Quantitation of Cell Shedding into Efferent Blood of Mammary Adenocarcinoma[1]

Thomas P. Butler and Pietro M. Gullino[2]

Laboratory of Pathophysiology, National Cancer Institute, NIH, Bethesda, Maryland 20014

SUMMARY

The rate of tumor cell shedding into efferent tumor blood was measured in growing and regressing MTW9 rat mammary carcinomas. The hormone-dependent tumor, grown as an "isolated" preparation (8), permitted collection of all of the efferent blood. Regression was induced by reduction of mammotropin level in the host. Tumor cells were differentiated from normal leukocytes by indirect immunofluorescence. Growing tumors shed 3.2×10^6 and regressing tumors shed 4.1×10^6 cells per 24 hr per g tissue. Cell shedding rates of growing *versus* regressing tumors were not significantly different over a tumor size range of 2 to 4 g. The number of tumor cells in the arterial blood was 12-fold smaller than in the efferent tumor blood. It is concluded that: (a) cell shedding via blood probably plays only a minor role in the total cell loss by growing MTW9 carcinomas; (b) hormone-induced tumor regression does not depend on increased cell shedding; (c) tumor cells are rapidly cleared from circulating blood; and (d) a 2-g MTW9 carcinoma pours enough cells into the host circulation to transplant the tumor every 24 hr.

INTRODUCTION

Since Engell's documentation of tumor cells in blood in 1955 (3), the problem of identifying these cells in the blood of tumor-bearing animals has been approached by many workers using cytological (7, 14) or immunological methods (12, 25). However, there has been no agreement concerning the reliability of these methods or their applicability to human cancer therapy (15, 27). In none of these experiments with blood drawn from peripheral veins (2, 27), vena cava (6), or tumor efferent veins (3, 20, 26) has there been a quantitation of the rate of neoplastic cell shedding from the entire tumor. Identification and quantitation of these blood-borne cells would help to clarify the role of whole cell shedding into blood in the processes of tumor growth and regression, as opposed to cell loss by intracellular processes described by Gullino et al. (9-11).

The purpose of this study was therefore 3-fold: (a) to differentiate tumor from nontumor cells in the bloodstream; (b) to quantitate the number of cells shed by entire tumors; and (c) to compare rates of cell shedding in growing and regressing tumors.

MATERIALS AND METHODS

Tumors. MTW9 mammary carcinomas were transplanted in Wistar-Furth inbred female rats. These tumors grow only in hosts with high levels of mammotropins which are normally induced by transplanting a 2nd tumor of pituitary origin in a region removed from the mammary carcinoma. Upon castration and ablation of the pituitary tumor, 3- to 5-g MTW9 carcinomas consistently regressed to about one-half of their initial size within 3 days. Induction of MTW9 (13) and physiopathological characteristics of the regression process (10) have been reported.

MTW9 mammary carcinomas were transplanted at the distal end of an ovarian pedicle and were grown in an s.c. pouch. The tumor was enveloped in a paraffin sack and completely isolated from surrounding tissues. Thus, all of the efferent blood of the tumor was drained by a single vein and was collected and measured (8) (Chart 1).

Sampling Procedure. The heparinized rat (1 mg/200 g body weight) was anesthetized with urethan (1 mg/g, i.p.), the abdominal cavity was opened, and the tumor vein was
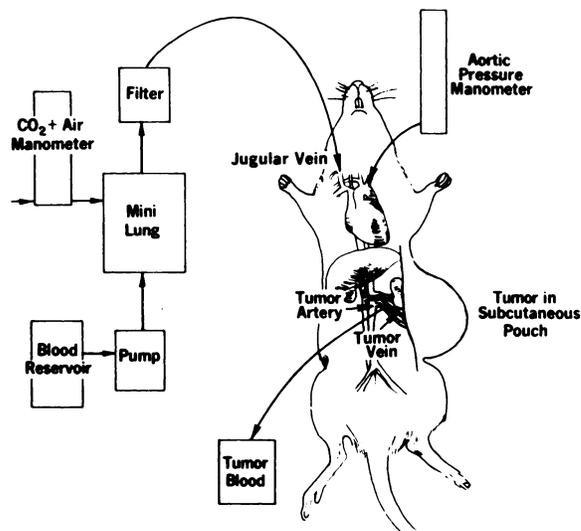

