some cultures showed drug sensitivity when evaluated at certain time points while evaluation at later time points showed only borderline drug effect or none at all. The potential utility of tumor colony growth curves in the clinical applications of tumor colony cultures is discussed.

INTRODUCTION:

The ability to grow primary human solid tumors in two-layer soft agar culture has provided a number of new opportunities in clinical oncology (Hamburger and Salmon, 1977; Hamburger, 1981; Salmon et al., 1978; Salmon, 1980). The initial clinical application of this technique was the in vitro testing of tumor sensitivity or resistance to chemotherapeutic agents, as a way both of tailoring chemotherapy regimens to individual patients and of testing new cytostatic agents. Newer clinical applications of the human tumor cloning system (HTCS) currently under investigation include monitoring of patients following therapy to assess the presence of persistent or recurrent tumor (Herman et al., 1983a) and prediction of in vivo tumor behaviour based on patterns of growth in vitro (Kirkels et al., 1982). The use of the HTCS to predict tumor response to cytostatic agents has shown a good correlation between in vitro and in vivo resistance (approx 90%) while correlation of in vitro with in vivo sensitivity has remained somewhat lower, 60-70% (Von Hoff et al., 1981; Selby et al., 1983).

As experience with the HTCS has accumulated in a number of laboratories , problems with the clinical applications of the system have been identified (see Selby et al., 1983, for review). Possibly the most serious problem preventing widespread clinical application of the HTCS is the relatively low percentage of primary human tumors which show evaluable growth in the system, varying from 25-50% depending on tumor type and source of starting material. In addition, preparation of a good suspension of single cells, generally considered an absolute necessity as starting material for the HTCS, is a persistent problem (Herman et al., 1976, 1979; Selby et al., 1983) for a number of solid tumor types.

In seeking solutions to these problems, as well as a better understanding of the process of tumor colony formation in soft agar, we have investigated the temporal patterns of growth of

both animal model tumors and primary human solid tumors in the HTCS.

MATERIAL AND METHODS:

In the Pathology Department, Radboud Hospital, all urologic and gynecologic tumors received, and tumors from other departments in cases of special interest, are cultured by the double-layer soft-agar method. All tumor samples, routine surgical specimens, urines, bladder irrigation fluids, pleural fluids and ascites, are brought sterile and unfixed to the Pathology Department. Urines and irrigation fluids from patients with transitional-cell carcinoma of the bladder who were undergoing transurethral resection of their tumor were collected in sterile urine bags through the cystoscope sheath before resection was started, as recently described (Kirkels et al., 1982). Pleural effusions and ascites were collecte' in sterile heparinized vessels.

After collection, urines, bladder irrigation fluids and effusions were transported to the Pathology Department within 15 min. The fluids were transferred to sterile 50 ml centrifuge tubes and spun for 15 min at 400 g, then the supernatant was discarded and 10-20 ml McCoy's 5A medium (GIBCO Europe) with 10% fetal calf serum and 1% pen/strep (McCoy's wash) were added to the cell pellet. After thorough vortexing, the cell suspensions were combined into one tube. After another washing of the cells, McCoy's wash was added to the cell pellet, 2-5 ml according to the estimated number of cells, and the specimens were processed further as described below for cell suspensions from solid tumor.

Solid tumor specimens were received sterile and unfixed at the Pathology Department and examined by a pathologist using ste rile technique. Material was selected for soft-agar culture and for routine histopathology. Tissue chosen for soft-agar culture was further processed according to the detailed description recently published (Salmon, 1980). Briefly, the tissue was minced

with double-blunt scissors in a sterile 50-ml centrifuge tube for 10 min in McCoy's wash medium. After centrifugation of the tumor mince (10 min, 400 g), the supernatant was discarded and the remaining tumor mass treated with collagenase Type II and DNase Type I for 2 h at 37°C (Slocum et al., 1981). Following incubation, McCoy's wash was added and the cell suspension was first passed through a metal sieve of 60 holes per inch. The cell suspension was centrifuged, the supernatant discarded and the cell pellet resuspended in McCoy's wash. This cell suspension was then passed through a gas-sterilized nylon filter with 70- to 100-μm holes (Ortho Diagnostics, Belgium). The cells were then washed again and the cell pellet resuspended in 2-5 ml McCoy's wash according to the estimated number of cells. Trypan-blue-excluding cells were determined using 0.1 ml of the cell suspension. The concentration of large nucleated cells was determined in a hemocytometer and then adjusted to 3×10^6 cells per ml McCoy's wash.

For in vitro drug testing, cells were incubated for 1 h in drug solutions made up in McCoy's wash to approximately 10% of the in vivo attainable peak plasma level (Salmon, 1980). The cells for the control group were incubated with McCoy's wash alone, omitting the cytotoxic drug. After drug incubation, the cells were centrifuged and seeded in 35-mm culture dishes with 5×10^5 cells per culture dish in double-layer soft-agar exactly as described elsewhere (Salmon et al., 1979) except that conditioned medium was not used. The cultures were then incubated at 37°C, 6% CO_2 in a humidified atmosphere.

All colony counting in the present study was performed using the Omnicon automated colony counter (Bausch and Lomb Inc, Rochester, NY, USA). This instrument and the choice of its operating parameters have been described in detail previously (Kressner et al., 1980). Briefly, culture plates are illuminated with a white-light source and a television camera produces high-contrast images for analysis by a solid-state picture processor. This picture processor examines all objects in the agar and analyzes their optical density, size and shape. On the

basis of preprogrammed logic using these three features, colonies of tumor cells are identified and counted in six size categories while artifacts are rejected. A 36-dish computer-controlled stage allows walk-away operation with 36 dishes taking approximately 45 min to count. The Omnicon has been demonstrated to be accurate and more reproducible than manual colony counting (Herman et al., 1983b).

Colony counting for the present study was begun within 1-2 days of plating of the tumor specimen and was thereafter repeated 2-3 times per week for up to 6 weeks, depending on the pattern of growth seen in culture. For each time point, at least two culture dishes if available were removed from culture, the number of colonies determined and the culture dishes fixed in glutaraldehyde for further morphologic and/or histochemical study. Culture dishes were not in general returned to the incubator following counting because of the risk of infection both of the counted dishes themselves and of other dishes in the incubator. Occasionally, when insufficient numbers of culture dishes were available from a given tumor, the culture dishes were sealed with porous tape and used for multiple counting with re-incubation after each count.

The present report is based on 117 tumor samples. Of these 117, 25 samples provided enough tumor cells to evaluate drug-pretreated as well as control dishes with 1-11 different drugs. For each growth curve, control or drug, 6-24 culture dishes, generally 24, were used.

Air-dried Papanicolaou-stained smears of aliquots of the cell suspension used for plating of the cultures were made from all specimens. These smears allowed morphologic assessment of the cell composition and morphology of the cell types brought into culture. Following colony counting of the culture dishes, detailed morphologic assessment of the colonies as well as other cells and particles in the agar with increasing time in culture was carried out on agar layers removed from the culture dishes, mounted on standard glass slides (Salmon and Buick,

1979), and stained with the Periodic-acid Schiff (PAS) reaction. From selected cultures, formalin-fixed paraffin blocks were made and 4- to 6-um sections were prepared as in routine histologic preparations (Pavelic et al., 1981). These sections were stained with hematoxylin and eosin as well as other routine histologic stains as appropiate for the type of tumor.

RESULTS:

Figure 1 demonstrates the growth in soft-agar of an undifferentiated rat prostate adenocarcinoma, MatLyLu, maintained in our laboratory. As in all the Figures, three curves are presented, representing all tumor cell colonies over 60 um in diameter (▲), over 86 um in diameter (●) and over 124 um in diameter (□). Standard error bars are absent if only one culture dish was available for counting at a given time point. The pattern of growth demonstrated in Figure 1 is typical of what is observed in tumor cell lines, either human or animal. A single-cell suspension is easily prepared and day 1-2 counts show few or no "colonies". Growth begins rapidly after plating, maximum colony counts being seen within 2 weeks of plating. Following the peak colony count, the number of counted colonies decreases, due to decreased optical density of necrotic colonies, which prevents their recognition by the Omnicon. The necrotic colonies are easily demonstrated morphologically in the PAS-stained agar layers.

To verify that the development of colonies in agar was real, an aliquot of the same tumor-cell suspension was pre-fixed for 1 h in 10% neutral buffered formalin, washed in McCoy's wash and seeded into agar culture in exactly the same way as the unfixed tumor cells. Colony counts from these cultures containing formalin-fixed tumor cells are presented in Figure 1 as open circles, showing no development of "colonies". Further data from this same culture showed no "colony" development up to 3 weeks after seeding of the formalin-fixed tumor cells. These data provide support for interpreting the growth curves presented here

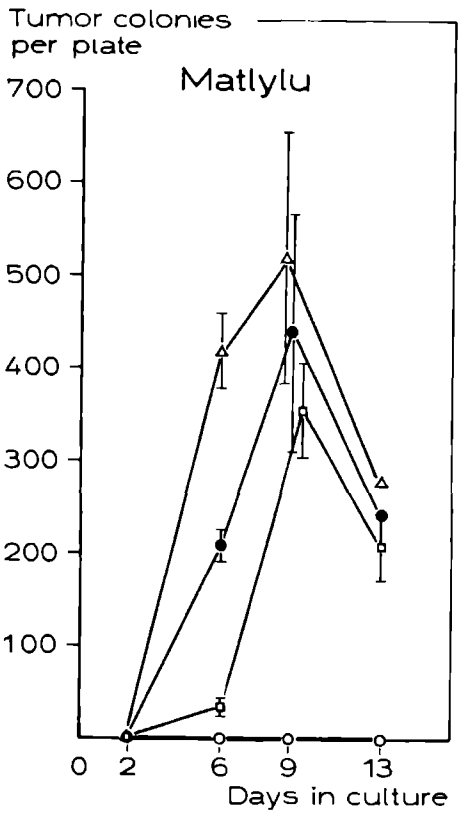


FIGURE 1 - Growth of rat prostate carcinoma cell line, MatLyLu, in soft-agar culture. In Figures 1-4, three curves are presented for each tumor: all colonies over 60 um in diameter (△), all colonies over 86 um in diameter (●), and all colonies over 124 um in diameter (□). In addition, an aliquot of this tumor cell suspension was fixed with 10% neutral buffered formalin before plating in soft-agar (0). Each point with standard error bars represents two or more culture dishes. Standard error bars omitted from day 13 points for visual clarity.

as representing real multiplication of cells during incubation after plating, rather than as artifacts of the agar culture system.

