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Introduction of Metastatic Heterogeneity by Short-Term In Vivo Passage of a Cloned Transformed Cell Line

James E. Talmadge, Jean R. Starkey, William C. Davis, and Arthur L. Cohen

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164 (J.E.T., J.R.S., W.C.D.), and Electron Microscope Center, Washington State University, Pullman, Washington 99164 (A.L.C.)

An experimental system for the study of metastasis has been developed using an epithelioid cell line of hepatic origin which had previously been chemically transformed in vitro. These metastatic cells were studied in the syngeneic rat strain. The cloned parent cell line metastasizes only to the lungs following intravenous, subcutaneous, or intraperitoneal injection. The metastatic phenotype is stable during in vitro passage, and subclones from the parent clone have a metastatic capacity statistically similar to that of the parent clone. Following ascites passage of the parent cell line, the cell population obtained exhibits the same metastatic ability as the parent clone. However, subclones obtained from the ascites-passaged population exhibit metastatic heterogeneity. This heterogeneity is introduced by the host passage and not by in vitro culture or subcloning. In the case of the two metastatic variants examined, the difference in the metastatic phenotype is found not to be due to differences in arrest or trapping of the cells but appears to be related to long-term survival and proliferation of the tumor cells following their arrest in the lungs. Morphologically the variants are very similar, and growth of the metastatic foci provokes a vigorous inflammatory response by the host.

Key words: cloned hepatic cell line, isolation of metastatic variants, metastatic heterogeneity introduced by ascites passage, metastatic homogeneity and stability, quantitative lung colony assay, scanning electron microscopy, tumor cell arrest and survival

One of the most important events in the pathogenesis of cancer is metastasis. During the development of a tumor it is the process of metastasis (growth of secondary foci at sites distant from the primary tumor) that most commonly defeats therapeutic efforts.

Within the population of cells in the primary tumor, there are subpopulations of cells with differing growth capabilities, degrees of tumorigenicity, and karyotypes [1]. This heterogeneous nature of a tumor cell population may also include differences in drug sensi-

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tivity [2], antigenicity [1, 3, 4], immunogenicity [5], and cytotoxic lymphocyte specificity [6]. The existence of metastatic heterogeneity within parent tumor cell populations was first demonstrated with the B-16 melanoma [7, 8] and, subsequently, in a UV-induced fibrosarcoma (UV-2237) [9] and a methylcholanthrene-induced fibrosarcoma [10].

Methods other than the cloning of variants from the parent cell population have been devised to obtain metastatic variants. These methods have included the selection of successive tumor lines varying in metastatic capabilities by altering cycles of in vivo–in vitro growth [11, 12], and the adaptation of a tumor to ascites growth in a syngeneic [13] or allogeneic host [14]. Variants which metastasize selectively to various organs [15] have been selected from the parent tumor cell population. Another technique which has been used to develop metastatic variants has been to direct in vitro selective pressures against a cell population. The selective pressures used have included selection of lectin-resistant variants [16], selection of variants with decreased adherence to immobilized lectins [17], and variants which are resistant to specific lymphocyte cytotoxicity [18].

In this report, we examine the metastatic diversity introduced by ascites passage of a cloned subline derived from a chemically transformed epithelioid cell line of hepatic origin.

MATERIALS AND METHODS

Cell Lines

IAR6-7, IAR6-1, and IAR6-1-RT7 cell lines were generously donated by Dr. R. Montesano (International Agency for Research on Cancer, Lyons, France). The cell line IAR6-7 is an epithelioid cell line which originated from a primary liver cell culture initiated from a BD-IV rat. IAR6-1 was derived from IAR6-7 following carcinogenic transformation with dimethylnitrosamine. The cell line IAR6-1-RT7 is an intraperitoneal passaged population of IAR6-1 cells. IAR6-1-RT7 has a shorter (3 weeks) average latent period than IAR6-1 (9 weeks). Both lines give rise to pulmonary metastases [19–21].

Animals

BD-IV (H-1^d) and BD-IX (H-1^d) [22] rats were bred in our animal facility by brother-sister mating. Pedigree stock of BD-IX rats were obtained from Dr. Mayo, Frederick Cancer Research Center. Pedigree BD-IV rats were kindly donated by Professor Rajewsky, Institute for Cell Biology, University of Essen, Germany, and by Dr. Montesano. Animals 6–12 weeks of age were used for these studies. The BD-IV and BD-IX strains are not syngeneic but do have the same major histocompatability loci, and each permanently accepts skin grafts from the other strain [22].

Culture Conditions

The cell lines were grown in Ham's nutrient mixture F-12 (Gibco) supplemented with 10% prescreened fetal bovine serum FBS (Gibco), penicillin (100 units/ml), and streptomycin (100 μg/ml). This was designated complete medium. Other media and buffers used were Dulbecco's modification of Eagle's minimum essential medium (Gibco) supplemented with 5% heat-inactivated (56°C for 30 min) FBS; Tyrode's balanced salt solution (TBSS); and Ca^{2+}, Mg^{2+}-free Tyrode's balanced salt solution (CMF). The cells were subcultured by gentle trypsinization (0.1% trypsin in CMF) for 6 min at 37°C followed by the addition of complete medium to inhibit further trypsin activity. The cells were grown in 100- × 20-mm tissue culture dishes or 75-cm^2 tissue culture flasks (T-75), both from Falcon. The cell lines

were subcultured every 7 days with a split ratio of 1:4. The cultures were incubated at 37°C in a humidified atmosphere of air and 7% CO_2.

Cloning Procedures

Cells were harvested by trypsinization, complete medium was added, and the cells were gently pelleted. The cells were resuspended in CMF, counted, and further diluted in CMF to 500 cells per milliliter. Aliquots of 1 ml were added to each of several 100- × 20-mm dishes, and 10 ml of complete medium was added to the cell suspension with agitation. After incubation for 7 days, the positions of several well-isolated colonies were marked on the underside of each dish, the medium was aspirated, cloning cylinders were placed over the colonies, and the cells were removed with trypsin. Each clone was recloned and reisolated a second time to insure the generation of clones of single cell origin.

Primary Cultures

Cultures of transformed cells were initiated from pulmonary nodules as follows: Lungs were aseptically removed from rats 3 weeks after IV injection of IAR6-1-RT7 cells. The metastatic nodules were located with a dissecting microscope and removed aseptically. The nodules from one rat were pooled, minced, and washed twice in complete medium, and small clumps of cells were allowed to attach to 100- × 20-mm tissue culture dishes. Previous experience with IAR6-1-RT7 had demonstrated a marked sensitivity of these cells to dislodgement from the substratum by trypsin treatment. A pure culture of putative tumor cells was obtained by selective detachment from the culture vessels with trypsin treatment for 6 min at room temperature. The resultant detached cells were washed in complete medium and then allowed to attach to tissue culture dishes for 30 min at 37°C, at which time the cultures were washed twice with complete medium and refed. This technique resulted in the growth of a monolayer of cells morphologically similar to cultures of IAR6-1-RT7 cells.