Chart 1. Diagram of the experimental arrangement for collection and measurement of tumor efferent blood. See "Sampling Procedure" described under "Materials and Methods."

cannulated with a PE 50 catheter (Clay-Adams, Parsippany, N. J.). The tumor remained in the s.c. pouch and was not disturbed during surgical manipulations. A 2nd catheter, inserted into the thoracic aorta through the left common carotid artery, was connected to a manometer for continuous monitoring of arterial pressure; from this catheter, aortic blood was drawn as needed. A 3rd catheter, introduced into the right external jugular vein, was connected to a reservoir of heparinized blood drawn from normal intact female rats. Replacement of blood lost through the tumor vein was accomplished by a pump adjusted to maintain constant aortic pressure during the sampling period. Blood entering the animal flowed through a minilung system that adjusted pH and pCO_2 to physiological levels, thus avoiding acid-base imbalance in the host. This perfusion system has been described in more detail (8).

Each blood sample consisted of 6 to 10 ml collected continuously within 15 to 30 min. Aortic blood (1.0 ml) was withdrawn at about midinterval of each venous sample. Hematocrits of venous and arterial samples were measured; leukocytes were counted in a hemocytometer and classified from May-Grünwald-Giemsa-stained slides of whole blood. Tumor blood flow rates were calculated for each collection.

Preparation of Blood Samples. Leukocytes and neoplastic cells from tumor efferent and aortic blood samples were concentrated by a modification of the dextran sedimentation method of Skoog and Beck (22). A 3% solution of dextran (M.W. 200,000 to 300,000) in 0.45% NaCl solution was added to blood (2:1), and erythrocytes were removed by two 45-min sedimentations at $1 \times g$. Pellets were washed 3 times with 0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4, and the remaining erythrocytes were removed by hypotonic lysis. Four ml of distilled water were added to 4 ml of erythrocytes in 0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4; the mixture was shaken vigorously for 45 sec and then brought back to isotonicity with 12 ml of 3.6% NaCl solution. The remaining cells were centrifuged at $100 \times g$ for 20 min, washed 2 times in 0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4, counted, and diluted to 5000 cells/μl. Twenty-μl aliquots (100,000 nucleated cells) were placed on glass slides to evaporate, fixed in methanol for 20 min, and stored at 4° until used for immunofluorescence.

Preparation of Antiserum. The ascites form of MTW9 carcinoma was 1st obtained from the solid tumor and whole ascites cells were used as antigens. Ascites fluid was processed by dextran sedimentation and hypotonic lysis to remove erythrocytes, as with the blood samples. MTW9 ascites cells (5×10^7) were injected at weekly intervals in 2 ml 0.15 M NaCl solution into 3-kg female New Zealand rabbits. A total of 4 injections were given, the 1st 2 i.v. followed by 2 s.c. in complete Freund's adjuvant. Serum obtained 7 days after the last injection was inactivated at 56° for 30 min. γ-Globulins were precipitated with ammonium sulfate, dialyzed, absorbed (6 times against normal rat leukocytes, 4 times against rat bone marrow, and 2 times against rat liver), and titrated for activity and specificity against MTW9 cells. The best antiserum, diluted 1:8 with normal NaCl solution, was chosen for use. FITC-Ab[3]

---

[3] The abbreviation used is: FITC-Ab, fluorescein isothiocyanate-conjugated anti-rabbit globulin.

(Baltimore Biological Laboratories, Baltimore, Md.) was diluted 1:10 in 0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4 and absorbed 1 time against normal rat liver and 2 times against leukocytes before use.

Preparation of Slides for Immunofluorescence. Slides were hydrated in 0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4, incubated with rabbit anti-MTW9 globulin for 30 min, washed 3 times in 0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4, incubated with FITC-Ab for 30 min at 37°, washed 3 times with 0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4, counterstained with 0.01% Evans blue, mounted in 9:1 glycerol-0.15 M NaCl + 0.01 M phosphate buffer, pH 7.4, and covered with cover slips. The slides were examined for immunofluorescent cells with an American Optical Fluorolume Microscope and photographed on a Zeiss 35-mm photomicroscope with barrier filters 50 and 44, excitor filter II, and Ansco 500 film.