Of the 117 primary human tumors studied, 30 showed growth in the HTCS using two criteria: (1) presence of more than 20 colonies over 60 μm in diameter at the peak of growth and (2) a maximum colony number at peak growth at least twice the day 1 count or twice the minimum count achieved after deterioration of clumps seeded into culture (see below). An additional 13 tumors showed growth in the HTCS by one or the other of the two criteria. Seventy-four tumors did not fulfill either criterion and thus were interpreted as showing no growth.

Growth of primary human tumors in soft-agar culture presents several aspects not seen in the growth of cell lines. Figure 2a shows the growth of a breast carcinoma from pleural fluid. The development of colonies in soft agar is rapid with peak colony counts at day 7-11, but following the peak, the deterioration of colonies seen in cell lines (Fig. 1) is frequently absent or less dramatic, with a relatively long plateau of stable colonies. This long plateau is seen best in Figure 2a for the colonies $>$ 86 μm and $>$ 124 μm diameter. The relative predominance of colonies of smaller size is also more marked with primary human tumors.

One of the most significant differences between human tumors and cell lines is the presence of significant numbers of clumps seeded into the agar. Figure 2a shows that more than 100 clumps exceeding 60 μm in diameter were seeded into the agar. This finding correlates well with the cytologic appearance of a number of epithelial tumors growing in effusions or on the surface of cavities. These tumors grow as tight clusters of cells with nuclear and cytoplasmic molding and vague to absent cell boundaries, the appearance suggesting very tight bonding or even syncytium formation. This morphologic appearance is identical to that of human tumor colonies developing in soft agar culture, thus preventing reliable distinction between colonies de-

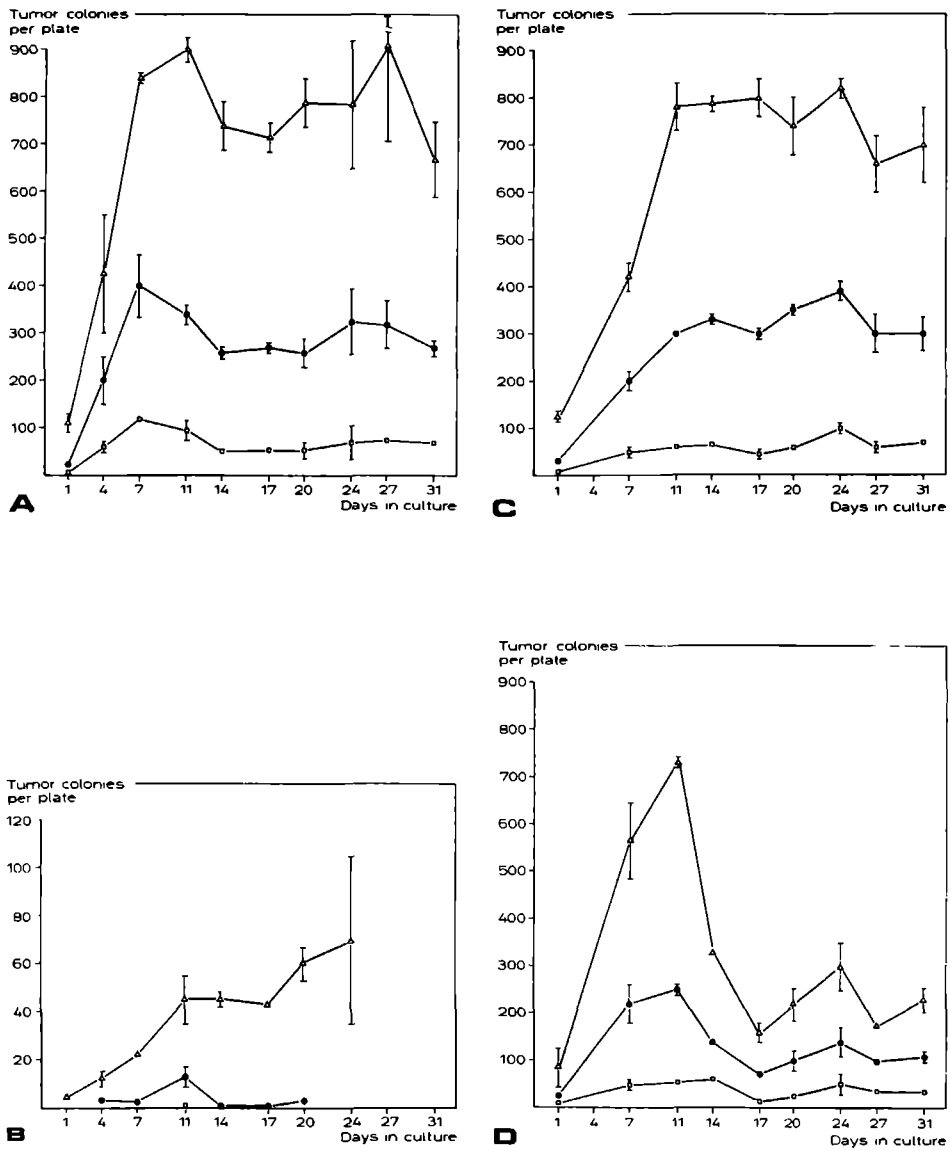


FIGURE 2 – Breast carcinoma from pleural fluid (*a*) Control (non-drug-treated), (*b*) same cell suspension as (*a*) but with additional incubation with trypsin (0 25% in 0 1% EDTA) before plating of culture, (*c*) and (*d*) same cell suspensions as in 2*a*, but incubated with 5-FU (*c*) or adriamycin (*d*) for 1 h before plating

veloping in culture and clumps seeded into culture if only a single time point of growth is evaluated. Examples of these tumors include breast and ovarian cancers growing in effusions and papillary transitional-cell carcinoma recovered from urine. Figure 2a demonstrates that growth of real tumor cell colonies can be recognized in soft agar using growth curves, even in the presence of significant numbers of clusters seeded into agar. Of the 117 tumors used in the present study,"Colony" counts 1 or 2 days after plating showed that 43 tumors contained 0-10 clumps over 60um in diameter seeded into the agar. Forty-nine samples contained 10-100 clumps and 25 contained more than 100 clumps seeded in the "single-cell" suspension.

An alternative solution to the problem of clumps seeded into agar is demonstrated in Figure 2b. Because of the marked clumpiness of the specimen shown in Figure 2a, an aliquot of the original cell suspension was further treated with trypsin digestion (0.25% in 0.1% EDTA; Flow Laboratories) before being plated in agar. Although the trypsin did not significantly change the percentage of trypan-blue-excluding cells, the growth of the tumor was significantly different from the growth without trypsin pre-digestion. The number of tumor cell clumps seeded into culture was markedly reduced. However, the time to peak colony growth was markedly prolonged: 24 days after trypsin compared with 11 days without trypsin. In addition, the growth of larger colonies ($>$ 86 um diameter) was almost completely eliminated by trypsin treatment.

A similar phenomenon is seen in Figure 3 from an ovarian cystadenocarcinoma cultured from peritoneal irrigation fluid. Figure 3a demonstrates that a large number of clusters were seeded into culture. However, by assessing "colony" counts at multiple time points, deterioration of these clumps over 3 weeks could be recognized. Finally at 3 weeks, real growth of tumor-cell colonies could be demonstrated with subsequent deterioration of the colonies over the following 2 weeks. Trypsin pre-digestion of the same single-cell suspension (Fig. 3b) markedly reduced the number of clumps seeded into the agar as well as the total number of colonies developed in all three size categories.

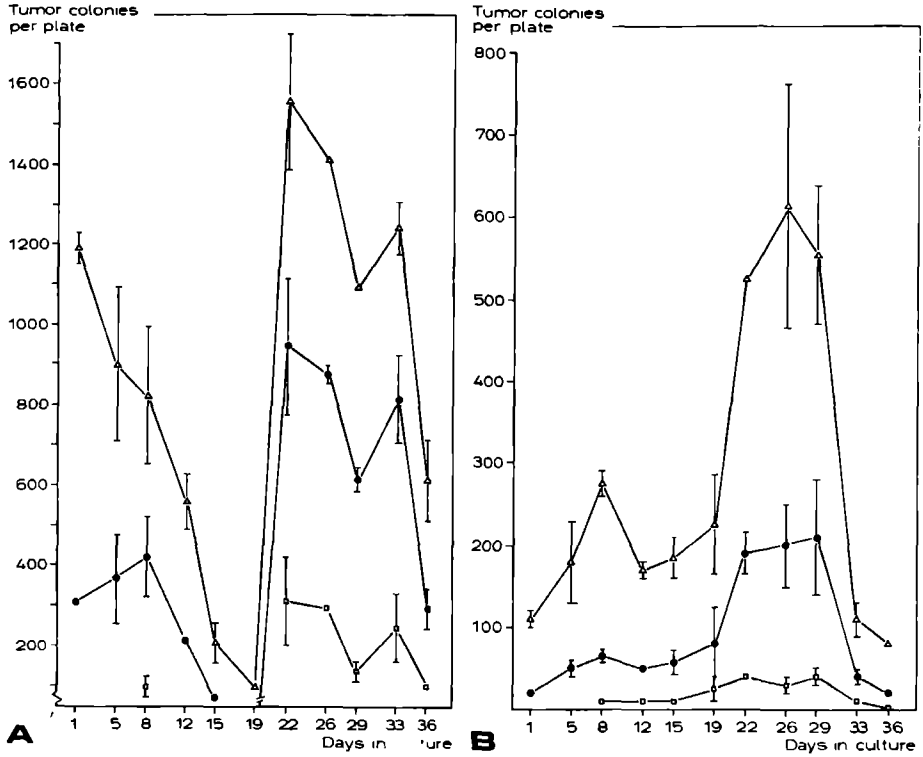


FIGURE 3 – Ovarian carcinoma from peritoneal washing (*a*) Cell suspension plated directly after preparation, (*b*) an aliquot of the same cell suspension as in 3*a*, but treated with trypsin before plating

