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

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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 μm diameter colonies to >149 μm 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 cytostatic 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 sometimes different if pure single-cell suspensions 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.

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.*, 1983*a*) and prediction of *in vivo* tumor behavior 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 collected 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 sterile 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-

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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.*, 1983*b*).

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 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-μm 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 appropriate for the type of tumor.

RESULTS

Figure 1 demonstrates the growth in soft-agar of an undifferentiated rat prostate adenocarcinoma, MatLy-Lu, maintained in our laboratory. As in all the Figures, three curves are presented, representing all tumor cell colonies over 60 μm in diameter (△), over 86 μm in diameter (●) and over 124 μm 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 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

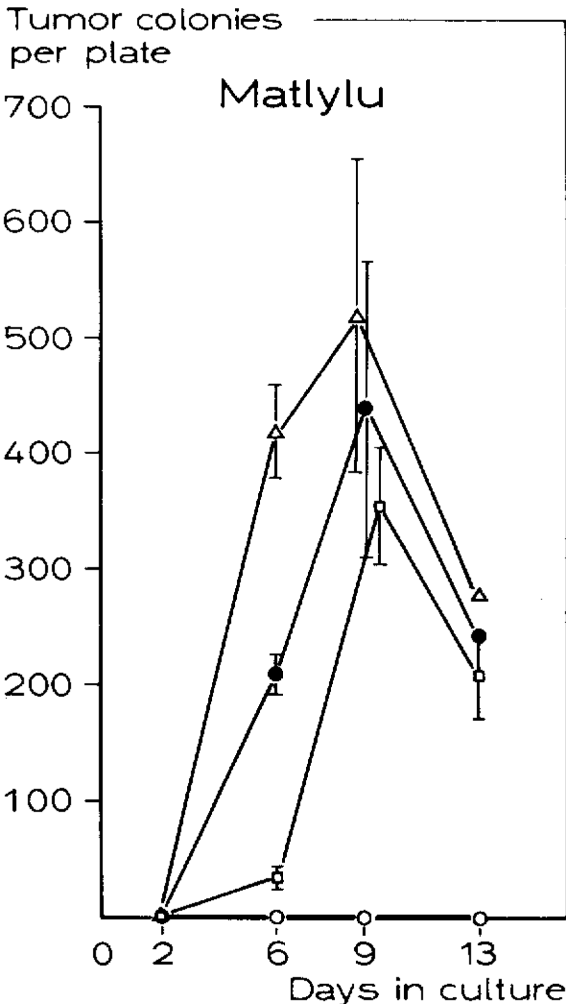


FIGURE 1 – Growth of rat prostate carcinoma cell line, MatLyLu, in soft-agar culture. In Figures 1-4, three curves are presented for each tumor: all colonies over 60 μm in diameter (△), all colonies over 86 μm in diameter (●), and all colonies over 124 μm in diameter (□). In addition, an aliquot of this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

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 2*a* 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 2*a* 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.

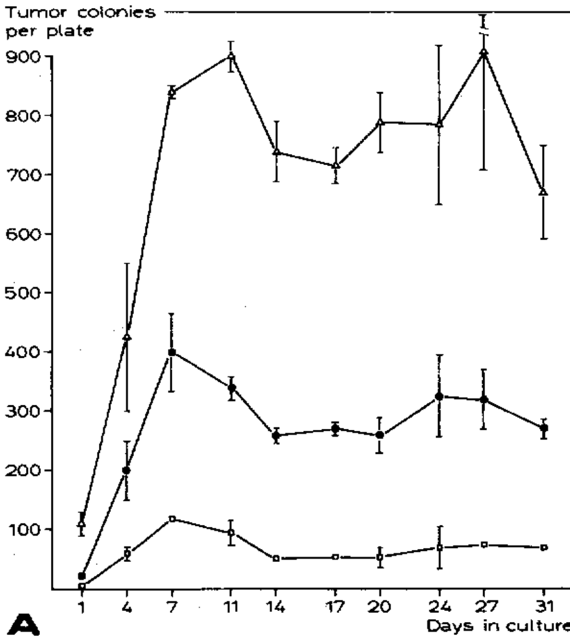
One of the most significant differences between human tumors and cell lines is the presence of significant numbers of clumps seeded into the agar. Figure 2*a* shows that more than 100 clumps exceeding 60 μm in diameter were seeded into the agar. This finding correlates well with the cytologic appearance 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 developing 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 2*a* demonstrates that growth of real tumor cell colonies can be recognized in soft agar culture using growth curves, even in the presence of significant numbers of clusters seeded into the 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 60 μm 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.

