Improved Plating Efficiencies for Human Tumors Cloned in Capillary Tubes versus Petri Dishes

Daniel D. Von Hoff, Barbara J. Forseth, Mai Huong, Joy B. Buchok, and Bernd Latham

ABSTRACT

As now constituted, the human tumor cloning assay performed in Petri dishes has several limitations including: (a) not all patients' tumors form colonies in the assay; (b) the plating efficiencies (number of colonies formed/number of cells plated) are low; and (c) a large number of tumor cells are required to perform drug sensitivity testing. In this study the use of capillary tubes, as vessels in which to clone human tumors, is compared to the use of 35-mm Petri dishes. In 100-μl capillary tubes the optimal plating efficiencies are found with 50,000 cells/vessel (500,000 cells/ml), while in 35-mm Petri dishes the optimal plating efficiencies are found with 500,000 cells/vessel (250,000 cells/ml). In head to head comparisons of plating efficiencies of 183 human tumors (18 different histological types), the median plating efficiency was 5-fold higher (range, 1.16–37.00) for the capillary tubes than for the Petri dishes. This improved plating efficiency was noted for nearly all of the histological tumor types examined. The improved plating efficiencies noted with the capillary system indicate that the Petri dish method may be too selective and not reflect the total number of clonogenic units in a human tumor. In addition, the higher plating efficiencies noted with the capillary system may be exploited to solve some of the problems noted with the conventional Petri dish method.

INTRODUCTION

One of the major problems in clinical oncology is our inability to predict in advance which patient will or will not respond to a particular chemotherapeutic agent. Instead of the totally empiric system used for drug selection today, a system must be devised which will enable intelligent selection of drugs to be used for an individual patient. Such a system would maximize the response of the patient's tumor and allow avoidance of unnecessary toxicities of drugs that have no chance of working for the patient.

In 1977 Hamburger and Salmon (1, 2) introduced a two layer soft agar system for cloning tumors taken directly from patients. The same group proposed that the two layer soft agar system be utilized to select the most appropriate chemotherapeutic for an individual patient's tumor in much the same way in which the most appropriate antibiotic is chosen for a particular patient's infection (3). Subsequent to that initial work, a number of investigators have confirmed that the two layer soft agar cloning system has the potential for predicting which drug will or will not work against a particular patient's tumor. In both retrospective and prospective clinical trials true positive rates for the cloning system have ranged from 47 to 82%, while true negative rates have ranged from 84 to 98% (4–9). However, convincing trials which prospectively randomize patients between a selection of drug by the assay or a selection of drug by the clinician have not yet been performed (10, 11). Such a definitive prospective trial has not yet been performed with the assay because there are still a variety of problems which plague the system.

The major problems with the two layer agar system include: (a) inability to grow the majority of patients' tumors (overall, only 30 to 50% of patients' tumors form sufficient colonies in the Petri dish system); (b) the plating efficiencies (number of colonies formed/number of cells plated) are very low, which has led to speculation that the colonies which do form may not be representative of the patient's tumor; and (c) there is a need for very large numbers of tumor cells for performing each drug sensitivity study (1.5 × 10^6 cells/drug test). Clearly, with these limitations, the conventional assay system has only limited use in the clinic.

A variety of methods have been utilized to address these major problems including use of conditioned media or special media supplementation (12–15) to improve colony formation. However, most of these efforts have not consistently improved the percentage of specimens which form colonies and are evaluable for drug sensitivity testing.

In this paper we describe a system which utilizes capillary tubes for cloning human tumors. Human tumor cells growing in these smaller vessels (i.e., capillary tubes) appear to have a higher cloning efficiency than cells growing in 35-mm Petri dishes. This capillary cloning system has the potential for solving some of the problems noted with the conventional two layer agar Petri dish system.

MATERIALS AND METHODS

To compare the cloning efficiencies of the new capillary system versus the conventional two layer Petri dish system two separate approaches were used: (a) a variable number of tumor cells were plated in each system (1 × 10^4–1 × 10^6) with determination of the optimal number of cells which should be seeded in each system; (b) a fixed number of tumor cells (optimal number) were plated in each system [5 × 10^5 (5 × 10^3/ml) in each capillary tube and 5 × 10^5 (2.5 × 10^6/ml) in each Petri dish] with a direct comparison of the cloning efficiencies in each system.

