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Development of an in vivo Clonogenic Cell Assay for Rat Prostate Metastatic Tumor – R3327-MatLyLu

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Summary. The effects of intravenous (iv) inoculation of rat prostate R3327-MatLyLu tumor cells in syngeneic Copenhagen rats have been investigated both qualitatively and quantitatively in order to establish an in vivo clonogenic cell assay. Intravenous injection of tumor cells resulted in formation of tumor nodules only in the lungs. A linear relationship existed between the number of tumor cells injected and the number of measured lung nodules. The distribution patterns of iv injected radiolabelled tumor cells and of non-labelled tumor cells injected in both arterial and venous blood vessels confirmed the major role of the lungs as recipient organ for injected tumor cells. Although a similar distribution pattern was observed after iv injection with radiolabelled fibroblasts, no lung nodules were observed even one year after injection. The sensitivity for measuring drug treatment effects by this in vivo clonogenic cell assay has been compared with that of the in vitro clonogenic cell assay. Information obtained using both in vitro and in vivo clonogenic cell assay will provide a better understanding of efficacy of treatment modalities.

Key words: Prostate tumor, Lung colony, Clonogenic cell, Therapy, R3327-MatLyLu.

Introduction

Considerable efforts have been made to establish in vitro cell culture techniques in which the self-replicative and proliferative potential of tumor cells can be measured (clonogenicity). Tumor cells exhibiting colony-forming capacity in vitro were considered to be progenitor or stem cells which would be responsible for the phenomena of tumor recurrence and metastasis [9]. Eradication of these tumor cells should be the goal of any anticancer therapy. Tumor clonogenic cell assays have been developed and used for the quantitation of anticancer drug sensitivity of human tumor biopsy specimens. Such an assay could be predictive of drug responsiveness and unresponsiveness in vivo [8]. We developed in vitro clonogenic assays for the Copenhagen rat R3327 prostate tumor variants and used them for screening a number of hormonal and chemotherapeutic agents [1, 7].

In spite of the promising potential value of these techniques, the relationship between the clonogenicity under in vitro conditions and the actual proliferation and replication of tumor clonogenic cell population under in vivo conditions remains to be elucidated. Therefore it is essential to correlate the in vitro and the in vivo tumor cell clonogenic potenial.

Tumor colonization experiments in vivo have been performed while studying part of the metastatic process, by intravenous injection of viable tumor cells into animals [5, 6]. Depending on the kind of tumor cells injected and the host animal studied, in general a specific distribution pattern of the injected tumor cells was observed, which eventually led to growth of tumor nodules in a particular target organ [3]. The use of immune-compromised hosts like nude mice or irradiated animals [11] in this type of investigations suffers from the disadvantages of a non-syngeneic system.

In the present study we used transplantable and metastatic Copenhagen rat prostate tumor cells to investigate the effects of intravenously injected isogeneic carcinoma cells. This procedure is proposed as an alternative method of measurement of clonogenic tumor cells, which can be used as an appropriate in vivo drug screening assay to complement the results obtained with in vitro clonogenic cell assay.

Material and Methods

Animals and Tumor Cells

Copenhagen rats, originally obtained from the Mammalian Genetics and Animal Production Section, National Cancer Institue, Bethesda,

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Md., were bred (brother x sister) in our animal facilities. In all experiments only male rats older than 90 days were used unless otherwise specified. The R3327-MatLyLu tumor variant was originally obtained from Dr. J. T. Isaacs (John Hopkins School of Medicine, Baltimore, Md.) and has been maintained in castrated male Copenhagen rats by trocar transplantation. Preparation of monodispersed cell suspension and establishment of cell culture lines, starting from tumor material obtained from tumor bearing rats have been described earlier [1]. For the experiments described herein either cell suspensions prepared freshly from tumor, or tumor cells from established cell lines (passages 10 to 20) were used.

