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Use of a reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells

(metastasis/chemotaxis/migration/laminin/type IV collagen)

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ABSTRACT **Malignant cells must traverse basement membranes during their migration to sites distant from the primary tumor. Since basement membranes are thought to be a critical barrier to the passage of tumor cells, we have constructed a model basement membrane–stromal matrix consisting of laminin and type IV collagen reconstituted onto a disk of type I collagen for use in an *in vitro* assay of invasiveness. Metastatic tumor cells and leukocytes are able to cross this barrier, whereas nonmetastatic tumor cells, fibroblasts, and epidermal cells cannot penetrate it. Those tumor cells that penetrate the barriers were found, when isolated and subcultured, to be more invasive and to produce more metastases than the parental population. This assay system should be useful for studying the invasiveness of tumor cells and for isolating highly invasive variants.**

After escaping from the primary lesion, tumor cells enter the lymphatic or circulatory system where some arrest in small vessels, invade the vessel wall, and grow into new tumors. Those cells that are capable of completing the entire process represent only a small subpopulation of the cells within the bulk tumor (for review see refs. 1 and 2). Consequently, these are the cells that increase the morbidity of patients with metastatic disease.

The interaction of invasive cells with normal tissues and extracellular matrix elements may be critical in the formation of metastases (for review see refs. 3 and 4). Basement membranes line most blood vessels, creating a physical barrier to the passage of cells. Therefore, a necessary component of the metastatic process must be the ability of tumor cells to bind to and degrade basement membranes (3–6). Indeed, many metastatic cells bind preferentially to basement membrane (type IV) collagen by using laminin as an attachment protein (7, 8). These cells also produce a distinct collagenase that is able to degrade basement membrane collagen (5). Such activities are correlated with the metastatic activity of the cells *in vivo* (6, 8, 9).

Various normal connective tissues have been used in assays as barriers to test the invasiveness of tumor cells *in vitro*. These include chicken chorioallantoic membrane (10, 11), mouse urinary bladder (12), the human amnion (13) and other placental membranes (14), and the lens capsule of the eye (15, 16). Such assay systems can be used to establish whether or not a cell is invasive. With the human amnion or lens capsule as barriers, the numbers of cells crossing the tissue were found to correlate with their metastatic activity *in vivo* (3, 16). However, leukocytes were also able to cross the amnion when directed by a gradient of a formylmethionyl peptide chemoattractant (17, 18). In addition, the melanoma cells that penetrated through urinary bladder wall *in vitro* were found to have greater metastatic activity than the original line, suggesting that highly invasive cells could be selected for and established as a line based on their invasiveness (12). Since the tissues used as barriers to tumor cell movement are devoid of cells but have a layer of basement membrane and consist predominantly of extracellular matrix, these findings suggest that extracellular matrix is a major barrier to the passage of the cells.

To achieve a defined barrier, we have reconstituted a basement membrane-like matrix by layering laminin and type IV collagen on a type I collagen disk. These matrices form a barrier to the passage of normal and noninvasive tumor cells but permit invasive cells and leukocytes to pass. Tumor cells passing through the barrier have been subcultured and found to be more invasive *in vitro* and more metastatic *in vivo* than the parental cell population.

MATERIALS AND METHODS

Matrix Barriers. Type I collagen purified from bovine tendon was used as the major structural component of the barriers. Fibrillar dispersions of this collagen were reconstituted into mats with open lattices (Helisert U137 and U138, Helitrex, Princeton, NJ), and in most cases the mats were compressed under high pressure to increase their density (U171). For use in the invasion assay, type I collagen mats were used as produced or their top surfaces were evenly coated first with 0.2 ml of a 1 mg/ml solution of type IV collagen (19) and then with 0.2 ml of a 1 mg/ml solution of laminin (20). There was little retention of laminin when type IV collagen was omitted. These mats are termed "layered." In other cases the mats were prepared by mixing a dispersion of type I collagen fibers with an equivalent amount of type IV collagen and laminin prior to molding. These mats are termed "coformed."