Reliability of Measurements. The immunofluorescent reaction was considered "positive" only when the cell margin appeared as an unbroken fluorescent ring. Cells having only sector or spot fluorescence were not counted as positive.

No fluorescein-positive cells were found on slides of leukocytes, marrow cells alone, or MTW9 tumor cells that lacked treatment with either rat anti-MTW9 globulin or goat FITC-Ab (Table 1).

To determine the sensitivity of this method, slides were prepared from MTW9 cells, either alone or mixed with leukocytes. In slides of MTW9 cells alone, 20 to 25% exhibited unbroken ring fluorescence; the remainder showed sector, spot, or negligible fluorescence. To duplicate more closely the actual assay conditions, MTW9 cells were mixed with normal rat leukocytes in 1:100 and 1:200 ratios. From this mixture, slides were prepared with aliquots of 100,000 nucleated cells, containing 1,000 or 500 tumor cells, respectively. Tumor cells were stained by the immunofluorescence technique and counted.

An average of 18.7% of tumor cells could be positively identified using the strict criterion of ring fluorescence. Such a factor was determined for each tumor perfusion and was used to correct the number of tumor cells observed in blood samples to the actual number of tumor cells present. To ensure that tumor efferent and arterial blood samples were handled in the same way as controls, each slide tested contained a 100,000-cell aliquot of tumor efferent or arterial cells and a 100,000-cell control aliquot. For every tumor perfusion, cell counts were made of 6 to 10 samples each of aortic, tumor efferent blood, and 2 controls (1:100 and 1:200 ratios).

To determine tumor cell recovery, whole blood, containing both normal rat leukocytes and suspensions of tumor cells, was subjected to the dextran sedimentation and hypotonic-lysis procedures. Cell counts on a hemocytometer showed 60% recovery of both leukocytes from whole blood and tumor cells from the suspension. Furthermore, 10^6 tumor cells were added to whole blood containing 10^8 leukocytes, and samples of this mixture were subjected to the entire cell concentration procedure and immunofluorescence reaction. Recovery of both tumor cells and leukocytes was again approximately 60%, as determined by immuno-

Table 1
Immunofluorescence controls

	Negative	
MTW9 cells	0.15 M NaCl	0.15 M NaCl
MTW9 cells	0.15 M NaCl	FITC-Ab
MTW9 cells	Normal rabbit serum	FITC-Ab
MTW9 cells	Rabbit anti-MTW9 absorbed *versus* MTW9	FITC-Ab
Normal rat WBC	Rabbit anti-MTW9	FITC-Ab
Normal rat bone marrow cells	Rabbit anti-MTW9	FITC-Ab
	Positive	
MTW9	Rabbit anti-MTW9	FITC-Ab
MTW9 cells 1:4 with WBC	Rabbit anti-MTW9	FITC-Ab
MTW9 cells 1:100 with WBC	Rabbit anti-MTW9	FITC-Ab
MTW9 cells 1:200 with WBC	Rabbit anti-MTW9	FITC-Ab

fluorescent and hemocytometer counting, respectively. More importantly, the ratio of tumor cells to normal leukocytes was not altered by the cell concentration method used, whether afferent or efferent blood was analyzed. Thus the number of tumor cells was expressed per 100,000 leukocytes (number counted corrected for the sensitivity of detection). The number of tumor cells per ml blood and rate of cell shedding per hr were calculated as follows:

(No. of tumor cells detected/100,000 cells)

$$\times \text{(No. of leukocytes/ml)} = \text{tumor cells/ml}$$

(tumor cells/ml) × perfusion rate (ml/hr/g)

$$= \text{tumor cells/hr/g}$$

(This number was extrapolated to 24 hr to yield a rate of tumor cells shed per 24 hr per g tumor.)

RESULTS

MTW9 carcinomas in the weight range of 2 to 4 g showed no central necrosis and a blood supply that was within the limits previously reported (10). An increased blood flow was often observed in regressing tumors but, generally, the values did not differ significantly from the blood flow of growing tumors (Table 2). Hematocrits and leukocyte counts of growing and regressing tumors were also similar. However, an unexpected difference in both parameters was found between afferent and efferent blood of the same tumor. A comparison of paired samples of arterial and tumor venous blood from each animal showed that the differences in hematocrits and leukocyte counts were consistent and significant at a level of $p < 0.01$ for both growing and regressing tumors.