Cultures of intraperitoneally passaged tumor cells were initiated from tumor ascites fluid which was collected aseptically with a 5-ml syringe and an 18-guage needle. The cells obtained from the ascites fluid were washed three times in complete medium and then plated in 100- × 20-mm tissue cultures dishes. The medium and putative tumor cells were removed after 10 min incubation at 37°C and replated, leaving the majority of the peritoneal macrophages attached to the first culture vessel. The cultures of tumor cells were washed and fed every 24 h for the first 4 days of incubation.

Isolation of Metastatic Variants

Monodispersed IAR6-1-RT7 cells (10^5) obtained from cell cultures in the early log phase of growth were injected into the lateral tail vein of a syngeneic rat, and 3 weeks later metastatic pulmonary nodules were removed and established in primary culture. Several clones were isolated from this metastatic tumor cell population and these were recloned twice to ensure genetic homogeneity. Clone RT7-4b was chosen for use on the basis of its intermediate metastatic capabilities and was passaged three times by intraperitoneal injection in adult BDIX rats. The RT7-4b cells (2×10^7–5×10^7) were injected intraperitoneally and harvested after 5 days. The ascites-passaged cells were propagated in vitro for 2 weeks until sufficient numbers were obtained for a second ascites passage. After the third such passage the cells were somewhat adapted to ascites growth and presumed to have undergone some inductive/selective pressure(s). Clones were isolated from this partially ascites-adapted cell population in order to obtain metastatic variants.

Quantitative Lung Colony Assay

The transformed cells were harvested from subconfluent (60–80% confluence) cultures growing in T-75 flasks by rinsing the cultures once with CMF and then trypsinizing them for 6 min at 37°C. The flasks were then tapped sharply to dislodge the monolayer and complete medium was added. The cells were pelleted, washed with CMF, resuspended in CMF, and counted. This technique of cell harvest resulted in a suspension of single cells with greater than 95% viability (trypan blue exclusion). The suspension was diluted to 10^5 cells per 0.2 ml CMF, which was the inoculum injected into each rat. This cell number resulted in an easily countable number of pulmonary metastases for most cell lines examined in this study.

Rats between 6 and 12 weeks of age were used for the lung colony assay. Prior to injection, the rats were warmed at 37°C for 30 min. The tumor cell suspension was then injected in the lateral tail vein using a 27-guage needle and a 0.25-ml syringe. After 5 weeks the rats were killed and necropsied. Tissues with suspect metastases were rinsed in TBSS and fixed overnight in Bouin's fixative for gross and histologic examination. Superficial nodules were counted using a dissecting microscope. This experimental metastasis assay was repeated a minimum of two times with a minimum of five animals in each assay. A few animals died following the tumor cell injection, or at times more than two groups of animals were injected; therefore the total number of animals necropsied for each assay did not always total 10.

Quantitative Analysis of Tumor Cell Arrest and Survival

Tumor cells were prelabeled by the addition of 0.5 μCi ^{125}IUDR per milliliter of complete medium to subconfluent cultures of cells in T-75 flasks, and incubating for 24 h. This resulted in the incorporation of 1 cpm of ^{125}I per 5–7 cells. These labeled cells were harvested and prepared for inoculation as described above. Representative samples from each labeled cell line were counted in a Beckman 300 gamma counter. Rats were injected with 10^5 viable labeled cells into the lateral tail vein as described earlier. Five rats from each group were killed at intervals from 10 min to 24 h post injection. Lungs, liver, spleen, kidneys, and 0.5 ml of blood were collected from each rat and the organs placed in vials containing 70% ethanol. The ethanol was replaced daily for 5 days to remove ethanol-soluble ^{125}I. The remaining ethanol-insoluble radioactivity is known to be incorporated into the DNA of tumor cells which are viable at the time of organ removal [23]. Cultures of the cell lines RT7-4bA and RT7-4bE labeled in this manner with ^{125}IUDR were found to have greater than 95% viability by trypan blue exclusion, but in comparison to unlabeled control cells they had a somewhat lengthened doubling time in cell cultures.

Scanning Electron Microscopy

Cells to be examined were seeded in 35-mm tissue culture dishes (Falcon) containing two acid-washed 12-mm-diameter round coverglass slips. The cultures were then incubated with complete medium for 48 h. Following incubation the coverglass slips were quickly rinsed in physiologic saline, and the attached cells were fixed for 30 sec in 4% OsO_4 fumes, rinsed in physiologic saline, and further fixed for 1 h in 1.75% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.3). The cells attached to the coverslips were then rinsed three times in cacodylate buffer and postfixed for 1 h in 1% OsO_4. Following postfixation, the coverslips were rinsed in water and stacked in critical-point dryer containers. The coverslips were gradually dehydrated in ethanol to 100% and flushed with Freon T.F [24]. The dehydrated coverslips were dried in an Omar SPC 1500 critical-point dryer with liquid CO_2. After dry-

ing, the coverslips were attached to aluminum stubs, and a drop of conductive paint was placed on the edge of each coverslip and in contact with the stub, thereby greatly increasing conductivity between the coverslip and the stub after coating (Cohen, unpublished observation). The cells attached to the coverslips were coated with gold-palladium using a Technics hummer sputtering device and examined in an ETEC Autoscan SEM at 20 kV.

RESULTS

Development of the Parent Clone

This experimental tumor metastasis system was developed from the transformed hepatic cell line IAR6-1-RT7. We choose to use a cell population that would preferentially seed to the lungs to facilitate enumeration of the metastatic foci. Following intravenous injection and cell growth for 3 weeks, pulmonary metastatic nodules were excised and established in culture. Among the cell types found in these cultures were fibroblasts and macrophages, islands of ciliated bronchial epithelium, normal epithelioid cells of undetermined origin, and putative epithelioid tumor cells with a high nuclear-to-cytoplasmic ratio. The putative tumor cells (selected as described in Methods) were then seeded into tissue culture dishes at very low density, and well-isolated clones were later ring-isolated. The experimental metastatic capabilities of the isolated clones were tested in the lung colony assay, and one clone which had an intermediate metastatic ability, RT7-4, was chosen for further study. After recloning to ensure single-cell origin this clone was designated RT7-4b.