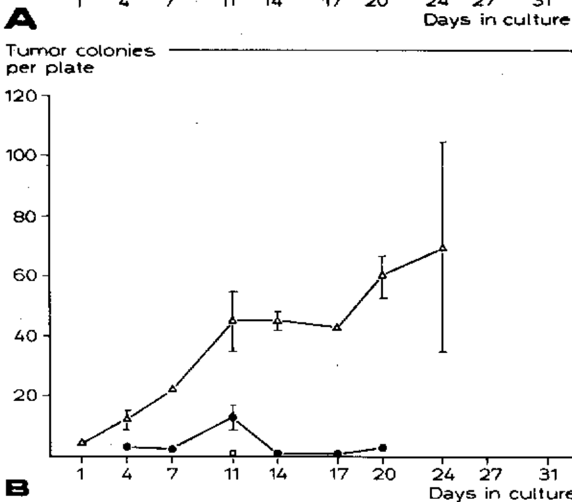
An alternative solution to the problem of clumps seeded into agar is demonstrated in Figure 2*b*. Because of the marked clumpiness of the specimen shown in Figure 2*a*, an aliquot of the original cell suspension was further treated with trypsin digestion (0.25% in 0.1% EDTA; Flow Laboratories) before 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 μm diameter) was almost completely eliminated by trypsin treatment.

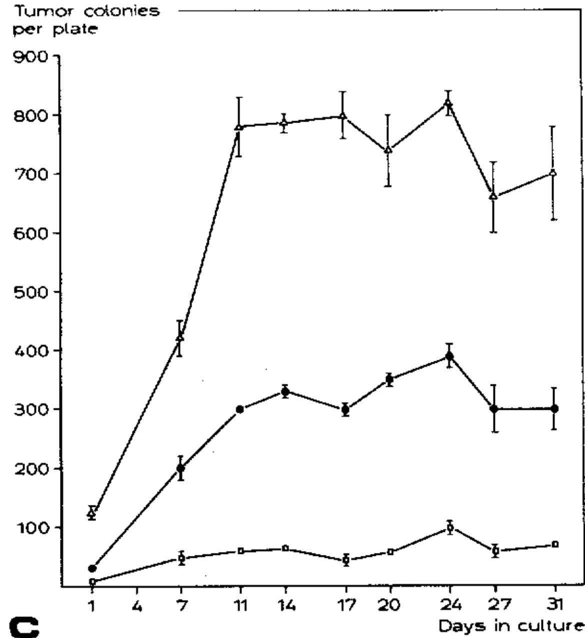
A similar phenomenon is seen in Figure 3 from an ovarian cystadenocarcinoma cultured from peritoneal irrigation fluid. Figure 3*a* demonstrates that a large number of clusters were seeded into culture. However, by assessing "colony" counts at multiple time points, deterioration of these clumps over 3 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. 3*b*) markedly reduced the number of clumps seeded into the agar as well as the total number of colonies developed in all three size categories. However, in contrast to Figure 2*a* 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.







