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Tumor Implantation and Invasion at Metastatic Sites

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I. Introduction

Most primary tumors can be treated successfully by surgery alone or in combination with chemotherapy, immunotherapy, or radiotherapy. However, the treatment of tumors once they have spread to metastatic sites is a much more difficult and complex problem. By the time cancer is diagnosed in many patients they often possess multiple metastases, and often these are small lesions which cannot be easily detected. Effective treatment of multiple small metastases by surgery or radiotherapy is often impossible due to their widespread distribution in major organs. In addition, it is now known that malignant tumors possess cell subpopulations with widely differing sensitivities to the most commonly used forms of therapy, and this can translate into therapeutic failure if multiple adjuvant therapies are not utilized.

In addition to the extensive variation in the susceptibility of cell subpopulations in a tumor to various therapies, metastatic cells also show considerable diversity in many other properties. These include differences in cell surface charge and cell partitioning behavior; expression of cell surface glycoproteins, glycolipids, antigens, enzymes, and other receptors (and the release of these components); and cell morphology, karyotype, and recognition structures (reviewed in Hart and Fidler, 1981; Fidler and Nicolson, 1981; Nicolson, 1982a; Poste, 1982a; Nicolson and Poste, 1982, 1983).

Tumor invasion and metastasis occurs by way of a complex series of sequential steps in which malignant cells first invade and occupy adjacent tissues (primary invasion) and penetrate into body cavities, the lymphatics

TABLE I
TUMOR AND HOST PROPERTIES IN MALIGNANT INVASION

Tumor properties	Host properties
Tumor growth	Tissue and stromal barriers
Tumor mechanical and osmotic effects	Tissue cell cohesion
Tumor cell adhesive properties	Tissue and serum enzyme inhibitors
Tumor cell locomotion and chemotaxis	Inflammatory and noninflammatory reactions
Tumor cell degradative enzymes	Specific and nonspecific immunological responses
Tumor cell chemotactic substances	Vascularization, nutrition, and oxygenation
Tumor cytolytic substances	Tissue growth inhibitors

and/or blood circulatory systems. Once malignant cells enter into these new compartments they can separate from the primary tumor mass (detachment) and be transported passively (dissemination) to near and distant sites where they must arrest (implantation), invade into surrounding tissues (secondary invasion), survive, and, finally, proliferate to form new metastatic colonies. This sequence is discussed in more detail elsewhere (see previous references and Weiss, 1977; Fidler *et al.,* 1978; Baldwin, 1978; Poste and Fidler, 1980).

Current knowledge of the mechanisms of malignant cell implantation and invasion at secondary sites will be surveyed in this contribution. We will also deal with the properties of metastatic cell subpopulations, their cellular diversity, and possible regulation at different stages of malignant cell growth. This review will focus on the use of rational experimental models of tumor implantation and metastasis and their use in studying the tumor cell and host characteristics which may be important in these two steps of the metastatic process (Table I). We have purposely concentrated on the events occurring at secondary rather than primary sites of tumor growth, and readers should refer to several excellent reviews and monographs which have focused on this latter topic (Easty, 1975; Armstrong, 1977; Mareel, 1979, 1980; Hart, 1981; Hart and Liotta, 1982; Poste, 1982b).

II. The Clinical Problem of Cancer Spread

A. SOME CLINICAL CONSIDERATIONS

Two of the most widely studied properties of malignant tumors are their abilities (1) to grow and (2) to invade adjacent host tissues at the primary site. Sugarbaker (1979, 1981) has reviewed the literature on the growth rates of malignant tumors and their relationship to host prognosis. Although there are numerous examples of rapidly growing malignant lesions which

aggressively metastasize and kill their hosts at very rapid rates, there are also other examples in which highly invasive and infiltrative cancers rarely metastasize. The classic example is basal cell carcinoma, where extensive primary invasion can occur without evidence of local or distant metastases.

Sugarbaker (1979) has questioned whether more rapidly growing primary malignant neoplasms have greater propensities for metastatic spread or whether they metastasize at the same rate but grow more rapidly at secondary sites, or whether a combination of these properties is more common. His studies can be summarized as follows. Although the incidence of metastasis formation appears to increase statistically with increased tumor size in many types of cancer, cancers of the same histologic type often produce quite diverse survival patterns for individual patients. This suggests that even in the same histologic class of cancer there is no simple relationship between the size of the primary tumor and the expected incidence or size of metastatic lesions. However, in many of the more common human cancers, such as squamous cell carcinoma of the lung and adenocarcinoma of the colon and breast, among others, the overall incidence of metastasis increases statistically with tumor size (Sugarbaker, 1979). Because of this general relationship, primary tumor size is an important datum in the clinical staging of certain cancers (Sugarbaker, 1981).

Wide variations exist in the metastatic potentials of various human malignancies. For example, Lindberg (1972) has examined primary squamous tumors of the head and neck and has related the presence of lymphatic metastases to the size and site of origin of the primary tumor. In this study of 2044 patients at the M. D. Anderson Hospital and Tumor Institute with head and neck carcinomas, there were dramatic differences in the abilities of different carcinomas in the same general anatomic region to metastasize. Although carcinomas of the tongue, soft palate, and mouth floor had higher incidences of metastases in patients with larger primary lesions, there were notable exceptions. Nasopharyngeal carcinoma showed a very high incidence of metastasis, even in the smallest lesions measured (Lindberg, 1972). In addition, nasopharyngeal cancers spread lymphatically to bilateral sites, frequently in the neck, and often give rise to blood-borne metastasis to systemic organs. On the other hand, carcinoma of the lip was considered to be of very low metastatic potential, because the infrequent metastases found in this form of cancer were almost always localized in the first set of draining lymph glands (Lindberg, 1972).

Of the various classes of neoplasms it appears that rapidly disseminating malignancies, such as the leukemias and many of the lymphomas, have high metastatic potentials. These types of cancers appear to disseminate rapidly and widely, even when the primary tumor mass is small shortly after the inception of the disease (Sugarbaker, 1981).

3. Patterns of Metastatic Spread

Of the cancers classified by Sugarbaker (1981) as having "moderate metastatic potentials," most spread initially to regional lymphatic and/or other regional sites rather than to distant sites. The regional location of metastases can be explained, for the most part, by anatomical or mechanical considerations, such as efferent venous circulation and/or lymphatic drainage to regional lymph nodes. For example, in several of the squamous carcinomas of the head and neck, the most common metastatic sites of involvement are the lymph nodes related anatomically to the site of the primary tumor and to the anatomy of the draining lymphatics (Lindberg, 1972). Thus, the locations of a majority of the initial metastases produced from many cancers appear to be determined simply by regional anatomy.

Consistent with anatomical-mechanical theories of distant metastasis, the most frequent organ site of blood-borne metastatic involvement is the first organ encountered by the blood-borne tumor cells. However, metastasis formation occurs often in organs distant from the initial organ encountered and, as discussed elsewhere (Nicolson, 1982a; Sugarbaker, 1981; Nicolson and Poste, 1982), many cancers display unique organ colonization patterns that do not fit the anatomical-mechanical theory of cancer dissemination. For example, adenocarcinoma of the breast metastasizes to bone, brain, and adrenals in a large percentage of patients with metastatic disease, along with the expected high incidence of metastasis to lung (Viadina *et al.*, 1973). Similarly, one of the most common sites of metastatic involvement of carcinoma of the prostate is bone, an unexpected site of colonization if only anatomical-mechanical considerations determine metastatic locations (Prout, 1973). Further, anatomical-mechanical theories do not explain the high frequency with which malignant melanomas colonize liver, brain, and bone (Einhorn *et al.*, 1974; Patel *et al.*, 1978).

In contrast to preferential metastasis of particular cancers to certain organ sites, some sites may show a statistically significant lower frequency of metastatic involvement. For example, despite their extensive lymphatic and circulatory networks, muscles rarely serve as the initial sites of distant metastases. Of the major organs the kidneys are also less frequently involved in tumor metastasis compared to other major organs, even though the kidneys receive approximately one-fourth of the cystolic output of the heart.

In order to explain the metastatic colonization that could not be explained by lodgment of malignant cells in the draining regional lymph nodes or blood capillaries, Paget (1889) proposed the "seed and soil" hypothesis. This hypothesis proposes that the microenvironment of individual organs ("the soil") influences the implantation, survival, and growth of certain tumor cells (the "seeds"). It follows from this argument that *both* the tu-

mor cells and the organ colonized must have unique properties. Paget (1889) made his proposal on the basis of a comparison of the dissemination of bacteria with that of breast cancer cells. He found that the spleen was a common site of bacterial abcesses but not of metastatic breast cancer. He concluded that the spleen is not more frequently involved in tumor colonization because it is not a favorable site for the survival and growth of cancer cells that circulate to the spleen. It follows that cancer cells that lodged in the spleen do not have the correct properties for survival and growth at that site.

In contrast to the seed–soil hypothesis, Ewing (1928) proposed that cancer invasion and metastasis can be explained strictly on mechanical–anatomical considerations. These include the locations of the primary tumor and its relation to tissue barriers, zones of weakness in tissues surrounding the primary tumor, and barriers to lymphatic and blood-borne circulation, such as draining regional lymph nodes and blood capillaries at the first anatomical barrier encountered.

In distinguishing between these two hypotheses, it should be noted that many malignant diseases are best explained by a combination of the two theories. In the case of the formation of distant metastases, the lungs, which are the first organs encountered by most circulating metastatic cells released from peripheral sites, are usually the most frequent sites of blood-borne metastatic colonization. Although metastases often occur at sites beyond the lungs, these organs remain the most common site of distant blood-borne secondary tumors for many of the most common types of cancer. The most reasonable interpretation is that both the mechanical–anatomical and seed–soil theories contribute to the observed patterns of distant metastatic spread, but that their individual importance can vary widely in different tumor systems (Proctor, 1976; Nicolson, 1982a; Sugarbaker, 1981).

C. Modes of Metastatic Spread

1. *Lymphatic Systems*

The most common mode of invasion and of metastasis of many sorts of human cancer cells is through lymphatic channels (van de Velde and Carr, 1977; del Regato, 1977). It is thought that the progressive growth of tumor cells in a restricted space results in an increase in tissue pressure. This mechanical pressure, coupled with enzymatic processes mediated by the presence of tumor (and/or host) degradative enzymes results in invasion and penetration of lymphatic vessels and the subsequent release of tumor cells into the lymph fluid. Alternatively, lymphatic vessels are known to have thin walls with many endothelial fenestrations, and malignant cells may

passively enter the lymph through these discontinuities (I. Carr and Carr, 1982). Casley-Smith (1976) has estimated that about 2% of the lymphatic vessel borders are composed of open junctions and approximately 10% of closed junctions. Since the lymphatic vessels are not surrounded by a thick basal lamina or basement membrane, modifications in fluid pressures can easily push open the lymphatic vessel walls in the regions of open junctions. In addition, the number of open junctions in the lymphatics increases at centers of inflammation and high interstitial fluid pressures (Casley-Smith, 1977), both of which occur commonly at sites of tumor growth. Carr *et al.* (1976) have examined the passage of metastatic tumor cells into lymphatic vessels by electron microscopy and have noted that single migrating tumor cells penetrate through endothelial junctions and enter lymph similar to macrophages. Ultrastructurally, this penetration occurs first by the protrusion of tumor cell processes through open gaps between endothelial cells, and then by distortion of the tumor cells as well as distortion of the lymphatic endothelium into flaplike structures which presumably resume their original shapes after release of the malignant cells into the lumen (van de Velde and Carr, 1977). In some cases, cancers grow into the lymphatics. Extension and growth of cancer in the lymphatics was recognized long before the development of modern cellular theories of cancer invasion (Handley, 1922).

Cancer cells in the lymph inside lymphatic vessels are transported passively to the first draining lymph node. For a long time, the draining lymph nodes of tumors were thought to act as a "barrier" to the spread of cancer (reviewed in van de Velde and Carr, 1977). However, it is now generally considered that such a barrier is at best temporary since some tumor cells can quickly pass through the first lymph node they encounter. Thus, draining lymph nodes are thought to be more of a "filter" than a barrier. Zeidman and Buss (1954) utilized the rabbit VX2 carcinoma to show that some tumor cells can be temporarily arrested in the lymph nodes and remain there for several weeks, while others pass through rapidly. Indeed, in other studies, tumor cells were found to pass quickly through the lymphatics to distant lymph nodes (Carr and McGinty, 1974; B. Fisher and Fisher, 1965). To demonstrate this, Zeidman (1955) injected VX2 carcinoma cells into the thoracic ducts of rabbits and observed that these carcinoma cells could easily travel to the more distant intercostal, mediastinal, and supraclavicular lymph nodes. Once tumor cells arrive at the draining lymph node, they can proliferate, die, remain dormant, or enter the blood circulation.

There is evidence that malignant cells can be destroyed in the lymph nodes via host mechanisms in the absence of any anticancer therapy (Zeidman, 1965; Carr and McGinty, 1974). In their studies on the destruction of allogeneic VX2 carcinoma cells in regional lymph nodes to a growing subcutaneous VX2 carcinoma, Herman *et al.* (1976) found that the nodes

showed signs of transplantation immunity and some tumor cell destruction within 10–14 days after subcutaneous tumor cell implantation. Carr and McGinty (1974) utilized the rat Rd/3 tumor system to study the fate of malignant cells in regional lymph nodes. When Rd/3 cells reached the subcapsular and medullary sinuses of a lymph node and began to proliferate, histiocyte infiltration and stromal hyperplasia were observed. Fisher *et al.* (1972) have examined and compared, by means of *in vitro* cytolysis assays as well as by Winn assays *in vivo,* the abilities of lymphocytes from regional and distant lymph nodes of tumor-bearing animals to kill tumor cells. The immunocytes from regional lymph nodes were more effective in these assays, a result that suggests that antitumor responses can occur in the tumor-draining lymph nodes. However, such results are not always obtained, notably in tumors that arise as spontaneous or chemically-induced primary lesions (Alexander *et al.,* 1967).

The kinetics of spontaneous metastasis of squamous cell carcinoma to regional lymph nodes of mice has been studied by Hewitt and Blake (1975, 1977). These authors found that tumor growth in the regional lymph nodes did not parallel the growth of the primary tumor in a system which was considered to be of low immunogenic potential. They concluded that many tumor cells capable of reaching the lymph nodes did not survive, even in the absence of demonstrable immunologic responses.

2. *Blood Circulation*

Malignant cells can enter the blood by direct invasion of blood vessels (intravasation) or via lymphatic channels across lymph nodes. Invading cancer cells have often been observed growing along large veins for some distances, as well as penetrating into blood vessels and proliferating intravascularly (Willis, 1973). The frequency of invasion of blood vessels by malignant cells appears to be fairly high. Willis (1973) found that 143/500 necropsies from all types of cancer showed evidence of venous invasion. He estimated that greater than half of all fatal cases of malignant disease would probably show neoplastic penetration into blood vessels if subjected to detailed histologic examination. Once in the veins, some cancers, particularly carcinomas, have been observed to extend for long distances inside unoccluded blood vessels.

When cancers reach a certain size, usually 1–2 mm, their growth is slowed due to the lack of sufficient oxygen and nutrients and to the build-up of toxic waste products (reviewed by Folkman, 1974, 1975). Once vascularization (angiogenesis) of the tumor has occurred, rapid rates of neoplastic growth are resumed. Angiogenesis appears to be controlled by substance(s) released from tumor cells that stimulate vascular endothelial cell proliferation and movement and rearrangement of basal lamina. Tumor angiogen-

esis promoting factor (tumor angiogenesis factor, TAF) and possibly other factors appear to be involved in the neovascularization of cancers. This will be discussed in more detail in Section VII,B.

Malignant cells that enter blood vessels can also be detached and transported to distant sites. The rates at which malignant cells are shed into the circulation have been determined for a few tumor systems. Tumor cell shedding into the afferent venous circulation has been measured by Butler and Gullino (1975). Using rat MTW9 mammary carcinoma transplants into rat ovaries which were in turn grafted at a subcutaneous site, these investigators found that tumors in the range of 2–4 gm released up to 4×10^6 cells per gram of tumor tissue per day. It is likely that the overwhelming majority of the carcinoma cells released into the circulation was destined to die and would not survive to lodge, invade, and grow at secondary sites. The heterogeneous nature of malignant cells and the fact that probably only very few cells are capable of completing the metastatic cascade will be discussed later in this review (Section IV,A).

The rates of tumor cell release into the blood appear to correlate with the size of the primary tumor, at least in the systems which have been carefully analyzed. Liotta *et al.* (1976) have studied the relationship of tumor size and tumor cell release into the circulation. When the T241 fibrosarcoma was implanted subcutaneously in the legs of syngeneic mice, these authors found the rate of tumor cell release to increase with the size of the subcutaneous tumor. Using this same system, Kleinerman and Liotta (1977) found that as the tumor implant grew, the size distributions of tumor cell clumps remained relatively constant. However, in the larger blood vessels tumor cell clumps were of much greater size than in the small vessels in the immediate vicinity of the tumor.

It is now apparent that most circulating tumor cells are rapidly eliminated from the blood stream (Fidler, 1970, 1976b; Butler and Gullino, 1975). After an exhaustive survey of the clinical literature, Salsbury (1975) concluded that the presence of tumor cells in the circulation is not of prognostic significance, and he thought that most of these tumor cells fail to form secondary tumors. Malmgren (1967) also noted that the incidence of metastases in patients did not correlate with the presence of tumor cells in the blood.

Since most tumor cells in the circulation apparently do not survive to form distant metastases, researchers have looked for mechanisms which result in the destruction of tumor cells during their circulation. It is here that nonspecific immune mechanisms mediated by natural antibodies, natural killer (NK) cells, or natural cytotoxic (NC) cells appear to be important. The evidence in favor of these host-mediated surveillance systems will be discussed in Section VII,B.

The success of blood-borne metastasis is determined by a complex inter-

play of both tumor and host factors, including the abilities of circulating malignant cells to survive the shear forces and trauma in the circulation, to evade destruction by host defense mechanisms, to implant or attach to endothelial cells in distant vessels, to become extravasated or undergo secondary invasion, and, of course, to survive at the secondary tumor site. These aspects of blood-borne implantation, invasion, survival, and growth will be discussed in Sections V and VI.

3. *Serous or Coelomic Cavities*

Once malignant cells invade a serous cavity, such as the peritoneal, pleural, or pericardial space, single cells or clumps of tumor cells can break off and seed over the serous membranes. This event is a major clinical problem during metastasis of ovarian carcinoma where the invasion and release of tumor cells into the peritoneum can result in multiple tumor growths at sites of implantation. Similarly, carcinomas of the prostate, pancreas, uterus, and kidney can, on occasion, disseminate into the peritoneal cavity, and carcinomas of the lung occasionally invade the thoracic cavity and release tumor cells or tumor cell clumps into it. Pericardial implants have only rarely been seen, but they do occur (Willis, 1973).