Invasion Assay. Two-compartment (Boyden) chemotaxis chambers constructed from Teflon were designed to accommodate 13-mm-diameter barriers (Fig. 1). The barriers were placed in contact with a type IV collagen-coated Nuclepore filter (0.1-μm pore size) on which the cells that passed through the barrier were collected. A Nuclepore filter and a connective tissue barrier then were placed over a porous support ring in the chamber so that the upper and lower compartments were separated. A hollow-center screw was used to clamp the combination in place inside the chamber.

In some experiments, the chemoattractants, *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe, a gift from E. Schiffmann, National Institutes of Health) or platelet-derived growth factor (a gift from G. R. Grotendorst, National Institutes of Health) were introduced into the lower com-

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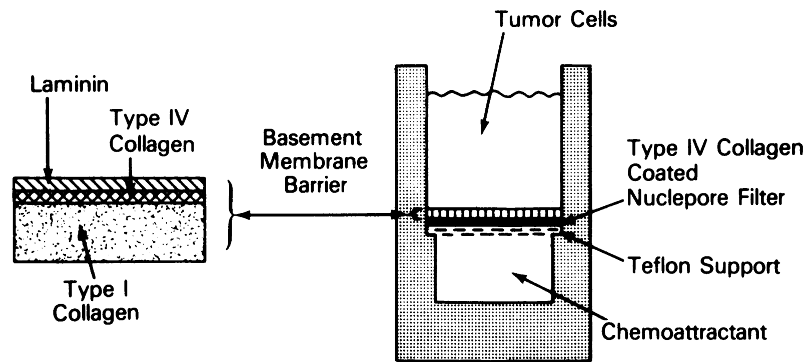

FIG. 1. Diagram of layered laminin–type IV collagen, type I collagen barrier, and of the invasion-assay chamber.

partment. All invasion assays were carried out in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin, nonessential amino acids, 25 mM Hepes buffer, and 2% acid-treated NuSera (Collaborative Research, Waltham, MA), pH 7.4. The chambers were incubated for various times in a humidified, 5% CO_2 atmosphere at 37°C; under these conditions the cells were viable for at least 2 days. In this assay, the cells penetrate through the matrix and attach to the surface of the underlying type IV collagen-coated Nuclepore filter. Quantitation of cell penetration was determined by removal of the Nuclepore filter and by staining it with hematoxylin and eosin. The cells in 10 microscopic fields (×400) per filter were counted; results are reported as the means for triplicate or quadruplicate assays.

Selection Assay. In other experiments, after fixed periods of incubation, the type IV collagen-coated Nuclepore filters were removed from the chambers under sterile conditions. The adherent cells on 10 Nuclepore filters were removed with 0.1% EDTA in 100 mM NaCl/60 mM mannitol/25 mM Hepes/10 mM $NaHCO_3$/6 mM K_2HPO_4, pH 7.4, and the resulting suspension was diluted with an equal volume of Dulbecco's modified Eagle's medium. The cells ($\approx 2 \times 10^3$) were collected by centrifugation and grown to confluence in medium supplemented with 10% fetal bovine serum. After not more than three passages, cells were used for injection into mice. For the *in vivo* metastasis assay, 2.5×10^5 cells were injected into the tail vein of syngeneic mice (25 mice per group). The mice were sacrificed 4 weeks after injection and pulmonary metastases were counted (21). Each experiment was performed at least four times.

Cell Lines. The B16F1 and B16F10 murine melanoma cell lines were provided by I. J. Fidler (Department of Cell Biology, M. D. Anderson Hospital and Tumor Institute, University of Texas System Cancer Center, Houston, TX). These cells are metastatic *in vivo* (21–23) and are invasive *in vitro* with human amnion as a barrier (21). The human rhabdomyosarcoma (RMS) cells were obtained from Meloy Laboratories (Springfield, VA). These cells were found to be invasive *in vitro* and by karyotypic analysis to be human in origin (24). Rabbit polymorphonuclear leukocytes obtained from peritoneal exudates were provided by E. Schiffmann (NIH). These cells have been shown to penetrate through the whole human amnion when the chemoattractant fMet-Leu-Phe is placed in the lower compartment of a modified Boyden chamber (17). Normal human skin fibroblasts (CRL 1507 and CRL 1477) and a fibroblast line (CRL 1260) from a xeroderma pigmentosum patient were obtained from the American Type Culture Collection.