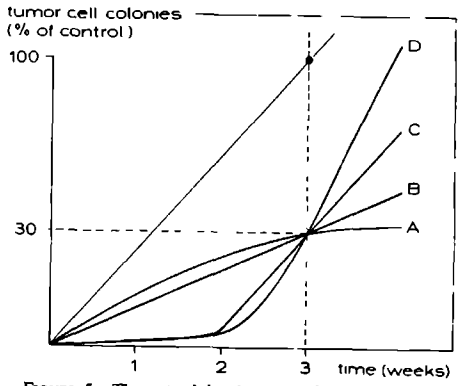


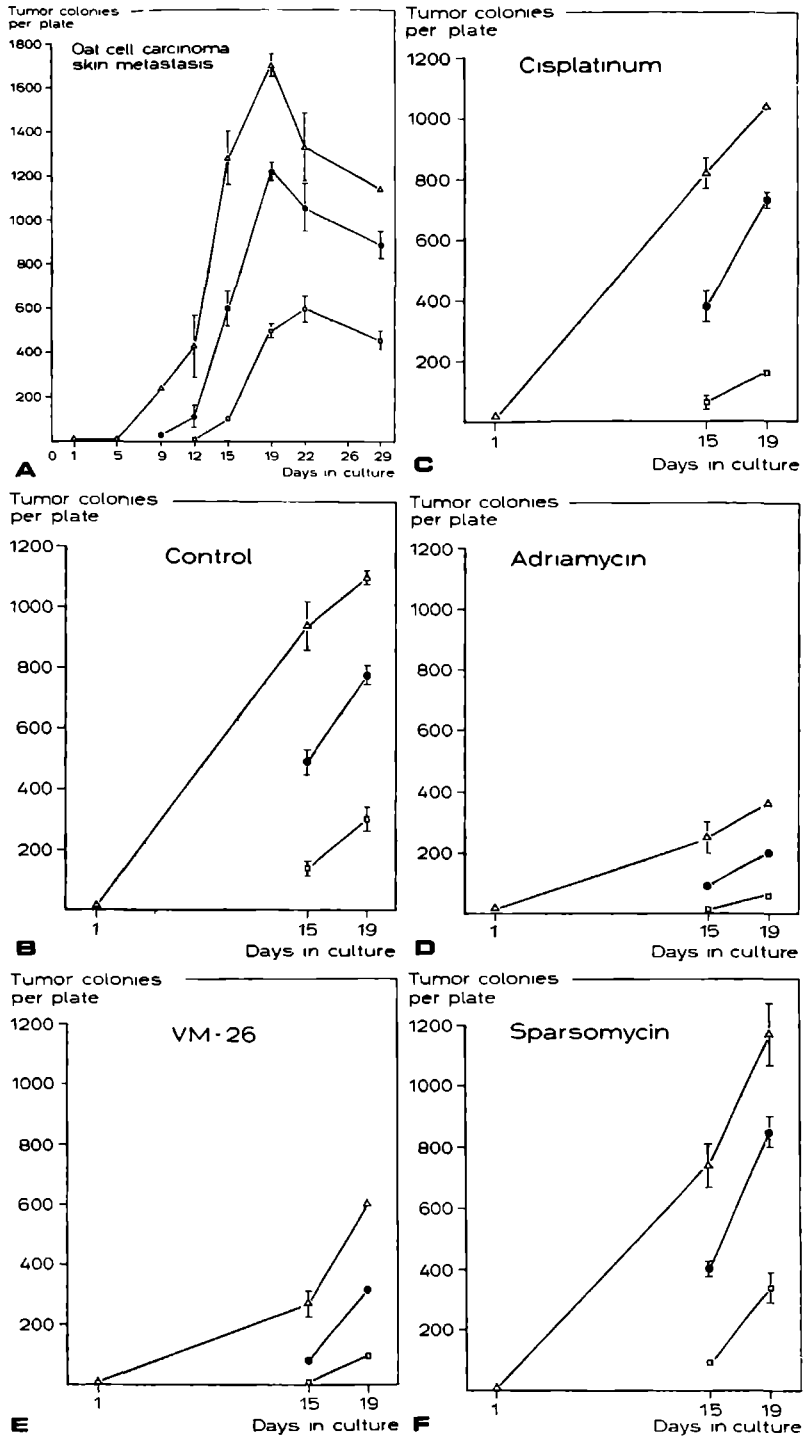
FIGURE 5 – Theoretical development of tumor colonies in soft-agar culture without (45° line) or after drug incubation Four different patterns of colony growth which could produce a 70% reduction in number of colonies at 3 weeks compared with control cultures For further details, see "Discussion"

However, in contrast to Figure 2a and b, the time to maximum colony growth was not altered by the trypsin treatment, being 22-26 days after plating of the tumor in both cases.

A morphologic difference between clumps seeded into culture and colonies developing in agar was suggested by study of the PAS-stained agar layers of this tumor. The clumps demonstrated early in culture (Fig. 3a, days 1-15) showed a morphology similar to paraffin block material from the original tumor with homogeneous, relatively dense cytoplasm. In contrast, the colonies developing in agar (Fig. 3a and b, later than day 22) contained cells with foamy cytoplasm and nuclei, suggesting phagocytosis of agar during growth.

Patterns of colony growth after drug treatment:

Twenty-five of the 117 tumors studied yielded enough cells to allow incubation of aliquots of the single-cell suspension with 1 to 11 cytostatic drugs before plating in agar. Of these 25 tumors, 14 also produced growth in the non-drug-treated (control) plates which met both criteria for growth as stated above. From these 14 tumors, a total of 89 growth curves after drug incubation were obtained. Seventy-two of the 89 samples showed no effect of the drug on the time course of colony development. Seventeen growth curves showed some effect of the cytostatic agent. The effects of drugs on the time course of tumor colony growth in vitro showed several patterns. Figure 2c shows the development of colonies from the breast carcinoma shown in Figure 2a, following 1 h pre-incubation with 5-FU. Although clearly resistant to 5-FU, the tumor shows some response to the drug in the delayed development of larger colonies ($\geq$ 86 um; $\geq$ 124 um) compared with the control, non-incubated sample. Figure 2d shows tumor colony development from the same tumor following 1 h incubation with adriamycin. Colony development to 11 days is similar to that of the control sample (Fig. 2a) but then a rapid decline in colony count ensues, followed by a minor second wave of growth during the third week in culture. This rapid decline in colony count between 11 and 17 days



◀▲ FIGURE 4 – Skin metastasis of oat-cell carcinoma (*a*) Plated directly from single cell suspension after preparation, (*b*) plated after 1 h incubation with McCoy s wash medium at 37° C as control for drug incubated specimens *c-f*, (*c f*) 1 h incubation with the indicated drugs before plating

could be recognized in PAS-stained agar layers as a loss of density of the colonies due to necrosis.

The real value of assessing growth dynamics in culture is suggested by Figure 4, cultures of an oat-cell carcinoma growing as a skin metastasis. Figure 4a shows a full growth curve of this tumor, demonstrating a lag phase of 5 days followed by rapid growth of colonies to a peak at 19 days and then a decline in the number of counted colonies. Figure 4b-f shows abbreviated growth curves of this tumor for non-drug-incubated (b) and four drug-incubated (c-f) samples. It should be noted that samples used only for growth curves, that is plated directly after preparation of the single-cell suspension as in Figure 4a, and aliquots from the same cell suspension incubated for 1 h before plating with McCoy's wash as a control for drug-treated samples as in Figure 4b, frequently showed quantitative and occasionally qualitative differences in the patterns of growth. When either day 15 or day 19 colony counts of this tumor were used, cis-platinum clearly showed no effect (Fig. 4c) while adriamycin was clearly effective in vitro ($>$70% inhibition of colony growth compared with control; Fig. 4d). VM-26 showed a more disturbing pattern of colony growth (Fig. 4e). At day 15 the number of colonies after drug treatment compared with control would be interpreted as showing drug sensitivity ($>$70% inhibition). However, the rate of colony growth between days 15 and 19 was faster after VM-26 treatment than in the control plates so that at day 19, in vitro drug sensitivity was no longer seen. A similar acceleration of growth between 15 and 19 compared with control was also seen in cultures incubated with sparsomycin, an experimental drug under investigation in our institution (Fig. 4f).

Evaluation of tumor colony development in soft-agar over time may provide new insight into the human tumor cloning system (HTCS) and may in addition contribute to solving some of the problems identified in the clinical applications of the HTCS. The development of tumor-cell colonies of different sizes after plating in agar can be demonstrated (Figs. 1-4) with va-

rying rates of growth to maximum number of colonies and sometimes with secondary growth periods (e.g. Fig. 2d). That this colony growth is real and not dependent on artifacts in the HTCS has been demonstrated (Fig. 1). The temporal pattern of growth of colonies in agar may yield additional information bearing on the expected behavior of the tumor in vivo (Kirkels et al., 1982) and also provide a more sensitive mechanism for recognizing tumor colony development as a way of monitoring patients after therapy (Herman et al., 1983a). However, there are frequently marked differences in growth dynamics between model tumor systems (Fig. 1) and primary human tumors (Figs. 2-4). These differences suggest that modifications of the HTCS based only on results from animal tumors or tumor cell lines should be implemented with great caution and only after extensive testing on primary human tumors.

The fact that real tumor colony development in agar can be recognized even when the tumor-cell suspension used for seeding the culture plates contain a significant number of clumps is potentially important (Fig. 2a,c,d; Fig. 3). However, this phenomenon demands further investigations for both practical and theoretical reasons. In practical applications of the HTCS, the fact that clumpy specimens may show different patterns of growth compared with pure single-cell suspensions (Fig. 2a,b) suggests that interpretations of in vitro chemosensitivity results must be made with caution. Even monitoring of patients after therapy for persistent or recurrent tumor may produce different results depending on whether the starting cell suspension contained clumps or not.

From the theoretical viewpoint, the HTCS is based on a "stem-cell" theory of tumor growth and the requirement that colonies developing in soft agar are the progeny of single tumor cells, that is, true clones (Mackillop et al., 1983). Clearly, the development of colonies in soft agar from specimens containing multi-cell spheroids introduces a fundamental change in the theoretical basis of the HTCS. Nevertheless, tumors in patients grow as aggregates of cells with ample cell-cell interaction. Thus it is not predictable whether in clinical applications of

HTCS the growth of tumor cell colonies from clumpy specimens will provide a better or poorer model on which to base decisions concerning predicted in vivo drug response, prognosis, or tumor persistence or recurrence. In working further with this system, it should be remembered that the growth of tumor colonies from clumps may be produced by a very limited number of cell divisions, particularly in those specimens in which the colonies produced are of the smallest acceptable diameters (60-72 um). Conceivably, "growth" might also be simulated by cell swelling so that clumps smaller than 60 um diameter could increase their size to acceptable colony size by cytoplasmic growth alone without any cell divisions.

