Assignment of Methylene Proton Resonances in NMR Spectra of Embryonic and Transformed Cells to Plasma Membrane Triglyceride*

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Some biological characteristics of cancer cells and solid tumors are identifiable by the high resolution NMR relaxation behavior of their nonaqueous components. Chemical analysis and two-dimensional scalar correlated (COSY) NMR spectroscopy show these resonances arise from neutral lipid in the plasma membrane. Triglyceride is shown to be the main plasma membrane component giving rise to the NMR spectrum, while soluble nonmembrane components account for 90% of the remaining resonances in the spectrum of intact cells. The presence of triglyceride has been detected by chemical analysis in highly purified plasma membranes from two different cell lines. The COSY spectra of cancer cells are comparable with that obtained for the triglyceride-rich very low density human lipoprotein.

The high resolution 1H NMR spectrum of a suspension of cancer cells or an excised solid tumor is characteristic of lipids (Mountford et al., 1980, 1982b). Lorentzian-Gaussian resolution enhancement of the lipid methyl proton resonances near 1.2 ppm resolves four peaks, at 1.22, 1.23, 1.25, and 1.28 ppm, each of which can be examined separately in an NMR relaxation experiment. In transverse relaxation measurements, each of these shows several relaxation times, \( T_2 \). We recently reported that the resonance at 1.25 ppm, which shows a multiphasic plot, has a longest \( T_2 \) (denoted \( T_{2h} \)) of the order of 400–800 ms when the cells were known to have the capacity to generate secondary growths in other parts of the body (metastasize). In contrast, cells which remained as malignant but nonmetastatic primaries gave a \( T_{2h} \) of less than 200 ms for the same resonance (Mountford et al., 1984a, 1984b). Furthermore, the behavior of these four methylene resonances is indicative of the exposure of the cancer cells to vinca alkaloid and other related anticancer drugs. Identification of the chemical species which give rise to the NMR spectrum, and hence provide an indication of the biological capabilities of the cells, is therefore important.

We have established previously that these lipids are in or attached to the plasma membrane, tumble isotropically, and are in domains which are not in diffusive exchange with other lipids in the plasma membrane bilayer (Mountford et al., 1984a, 1984b).

Chemical analysis of a crude plasma membrane preparation (enriched in plasma membrane markers 4-fold) from the rat mammary adenocarcinoma cell line 13762 and J clone showed a significant quantity of triglyceride present in both cell lines, with the metastatic line 13762 having significantly more cholesterol ester. Other rapidly dividing cells have been shown to give a similar NMR spectrum (Block, 1973; Nicolau et al., 1975, 1978; Mountford et al., 1982b) and we have now extended the analytical studies to verify the presence of triglycerides in the crude plasma membrane preparations of all the rapidly dividing cells which we have investigated that give the NMR spectrum. In addition a highly purified plasma membrane, which is enriched 45-fold in plasma membrane markers, has been analyzed to ensure that the presence of triglyceride is not due to a contaminant.

The one-dimensional 1H NMR spectra of cancer cells have narrow lines (≤10 Hz). However, assignment of the resonances to specific protons is difficult from one-dimensional spectra. In contrast, two-dimensional scalar correlated spectroscopy (COSY) provides a great deal of information not available from the one-dimensional spectrum (Wagner et al., 1981; Arseniev et al., 1982) by generating spectra which allow spin system assignments based on spin-spin (scalar) coupling. Using a modified pulse sequence (Cross et al., 1984) we have found that suspensions of intact viable cancer cells may be studied by two-dimensional NMR methods. Two-dimensional scalar correlated spectroscopy has verified that the NMR spectrum arises predominantly from triglyceride in the plasma membranes and from soluble non-lipid components.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture

Human peripheral blood lymphocytes, stimulated by pokeweed mitogen, were supplied and grown as previously described (Mountford et al., 1982b). The preparation and cultivating of chicken embryo fibroblasts followed reported procedures (Mountford et al., 1982a). VBL20 is the acute lymphoblastic leukemic T cell line (CCRF-CEM) which has been made resistant to 30 ng/ml of the anti-cancer drug vinblastine. The cultured rat mammary adenocarcinoma metastatic cell line 13762 and the nonmetastatic clone, J clone, were supplied by Dr. I. Ramshaw, Australian National University and grown as previously described (Ramshaw et al., 1982). All cells were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were maintained and studied in the logarithmic phase of growth. The doubling time was 24–36 h.