Collection and Preparation of Tumor Cells. After obtaining informed consent in accordance with federal and institutional guidelines, malignant effusions, ascites, bone marrow-containing tumor cells, and solid tumor specimens were collected from patients undergoing procedures done as part of a diagnostic work-up or as part of treatment for their disease. Tumors from patients with 18 different histological types of malignancy were obtained (see below). No major surgical procedures were performed solely to obtain specimens for drug sensitivity testing. Solid tumors or lymph nodes were minced into 2- to 5-mm fragments in the operating room and were immediately placed in McCoy's Medium 5A plus 10% heat-inactivated newborn calf serum plus 1% penicillin and streptomycin (all from Grand Island Biological Co., Grand Island, NY) under aseptic conditions. Within 4 h, these solid tumors were mechanically dissociated with scissors, forced through a No. 400 stainless steel mesh, and then washed with Hanks' balanced salt solution as described previously (1, 2, 4, 9, 16, 17). Ascitic, pleural, and pericardial fluids and bone marrows were obtained by standard techniques. The fluid or marrow was placed in sterile containers containing 10 units of preservative-free heparin (Grand Island Biological Co.) per ml of malignant fluid or marrow. After centrifugation at 150 × g for 10 min the cells were harvested and washed twice in Hanks' balanced salt solution plus 10% heat-inactivated fetal calf serum. The viability of cell
suspending cells was determined in a hemocytometer with trypan blue. Viability of cells derived from solid tumors ranged from 0 to 100% (median, 90%), while viability of cells from effusions and bone marrows ranged from 0 to 100% (median, 90%).

Positive Control. To assure the presence of an excellent single cell suspension for both the capillary and the Petri dish techniques, a positive control consisting of chromomycin A₃ (Sigma Chemical Company) at a concentration of 100 µg/ml was utilized. In order for an experiment to be considered evaluable in either system, the chromomycin had to produce a <30% survival of colony-forming units. The use of a positive control has been shown to greatly increase the reproducibility of the human tumor cloning assay (18).

Culture of Cells in Two Layer Agar Petri Dish System. The culture system used in this study has been extensively described elsewhere (1–9, 16, 17). In brief, cells to be tested were suspended in 0.3% agar in enriched Connaught Medical Research Laboratories Medium 1066 (Grand Island Biological Co.) supplemented with 15% horse serum, penicillin (100 units/ml), streptomycin (2 mg/ml), glutamine (2 mM), CaCl₂ (4 mM), and insulin (3 units/ml). Prior to plating, asparagine (0.6 mg/ml), DEAE-dextran (0.5 mg/ml; Pharmacal Fine Chemicals, Inc., Piscataway, NJ), and freshly prepared 2-mercaptoethanol (final concentration, 50 mM) were added to the cells. One ml of the resultant mixture was pipetted onto 1-ml feeder layers in 35-mm plastic Petri dishes (Falcon Plastics). The final concentration of cells in each plate ranged from 1 x 10⁶ to 1 x 10⁷ cells in the top 1 ml of agar medium (see experiments below). The feeder layers used in this study consisted of McCoy’s Medium 5A plus 15% heat-inactivated fetal calf serum and a variety of nutrients described by Pike and Robinson (19). Immediately before use, 10 ml of 3% tryptic soy broth (Grand Island Biological Co.), 0.6 ml of asparagine, and 0.3 ml of DEAE-dextran were added to 40 ml of the enriched underlayer medium. Agar (final concentration, 0.5%) was added to the enriched medium, and underlayers were poured into 35-mm Petri dishes. After addition of the top layer of agar with cells, the plates were then incubated at 37°C in a 7% CO₂ humidified atmosphere.

Colonies (>50 cells) usually appeared by Day 14 of culture, and the number of colonies on the plates was determined by counting the colonies on an inverted stage microscope at x30. To be considered evaluable for colony growth, the plates had to have excellent single cell suspensions which were defined as a >30% survival of the number of colonies in the six chromomycin A₃-treated capillary tubes compared to the six control capillary tubes.