Perhaps the most significant application of the tumor colony growth curves is the recognition of different patterns of tumor response to drugs. This is illustrated in Figure 5. If a tumor is evaluated for drug response in the HTCS, present practice dictates that the drug-treated growth is compared at some arbitrary time point (e.g. 3 weeks) with control (non-drug-treated) growth. If growth at the chosen time point is 30% or less of the growth in control plates, the tumor is considered as sensitive to that drug. However, as illustrated in Figure 5, there are several growth patterns in culture which could produce 70% growth inhibition at 3 weeks. The most favorable pattern is illustrated in curve A of Figure 5 In this pattern, colony development in the plate has reached a plateau at 3 weeks after having a relatively slower rate of colony growth compared with the hypothetical control curve, the 45° line. A somewhat less favorable pattern of growth is seen in curve B where the colony growth does not reach a plateau but the rate of colony development is slower than in the control cultures. However, very different patterns of colony development are seen in curves C and D where the drug incubation produces a lag phase in colony development, after which the tumor cell colonies begin to develop at the same (C) or a faster (D) rate compared with the control cultures. Growth curves analogous to these theoretical curves are suggested by actual data shown in Figures 2 and 4. It is far too early to

determine whether specific tumors show specific patterns of colony growth dynamics or whether specific drugs cause reproducible changes in the temporal pattern of tumor growth in vitro. Considerably more experience with the use of growth curves in vitro must be accumulated before questions of this kind can be answered and the contribution of in vitro measurement of tumor growth dynamics to clinical applications of the HTCS can be evaluated.

ACKNOWLEDGEMENTS:
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CHAPTER 7.

IN VITRO CHEMOSENSITIVITY TESTING WITH THE HUMAN TUMOR CLONING SYSTEM (HTCS) IN UROLOGICAL CANCER.

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INTRODUCTION:

The recent development of short-term culture, derived from research on hematological malignancies, applied to human primary tumors (1) has offered the clinical laboratory the possibility of monitoring dynamic rather than static properties of human tumor cells. Specifically, the tumor cloning system (HTCS) applied to solid tumors by Salmon et al allows the growth in the two-layer soft agar system of tumor colonies from clonogenic, presumptive "stem" cells.(2,3)

The first proposed application of this system was the in vitro determination of chemotherapeutic sensitivity or resistance of tumors to allow individualizing of chemotherapeutic regimens. (2) Further clinical applications have been proposed, including post-therapy evaluation of persistent or recurrent tumor (4) and evaluation of grade and stage of tumor, based on in vitro growth characteristics.(5,6)

The first goal of the study presented here was to determine the value for clinical management of patients with transitional cell carcinoma and renal cell tumors who were to be treated with cytotoxic drugs for metastatic disease or in case of superficial transitional cell carcinoma of the bladder with intravesically applied cytotoxic drugs to prolong the recurrence free interval after transurethral resection of the tumor. In other words can the sensitivity or resistance to cytotoxic drugs of these tumors be predicted with sufficient accuracy with the in vitro tumor clonogenic cell culture system and can the management of the patients be improved by application of this laboratory test method?

MATERIALS AND METHODS:

We used the two-layer soft agar method (3) in the Dept. of Pathogy in the Radboud Hospital. Routine surgical specimens of solid

tumors were brought sterile and unfixed to the Dept. of Pathology. Using sterile technique, a pathologist examined the material and chose tissue for soft agar culture (approximately 1-2 gr) and routine histopathologic study. In case of transurethral resected bladder tumors the tissue for soft agar culture was chosen in the operating room during resection, mostly the exophytic part of the tumor. The material was brought to the Pathology Dept. within minutes. Care was taken that enough representative tumor tissue was left for histopathology. Tissue selected for soft agar culture was further processed according to the detailed description recently published.(2) In brief, the tissue was minced with scissors for 5-10 minutes through a metal sieve of approximately ten holes per centimeter into McCoy's wash medium, later the tumor tissue was minced in a centrifuge tube with double blunt scissors. The cell suspension with the tumor mince was incubated for 2 hours with a mixture of collagenase type II (Worthington) and DNAase type I at 37°C and 6% CO_2.(7) After incubation, McCoy's wash was added and the cell suspension passed through a gas-sterilized nylon filter with 70-100 um holes (Ortho Diagnostics, Belgium). The cells were then washed again and the cell pellet resuspended in 2-5 ml McCoy's wash according to the estimated number of cells. Trypan blue excluding cells were determined using 0.1 ml of the cell suspension. The concentration of large nucleated cells was determined in a haemocytometer and then adjusted to $3x10^6$ cells per McCoy's wash. For in vitro drug testing, cells were incubated for 1 hour in drug solutions prepared in NaCl 0.9% or water as required for the specific drug and diluted in McCoy's wash. Drug concentrations were approximately 10% of the in vivo attainable peak plasma level. (3) The cells for the control group were incubated with McCoy's wash alone, without a cytotoxic drug. After incubation the cells were centrifuged and seeded in 35 mm culture dishes with $5x10^5$ cells per culture dish in double layer soft agar exactly as described elsewhere (2), except that conditioned medium was not used. The cultures were then incubated at 37°C 6% CO_2 in a humidified atmosphere.

ALTERNATIVE SOURCES OF TUMOR CELLS FOR CULTURE:

In an attempt to obtain good quality tumor cells for culture with minimal trauma to the patient we tried to culture transitional cell carcinoma colonies from urine specimens of patients with transitional cell carcinoma collected before transurethral tumor resection was started.(5,6) The use of effusions and irrigation fluids had previously been reported (8,9,10), however, culture of tumor cells derived from urine samples had not yet been published. Growth results from urine samples were comparable with cultures of solid tumor samples of TCC. Only the numbers of colonies produced in cultures of cells derived from urines were not sufficient to test cytotoxic drugs where at least 30 colonies had to be grown in the control dishes. But the possibility to culture transitional cell tumor colonies from urine, which can be collected easily and repeatedly without much inconveniece for the patient, is the bases for an additional application of the soft agar tumor cell culture system that will be discussed later.

COLONY COUNTING PROCEDURES:

Colony counting plays an important role in the quantification of the tumor cell colonies in culture. Initially, colony counting was performed with an inverted microscope, a time consuming procedure, which lacks reproducibility. When an automated tumor colony counter (Omnicon Feature Analysis System II, Bausch and Lomb Inc., Rochester NY, U.S.A.) became available we started investigations on reproducibility and accuracy of this system.(11) The theory and operation of this instrument has been published.(12) The instrument used in our laboratory was similar to that previously described except that it was fitted with an inverted microscope and a fully automated 36-dish microscope stage. This allowed

walk-away operation with counting of 36 culture dishes taking an average of 40-45 minutes.

For determination of within day rum-to-run reproducibility, sets of 36 glutaraldehyde fixed culture dishes were counted twice in succession with no change in position of the dishes, settings of the instrument controls etc. For determination of day-to-day reproducibility, the fixed culture dishes were counted 5 times on 5 or 6 different days over a period of 8 days with demounting and remounting of the dishes as well as readjustment of the illuminator source and scanner sensitivity each day. Duplicate Omnicon counts on the same day, showed a modal correlation coefficient (r) $\geq$ 0.990; the median was $\geq$ 0.985. The modal day-to-day r value of automated colony counting was $\geq$ 0.990 and the median r $\geq$ 0.980. The distribution of run-to-run and day-to-day r values showed considerable left skewness. The coefficient of variation (c.v.) for each culture plate counted on 5 or 6 days was evaluated individually. The modal c.v. was 6%, the median c.v. 10%, and 61 of 79 evaluable culture dishes showed a c.v. of automated counting less than 20%. Comparing the mean number of colonies per dish with the c.v., a tendency was seen to lower c.v. with higher colony counts.

The reproducibility of manual counting was assessed by comparing the intra- and inter-observer variability. Great differences were seeen between the results of colony counting by 3 observers. Intra-observer reproducibility varied from r = 0.943 to r = 0.733. Inter-observer reproducibility varied from r = 0.831 to r = 0.478, suggesting that results from 2 different observers in one laboratory can not be reliable compared, and even greater differences can be expected for comparison of results of two different laboratories counting colonies and publishing their results.

EVALUATION OR GROWTH IN CULTURE:

Automated instead of manual counting and sizing of tumor colonies growing in soft agar allows accurate and reproducible analysis of the in vitro growth dynamics of tumor colonies. (11,13) This analysis demonstrated differences in the time course of tumor response to cytostatic agents as well as differences in the time course of development of various sizes of colonies from tumors of the same histologic type. The routine use of tumor colony "growth curves" (see fig. 1 and 2) may decrease the relatively high false positive rate of soft agar culture measurement of tumor drug sensitivity. In addition, the temporal pattern of the tumor colony development in agar may provide valuable new information on the prognosis of individual tumors.

RESULTS:

In the study here presented 64 evaluable samples are included, obtained from 39 patients with transitional cell carcinoma and 14 samples of renal cell tumors obtained from 14 patients.

The results from soft agar culture of human transitional cell carcinoma tumor colonies from urine, irrigation fluid and tumor samples are shown in table I, using the Omnicon FAS II automated colony counter for colony development evaluation. Growth in culture is described with "growth curves" from controls (non drug treated cultures) and drug pretreated cultures for drug sensitivity testing. Table I shows the growth results of 64 samples that were evaluable, obtained from 39 patients. Infected cultures and cultures not evaluable because of poor quality of the culture dishes are not included.
Twenty-seven of 64 samples (42%) showed growth which means that 2 criteria were met, arbitrarily chosen for growth evaluation. These criteria are: 1. presence of more than 20 colonies over 60 um diameter at peak of growth and 2. a maxi-

mum colony number at peak growth at least twice the day one count or twice the minimum count achieved after deterioration of clumps seeded in to culture.

TABLE I. GROWTH OF TRANSITIONAL CELL CARCINOMA IN SOFT AGAR CULTURE.