Experimental Metastatic Homogeneity and Stability of the Parent Clone

The parent clone RT7-4b was seeded at low density and well-isolated clones were removed and propagated. The 10 subclones chosen for further study, RT7-4b (1–10), were examined for their experimental metastatic ability by the intravenous injection of 10^5 cells of each clone into a minimum of two different sets of five rats on different days. The metastatic capabilities of these clones were compared with the metastatic capability of the parent clone (RT7-4b) (Table I). The Mann-Whittney U test was used to evaluate statistical significance. No differences in experimental metastatic abilities were demonstrated between the subclones and the parent clone. It was concluded that the cloned parental cell population was homogeneous with respect to its experimental metastatic capability in the lung colony assay, and this in vivo tumor phenotype was not perturbed by the cloning procedures used. The stability of the metastatic phenotype was evaluated by examining the behavior of the parent clone, RT7-4b, in the lung colony assay at various in vitro passages (Table II). The parent clone exhibited a stable metastatic phenotype over 23 tissue culture passages made at a 1:4 split ratio. This in vitro propagation directly followed its initiation as a clone and took place over a period of 9 months.

Development of Metastatic Heterogeneity During Ascites Growth

The parent clone RT7-4b was subjected to three ascites passages of five days each, separated by several days of in vitro propagation. The resulting cell population (thrice ascites-adapted cell line, 3XAA-RT7-4b) was seeded at clonal density, and clones were isolated and propagated. The experimental metastatic abilities of these variant clones were compared to that of the parent clone, RT7-4b, and the uncloned ascites-adapted population, 3XAA-RT7-4b (Table III). Statistically (Mann-Whitney U test) the uncloned ascites-adapted cell line and the original parent clonal cell line, RT7-4b, had the same metastatic capabilities as determined by the lung colony assay. However, a wide range of experimental meta-

static capabilities was found among the subclones from the ascites-adapted cell line, 3XAA-RT7-4b. The data presented in Table III are from lung colony counts made on the left lobe only. This was done because the very high number of pulmonary nodules which developed from several of the variant clones made counting the metastatic nodules present in the whole lung tedious. We had previously demonstrated that comparisons obtained from counts on the left lobe are proportional to those for the whole lung (Talmadge, unpublished results).

The parent clone, RT7-4b, produced 110 ± 53 metastatic nodules in the main pulmonary lobe per 10^5 cells injected. The metastatic capabilities of subclones ranged from an average lung colony count of 396 ± 176 metastatic nodules in the left lobe per 10^5 cells injected for RT7-4bE to an average lung colony count of 12 ± 11 metastatic nodules in the left lobe per 10^5 cells injected for RT7-4bA. RT7-4bL, which produced 205 ± 60 metastatic nodules in the left lobe per 10^5 cells injected, was an example of a clone with intermediate metastatic ability compared to RT7-4bA and RT7-4bE.

Of the 10 subclones examined, 3 were statistically indistinguishable from the parent clone, RT7-4b, in the lung colony assay. For example, RT7-4bM produced 89 ± 62 metastatic nodules in the left lobe per 10^5 cells injected. Thus 70% of the subclones were significantly different from the parent clone, RT7-4b, in the lung colony assay. We found that following multiple brief intraperitoneal passages of the cloned, metastatically homogeneous, parental cell population, the character of the tumor cell population was changed such that metastatic heterogeneity was present and metastatic variant subclones could be isolated.

Quantitative Analysis of Tumor Cell Arrest and Fate

To determine whether the differences in metastatic capabilities of the subclones isolated from the ascites-passaged population were due to differences in tumor cell arrest, trapping, and subsequent short-term survival, ^{125}IUDR-labeled tumor cells were traced in vivo following lateral tail vein injection. This was studied using the two clones which had the greatest difference in their metastatic capabilities: RT7-4bA and RT7-4bE (Table III). Tumor cell arrest, trapping, and short-term survival for these two variants is shown in Table IV. At 10 min post injection, no differences were found in the initial trapping rates of the tumor cells in the capillary beds of the various organs examined.

The highly metastatic variant subclone, RT7-4bE, had 74% of the originally injected cells viable and trapped in the lungs at 10 min post injection, while the poorly metastatic variant subclone, RT7-4bA, had 80% of the originally injected cells viable and trapped in the lungs. At 24 h following the injection of the cells, there was still no significant difference in trapping and survival between the two metastatic variant subclones in the lungs. From the originally injected highly metastatic RT7-4bE cells only 2.3% were still viable and trapped in the lungs compared to 2.0% of the poorly metastatic variant subclone RT7-4bA at 24 h. Therefore, there appeared to be no difference in the initial arrest, trapping, and short-term survival characteristics of the metastatic variant subclones examined. The difference in metastatic capabilities could therefore be attributed to a combination of the long-term survival of a small number of cells and their subsequent proliferation to form gross metastases.

Morphology of the Clonal Lines In Vitro

The morphology of the cells in vitro was studied using unfixed cultures by phase contrast microscopy; and methanol-fixed cells stained with hematoxylin. At the midlog phase of growth no difference in morphology could be discerned between the variant

TABLE I. Pulmonary Metastases Resulting From Intravenous Injection of Cells From the Hepatic Origin Tumor Cell Line RT7-4b and Subclones of RT7-4b

Hepatocyte cell line	No. of rats injected	Median No. of metastases	Mean No. of metastases ± SD[a]	Range	Significance[b]
Parent clone RT7-4b	24	318	370 ± 159	152–743	
Subclone 1	8	321	316 ± 52	253–381	NS
2	16	340	362 ± 166	100–437	NS
3	10	415	429 ± 91	315–609	NS
4	8	321	277 ± 202	79–467	NS
5	11	289	349 ± 165	124–635	NS
6	9	251	284 ± 109	120–573	NS
7	18	322	360 ± 116	185–472	NS
8	10	365	347 ± 139	62–507	NS
9	11	357	364 ± 123	257–460	NS
10	13	406	409 ± 123	266–616	NS

[a]Mean number of metastases per rat ± standard deviation obtained by averaging the results for a minimum of two different sets of five animals each.
[b]Probability of difference from pooled data for parent clone RT7-4b (Mann-Whitney U test). NS, no significant difference between parent clone RT7-4b and subclones. Probability more than 0.05.