PREPARATION OF SAMPLES FOR NMR SPECTROSCOPY

Cell samples were prepared for NMR experiments as previously...
described (Mountford et al., 1982a, 1984a). The samples were kept at 37 °C in the NMR tube for 1 h prior to commencement of the experiments to allow establishment of a stable state following increase in cell-cell contact. Cell viability was measured before and after each experiment. Only those data obtained from cells with at least 90% viability were used. The longest time for which this could be achieved was 4 h.

Triolein (Sigma) was dissolved (20 mg/ml) in CDCl₃ (99.96% isotopic purity, supplied by Wilmad Glass Co.) and the solution was deagitated while freeze-thawing four times with liquid nitrogen.

Very low density lipoprotein (VLDL) from healthy human donors was isolated by previously reported methods (Hatch and Lees, 1968). The particle size was measured by electron microscopy to be 27–32 nm, with a total variation of 22–54 and 31–78 nm on two experiments, which is within the normal range for human VLDL. The preparations were negatively stained with 1% ammonium molybdate solution and the electron micrographs were obtained with a Philips 400 EM operating at 100 keV and a working magnification ×92,800.

NMR Spectroscopy

1H NMR spectra were recorded at 37 °C (cells, supernatant, and VLDL) or 25 °C (triolaen) using a Bruker WM 400 spectrometer. Peaks were referenced to aqueous sodium 3-(trimethylsilyl)propanesulfonate or to the residual CHCl₃ resonance at 7.28 ppm as an external chemical shift reference. The Meiboom-Gill modification of the Carr Purcell pulse sequence (CPMG) (Meiboom and Gill, 1958) was used for spin-spin relaxation measurements as described (Mountford et al., 1984a) and the COSY spectra were recorded with a pulse sequence modified to compensate for radiofrequency inhomogeneity (Cross et al., 1984).

Membrane and Supernatant Preparations

Supernatant from VBL20 Cells—Washed cells (2.0 × 10⁹) were lysed for 20 min at 4 °C in 2 ml of D₂O. The homogenate was centrifuged at 130,000 × g for 60 min and the supernatant fraction was removed.

Membrane Isolation—The preparation of crude plasma membranes was accomplished as reported (Mountford et al., 1984b) with cell disruption by nitrogen cavitation at 800 p.s.i. for 15 min. The purification of the plasma membrane similarly involved cell disruption by nitrogen cavitation at 800 p.s.i. (15 min) of cells (4.5 × 10⁹) washed three times in saline. The disruption was effected in a Parr pressure bomb with the cells suspended in 4 volumes of buffer consisting of 100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10% (w/v) sucrose, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was centrifuged at 1000 × g for 5 min and the pellet was disrupted by nitrogen cavitation at 800 p.s.i. for 15 min. The combined lysate (total homogenate) were again centrifuged at 100,000 × g for 5 min. This pellet of unbroken cells and nuclei was discarded. The membranes were then sedimented by centrifugation of the 1000 × g supernatant at 140,000 × g for 1 h (4 °C) and resuspended in phosphate-buffered saline, pH 7.4. The suspension was further fractionated according to the method of Finck et al., 1984. The membrane bands from the sucrose density gradients were removed by aspiration and diluted 1:4 with 10 mM HEPES, pH 7.2. All marker enzyme activities except 5’ nucleotidase and NADPH cytochrome c reductase were assayed on membranes which had been frozen overnight at −70 °C.