		Growth curves based upon colony development in 24 culture dishes.*		Growth curves based upon colony development in 1-3 culture dishes.**	
	***	growth pos.	growth neg.	growth pos.	growth neg.
urines	23	7	7	3	6
irrigation fluids	19	5	7	1	6
urine + irrigation fluids	2	1			1
tumor samples	20	10	10		
total	64	23	24	4	13

* For every time point 2 dishes were removed from culture and colony number determined without reincubation of the dishes.(see fig. 1)

** At regular time intervals the dishes were sealed with porous adhesive tape to prevent infection, the number of colonies counted and the plates reincubated after colony counting.

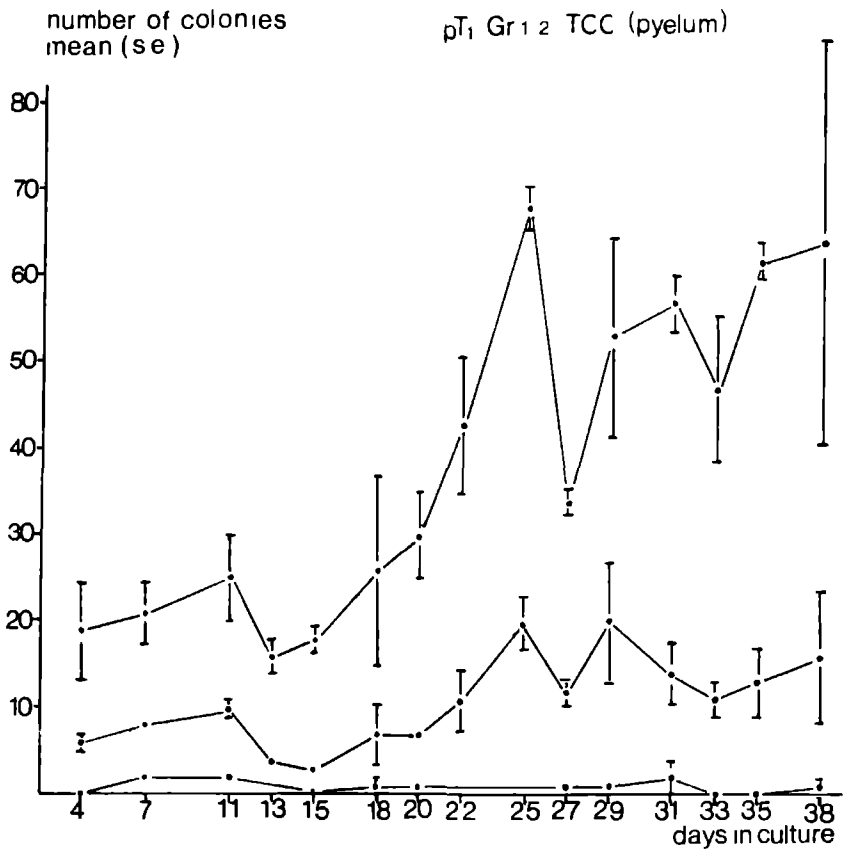
*** For growth criteria see text below.

As shown in fig. 1 this growth curve is growth positive. Some other facts are illustrated in this growth curve which are almost general in this type of culture. Firstly, at day one colonies are counted, which is not very likely to be true. The structures counted at day 1 are most likely to be clumps seeded into culture. This has major implication on the colony development. In the case clumps are plated, growth in soft agar culture is not clonal as it would be if an absolute single cell suspension could be plated. It has been posed that no growth in culture is possible if only single cells are plated in the culture dishes.(14,15) This means that eventually the growth obtained in the culture system is not derived from real "stem" cells but possibly originates from clumps of cells with limited reproductive potential.(16) With only a small number of cell divisions the size criteria of a colony may be reached. What impact these theoretical considerations have on the evaluation of drug sensitivity testing with "clonogenic" assays has to be determined.(13)
Secondly, the lag time in colony development is apperent, followed by a logarithmic growth phase. If the sensitivity test is evaluated too early, erroneous results would be obtained. Thirdly, the large interplate variability is shown, described by the standard error values. This interplate variability will make the evaluation of a drug test more difficult.

A total of 10 samples, 5 tumor samples, 4 irrigation fluids and 1 urine sample, yielded enough cells for cytotoxic drug testing, 1-4 drugs were tested. One tumor sample showed histologically no transitional cell carcinoma, only atypia of the cells; this sample showed no growth in culture. One irrigation fluid culture was lost for evaluation due to infection.

Of a total of 29 evaluable growthcurves of drug pretreated cells no significant growth inhibition was obtained. Drugs tested, were cisplatin, adriamycin, 5-fluorouracil and methotrexate.

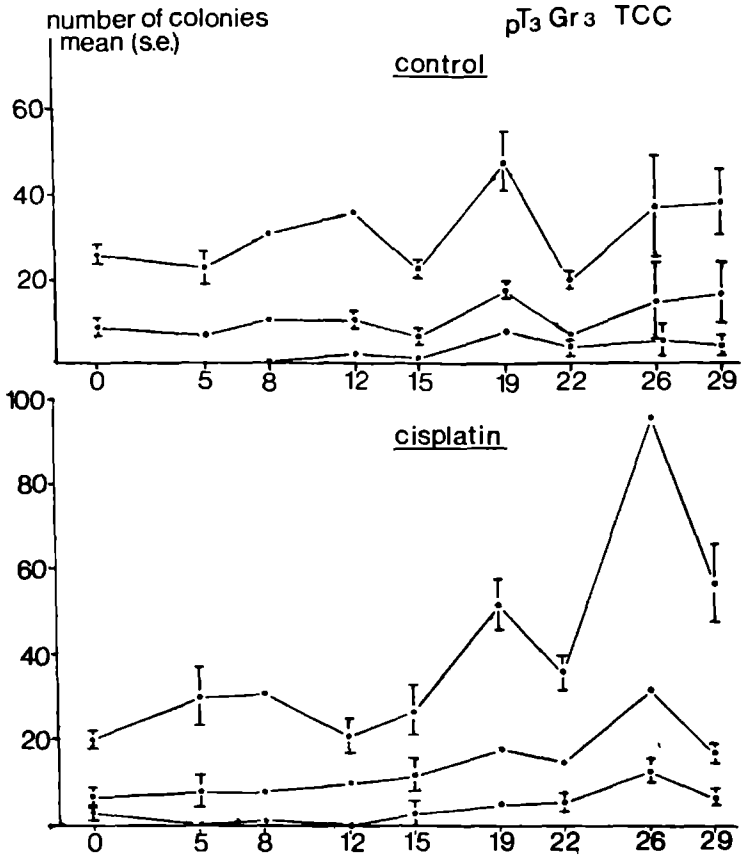
Figure 1.



Growth of T_1 G_{1-2} TCC (pyelum) in soft agar culture. For each time point the mean and S.E. of colony counts of 2 dishes are shown.
The upper line shows all colonies over 60 um in diameter, the middle line all colonies over 86 um in diameter and the lower line all colonies over 124 um in diameter.

As shown in fig. 2 some drugs caused growth stimulation compared with growth in the control dishes. What impact this stimulation in vitro has in vivo, if the patient is treated with this drug, is not yet clear. Probably it is best not to treat the patient with the drug which showed stimulation in vitro.

Figure 2.



Growth in control cultures (upper growth curve) and drug pretreated cultures after 1 hour exposure to cisplatin at 10% of the in vivo achievable peak plasma concentration (lower growth curve).

The growth results of 14 renal cell tumors are as follows. Eight out of 14 samples (57%) showed colony growth in soft agar culture, according to the criteria described above. Ten samples yielded enough cells for in vitro cytotoxic drug testing; 4-9 drugs were tested, with a total number of 55

drug tests. Thirteen of these 55 drug tests showed some growth inhibition which was not significant.

Until now it was not yet possible to make any in vitro in vivo correlations because none of the patients from whom tumor samples were obtained and tested with cytotoxic drugs had metastatic disease, needing systemic cytostatic treatment.

It can be concluded from our experiences that cytotoxic drug sensitivity testing on transitional cell carcinoma and renal cell tumors cannot be routinely applied to all patient's tumors. The low growth potential and the low cell yield make it impossible to test cytotoxic agents on all samples.

The lack of effective cytotoxic agents against renal cell tumors makes it difficult to identify a drug that shows sensitivity in vitro. For clinical application, the long culture period needed to obtain sufficient growth, if growth is development at all, is a major roblem for clinicians who want to start chemotherapy as soon as possible after the operation and diagnosis.

Additional applications of the soft agar culture system are the follow-up investigation of patients during and after intravesical chemotherapy or radiation for transitional cell carcinoma of the bladder. Patients with apparently no tumor in the bladder showed positive growth curves from cells obtained from urine samples, during and after therapy. Some patients already developed recurrences, another group of patients had positive urine growth curves but did not yet develop recurrences. Our hypothesis is that this group has a higher risk to develop recurrences than the patients without positive growth curves. Prospective randomized studies have to be performed to determine if this follow-up investigation will provide an improvement of patient treatment.

Another application of the soft agar culture system is the grading of the malignant characteristics of transitional cell carcinoma and renal cell tumors not only based on morphologic investigations but additionally by determination of the biological properties of the tumors with growth curves, to predict the growth potential in vivo and metastatic behaviour in the future of the tumors under investigation.

In conclusion, the soft agar culture system is valuable as an investigational tool to learn about biological properties of tumors. The initially proposed routine laboratory test for cytostatic drugs on all patient's tumors meets with practical and theoretical problems, which make the test not practicable in a routine laboratory setting.

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CHAPTER 8.

REPEATED URINE CELL CULTURE IN SOFT AGAR: POTENTIAL ROLE IN FOLLOW-UP OF PATIENTS WITH TRANSITIONAL CELL CARCINOMA.

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ABSTRACT:

To evaluate the persistence of cells with clonogenic properties in patients being treated for transitional cell carcinoma, 272 urine samples were collected from 75 patients and cultured in a double layer soft agar "cloning" system.