TABLE II. Stability of the Metastatic Phenotype of the Hepatic Origin Tumor Cell Line RT7-4b, Following In Vitro Propagation

Cell line	No. of rats injected	No. of culture passages[a]	Median No. of metastases[b]	Range
RT7-4b	5	2	318	178–391
RT7-4b	5	9	329	172–362
RT7-4b	5	10	452	143–743
RT7-4b	5	11	288	182–407
RT7-4b	4	18	487	200–620
RT7-4b	5	23	351	211–407

[a]Number of cell culture passages following initiation of RT7-4b as a clone. The cell cultures were subcultured every 7 days at 1:4 ratio.
[b]Median number of pulmonary metastases per rat.

clones (Fig. 1). In general the cells exhibited a high nuclear-to-cytoplasmic ratio and an occasional multinucleated cell was noted. The nuclei usually contained 2–3 nucleoli and often a slight indentation was found on one side of the nucleus. The cytoplasm was slightly granular. Overall the metastatic variant cells presented an appearance of well-attached "pavement block" epithelioid cells, appearing much the same as the parent line, IAR6-1-RT7. Abnormal mitotic figures were not common.

Morphology of the Metastatic Lesion

The morphology of the pulmonary metastases was examined using periodic acid-Schiff (PAS) and hematoxylin-eosin-stained sections. The metastatic nodules (Figs. 2 and 3) were usually present at the periphery of the lung, although in the case of animals with large numbers of metastases, occasional nodules were present in deeper areas. There was a marked inflammatory response to the tumor which was manifested as mononuclear cell perivascular cuffing and mononuclear cell infiltration of the metastatic nodules. Foamy macrophages were often numerous within, and adjacent to, the metastatic nodules. Invari-

TABLE III. Pulmonary Metastases Resulting From Intravenous Injection of Subclones Isolated From the Hepatic Origin Tumor Cell Population 3XAA RT7-4b (obtained following triple adaptation of the parent clone to ascites culture)

Cell line	No. of rats injected	Median No. of metastases	Mean No. of metastases ± SD[a]	Range	Significance[b]
Parent clone RT7-4b	24	98	110 ± 53	46–227	
3XAA RT7-4b	12	104	105 ± 31	57–163	NS
3XAA RT7-4b-A	10	8	12 ± 11	0–29	>0.0001
B	9	176	217 ± 111	151–361	0.0143
E	9	406	396 ± 176	195–694	>0.0001
F	9	195	205 ± 121	49–454	NS
G	12	212	192 ± 72	64–290	0.0015
K	10	192	221 ± 93	118–337	0.0003
L	10	204	205 ± 60	115–286	0.0002
M	9	61	89 ± 62	23–203	NS
N	8	139	129 ± 39	73–176	NS
O	8	210	209 ± 55	130–297	0.0008

[a]Mean number of pulmonary metastases per rat ± standard deviation (left lobe only) obtained by averaging the results from at least two different sets of five animals each.
[b]Probability of difference from pooled data for parent clone RT7-4b (Mann-Whitney U test) P values. NS, no significant difference between parent clone RT7-4b and subclones. Probability more than 0.05.

ably, the metastatic nodules were associated with the microvasculature of the lung, either capillaries or venules. The anaplastic tumor cells were often present within remnants of the pulmonary stroma giving a histologic impression of glandular structure. This stromal outline was observed best with PAS staining, which we have found to be optimal for scanning slides. The majority of the metastatic clones gave rise to morphologically identical lesions to those described above, differing only in the frequency of metastatic nodules observed. The least metastatic clone, RT7-4bA (Fig. 3), differed slightly; histologic lesions were very rare and small, and were not associated with mononuclear cell perivascular cuffing, although there was a mononuclear and foamy macrophage infiltrate present within the metastatic nodules. The tumor cells, in all the histologic sections examined, had indented nuclei, exhibited multiple nucleoli, and were occasionally multinucleated. They had the same general morphology in vivo (Fig. 4) as they did in tissue culture (Fig. 1). Mitotic figures were not frequently seen and abnormal figures were rare in vivo. Margination of the chromatin was common in these cells, although it was not marked. A few PAS-positive granules were present in the cytoplasm. Cells from the different variant subclones could not be distinguished from one another histologically. Morphologically, the metastatic lesions produced by the different variant clones were remarkably similar.

Scanning Electron Microscopy

Scanning electron microscopy was used to examine the surface morphology of the cloned cell lines in culture. Comparisons were made of cells in areas of comparable densities. Cultures of RT7-4b, RT7-4bA, RT7-4bE, RT7-4bL, and RT7-4bM cells were examined. Mycoplasma were not seen by scanning electron microscopy in any of these cultures.

The poorly metastatic subclone, RT7-4bA (Figs. 5 and 6), had the most distinctive surface morphology. Occasionally a large bleb was seen on the surface of a cell while mitotic cells had numerous blebs (Fig. 5). At higher magnification numerous microvilli and filamentous strands were present (Fig. 6). The majority of the cellular margins were free of

TABLE IV. Arrest, Distribution, and Survival of 100,000 Viable Tumor Cells Injected IV Into Normal BD-IX Rats

Time after tumor cell injection	Lung	Spleen	Liver	Kidney	Blood[a]
RT7-4bA					
10 minutes	80,200 ± 10,500	000 ± 00	2,000 ± 706	173 ± 52	434 ± 116
1 hour	38,400 ± 4,100	153 ± 43	827 ± 717	48 ± 44	266 ± 79
2 hours	16,900 ± 4,800	89 ± 29	726 ± 348	36 ± 8	288 ± 63
4 hours	14,800 ± 3,800	32 ± 7	674 ± 217	48 ± 14	209 ± 60
6 hours	11,400 ± 3,000	24 ± 2	621 ± 106	69 ± 10	198 ± 24
24 hours	2,000 ± 400	20 ± 3	117 ± 97	65 ± 10	82 ± 85
RT7-4bE					
10 minutes	73,900 ± 9,800	161 ± 18	389 ± 361	212 ± 60	240 ± 66
1 hour	37,100 ± 2,500	576 ± 88	1,550 ± 915	110 ± 32	624 ± 152
2 hours	14,300 ± 5,500	153 ± 15	1,212 ± 680	000 ± 00	488 ± 153
4 hours	12,600 ± 6,000	147 ± 10	1,280 ± 227	000 ± 00	488 ± 116
6 hours	8,500 ± 2,600	160 ± 27	971 ± 134	000 ± 00	324 ± 87
24 hours	2,300 ± 800	153 ± 20	176 ± 67	000 ± 00	121 ± 217

Data are average number of originally viable injected tumor cells still present ± SD. Five rats per time interval.
[a]0.5 ml of blood per rat.

contact with other cells. The marginal contacts with other cells were overlapping. Ruffling occurred only at marginal cell-cell contact and was slight. Clefts were found between the individual cells. The majority of these clefts had cellular pseudopodia or filamentous strands extending across or into the clefts, suggesting that they were not artifacts. However, in some cases, rifts, an artifact of preparation, were seen as a "jigsaw puzzle" appearance of the separated cells.