Marker Enzymes

The purity of the plasma membrane preparation was assessed by calculating the specific activity of the marker in the membrane and dividing by that in the homogenate. Protein content was measured with the Bio-Rad protein assay kit using bovine serum albumin as a standard, The following marker enzymes were assayed according to methods which have already been described: 5’ nucleotidase (Newby et al., 1975), Na⁺, K⁺ adenosinetriphosphatase (Ames, 1965), and γ-glutamyltranspeptidase (Jacobs, 1971) as plasma membrane markers; acid phosphatase (Ames, 1965) as the marker for lysosomes; lactate dehydrogenase (Boehringer Mannheim GmbH) as a cytoplasmic marker; NADPH-cytochrome c reductase (Sottocasa et al., 1967) as the marker for endoplasmic reticulum; and cytochrome c oxidase (Wharton and Tzagoloff, 1967) as the mitochondrial marker. DNA was used as the nuclear marker (Brunk et al., 1979).

RESULTS AND DISCUSSION

The 400-MHz COSY spectrum of triolein in CDCl₃ is compared with that obtained from a suspension of cells in Fig. 1, A and B. The off diagonal cross-peaks, labeled A–G, indicate spin-spin coupling between protons on adjacent carbon atoms. The connectivities corresponding to each acyl chain cross-peak (A–F), summarized in Structures 1 (phospholipid) and 2 (triacylglycerol), can be seen in the spectrum of the leukemic T cell line VBL20 (Fig. 1B) confirming the assignment of lipid acyl chain resonances in the spectrum of cancer cell plasma membranes.

Two-dimensional NMR also identifies the lipid acyl chains as components of triglyceride. Triglyceride possesses a unique cross-peak G' at 4.3 ppm resulting from the geminal protons of carbons 1 and 3 of the glycerol backbone (Structure 2 and Fig. 1A). This resonance is 0.1 ppm downfield from the corresponding glycerol resonance (Structure 1) in the phosphatidylcholine spectrum. Furthermore, no cross-peaks from a glycerol methylene adjacent to a phosphatidylcholine group, H, (Cross et al., 1984) are in evidence in the cell spectrum, nor indeed is the cross-peak d due to the choline head group. The cross-peak D is not present in Fig. 1A as triolein has only one olefinic group in each acyl chain. However, the absence of C in the cell spectrum (Fig. 1B) is unexpected and while this cannot be due to the complete absence of carbon-carbon double bonds (as evidenced by the presence of D) it may suggest a low level of olefinic groups or that this cross-peak is not observed for dynamic reasons. The cross-peak G has never been observed for a cell system, possibly because of restricted motion of the glycerol backbone. Restricted motion may also explain the absence of cross-peaks from phospholipid headgroups and backbone.

In order to determine which cross-peaks in the cellspectrum originate from nonmembrane components such as metabolites, we lysed the cells. After the membranes and nuclei are spun down, the supernatant, which contains only soluble components, accounts for all of the remaining cross-peaks not generated by the triglyceride molecule in this cell line except that connecting resonances at 1.7 and 3.0 ppm (Fig. 1C). This latter cross-peak has been tentatively assigned to a resonance arising from the oligosaccharide head group of gangliosides, which display the same cross-peak.

The amounts of free cholesterol and cholesterol ester were variable in the crude plasma membrane preparations (Table 1). A cross-peak connecting resonances at 0.9 and 1.4 ppm from the methyl and methine protons of the alkyl side chain of the cholesterol ring system, denoted Z in Fig. 2B, can be observed in the spectra of many cancer cells. This suggests the presence of cholesterol and/or cholesterol ester in the neutral lipid domain of the rat mammary adenocarcinoma cell line 13762 (Fig. 2B). In contrast, the VBL20 cell line (Fig. 2B).
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**A.**

![Symmetrized COSY spectra with cross-peaks](image)