Free tumor cells or tumor cell clumps have often been observed in serous fluids. Various early reports (reviewed in Willis, 1973) have documented that a variety of tumors can metastasize by release into serous fluids, and by implantation on and invasion through serous membranes. This is particularly apparent after tumor cell release into ascites fluid, secondary to intraperitoneal invasion of cancer. Once the tumor cells have implanted, they can grow on the serous membrane surface or invade into the subjacent tissues.

4. *Epithelial Cavities*

Although rare, there are a number of documented instances in which metastasis has occurred by "contact" implantation of detached tumor cells on epithelial surfaces. Carcinomas have been transferred by direct contact from one epithelial surface to another; for example, from one lip to the other, or from the vaginal wall to the cervix or to the opposite vaginal wall. In some cases, mechanical trauma results in the release of cells and their subsequent implantation and growth at distant sites. Brøyn (1972) implanted Walker 256 carcinoma cells in the colonic mucosa of rats and studied their trauma-induced transplantation to other regions of the colon. However, even without mechanical trauma, metastasis occurred in approximately 15% of the animals. In other spaces, such as those in the lungs, aspiration appears to cause at least some metastases. This has been indirectly documented by examining the incidence of metastases of basal cell carcinoma to the lungs. Basal cell carcinoma of the skin normally has a

very low incidence of metastasis, although it can be highly invasive. Pickren and Katz (1958) proposed that in certain cases lung metastases result from aspiration of viable tumor cells from the skin. This has been confirmed; for example, in the case reported by Guillan and Johnson (1978), the patient exhibited recurrent basal cell carcinoma of the face, difficulty in swallowing, and apparent aspiration accompanied by choking and coughing. At autopsy, basal cell carcinoma was found in bronchi. Histologic examination revealed structural similarities between the primary skin tumor and the lung metastases. No other evidence of metastasis was found. The potential for metastasis by inhalation of neoplastic cells through respiratory passages was demonstrated earlier by Furth (1946).

III. Animal Tumor Models of Implantation, Invasion, and Metastasis

It is instructive to clarify the terminology used to classify tumor cells and their behavior before discussing the choice of tumor systems and selection procedures to obtain invasive and metastatic cells. For the purposes of our discussion, the terms "benign," "invasive," or "malignant" will be used to describe the inability (benign) or ability of tumor cells to invade (invasive) and metastasize (malignant). Although invasion is a requirement of malignant tumors, invasiveness and metastatic ability are not always correlated (see discussion in Section II).

Another term, "transformation," was first used to describe morphologic changes in cell populations *in vitro* whether they occurred spontaneously or after treatment with chemicals, viruses, or radiation. A number of different cellular properties are known to be modified by transformation *in vitro* (see Nicolson, 1976a,b; Nicolson and Poste, 1976; Hynes, 1976; Roblin *et al.,* 1975). Unfortunately, most of the properties that define the transformed state *in vitro* have been used uncritically to determine that a cell population has undergone neoplastic conversion. Obviously the latter property must be assayed *in vivo* to determine if the transformed cells are tumorigenic. Indeed, many established heteroploid cell lines have been called "normal" in order to compare them with various transformed derivatives. However, in many cases the untransformed cells are themselves tumorigenic, which confuses the whole issue of the relationship between transformation and tumorigenicity.

In order to relate transformed properties of cells with events *in vivo* many investigators have utilized the term "malignant transformation." Unfortunately, this term is still being used widely to describe *in vitro* criteria, and in many instances the tumorigenic, invasive, or metastatic potential of the cells to which this term is applied is completely unknown.

A. Some Experimental Considerations

One of the major problems in examining human tumors for their invasive and metastatic properties has been the lack of an appropriate *in vivo* assay system. In order to overcome this problem, immunosuppressed animals have been used as recipients of human tumor xenografts, or, alternatively, human tumor cells have been implanted at immunologically privileged sites in animals. Unfortunately, it is quite difficult to maintain large numbers of immunosuppressed animals for such experiments, and the grafting of human tumor cells into nonimmunosuppressed animals has not been widespread.

The congenitally athymic nude mouse has been the most popular experimental tool for studying the *in vivo* growth of human tumors. However, the main problem in utilizing nude mice is that most malignant tumors are rarely metastatic in these animals. More recently it has been observed that even highly metastatic murine tumor cells show very low incidences of metastasis or blood-borne implantation, survival, and growth in nude mice (Fidler and Nicolson, 1978; Fidler *et al.,* 1977; Hanna, 1980). Hanna and Fidler (1981) found that a variety of malignant tumors which are nonmetastatic in adult nude mice were capable of metastasizing at high frequencies in newborn or 3-week-old nude mice, or in nude mice after X-irradiation. The interpretation of their results was that the immune system in young nude mice must undergo further maturation before it is fully competent in inhibiting metastatic processes. The component of the immune system that appears to be responsible for preventing tumor metastasis in adult nude mice is the natural killer system which, in older animals, appears to render them resistant to the spread of most metastatic tumor cells (Hanna and Fidler, 1981; Hanna, 1982). The newborn nude mouse is thus a suitable recipient for evaluating the metastatic potential of human tumors.

Important questions have been raised concerning the transplantation of human tumors into nude mice. Since nude mice appear to be somewhat immunologically competent and may be able to mount at least feeble natural responses against tumor xenografts, immunoselection may occur during the transplantation of human tumor cells in such recipients. Information is not readily available on nonimmune host mechanisms that can affect tumor cells. For example, changes in tumor–stromal requirements may result in the selective survival of human tumor cells with particular properties. Another important question concerns whether the cellular diversity present in the original human tumors can be maintained in the xenogeneic grafts. Also, the potential biohazards associated with the transplantation of human xenografts in nude mice are often only casually considered by researchers (Poste, 1982a).

The large-scale establishment of cell lines or strains from solid human

tumors and the development of cell lines from both primary and metastatic lesions from the same patient have not been achieved. This has been due mainly to the difficulties encountered in obtaining enough fresh tissues immediately after surgery, the lack of appropriate facilities for their culture, and the use of suboptimal conditions for their growth, *in vitro* or for their transplantation *in vivo*.

The most worrisome problem, however, may occur during transplantation and may plague all such xenograft experiments. This is the possibility that human tumor cells, once in a recipient such as the nude mouse, can fuse with normal surrounding cells resulting in the formation of heterokaryons. Many investigators have found that the karyotypes of tumor cells obtained from human tumor xenografts are hybrids of human and nonhuman cells, and may even be apparently nonhuman. These events may also have been responsible to some degree for the incorrect classification of nonhuman tumor viruses as being of human origin.

The practical problem in conducting studies with animal tumor tissue (especially with human tumor tissue) is the lack of directly comparable primary and secondary metastatic lesions. This problem is compounded in human cancer when metastases may be detected clinically months, or even years, after the primary tumor has been removed surgically. Irrespective of the financial problems in establishing the facilities necessary for primary and secondary human tumor culturing and transplantation, there are certain types of human cancer cells, such as human lymphoid tumors, that are readily available for experimental study. Unfortunately, this has not been the case for cell lines or strains established from "solid" tumors derived from both primary and metastatic lesions in the same patient.

B. Choosing Animal Tumor Models

1. *Criteria for Animal Tumor Models*

Animal tumor models that mimic human metastatic disease are available for studying tumor implantation, survival, and growth at metastatic sites in syngeneic recipients. In the last section we discussed some of the problems inherent in studying human xenograft tumors in nude mice. In the case of animal tumor models, the extensive use of experimental tumors transplanted in their syngeneic hosts has provided invaluable information on the role of tumor cell and host properties in metastatic processes. In addition to their importance to the gathering of information about basic mechanisms of invasion and metastasis, the animal tumors have also been useful in developing new procedures for the detection, prevention, and cure of human cancer.

The animal tumor models of metastasis currently in use may be of only

limited value in answering certain questions about the metastatic process. On the other hand, some animal tumor models may be quite valid for studying a wide range of different tumor and host characteristics important in metastasis. The usefulness of each model in suggesting the possible answers to questions posed by clinical diseases will, of course, depend on how closely the animal tumor model mimics the human counterpart disease. Unfortunately, it can be argued that many of the most widely used transplantable animal tumor models do not reflect the events that occur when a spontaneous human neoplasm progresses to malignancy and metastasizes in its natural host (Hewitt, 1976, 1978).

It is astonishing that articles exist in the literature which claim that particular tumor properties are associated with malignancy or metastasis when the tumors under examination were incapable of metastasis in their syngeneic host. A most obvious requirement is that the animal tumor must be metastatic in its syngeneic host. A more difficult question is whether spontaneous transplanted tumor should be used to study metastasis. Spontaneously arising tumors are certainly more relevant to the metastatic process. The problem here is that most spontaneous neoplasms arise only rarely in laboratory animals, a fact that makes it extremely expensive and time-consuming to accumulate enough experimental data. There are other criteria which should also be fulfilled by animal tumor models of metastasis (Table II).

Most of the available animal tumor models were established originally from relatively common animal tumors that have been adapted to grow *in vitro* (Table III). Although many of the animal tumor models utilized in studies of metastasis are sarcomas, the majority of metastatic human tu-

TABLE II

Essential Criteria for the Establishment of an Animal Tumor Model for Invasion and Metastasis[a]

The origin and subsequent passage history of the tumor should be known before it has been received in the investigator's laboratory
The tumor should be transplantable in syngeneic hosts and produce a consistent pattern of metastatic disease in defined organs
The tumor must have growth properties in *vitro* similar to those found *in vivo*
The phenotypic properties expressed by the cells in tissue culture must persist *in vivo,* and vice versa
Procedures for staging tumor spread and for the quantitation of metastatic burden should be available and standardized
Other similarities to the human disease, such as cell and organ of origin, pattern of tumor spread, and pathogenesis of metastasis, should be present
For the development and screening of therapeutic agents, the animal tumor should be susceptible to the same range of therapeutic modalities as its human counterpart

[a]From Poste (1982b).

TABLE III
SOME ANIMAL TUMOR MODELS FOR STUDYING METASTASIS[a]

Species	Tumor	Major site(s) of metastic colonization
Mouse	MCA-10 sarcoma	Lung
	MDAY-D2 undifferentiated tumor	Lung > liver, spleen
	JR-5 fibrosarcoma	Lung
	Type A reticulum cell sarcoma	Liver > spleen
	M5076 reticulum cell sarcoma	Liver, spleen
	SV3T3 SV40-transformed sarcoma	Lung
	KHT sarcoma	Lung
	CMT-64 tumor	Lung
	MCA-3256 fibrosarcoma	Lung
	MS-2 Moloney sarcoma virus-transformed tumor	Lung > spleen, liver, brain
	PMT fibrosarcoma	Lung
	FSA fibrosarcoma	Lung
	UV2237 fibrosarcoma	Lung $\gg$ other sites
	B16-F10 melanoma	Lung > ovary > other sites
	B16-013 ovary-selected melanoma	Ovary, lung
	B16-B15b brain-selected melanoma	Brain, lung
	B16-BL6 invasion-selected melanoma	Lung, lymph nodes
	S91 Cloudman melanoma	Lung
	K1735 melanoma	Lung
	X5563 plasmacytoma	Spleen
	Kobayashi plasmacytoma	Bone
	Eb lymphoma	Liver
	RAW117-H10 liver-selected lymphosarcoma	Liver, spleen
	RAW117-L17 lung-selected lymphosarcoma	Lung, liver
	CT26 colon carcinoma	Lung
	2661 mammary carcinoma	Lymph node, lung
	Lewis lung carcinoma	Lung
	M109 carcinoma	Lung
	Colon 36 carcinoma	Lung
	MC mammary carcinoma	Lung
	2661 mammary carcinoma	Lung
Rat	13762 mammary adenocarcinoma	Lung, lymph nodes
	ARG-1-RT7 hepatocarcinoma	Lung
	Flexner-Jobling carcinoma	Kidney, adrenals
	Walker 256 carcinoma	Lung
	AH Yoshida ascites hepatoma	Lung
	Line 1 colon adenocarcinoma	Lymph node, lung
	R39 sarcoma	Kidney, adrenals
	PA III prostate adenocarcinoma	Lung
	SP4 carcinoma	Lymph node, lung
	Yoshida sarcoma	Lymph node, lung

(*continued*)

TABLE III (*cont.*)

Species	Tumor	Major site(s) of metastic colonization
Hamster	Schwanian sarcoma	Lung
	Melanotic melanoma	Lung
Guinea pig	Line 10 hepatocarcinoma	Liver, lymph nodes
Rabbit	V_2 carcinoma	Liver, lung
Chicken	Herpesvirus-transformed AL.2 lymphoma	Liver

[a]For references see Nicolson (1982a).

mors are carcinomas. Almost all of the animal tumor models listed in Table III grow rapidly and kill their hosts within 3 to 6 weeks. These rapid growth rates reflect short cell cycle times, high growth fractions, and relatively low cell loss rates. Unfortunately, these characteristics do not appear to be comparable to slowly growing primary human or animal tumors, which often take years to grow to clinically detectable sizes and even more time to metastasize. Often the animal tumors are extremely anaplastic and display considerable variation in their karyotypes. Thus, most animal models can only be considered useful for mimicking the most rapidly growing aggressive human cancers.

The most common spontaneous animal tumors used for studies of metastasis are those that arise at high incidence in specific strains of laboratory rodents. While it is not true that animal tumors from larger animals, such as dogs, cats, pigs, and cattle, are not useful, it is a fact that detailed analysis of such tumors is financially demanding and thus unattractive. Although outbred populations of large farm animals can be expected to be closer to the situation in humans than to that in mice or rats, the use of inbred rodents for the induction of tumors and for studies of tumor metastasis permits study in multiple syngeneic hosts.

2. In Vivo *Assays for Implantation, Invasion, and Metastasis*

Assays for invasive or metastatic properties of tumor cells *in vivo* should reflect or mimic the natural events as closely as possible. This has sometimes proved difficult, even with almost identical tumor cell populations and uniformly matched syngeneic hosts, perhaps because even minor modifications in tumor cell and/or host properties can result in marked changes in invasive and/or metastatic properties *in vivo* (Fidler, 1978a). Stringent, uniform protocols are necessary to maintain uniformity in tumor cell

preparations, as well as in host animals. The animals used for assays of invasion and metastasis must be matched for age, weight, sex, and colony, and they should be maintained in an excellent state of health under stable environmental conditions. In our laboratories, we routinely screen for such pathogens as mycoplasma and viruses, and serum samples are collected and maintained frozen for future screening purposes. It is extremely important that animal tissues are assayed for a variety of viruses and other pathogens. It is our experience that modifications in host environment, infections, and trauma can result in differences in the metastatic assays. These factors can modify host immune responses, which often have complex relationships with invasion and metastasis (see Section VII).

The two most widely utilized assays for determining the invasive and/or metastatic properties of tumors are the so-called "spontaneous" and "experimental" invasion and metastasis assays. In the spontaneous metastasis assay, transplantable tumors or tumor cells are implanted subcutaneously or intramuscularly, and the ability of the implanted cells to invade surrounding tissue or to metastasize to regional lymph nodes or distant organs is then monitored at various times. In experimental assays of metastasis, the initial steps of primary invasion and dissemination are eliminated by injecting tumor cells directly into the circulation. Tumor colonies in various organs are measured and scored after blood-borne implantation, invasion, survival, and growth. This experimental metastasis assay method introduces the risk that in various organs tumor colonies may be formed from cells that would not have been invasive at implant sites or able to disseminate rapidly into the lymphatics or blood circulation.

The spontaneous and experimental metastasis assays have been compared in disparate tumor systems, with differing results. In some of these animal tumor models, such as the B16 melanoma (Giavazzi *et al.*, 1980; Stackpole, 1981), F9-4 rhabdomyosarcoma (Sweeney *et al.*, 1982), and the M5076 reticulum cell sarcoma (Giavazzi *et al.*, 1980), the abilities of different tumor cell clones or sublines to form lung tumor colonies varied after subcutaneous or intravenous injections of tumor cells. Closer examination of the B16 melanoma system by means of a series of B16 clones revealed that some of the clones that metastasize spontaneously from subcutaneous sites consistently produced lung tumor colonies after intravenous injection; however, other clones that were fully capable of metastasizing in the spontaneous metastasis assays were not always able to metastasize readily from subcutaneous sites (Stackpole, 1981; Poste 1982a).

There are animal tumor models that show a nice correspondence of the spontaneous and the experimental metastasis assays. Kripke *et al.* (1978) examined the metastatic behavior of 21 clones of the UV2237 fibrosarcoma in three assays for metastasis, i.e., the intravenous experimental metastasis assay, the spontaneous or subcutaneous assay, and the survival times of animals injected subcutaneously. These assays were scored by determining

the number of lung tumor nodules at a fixed time or by recording time of death. A good correlation was found between the various assays when 21 clones were ranked in order of increasing metastatic potential. However, there were exceptions, and a few of the clones did not have exactly the same rank order in each of the assays.

Comparisons of the spontaneous and experimental metastasis assays have been made in different ways with cell clones of the rat 13762 mammary adenocarcinoma (Welch *et al.,* 1983b). By examining the number of lung tumor colonies and the total tumor burden (total tumor volume) the two assays were quantitatively comparable for a number of tumor clones. The data (Table IV) indicate that there is an excellent correspondence in this system between the rank order of tumor cell clones in the two assays, both in terms of the number of lung lesions formed and in terms of the total volume of tumor tissue in the lungs. Like Kripke *et al.* (1978), we found that one of the 13762NF tumor clones did not always yield the same rank order position for metastatic potential in the various assays. These studies are instructive, however, because they indicate that in certain animal tumor models assays of experimental metastasis are just as valid as assays of spontaneous metastasis since both yield the same results.

It is quite reasonable that in certain animal tumor models cell sublines or clones are capable of producing experimental tumor colonies after intravenous injection of tumor cells, but are incapable of generating tumor colonies in various organs after subcutaneous tumor implantation of tumor cells. In order for tumor cells to metastasize from implant sites, they must possess all of the properties important for blood-borne implantation, invasion, survival, and growth, and they must also be competent to complete primary invasion, penetration into blood vessels, and dissemination in the blood in large numbers so as to form blood-borne tumor colonies in distant organs. If a locally growing tumor has a low rate of entry into blood vessels, detachment, and dissemination, its spontaneous metastatic potential may be negligible (Tarin and Price, 1979).