Other cell lines tested for invasive potential included murine melanoma cells (B16BR2 and B16BL6) and murine reticular-cell-sarcoma cells (M50-76) (I. Hart, Imperial Cancer Research Fund, London); murine fibrosarcoma cells (T241-PM2) and murine fibrosarcoma (C3H) (L. A. Liotta, NIH); murine endothelial cells (TR-1) and canine kidney cells (MDCK) (G. R. Grotendorst, NIH); murine epidermal cells (PAM-212) (S. Yuspa, NIH); human breast carcinoma cells (MCF7 and ZR-75-1) (Michigan Cancer Foundation); and human cervical squamous-cell-carcinoma cells (A431) (D. Salomon, NIH).

Attachment, Laminin-Binding, and Collagenase Assays. The attachment assay was adapted from that described by Klebe (26) as modified by Terranova *et al.* (27). The assay for laminin binding to cells has been described (28), as has the type IV collagenase assay (5).

RESULTS

The compressed type I collagen disks were observed by scanning electron microscopy to be formed of fine fibers (Fig. 2*A*). Addition of type IV collagen and laminin to the surface of the type I collagen generated a film-like coating that ranged in thickness from 200 to 600 μm (Fig. 2*B*). Immunofluorescence studies on sectioned filters, using antibody to type IV collagen [prepared as described (34)], showed the protein to be distributed in a rather even fashion across the surface of the disk (Fig. 2*C*). It was our experience that laminin was not retained well on the barrier when added without type IV collagen (data not shown). It is known that laminin binds to type IV collagen but not to type I collagen (27).

A variety of tumor cells were tested for their ability to cross the reconstituted basement membrane-type I collagen barrier. The rate at which some of these cells penetrated the barrier is shown in Fig. 3. Highly metastatic cells, such as human rhabdomyosarcoma (RMS) and mouse melanoma (B16F10, B16BR2, B16BL6), penetrated more rapidly than the less metastatic cells. Various other tumor cell lines also were tested, and cells known to be invasive and metastatic *in vivo* were able to penetrate through the barrier, including fibrosarcoma cells (T241-PM2) reticular-cell sarcoma cells (M50-76), breast carcinoma cells (MCF7, ZR-75-1) and uterine carcinoma cells. C3H cells, from a tumorigenic but nonmetastatic fibrosarcoma, did not penetrate the barrier, nor did epidermal cells (PAM-212), endothelial cells (TR-1), kidney epithelial cells (MDCK), and human skin fibroblasts (CRL 1507 and 1477). Such differences were not observed when the barrier consisted of the type I collagen matrix alone, which was more readily penetrated by all tumor cells (data not shown).

In vivo, polymorphonuclear leukocytes penetrate basement membrane to reach sites of infection. Therefore, we tested the ability of these cells to traverse the barrier when a leukocyte chemoattractant, fMet-Leu-Phe, was placed in the lower compartment. These studies show that although some leukocytes penetrate the basement membrane barrier without any attractant, the formylmethionyl peptide increased the number of penetrating cells (Fig. 4). Under the same conditions, B16F10 cells showed a high penetration in the absence of formylmethionyl peptide and little stimulation by addition