**B.**

![Symmetrized COSY spectra with cross-peaks](image)

**C.**

![Symmetrized COSY spectra with cross-peaks](image)

**FIG. 1.** Symmetrized COSY spectra. A, triolein in CDCl₃ solution (20 mg/ml). Lipid acyl chain and glycerol backbone connectivities are indicated. The assignments of the cross-peaks are shown in Structure 2 which is described in the text. A Sine-bell window function was applied in both the t₁ and t₂ domains. B, a suspension of VBL20 cells (1 x 10⁶ cells) in phosphate-buffered saline in D₂O. Spectra were obtained at 37 °C with the sample spinning and suppression of the residual HOD peak by gated irradiation. Sine-bell and Gaussian (line broadening = 0.22) window functions were applied in the t₁ and t₂ domains, respectively (Cross et al., 1984). Lipid acyl chain and glycerol backbone connectivities are indicated. (VBL20 is the acute lymphoblastic leukemia T cell line CCRF-CEM which has been made resistant to 20 ng/ml of vinblastine). C, supernatant from VBL20 cells (2.0 x 10⁶). After washing, cells were lysed for 20 min at 4 °C in 2 ml of D₂O. The homogenate was centrifuged at 130,000 x g for 60 min and the supernatant was removed. The COSY spectrum was recorded as for VBL20 cells.

**Table I**

Neutral lipid composition of membranes of various cell lines

Crude plasma membranes from 2.5 x 10⁶ cells were prepared as described by Mountford et al., 1984b. The 13762 and J clone cell lines were enriched 4-fold and the other cell lines were prepared similarly. The analyses were carried out as described under “Experimental Procedures,” and the values represent the mean ± S.E. as determined from assays run in duplicate on the number of experiments indicated in parentheses. Data are expressed as nmol/mg of lipid. The abbreviations used are: ALL, acute lymphoblastic leukemic; PBL, peripheral blood lymphocytes; PWM, pokeweed mitogen.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Triglyceride</th>
<th>Free cholesterol</th>
<th>Cholesterol ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat mammary adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic line 13762</td>
<td>(3) 86 ± 9</td>
<td>278 ± 21</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>Nonmetastatic J Clone</td>
<td>(3) 77 ± 6</td>
<td>236 ± 9</td>
<td>6 ± 6</td>
</tr>
<tr>
<td>All T cell CCRF-CEM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td>(5) 94 ± 5</td>
<td>182 ± 41</td>
<td>28 ± 11</td>
</tr>
<tr>
<td>Resistant to 20 ng/ml VBL</td>
<td>(6) 88 ± 16</td>
<td>167 ± 31</td>
<td>48 ± 23</td>
</tr>
<tr>
<td>Chicken fibroblast</td>
<td>(2) 163 ± 36</td>
<td>191 ± 22</td>
<td>55 ± 26</td>
</tr>
<tr>
<td>Human PBL stimulated with PWM</td>
<td>(2) 491 ± 25</td>
<td>256 ± 17</td>
<td>14-290*</td>
</tr>
</tbody>
</table>

*The range of values has been indicated due to large variation in results.

1, B and C) does not display this cross-peak, the absence of which may be accounted for either by dynamic effects or a difference in cholesterol and/or cholesterol ester composition.

Low levels of diglycerides have also been found in the membranes of some cancer cell lines. Their two-dimensional spectra are essentially indistinguishable from those of triglycerides since the acyl chain connectivities are identical.

The chemical analyses of the crude plasma membranes of some cancer cells and of other rapidly dividing cells known to give narrow NMR lines are shown in Table I. The membrane triglyceride content and composition are comparable in most

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cases, with chicken fibroblasts and stimulated human lymphocytes showing the greatest variation. Furthermore, cholesterol ester and free cholesterol levels display an even greater variation as evidenced by the standard errors.