The development of colonies was evaluated with growth curves based on repeated colony counting with an Omnicon automated colony counter at regular time intervals. Forty-eight patients had at least one evaluable culture. Comparing the results of colony development in culture with the clinical evaluation of the patients, 9 patients had a histologically proven recurrence preceded or accompanied by tumor colony growth in urine culture. One patient had tumor recurrence with growth negative urine culture (false negative). Fifteen patients have had growth negative urine culture with a negative follow-up (mean 19.5 mos.). Twenty-one patients have had growth positive urine cell cultures wiht no recurrence in thei follow-up (mean 18.7 mos.).

Although the follow-up times are at present relatively short, the present study suggests that repeated soft agar urine culture of patients with low-grade, low-stage bladder carcinoma may provide a means for identifying those patients at a higher risk for recurrence / progression of their disease.

INTRODUCTION:

Superficial transitional cell carcinoma of the bladder is usually treated by local resection followed by intravesical chemotherapy (1). Post-therapy evaluation of these patients usually requires repeated urine cytologies, cystoscopies and transurethral biopsies. Because of the effects of therapy, cystoscopy and even cytology can be equivocal in evaluating the response to treatment and the development of recurrent disease.

The use of the two layer soft agar culture as a means of evaluating treated patients for persistent or recurrent tumor has recently been proposed (2). It has been shown that transitional cell carcinoma cells present in urine form colonies in this system (3,4) while normal urothelial cells do not grow (5,6).

The present study was therefore designed to evaluate the potential utility of repeated soft agar culture of urine as a way of monitoring the status of the urothelium in patients treated for low-grade, low-stage bladder carcinoma. Since single time-point counting of colonies may be misleading (7), the present study uses growth curves of colonies developing in agar over 2-5 weeks as a way of evaluating the clonogenic properties fo transitional cells. Preliminary results suggest that soft agar culture may be a useful adjunct to other follow-up methods in identifying patients with recurrence of transitional cell carcinoma after therapy.

MATERIALS AND METHODS:

Seventy-five patients treated for transitional cell carcinoma were included in this study. These patients had been or were being treated by intravesical chemotherapy and were being routinely evaluated by urine cytology, cystoscopy and when indicated, transurethral biopsies. Also included in the study were 2 patients who received intravesical Adriamycin for persistent CIS, and 3 patients treated with external beam radiation for invasive (pT2-pT4) bladder carcinoma.

The patients were asked to come to the outpatient department with a full bladder and urine was then collected into heat-sterilized polypropylene bottles in one of three ways: 1) by spontaneous voiding; 2) through a cystoscope sheath if the patient was undergoing a cystoscopy or 3) from catheterization if the patient was receiving intravesical chemotherapy. After collection, the urine samples were transported to the Pathology Department and processed as recently described in detail (3,4). $5x10^5$ Cells were seeded per 35 mm culture dish in double layer soft agar. If fewer than $5x10^5$ cells were available, all cells were seeded into one 35 mm culture dish. The culture dishes were incubated at 37°C, 6% CO_2 in a humidified atmosphere for up to 5 weeks, to allow well-formed colonies to develop.

All colony counting in the present study was performed using the Omnicon FAS II automated colony counter (Bausch and Lomb Inc., Rochester NY, USA). This instrument has been described in detail previously (8) and its operating characteristics reported (9). Colony counting was begun within 1-2 days after plating the cell specimen. Because most of the urine samples yielded only enough cells to plate one 35 mm culture dish, the dishes were counted once a week, and reincubated after counting. To prevent airborne infection during counting, the culture dishes were sealed with porous tape. Occasionally, when enough cells were available to seed a series of culture dishes, a growth curve as routinely used in our laboratory was evaluated, removing 2 dishes from culture for each time point to determine the number of colonies starting the first day after plating without reincubation.

A growth curve was accepted as showing colony growth if it met 2 criteria: 1) at least 90-100% increase in colonies comparing the day 1 colony count with a maximum colony count, or after an initial decrease, at least 90-100% increase above the minimum colony count and 2) a sustained consistent increase over time in the number of colonies. If the percent increase criterion was not met the culture was said to be growth negative. If the growth pattern was not consistent, the culture was said to be not evaluable. Most cultures that were considered growth positive showed

considerably more than 100% increase in colony number.

After termination of the culture, detailed morphologic assessment of the colonies as well as other cells and particles in the agar was carried out on agar layers removed from the culture dishes, mounted on standard glass slides (10) and stained with the Periodic Acid Schiff (PAS) reaction.

RESULTS:

272 Urine samples were collected from 75 patients. 27 Patients were not further evaluable because of infections in culture or other technical reasons (see table 1).
Of the 48 evaluable patients, 32 had at least one culture that showed colony growth: 16 patients had no growth positive cultures. The day 1 counts of 81% of 54 growth positive cultures were less than 30 "colonies". Conversely the peak counts of the growth positive cultures was above 30 colonies in 65% of the cultures (see table 2).

In table 3, the results of the urine colony cultures are compared with the clinical course or the patients. In group 1, 9 patients are listed who developed a histologically proven recurrence and in whom at least one urine culture showed growth. Eight urine samples that were growth positive were collected at a time when cystoscopy revealed a recurrence. Ten urine samples obtained from 6 patients were growth positive 1-6 months (1,2,2,5,6,6) before a diagnosis of recurrent TCC was made. Group 2 consists of 21 patients who have not yet shown evidence of recurrent disease. Group 3 consists of 1 patients who developed several recurrences, but the 2 evaluable urine cultures showed no growth. As can be seen in table 3, 9 of 11 cultures of this patients were not evaluable, primarily due to infection. Group 4 consists of those patients with only growth negative urine cultures and who had not developed a recurrence.
Of the 30 in Groups 1 and 2, 16 had at least 2 evaluable cultures. The second culture was also growth positive in 14 of the

Table 1. 272 urine cultures from 75 patients with transitional cell carcinoma.

	TOTAL	EVALUABLE*	NOT EVALUABLE
PATIENTS:	75	48	27
CULTURES:			
not evaluable**	93	68	25
infected	72	42	30
growth pos.	58	58	
growth neg.	49	49	
total	272	217	55

* These patients had at least one evaluable culture.

**Not evaluable cultures:

-degeneration of agar layers

-lack of multiple time points for counting

-no consistent pattern in the growth (see methods)

16 patients, suggesting reasonable reproducibility.

The duration of follow-up after the last transurethral resection of a histologically proven TCC is 18.7 months ± 14.5 (mean ± SD) for group 2 and 19.5 months ± 15.1 for group 4, not significantly different. This similarity in duration of follow-up suggests that the development of recurrences in group 4 compared with group 2 is not due merely to differences in follow-up time after the initial diagnosis of TCC treated with transurethral resection.

Omitted from table 3 are 2 patients each of whom had one positive urine culture but who have been lost for follow-up.

In those urine cultures that showed growth by the criteria listed in Materials and Methods, the PAS stained agar layers contained structures which could be recognized as tumor cell colonies of transitional cell carcinoma (cf Herman et al,(2)).

The comparison of the growth results and the results of cytological investigation of urine samples shows that in all patient groups most of the cytology results reported atypia of the cells found in the urine (group 1:3/4; group 2:18/22; group 3:2/2; group 4:9/12). The other 8 cytology specimens of a total of 40 were suspicious for, but not diagnostic of malignant lesions.

DISCUSSION:

The follow-up of patients with bladder cancer who have received treatment other than radical cystectomy remains a difficult problem. The natural history of the disease is variable and patients with similar grade and stage of tumor may show markedly different clinical course. The least invasive follow-up technique, urine cytology, is frequently indecisive as a guide to further management.

Table 2. Colony counts of growth positive cultures on first day after plating, at minimum number (see text) and at peak of colony growth.

colony numbers	day 1 count	minimum count	peak count
0-5	12 (22%)	16 (30%)	0
6-30	32 (59%)	29 (53%)	19 (35%)
31-100	9 (17%)	8 (15%)	19 (35%)
101-	1 (2%)	1 (2%)	16 (30%)
	54	54	54

Table 3. Comparison of urine culture colony growth and the clinical course of 48 evaluable patients.

	Histologically proven diagnosis of recurrent TCC/CIS after TUR.	No recurrenc after TUR of TCC.
	GROUP 1. (1)	GROUP 2.(2)
at least one cell culture growth positive	9 patients (1-10 cultures, 4.3 ± 2.5*) follow-up 12.6 ± 10.5 mos.*	21 patients (1-12 cultures, 5.1 ± 3.5*) follow-up 18.7 ± 14.5 mos.*
	GROUP 3.(3)	GROUP 4.(4)
no growth positive urine cell culture	1 patient (11 cultures)	15 patients (1-8 cultures, 3.7 ± 2.6*) follow-up 19.5 ± 15.1 mos.*

Duration of follow-up periods of groups 1, 2 and 4 are not significantly different (Student's t-test).

* mean ± S.D.

(1) Total of 24 evaluable cultures from 9 patients: 19 growth positive, 5 negative.
(2) Total of 51 evaluable cultures from 21 patients: 39 growth positive, 12 negative.
(3) Total of 2 evaluable cultures from 1 patient: 2 growth negative.
(4) Total of 28 evaluable cultures from 15 patients: 28 growth negative.

Soft agar culture of urothelial cell tumor colonies from bladder irrigation specimens has been shown to correlate with the general status of the urothelium (6) and the risk of tumor recurrence (11,12). Recently, we have shown that transitional cell carcinoma cultured from urine forms colonies in soft agar (3,4). Normal transitional cells do not show growth in this system (5). We have further suggested that the use of soft agar culturing may provide a means of identifying persistent of recurrent disease in tumor patients after treatment (2). The present study is the first attempt to demonstrate the application of this method.

In this series, only 48 of 75 (64%) patients provided evaluable soft agar urine cultures. Although disappointing, this percentage is not out of line with the results obtained from routine bacteriological cultures of blood from patients with endocarditis or other infectious illnesses caused by fastidious microorganisms.
Nevertheless, routine bacteriological cultures are well accepted as important clinical laboratory tools.

Of the 48 evaluable patients, 9 patients showed tumor colony growth in soft agar. In 6 of these patients, the positive cultures preceded histological proof of recurrence by up to 6 months. Conversely, only one of the 48 patients showed false negative urine culture, developing tumor recurrence after 2 negative urine cultures.

The remaining patients provide a more interesting group. Twenty-one of these patients have shown at least one positive urine culture while 15 patients have had no positive urine cultures. All of these 36 patients remain disease free after mean follow-up times of 19 and 20 months respectively. Although these follow-up times are short in the context of the natural history of transitional cell carcinoma, longer follow-up of these patients eventual prospective clinical trials should answer the question whether repeated soft agar culture of urine can identify a subpopulation of transitional cell carcinoma

patients at significantly higher risk for tumor recurrence and/or progression.