Evidence of Malignancy. There has been adequate evidence that the cells which comprise the colonies which form in soft agar in 35-mm Petri dishes are indeed malignant cells (1–4, 16, 17, 20, 21). To document that the colonies which formed in the capillaries were indeed composed of malignant cells, two approaches were utilized. These approaches included routine histological study as well as karyotype analysis. For preparation of slides for histological study, the agar was extracted from the capillary tube onto a microscope slide by removing the clay (breaking the ends and applying positive pressure on one end of the tube). The agar was allowed to dry on the slide and stained with Harleco Diff Quick Stain (a hematoxylin stain; American Scientific, Grand Prairie, TX; No. R4132-1) or with Papanicolaou stain. The karyotype analysis was performed utilizing a method described by Trent (20) and colleagues.

Data Analysis and Statistical Considerations. The percentage of cloning efficiency in each system was calculated as

\[
\text{No. of colonies/vessel} \times 100
\]

No. of cells plated

To compare results from different groups, a Student’s paired t test method was utilized.

RESULTS

Plating of Variable Number of Tumor Cells in Each System. To determine the optimal cell concentration in each vessel (capillary versus Petri dish) and to directly compare the cloning efficiency of both systems at optimal concentrations, a variety of concentrations of cells were placed in the capillary tubes and in the Petri dishes. These cell concentrations, per vessel, included 1 x 10⁵, 2 x 10⁵, 4 x 10⁵, 6 x 10⁵, 8 x 10⁵, 1 x 10⁶, 1.2 x 10⁶, 1.5 x 10⁶, 2.0 x 10⁶, 3 x 10⁶, 5 x 10⁶, 6 x 10⁶, 7.0 x 10⁶, and 1 x 10⁷. Fig. 3 details a representative result for the comparisons of the cloning efficiencies in each vessel. As noted in Fig. 3, 30 colonies formed in the 100-µl capillary tube at a cell concentration of 80,000 cells/capillary tube (8 x 10⁴ cells/ml). For the Petri dish there had to be 500,000 cells/dish (2.5 x 10⁵ cells/ml) to form the same number (30 colonies) of colonies. Based on these data, the cloning efficiency in the

Ends Sealed with Clay

Colonies Growing in Agar in Tube

Fig. 1. Schematic diagram of a capillary cloning tube.

Fig. 2. Colonies of human breast cancer growing in 100-µl glass capillary tube viewed under an inverted microscope. ×200.
capillary tube was 0.038% (30/80,000) and in the Petri dish was 0.006% (30/500,000). This represents a 6.3-fold increase in cloning efficiency with the capillary system. Table 1 summarizes the data for 22 head to head comparisons of the two systems with variable numbers of cells plated. To obtain these 22 evaluable direct comparisons a total of 44 human tumor specimens were plated in each system. For the capillary tube method, there were 34 evaluable experiments [acceptable positive control and some colony formation (≥3 colonies/vessel at any cell concentration)] while for the Petri dish method there were 22 evaluable experiments [acceptable positive control and ≥3 colonies/vessel at any cell concentration]. Thus, there were 22 evaluable experiments in both systems. As can be noted from the data in Table 1, the optimal cell plating density for the capillary ranges from 50,000 to 100,000 with a median of 50,000 cells. For the Petri dish, the optimal plating density ranged from 500,000 to 1,000,000 cells with a median of 500,000 cells. The median number of colonies per capillary tube at the optimal plating density was 18 (mean, 42) while the median number of colonies at the optimal plating density per Petri dish was 40 (mean, 128). The median percentage of plating efficiency was 0.036% for the capillaries and 0.007% for the Petri dish system (5.14-fold increase in median plating efficiency for the capillary tube versus the Petri dish system). In all 22 of the head to head comparisons, the ratio of the plating efficiency in the capillaries to the plating efficiency in the Petri dishes was always greater than one indicating a superior plating efficiency in the capillary tubes.

Plating of Fixed Numbers of Tumor Cells in Each System. To further compare the cloning efficiencies in the capillary versus the Petri dish system, a total of 226 consecutive tumors were simultaneously placed in each cloning system. The cell concentration in the 100-μl capillary tubes was fixed at 5 x 10⁶ cells/0.05 μl (5 x 10⁶ cells/ml) while the cell concentration in the 35-mm Petri dishes was 5 x 10⁴ cells/2 ml of agar (2.5 x 10⁴ cells/ml) (cell concentrations derived from optimal values in Table 1).