Another criticism of the experimental intravenous metastasis assay is that tumor cells injected intravascularly by different routes often produce different patterns of tumor colonization (Roos and Dingemans, 1979; Conley, 1982). In certain cases, this may be caused by the mechanical trapping of tumor cell emboli in the first organ bed encountered. Alternatively, it has been apparent for a number of years that malignant cells or their emboli can undergo transorgan passage at high efficiencies and recirculate to other sites (Zeidman and Buss, 1952; Zeidman, 1961). Fidler and Nicolson (1976) compared lung-colonizing B16 melanoma sublines for their abilities to form lung and other organ tumor colonies after intravenous or intracardiac injection. They found that the same number of gross lung tumor colonies was formed by either injection route, even though after intracardiac injection

TABLE IV

COMPARISON OF "SPONTANEOUS" AND "EXPERIMENTAL" METASTASIS ASSAYS USING CELL CLONES OF THE RAT 13762NF MAMMARY ADENOCARCINOMA[a]

13762NF clone	*In vitro* passage number	Clonal origin	Sample number[b]	Average number of surface lung metastases mean; (95% C.I.)	Rank order	Average volume of lung metastases (mm^3) mean; (95% C.I.)[c]	Rank order
Spontaneous metastasis assays[d]							
MTC	9–11	Local tumor	20	0 (—)	1	0 (—)	1
MTF7	31	Local tumor	32	0.69 (0,1.64)	2	0.36 (0,0.86)	2
MTLn2	35–44	Lung metastases	55	7.00 (0,16.26)	3	3.67 (0,8.51)	3
MTLn3	39–41	Lung metastases	39	28.59 (3.89,53.24)	4	14.97 (2.04,27.90)	4
MTC	14–21	Local tumor	60	29.18 (15.43,42.83)	5	15.28 (8.14,22.42)	5
MTF7	10–12	Local tumor	100	34.19 (20.71,47.67)	6	17.90 (10.84,24.96)	6
MTLn3	14–15	Lung metastases	60	68.03 (43.85, 92.21)	7	35.62 (22.96,48.28)	7
MTA	10–18	Local tumor	39	89.51 (60.88,118.15)	8	46.87 (31.88, 61.86)	8
MTLn3	44–47	Lung metastases	48	132.90 (97.23,168.56)	9	69.58 (50.91,88.26)	9
Experimental metastasis assays[e]							
MTC	9–13	Local tumor	68	0.13 (0.01,0.25)	1	0.07 (0.01,0.13)	1
MTC	15–20	Local tumor	55	0.18 (0,0.36)	2	0.10 (0,0.19)	2
MTLn2	39–41	Lung metastases	44	5.17 (2.25,8.10)	3	8.17 (1.52, 14.82)	3
MTLn3	15–18	Lung metastases	50	10.06 (6.67,13.45)	4	75.69 (43.94, 107.44)	6
MTF7	13–17	Local tumor	50	50.09 (27.91,72.27)	5	41.42 (23.56, 59.28)	4
MTF7	31	Local tumor	60	83.92 (55.18,112.66)	6	62.03 (41.02, 83.03)	5
MTLn3	41–46	Lung metastases	35	102.95 (67.67,138.23)	7	263.38 (145.29, 381.47)	7

[a] Data of Welch *et al.* (1983b).

[b] Pooled from a minimum of three independent experiments.

[c] Volume based on the assumption that lung metastases are spherical.

[d] 1×10^6 cells were injected subcutaneously into the left inguinal mammary fat pad of 6- to 9-week-old female Fischer 344/CRBL rats. Animals were sacrificed 23 days after injection, and the numbers and sizes of lung metastases were recorded.

[e] 5×10^4 cells were injected intravenously into the lateral tail vein of 6- to 9-week-old female Fischer 344 rats. Animals were sacrificed 23 days after injection, and the numbers and sizes of the lung metastases were recorded.

the tumor cells had to escape the extrapulmonary organ sites and recirculate to the lung to form pulmonary tumor colonies. In a later experiment, parabiotic animals were used (Fidler and Nicolson, 1977) and it was shown that injection of lung-colonizing B16 melanoma cells into one parabiont of a pair of parabiotic mice resulted in tumor colony formation in the lungs of the uninjected partner. This experiment confirmed that melanoma cells destined to colonize the lungs could recirculate after initial arrest in the lung of the injected partner and rearrest in the lung of the uninjected one after passage through the site of surgical anastomosis. However, in other experiments with B16 melanoma cells, Kawaguchi *et al.* (1983a) found differences in the assays of experimental metastasis after brain-colonizing B16 cells were injected intravenously, intracardially, or via the internal carotid artery. As expected, more brain tumor colonies formed after injection of B16 cells into the internal carotid artery.

One of the major problems in comparing results from different laboratories using the same animal tumor system may arise from modifications in cell growth conditions that lead to divergent cell populations. The use of short-term cultures and routine, parallel, biologic assays of invasion and metastasis can alleviate problems associated with phenotypic divergence or drift during prolonged growth *in vitro* (reviewed by Poste, 1982a; Nicolson, 1982a). In addition, growth in culture is probably selective, and it can result in the loss of certain phenotypic properties (see Nicolson, 1982a). To minimize the extent of phenotypic drift of tumor cell properties during growth *in vitro,* it is essential that routine passaging of tumor cells be conducted under standardized conditions which stabilize the tumor cell populations. It is also important to prepare frozen stocks at 2- to 4-week intervals because of the possibility of a change of behavior of the tumor cell strains or lines upon continued growth (Poste, 1982a; Nicolson and Poste, 1982).

Prolonged serial passage *in vivo* using tumor fragments rather than single cell suspensions can also result in selection of tumor subpopulations due to zonal variations in the cells populating tumors (Fidler and Hart, 1981a). Repeated passage of tumor cells or cell clones through the peritoneal cavity is known to result in population drift or selection of more malignant subpopulations (Klein, 1955). Prolonged passage in tissue culture can also result in drastic alterations of the metastatic phenotypes of cell lines and clones (Miner *et al.,* 1982; Neri and Nicolson, 1981) (this problem will also be considered in Section IV,B).

C. Selection of Invasive/Metastatic Variants

One of the more common characteristics of malignant tumors is that they are composed of heterogeneous cellular populations. This has been reviewed in detail elsewhere by Hart and Fidler (1981), Nicolson (1982a),

Poste (1982a), and Nicolson and Poste (1982), and will be discussed in Section IV,A. Although the number of different cell subpopulations within a tumor cannot be accurately determined, it is probably dynamic and fluctuates in response to small changes in the host environment (Poste, 1982a; Nicolson and Poste, 1982, 1983).

The actual number of invasive and/or metastatic cells in a tumor cell population is probably small. For example, Fidler (1976) found that only a very small proportion (<0.1%) of B16 melanoma cells successfully implanted, survived, invaded, and grew to form gross pulmonary tumors after intravenous injection. Considering the number of different and diverse tumor cell properties that are probably important in the formation of metastases, it is reasonable to suppose that highly invasive and/or metastatic cells comprise a rather small proportion of the cell subpopulations within a tumor. It is also likely that a variety of different tumor cell subpopulations exist which can complete many, but not all, of the steps required for successful invasion and metastasis. Indeed, Poste *et al.* (1980) have isolated tumor cell subpopulations that were invasive through tissue *in vitro;* however, when tested *in vivo,* invasive subpopulations selected in certain tissues were found to be highly metastatic, but subpopulations selected for invasiveness in other tissues were found to be nonmetastatic, findings which indicate that these invasive tumor cells may have some, but not necessarily all, of the properties necessary for metastasis.

Invasive and/or metastatic cell subpopulations may possess certain deficiencies that prevent them from efficiently completing certain steps of the metastatic cascade. However, such malignant cells may overcome these deficiencies by being much better at other steps in the metastatic pathway. Thus, a direct correlation between a given property and metastatic potential may not always be found in a metastatic tumor system. Yet, a threshold level of a given property may be sufficient to allow malignant cells to complete a certain step and eventually gain metastatic status.

For the most part, three different approaches have been used to obtain tumor cell subpopulations with different invasive and/or metastatic properties:

1. Selection and sequential enrichment of metastatic cell subpopulations *in vivo*.
2. Sequential selection, either *in vivo* or *in vitro,* of tumor cell subpopulations with characteristics considered important in invasion and/or metastasis.
3. Single cell cloning of tumor cell populations to obtain clones differing in their invasive and/or metastatic properties.

In some cases, combinations of the above schemes have been used to yield tumor sublines or clones with particular characteristics.

Koch (1939) used sequential enrichment of tumor cell subpopulations to obtain cells with more metastatic phenotypes, and a similar procedure was utilized by Klein (1955). Klein (1955) repeatedly selected for survival and growth of tumor subpopulations in ascites fluid. Upon assaying the ascites-selected carcinoma line, she found that it possessed increased potential of metastasis to distant sites.

One of the most widely used procedures for obtaining cell sublines that differ in their experimental metastatic properties was developed by Fidler (1973b) to select B16 melanoma cells sequentially for their abilities to implant, invade, survive, and grow to form macroscopic pulmonary tumor colonies in syngeneic mice. Since Fidler's first experiments, a whole series of different B16 melanoma sublines showing differing preferences for colonizing individual organs have been selected sequentially after intravenous injection of suspensions of single tumor cells. Sublines are now available with enhanced abilities to colonize brain parenchyma (Brunson *et al.,* 1978; Raz and Hart, 1980), brain meninges (Miner *et al.,* 1982), liver (Tao *et al.,* 1979), and ovary parenchyma (Brunson and Nicolson, 1979). Alternatively, sequential selections *in vivo* can be made by inoculating tumor cell suspensions subcutaneously and allowing the tumor cells to metastasize spontaneously (McGuida *et al.,* 1980; Neri *et al.,* 1982). All of these procedures yield cell sublines that remain heterogeneous (polyclonal), although probably less heterogeneous than the original parental tumor from which they were selected (Reading *et al.,* 1980b; Fidler and Nicolson, 1981; Miner *et al.,* 1982; Poste *et al.,* 1982b).

A second commonly used method for isolating cell sublines with differing invasive and metastatic properties has been to select for subpopulations which exhibit certain properties considered important for invasion and/or metastasis. Thus, tumor cells have been selected for enhanced tissue invasiveness, increased blood-borne arrest properties, increased rates of attachment to endothelial basal lamina, increased production of degradative enzymes, enhanced resistance to host-mediated defense mechanisms, and other properties thought to be important in invasion and/or metastasis (reviewed by Nicolson, 1982a; Poste, 1982a; Nicolson and Poste, 1982). Tumor cell subpopulations that possess or lack certain properties of interest are recovered and then assayed *in vivo* in order to determine if their invasive and/or metastatic potentials have been modified concomitant with the acquisition or loss of the property of interest. By *in vitro* techniques, variants have been selected in quite different tumor systems for loss of sensitivity to lymphocyte-mediated cytotoxicity (Fidler *et al.,* 1976), resistance to complement-dependent antibody-mediated cytotoxicity (Frost and Kerbel, 1981), increased resistance to lectin toxicity (Dennis and Kerbel, 1981; Tao and Burger, 1977), decreased binding to immobilized lectins (Reading *et al.,* 1980a), increased ability to invade chorioallantoic membrane (Hart, 1979;

Poste *et al.,* 1980) or intact blood vessels (Poste *et al.,* 1980), increased resistance to NK cell-mediated killing (Hanna and Fidler, 1981; Gorelik *et al.,* 1982a), increased adhesion to collagen (Liotta *et al.,* 1980a), or increased detachment from tissue culture substrata (Briles and Kornfeld, 1978). The selected variants, when assayed *in vivo,* often show alterations in their invasive and/or metastatic properties consistent with their altered phenotypes.

A major strategy for obtaining metastatic cell subpopulations has been the use of cell cloning techniques. Once tumor cell clones have been obtained, they can be analyzed directly, assuming that their phenotypic properties remain stable during their propagation *in vitro.* This latter point is not trivial, and the instability of clonal tumor cell populations is notable (see Section IV,B). However, the instability of clonal tumor cell subpopulations can be utilized to confirm the importance of the given property in invasive and/or metastatic processes. For example, cell clones of a meninges-colonizing B16 melanoma variant have been followed during propagation *in vitro* with respect to their cell surface and brain-colonization properties (Miner *et al.,* 1982). In this case we were particularly interested in a cell surface glycoprotein with a molecular weight of 90,000 that was detected by surface-labeling techniques. This glycoprotein increased in degree of exposure and/or amount during three independent sequential selections for brain colonization. When the B16 cell clones of high brain-colonization potentials were examined for the 90,000 MW component, we found that clones with high brain-colonization potential displayed increased surface exposure or amount of this component, while clones of low colonization potential did not (Fig. 1). Since several of the brain-colonizing clones were found to be unstable during serial passage *in vitro,* we examined these clones at various times during growth in tissue culture for the presence of the 90,000 MW component. In almost every case we found losses in amounts or exposure of the 90,000 MW component as the unstable clones lost their abilities to colonize meninges (Miner *et al.,* 1982). This particular glycoprotein is interesting since it may be related to an oncofetal antigen (Nishio *et al.,* 1982) that could be important in brain colonization and/or tumor cell survival (Miner *et al.,* 1982).

D. Adaptation versus Selection in the Origin of Metastatic Variants

Distant metastasis may result from random arrest and survival of tumor cells in distant organs, or alternatively it could result from nonrandom, preferential implantation, survival, invasion, and growth of tumor cell subpopulations at specific sites. The ability to select variant sublines of tumor cells that show quite different patterns of organ colonization argues against

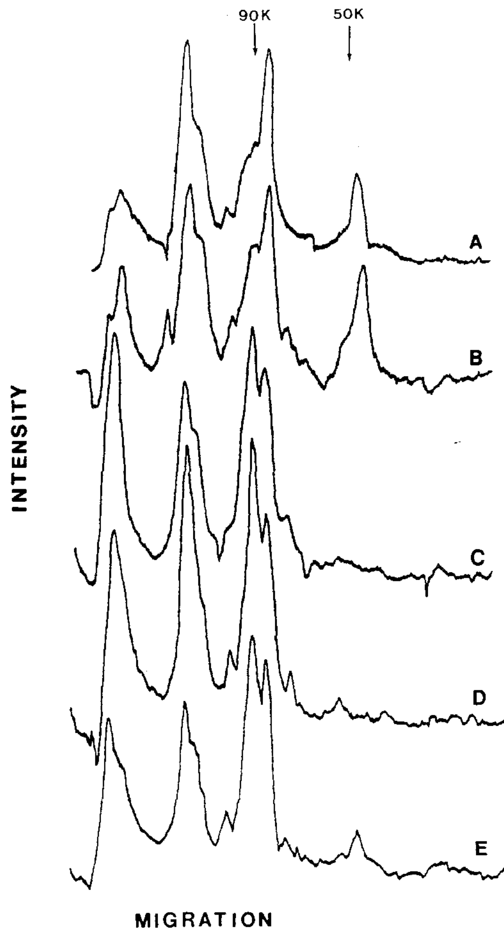

FIG. 1. Exposures of cell surface proteins on murine B16 melanoma sublines by lactoperoxidase-catalyzed iodination. Cell samples were surface labeled with ^{125}I, solubilized in detergent, and electrophoresed in the presence of sodium dodecy sulfate on polyacrylamide slab gels, followed by autoradiography and densitometric scanning of the autoradiograms. Scan intensities indicate relative incorporation of ^{125}I into cell surface proteins of (A) B16-F1 cells; (B) B16-F10 cells; (C) B16-B14b cells; (D) B16-B14b clone 9 (passage 7) cells; and (E) B16-B14b clone 22 (passage 5) cells A-B are low and C-E are high brain-colonizing lines and clones. (Reproduced with permission from Miner *et al.*, 1982.)

theories based entirely on the random arrest and survival of circulating malignant cells. In the B16 melanoma system the same colonization patterns have been obtained after introducing tumor cells into the blood via different injection routes (Fidler and Nicolson, 1976) or by use of parabionts surgically joined together so that tumor cells are required to recirculate from the injected parabiont to the uninjected parabiont in order to form tumor colonies in various organs (Fidler and Nicolson, 1977).

The most compelling data indicating that nonrandom blood-borne tumor cell colonization occurs have been obtained with animals carrying ectopically implanted organ fragments (Kinsey, 1960; Sugarbaker *et al.,* 1971; Hart and Fidler, 1980). Hart and Fidler (1980) have shown that when transplanted lung grafts were used as target sites for blood-borne colonization, intravenous injection of animals with lung-colonizing tumor cells resulted in the formation of tumors not only in the lungs, but in the ectopic lung grafts as well. Of course, in the latter case, the arrest, detachment, and escape of tumor cells from an initial organ bed after intravenous injection presumably occurred before the ectopic organ site was reached. In control experiments, nontarget organ grafts in mice were not colonized by the blood-borne tumor cells. Thus, mere surgical trauma or the presence in the grafts of an improper tissue for colonization were insufficient for colonization of the ectopic organ tissue.

To explain the unique organ colonization properties of metastatic tumor cells, Weiss (1980) has put forward the concept that the adaptation of tumor cells to specific organ microenvironments is critical in metastasis, and that metastatic cells possess transient properties that are also important in determining their metastatic phenotypes. This follows from Weiss and Harlos' (1979) results on the electrophoretic mobilities of Walker carcinoma cells removed from different organ sites *in vivo.* These authors found differences in tumor cells growing at different organ sites, as well as differences between these cells and the original tumor cell population. However, Weiss and Harlos (1979) did not control for the nonspecific absorption of tissue-stromal materials, such as proteoglycans, to their Walker carcinoma cells. These highly charged stromal molecules could transiently modify the electrophoretic mobilities of tumor cells removed from various organ sites. It has not been clearly stated in this hypothesis, beyond vague statements on energy barriers inhibiting collisions between tumor and endothelial cells in the circulation, what role cell surface charge alterations play in tumor cell phenotypic properties.

In another attempt at transiently modifying properties such as the growth rates of various tumor variant cells, Hart (1982) has used treatments of tumor cells with soluble organ extracts. Although these heterogeneous extracts had variable effects on malignant cell growth *in vitro,* Hart (1982) did find that extracts from organs such as brain could inhibit the growth of B16 melanoma cells. Unfortunately, this result was not regularly obtained, and the extracts were admittedly crude and probably contained a number of toxic materials, such as lysosomal enzymes (see Section V).

The question of the role of adaptation versus selection in the formation of metastases has been approached by attempting to select B16 melanoma cells sequentially for the ability to survive and grow at various organ sites. Brunson and Nicolson (1980) undertook to select B16 melanoma cells sequentially for survival and growth in brain parenchyma after direct intra-

cerebral injection. B16 melanoma tumors formed in the brain were removed, cultured *in vitro,* and reinjected back into the brain for readaptation *in vivo.* After 10 cycles of adaptation for brain growth, the final brain melanoma tumor was placed in culture, and the resulting cell subline was then examined for its ability to undergo blood-borne dissemination, implantation, survival, and growth in the brain. Brunson and Nicolson (1980) found that the 10-times brain-adapted B16 subline did not differ in its brain implantation and survival properties from the original B16 parent line. This was in marked contrast to the results obtained from *in vivo* selection methods, where the heterogeneous parent cell population selected for the ability of single cell suspensions of blood-borne B16 cells to implant, survive, invade, and grow to form macroscopic tumor colonies in the brain was used instead (Brunson *et al.,* 1978; Miner *et al.,* 1982). In the latter experiments, B16 melanoma sublines selected sequentially 10 to 14 times for brain colonization formed significantly more brain metastases in experimental assays than the parental tumor cells or cells sequentially adapted for brain survival and growth.