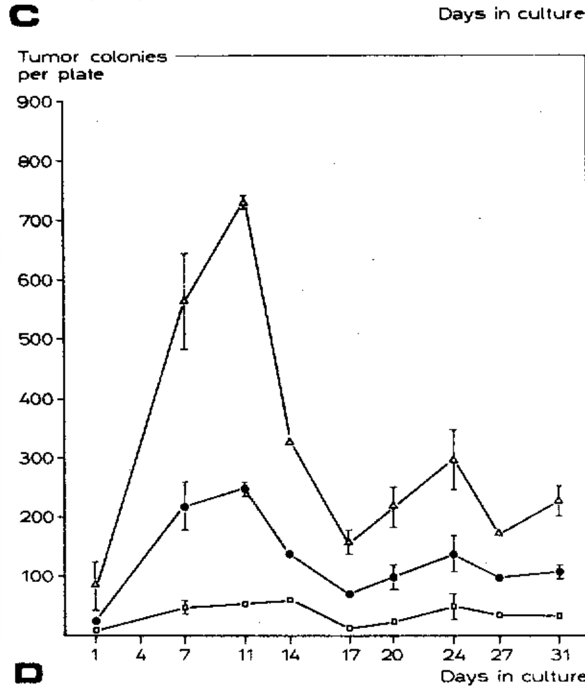


FIGURE 2 – Breast carcinoma from pleural fluid. (*a*) Control (non-drug-treated); (*b*) same cell suspension as (*a*) but with additional incubation with trypsin (0.25% in 0.1% EDTA) before plating of culture; (*c*) and (*d*) same cell suspensions as in 2*a*, but incubated with 5-FU (*c*) or adriamycin (*d*) for 1 h before plating.

3*a*, 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. 3*a* 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 2*c*