The major contamination appears to stem from endoplasmic reticulum as evidenced by the enrichment of NADPH cytochrome c reductase activity. Even so, the ratio of plasma membrane to endoplasmic reticulum markers in Band 1 is 11:1. Bands 2–4 are mixed membrane bands containing varying levels of mitochondrial, endoplasmic reticulum, lysosomal, and/or nuclear membranes in addition to plasma membrane. Chemical analysis of the major lipids (Table IIB) demonstrated that the levels of free cholesterol in the mixed membrane bands (B2 and B3) were in agreement with those found for the crude membrane preparations of VBL20 cells (Table I). In contrast, the level of free cholesterol in the B1 fraction was considerably higher and no cholesterol ester was detected in any of the bands. The presence of large amounts of free cholesterol is in itself an indication of membrane purity (Haeffner et al., 1982) since the plasma membrane is the major site of cholesterol accumulation. The decrease in free cholesterol from B1 to B4 reflects the level of plasma membrane enrichment. In contrast, the cholesterol-to-phospholipid ratio shows an increase over the homogenate in bands 1–3. The levels of triglyceride in the bands (Table IIC) are most interesting since two of the mixed membrane bands (B2 and B3) again show agreement with those measured in the VBL20 crude membrane preparations (Table I) while triglyceride in B4 is slightly elevated and the purest plasma membrane-containing fraction (B1) has the highest levels of triglyceride. It is very unlikely that triglyceride has been trapped in the membrane bands from the triglyceride-rich floaters and supernatant fractions since for B1 the ratio of the plasma membrane marker 5’ nucleotidase to the cytoplasmic marker lactate dehydrogenase is 5000:1. The data further suggest that triglyceride may be present in all the membranes and indeed increased amounts of triglyceride or diglyceride have been found recently in mitochondrial membranes of rat liver, the rapidly dividing fetal cells containing four times as much as the adult cells (Harsas et al., 1985). It has been suggested by others (Verkleij, 1984; Sen et al., 1981) that membranes containing increased proportions of neutral lipids such as triglycerides would exhibit changes in their permeability properties reflecting a decrease in the bilayer content of the membranes.

We have also found elevated levels of triglyceride in a 9-fold enriched plasma membrane preparation from a rat mammary adenocarcinoma 13762 subline. Chemical analysis shows the B1 fraction to contain 376 nmol/mg of lipid. The data thus support the NMR assignments in Fig. 2.

The T₂ of greater than 600 ms, found to correlate with the presence of malignant cells with the capacity to metastasize, is very long and can only be accounted for by fast molecular motion in an unusual environment (Mountford et al., 1984a). The location and environment of the triglyceride in the membrane has been investigated by spin–spin relaxation experiments. Different solvent systems have been studied in an attempt to determine the type of environment conducive to this type of relaxation mechanism.

Triglycerides (and diglycerides) as well as cholesterol esters of differing acyl chain content in CDC₁₀ were found to give a single exponential decay with the T₂ varying from 1 s to 300 ms. (The acyl chains from cholesterol esters and triglycerides

![Symmetrized COSY spectra](image-url)
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**TABLE IIA**

Marker enzyme activity

<table>
<thead>
<tr>
<th>Enzyme marker</th>
<th>Band 1</th>
<th>Band 2</th>
<th>Band 3</th>
<th>Band 4</th>
<th>Floaters</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase</td>
<td>44.9 ± 17.7</td>
<td>19.4 ± 5.0</td>
<td>14.6 ± 5.3</td>
<td>0.46 ± 0.09</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>γ-Glutamyl/transferase</td>
<td>12.6 ± 3.3</td>
<td>11.1 ± 2.9</td>
<td>0.6 ± 0.6</td>
<td>ND</td>
<td>0.15 ± 0.15</td>
<td>ND</td>
</tr>
<tr>
<td>Na⁺/K⁺ adenosinetriphosphatase</td>
<td>10.5 ± 3.4</td>
<td>14.4 ± 5.0</td>
<td>2.4 ± 0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>1.2 ± 0.4</td>
<td>1.9 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>2.0 ± 0.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>0.009 ± 0.001</td>
<td>0.008 ± 0.01</td>
<td>0.035 ± 0.004</td>
<td>2.5 ± 0.7</td>
<td>1.5 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase</td>
<td>4.0 ± 0.2</td>
<td>6.8 ± 1.4</td>
<td>6.7 ± 2.6</td>
<td>0.9 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>0.39 ± 0.3</td>
<td>3.5 ± 0.7</td>
<td>6.4 ± 0.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DNA</td>
<td>0.079 ± 0.007</td>
<td>0.11 ± 0.02</td>
<td>0.39 ± 0.03</td>
<td>0.021 ± 0.004</td>
<td>0.015 ± 0.003</td>
<td>ND</td>
</tr>
</tbody>
</table>