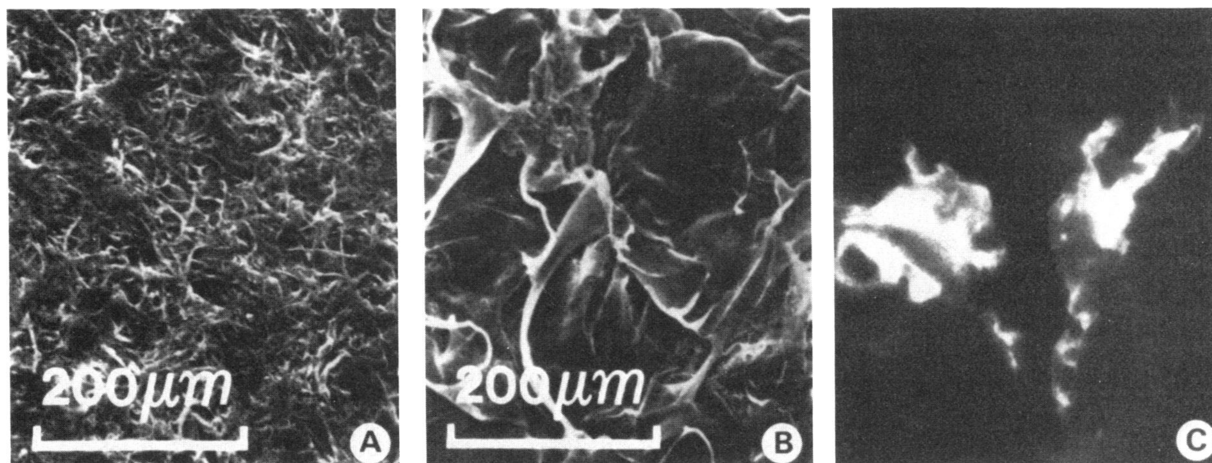

FIG. 2. (*A* and *B*) Scanning electron micrographs of a compressed type I collagen disk (*A*) and a type I collagen disk that was coformed with type IV collagen and laminin (*B*). (*C*) Photomicrograph of the distribution of type IV collagen as determined by indirect immunofluorescence (25). (×400.)

of the peptide. These results indicate that chemoattractants with different cellular targets can be assessed in this system.

Various cells from normal tissues were tested for their ability to cross the barrier. These included epidermal cells, endothelial cells from testes, kidney epithelial cells, and various human skin fibroblasts. After 48 hr of incubation, no cells were detected on the Nuclepore filter. A strain of human fibroblasts (CRL 1260) was studied further. Barriers were chosen either with or without basement membrane components. These studies showed that the fibroblasts penetrated through type I collagen mats lacking type IV collagen and laminin when platelet-derived growth factor was placed in the lower compartment (Fig. 5). Fibronectin (10 μg/ml) also stimulated fibroblast penetration when placed in the lower compartment (data not shown). However, when using barriers coated with laminin and type IV collagen in the presence of a chemoattractant, we observed little or no migration of cells (Fig. 5). These studies suggest that basement membrane proteins form the critical barrier to cell passage.

Isolation of Invasive Tumor Cells. Cells from two different tumor lines that penetrated the basement membrane barrier were subcultured and several of their characteristics were measured and compared with those of the parent-cell population (Table 1). The B16F1 cells derived from those that migrated through the matrix had a 58% higher level of attachment to type IV collagen than the parental cells and bound three times the amount of ^{125}I-labeled laminin, but with a similar receptor affinity as judged by Scatchard analysis (data not shown). The conditioned medium from these cells also had the capacity to degrade type IV collagen at twice the rate for medium from the parental population. Further, when

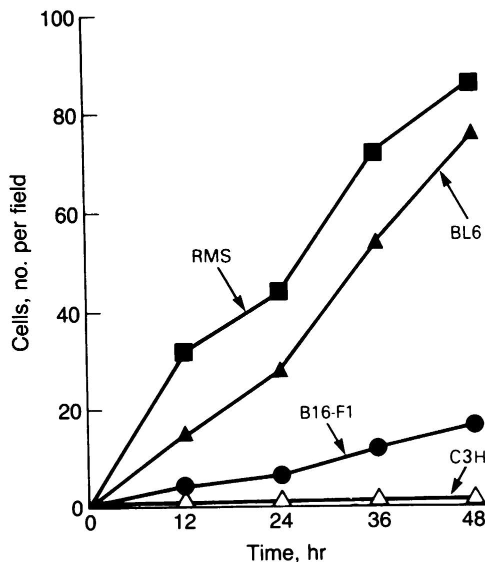

FIG. 3. Penetration of tumor cells with differing degrees of invasiveness through the coformed barriers. Cells (5×10^5) were added to the upper compartment in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin, 1% nonessential amino acids, 25 mM Hepes, and 2% acid-treated NuSera, pH 7.4. At 12, 24, 36, and 48 hr, the chambers were removed from incubation and the type IV collagen-coated Nuclepore filters were stained with hematoxylin and eosin. Cells in 10 high-power fields (×400) per filter were counted. Means of the triplicate assays did not differ by more than 10%.