For all tumors the number of neoplastic cells in the efferent blood was many times higher than the number of neoplastic cells in the arterial blood entering the tumor (Table 3). Both in terms of number of cells per ml of efferent blood and total number of cells lost by each tumor over a 24-hr period, the regressing tumors did not differ from their growing counterpart. Thus, regression could not depend on a substantial increase of neoplastic cell loss via blood. No correlation was found between the rate of cell shedding and either tumor size or regression time.

Attempts to evaluate the viability of neoplastic cells isolated from the blood with our procedure have been fruitless. The dye-exclusion test suggested by Yip and Auersperg (28) using glutaraldehyde fixation and Alcian Blue yielded extremely variable results and poor quality preparations with our cells. From the experience gained in handling MTW9 ascites cells, we concluded that treatment of the neoplastic cells by the dextran concentration proce-

Table 2
Characteristics of tumor blood

	Blood flow (ml/hr/g)	Tumor wt (g)	Hematocrit of aortic blood (%)	Hematocrit of tumor efferent blood (%)	Leukocytes of aortic blood (cu mm)	Leukocytes of tumor efferent blood (cu mm)
Growing tumor	8.0 ± 2.4[a]	3.7 ± 0.3	30.9 ± 1.4	32.5 ± 1.3	14,600 ± 1,650	16,020 ± 1,530
Regressing tumor	11.7 ± 2.7	2.6 ± 0.3	31.3 ± 1.0	35.2 ± 1.8	13,400 ± 850	15,300 ± 920
p value	>0.2	<0.05	>0.02	>0.02	>0.02	>0.02

[a] Mean ± S.E.

Table 3
Tumor cells in blood

	Afferent blood (no./ml)	Efferent blood (no./ml)	Total cells released (no./24 hr/g)
Growing tumor[a]	1,290 ± 339[b]	16,830 ± 7,590	3.18 ± 1.45 × 10⁶
Regressing tumor[a]	936 ± 2.74	20,270 ± 4,970	4.05 ± 0.23 × 10⁶

[a] Seven experiments with growing tumors and 7 experiments with regressing tumors.
[b] Mean ± S.E.

dure and hypotonic lysis inflicted such damage that the test of cell viability was meaningless.

DISCUSSION

The principal objective of this study was a quantitation of neoplastic cells shed into the efferent blood by mammary carcinomas studied during growth or regression produced by hormonal deprivation of the host. The experimental approach utilized a preparation in which blood from the entire tumor was collected by a single vessel cannulated without manipulation of the tumor itself. Identification of neoplastic cells was made by immunofluorescence, because distinction between neoplastic and mononucleated cells of host blood by normal cytological techniques was not satisfactory. Three conclusions can be drawn from the data obtained: (*a*) MTW9 mammary carcinomas lost 3 to 4 million cells/day for each g of tissue when tumors were 2 to 4 g in size; (*b*) cell loss into blood did not change during hormone-induced tumor regression; and (*c*) neoplastic cells were rapidly cleared from the circulation.

MTW9 carcinomas of the size utilized regressed at a rate of about 1 g/day. The average 24-hr cell loss via blood for whole regressing tumors was calculated as follows:

$$\frac{4.05 \times 10^6 \text{ cells}}{24 \text{ hr/g}} \times \frac{2.6 \text{ g}}{\text{tumor}} = 10.53 \times 10^6$$

tumor cells per 24 hr per whole tumor

If it is assumed that 1 g of tumor contains approximately 100 million cells, then cell loss via blood comprised about 10% of tumor weight loss. At the time the measurements were made, growing MTW9 mammary carcinomas gained about 1 g in weight every 24 hr. This growth represented both cell proliferation and cell loss. The rate of cell loss as a fraction of the rate of cell production in a tumor has been calculated by Steel (23, 24) and others (1, 21) for a number of tumors. Detailed cell cycle studies have not been reported for MTW9; therefore, the cell loss factor cannot be calculated. However, MTW9 is similar to the 7,12-dimethylbenz(a)anthracene-induced mammary carcinomas of rats, although the latter grow more slowly than does the MTW9 tumor. Steel (24) has estimated from kinetic analysis that 7,12-dimethylbenz(a)anthracene carcinomas have an 86% cell loss and hence that growth of 1 g would require mitotic production of 710 × 10⁶ cells while 610 × 10⁶ cells are being lost. If these rates apply to the MTW9, then only 11.8 × 10⁶ cells or 2% of cell loss occurs by intact