For the capillary tube method there were 191 evaluable experiments (defined as an acceptable positive control of <30% survival in chromomycin-treated tubes), while for the Petri dish method there were 183 evaluable experiments (defined as an acceptable positive control of <30% survival in chromomycin-treated plates). The median coefficient of variation for the number of colonies in the 6 capillary tubes was 28% while for the 3 Petri dishes it was 14%. The median percentage of survival in the chromomycin-treated tubes and plates was 9%. For these experiments there were no minimal numbers of colonies required because all experiments were assessed for colony formation. Overall, there were 183 specimens (18 different histological tumor types) which were evaluable in both systems and could therefore be compared. Table 2 details the comparison of plating efficiencies in the capillary system versus the Petri dish system. Both the mean and the median plating efficiencies were higher in the capillary system. However, when the plating efficiencies for all 183 specimens were compared with a paired t test the improvement was significant at only the P = 0.10. The average and the median numbers of colonies per 50,000 cells plated were also higher in the capillary system than in the Petri dish (see Table 2).

To determine if there were certain tumor types in which the plating efficiencies were the most favorable in the capillary tubes, an analysis of mean plating efficiencies by tumor type...
CAPILLARY CLONING SYSTEM

Table 2 Comparison of percentage of plating efficiency in capillary system versus Petri dish system

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Capillary tubes</th>
<th>Petri dish</th>
<th>Capillary tubes</th>
<th>Petri dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean % of plating efficiency</td>
<td>0.0326&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0176&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>Median % of plating efficiency</td>
<td>0.0150&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0030&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Range of % of plating efficiencies</td>
<td>0-0.9320</td>
<td>0-0.7238</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Av. no. of colonies/50,000 cells plated</td>
<td>27.1</td>
<td>10.2</td>
<td>2.66</td>
<td></td>
</tr>
<tr>
<td>Median no. of colonies/50,000 cells plated</td>
<td>7.5</td>
<td>1.5</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Range of no. of colonies/50,000 cells plated</td>
<td>0-466</td>
<td>0-362</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data base is 183 patients' tumors of 18 histological types.

The precise reasons for this improvement in plating efficiencies are unclear. One possibility is that, in the reduced volume of the capillary tubes, the cells have more space to grow and less diffusion limitations. Another possibility is that the capillary tubes provide a more controlled environment, allowing for better control of nutrient delivery and waste removal. Further studies are needed to determine the exact mechanisms involved.

DISCUSSION

Based on the above results, utilization of capillary tubes as vessels for cloning human tumors offers an advantage over the conventional two layer Petri dish system. The plating efficiencies of individual tumors are greater in the capillary tubes. This improvement in plating efficiencies with the smaller volume vessel lends support to the argument that the Petri dish may be too selective and underestimates the true percentage of clonogenic or stem cells in a tumor population. The median increase in cloning efficiencies with the capillary tubes was modest (5-fold) but was indeed significant. This improvement in colony formation clearly indicates that further improvement in the cloning of human tumors can be accomplished, and that we may not yet have an assay which is ideal for growth of all tumor stem cells.

Utilization of capillary tubes for culturing human tumors has been reported by other investigators. In 1948 Sanford et al. (22) used a capillary tube to attempt to start a cell line from an individual tumor cell. They observed that growth of an individual cell required the presence of other cells. They reasoned that if they reduced the amount of culture medium to that volume which a single cell could adjust or condition by its own metabolic activity that might allow the single cell to proliferate into a continuous cell line. In their studies, they were unable to obtain cell growth with a single cell but were able to successfully start a cell line when four cells were present in the smallest capillary they could make. In addition, with the use of medium that was conditioned by other cells they were able to grow a continuous line from a single cell.

The next mention of a capillary system to grow tumor cells was by Knazek et al. in 1972 (23) who utilized a bundle of porous capillaries inside a tube. In that system the cells were placed in the tube surrounding the porous capillary bundle. Medium was then delivered through the porous capillary tubing to the cells growing around the capillary bundle. Unfortunately, that system cannot be utilized for growing primary human tumors because fibroblasts would preferentially grow on the outside of the capillary tubes and preclude growth of tumor cells.