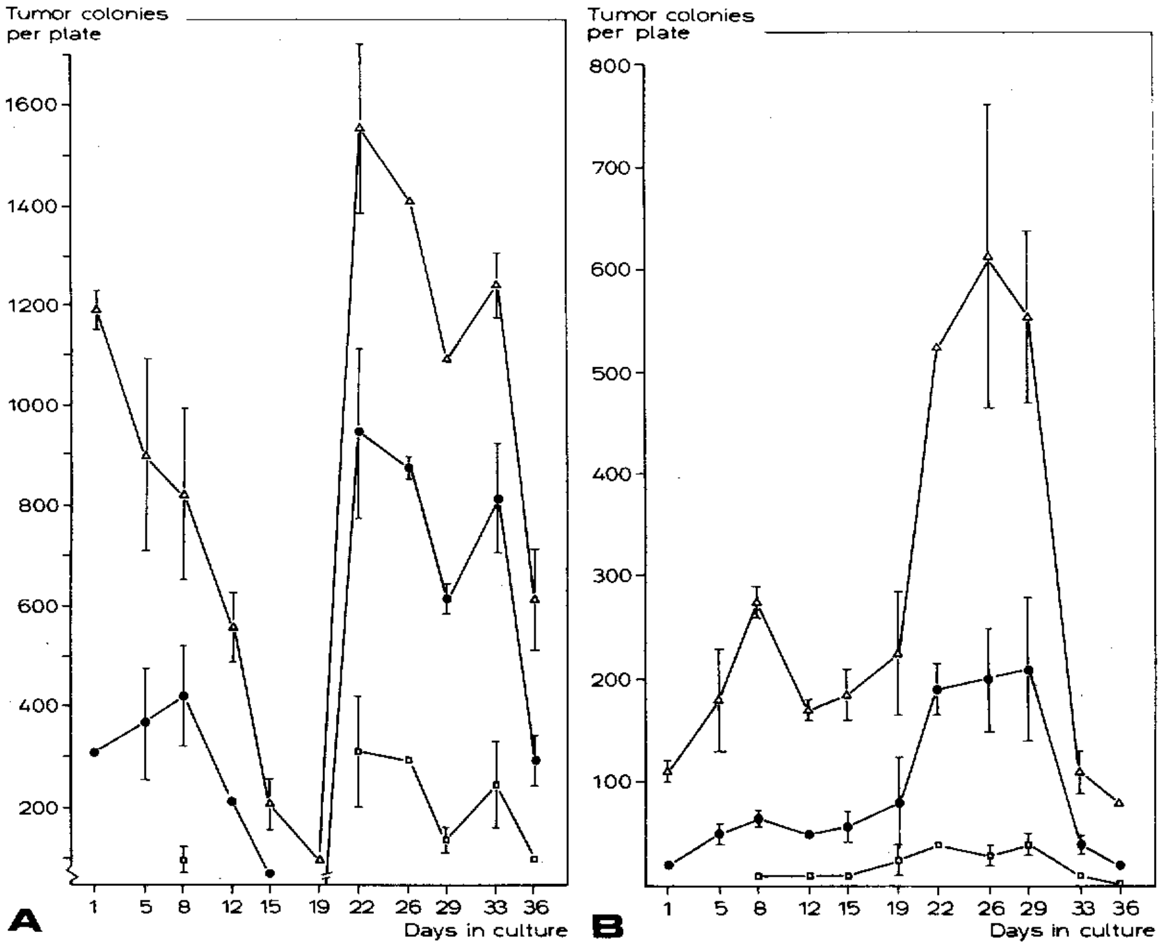


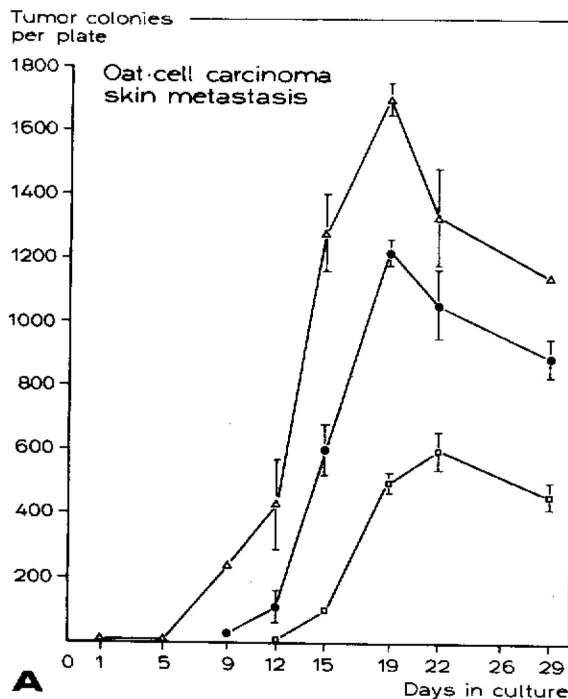
FIGURE 3 – Ovarian carcinoma from peritoneal washing. (*a*) Cell suspension plated directly after preparation; (*b*) an aliquot of the same cell suspension as in 3*a*, but treated with trypsin before plating.

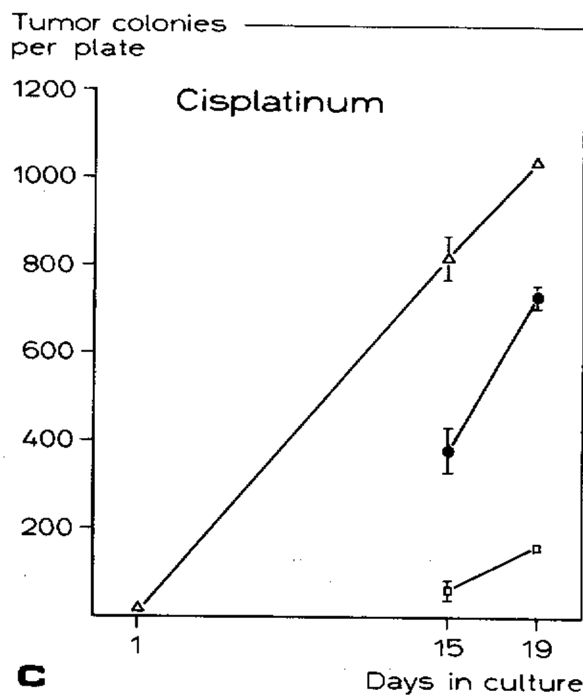
shows the development of colonies from the breast carcinoma shown in Figure 2*a*, following 1 h pre-incubation with 5-FU. Although clearly resistant to 5-FU, the tumor shows some response to the drug in the delayed development of larger colonies (>86 μm; >124 μm) compared with the control, non-incubated sample. Figure 2*d* 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. 2*a*) 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 could be recognized in PAS-stained agar layers as a loss of density of the colonies due to necrosis.