Lung Colony Assay

Monodispersed tumor cell suspensions were injected intravenously (in aliquots of 0.2 ml Hanks salt solution) into the lateral tail vein of rats under light ether anaesthesia. At different times after injection (day 6, 8, 10, 11, 13, 15, 17) animals were killed and autopsied. The lung lobes were fixed in Bouin solution to increase contrast between tumor nodules (white) and lung tissue (gray-yellow). Macroscopically visible lung colonies were counted using an illumination-lens combination. Inflation of lungs using India ink to bring out more contrast between the tumor nodules and the lung tissue [13] was found to be unnecessary.

Semisolid Agar Assay

The number of in vitro clonogenic cells in a given cell suspension was determined as described earlier [1]. Samples of a cell suspension and a sterile agar solution in Phosphate Buffered Saline (PBS) were mixed at 37 °C (final agar concentration 0.25%) and layered above an underlayer of semisolid agar (0.375%). After 10 days culturing the number of in vitro colonies was quantitated.

Determination of Surviving Fraction

Both in the lung colony assay as well as in the semisolid agar assay the effect of a preceeding exposure of tumor cells to Cisplatinum was determined. In order to do this tumor cell suspension (0.25×10^6/ml) was incubated for 1 h at 37 °C in a closed tube in a shaker water bath with Cisplatinum (5 mg/ml, freshly prepared in PBS). After incubation and careful washing by repeated centrifugation steps, samples were plated for in vitro clonogenic cell assay and injected iv into animals for in vivo lung colony assay. Samples of the untreated control cell suspension were also plated for in vitro assay and injected into animals for in vivo assay. The mean number of in vitro colonies in 6 replicative wells containing drug treated tumor cells, was determined and compared with the mean number of in vitro colonies in 6 wells containing untreated control cell samples. The surviving fraction was calculated as the ratio between the latter and the former numbers. Likewise the surviving fraction was calculated comparing the mean number ($n = 6$) of lung colonies arising in animals injected with drug treated tumor cells, and the mean number ($n = 6$) of lung colonies in animals injected with untreated control tumor cells.

Tumor Cell Inoculation in Different Venous and Arterial Blood Vessels

Groups of 6 male rats were injected under anaesthesia (Hypnorm, Duphar b.v. Holland; sc 1 ml/kg) with monodispersed tumor cell suspension (1×10^6 viable cells in 0.2 ml Hanks salt solution) either in the lateral tail vein, or in the hepatic artery or in the left femoral artery. After 10 days animals were killed and complete autopsy was performed. Lung lobes were prepared free and placed in Bouin fixative. Samples of lung lobe and lymph node tissue were prepared for histological confirmation for the presence or absence of tumor cells.

Radiolabelling of Cells and Autoradiography

Monodispersed tumor cell suspension from in vitro cultured MatLyLu cell line (passage 10 to 20) and a cell suspension of a fibroblast line derived from subcutaneous connective tissue from the flank region of a male Copenhagen rat (passage 15 to 20), were radiolabelled with 6-^{3}H Thymidine (Amersham International Ltd, Amersham GB; specific activity 26 Ci/mmole). The medium was replaced by fresh Minimal Essential Medium containing 10% fetal calf serum and deoxyribonucleotides (dATP, dCTP and dGTP; Sigma, St. Louis, USA), were added at a final concentration of 1 μg/ml, one hour before the addition of ^{3}H-Thymidine to cell cultures. The cultures incubated with 1 μCi/ml culture medium for 1 h at 37 °C in an incubator under 5% CO_2 and saturated humidity. After careful washing by repeated centrifugation steps and checking of the labelling effeciency, cell suspensions were collected in Hanks salt solution, checked for monodispersion and viability using trypanblue exclusion test under the microscope, and counted. Samples were prepared from these ready to inject cell suspensions for cytology using a cytocentrifuge. The preparations were processed for autoradiography using Kodak AR10 stripping film. After exposure for 2 weeks at 4 °C in the dank autoradiographs were developed and stained with Haematoxylin.