Another experimental approach has been used to examine the role of adaptation in tumor organ colonization. Unselected parental B16 melanoma cells were grown attached to 180-μm-diameter Bio-Carrier beads which were then injected intravenously into syngeneic mice. These cell-coated beads are too large to pass easily through the microcirculation, and they lodge mechanically in the lungs (Nicolson and Custead, 1982). When the mechanically arrested B16 cells grew into gross lung tumor nodules, these latter were excised and established in tissue culture. The cultured B16 cells were then reattached to new microcarrier beads, which were injected into mice to repeat the *in vivo* adaptation sequence. After repeating this sequence nine times, the resulting lung-adapted B16 cells were tested for their metastatic properties after intravenous or subcutaneous injection. The lung-adapted B16 cells remained low in their metastatic potentials, similar to the parental tumor cells, and they were not more metastatic or capable of colonizing lungs than the cell population from which they were derived. This was in contrast to the results obtained when B16 sublines selected for blood-borne implantation, invasion, and survival were tested in parallel experiments (Nicolson and Custead, 1982).

A different tactic was used by Raz *et al.* (1981) to test the role of adaptation in the organ colonization of malignant cells. Raz *et al.* (1981) compared cells from a subcutaneously growing fibrosarcoma with five different cell strains obtained from individual spontaneous lung metastases derived from the same tumor growing locally. In experimental metastasis assays, each of the cell strains established from the spontaneous metastases was found to colonize lungs with significantly greater efficiency than cells established from the parent tumor. This was in contrast to a cell clone derived

from the parental tumor that was capable of producing only a few lung tumor colonies after intravenous injection. A tumor cell strain was established from a rare lung metastasis produced after subcutaneous injection of the clone, and this was compared to the original low metastatic parental clone. No significant differences were found in metastatic potentials when these were assayed in parallel experiments (Raz *et al.,* 1981). Thus, one cycle of adaptation to the lung environment was insufficient to produce a more metastatic phenotype from a cell clone of low metastatic potential.

Collectively, the negative results obtained in the organ adaptation experiments described above argue against an important role for adaptation in metastatic organ colonization. The available data are more consistent with the alternative possibility that metastasis involves a selection process whereby metastases are caused by subpopulations of tumor cells with enhanced potential for blood-borne implantation, invasion, survival, and growth, and that organ-specific colonization is produced by tumor cell subpopulations that have enhanced abilities to implant, survive, and proliferate at specific sites. Thus, metastasis to various organs does not appear to be a random process, nor does it involve a process of gradual adaptation of tumor cell populations to particular organ microenvironments.

Even if the formation of distant metastases is a nonrandom, selective process, random events are probably quite common during the metastatic process. Throughout the metastatic cascade, chance, random events will occur that can affect the colonization of organ sites. These random events include the collision of tumor cells with normal blood cells and vessel walls, and the nonspecific lodgment of tumor cells in hostile or unsuitable microenvironments. Also, alterations in tumor cell nutrition and metabolism could be caused by a variety of organ-specific and even transient events. Weiss (1980) has argued that these "transient metastatic compartments" could modulate malignant cell properties and produce preferential growth of tumor cells at certain sites. Although transient processes may be important in the preferential organ growth of certain tumor systems, their generality remains unproved and rather speculative.

IV. Tumor Cell Heterogeneity and Implantation, Invasion, and Metastasis

A. Evidence for Phenotypically Diverse Malignant Cell Subpopulations

The heterogeneity of malignant neoplasms has been recognized for some time in numerous clinical publications on human cancer (Willis, 1973). It is now well known that in addition to morphological evidence of hetero-

geneity among cells populating the same tumor, cell subpopulations can be distinguished on the basis of heterogeneity in their growth characteristics, biochemical properties, karyotypes, immunogenicities, and antigenicities, and in their sensitivities to chemotherapeutic drugs, radiation, hyperthermia, and host-mediated immunity (for a discussion, see Poste, 1982a; Nicolson, 1982a; Nicolson and Poste, 1982, 1983). Important to the discussion here is that studies on histologically diverse types of malignant tumors have revealed wide variations in the metastatic potentials of cell subpopulations isolated from the same tumor or tumor cell line. These findings have been extensively reviewed elsewhere (Hart and Fidler, 1982; Poste, 1982a; Nicolson, 1982a) and will be discussed only briefly here.

Malignant variants or subpopulations are thought to arise spontaneously during progressive growth of a malignant neoplasm when the tumor cell populations are subjected to various host selection pressures. With time, this process generates tumors that contain phenotypically diverse cell subpopulations. In his extensive studies on mouse mammary tumors, Foulds (1956a,b) described what he termed "neoplastic evolution." This term was coined to explain the process whereby each tumor gradually gains autonomy from its host while at the same time acquiring or losing certain cellular characteristics. Thus tumor evolution was considered to involve a series of permanent, irreversible changes in cellular properties that occurred independently of events in other types of tumors. Medina (1975) has concluded that virtually all characteristics of tumors are subject to variation and can thus contribute to the evolution of cellular diversity during progressive tumor growth. This can result eventually in cellular changes and gradual escape from growth controls and other host regulatory processes which may tend to restrain the emergence of new phenotypes.

Tumor evolution and progression, and the resulting emergence of rare tumor cell subpopulations with altered malignant potentials, has been proposed by Nowell (1976) to be due to genetic alterations. Alternatively, some tumors may be polyclonal due to initial transformations of different cells (clones) that result in neoplasms with diverse cellular phenotypes (Fig. 2). Nowell's hypothesis of tumor progression circumvents the argument of whether tumors have a clonal or a polyclonal origin. The only requirement is that malignant cells acquire genetic variability or instability that results in the emergence of cell variants or subpopulations. Tumors of monoclonal or polyclonal origin will diversify with growth. Thus the tumor cell subpopulations present in advanced tumors may or may not have any obvious phenotypic resemblance to the cells from which the tumor originated. As a whole, these changes may result in an evolution of tumor cell characteristics and in properties that progress toward those which are most favorable for survival and growth in the face of various host pressures.

Evidence for the coexistence, within the same tumor, of heterogeneous

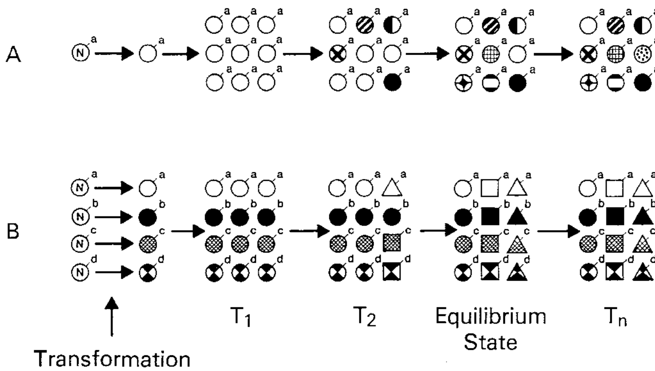

FIG. 2. Schematic representation of possible events in the generation of cellular diversity and metastatic heterogeneity during tumor progression. After transformation of clonal (A) or polyclonal (B) normal cells, tumors are formed that undergo rapid initial proliferation (T_1). In time, cell variants are generated (T_2) with differing metastatic phenotypes. The formation of new metastatic variants may not proceed indefinitely, and in cellular populations containing multiple metastatic phenotypes, an "equilibrium" or "static" state may be reached where the rate of generation of new metastatic phenotypes is "restricted" with time (T_n) by polyclonal cellular interactions. Superscripts on symbols indicate that other cellular or genetic properties may be stable and not susceptible to phenotypic diversification. (Modified from Poste, 1982a.)

tumor cell subpopulations that differ in their cellular characteristics, including metastatic potentials, has been obtained in experiments using single cell cloning and fluctuation analysis methods. Fidler and Kripke (1977) were the first to use these procedures in analyzing tumor cell subpopulations. They prepared cell suspensions from a subcutaneously growing B16 melanoma tumor and assayed one sample of cells for its ability to form experimental pulmonary metastases following intravenous injection, while a replicate sample was used to isolate 17 different tumor cell clones. The B16 melanoma clones were then tested separately for their ability to produce lung tumor nodules. If the parental tumor was composed of cells with similar metastatic potentials, then each cell clone should have produced similar numbers of experimental metastases, i.e., the same number as the uncloned cells from the parent cell population. However, Fidler and Kripke (1977) found that although the number of experimental metastases produced by any given clone was constant in separate experiments, each clone showed wide variability in its metastatic potential compared to other clones and the parental cells. Fluctuation assay techniques using controlled subcloning experiments demonstrated that the cloning process itself was not responsible for generating heterogeneity during clonal cell growth *in vitro*. Fidler and Kripke (1977) concluded that metastatic cell subpopulations existed in the parental tumor before cloning and that they expressed heterogeneous met-

astatic potentials. Although it could be argued that the metastatic heterogeneity of B16 melanoma subpopulations was the result of the long-term growth of the B16 tumor in animals or in tissue culture, recent studies with tumors of more recent origin suggest that this was not the case (Kripke *et al.,* 1978; Reading *et al.,* 1980b).

The question of whether cellular metastatic heterogeneity found in transplanted tumors is simply the result of long-term growth was approached by the following experiment. Fidler *et al.* (1981) induced a melanoma in a C3H mouse by UV irradiation and applications of croton oil to the skin. After 2 years of this treatment, a primary melanoma arose, and this was transplanted immediately into immunosuppressed mice to eliminate the possibility of immunoselection of cell subpopulations within the new tumor. The tumor was quickly established in tissue culture and cloned within five passages. Each clone was then examined and found to differ dramatically in cell size, growth rate, karyotype, pigmentation, and metastatic potential.

In the experiments just described, the cloning and subcloning procedures did not involve the long-term cultivation of potentially unstable clonal cell populations. Using a fluctuation assay, Fidler and Kripke (1977) found that B16 melanoma cell clones in short-term cultures were stable. However, it is now known that individual B16 cell clones will eventually diverge into heterogeneous cell populations during prolonged cultivation *in vitro* (Fidler and Nicolson, 1981; Poste *et al.,* 1981).

The possibility that a polyclonal origin of tumors results in immediate phenotypic diversity was ruled out by Fidler and Hart (1981b). Their experiment was based on a report that individual clones of murine sarcoma virus (MuSV)-transformed 3T3 fibroblasts differ in their metastatic potentials (Nicolson *et al.,* 1978). Fidler and Hart (1981b) infected a single 3T3 fibroblast cell clone with a cloned murine sarcoma virus preparation. They then allowed individual cell colonies to grow without selection, and six colonies were chosen at random and expanded as clonal populations. Within 24 days after the initial virus infection, these cloned cell populations were injected either subcutaneously or intravenously into groups of mice in order to determine their metastatic potentials. The subcutaneous injection of the transformed clones resulted in tumors that regressed within approximately 4 weeks. However, the clones injected intravenously produced tumor nodules in the lungs. These clones were dramatically different in their lung colonization potentials, indicating that the original clonal cell population had diverged rapidly into subpopulations with widely differing phenotypes.

The divergence of clonal populations into multiple subpopulations with altered phenotypic properties can also occur *in vivo* at metastatic sites. Poste *et al.* (1982b,d) examined individual lung metastases obtained from the same animal after intravenous or subcutaneous injection of B16 melanoma or

UV2237 fibrosarcoma cells. Initially, the majority of the individual metastases were found to be populated by tumor cells with indistinguishable metastatic properties, but clones isolated from different metastases exhibited diverse metastatic phenotypes. A few metastases were found, however, that yielded clones with significantly different metastatic properties, but the range of clonal diversity found for this type of tumor colony was far less than that of the original parent tumor cell population. Since in the latter case malignant cells could have undergone homotypic aggregation to produce polyclonal cell populations during the metastatic process (Fig. 3) (for example, while in the circulation, or after arrest in the microcirculation), the evidence suggests that the majority of the lung metastases formed in these experiments were of clonal origin. As the experimental metastases grew progressively at lung sites, however, widely divergent subpopulations eventually emerged (Poste *et al.,* 1982b).

The variability in metastatic properties of subpopulations isolated from the same tumor has important implications for the analysis of cellular properties associated with metastasis. It is clear that analyzing tumor cells isolated at random from cultured tumor cell sublines or from tumor biopsies should be acceptable only if the tumor cell populations are uniform and possess similar metastatic characteristics. In tumors that contain numerous,

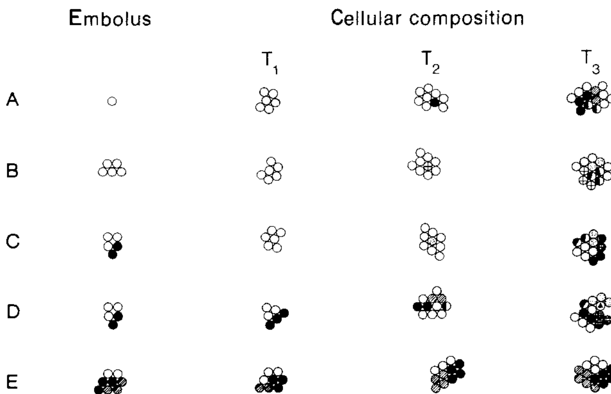

FIG. 3. One possible mechanism for generating malignant phenotypic diversity within individual metastases during blood-borne spread. Metastases can be formed by the implantation of a single tumor cell (A) or multicell emboli of clonal (B) or polyclonal (C–E) origin. With time (T_2, T_3, etc.) and progressive growth, phenotypic diversification can occur, leading to cellular heterogeneity at the metastatic site. The rate of generation of phenotypic diversity may also differ between tumor cell populations. In scheme (D), new variants are quickly generated, as compared to scheme (E) where a metastasis would be heterogeneous due to the implantation, invasion, and growth of polyclonal cells within a blood-borne embolis. (Reproduced with permission from Poste, 1982b.)

phenotypically diverse cellular subpopulations, such as those where the highly metastatic cells represent only a small portion of the total cell population, random analysis of the entire cell population may obscure important characteristics due to the presence of a wide variety of cells with different metastatic potentials. Therefore, studies devised to analyze metastatic properties must concentrate on examining cells which are highly metastatic, and compare these to nonmetastatic (yet tumorigenic) subpopulations isolated from the same parent tumor or parent cell line.

When one is studying various properties that may be important in specific events during the metastatic cascade, tumor cell subpopulations can be defined by their abilities to complete only specific steps of the metastatic process. For example, if invasive properties of tumor cells are to be studied, invasive variants can be isolated and studied in relation to this event without involving the entire sequence of metastasis.

B. Phenotypic Stabilities of Malignant Tumor Cell Subpopulations

In the preceding section we mentioned that clonal populations of malignant cells may undergo phenotypic diversification with time (Fidler and Nicolson, 1981; Poste *et al.,* 1981; Neri and Nicolson, 1981; Nicolson *et al.,* 1983). This suggests that cellular heterogeneity within neoplasms could arise from the phenotypic drift of individual cell clones in the tumor cell population. Evidence for such phenotypic drift in various tumor properties has been obtained with cell clones derived from B16 melanoma (Fidler and Nicolson, 1981; Miner *et al.,* 1982; Poste *et al.,* 1981, 1982b,d), hepatocarcinoma (Talmadge *et al.,* 1979), lymphosarcoma (Nicolson *et al.,* 1982b), and adenocarcinoma (Neri *et al.,* 1982; Neri and Nicolson, 1981; Tomasovic *et al.,* 1982; Welch *et al.,* 1983b).

Certain rat 13762NF mammary adenocarcinoma cell clones derived from locally growing tumors or from spontaneous lung metastases drift phenotypically when grown in tissue culture. Although some 13762NF clones were found to be stable in culture, others drifted to increased metastatic potentials with time. Still others showed decreasing metastatic potentials during growth *in vitro.* Interestingly, these changes were nonrandom and occurred reproducibly after a specific number of culture passages of the same frozen cell stocks. In addition to differences in metastatic properties, differences were also found in cell morphologies, expression of certain membrane glycoproteins, (such as fibronectin) (Neri *et al.,* 1981, 1982), and sensitivities to chemotherapeutic drugs (Welch and Nicolson, 1983), radiation (Welch *et al.,* 1983a), and hyperthermia (Tomasovic *et al.,* 1982). These changes all occurred concomitantly with the divergence in the spontaneous meta-

static potentials of the individual clones (Neri and Nicolson, 1981; Neri *et al.,* 1981, 1982).

Phenotypic drift in clonal tumor cell populations, resulting in either decreased or increased metastatic potentials, may explain the observation that clones of low metastatic potential can be obtained from highly metastatic tumors (Fidler and Nicolson, 1981; Miner *et al.,* 1982; Reading *et al.,* 1980b). In this case, the generation of cells with low metastatic potential can be followed using cell cloning techniques, but *in vivo* these cells would be difficult to find.

Since phenotypic variants arising *in vivo* can revert to a more normal phenotype, they might be more susceptible to nonimmune and immune control or surveillance mechanisms in the host. Clones under increasing control of host mechanisms could become dormant, allowing other tumor cells with more malignant phenotypes to proliferate and outgrow them. Dennis *et al.* (1981) used a series of lectin-resistant variants of the MDAY-D2 tumor to study the potential of more malignant cell phenotypes for overgrowing a tumor cell population. One lectin-resistant variant was isolated that had a low metastatic potential in comparison to the parental tumor line, and that rarely formed metastases after subcutaneous injection. A few of these rare metastases were established in culture and were found to be populated by lectin-sensitive revertants. The authors suggested that the lectin-sensitive, highly malignant phenotypes were generated *in vivo* and that these more malignant "revertant" cells were the ones that metastasized spontaneously. However, since clonal cell populations were not used in this study by Dennis *et al.* (1981), it is possible that rare, unstable clones existed, even in the lectin-resistant population. It is even more likely that the lectin-resistant variant cells had drifted phenotypically during growth *in vivo* so as to generate some highly malignant cells.

The existence of phenotypic heterogeneity and the rapid generation of phenotypic diversity *in vivo* were observed when cells growing *in vitro* were compared to similar cells growing *in vivo.* Chow and Greenberg (1980) found that growth *in vivo* resulted in more rapid diversification of different cell phenotypes. Using subcloning techniques to follow the instabilities of single clones of differing metastatic potentials during serial tissue culture passaging, Talmadge *et al.* (1979) compared clones growing in culture with similar clones proliferating in ascites fluid *in vivo.* Upon growth *in vitro* clones and various subclones derived from them did not vary significantly during the experiment, in contrast to the comparable clones grown *in vivo.* When the clones growin *in vivo* were subcloned, approximately 1/3 of the subclones differed significantly in phenotype from the parent clones, which suggests that growth in ascites fluid *in vivo* may have stimulated phenotypic diversity.