**TABLE IIIB**

Cholesterol and phospholipid composition

The homogenate and four membrane bands from the density gradient centrifugation were analyzed according to the methods described under "Experimental Procedures" and the values represent the mean ± S.E. as determined from assays run in duplicate on two different extractions. No cholesterol ester was detected in the bands. Data are expressed as nmol/mg of lipid.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Free cholesterol</th>
<th>Phospholipid</th>
<th>Cholesterol-to-phospholipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>110 ± 16</td>
<td>1101 ± 15</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>B1</td>
<td>122 ± 12</td>
<td>1010 ± 16</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td>B2</td>
<td>184 ± 10</td>
<td>856 ± 95</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>B3</td>
<td>157 ± 30</td>
<td>922 ± 301</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>B4</td>
<td>75 ± 5</td>
<td>1101 ± 15</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

...were found to behave identically. Neutral lipids with one double bond at position 9 generate two clusters of resonances at 1.28 and 1.30 ppm from the nuclei at positions 2–7 and 12–17, respectively, in the chain (Frost and Gunstone, 1975). The results of a CPMG experiment, over the delay range 0–2 s for triolein (18:1) in chloroform, is shown in Fig. 3A.

The metastatic 13762 (Fig. 3B) and nonmetastatic J clone lines (Fig. 3C) both have four resonances under the broad methylene envelope at 1.2 ppm. CPMG experiments over the delay range 0–200 ms generate two rates of decay for each of the four resonances. A long T2 for the resonance at 1.25 ppm identifies the metastatic line 13762 from its nonmetastatic counterpart J clone. In contrast to the triglyceride in CDCl₃, these resonances cannot readily be followed over the delay range 200–2000 ms due to lack of signal intensity. Other cell lines, including human ovarian and colon cells (Mountford, et al., 1984b), have been found to demonstrate a similar pattern whereby the metastatic cells generate a T2 of greater than 400 ms while their nonmetastatic counterparts have a T2 of less than 200 ms. Triolein dispersed in D₂O does not yield a T2 in excess of 200 ms.

Human lipoproteins are rich in cholesterol ester and triglyceride and have been shown to give narrow line ¹H NMR spectra. A possibility for the anomalously narrow ¹H NMR lines in our cells is that the neutral lipid domains may be akin to those in lipoproteins. The COSY spectra in Fig. 2 show...
the oily membrane domain of the type described above. If this domain were large enough to traverse the plasma membrane, it could be a method of transporting many different types of molecules, including anesthetics and drugs into the cell.

Acknowledgments—We are indebted to Prof. Martin Tattersall for the suggestion that we study cancer metastasis and for his continued interest and support. We gratefully acknowledge Prof. Myer Bloom and Dr. John Saunders for their considerable contribution of time and thought to this program, and Dr. Ian Ramshaw for supplying the original metastasis model and his continued interest and advice. In addition we would like to thank Marlen Dyne for electron micrographs and technical assistance, Liz Kean for the tissue culture, Pat Gregory for animal facilities, and Judy Hood for her enthusiastic typing of the manuscript.

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