Measurement of the Distribution Kinetics of Injected Radiolabelled Cells

Radiolabelled tumor cells and radiolabelled control fibroblast cells were injected as monodispersed cell suspension (0.6×10^6 per animal in a 0.2 ml aliquot) into male rats via the lateral tail vein under light ether anaesthesia. At 0.5, 1, 2, 3 and 24 h, groups of 4 animals each were killed and from the following organs and tissues samples were collected: blood, lungs, liver, spleen, kidney, small intestine, testis, prostate, thigh muscle and bone marrow. After weighing the removed organs and the tissue samples, the tritium content in the tissue was extracted using a sample oxidizer (306 Tri-Carb, Packard Instr. Co) The efficiency of the oxidizer was over 97%. The radioactivity measured in each organ was expressed as the percentage of injected radioactivity.

The radioactivity measured in blood was calculated, assuming a total blood content of 13 grams in these rats.

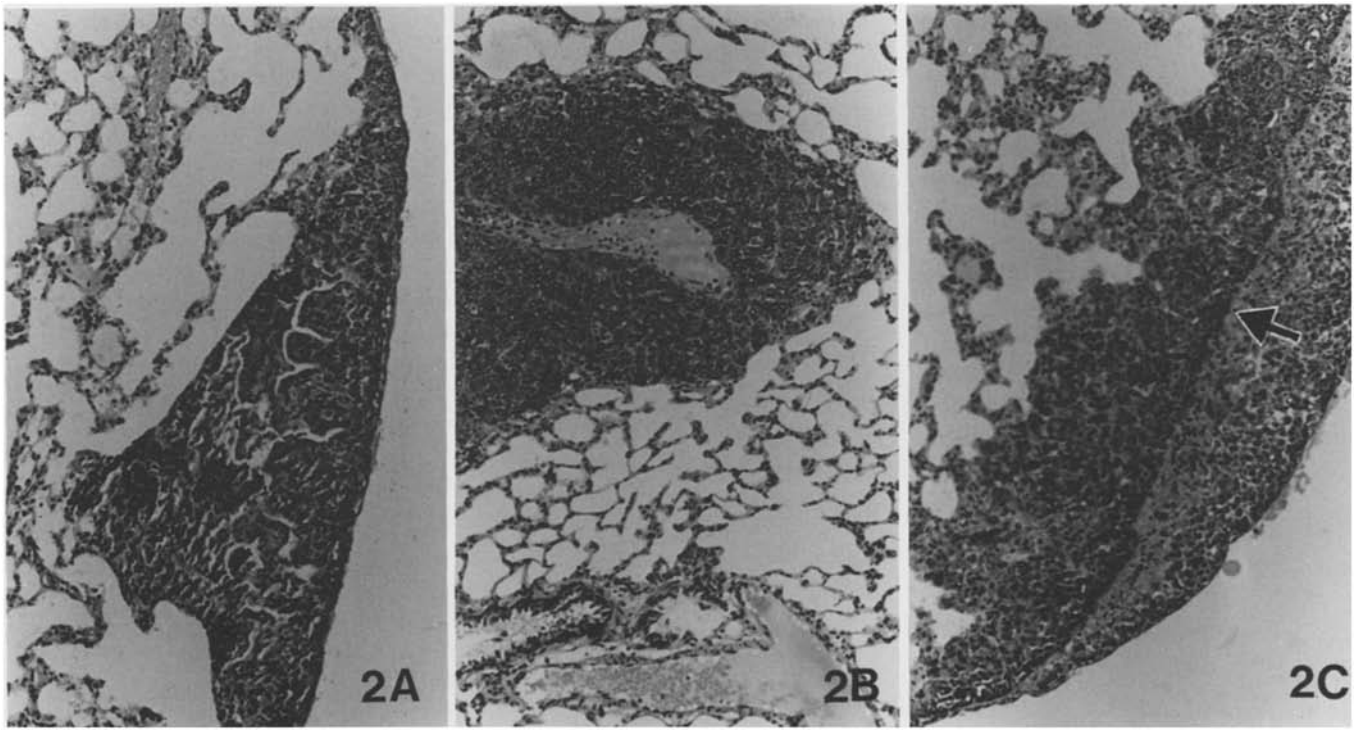
Results

Effects of iv Injection of Tumor Cells

Seven to eight days after tail vein inoculation of tumor cells small macroscopically recognisable tumor nodules were observed in the lungs. These nodules became readily visible 10 days after injection and they became prominent after fixation of lung lobes in Bouin fixative for 24 h (Fig. 1). Tumor growth was not detected during autopsy at any other site than lungs. Also histological screening of different tissues showed the absence of tumor tissue in organs other than the lungs. At 15 days after injection, grossly visible round or oblong lung colonies (diameter about 4 mm) were observed at the lung surface. At that time an increasing number

Fig. 1. Tumor nodules in male Copenhagen rat lungs, 10 days after intravenous injection of R3327-MatLyLu tumor cell suspension. Lung lobes were fixed in Bouin solution (24 h). Magnification x2