By contrast to the rapid diversification of different cell phenotypes from

single cell clones growing *in vitro* or *in vivo,* polyclonal populations appear to be relatively stable. Poste *et al.* (1981) examined the phenotypic stabilities of polyclonal and monoclonal B16 melanoma tumor cell populations during growth *in vivo* or *in vitro* and found that cultures of individual clones were phenotypically unstable (Fig. 4). But when these same populations were cocultivated with different cell clones, phenotypic diversification was inhibited. Similar results have been reported by Miner *et al.* (1982) for brain-colonizing B16 melanoma clones. The latter authors also found that single-cell clones were unstable while growing *in vitro,* but could be stabilized by cocultivating three different clones together (Fig. 5). The results of Poste *et al.* (1981) and Miner *et al.* (1982) suggest that interactions may occur between various cell subpopulations in polyclonal cell mixtures, resulting in the stabilization of each clone within the population, and also of the overall metastatic properties of the populations. Poste *et al.* (1981) have proposed that when clonal diversity is conserved, the domination of one or of a few cell subpopulations over the entire cell population is prevented.

Evidence has been obtained that similar clonal interactions can occur *in vivo* at metastatic sites (Poste *et al.,* 1982b). This has been shown by isolating several cell clones from individual lung tumor colonies produced by

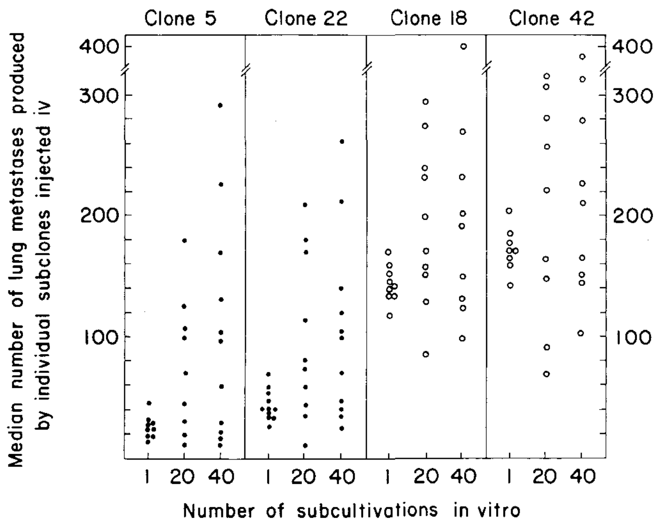

FIG. 4. Instability of the lung-colonization properties of individual cell clones obtained from the lung-colonizing murine B16-F10 melanoma subline. Cell clones were derived from a B16-F10 cell culture and subcloned at various intervals during serial subculturing. Experimental metastatic potentials were determined by injecting 2.5×10^4 cells intravenously into C57BL/6 mice and counting the number metastases formed 18 days later. (Reproduced with permission from Poste *et al.,* 1981.)

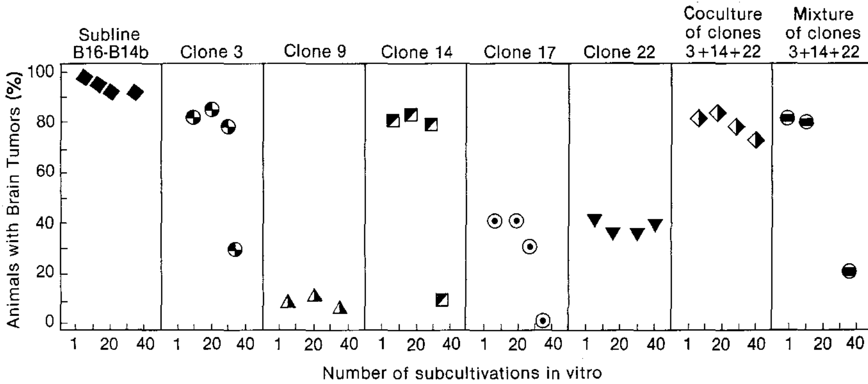

FIG. 5. Instability of the brain-colonization properties of individual cell clones obtained from the brain meninges-colonizing murine B16-B14b melanoma subline. Cell clones were derived from a B16-B14b cell culture and subcultured for various intervals. Some cultures were established with equal numbers of cells from three different clones ("coculture"), and as a control for this type of experiment equal numbers of cells from the three clones grown separately ("mixture") were combined just before the *in vivo* assay. Experimental metastatic brain colonization was assessed by injecting 2×10^4 cells intravenously and determining organ colonization 5–8 weeks later. (Data are from Miner *et al.*, 1982.)

lung-colonizing B16 melanoma cells. Clones established from individual experimental lung metastases excised after 18 days of growth at pulmonary sites were found to exhibit similar metastatic phenotypes in approximately 80% of the metastatic lesions examined (we will call this "intralesional clonal homogeneity"). By contrast, tumor clones isolated from different experimental metastases in the same animal exhibited different metastatic phenotypes ("interlesional clonal heterogeneity"). This situation, however, changed dramatically with progressive growth of the metastases. Within 40 days of growth of the metastatic lesions in the lungs, intralesional clonal homogeneity was found in only about 30% of the metastases, and the remainder of the lung tumor colonies contained two or more clonal subpopulations of cells with differing metastatic phenotypes "intralesional clonal heterogeneity" (Poste *et al.,* 1982b). The interpretation of these experiments is that while most experimental metastases were populated initially by cells with similar metastatic phenotypes, presumably single-cell clones, in the absence of the stabilizing influence of other clonal subpopulations cells in the metastases were able to undergo phenotypic diversification that resulted in the rapid generation of intralesional clonal heterogeneity. Miner *et al.* (1982) noted similar results during growth *in vitro* and *in vivo* of brain-colonizing B16 melanoma cells.

There are also reports suggesting that interactions between different tumor cell subpopulations can occur over long-range distances *in vivo*. Miller

et al. (1980) found that mouse mammary tumor cell subpopulations growing at subcutaneous sites can impose regulatory restraints on the growth of other mammary tumor cell populations at distant sites. The reports indicating that primary tumors in some way can regulate the growth of their metastases may be examples of the same phenomenon (DeWys, 1972; Ketcham *et al.,* 1961).

The mechanisms underlying the generation of phenotypic instability and its possible control have not been determined. Schirrmacher (1980) has speculated that preformed genetic programs could be activated by the presence of environmental signals (or their absence) and that this could lead to widespread changes in genetic expression and eventually to variations in phenotypic properties. One mechanism that might be important in stabilizing polyclonal populations or in generating phenotypic diversity is the ability of tumor cells to undergo somatic cell hybridization *in vivo.* Goldenberg and his collaborators (1974) have speculated that cell hybridization *in vivo* could lead to tumor progression and the emergence of more malignant cell phenotypes. It is apparent from the above discussion that such hybridization could also lead to an "equilibrium" situation where genetic information has been "shared" by the tumor cell population.

The rapid generation of phenotypic variants, presumably through phenotypic (genotypic?) instability, may be related to the ability of tumor cells to undergo spontaneous mutation. Cifone and Fidler (1981) have examined the rates of spontaneous mutation(s) to drug resistance in sublines and clones isolated from three different metastatic tumor systems. Comparison of sublines and clones with low and high metastatic potentials revealed that the more metastatic cells had higher rates (up to sixfold) of spontaneous mutation. It would be of interest to use this system to examine the rates of spontaneous mutation in highly metastatic clones during monoclonal or polyclonal growth, and to determine if polyclonal interactions can modulate the rates of spontaneous mutation.

The emergence of cell variants within tumor subpopulations appears to be an inevitable and unrelenting feature of progressive tumor growth. That tumor progression can be explained by a series of multiple, yet independent, changes in different cellular properties, as proposed by Nowell (1976), could account for the rapid production of variant subpopulations with widely different phenotypic properties. Tumor progression toward autonomy from host control may also proceed simultaneously along several pathways which, in turn, could be independently influenced by changes in microenvironment, host surveillance, and other selective processes, resulting ultimately in myriad subpopulations of differing phenotypes. Thus, the extent of phenotypic diversity and the number of different subpopulations present in a tumor may vary with time depending upon changes in the environment *in vivo.* Modifications in tumor microenvironment, host surveillance mech-

anisms, and changes in cell nutrients, hormones, and other factors could each contribute to the generation of phenotypic diversity. In addition, phenotypic diversification can also be influenced by external factors such as antitumor therapy (see next section).

The generation of phenotypically diverse subpopulations of cells during metastatic processes could occur by different mechanisms during each step of the metastatic cascade. For example, metastatic lesions could be formed by local invasion, detachment, dissemination, implantation, secondary invasion, survival, and proliferation of either a single malignant cell or a clump of cells (Fig. 3). Thus, phenotypic diversity can result from subsequent diversification of monoclonal or polyclonal populations, and the initial cellular composition of metastases will depend on whether the individual tumor cells or their cell aggregates possess similar or dissimilar cellular phenotypes (Fig. 3). One conceivable reason why blood-borne tumor metastases are often quite homogeneous is that cell aggregates that arise from the detachment of multiple cells from invading tumors can have their origin within a single homogeneous cellular zone of a tumor (Fidler and Hart, 1981a). Alternatively, a blood-borne embolus could be derived from an area containing a mixture of different cell phenotypes and could produce a metastasis that is phenotypically heterogeneous; or blood-borne tumor emboli containing cells with different phenotypes could implant, but the selective survival of a single-cell clone and its subsequent invasion and growth could also result in initial intralesional clonal homogeneity (Fig.3) (see Poste, 1982a).

There appear to be wide variations in the metastatic potentials of various human malignancies. For example, certain cancers carry high risks of metastatic disease at an early stage, whereas others can grow for rather long periods of time and reach substantial sizes without evidence of metastasis (Sugarbaker, 1979). Thus, in malignant cancers that carry a high risk of early metastasis, the emergence of highly metastatic phenotypes must not only occur at an early stage of the primary tumor, but they must also be expressed.

C. Therapeutic Implications of Malignant Cell Heterogeneity and Stability

For the most part, random screening procedures have been used to develop new techniques and agents to inhibit metastasis or to destroy metastatic tumors. However, new approaches will have to be developed to deal with metastatic disease in particular. Metastatic disease with its potential for generating phenotypic diversity among cell subpopulations, both within the same lesion and among different lesions, represents a major obstacle to

the development of new therapeutic strategies for the treatment of cancer. Since individual tumor cell subpopulations differ significantly in their sensitivities to various therapeutic approaches and agents, future therapies will have to circumvent phenotypic diversity among tumor cells somehow and overcome the problem of residual resistant subpopulations.

The development of most anticancer agents or procedures, as well as the treatment protocols by which they are administered, have in general failed to take into account the necessity of destroying established metastases. With the dual problems of heterogeneity in the sensitivities of cellular subpopulations to different therapies and the rapid generation of phenotypic diversity, a new treatment or agent for primary tumor therapy may be ineffectual in stopping the disease if metastases continue to form and proliferate. As we have mentioned previously, metastatic cell subpopulations are often present as a minor fraction of the total tumor cell population. Obviously, the therapeutic agents which can circumvent tumor cell diversity and heterogeneity and destroy metastatic cells are the ones that will have the highest chance of success in treating clinically detectable metastases.

One assay for screening the susceptibility of tumor cell subpopulations to therapeutic agents has been to disperse cells from surgical samples and place them into a tumor stem cell assay, usually in semisolid medium (Hamburger and Salmon, 1977). Unfortunately, successful use of stem cell or clonogenic assays in identifying agents that will be effective in treating individual cancers has not yet been achieved. This is due to the fact that very few cells, usually less than 0.01%, can actually proliferate in the stem cell assays as they are currently performed. Thus, the tumor cells that grow in the assay may represent extremely minor cell subpopulations within the tumor, and the assumption is that these are the same subpopulations that are the most likely to grow and to metastasize in their host. However, if metastatic cell subpopulations vary significantly in their clonogenic potentials, and metastasis formation bears no relationship to the cells which are able to grow in semisolid medium, then the evaluation of therapeutic responses of tumor cell subpopulations in the clonogenic assay may not encompass cells that fail to grow in the *in vitro* assay but have a high potential to metastasize *in vivo*.

Single-cell growth in the clonogenic assay may also eliminate polyclonal interactions among tumor cell subpopulations. Most clonogenic assays do not take this fact into account, and, in particular, little attention is usually paid to the stabilities of cell clones in semisolid medium. We have found that cell clones growing in semisolid medium, such as agarose, are highly unstable and can generate diverse subpopulations within the short period of time (14 days) of the assay. This experiment was performed by growing doubly cloned mammary tumor cells of varying metastatic potentials at subcutaneous sites *in vivo*, in tissue culture, or in 0.3% agarose. By contrast

to the other growth environments, the cloned cells in semisolid medium underwent rapid diversification and eventually yielded tumor cell colonies that differed widely in metastatic potentials and cell surface properties (Fig. 6) (Nicolson *et al.,* 1983). This finding may explain why the clonogenic assays have not proved to be as accurate as originally expected. However, their principal value may not be as a predictive test for identifying agents that are effective in inhibiting tumor growth, but instead they may be useful in predicting whether agents will be of limited value in inhibiting tumor cell growth because of the presence of resistant cells (Nicolson and Poste, 1983). Extensive use of the clonogenic assay may help in defining resistance to particular agents, although problems of tumor uniqueness and cellular diversity within tumors may not allow meaningful correlations to

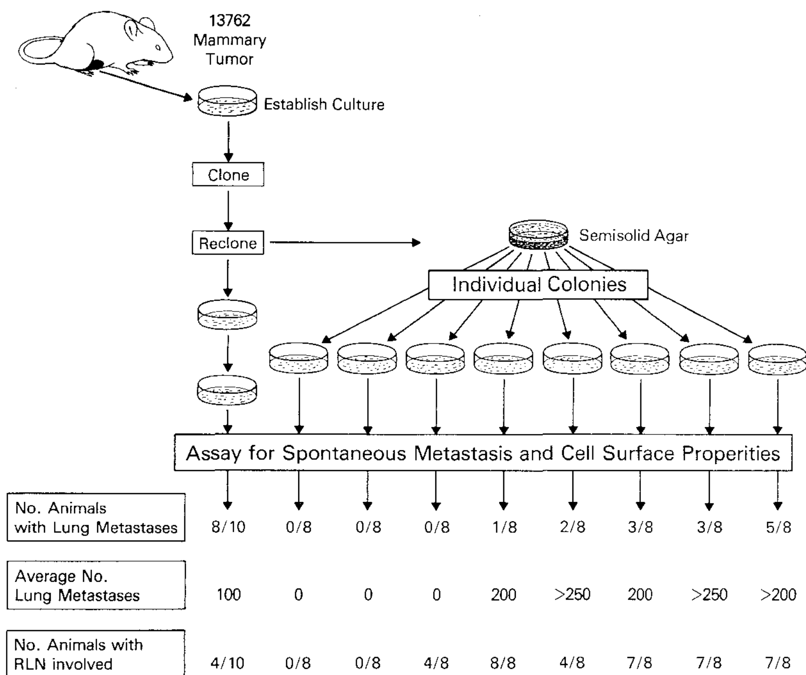

FIG. 6. Phenotypic drift of mammary carcinoma cells in the stem cell or clonogenic assay. A rat mammary tumor (Neri and Nicolson, 1981; Neri *et al.,* 1982) was established in tissue culture and twice cloned to ensure single-cell origin. Cell clones were seeded into culture plates with liquid or semisolid (agarose) medium, allowed to grow, and individual colonies were expanded and assayed for their spontaneous metastatic properties *in vivo.* Each cell colony derived from the semisolid clonogenic medium diverged in phenotype as compared to similar cells grown on tissue culture plates for an equivalent period of time. RLN, regional lymph node. (Reproduced with permission from Nicolson and Poste, 1983.)

be established between clinical prognosis and tumor cell sensitivity or resistance in the clonogenic assay.

The role of polyclonal interactions between different tumor cell subpopulations in regulating the stability of the metastatic phenotype and in controlling the emergence of variant subpopulations is rarely taken into account during the development of anticancer therapies. Since tumor cell subpopulations influence the behavior of one another, modifications in cell subpopulation diversity can affect the rate at which new metastases emerge or become dominant in a cell population. This could prove to be clinically important in the establishment of new therapeutic strategies (Nicolson and Poste, 1983).

One aspect of tumor cell subpopulation diversity that may be critical in determining whether a tumor can be eliminated is the timing of the therapy. If therapy results in the survival of minor tumor cell subpopulations that were originally in "phenotypic equilibrium" (where the overall rate of generation of phenotypic variant cell subpopulations is reduced), the surviving subpopulations could become "phenotypically unstable," possibly because of the lack of "proper interactions" between different cell subpopulations within the residual tumor that favor phenotypic stability over phenotypic diversification. Loss of these regulatory interactions could lead to deregulation and the generation of phenotypic diversity. Thus, therapy may modify the so-called "equilibrium state" by restriction of subpopulation diversity, and this, in turn, could rapidly result in the generation of new tumor cell variants within the surviving cell subpopulations (Poste, 1982a) (Fig. 7). The generation of new tumor cell variants would be expected to continue until complete subpopulation diversity is achieved, resulting in the imposition of a new equilibrium on the tumor cell population through polyclonal interactions. In the second cycle of treatment, this scheme could be repeated with a new therapeutic agent or regimen, eliminating further the surviving subpopulations before they have time to generate new variants. As the different subpopulations are destroyed, the chance of phenotypic diversity is reduced, and the chance that the tumor will be eliminated is increased (Fig. 8).

The importance of timing in this sequence is obvious. In order to achieve complete tumor kill, it would be necessary to reduce the time interval between successive treatments with different agents. Use of a series of different treatment regimens in rapid succession in order to eliminate tumor cell subpopulations which survive each treatment should result in the elimination of new variant cell subpopulations as they arise, but before they can undergc rapid phenotypic diversification (Fig. 8) (Poste, 1982a; Nicolson and Poste, 1983).

In the usual clinical setting, sequential treatment with a variety of agents or therapies is not used if the initial protocol is successful in producing a

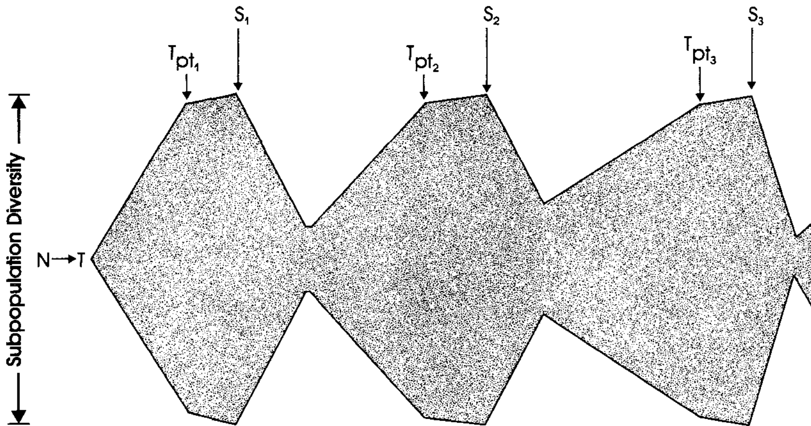

FIG. 7. Schematic illustration of possible fluctuations in the phenotypic diversity of tumor cell subpopulations at different stages during the progressive growth of a malignant neoplasm. Neoplastic transformation of a normal cell (N) to a tumor cell (T) is followed by the emergence of variant tumor cell subpopulations at a relatively constant rate to generate a phenotypically diverse tumor. Once substantial diversity has been achieved (T_{pt_1}), the overall rate at which new variants are formed is reduced. However, if a tumor is exposed to a selection pressure (S_1) that significantly reduces subpopulation diversity within the tumor, the rate at which surviving subpopulations generate new variants increases until extensive subpopulation diversity (T_{pt_2}) is restored. The nature of the clonal subpopulations present in the tumor at T_{pt_2} is different from that present at T_{pt_1}. New selection pressures (S_2) are expected to repeat another cycle of subpopulation restriction and subsequent phenotypic diversification. No absolute values are implied for subpopulation diversity (*y*-axis) or time scale (*x*-axis), and the extents of subpopulation diversity and its restriction are unknown. (Modified from Poste and Greig, 1982).

clinical remission or rendering the tumor undetectable. If residual disease is detectable, then additional treatment is often applied, frequently with the same agent. Unfortunately, by the time recurrent disease is clinically evident, phenotypic diversification of the surviving tumor cell subpopulations may have resulted in a new, more diverse set of malignant cell subpopulations which possess quite different sensitivities to the therapeutic agents used. In the future, routine implementation of further clinical treatment in the absence of detectable disease may become attractive, and not just in cases where there is clear evidence of tumor recurrence. As emphasized above, in recurrent disease the constituent cell subpopulations may have diverged phenotypically and the cellular subpopulations present in recurrent lesions may not exhibit the same therapeutic sensitivites as the original tumor.