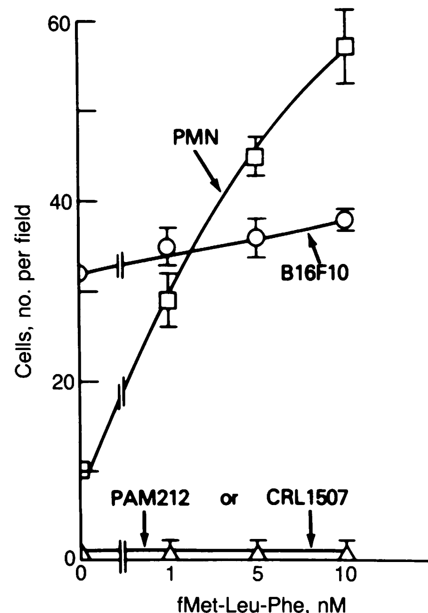

FIG. 4. Penetration of rabbit polymorphonuclear leukocytes (PMN) through the coformed barriers as a function of fMet-Leu-Phe concentration. PMN (2×10^6) were placed in the upper compartment for 24 hr. After this time, filters were removed and the number of cells that penetrated were quantitated. Also tested were murine melanoma B16F10, murine epithelial cell line PAM-212, and human fibroblast line CRL-1507. Standard error of the mean is given for quadruplicate samples.

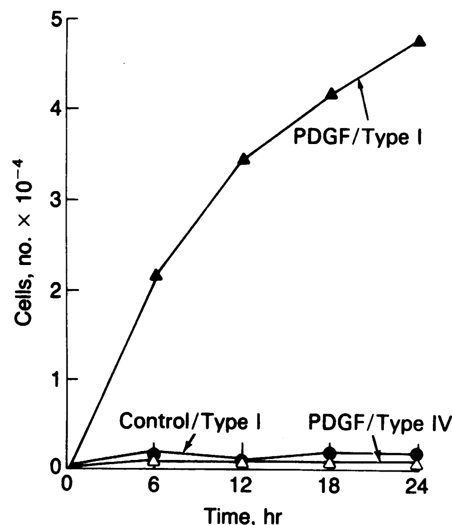

FIG. 5. Penetration of human skin fibroblasts (CRL 1260) through the type I collagen disk (▲), but not through the coformed barrier with type IV collagen and laminin (△), when platelet-derived growth factor (PDGF, 5 units/ml) was used as a chemoattractant in the lower compartment. Results are expressed as means of quadruplicate assays. Cells on the entire filter were counted to obtain total number of cells that had penetrated.

cells that had migrated through the matrix were placed back into the assay chambers, they penetrated new barriers 4-fold better than the parental population (Table 1). Those MCF-7 cells derived from the cells that penetrated through the barrier had a 40% increase in attachment to type IV collagen, a 5-fold increase in laminin binding capacity, a 2.5-fold increase in the amount of type IV collagen degraded, and a 6-fold increase in their invasive activity *in vitro* (Table 1). Similar differences were observed after selecting the invasive B16F10 cells (data not shown), whereas no such changes were noted in the population derived from tumor cells that had passed through the type I collagen matrix alone.

Some differences were found in metastatic activity between the parental cells and the population derived from invasive cells (Table 2). The B16F1 cells that had been selected for invasiveness produced about 3 times as many metastases as did the parental line. Cells selected for invasiveness from the B16BR2, B16BL6, and T241-PM2 lines also showed some increase in metastatic activity. However, a second round of selection did not produce an equivalent increase in metastatic activity.

Table 1. Differences observed between parental cells and cells derived from those that penetrated the reconstituted basement membrane matrix

	% attachment to type IV collagen	Laminin binding, cpm per 10^4 cells	Type IV-collagen degradation, cpm per 10^4 cells	Invasion *in vitro*, no. of cells per field
B16F1				
Parental	38	185	50	12
Selected	62	575	105	52
MCF7				
Parental	68	160	112	49
Selected	95	775	280	298

Attachment assays (8, 27), laminin binding assays (28), and type IV collagenase assays (5, 6) were performed as described. Invasion assays were as described in *Materials and Methods*.