Fig. 2A–C. Histology of tumor nodules in the lungs, 10 days after intravenous injection of tumor cells. Paraffin sections (6 μ) Stained H. E., magnification x100) **A** tumor nodule under pulmonary pleura. **B** tumor nodule in lung parenchyma surrounding a large blood vessel. **C** "double layered" pleural tumor nodule, with tumor tissue outside the pulmonary pleura (*arrow* indicates pleura)



of tumor nodules also appeared at the surface lining of the pleural cavity. Eventually (17 to 20 days) the tumor cell injected animals died due to a progressive tumor growth within the lung parenchyma and a pleural cavity filled with tumor tissue. Tumor growth was observed at no time in any other site.

Histological screening of tissue samples after iv injection confirmed the confinement of tumor tissue exclusively to the lungs and pleural cavity. The histology of lungs of tumor cell injected animals showed tumor nodules mainly under the pulmonary pleura but also some scattered throughout the lung parenchyma (Fig. 2a, b). From about the 9th day onward an increasing number of pleural colonies also showed a local extension of the tumor nodule outside the pleural lining, resulting in a sort of double layered pleural colonies (Fig. 2c). No difference could be observed in the patterns of tumor growth between rats injected with cells freshly prepared from a tumor and rats injected with cells maintained in in vitro culture.

Intravenous inoculation of cultured fibroblast cells on the other hand never resulted in any observable nodule either in the lungs or in other organs. Lung histology sections were completely free of any recognisable extraneous cell. A similar result was obtained in animals killed and examined one year after the injection of fibroblast cells.

Relation Between Number of Injected Tumor Cells and Number of Lung Colonies

The absolute number of colonies arising in the lungs after iv injection varied between experiments. However, a linear relationship between the number of cells injected and the number of lung colonies arising after 10 days was always

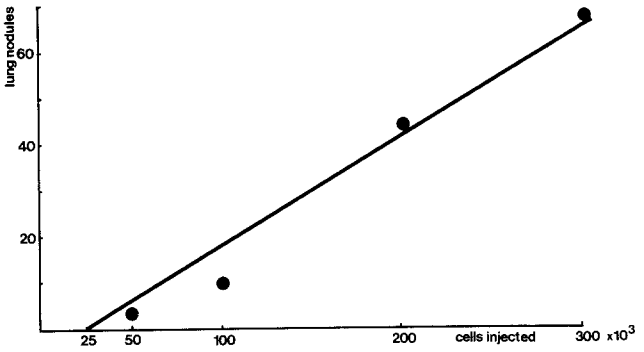


Fig. 3. Relationship between the numbers of tumor cells injected intravenously and the numbers of resulting tumor nodules in the lung