Although there is a possibility that anticancer therapy may stimulate phenotypic diversification and result in increasingly unstable, more malignant cell variants, there is no reason to abandon currently used therapies

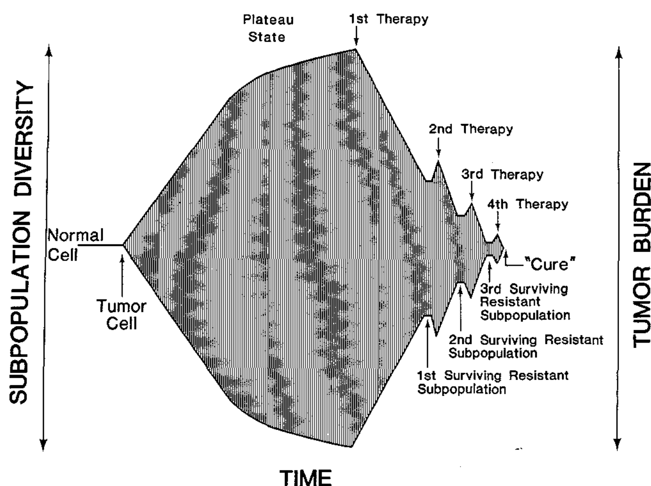

FIG. 8. Illustration of a successful therapeutic regimen on tumor cell subpopulation diversity and tumor burden. At initial clinical detection the neoplastic lesion contains multiple tumor cell subpopulations such that an "equilibrium" or "static" state exists in which "interactions" between the tumor cell subpopulations limit the rate at which new phenotypic variants are generated. Upon exposure to therapy some (susceptible) subpopulations are killed and others survive. This restriction of subpopulation diversity may render the surviving subpopulations phenotypically unstable, thus generating a new panel of tumor cell variants with widely differing phenotypic properties. With increasing diversification a new "equilibrium" or "static" state will again be imposed, and on subsequent therapeutic treatments the cycle of subpopulation restriction and diversification will be repeated until either subpopulations resistant to all therapeutic efforts kill the host or a therapeutic modality is identified that can destroy residual cell subpopulations within a lesion, resulting in a "cure." (Reproduced with permission from Poste and Nicolson, 1983.)

that have been successful in treating cancer. The concepts discussed here suggest, however, that we may need to reassess the impact of cancer therapies on phenotypic diversification and tumor cell heterogeneity with the aim of making the overall therapy more effective in preventing the diversification of tumor cell subpopulations that give rise to recurrent disease.

V. Dissemination, Implantation, and Invasion of Metastatic Tumor Cells

A. DISSEMINATION OF METASTATIC TUMOR CELLS

Tumor cell dissemination, or the detachment of malignant cells from an invading tumor mass and their release into lymph, blood, or body cavities, is an important requirement for metastasis. Often tumor cell detachment

has been confused with tumor cell adhesion or cohesion. However, tumor cell detachment, as opposed to tumor cell adhesion or attachment, must be considered a separate phenomenon (Weiss, 1978). It was Coman (1961) who first differentiated between what he called ''adhesiveness'' (for detachment properties) and ''stickiness'' (for attachment properties). Coman (1961) measured the detachment of malignant cells from one another *in vitro* by determining the forces required to separate bound cells or the rates at which cells could be separated at a given mechanical force. In these experiments, malignant cells were more quickly and easily separated from solid tumors than normal cells from their corresponding tissues (Coman, 1961; McCutcheon *et al.,* 1948). From this evidence it was proposed that malignant cells were less adhesive than their normal cell counterparts. This unwarranted generalization has persisted in the literature for a remarkable length of time and is still cited as a general feature of cancer cells in many modern textbooks of pathology and medicine. Moreover, the techniques used by Coman (1961) result in cell damage. In his experiments cells were impaled with micromanipulators and physically torn apart, and the subsequent viabilities of the cells during and after these manipulations were not known.

Although it has been speculated that malignant cells should have reduced homotypic adhesive properties that aid in their release from the primary tumor mass, most of the experiments designed to test this hypothesis have not assayed cell *detachment,* but instead have measured cell *attachment* (see review by Nicolson, 1982a). However, there are a few experiments in which investigators have attempted to measure tumor cell detachment. In most of these experiments, quantitative data were not gathered. For example, Criborn *et al.* (1974) used an aspiration technique to remove cells from various tumors and tumor biopsies. They noted that aspirates from malignant tumors, such as carcinomas, contained more free cells than similar aspirates from benign tumors. In other experiments, agitation was used to release single cells or cell clumps from tumor masses. In general, the results indicated that cells of greater malignant potential are more easily released from their corresponding tumors than cells of lower malignant potential (Coman, 1961; McCutcheon *et al.,* 1948).

The detachment of tumor cells from a tumor mass may involve enzymatic actions, such as those provided by lysosomal enzymes (see reviews by Poste and Weiss, 1976; Nicolson, 1982a; Poste, 1982b). It is known that lysosomal enzymes can enhance the detachment of cells from each other or from their extracellular matrix (Weiss, 1978; Sylvén, 1968). In order for this to happen, these intracellular enzymes must be released from malignant cells or released in the tumor area as a consequence of cell damage or death. Since these enzymes are also present in normal cells in tumor tissues, such as immunocytes, fibroblasts, and endothelial cells, an alternative suggestion is that lysosomal enzymes may be released, at least in part, from normal cells in or around the tumor.

The importance of lysosomal enzymes in cell detachment has been studied by Weiss (1977). Using Walker 256 carcinoma cells, Weiss found that cell detachment was facilitated by fluids from the necrotic regions of tumors. These regions are known to be high in intercellular lysosomal enzymes, and extraction of necrotic and other regions of tumors in saline has yielded solutions rich in lysosomal enzymes that have been used to detach tumor cells from their growing mass. In his work on the tumor detachment process, Weiss (1978) used extracts of necrotic and nonnecrotic parts of tumors to examine the detachment of Walker 256 from artificial surfaces *in vitro*. The necrotic extracts, which were richer in lysosomal enzymes, facilitated cell detachment, and Weiss found that lysosome stabilizers, such as hydrocortisone, could partly inhibit detachment. Detachment of malignant cells by means of extracts of necrotic tumor tissue and lysosomal enzymes would be analogous to the phenomenon of "sublethal autolysis" (Poste, 1971) in which release of lysosomal enzymes can modify the surface properties of cells and thus result in altered adhesiveness and cohesiveness without injuring the cell.

The above explanation is reasonable because several studies have revealed the presence of higher levels of various proteases, particularly of lysosomal proteases, in invasive tumor tissues than in surrounding normal host tissues (Poole *et al.*, 1980; Nicolson, 1982a; Liotta *et al.*, 1982a,b). In most of these studies it was difficult to determine the relative contributions of tumor-associated and of host cell enzymes within the tissues examined. Although there are exceptions (see Sträuli and Weiss, 1977), it has been generally assumed that the proteolytic enzymes found in tumor tissues are of tumor origin. High levels of neutral proteases, aminopeptidase, proline hydroxylase, and collagenase have been found at the peripheries of invasive tumors or in tumor necrotic regions (Hashimoto *et al.*, 1972; Yamanishi *et al.*, 1973; Koono *et al.*, 1974; Sylvén *et al.*, 1974; Zimmerberg *et al.*, 1975; Recklies *et al.*, 1980). Nonetheless, Sträuli and Weiss (1977) have found exceptions to these general findings in which the presence of high levels of these tissue-degradative enzymes did not always correlate with tissue invasion.

Specific lysosomal enzymes from highly metastatic, lung-colonizing murine melanoma cells have been measured by Sloane *et al.* (1981, 1982). These authors correlated the levels of tumor lysosomal cathepsin B with the metastatic potentials of two B16 melanoma sublines. Cathepsin B activities were several times higher in cells dispersed from tumors of the high lung-colonizing B16-F10 subline and four times higher in the lysosomal preparations from B16-F10 tumor cells than in similar preparations from the low metastatic B16-F1 line. The possibility that these higher cathepsin B activities were due to host cell contamination of the melanoma tumors was ruled out by separation of the B16 cells from other cells within and around the grow-

ing tumors. Although Poole *et al.* (1978) failed to find differences in the *total* amounts of cathepsin B or cathepsin D in homogenates from malignant and benign breast cancer and from normal breast tissue, they did note a dramatic difference in the relative amounts of the secreted forms of the enzymes. Malignant breast tissue released up to 11 times the activity of cathepsin B released by benign or normal tissue. Similarly, in animal models, Drewa *et al.* (1978) noted high activities of cathepsin B, β-glucuronidase, acid phosphatase, and arylsulfatases in the more invasive hamster melanomas.

The cathepsins have the potential to degrade the extracellular matrices surrounding tumors, and some can activate other latent tumor enzymes (Fig. 9). With its low pH optimum, cathepsin D may not be as important as cathepsin B, which has significant activity near neutral pH, in the degradation of the extracellular matrix (Mort *et al.*, 1980). In contrast to cathepsins C and D, cathepsin B can activate latent collagenase to a fully active enzyme (Barrett and McDonald, 1980). The possible role of metastatic cell proteases and glycosidases in the destruction of the basal lamina in capillaries during extravasation will be considered in Section V,B.

Several investigators have examined the levels of various proteases in malignant tissues in comparison to the surrounding host tissues (reviewed by Nicolson, 1982a; Liotta *et al.*, 1982a,b). In addition to the proteases already mentioned, the collagenases are thought to be an important tumor cell product, and collagenase levels have been correlated with tumor invasion of surrounding normal tissues (Wooley *et al.*, 1980; Liotta *et al.*, 1982b). There are also notable examples in which the levels of collagenase released by tumor correlate with metastatic potentials (Liotta *et al.*, 1980b, 1982b). However, collagenase activities are often measured by proteolytic activation of latent collagenases after their extraction from malignant cells or tissues. It is usually assumed in such studies that tumor-released latent collagenases are activated *de novo* by other proteolytic enzymes released from the malignant cells (Fig. 9). Enzymes such as plasminogen activator are of interest, because this enzyme can activate plasminogen to plasmin which, in turn, can activate latent collagenases (Moscatelli *et al.*, 1980).

The role of plasminogen activators in tumor invasion and metastasis has been controversial and is reviewed elsewhere (Nicolson, 1982a). Although the secretion of plasminogen activator generally correlates with the transformed phenotype, the relationship between plasminogen activator secretion and metastasis is unclear. In several metastatic models no obvious relationship between plasminogen production and metastatic potential has been observed (Nicolson *et al.*, 1976; Roblin, 1981; Talmadge *et al.*, 1981), but in other studies highly metastatic cells were found to release more plasminogen activator than less metastatic cells (Laug *et al.*, 1975; Wang *et al.*, 1980). The release of plasminogen activator and its activation of plasmin-

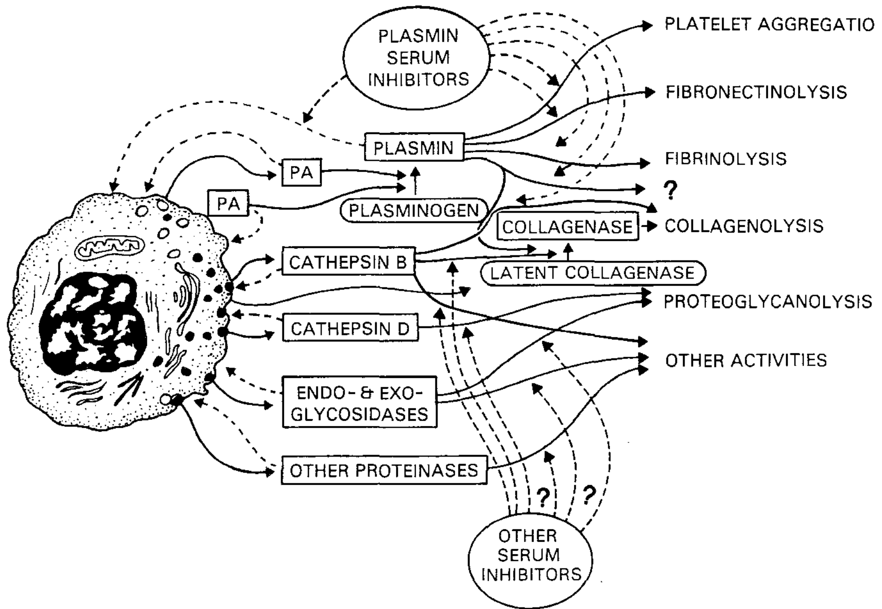

FIG. 9. Release or cell surface display of tumor-associated degradative enzymes and their possible roles in metastatic processes. The solid lines represent the possible direct or indirect effects of tumor-associated enzymes on biologic substrates that form a part of the barriers to malignant cell invasion. The broken lines represent possible feedback inhibitory or autolysis mechanisms. (Modified from Nicolson, 1982a.)

ogen to plasmin is thought to result in fibrinolysis, which can result in the dissolution of a fibrin deposit around tumor cells. Fibrin clots around tumor cells have been observed after invasion of malignant cells into blood vessels, and it is thought that dissolution of fibrin may result in enhanced detachment of tumor cells from the invading foci. In addition, many nonmalignant or normal cells produce high levels of plasminogen activator (Rifkin *et al.,* 1974; Tökés and Sorgente, 1976, Salo *et al.,* 1982).

High plasminogen activator levels have been found in a number of human cancers, including malignant lung tumors (Markus *et al.,* 1980) and cancers of the colon (Corasanti *et al.,* 1980) and of the prostate (Camiolo *et al.,* 1981). In the latter study, neoplastic prostate tissue contained approximately twice the concentration of plasminogen activator that was present in biopsies from hyperplasic prostates. Although abnormally high plasminogen activator levels have often been noted in various human cancers, the correlation between plasminogen activator activity and metastatic potential has been equivocal in studies using animal tumor models in which

nonmetastatic and highly metastatic tumor cell sublines were compared (reviewed in Roblin, 1981; Nicolson, 1982a).

There are a number of technical difficulties in determining the relationship between a tissue-degradative enzyme and the invasion and dissemination of malignant cells. First, a major problem is to determine whether the enzyme is derived or released from tumor cells or from infiltrating host inflammatory cells, endothelial cells, or other host cells. In only a few cases have these experimental problems been addressed adequately. Sloane *et al.* (1981, 1982) examined the contribution of normal host cells in producing the lysosomal enzymes measured in B16 melanoma tumor samples. By separating tumor cells from host cells with centrifugal elutriation and analyzing the purified cells, these authors found that the lysosomal enzyme activities measured were derived from malignant cells and not from contaminating host cells. In other studies a more complicated picture was apparent. For example, Urban (1981) found that the levels of lysosomal enzymes in tumors did not correlate with the invasiveness of the tumors, but rather with the extent of infiltration by cytolytic macrophages. Urban (1981) proposed that macrophage-mediated tumor cell cytolysis resulted in the release of lysosomal enzymes from the lysed tumor cells, although the elevated levels of lysosomal enzymes present in activated, tumoricidal macrophages might also be expected to make some contribution.

The second problem in defining functional correlates between the secretion of degradative enzymes and tumor invasion is that the sampling procedure itself might damage cells, resulting in nonspecific release of lysosomal enzymes. For example, the insertion of large-bore micropipets or microelectrodes, which have been used to measure the levels of lysosomal degradative enzymes in extracellular fluids, may yield erroneous results if cellular damage results in the nonspecific release of the enzymes (Poste and Flood, 1979).

The analysis of enzyme activities in tumor samples can also be complicated by the presence of a variety of enzyme inhibitors in tissues. For example, Hisazumi *et al.* (1974) found that tumors of the urinary bladder contain an inhibitor(s) of plasminogen activator. These authors noted that a correlation existed between the amounts of plasminogen activator-inhibitor(s) present in tumors and the malignant potential of the tumor cells. In their follow-up studies, Naito *et al.* (1981) examined 21 different human cancer cell lines for the presence of inhibitors of this enzyme and found that the majority possessed the inhibitors. The presence of such inhibitors suggests that attempts to measure the activities of tumor cell enzymes by sampling the fluid phase bathing the tumors, or by measuring enzyme activities after homogenization of tumor tissue, could well yield erroneous results if the inhibitors are not taken into account.

Finally, in many studies the measurements of degradative enzymes released by tumor cells are performed after the activation of proteolytic enzymes. The addition of activators proteolytic enzymes such as latent collagenase could thus mask actual enzyme activities *in situ* and produce falsely high values.

B. Implantation and Secondary Invasion of Metastatic Tumor Cells

The behavior of circulating tumor cells and the factors that determine their survival have been reviewed elsewhere (Fidler 1975a, 1976a, 1978a,b; Fidler *et al.,* 1978; Poste and Fidler, 1980; Fidler and Nicolson, 1981; Warren, 1981; Nicolson, 1982a; Poste, 1982a). Once in the circulation, a variety of cellular interactions and host properties can affect blood-borne arrest and survival, including the homotypic adhesion of tumor cells to form multicell emboli that are known to have increased implantation and survival characteristics (Fidler, 1973a; Liotta *et al.,* 1976). Heterotypic adhesive interactions, such as those between tumor cells and platelets (Gasic *et al.,* 1973; Pearlstein *et al.,* 1980), lymphocytes (Fidler, 1975b; Fidler and Bucana, 1977), endothelial cells (Kramer and Nicolson, 1979; Nicolson, 1982c), and soluble blood components involved in coagulation (Warren, 1973; Chew and Wallace, 1976; Chew *et al.,* 1976), are also important in blood-borne tumor cell implantation. In addition to these mostly cellular interactions, mechanical factors, such as the size and deformability of the circulating tumor cell emboli and the deformability of microcirculation (Zeidman and Buss, 1952; Zeidman, 1961), can affect the implantation of circulating malignant cells.