Table 2. Metastatic activity of parental cell lines and of cells derived from those that penetrated the reconstituted basement membrane matrix

	No. of pulmonary metastases		
Cell line	Parent	Selected once	Selected twice
B16F1	37 ± 9	110 ± 22	136 ± 30
B16BR2	70 ± 10	106 ± 21	122 ± 16
B16BL6	100 ± 10	163 ± 38	185 ± 27
T241-PM2	81 ± 14	156 ± 29	186 ± 35

In vivo pulmonary metastases assays were done as described (8, 21) after injection either of parental cells or of early-passage cells obtained after one or two rounds of selection for *in vitro* invasiveness. Cells were judged to be >98% viable by trypan blue exclusion and by incorporation of ^{14}C-labeled amino acids into acid-insoluble material (data not shown). Before injection, cell suspensions were passed through a nylon mesh (20-μm pore size) to remove clumps. Results shown are means ± SEM for four assays, each using 25 mice per cell line.

DISCUSSION

This study demonstrates that the addition of type IV collagen and laminin to a disk of type I collagen creates a selective barrier to the passage of cells. Scanning electron microscopy and immunofluorescence of the barrier showed that the basement membrane components formed a continuous layer on the surface of the type I collagen matrix, forming an artificial basement membrane. Presumably, it is this layer of basement membrane components that blocks the passage of noninvasive cells, as such cells readily crossed the barrier when the basement membrane components were omitted. Since metastatic cells are able to traverse the reconstituted basement membrane, it is likely that they are binding to laminin, for which they have a high affinity (21, 28, 29), and subsequently producing enzymes able to degrade the type IV collagen (5, 6). Chemoattractants increase the number of leukocytes (Fig. 4) and metastatic tumor cells (30), passing through the barrier. Presumably this requires not only increased cell mobility but also the induction of more active cells with a greater capacity to degrade the basement membrane barrier. Such factors would also be expected to function *in vivo* and may include laminin, which has been found to possess chemotactic and haptotactic activity for metastatic melanoma cells (31).

Many investigators have studied the differences between normal and transformed cells with the aim of developing more effective therapeutic regimens. However, with the exception of uncontrolled growth, there are few qualitative differences and no firm correlation to metastatic potential. This may be due to the diverse populations of cells that tumors contain (for review see refs. 32 and 33), of which the metastatic cells represent only a small subpopulation. Since invasiveness in our assay correlates with metastatic activity, it may be possible to assess tumors for the presence of cells with the metastatic phenotype, to isolate these populations of cells, and to test factors that may inhibit invasion. However, only modest increases in metastatic activity were observed in the cells studied here after a second selection cycle, which suggests that differences in invasiveness are only a limited part of metastasis formation. Immunogenicity, for example, would prevent invasive cells from being metastatic.

The assay described here may have other uses in tumor cell biology, including assaying for specific chemotactic factors localized in primary target tissues and studying directed movement of tumor cells, which may play a role in organ-specific metastasis (30). Further, modalities of treatment that are directed against the attachment of tumor cells to the subendothelial basement membrane or against the secretion

of degradative enzymes can be investigated. Preliminary studies indicate that human endothelial cells can be cultured as a monolayer on the barrier and used to study tumor-cell–endothelial-cell interactions (unpublished data). Factors that inhibit tumor cell attachment to endothelium might be useful in reducing metastasis by the hematogenous route.

In summary, we have developed a simple and reproducible method for assaying tumor cell invasiveness and one suitable for isolating tumor cell subpopulations with a highly invasive phenotype.