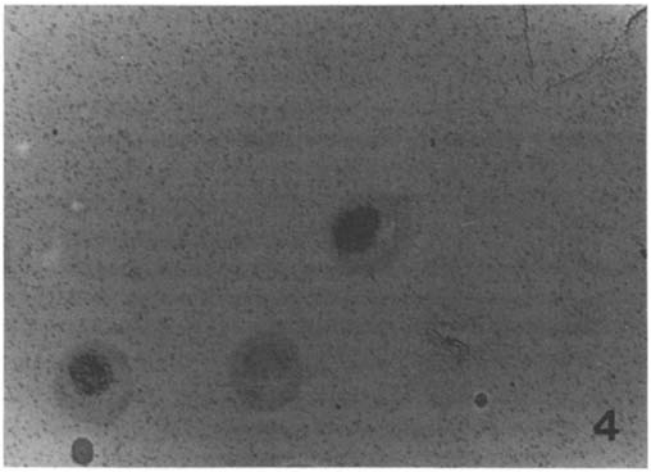


Fig. 4. Autoradiography of radiolabelled tumor cell suspension. Two labelled and one unlabelled cell are shown (Cytospin preparation, x360)

observed. A typical dose-response curve is shown in Fig. 3. In this experiment groups of 7 male rats were injected with different numbers of viable monodispersed tumor cells ($25–300 \times 10^3$). A linear relationship between the number of observed colonies in the lungs on day 10 and the number of cells injected was observed.

Comparison of Lung Colony Formation in Male and Female Rats

Groups of 5 male and 5 female rats (6 months old) were injected in the lateral tail vein with a monodispersed tumor cell suspension (10^6 cells in 0.2 ml). The number of resulting lung colonies after 10 days (mean ± standard deviation) of male rats was: 166.5 ± 67.3. The number of lung colonies in in female rats was: 246.2 ± 149.5. Using Student's t test no significant difference could be detected between the number of lung colonies formed in male and female rats (at $p = 0.05$). Variation in the number of lung colonies was more pronounced in female compared to male rats.

Injection of Tumor Cells in Arterial and Venous Blood Vessels

Monodispersed tumor cells from cultures were injected in aliquots of 0.2 ml (containing 0.2×10^6 cells) in groups of 6 male adult rats, either in hepatic artery, or in femoral artery or in the lateral tail vein. Animals were killed after 10 days and autopsied. No tumor nodules were observed either in the liver or in the femoral muscle. In all three experimental the groups tumor nodules were found only in the lungs.

Organ Distribution of Radiolabelled Cells After Intravenous Injection

Cell suspensions, after labelling with ^{3}H-Thymidine, were checked for viability, monodispersion and labelling index prior to injection into the animals. Viability and monodispersion remained constant during radiolabelling. Autoradiography showed the number of cells in which ^{3}H-thymidine was incorporated (Fig. 4). The labelling index of tumor cells was 62% while the labelling index of fibroblasts was 20%. At 0.5, 1, 2, 3, and 24 h after iv injection of radiolabelled tumor cells and radiolabelled fibroblasts, groups of 4 rats each were killed. The amount of ^{3}H labelling as a measure for the presence of injected cells was determined in blood, lungs, liver, spleen, kidney, small intestine, testis, prostate, muscle and bone marrow. The results (Fig. 5a) show that the highest amount of injected radioactivity was traced in the lungs, especially shortly after injection (30 min) and that the radioactivity in the lungs rapidly declined to 2% at 24 h. In blood and liver up to 10% of injected amount of radioactivity was measured. Unlike the lungs, no clear peaks of radioactivity were observed in other organs. The same distribution pattern of radioactivity was observed after injection of radiolabelled fibroblasts, however at a lower level of the total injected radioactivity. Besides lungs, liver and blood the radioactivity levels in other organs both after radiolabelled tumor cells and radiolabelled fibroblast injection were below the detection level. Animals injected with radiolabelled tumor cells developed lung nodules on day 10 similar to that injected with non-radiolabelled tumor cells. No radioactivity was detectable in the lung colonies.