The highly metastatic subclone, RT7-4bE (Figs. 7 and 8), exhibited several morphologic differences from the poorly metastatic subclone, RT7-4bA. The RT7-4bE cells had only a few stubby microvilli. Slender strands were found extending across adjacent cells. Blebs were not seen on these flat, well-spread cells. The mitotic cells exhibited only filopodia and ruffled membranes (Fig. 7). Extensive over-underlapping was found at the cellular margins and marginal ruffles were common at regions of cellular contact (Fig. 8).

The other subclones, as exemplified by RT7-4b (Fig. 9), had surface structures intermediate between the two types previously described for RT7-4bA and RT7-4bE. Blebs were occasionally seen in addition to marginal and internal ruffles. Microvilli were numerous and filamentous strands were found. The subclones RT7-4bL and RT7-4bM appeared by scanning electron microscopy to be very similar to RT7-4b. In addition, RT7-4bM had numerous small pits on the surface of each cell.

DISCUSSION

We have developed a cloned cell line of hepatic origin which exhibited a stable metastatic phenotype during in vitro culture. Ten subclones obtained from the parent clone all exhibited the same metastatic phenotype as the parent clone. However, when the cloned parent cell line was grown as an ascites tumor, heterogeneity appeared such that subclones could be isolated with marked differences in their individual ability to metastasize. The lack of metastatic variation found between subclones from the parent tumor line showed that the

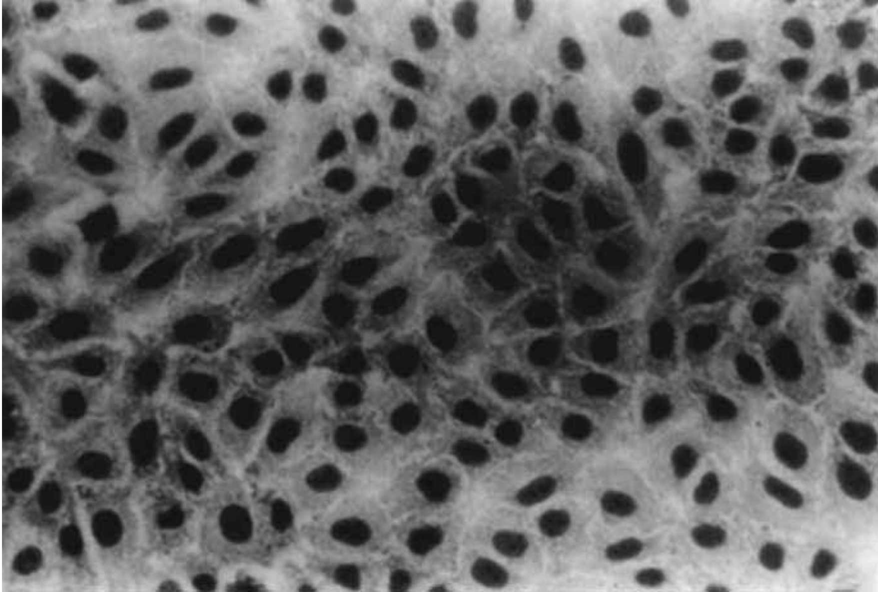

Fig. 1. Micrograph of the RT7-4b cell line. The cells are in log phase of growth. Multiple nucleoli are seen in the slightly indented nuclei. The cells present a "pavement block" pattern of epithelial cells. Hematoxylin; X 1,300.

parent line was homogeneous in this respect and that the high degree of heterogeneity demonstrable by clonal analysis of the ascites-passaged cell population must have resulted from in vivo factors associated with ascites growth, and was not due to subcloning. It is possible that other forms of in vivo passage may also act to introduce similar heterogeneity.

The metastatic capabilities of clones obtained from tumor cell populations has been studied by other workers using several different tumor cell lines. When B-16 melanoma cultures were cloned, the starting population was found to exhibit a heterogeneous metastatic phenotype in either the lung colony assay or following subcutaneous growth [7, 8]. However, subclones obtained from two of these clones exhibited the same metastatic phenotype as the parent clone [7]. Similar results were obtained with a UV-induced fibrosarcoma [8,9] which had been passaged once in a nude mouse and which had limited in vitro passage [25]. As was demonstrated in the B-16 cell system, subclones isolated from one of the fibrosarcoma clones had the same metastatic capability as the parent clone. In both of these tumors the starting tumor populations had previously undergone sufficient cellular proliferation to allow for the induction of genetic and epigenetic variation even without any additional selective pressures.

We introduced metastatic variance into the previously homogeneous tumor cell population, RT7-4b, by partially adapting it to ascites growth. The adaption to ascites growth was achieved by 15 days of intraperitoneal growth, during which time mutational or epigenetic events could have occurred. However, owing to the limited period of ascites growth and the initial poor survival of the tumor cells in this environment, it is likely that restricted numbers of cell divisions occurred in vivo and only a few mutational events would have resulted. Thus, the considerable introduced variance observed is more likely to be of epigenetic origin.

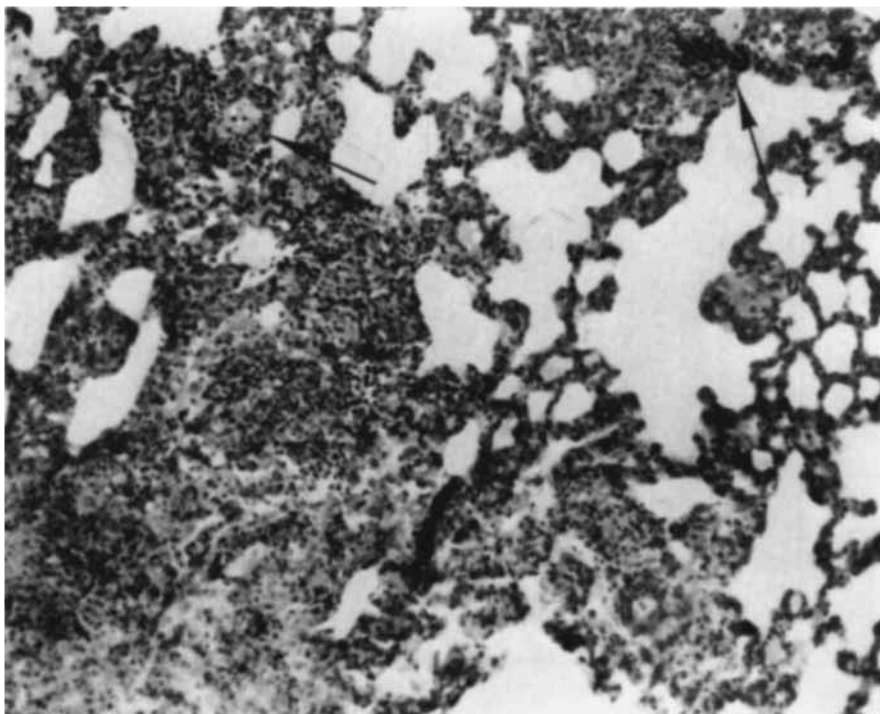

Fig. 2. Histologic appearance of a rat lung 5 weeks after the injection of 100,000 RT7-4b cells into the lateral tail vein. Mononuclear perivascular cuffing (arrow) foci are seen in several areas. The main metastatic nodule, seen here as a dense cellular area, contains tumor cells and a mononuclear infiltrate. Periodic acid-Schiff × 200.