1. Hart, I. R. & Fidler, I. J. (1981) *Biochim. Biophys. Acta* **651,** 37–50.
2. Poste, G. (1982) *Cancer Metastasis Rev.* **1,** 141–199.
3. Liotta, L. A., Rao, C. N. & Barsky, S. H. (1983) *Lab. Invest.* **49,** 636–649.
4. Liotta, L. A. (1984) *Am. J. Pathol.* **117,** 339–348.
5. Liotta, L. A., Abe, S., Gehron-Robey, P. & Martin, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2268–2272.
6. Liotta, L. A., Thorgeirsson, U. P. & Garbisa, S. (1982) *Cancer Metastasis Rev.* **1,** 277–297.
7. Murray, J. C., Liotta, L. A., Rennard, S. I. & Martin, G. R. (1980) *Cancer Res.* **40,** 347–351.
8. Terranova, V. P., Liotta, L. A., Russo, R. G. & Martin, G. R. (1982) *Cancer Res.* **42,** 2265–2269.
9. Barsky, S. H., Siegal, G., Jannotta, F. & Liotta, L. A. (1983) *Lab. Invest.* **49,** 140–148.
10. Easty, D. M. & Easty, G. C. (1974) *Br. J. Cancer* **29,** 36–49.
11. Poste, G., Doll, J., Hart, I. R. & Fidler, I. J. (1980) *Cancer Res.* **40,** 1636–1644.
12. Hart, I. R. (1979) *Am. J. Pathol.* **97,** 587–600.
13. Liotta, L. A., Lee, W. C. & Morakis, D. J. (1980) *Cancer Lett.* **11,** 141–147.
14. Schleich, A. B., Frick, M. & Mayer, A. (1976) *J. Natl. Cancer Inst.* **56,** 221–237.
15. Starkey, J. R. & Stanford, D. (1984) *J. Cell Biochem.*, Suppl. 8B, 303.
16. Starkey, J. R., Hosick, H. C., Stanford, D. R. & Liggitt, H. D. (1984) *Cancer Res.* **44,** 1885–1894.
17. Russo, R. G., Thorgeirsson, U. P. & Liotta, L. A. (1982) in *Tumor Invasion and Metastasis*, eds. Liotta, L. A. & Hart, I. R. (Nijhoff, The Hague, Netherlands), pp. 173–187.
18. Terranova, V. P., DiFlorio, R. M., Hujanen, E. S., Lyall, R. M., Liotta, L. A., Thorgeirsson, U., Siegle, G. P. & Schiffmann, E. (1985) *J. Clin. Invest.*, in press.
19. Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K. & Martin, G. R. (1982) *Biochemistry* **24,** 6188–6193.
20. Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M. & Martin, G. R. (1979) *J. Biol. Chem.* **254,** 9933–9937.
21. Terranova, V. P., Liotta, L. A., Williams, J. E. & Martin, G. R. (1984) *Science* **226,** 982–985.
22. Fidler, I. J. (1973) *Nature (London)* **242,** 148–149.
23. Giavazzi, R., Alessandri, G., Spreafico, F., Garattini, S. & Mantovani, A. (1980) *Br. J. Cancer* **42,** 462–472.
24. Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I. R., Foltz, C. M. & Shafic, S. (1980) *Nature (London)* **284,** 67–68.
25. Foidart, J. M., Bere, E. W., Yaar, M., Rennard, S. I., Guillino, P. M., Martin, G. R. & Katz, S. I. (1980) *Lab. Invest.* **42,** 336–342.
26. Klebe, R. J. (1974) *Nature (London)* **250,** 248–251.
27. Terranova, V. P., Rohrbach, D. H. & Martin, G. R. (1980) *Cell* **22,** 719–728.
28. Terranova, V. P., Rao, C. N., Kalebic, T., Margulies, I. M. & Liotta, L. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 444–448.
29. Malinoff, H. & Wicha, M. S. (1983) *J. Cell Biol.* **96,** 1475–1481.
30. Hujanen, E. S. & Terranova, V. P. (1985) *Cancer Res.* **45,** 3517–3521.
31. McCarthy, J., Palm, S. & Furcht, L. (1983) *J. Cell Biol.* **97,** 772–779.
32. Nicolson, G. L. & Poste, G. (1982) *Curr. Problems Cancer* **7,** 4–83.
33. Nicolson, G. L. & Poste, G. (1983) *Int. Rev. Exp. Pathol.* **25,** 77–181.