Comparison of Cisplatinum Effect on Tumor Cells Using in vitro and in vivo Clonogenic Cell Assay

A sample from a Cisplatinum-treated cell suspension was taken, carefully washed and plated in semisolid agar (6 wells) while another sample from the same suspension was washed and injected intravenously in male rats ($n = 6$). As controls, samples of an untreated cell suspension also were both plated in agar (6 wells) and injected into the animals ($n = 6$). The number of surviving colonies arising from the Cisplatinum treated cell suspension was determined in both

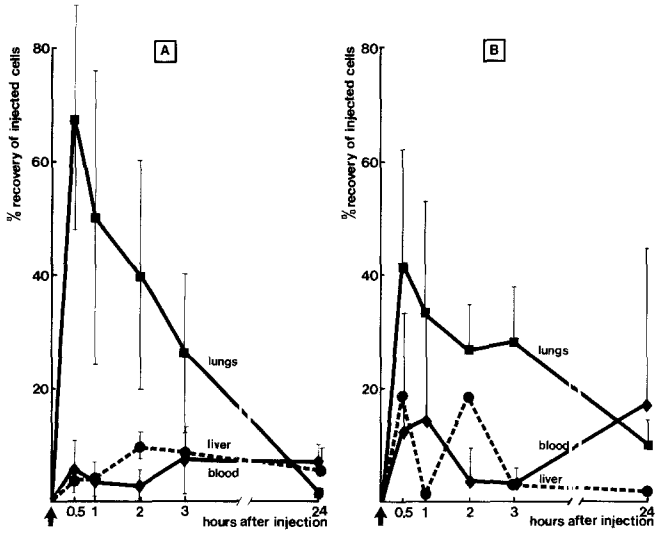


Fig. 5A, B. Distribution pattern of radiolabelled cells in lungs, blood and liver of rats at different times after intravenous injection. A Tumor cells injected. B fibroblast (control) cells injected

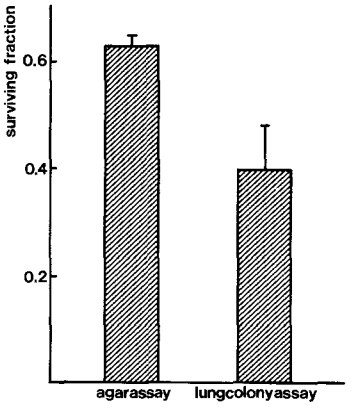


Fig. 6. Cisplatinum induced reduction in the number of surviving clonogenic cells measured in in vivo lung colony assay and in in vitro agar assay

in vitro and in vivo assays, and compared with the number of colonies arising from the untreated control cell suspension (Fig. 6). There was a significant increase in Cisplatinum treatment effect as measured in the lung colony assay compared to the agar cell assay (Student's t test, $p < 0.05$). Variation in the lung colony assay, however, was slightly higher compared to the agar assay.

Discussion

The results of the study presented clearly show that intravenous injection of tumor cells leads to formation and growth of tumor nodules which appeared exclusively in the lungs. Outgrowth of tumor tissue outside the lungs in the pleural cavity at a later stage may be due to disruption of the pulmonary pleura by rapidly growing tumor tissue and subsequent shedding of tumor cells in the pleural cavity. This hypothesis is strengthened by our histological observation of "double layered" colonies from the 9th day after injection. The composition of these colonies can be explained as a layer of tumor tissue above the boundary of the pulmonary pleura and the original colony underneath the pleura. The observed lung nodules were quantified readily and the kinetics of their appearance investigated. Literature reports have dealt with this lung colony formation after iv injection as an experimental model for understanding the metastatic process [5, 6]. Our results show that this system also can be used as an in vivo clonogenic cell assay. This assay closely resembles the in vivo clonogenic assay developed by Till and McCulloch [10], in which spleen colonies can be monitored macroscopically as a measure for quantitation of hematopoietic stem cells (CFUs).