Common to most, if not all, of the malignant cells that circulate in the blood is that they perish at high rates (for example, see Fidler, 1970, 1976b). The high tumor cell death rates in the circulation are thought to be due to both passive events and natural host responses. Most circulating malignant cells cannot tolerate the high shear forces and mechanical distortions that occur during circulation (Sato and Suzuki, 1976; Zeidman, 1961; Zeidman and Buss, 1952), and often these tumor cells lack the proper cellular structures to withstand the trauma of blood flow and transcapillary passage. The transcapillary forces that confront malignant cells could alone account for the wide range in tumor cell viabilities found when circulating malignant cells are recovered from various organs after intravenous injection. Weiss (1980) has proposed that differences exist between tumor cell viabilities in organs where the cells lodge first and in organs that are reached only after tumor cells have had to first pass through microcirculatory networks. Weiss claims that the latter cells are more susceptible to death in the next capillary

bed encountered because these malignant cells are "processed" before their arrival at another organ site. However, the data could also be explained by the simple fact that any given malignant cell may only survive a given amount of shear force or trauma. Thus, there would be a random statistical chance of survival as the cells reached each subsequent capillary bed.

The additive effect of repeated mechanical trauma on tumor cells should result in decreasing the survival percentages of the cells capable of recirculating. Clearly this process could vary substantially between cells derived from different tumors or the same tumor, and it could also be determined by the capillary systems that are encountered first. In experimental systems, widely different tumor cells survive at quite different rates after they pass through the lung microcirculation. For example, B16 melanoma cells are arrested at high rates in the lungs after intravenous injection, and few survive to form experimental metastases in the lungs or in other sites (Fidler, 1970, 1976a; Fidler and Nicolson, 1976). In contrast, cells of the murine RAW117 large cell lymphoma pass quickly through the lung microcirculation without specific implantation and subsequent pulmonary colonization, surviving at a high rate to implant, invade, and survive in the liver to form large numbers of hepatic tumor nodules (Reading *et al.*, 1983).

Host-mediated destruction of circulating tumor cells is thought to be an important, albeit nonspecific, host surveillance mechanism. It is of interest to note that the role of *specific* host immunity in the destruction of blood-borne tumor cells is undocumented. This is due to the fact that the arrest and the survival of tumor cells in immunized as compared to unimmunized animals are often similar (for example, see Glaves and Weiss, 1977). In addition, nonspecific or natural mechanisms mediated by natural antibodies (Lewis, 1974; Vaage and Agrawal, 1976; Vaage, 1973, 1978) or natural killer cells (Gorelik *et al.*, 1979; Hanna and Fidler, 1980; Hanna, 1980; 1982) are important in destroying circulating tumor cells.

Natural antibodies against metastatic murine cells have been found in the serum of "naive" animals (Vaage, 1973, 1978; Vaage *et al.*, 1974; Vaage and Agrawal, 1976), and these natural antibodies may be involved in complement-mediated cytolysis or cytostasis of blood-borne tumors. Alternatively, natural antibodies may opsonize tumor cells which can then be destroyed by antibody-dependent, cell-mediated mechanisms (Chow *et al.*, 1979). Vaage (1978) has found that natural antibodies plus complement can produce cytolysis of malignant murine carcinoma and ovarian carcinoma cells *in vitro,* and these immunoglobulins can presumably do the same *in vivo*. Interestingly, the levels of natural antitumor antibodies in tumor-bearing animals have been found to be lower than those in naive animals (Vaage, 1978).

Natural killer or natural cytotoxic cell-mediated activities against circulating tumor cells are thought to be extremely important in surveillance

against the formation of metastases (reviewed by Hanna, 1982). Hanna and Fidler (1980) found that NK cells can effectively kill metastatic melanoma cells within the bloodstream. By examining the abilities of B16 melanoma cells to implant, survive, and grow in animals with low or high NK cell activities, such as in beige mice (Talmadge *et al.,* 1981) or in nude mice (Hanna and Fidler, 1980; Hanna, 1980), the role of NK cell-mediated destruction of tumor cells in the circulation was confirmed. On the other hand, not all malignant cells in the circulation are susceptible to NK cell-mediated destruction (Schirrmacher, 1981; Reading *et al.,* 1983), and some may escape because of differential NK cell sensitivity. For example, Gorelik *et al.* (1979) found that normal spleen cells can kill 3LL carcinoma cells obtained from locally growing tumors, but are less able to kill 3LL carcinoma cells established from spontaneous lung metastases. The antimetastatic action of the NK or NC cells was shown in experiments in which these cells were mixed with 3LL tumor cells and injected into the footpads of syngeneic mice. Spontaneous metastasis of the 3LL cells was prevented at high ratios (100:1) of spleen to tumor cells. However, when tumor cells obtained from 3LL lung metastases were utilized in similar experiments, similar ratios of spleen to tumor cells were less effective in inhibiting the formation of metastases. Hanna (1980) has found that stimulating the NK system enhances host resistance to experimental metastasis by murine melanoma cells and other metastatic murine tumors. LeGrue (1983) recently discovered that 1-butanol extraction can remove cell surface components that affect NK function. Noncytolytic concentrations of 1-butanol were used to treat MCA-F fibrosarcoma and B16 melanoma cells, and the treated, viable cells were then assayed for their experimental metastatic (and other) properties. The numbers of experimental pulmonary metastases were increased significantly by 1-butanol treatment without modifying the homotypic or heterotypic adhesive properties of the cells. This was attributed to an increase in the resistance of the 1-butanol-treated tumor cells to NK-mediated cytolysis.

The arrest of blood-borne tumor cells in the microcirculation by nonspecific mechanical trapping and lodgment of tumor cell emboli may be the simplest of the mechanisms that govern malignant-cell implantation, survival, and growth. It is well known that tumor cell emboli arrest much more efficiently in the capillary bed of the first organ encountered (Zeidman and Buss, 1952; Fidler, 1973b; Liotta *et al.,* 1976). The ability of circulating malignant cells to undergo deformation in the microcirculation and subsequently to survive may also play an important part in their ability to recirculate after initial lodgment. In fact, drugs which modify cell deformability and disrupt cytoskeletal organization can alter the kinetic distributions of circulating radiolabeled tumor cells and their ability to lodge, detach, and recirculate to other organ sites (Raz *et al.,* 1981). Thus, tumor

deformability is thought to be important in the survival and recirculation of cells which have lodged in the microcirculation.

The ability of tumor cells to be deformed, such as by transcapillary passage, and to survive appears to be correlated, at least in some systems, with their metastatic success. Attempts to characterize tumor cell deformability by measuring the abilities of tumor cells to pass through filters of defined pore sizes under positive pressure have been conducted by Sato and Suzuki (1976), with the assumption that the pressure drop across the filter pores is similar to the pressure drop encountered in the arterial-to-venous passage. Although Sato and Suzuki measured the ability of a series of hepatocarcinoma cells to survive the trauma of deformation under flow conditions, it is unlikely that their transfilter passage of tumor cells is a linear event even under different pressure-flow conditions (Lessin *et al.,* 1977). In addition, the actual pressures used in such experiments must be in the physiologic range, since the differences found under one set of conditions *in vitro* may be different from those encountered *in vivo.* Nonetheless, Sato and Suzuki established a relationship between the "deformability" (filterability) of hepatocarcinoma cells and their transcapillary survival and subsequent ability to form metastases at sites beyond the first organ of lodgment.

In other experiments tumor cell deformability has been measured by applying mechanical forces to the cell surface of single cells. This was accomplished with a micropipet that was brought to the surface of the cell; subsequently a negative pressure was applied to draw a portion of the cell into the tip of the pipet. The measurements were recorded as the degree of cell surface deformation for a given pressure (Weiss, 1976). Evans and Waugh (1980) have improved this technique considerably by using pressure transducers and video recording of cell deformations in order to obtain data on both the extent and the kinetics of deformation.

During blood-borne transit, tumor cells can undergo a number of intercellular interactions (see reviews by Fidler *et al.,* 1978; Fidler and Nicolson, 1981; Poste, 1982a; Nicolson, 1982a). Since formation of multicell aggregates results in enhanced implantation and survival in the circulation (Zeidman and Buss, 1952; Fidler, 1973a; Liotta *et al.,* 1976), the rates of homotypic aggregation of tumor cells possessing various metastatic potentials to form cell aggregates have been measured *in vitro.* In most studies tumor cells with higher metastatic potentials were found to exhibit higher rates of homotypic aggregation to form multicell emboli (Nicolson, 1978a,b; Nicolson *et al.,* 1976; Winkelhake and Nicolson, 1976). The studies used, for the most part, B16 melanoma sublines and measured the rates of homotypic aggregation; various B16 melanoma sublines which underwent homotypic aggregation at high rates were found to be of high potential for

producing blood-borne organ colonization. Similar results have been found with murine sarcoma sublines selected *in vivo* for organ implantation (Nicolson, 1978b). There are undoubtedly exceptions to this generalization, particularly in the case of malignant cells that disseminate and are arrested as single cells.

Heterotypic cellular interactions occurring during blood-borne transit can also lead to enhanced implantation and survival. These events can be both nonspecific, such as those involved in mechanical lodgment, and specific, such as those involved in specific adhesive interactions. Important host cells in blood-borne tumor interactions are platelets. Platelets are known to enhance blood-borne implantation of many types of malignant cells (Gasic *et al.,* 1973, 1976, 1978; Hilgard, 1973; Donati *et al.,* 1977), and it is well known that the incidence of metastasis in certain experimental systems can be reduced by inducing thrombocytopenia. This is usually accomplished by administration of antiplatelet drugs (Gasic *et al.,* 1972, 1973; Brown, 1973). Platelet–tumor cell heterotypic adhesive activities have been demonstrated in most malignant cells that can enter the circulation (Giraldi and Sava, 1982; Gasic *et al.,* 1977), though exceptions exist, such as in the 13762NF tumor system (Estrada and Nicolson, 1983). Platelet–tumor cell adhesion occurs at the cell surface, and platelet-aggregating activities have been found in cell membrane vesicles spontaneously shed from a variety of tumor cells (Gasic *et al.,* 1977, 1978). Platelet-adhesion activities have been purified from a variety of tumor cell lines (Gasic *et al.,* 1972, 1977; Pearlstein *et al.,* 1980), and these activities have also been found in isolated cell membrane vesicles released spontaneously from tumor and transformed cells (Pearlstein *et al.,* 1980; Gasic *et al.,* 1978). Platelet–tumor cell aggregation factors have been partially purified and found to be composed of protein, lipid, and carbohydrate (Hara *et al.,* 1980), suggesting that they are either lipoglycoproteins or membrane glycoproteins surrounded by some lipid, possibly in the form of lipid "droplets" or membrane fragments.

Malignant cells also form heterotypic aggregates with host lymphocytes during blood-borne transit. In the B16 melanoma system, Fidler (1975b) and Fidler and Bucana (1977) found that the high lung-colonizing B16-F10 variant cells adhered to host lymphocytes at higher rates than the B16-F1 cells of lower metastatic potential. Fidler *et al.* (1976) used sequential selection methods to recover B16 melanoma cell variants that were resistant to lymphocyte-mediated cytolysis. After six *in vitro* selections for resistance to lymphocyte-mediated cytolysis, the selected lymphocyte-resistant B16 sublines showed reduced lymphocyte reactivity and formed significantly fewer experimental lung metastases. Although tumor cells such as B16 melanoma appear to be sensitive to natural killer cells while they are in the circulation (Hanna, 1980, 1982; Hanna and Fidler, 1980), it is not known

whether specific immune mechanisms, such as those mediated by T lymphocytes, make any contribution to the destruction of circulating tumor cells.

When blood-borne malignant cells reach the microcirculation, they interact with cells of the vascular endothelium. These interactions include nonspecific mechanical arrest, as well as formation of specific stable adhesions between tumor cells and capillary endothelial cells. These interactions may be necessary to prevent the spontaneous detachment of the tumor cells and their subsequent recirculation during continued mechanical trauma. The use of model vessels or endothelium to study the interactions of malignant cells will be discussed in Section VI,B.

The suface of the malignant cell appears to be involved in blood-borne implantation, and procedures have been used to modify this process by blocking or modifying certain cell surface components. For example, specific enzymes have been used to modify blood-borne implantation and the subsequent experimental formation of metastases (Hagmar and Norrby, 1973; Fidler, 1978a; Sinha and Goldenberg, 1974). Antibodies have also been used to block organ-specific tumor cell colonization. For example, Shearman *et al.* (1980) found that the liver-colonizing abilities of avian MDCC-AL2 lymphoma sublines could be reduced and correlated with the amounts of a surface antigen recognized by a monoclonal antibody. Similarly, Nicolson *et al.* (1982b) found that an antigen similar to a hepatic antigen found on embryonic liver cells was present on adult murine lymphoma cells, and its expression was correlated with liver-colonization ability (McGuire *et al.,* 1983). Divalent or monovalent antibody fragments against the liver antigen, but not against unrelated antigens such as H-2, were found to completely inhibit blood-borne colonization of liver by the lymphoma cells (Fig. 10). Future investigations utilizing such techniques will focus on the role of specific cell surface components in endothelial cell interactions at specific organ sites.

The behavior and survival of circulating tumor cells in the microvasculature is also thought to be modified by the deposition of blood components, such as fibrin, on the arrested tumor cells (Warren, 1973; Chew *et al.,* 1976; Wood, 1971). The formation of a fibrin coating around arrested malignant cells appears to be related to their thromboplastic properties (Gasic *et al.,* 1976; Day *et al.,* 1969; Mootse *et al.,* 1965). Metastatic tumor cells that possess high thromboplastic and platelet-aggregating activities generally form large numbers of blood-borne metastases (Chew and Wallace, 1976; Gasic *et al.,* 1976, 1977; Tanaka *et al.,* 1977). The thromboplastic properties of malignant cells are in turn related to a substance which has been called thromboplastin (Gasic *et al.,* 1977). Crude thromboplastin has been prepared from the lung-colonizing AH130 hepatocarcinoma cell lines by Kohga and Tanaka (1979). Intravenous injection of this partially

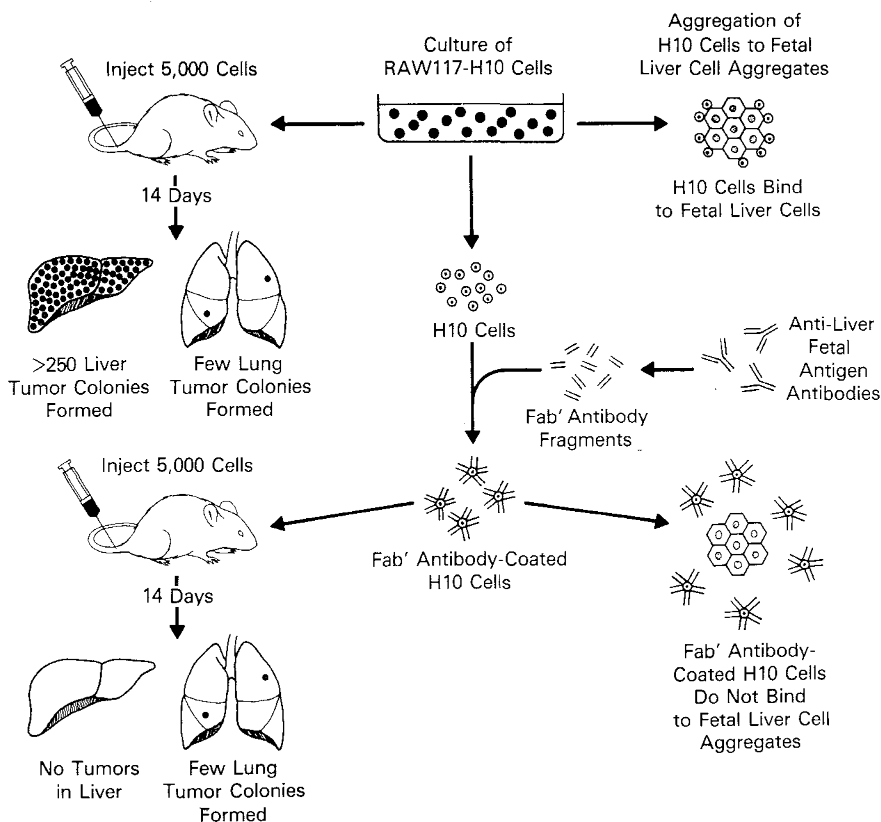

FIG. 10. The role of fetal antigens in the metastasis of murine RAW117 large cell lymphoma to liver. Tumor subline RAW117-H10, selected 10 times *in vivo* for enhanced liver metastasis, forms large numbers of liver tumors within 2 weeks after injection intravenously into syngeneic BABL/c mice. With antibodies against the murine fetal liver cell adhesion system, the aggregation of fetal liver cells and the binding of lymphoma cells to fetal liver cell aggregates can be blocked. The role of the fetal liver recognition system in the metastasis of RAW117 cells was assessed by treating the lymphoma cells with Fab′ fragments of these antibodies, injecting the cells intravenously, and assessing liver colonization. The Fab′ antibody fragments blocked tumor cell colonization in liver, but not in lung. (Adapted from Nicolson, 1982d; reproduced with permission from Nicolson and Poste, 1983a.)

purified preparation caused widespread thrombosis formation in lung capillaries, arterioles, and arteries. The identity of thromboplastin and its detailed structure are under investigation.

The thromboplastic and platelet-aggregation properties of tumor cells, though important, may not be essential for blood-borne implantation. The evidence for the role of platelet aggregation and fibrin formation has been

reviewed by Warren (1981). The evidence indicates that these activities are not required for metastatic spread of tumor cells, and in many cases a definite correlation cannot be defined between the presence of thromboplastin and platelet-aggregation activities and the ability to metastasize via a blood-borne route.

After implantation of malignant tumor cells in the microcirculation has occurred, the tumor cells escape from the vascular compartment by invasion of the blood vessel wall, or, alternatively, they simply grow expansively within the vessel until it ruptures (Nakamura *et al.*, 1977; Kawaguchi *et al.*, 1982, 1983b). Invasion of the blood vessel wall or extravasation usually starts with tumor cell migration through the endothelial cell layer (Fig.