This method of patient follow-up after therapy may also be of value in other tumors. Non-seminomatous testis tumors which present with bulky retroperitoneal disease are frequently treated by retroperitoneal lyphe node dissection after chemotherapy. The demonstration of clonogenic tumor cells by soft agar culture, persisting after chemotherapy in these patients as well as assessment of the persisting tumor's growth dynamics in culture could provide information useful to planning further therapy.

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CHAPTER 9.

GENERAL DISCUSSION.

DISCUSSION:

The first goal of the present study was to evaluate the possibility to culture transitional cell carcinoma in the double layer soft agar system. Because of the opportunity to compare our results with other major laboratories, we decided to follow the original protocol as described by Salmon. (1)
For the preparation of single cell suspensions of solid tumors we chose the protocol of Slocum, using Collagenase type II and DNase. (2)
For morphologic studies of the colonies formed in agar we made preparations on slides according to Salmon. (3)

Our first results showed the possibility to culture transitional cell carcinoma colonies from resected bladder tumor material, chapter 2 and 3, comparable with the results of others. (4) Seventy-seven percent of the transitional cell carcinoma tumor samples showed growth (defined as development of at least 5 tumor cell colonies). For an eventual cytotoxic drug sensitivity test at least 30 colonies had to be grown in control culture dishes, according to the criteria suggested by the Arizona group. Only 28% of our evaluable cultures of solid tumors showed this number of colonies, a percentage lower than that achieved by others culturing solid tumors. (4,5) Von Hoff published results on 510 tumors of which 372 tumors (73%) formed colonies. In that study, the percentage of tumors with more than 30 colonies per dish was not reported. (6) Benard reported 47% growth positive cultures (more than 5 colonies), but very few tumor samples grew enough colonies for drug sensitivity testing. (7) Kern cultured more than 1000 tumors, of which 68% produced at least 30 colonies. Tumor cells were cultured in a CEM medium instead of the original double enriched McCoy's 5A bottom layer and the double enriched CMRL 1066 for the top layer. (8,9) Bertelsen reported 1582 assays of which 68% produced more than 30 colonies per control plate. (10)

The fact that in urine cytology well preserved cells were obtained suggested the possibility of culturing cells from catheter urine, or from spontaneously voided urine as an

alternative source of tumor cells. This would make it easier to obtain transitional cell carcinoma cells for culturing purposes, with little or no problem caused to the patient who would not need to undergo any invasive procedures. As we have found, it is possible to culture cells from urine although the overall production of colonies is lower than with solid tumor specimens (chapters 2 and 3).

Culture of tumor cells from irrigation fluids of the bladder showed overall results which were slightly worse than from urine tumor cell cultures. The growth results from bladder irrigation fluids were comparable with the growth results achieved by others. (11,12)

In our experience, culturing transitional cell carcinoma of the bladder is attended by several problems, structural and theoretical.

Tumors in the bladder are often small and multifocal. Preserving a representative part of the tumor material for histopathologic investigation makes it often impossible to obtain a large enough piece of bladder tumor material for culture.

Furthermore, tumors in the bladder are often accompanied by bacterial infection (13) which makes soft agar culture of the tumor cells impossible because of bacterial overgrowth despite the antibiotics added to the culture media. Doubling the concentration of penicillin and streptomycin produced no improvement in infection rates. We thought it not wise to add more effective antibiotics because of development of resistant strains of bacteria in the laboratory and because of the risk of spreading infections, difficult to treat because fo resistance to antibiotics.
Because of infected material approximately 25% of the samples for culture were lost before culture was started or, if the infection was not evident in the material presented for culture, during the incubation period of 3 to 4 weeks.

In the preparation of the tumor cell suspension that ultimately will be plated in the culture dishes, the total number of real tumor cells had to be determined. The cells were counted in a hemocytometer. In a wet, unstained preparation tumor cells had to be identified and counted in a total number of cells, often containing several times more inflammatory cells. This was often due to earlier intravesically administered chemotherapy resulting in a cystitis. We chose the criterion of large nucleated cells to be counted as tumor cells. This often gives rise to the problem of a total number of cells per dish that grossly exceeds $5x10^5$ cells per culture dish. (1) This excessive number of total cells plated has certain implications for growth, most likely decreased total growth due to early exhaustion of the culture media. Unfortunately this effect was not measurable. Furthermore tumor cell counting lacked reproducibility due to differences in experience with judgment of cells in a preparation.

Colony counting plays an important role in the evaluation of growth in culture. It is a very time consuming and poorly reproducible procedure. Because of rather subjective criteria for tumor cell colonies, e.g. a three dimensional structure consisting of more than 30 tumor cells; and the differentiation that is needed between pre-excistent clumps seeded into culture and colonies grown, colony counting by human observers is subject to error both within one laboratory and especially among different laboratories. Even if observers in one laboratory have the opportunity to come to a common opinion about colony evaluation, the inter-observer variability is not negligible, possibly due to attention, concentration and fatigue (chapter 5).

In this context, investigations were undertaken to evaluate the performance of an automated colony counter, the Omnicon^R FAS II (Bausch and Lomb, Rochester, NY, USA) when the device became available in the Department of Pathology where this study was performed.
A colony is in terms of this apparatus a smooth, round structure of homogenous density against the background and with a diameter

of at least 60 µm. In chapter 5 a detailed description is given, based upon our own experience and earlier data of Kressner (14). The reproducibility value (r) for the Omnicon, counting dishes twice on one day were: modal r value > 0.990 and median r value > 0.985. The day to day colony counts on 5 subsequent days were: modal r value > 0.990 and median r value > 0.980. However, a considerable coefficient of variation (C.V.) was found depending on the number of colonies in a dish. There was a tendency to lower C.V. values with higher colony counts.

In a separate experiment the question if the Omnicon counts artifacts, e.g. cracks and bubbles in the agar layers, as colonies was examined. It was shown that living tumor cells are needed in culture to achieve colony growth; if formalin fixed cells were seeded into culture no colonies were counted by the Omnicon (chapter 5).
We concluded that the Omnicon^{R} FAS II automated colony counter is applicable for routine colony counting in the double layer soft agar culture system.

Growth in culture is a dynamic rather than a static process. In an attempt to describe growth in culture quantitatively, the concept of growth curves was applied to this culture system as described in chapter 6. Growth patterns were in our opinion a possibility to deal with cultures in which clumps of tumor cells had been seeded (so the day one colony counts were not equal to zero). Growth of clumps seeded into culture into colonies, departs from the stem cell theory underlying the concept of the soft agar culture system. (15,16,17)

Furthermore the low plating efficiency of the soft agar cultures means that many cultures can not be used for drug sensitivity testing. The description of growth patterns in cultures with growth curves offers a more sensitive method for evaluation of tumor cell colony growth and also for the evaluation of cytotoxic drug effects in soft agar culture.

In our laboratory we changed over from single point colony counting as described originally and a 70% decrease in colony

numbers in the drug pretreated dishes as a criterion for sensitivity, to the growth curve evaluation of cultures. Growth patterns were ultimately used to evaluate our cytotoxic drug sensitivity tests performed on transitional cell carcinoma. Results are described in chapter 7. In none of the cultures was drug sensitivity indicated with the soft agar culturing system. This can be expected because of the clinical resistance of transitional cell carcinoma to many cytotoxic drugs. Also in another report, this chemoresistant picture in culture was found.(4) It is noteworthy that Cisplatin, clinically used as a moderately active agent against transitional cell carcinoma did not show any growth inhibition in culture. In fact, Cisplatin caused in some instances growth stimulation. Elsewhere it is suggested that this drug resistance in culture may be due either to inappropriate incubation times of tumor cells before seeding into culture, or inadequate concentration because of incomplete knowledge of the pharmacodynamics of Cisplatin in culture media.(10)

The value of drug sensitivity testing with an in vitro system such as the double layer soft agar system is measured by weighting the chance to identify an effective drug against an individual patient's tumor that would not be chosen by an experienced oncologist, making a choice based on retrospective and prospective clinical trials.(17,18,19,20)

At this moment the role of soft agar tumor cell culture in choosing drug regimens for individual patients seems to be very limited. The favorable results presented by Von Hoff (21) and Alberts (22) were based on non-randomized clinical trials. Thus, randomized controlled prospective studies are still needed.(10)

The double layer soft agar tumor cell culture system is in our opinion an important tool to study dynamic tumor biology, not only centered on studying inhibitory effects of cytotoxic drugs on growth but also to investigate if after therapy residual viable tumor cells can be found. As is described in chapter 4, the pathologist has to answer the question of residual viable

tumor cells after therapy based on the morphologic appearance of cells in histologic slides. However, we have demonstrated in culture that cells can keep their morphologic appearance which suggests viability without giving evidence of growth potential. It can be of importance for an oncologist to know if there were cells with growth potential in the residual tumor mass after therapy (chapter 4).

This brings us to the question of selectivity of growth of malignant cells in culture or have certain benign cells also growth potential in soft agar culture? In the earliest reports it was shown that inflammatory and connective tissue cells do not show growth in this culture system. (1) With increasing experience in all laboratories some benign cell types have been recognized that did show growth in this culture system. For some cell types, this may represent pre-malignant properties in vivo. This will be difficult to determine because of the low incidence of this event.

A synthesis of our ideas about tumor growth potential monitoring and using alternative sources for tumor cells for culture is described in chapter 8. Here we describe the first results of a study performed to evaluate the possibilities of the soft agar culture system to predict recurrences of transitional cell carcinoma during and after intravesical chemotherapy, before urine cytology and cystoscopy give definite evidence.
Our first results offer the possibility of future studies using soft agar cultures to monitor and eventually predict in vivo behavior of cells with an apparently normal appearance with todays investigations. In this way differentiation may be possible to select those patients who need more aggressive treatment.

For future investigations, the double layer soft agar culture system can be used to study the clinical heterogeneity of transitional cell carcinoma (23) which was also identified in the soft agar culture system comparing drug sensitivity of tumor cells originating from different sites in one patient. (24) Isolated colonies grown in soft agar can be useful for further

investigations looking for differences in expression of cytoskeleton proteins.
It may also be possible to culture the colonies grown in soft agar in an attempt to establish tumor cell lines originating from one tumor cell.