Preconditions for establishing an appropriate clonogenic cell assay are:

1. a fixed relationship between the number of cells in assay, and the number of colonies arising;

2. that each colony arises as the result of the growth starting from a single clonogenic cell and

3. the kinetics of the clonogenic growth can be recorded and quantitated at different moments during growth.

It appeared that the lung colony assay fulfils the conditions mentioned above: relationship between cell number injected and lung colony number observed is linear in the range studied. The kinetics of colony formation can be established in an optimal phase. The monocellular origin of the colonies is not so readily understood because theoretically there is a chance of cellular clumps being responsible for the origin of lung colonies. Also the embolism of a tumor cell-blood clot could give rise to the appearance of lung colonies [12], with several cells contributing to the start of the nodule. However, since the injected tumor cells were checked and found to be monodispersed at the time of injection, it is assumed that cell clumps did not significantly contribute to the appearance of lung colonies. Also the fact that increasing the number of injected cells causes only linear increase in the number of resulting lung colonies, provides evidence against the relevance of tumor cell clotting during circulation and arrest in our experiments. Therefore it is reasonable to conclude that a single circulating tumor cell appears to invade the lung parenchyma to give rise to a growing lung colony of monoclonal composition.

The tracer studies using recovery of ^{3}H-labelled cells show an instantaneous influx of radioactivity in the lungs during the first 30 min. The premise that the radioactivity recovered equals the amount of injected cells without significant loss of label by cell death seems tenable within these short term experiments. After the first massive influx of cells in the lungs the recovered cell number in the lungs significantly reduces to about 2% at 24 h. As this still accounts for 12,000 tumor cells and the expected number of lung colonies arising from the injected cell number is between 100 and 400, only a small fraction of this 2% tumor cells left

at 24 h will ultimately give rise to lung colonies. The number of radiolabelled cells recovered in blood and in the liver constitutes a relatively low amount when the weight of total blood and of the liver is taken into account. This consideration renders the extent of recovery of radiolabelled injected cells in the lungs even more dramatic.

The fact that the majority of injected tumor cells and fibroblasts lodge in the lungs could be explained by the physical arrest of injected cells in the small lung blood vessels which are the first capillary network encountered following intravenous injection. However, the lodging of tumor cells in the lungs is more pronounced than that of fibroblasts. Moreover the release of tumor cells from the lungs after initial trapping also is an extremely rapid process. These observations support the idea of a selective homing of injected tumor cells to the lungs. This idea is strongly strengthened by our observation of colony formation exclusively in the lungs after injection in arterial blood vessels. Studies including autoradiographic recording of tumor cell features in the lungs and of tumor cell clearance from the blood are being performed in our laboratory.

Studies concerning organ-specific patterns of tumor spread often centered on the evolvement of experimental tumor variants by applying specific selection pressure [3]. The R3327-MatLyLu tumor variant arose as a highly metastatic variant from the anaplastic R3327-AT variant, which had a low rate of metastasis [4]. It was shown that the MatLyLu tumor metastasizes to the ipsilateral axillary lymph node first, followed by the later (hematogeneous) metastasis to the lungs. Therefore we conclude that once the cells from the tissue reach a blood vessel they preferentially migrate to the lungs. It must be pointed out that the MatLyLu tumor cells can be considered as androgen-independent cells, that proliferate equally well in castrated and intact male animals. Androgen-independency of MatLyLu tumor cells is additionally supported by our observation that there is no significant difference between the number of lung colonies formed in male and female animals.

Since this in vivo assay determines and quantitates the important population of tumor cells responsible for proliferation and selfreplication in the animal, it provides significant information in addition to the existing in vitro semisolid agar assay. Our comparison of the measurement results using both in vitro and in vivo clonogenic cell assay confirms their similarities in value in one situation of chemotherapeutic drug effect. Additionally evidence for an increased sensitivity for measuring these effects in the lung colony assay compared to the in vitro assay is provided.

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