In 1973 Abrams et al. (24) went back to attempting to grow cells inside the capillary tubes. They successfully grew granulocyte colonies from mouse marrow utilizing agar-filled capillaries. Since that description, Maurer et al. (25-28) have performed a considerable amount of additional work in perfecting the glass capillary system for growth of granulocyte and B-lymphocyte colonies.

Most recently Ali-Osman and Maurer (29, 30) have also reported utilization of capillary tubes for growing a variety of tumors taken directly from patients. However, they reported a comparison of plating efficiencies in capillaries versus Petri dishes for only one prostate cancer specimen. For that one specimen the plating efficiency was 9-fold higher in the capillary tubes than in the Petri dish (0.63% versus 0.07%).

The present study represents the first systematic head to head comparison of plating efficiencies in capillary tubes versus Petri dishes. The plating efficiencies in the capillary tubes are consistently superior to the plating efficiencies in the Petri dishes. The precise reasons for this improvement in plating efficiencies are unclear. One possibility is that, in the reduced volume of agar in the capillary, there may be confinement of autocrine growth factors secreted by the tumor cells. This explanation is particularly appealing in that, in the present study, two of the tumors with the most significant improvement in plating efficiencies in the capillaries were breast cancer and sarcoma. Both of these tumor types have clearly been proved to produce autocrine growth factors (31-33). At present we have no direct evidence that this is occurring. However, the amount of agar which the cells must condition in the capillary tube is less than...
in the Petri dish. At the optimal plating density of 50,000 cells/100-μl capillary tube the amount of agar per cell is 100 μl/50,000 cells = 0.002 μl/cell. At the optimal plating density of 500,000 cells/Petri dish (2 ml of agar) the amount of agar per cell is 2000 μl/500,000 cells = 0.004 μl/cell. The only problem with that explanation is that we should have seen optimal plating efficiencies with the Petri dish with the 1 x 10^5 cells in the plate (2000 μl/1 x 10^6 cells = 0.002 μl/cell) and this was clearly not the case (optimal plating efficiencies were noted at the 500,000-cells/dish seeding density). Therefore, if autocrine growth factors are indeed involved in the improved plating efficiencies, it is on the basis of something more complicated than an amount of medium the cells must condition.

Another possible reason for the improved plating efficiencies could be a difference in other conditions within the capillary tubes, including gas exchange, humidity, and temperature fluctuation. The tubes are sealed and probably are not as prone to fluctuations in temperature, humidity, or gas concentrations that occur frequently with the nonsealed Petri dishes in a CO₂ incubator. Work with microelectrodes for temperature and oxygen measurements is being performed to try to determine if a more stable environment or a more hypoxic environment is present in the capillary tubes versus the Petri dishes. The more hypoxic environment utilized in the Courtenay assay has been associated with improved growth of human tumors (34).

A third possible explanation is that the higher plating efficiency noted in the capillaries is an artifact caused by greater cell clumping in the capillary tubes than in the Petri dishes or caused by growth of nontumorous cells in the capillaries. The possibility of a clumping phenomenon has been eliminated in this study by the use of the chromomycin-positive controls in both the capillary and Petri dish systems. As far as growth of nontumorous cells is concerned, based on histological studies as well as on very limited karyotype analysis, we have been able to document that the colonies are composed of malignant appearing cells. This documentation could be more extensive and is being expanded to include electron microscopic and nude mouse studies (21, 35).

In addition, a method to document that the colonies contain tumor stem cells with a high capacity for self-renewal is also needed (36, 37).

Despite the lack of explanation for the increased plating efficiencies in the capillary tubes, this improved plating efficiency produces advantages which might be exploited at this time. These advantages could include a higher percentage of patients' tumors with adequate growth for drug sensitivity testing, as well as a smaller number of tumor cells (one-tenth as many) required for drug sensitivity testing. This higher percentage of patients' specimens with adequate growth, as well as the ability to test a larger number of drugs on each specimen, could lead to definitive prospective clinical trials of the cloning system. The results of such trials could define the place of the cloning system in the treatment of human tumors. The results of such trials could define the place of the cloning system in the treatment of human tumors.

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**REFERENCES**

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