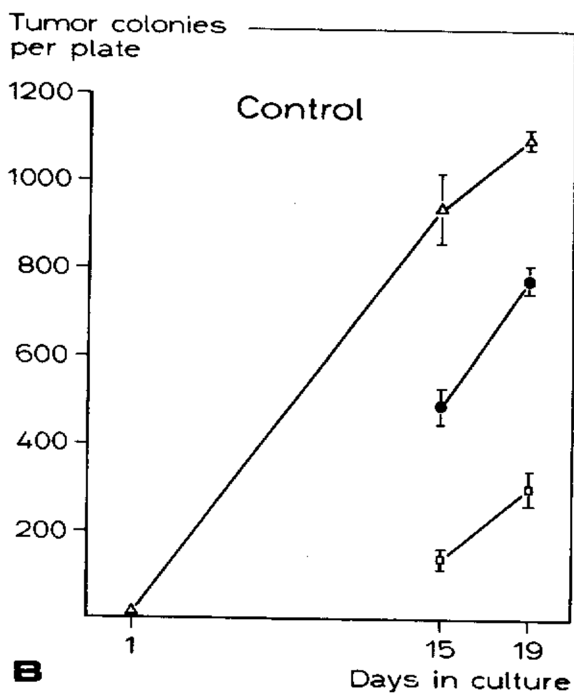
The real value of assessing growth dynamics in culture is suggested by Figure 4, cultures of an oat-cell carcinoma growing as a skin metastasis. Figure 4*a* shows a full growth curve of this tumor, demonstrating a lag phase of 5 days followed by rapid growth of colonies to a peak at 19 days and then a decline in the number of counted colonies. Figure 4*b-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 4*a*, 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 4*b*, 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. 4*c*) while adriamycin was clearly effective *in vitro* (>70% inhibition of colony growth compared with control; Fig. 4*d*). VM-26 showed a more disturbing pattern of colony growth (Fig. 4*e*). 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 days 15 and 19 compared with control was also seen in cultures incubated with sparsomycin, an experimental drug under investigation in our institution (Fig. 4*f*).