Ascites growth adaptation of cells in syngeneic [13] or allogeneic [14] hosts has been reported to increase the metastatic capabilities of tumor cell populations. However, we found that the ascites-passaged 3XAA-RT7-4b cell population gave, statistically, the same number of lung nodules, as the original non-ascites-passaged parent clone. This may be explained in this system by ascites growth being continued for only 15 days. This was probably insufficient time to have achieved a fully ascites-adapted subpopulation.

Subclones isolated from the ascites-passaged population could be expected to be either homogeneous in their metastatic capabilities, or heterogeneous in their metastatic capabilities as was, in fact, observed. The ascites-passaged cell population was heterogeneous in its metastatic capability, and it might, a priori, be expected that randomly isolated subclones would contain approximately equal numbers of subclones with metastitic capabilities above and below that noted for the ascites-adapted cell population in general. This, however, was not the case. Only one clone tested had a decreased metastatic capability compared to the general population. This would suggest that some selection might have been imposed on the ascites-passaged cells during the cloning process. Perhaps the poorly metastatic cells were less able to survive and grow from single cells in culture than the more metastatic cell lines, thus skewing the resultant isolated clonotypes. Other possibilities are cell interactions that may occur when the heterogeneous ascites-passaged cell population is used in a lung colony

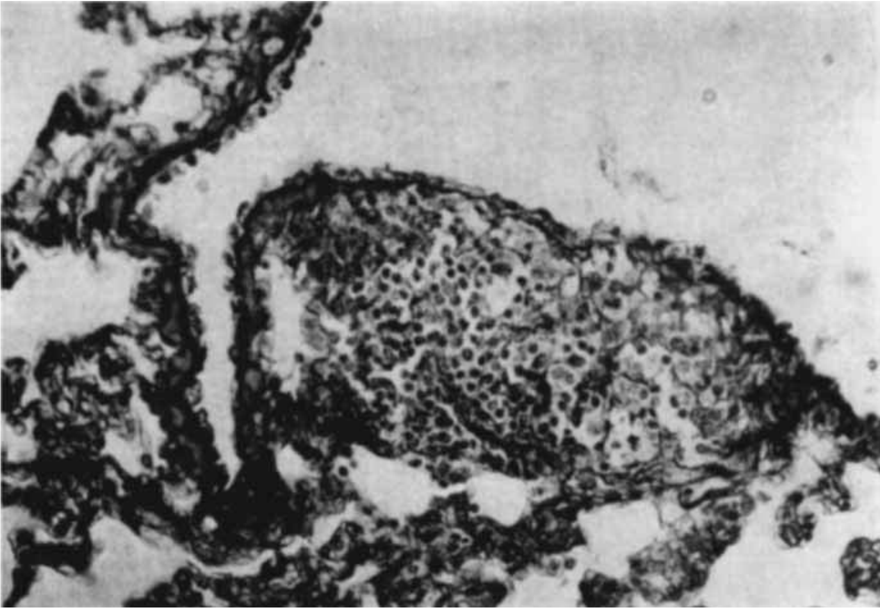

Fig. 3. Pulmonary metastasis from a rat 5 weeks after the intravenous injection of 100,000 RT7-4bA (poorly metastatic subclone) cells. The nodule is found on the periphery of the lung and there is associated with the nodule an infiltrate of inflammatory cells and foamy macrophages. Periodic acid-Schiff × 330.

assay. Such interactions could be postulated to decrease the metastatic expression of the highly metastatic subpopulations.

Adaptation to ascites growth of a tumor cell population appears to be due to the selection of a small (2.5×10^{-6}) subpopulation of phenotypically stable variants which possess a selective growth advantage as an ascites tumor but not as a solid tumor [26]. Adaptation of a tumor population to ascites form occurs at a rate dependent on the number of cells injected and any other enrichment for the ascites phenotype. The role of the ascites environment is selective, not inductive or adaptive, and it enriches for those cells with increased survival capacities in the peritoneal fluid [26].

One of the secondary attributes of tumor cells which can survive in the ascites fluid is an increased ability to metastastize [26]. Because of the short time in the peritoneal cavity and the limited number of serial transfers, the adaptation to ascites growth in our system was not complete nor was the entire cell population converted to being highly metastatic. Thus it appears that the ascites adaptation probably selected those rare cells from the original clonal cell population which had developed some diversity, either genetic or epigenetic, during proliferation.

Morphologically, cells from the subclones and the parent clone appeared quite similar in vitro and in vivo in histologic sections of pulmonary metastases. The vigorous mononuclear inflammatory response observed in the histologic sections is indicative of an immunologic reaction to the cells. This immunologic response was found regardless of the route of injection of the tumor cells (intravenous or intraperitoneal), sex of the animal, or strain of rat injected – BDIV or BDIX (both $H\text{-}1^d$ histocompatability type).

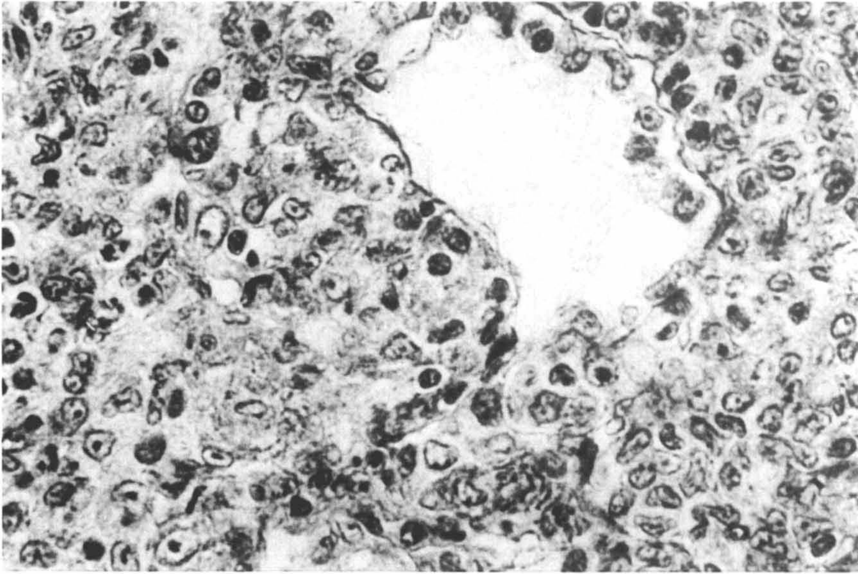

Fig. 4. Photomicrograph of tumor cells from a metastatic nodule in the lung of a rat 5 weeks following the injection of 100,000 RT7-4b cells intravenously. The anaplastic epithelioid cells exhibit slightly indented nuclei and multiple nucleoli and show some chromatin margination. Periodic acid-Schiff × 2,000.