cells being shed into the bloodstream. Even if the cell loss were only 20%, a low figure for solid tumors, 11.8 × 10⁶ cells is 8.3% of the loss rate. These calculations suggest that cell loss during both growth and regression of MTW9 is primarily the result of cell destruction or necrosis within the tumor and is consistent with the findings that the amino acid nitrogen lost by regressing MTW9 via blood can account for 60 to 90% of tumor protein loss during regression (11). The actual rate of tumor cell shedding into blood for each g of tumor tissue remains relatively constant whether the tumor is growing or regressing.

The usual inoculum needed to obtain 100% takes in MTW9 transplantation is 10 × 10⁶ tumor cells. Thus, 2- to 4-g tumors pour enough cells into the host circulation to transplant the tumor every 24 hr, assuming that the released cells are all alive, a fact that we were unable to ascertain.[4] On the other hand, MTW9 does not metastasize over a growth period of several weeks. Although the kinetics of tumor cell clearance from blood cannot be calculated by this method, the total number of circulating tumor cells calculated from arterial concentration represents about 3% of the hourly input or 0.1% of the average daily output of tumor cells into efferent blood. This is consistent with the data of Fidler (4), who found that only 1% of [¹²⁵I]5-iodo-2-deoxyuridine-labeled B16 melanoma cells survived for 24 hr after i.v. injection in mice.

Several investigators have reported finding circulating tumor cells in patients with nonmetastatic cancer (16—18). From our data it is obvious that the presence of large numbers of tumor cells in blood is not, by itself, a sufficient condition for metastasis to occur. Descriptions of neoplastic cells found in blood of patients have been reported under a variety of circumstances (2, 3, 5, 16—19, 27), but we have been unable to find a quantitation of the actual load of circulating neoplastic cells due to the presence of a tumor. Our data permit such a quantitation. The unexpected increase in concentration of erythrocytes and leukocytes in efferent *versus* afferent blood is probably due to hemoconcentration. Results of studies on this problem will be reported later.

REFERENCES

1. Denekamp, J. The relationship between the "Cell Loss Factor" and the Immediate Response to Radiation in Animal Tumors. European J. Cancer, *8:* 335–340, 1972.

[4] Cell viability test by injection of tumor efferent blood s.c. was impossible for MTW9, since a transplant inoculum of 10⁷ cells required injection of 500 ml blood.