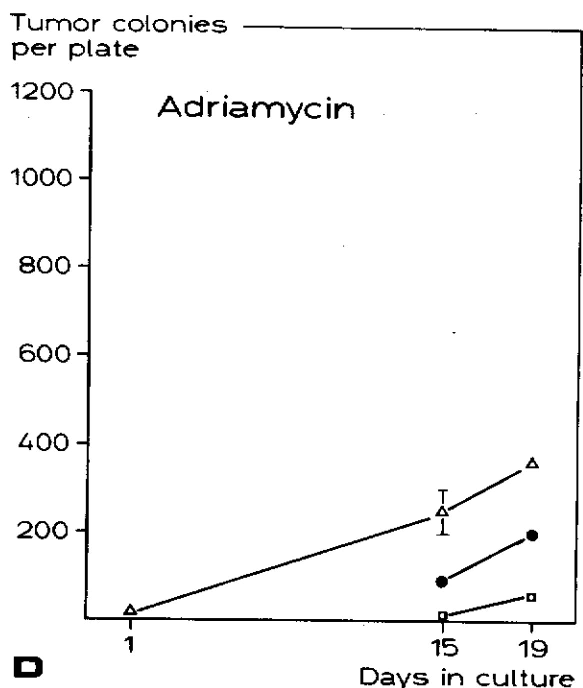
DISCUSSION

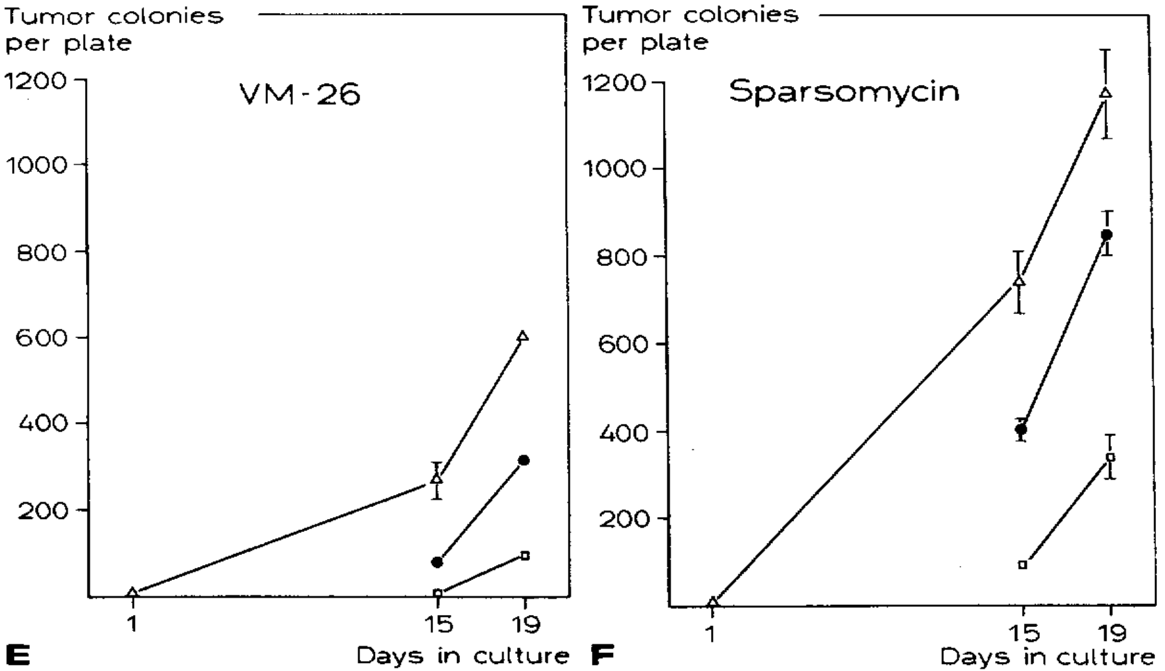
Evaluation of tumor colony development in soft-agar over time may provide new insight into the human tumor cloning system (HTCS) and may in addition contribute to solving some of the problems identified in the clinical applications of the HTCS. The development of tumor-cell colonies of different sizes after plating in agar can be demonstrated (Figs. 1-4) with varying rates of growth to maximum number of colonies and sometimes with secondary growth periods (*e.g.* Fig. 2*d*). 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.*, 1983*a*). However, there are frequently marked differences in growth dynamics between model tumor systems (Fig. 1) and primary human tumors (Figs. 2-











◀ ▲ 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.

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 contains a significant number of clumps is potentially important (Fig. 2*a,c,d*; Fig. 3). However, this phenomenon demands further investigation 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. 2*a,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.

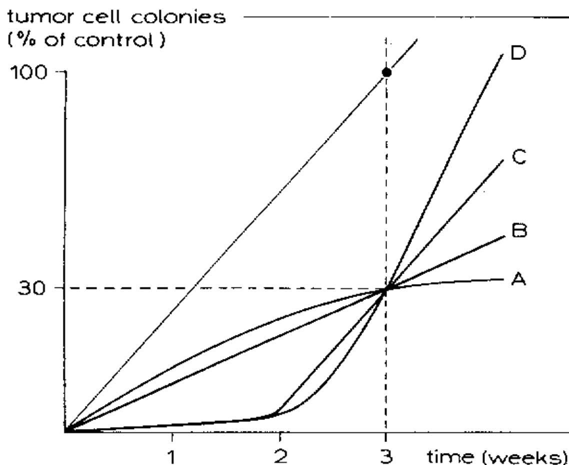


FIGURE 5 – Theoretical development of tumor colonies in soft-agar culture without (45° line) or after drug incubation. Four different patterns of colony growth which could produce a 70% reduction in number of colonies at 3 weeks compared with control cultures. For further details, see "Discussion".

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 multicell 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 recurrencxe. 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 μm). Conceivably, "growth" might also be simulated by cell swelling so that clumps smaller than 60 μm 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.

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