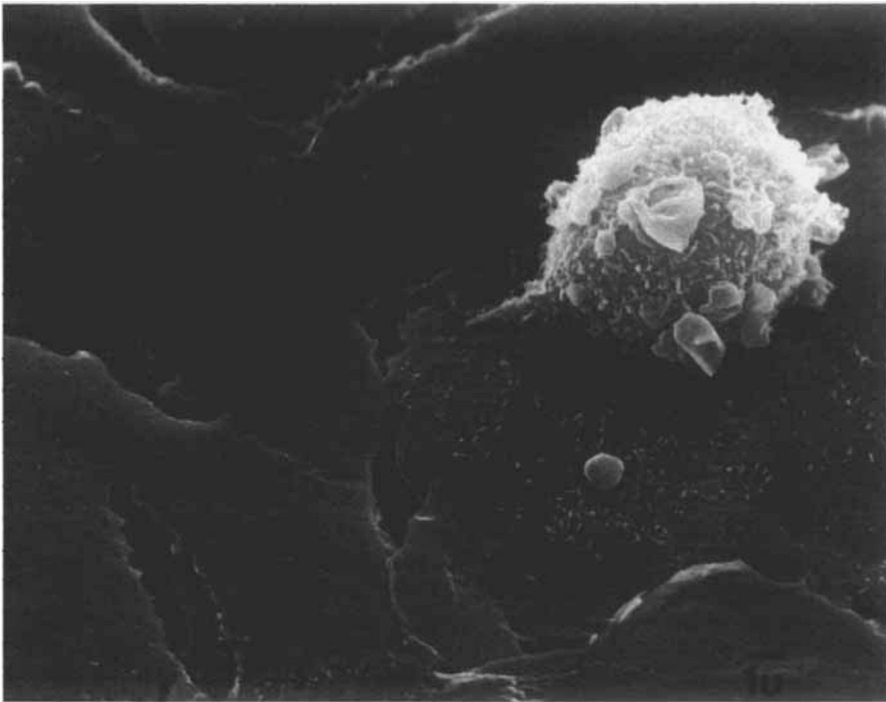

Fig. 5. Scanning electron micrograph of RT7-4bA cells; a poorly metastatic cell line fixed in situ. Note the numerous microvilli and blebs on the mitotic cell. Tilted 45°.

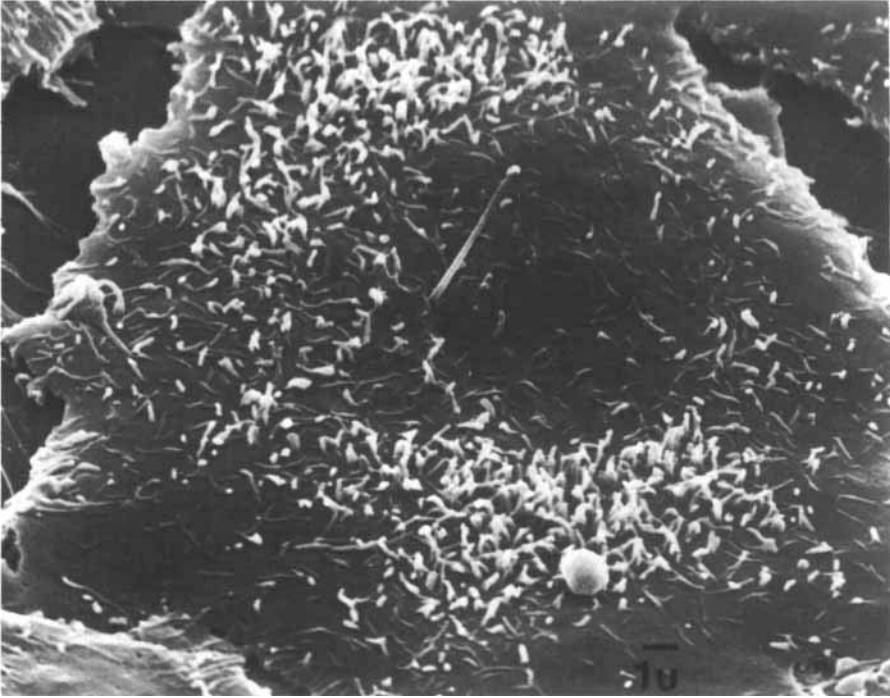

Fig. 6. Scanning electron micrograph of RT7-4bA, a poorly metastatic cell line, fixed in situ. Note the bleb, numerous microvilli, and the short filamentous strands. The cellular margins form spaces between the cells which are characteristic of this subclone. Tilted 45°.

There was one exception to the general histologic picture. Subclone RT7-4bA did not provoke the mononuclear perivascular cuffing found with the other subclones, possibly owing to limited antigenic stimulation following the restricted cell survival and growth. Since, in general, the variants appeared to provoke an active immunologic response as exemplified by the mononuclear and foamy macrophage infiltrate and the mononuclear perivascular cuffing, it is likely that the more metastatic cells are better able to "escape" immune effector cells and so survive to produce gross metastatic lesions. Since cells were well washed in Ca^{2+}-Mg^{2+}-free BSS, an immune reaction to medium components (FBS) is highly unlikely.

Scanning electron micrographs revealed a continuum of membrane morphologic variance which correlated with the subclones' metastatic abilities. The least metastatic subclones exhibited numerous membrane-associated structures including blebbing and extensive microvillus formation, which were indicative of an "active" cell membrane. The most metastatic subclone (RT7-4bE) exhibited less extensive membrane activity, which appeared as only a few stubby microvilli and marginal ruffling. Similar morphologic structures were seen in studies by other workers in which two different solid tumors and their respective ascites-adapted counterparts were examined using scanning electron microscopy [27]. The ascites-adapted cell lines had decreased membrane activity (blebbing, ruffling, and microvilli) compared to the solid tumor cells. Previously it had been reported that the ascites-adapted cells examined by these workers were more metastatic than the solid tumors [13].