2. Döbrössy, L., Sugár, J., and Tóth, J. Light and Electron Microscopic Study of Malignant Melanoma Cells, Isolated from the Peripheral Blood. Arch. Pathol. Anat. Physiol., *345:* 331–337, 1968.
3. Engell, H. C. Cancer Cells in the Circulating Blood. Acta Chir. Scand. Suppl., *210:* 10–70, 1955.
4. Fidler, I. J. Metastasis: Quantitative Analysis of Distribution and Fate of Tumor Emboli Labeled with ^{125}I-5-Iodo-2′-deoxyuridine. J. Natl. Cancer Inst., *45:* 773–782, 1970.
5. Fleming, J. A. Tumor Cells in the Blood in Carcinoma of the Breast. Proc. Roy. Soc. Med., *56:* 497–500, 1963.
6. Gazet, J. C. The Detection of Viable Circulating Cancer Cells. Acta Cytol., *10:* 119–125, 1966.
7. Goldblatt, S. A., and Nadel, E. M. Cancer Cells in the Circulating Blood: A Critical Review II. Acta Cytol., *9:* 6–20, 1965.
8. Gullino, P. M. Techniques for the Study of Tumor Pathophysiology. *In:* H. Busch (ed.), Methods in Cancer Research, Vol. 5, pp. 45-91. New York: Academic Press, Inc., 1970.
9. Gullino, P. M. Regression of Solid Tumors. *In:* S. Garattini and G. Franchi (eds.), Chemotherapy of Cancer Dissemination and Metastasis, pp. 89-95. New York: Raven Press. 1973.
10. Gullino, P. M., Grantham, F. H., Losonczy, I., and Berghoffer, B. Mammary Tumor Regression. I. Physiopathologic Characteristics of Hormone-Dependent Tissue. J. Natl. Cancer Inst., *49:* 1333–1348, 1972.
11. Gullino, P. M., and Lanzerotti, R. H. Mammary Tumor Regression. II. Autophagy of Neoplastic Cells. J. Natl. Cancer Inst., *49:* 1349–1356, 1972.
12. Herbeuval, R., Duheille, J., and Goerdert-Herbeuval, C. Diagnosis of Unusual Blood Cells by Immunofluorescence. Acta Cytol., *9:* 73–82, 1965.
13. Kim, U., and Furth, J. Relation of Mammotropes to Mammary Tumors. IV. Development of Highly Hormone-Dependent Mammary Tumors. Proc. Soc. Exptl. Biol. Med., *105:* 490–492, 1960.
14. Kiseleva, N. S., and Magamadov, Y. C. Hematogenous Dissemination of Tumour Cells and Metastases Formation in Ehrlich Ascites Tumour. Neoplasma, *19:* 257–275, 1972.
15. Leading article. Circulating Cancer Cells. Lancet, *2:* 84–85, 1967.
16. Morley, T. P. The Recovery of Tumor Cells from Venous Blood Draining Cerebral Gliomas. Can. J. Surg., *2:* 363–365, 1959.
17. Potter, J. F., Longenbaugh, G., Chu, E., Dillon, J., Romsdahl, M., and Malmgren, R. A. The Relationship of Tumor Type and Resectability to the Incidence of Cancer Cells in Blood. Surg. Gynecol. Obstet., *110:* 734–738, 1960.
18. Potter, J. F., and Malmgren, R. A. A New Technique for the Detection of Tumor Cells in the Blood Stream and its Application to the Study of the Dissemination of Cancer. Surg. Forum, *9:* 580–583, 1958.
19. Roberts, S., Jonasson, O., Long, L., McGrew, E. A., McGrath, R., and Cole, W. H. Relationship of Cancer Cells in the Circulating Blood to Operation. Cancer, *15:* 232–240, 1962.
20. Romsdahl, M. M., McGrath, R. E., Hoppe, H., and McGrew, E. A. Experimental Model for the Study of Tumor Cells in the Blood. Acta Cytol., *9:* 141–145, 1965.
21. Simpson-Herren, L., and Griswold, D. P. Studies of the Cell Population Kinetics of Induced and Transplanted Mammary Adenocarcinoma in Rats. Cancer Res., *33:* 2415–2424, 1973.
22. Skoog, W. A., and Beck, W. C. Studies on the Fibrinogen, Dextran, and Phytohemagglutinin Methods of Isolating Leukocytes. Blood, *11:* 436–454, 1956.
23. Steel, G. G. Cell Loss from Experimental Tumors. Cell Tissue Kinet., *1:* 193–207, 1968.
24. Steel, G. G. The Cell Cycle in Tumors: An Examination of Data Gained by the Techniques of Labeled Mitoses. Cell Tissue Kinet., *5:* 87–100, 1972.
25. Stevenson, J. L., and von Haam, E. The Application of Immunofluorescence Techniques to the Cytodiagnosis of Cancer. Acta Cytol., *10:* 15–20, 1966.
26. Stevenson, T. D., and von Haam, E. A Study of Factors Affecting Circulating Tumor Cells in Experimental Animals. Acta Cytol., *10:* 383–386, 1966.
27. Webster, D. R., and Sabbadini, E. The Prognostic Significance of Circulating Tumour Cells: A 5-Year Follow-up Study of Patients with Cancer of the Breast. J. Can. Med. Assoc., *96:* 129–131, 1967.
28. Yip, D. K., and Auersperg, N. The Dye-Exclusion Test for Cell Viability: Persistence of Differential Staining following Fixation. In Vitro, *7:* 323–329, 1972.

Quantitation of Cell Shedding into Efferent Blood of Mammary Adenocarcinoma

Thomas P. Butler and Pietro M. Gullino

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/35/3/512

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/35/3/512. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.