We can conclude that the double layer soft agar culture system is a useful investigational tool for tumor biology of transitional cell carcinoma. Drug sensitivity testing is still not possible in a routine set up for transitional cell carcinoma. Alternative sources for good quality tumor cells, urine and bladder irrigation fluid, were investigated with positive results. Growth pattern analysis has been described.
For future application a more detailed description of growth curves will be needed. Possibly, more mathematical description of the growth curves can make this way of describing growth in a culture system more sensitive for changes with time, also those changes induced by drug incubation of the cells.

The use of soft agar cell culture for the identification of patients with aggressive tumors or of patients who will readily develop recurrences during or after therapy is the most promising application for future investigations on the soft agar tumor cell culture system.

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CHAPTER 10.

SUMMARY.

SAMENVATTING.

SUMMARY:

This study was undertaken to investigate the possibilities to test cytotoxic drugs in vitro on transitional cell carcinoma with the double layer soft agar culture system. First goal was to determine the practicability of this in vitro test system in a routine laboratory setting.

We showed that in urine and bladder irrigation fluids from patients with a bladder tumor good quality tumor cells can be obtained for culture purposes.

For colony counting an automated image analysis system became available in the laboratory of the Department of Pathology, the Omnicon FAS II (Bausch and Lomb, Rochester, NY, USA). This system was integrated in the laboratory routine after extensive studies on reproducibility. We concluded that the overall performance of the Omnicon is more reproducible than the results obtained by human observers counting colonies.

Because a more dynamic evaluation of tumor cell colony growth seemed to be more sensitive for changes in colony development in culture, we started to evaluate growth patterns with "growth curves". Theoretical advantages are described to advocate the use of growth curve evaluation of colony growth instead of single point colony counting as was described originally.

We also concluded that tumor cell clumps seeded into the culture dishes can be evaluated for growth, but it is impossible to hold to the original concept of stem cells, single clonogenic cells, as a starting point of colony development. Theoretically, this change in concept will have major implications for cytotoxic drug testing with the double layer soft agar culture system.

Our results of drug sensitivity testing on transitional cell carcinoma are disappointing. No growth inhibition was obtained

in 29 drug sensitivity tests. Drugs tested were Cisplatin, Adriamycin, 5-Fluorouracil and Methotrexate. The largest part of the tumor cell samples obtained for culture purposes yielded insufficient cells for the rather extensive number of culture dishes to be seeded for a growth curve evaluation.

We have no data on the comparison of the in vitro drug resistance and the in vivo sensitivity or resistance of the tumors, because none of the patients was treated with systemic chemotherapy.

Additional applications for the double layer soft agar system were investigated. It seems possible to give an indication of viability of cells in a tumor specimen, based upon the growth potential under highly artificial growth circumstances as in the double layer soft agar system. The impact for the management of a patient's tumor remains to be defined.

As a synthesis of our ideas of viable tumor cells, growth evaluation with growth curves and the use of urine as a alternative source for tumor cells we have started a study to evaluate the value of repeated urine tumor cell cultures in the detection of those patients who will develop recurrences of their bladder tumor in the future. There was a relationship between growth in culture and the development of a recurrence. One group of patients showed growth in culture from urine tumor cells who have not yet developed a recurrence. These are the patients who need a very accurate follow up. The value of these findings has to be studied in prospective trials.

In the study on the heterogeneity of bladder tumors, the tumor cell colonines can be used for further investigations.

It can be concluded that the double layer soft agar culture system is a valuable tool for investigation in oncology. Further development and improvements of the technique, in combination with clinical trials are still needed.

SAMENVATTING:

Deze studie werd gestart met als doel de mogelijkheden te onderzoeken om met behulp van het "double layer soft agar tumor cell culture system" cytostatica te testen, werkzaam tegen het overgangscelcarcinoom van het urotheel. Het eerste doel was een antwoord te geven op de vraag of het in de praktijk mogelijk is dit testsysteem in een routinematige laboratorium opzet toe te passen.

We hebben aangetoond dat uit urine en blaasspoelvloeistof van patienten met een urotheelcelcarcinoom tumorcellen van voldoende kwaliteit verkregen kunnen worden voor kweekdoeleinden.

Voor de telling van de kolonies kwam een volledig geautomatiseerd beeldanalyse systeem, de Omnicon FAS II (Bausch and Lomb Inc., N.Y., U.S.A.), ter beschikking in het laboratorium van de afdeing Pathologische Anatomie waar het onderzoek werd uitgevoerd. Dit systeem werd onderworpen aan uitvoerige studies naar de reproduceerbaarheid van de telresultaten, voordat tot invoering in het laboratorium werd overgegaan. De conclusie was dat de reproduceerbaarheid van de telling van kolonies door de Omnicon beter is dan de reproduceerbaarheid van de telling van kolonies door het laboratorium personeel.

Omdat een dynamische evaluatie van de groei van kolonies gevoeliger lijkt voor geringe veranderingen in het verloop van de tijd, startten wij een evaluatie van groeipatronen in kweek met behulp van groeicurves. De theoretische voordelen werden beschreven die het gebruik van de evaluatie met groeicurves steunen, in plaats van de tot dusver gebruikelijke eenmalige telling van de kolonies.

Wij concludeerden eveneens dat in kweekschaaltjes waarin tumorcelklontjes werden opgebracht, evaluatie van de groei mogelijk is. De consequentie is wel dat onmogelijk aan het originele concept van "stamcellen", enkele, clonogene cellen als startpunt van de ontwikkeling van kolonies kan worden vastgehouden. Theoretisch zal deze verandering van concept grote implicaties hebben voor

het testen van cytostatica met behulp van het "double layer soft agar culture systeem".

Onze resultaten van de gevoeligheidstesten op het urothcelcel-carcinoom waren teleurstellend. Er werd geen significante groeiremming geconstateerd in 29 cytostatica gevoeligheidstesten. De middelen die werden getest waren Cisplatina, Adriamycine, 5-Fluorouracil en Methotrexate. Het grootste deel van de tumor specimens voor kweekdoeleinden verkregen, leverden onvoldoende cellen op voor het grote aantal kweekschaaltjes dat nodig is voor een groei evaluatie met behulp van een groeicurve.
Er kwamen geen data ter beschikking over de vergelijking van de in vitro resistentie in de in vivo resistentie of gevoeligheid van de tumoren voor de geteste cytostatica, want geen van de patienten werd behandeld met systemische chemotherapie.

Verder werden nog alternatieve toepassingen van het "double layer soft agar culture system" onderzocht. Het lijkt mogelijk een indruk te geven van de "viabiliteit" vna cellen in een tumorspecimen, gebaseerd op het groeipotentieel onder zeer kunstmatige omstandigheden voor groei zoals in het "double layer soft agar culture systeem". De implicaties voor de behandeling van patienten dienen nog nader onderzocht te worden.

Als een synthese van onze ideeën over viabele tumorcellen. evaluatie van groei in kweek met behulp van groeicurves en het gebruik van urine als een alternatieve bron voor tumorcellen, zijn we gestart met een studie ter evaluatie van de waarde van herhaalde kweken van cellen uit urine van patienten in de follow-up van een blaascarcinoom, voor de vroege detectie van die patienten die met grote waarschijnlijkheid een recidief zullen ontwikkelen in de toekomst. Er werd een relatie geconstateerd tussen groei in kweek en het ontstaan van recidieven. Eén groep patienten ontwikkelde gedurende de follow-up periode een recidief, waarbij eerder groei in kweek was geconstateerd, een andere groep patienten liet groei in kweek zien van tumorcelkolonies, maar deze patienten hebben nog geen recidief ontwikkeld. De verwachting is dat dit de patienten zijn die zeer nauwkeurige follow-up nodig hebben. De waarde van deze bevinding moet nog in prospectieve,

klinische studies verder worden geëvalueerd.

Voor de bestudering van de heterogeniciteit van blaastumoren lijkt het mogelijk tumorcelkolonies te isoleren die voor verder onderzoek kunnen worden gebruikt.

Concluderend kan gesteld worden dat het "double layer soft agar tumor cell culture system" een waardevolle methode is in het experimenteel oncologisch onderzoek. Verdere ontwikkeling en verfijning van de techniek zijn noodzakelijk evenals klinische trials om de waarde van de gevonden resultaten te toetsen.

CURRICULUM VITAE:

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1974. Eindexamen Atheneum B, St. Janscollege te Hoensbroek.

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STELLINGEN

1. Het is logischer de vitaliteit van cellen te beoordelen op basis van het groeipotentieel in kweek dan op basis van de morfologie van de cellen in gefixeerde toestand.

2. Laboratoriumbepalingen die gebaseerd zijn op subjectieve menselijke beoordeling zullen vervangen worden door objectieve, kwantitatieve bepalingen.

3. Een kolonie is zelden een kloon.

4. De preliminaire publicatie van positieve resultaten van een nieuwe onderzoeksmethode wekt valse hoop bij ernstig zieke patienten. Als er commerciële belangen in het spel zijn, geraakt een dergelijke methode voortijdig in discrediet.

5. "Het zou de wetenschap ten goede komen wanneer meer aandacht wordt besteed aan publicatie van negatieve resultaten" (Dr. R.C.Roozemond, G.U. Amsterdam, 1969) is een stelling die steeds belangrijker wordt.

6. Iedereen geeft slagpijn aan op de nierloges. Kloppijn op de nierloges heeft een heel andere betekenis.

7. Het inzagerecht van het medisch dossier kan een mondelinge uitleg aan de patient niet vervangen.

8. Het gebruik van de waarschuwingsknipperlichten maakt een verkeersovertreding niet legitiem.

9. Chest-X-ray films should not be ordered solely because of hospital admission. N.Engl.J.Med.,312: 209-213, 1985.

10. De voeten van diabetici worden ernstig bedreigd door de diabetische neuropathie waardoor geringe traumata niet worden opgemerkt en de slechte wondgenezing tengevolge van de trofische stoornissen door de diabetische neuropathie en de diabetische angiopathie.

11. Was eerder de promotie een afsluiting van een medisch wetenschappelijke carriere, tegenwoordig wordt het steeds meer een begin.

Nijmegen, 19 april 1985.
W.J. Kirkels.