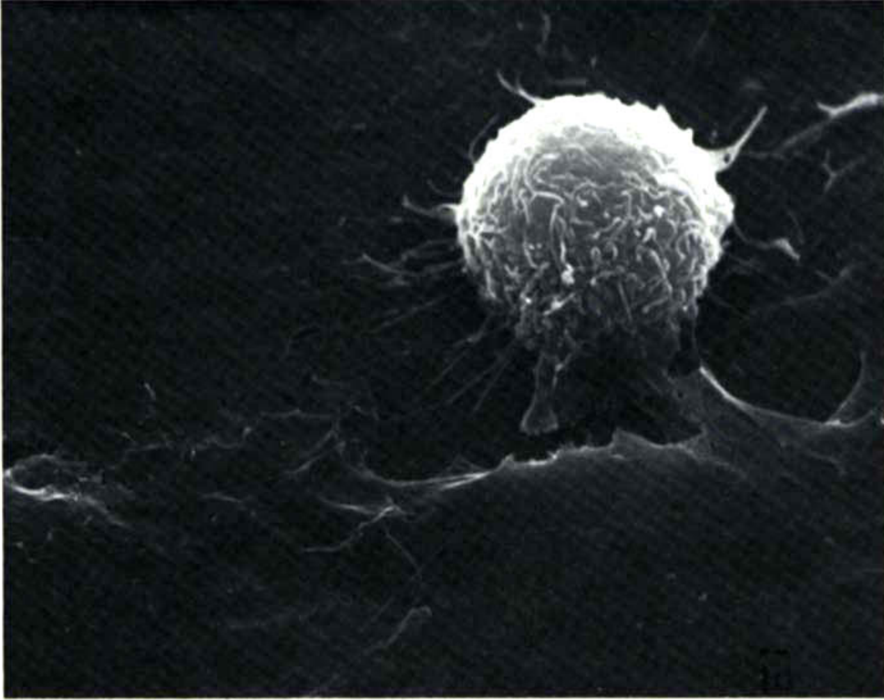

Fig. 7. Scanning electron micrograph of RT7-4bE, a highly metastatic subclone, fixed in situ. Mitotic figures lack blebs and do not exhibit extensive ruffles. The cells appear flat and very adhesive to the substratum. Few microvilli are seen and extensive over-underlapping is found with marginal ruffling. Tilted 45°.

Scanning electron micrographs of the metastatic variant subclones had an appearance suggestive of a tighter adherance to the substratum by the more metastatic subclone (RT7-4bE) as well as increased intercellular contact. The postulated increase in homotypic and substratum attachment suggests that these cells may have greater adhesiveness, which could serve to increase the metastatic capabilities of the tumor by increasing embolus arrest and trapping [28]. However, our studies of the arrest and trapping of ^{125}IUDR-labeled cells show no significant increases in those parameters for RT7-4bE cells over RT7-4bA cells.

Cells labeled with ^{125}IUDR allowed not only the study of tumor cell arrest and trapping, but also short-term tumor cell survival to be evaluated. Using such labeled cells, we studied the mechanism of metastasis of the two subclones (RT7-4bA and RT7-4bE) which exhibited the greatest difference in metastatic abilities. Despite the great difference between these variants in the ultimate yield of lung metastases, there was no discernable difference found in the rate of arrest, the number of cells arrested, or cell survival for 24 h following intravenous injection of the ^{125}IUDR-labeled tumor cells. This suggests that the final metastatic expression was not due to differences in the arrest or trapping of the tumor cells, but to long-term tumor cell survival subsequent to arrest and trapping. Such enhanced survival is postulated to be of particular importance in allowing cell growth to occur and gross metastases to be produced.

Fig. 8. Scanning electron micrograph of RT7-4bE, a highly metastatic subclone, fixed in situ. Only a few stubby microvilli are found. Extensive over-underlapping is seen with marginal ruffling. Tilted 45°.

Following ascites passage, metastatic diversity could be demonstrated to exist in a cell population which prior to ascites growth had a stable homogeneous metastatic phenotype. The expression of the variant metastatic capabilities does not appear to be due to differences in the arrest and trapping of the tumor cells, but rather to a difference in the subclone's ability to survive to form a gross metastatic nodule. This could to a large extent be due to an ability of the highly metastatic subclone to "escape" the hosts immunologic response [29].

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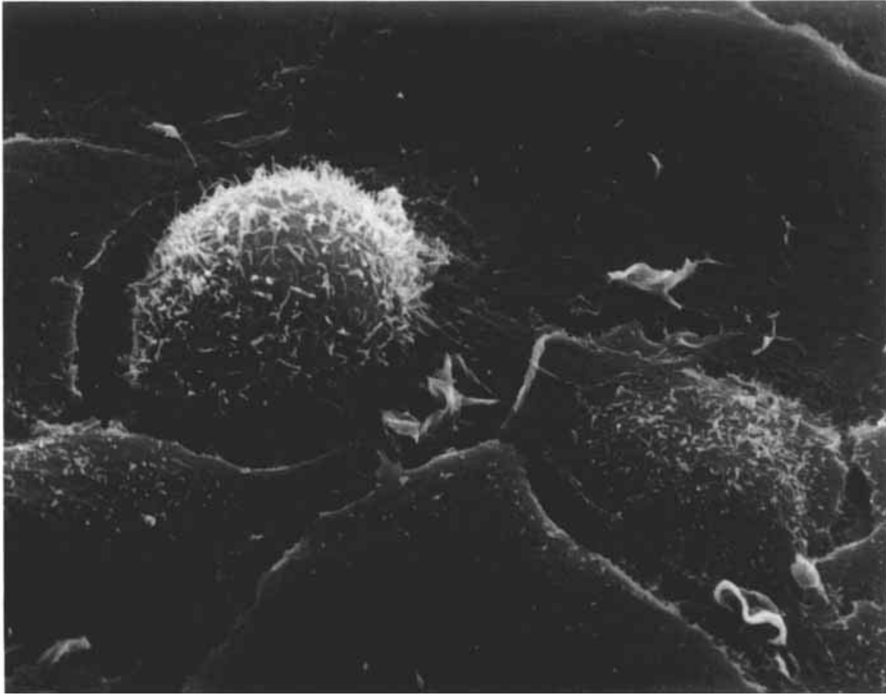

Fig. 9. Scanning electron micrograph of RT7-4b, metastatic parent clone, fixed in situ. Numerous microvilli are found with occasional blebs and internal and marginal ruffles. The prominent surface characteristic of the mitotic cells are microvilli. Spaces are found between the cellular margins. Tilted 45°.

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