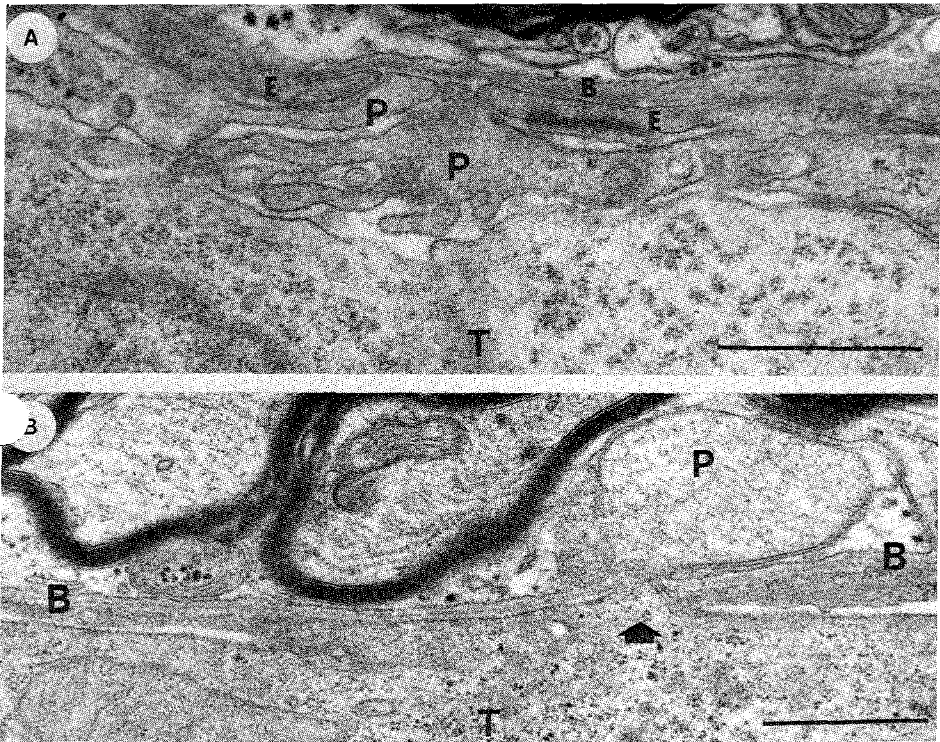

FIG. 11. Blood-borne implantation and invasion of brain vascular endothelium by rat ascites hepatoma cells injected into the carotid artery. In A, pseudopodia (P) from a malignant tumor cell (T) have caused retraction of adjacent endothelial cells (E), but the tumor cell has not yet penetrated the underlying basal lamina (B). In B, a tumor cell has attached to the basal lamina (B) and projected a pseudopodium (arrow; P) into perivascular tissue through a small pore (~ 0.16 μm in diameter) in the basal lamina. Bars = 1 μm. (Data of Kawaguchi *et al.*, 1982, with permission.)

11A). This can occur through endothelial cell gaps or defects (Wood, 1958), by tumor cell disruption, by invasion of the intercellular junctions between adjacent endothelial cells (Kramer and Nicolson, 1979; Chew *et al.,* 1976; Sindelar *et al.,* 1975; Kawaguchi *et al.,* 1982), or by penetration of the endothelial cell cytoplasm by tumor cell pseudopodia (Roos and Dingemans, 1979; Dingemans, 1974). This is followed by adherence to and destruction of the underlying endothelial basal laminar basement membrane (Wood, 1971; Warren, 1981). Often this produces only a local lesion in the basal lamina where malignant cells migrate out of the circulation (Fig. 11B) (Kawaguchi *et al.,* 1982). Endothelial cell models for studying this process will be discussed in Section VI,B.

Destruction of the host tissue stroma by invading malignant cells occurs during blood-borne metastasis. However, in many tumor systems massive host destruction by invading malignant cells does not occur, and it does not appear to be an essential prerequisite of the invasion process (Carr *et al.,* 1976; Roos and Dingemans, 1979; Warren, 1981). Tumor cells are often found associated with and extending along veins and other vessels, attached to apparently intact basal lamina (Fig. 12) (Kawaguchi *et al.,* 1983b). The synthesis and release of factors that inhibit invasion may be important in determining differences in the susceptibility or resistance of various tissues to invasion and destruction by metastatic tumors (De Vore *et al.,* 1980; Kuettner *et al.,* 1977; Sorgente *et al.,* 1975).

Stromal cell defense reactions may also limit metastatic cell invasion. De Vore *et al.* (1980) found that the invasion of small cell carcinoma at subcutaneous sites in nude mice was limited by the formation of a fibroblastic capsule around the tumors. They determined that the fibroblastic layer *per se* was not responsible for providing a mechanical barrier to invasion, but the fibroblasts were releasing inhibitors active against human collagenases and possibly other degradative enzymes of tumor cells.

The frequency of metastasis in specific organs suggests that the properties of circulating malignant cells, host capillary endothelial cells, and the organ environment may each affect the fate of tumor cells *in vivo,* rather than random events which should result in widespread metastasis or random colonization. That the simple distribution of circulating malignant cells does not totally determine the subsequent formation of metastases has been determined with radiolabeled tumor cells in kinetic distribution studies. Using [^{125}I]IUdR-labeled B16 melanoma cells injected intravenously into syngeneic mice, Fidler (1975b) found that the initial rates of arrest and subsequent survival in the lungs 1 day after injection of the high lung-colonizing B16-F10 subline were always greater than the rates obtained with the low lung-colonizing B16-F1 subline. Cells from either subline lodged or were arrested initially in the lungs after intravenous injection, but most of these cells subsequently died or recirculated to other sites. Twenty-four

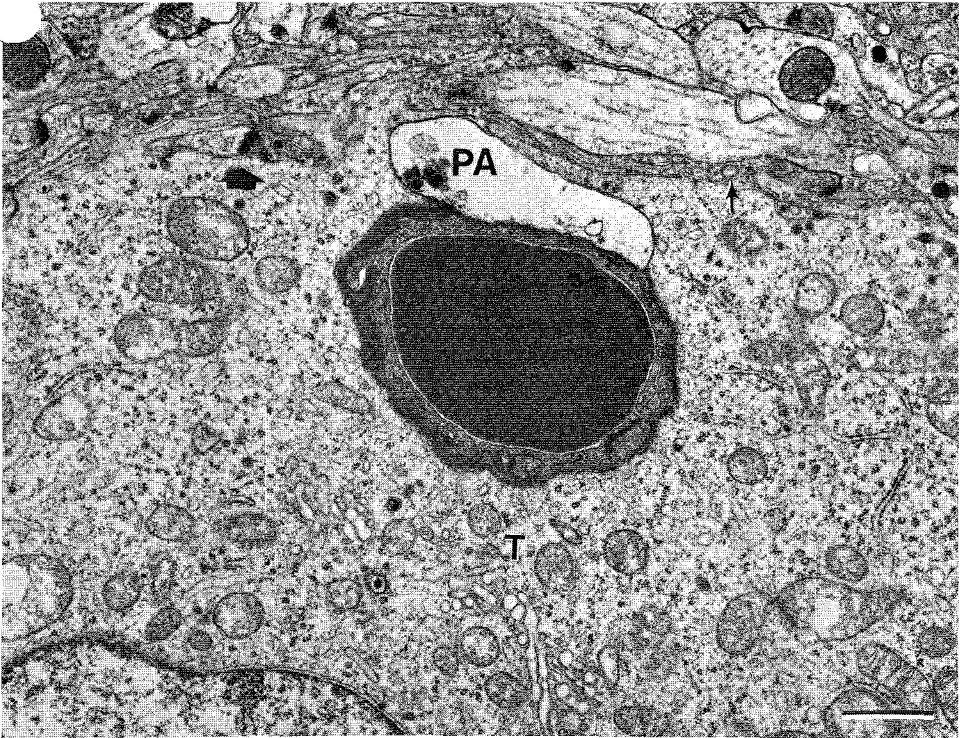

FIG. 12. A murine B16-B14b melanoma cell located extravascularly near the basal lamina of a cerebral capillary 14 days after injection of melanoma cells into the carotid artery. A tumor cell (T) is extending cytoplasmic protrusions (arrow head) and apparently engulfing a nerve synapse (large arrow) near a perivascular astrocyte process (PA). Bar = 1 μm. (Reproduced with permission from Kawaguchi *et al.*, 1983b.)

hours after injection of the radiolabeled B16-F10 cells, approximately one-half were still viable and were localized in the lungs. This contrasted with the B16-F1 cells, of which only about one-tenth remained viable in the lungs. The difference appeared to be due to a specific rather than nonspecific mechanism, such as simple trapping or lodgment, because administration of B16 melanoma cells by intercardiac injection resulted in similar numbers of pulmonary tumor colonies 2 weeks after their introduction into the circulation (Fidler and Nicolson, 1976). In these experiments, the formation of lung tumor colonies did not correlate with the initial patterns of tumor cell arrest in the lungs, which were much higher after the intravenous injection route, indicating that mere mechanical localization and retention in the pulmonary microcirculation are not sufficient for the formation of metastases.

Evidence exists that specific rather than nonspecific tumor cell properties

are responsible for metastatic colonization of particular organs. As mentioned above, specific enzyme treatments of tumor cell surface components can modify malignant cell implantation properties and the formation of metastases (Hagmar and Norrby, 1973; Sinha and Goldenberg, 1974; Fidler, 1978a). In addition, specific biosynthetic modification of tumor cell glycoproteins by glycosylation inhibitors can block metastatic cell implantation. Irimura *et al.* (1981) used tunicamycin to reversibly modify the biosynthesis of glycoproteins in B16 melanoma cells. Nontoxic treatment with tunicamycin resulted in morphologic changes of cells and changes in their adhesive properties, such as cell rounding and loss of adhesion to vascular endothelial cells *in vitro* (Fig. 13). However, these changes, as well as changes in implantation properties in the circulation, were reversed within 1 to 2 days after removal of the drug from the cultures in which the B16 cells had been modified by treatment. The modifications were found to correlate with the loss of a particular class of cell surface molecules (sialogalactoproteins) which were postulated to be involved in B16 cell implantation and adhesion properties (Irimura *et al.,* 1981; Irimura and Nicolson, 1981).

Another important line of evidence indicating that specific tumor cell properties are involved in blood-borne implantation at particular sites comes from the transfer of portions of plasma membrane from B16-F10 to B16-F1 cells. B16 melanoma cells spontaneously shed closed plasma membrane

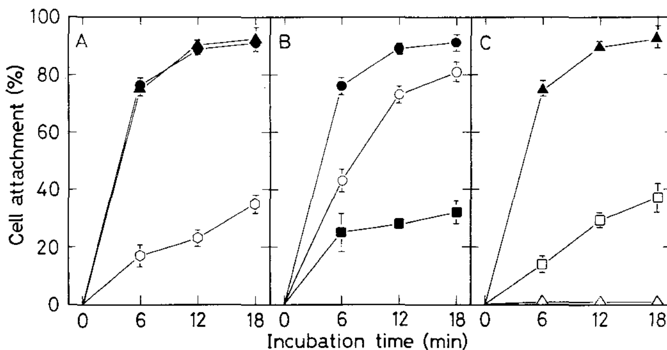

FIG. 13. Rates of attachment of radiolabeled murine B16-F10 melanoma cells to endothelial cell monolayers, to basal lamina-like extracellular matrix produced by endothelial cells, or to immobilized fibronectin *in vitro.* A, Adhesion of untreated B16-F10 cells to untreated endothelial cell monolayers (○), endothelial matrix (●), or immobilized fibronection (▲); B, adhesion of untreated (○,●) or tunicamycin-treated (0.5 μg/ml for 24 hours) (■) B16-F10 cells to endothelial extracellular matrix or matrix pretreated with 400 μg/ml purified antifibronectin antibody (○); C, adhesion of untreated (△, ▲) or tunicamycin-treated (0.5 ~gm/ml for 24 hours) (□) B16-F10 cells to immobilized fibronectin or immobilized fibronectin pretreated with 400 μg/ml antifibronectin (△). (Reproduced with permission from Irimura *et al.,* 1983b).

vesicles *in vitro* and *in vivo*. The B16-F10 vesicles were harvested, purified, and added back to the B16-F1 cells in the presence of fusing agents to insert B16-F10 plasma membrane components by fusion into the cell membranes of the low lung-colonizing B16-F1 line. The vesicle-modified B16-F1 cells showed enhanced blood-borne lung implantation and experimental metastatic properties, but only when the membranes transferred were from the high lung-colonizing B16-F10 line (Poste and Nicolson, 1980). Controls demonstrated that the transferred plasma membrane components were integrated into the cell surface membranes of the recipient cells. These changes were transient, and their loss correlated with the natural turnover and degradation of plasma membrane glycoproteins (Poste and Nicolson, 1980).

One of the most interesting experiments examining the abilities of metastatic cells to implant, survive, and grow at particular organ sites such as lung was performed with ectopic lung implants. By implanting neonatal lung tissue into the thighs of syngeneic mice, Kinsey (1960) found that a lung-colonizing melanoma line could colonize both the natural lung and the grafted ectopic lung tissue. An important control for this experiment was to show that nontarget organ tissues grafted into similar sites were not colonized by the melanoma cells. Hart and Fidler (1980) repeated this experiment with lung-colonizing B16 melanoma cell variants. After intravenous injection into syngeneic mice bearing ectopic lung implants, they found that experimental metastases developed in the lungs, as well as in the lung implants, but the metastases did not develop in ectopic implants of other tissues. Examination of the kinetic distributions of [^{125}I]IUdR-labeled B16 cells in these experiments revealed no significant differences in the initial patterns of B16 cell arrest in implanted fragments of either lungs or kidneys, suggesting that the location of the cells in these implants and their ability to extravasate may determine whether colonization and tumor growth occurs at the implant site, or that events subsequent to the arrest and survival of malignant cells are important in determining tumor growth at the ectopic sites. Hart and Fidler (1980) also found that liver-colonizing M5076 carcinoma cells failed to grow in either the lungs or the ectopic lung implants, consistent with the specificity of this tumor line to colonize liver and not lung. It was not determined whether proper extravasation or other tissue factors were involved in allowing growth of the lung-colonizing B16 cells to form tumor colonies at the ectopic lung sites.

C. Survival and Growth of Metastatic Tumor Cells

Once malignant cells arrive at a distant site and invade into the surrounding extravascular organ parenchyma, they must survive and proliferate to form a clinically detectable metastasis. There are many environmental factors that appear to play a role in the formation of metastases, such as the

endocrine status of the host. A survey of the clinical literature indicates that hormonal imbalances can influence the formation of metastases. This is often seen in human malignant melanoma, where primary tumors have been observed to grow more slowly and to metastasize less frequently in female than in male patients. This observation has been attributed to variations in hormones between male and female melanoma patients (Cochran, 1973). In addition, patterns of experimental metastasis in female mice differ from those in male mice (Proctor *et al.,* 1976). Proctor *et al.* found that surgical removal of female endocrine organs abolished differences between the formation of metastases in female and male mice. In other studies, addition of hormones was found to modify the metastatic process. Shafie and Liotta (1980) found that ectopic implants of estrogen-containing pellets enhanced the spontaneous metastasis of human MCF-7 mammary carcinoma cells to lung, liver, and spleen of nude mice. The same authors also showed that the metastatic behavior of the MCF-7 tumor can be dramatically inhibited in female mice by mastectomy and induction of diabetes with streptozotosin.

It it well known that trauma, tissue damage, or inflammation can modify local tissue environments and facilitate the implantation and survival of blood-borne malignant cells. For example, metastases often form at sites of injury or inflammation (Smith *et al.,* 1958; Sugarbaker *et al.,* 1971; Fisher *et al.,* 1967). Metastases can develop at the sites of surgical incisions, and this can occur at long distances from the sites of tumor resection (Der Hagopian *et al.,* 1978). Since sites of tissue injury, such as surgical injury, contain damaged blood vessels, these should naturally allow circulating malignant cells to escape the vascular system at the point of injury.

Inflammation may aid malignant cells to invade and metastasize. Host defense cells, such as neutrophils, lymphocytes, or macrophages, can release a number of tissue-degradative enzymes, growth factors, and other substances at the sites of inflammation (Sugarbaker, 1981; Nicolson, 1982a). However, this does not always happen. For example, malignant cells can release substances that impair leukocyte chemotaxis, thus inhibiting the accumulation of leukocytes in the tumors and possibly preventing subsequent degranulation and release of lysosomal enzymes (Hamby and Barrett, 1977; Snyderman and Pike, 1976).

Cancer therapies, including radiation, hormones, chemotherapy, hyperthermia, or even immunotherapy, can modify the host's microenvironment. For example, X-irradiation can result in an increased number of metastases restricted to the radiation field (Sugarbaker, 1981). This has also been seen in experimental animals in which X-irradiation of the thoracic cavity enhanced the implantation and formation of metastases of melanomas (Fidler and Zeidman, 1972) and fibrosarcomas (Withers and Milas, 1973). Administration of hormones has also been shown to affect clinical

metastatic patterns. Administration of corticosteroids to breast cancer patients results in the increased formation of metastases in the spleen (Sherlock and Hartmann, 1961; Iverson and Hjort, 1958). An immunotherapeutic regimen to treat malignant melanoma resulted in an increased incidence of cerebral metastases in several patients (Grooms *et al.,* 1977).

Other therapeutic regimens can also affect the incidence and location of metastases. Van Putten *et al.* (1975) examined the effect of more than 30 chemotherapeutic drugs utilized in cancer chemotherapy on experimental blood-borne metastasis and found that most of the drugs caused alterations in the number of lung metastases. The most dramatic effect was produced by treatment with cyclophosphamide, which resulted in a dramatic increase in the number of pulmonary tumor colonies. Cyclophosphamide has been shown to increase lung colonization by blood-borne malignant cells in other systems (Carmel and Brown, 1977; Peters and Mason, 1977). This may occur because of damage to the pulmonary microvascular system, or, alternatively, the treatment with cyclophosphamide could result in the release of tissue-degradative enzymes or growth factors, or it could affect the host's immune system.

Host immunity can dramatically modify the formation of metastases in at least certain systems (this will be discussed in detail in Section VII,C). It has been proposed that there may be organ differences in host response to metastatic cells (Fidler *et al.,* 1978; Vaage *et al.,* 1971), and this could be important in certain organs that possess large numbers of reticuloendothelial cells.

Tumor cells appear to be capable of modifying their microenvironment by the secretion or release of biologically active molecules. These molecules could be any number of substances, such as growth factors, hormones, and hormone-like molecules. For example, prostaglandins are known to be secreted by many malignant tumors (reviewed by Nicolson, 1982a). E-type prostaglandins are thought to be responsible for tumor-induced, osteoclast-mediated bone resorption (Galasco, 1976) and for inhibition of effector activities by activated macrophages (Schultz *et al.,* 1978). Prostaglandins of the D series are known to modify platelet aggregation (Nishizawa *et al.,* 1975; Smith *et al.,* 1974), and these, in turn, can affect malignant cell implantation properties. Fitzpatrick and Stringfellow (1979) have found that B16 melanoma cells produce prostaglandin D_2. Since the low lung-colonizing B16-F1 variant produced more prostaglandin D_2 than the B16-F10 subline, they proposed that the production of this class of prostaglandin modified the platelet-aggregation abilities of B16-F1 cells.

Prostaglandins of the E series have been found to be produced by tumor cells (Bennett *et al.,* 1976, 1977). Since prostaglandins of the E type can cause the activation of osteoclasts, they may be important in the destruction of bone by metastasizing cells. Evidence for such an involvement in osteo-

lysis is that bone resorption caused by tumor cells was retarded by prostaglandin inhibitors such as indomethacin (Powles *et al.,* 1973). Indeed, human breast carcinomas have a high propensity for metastatic colonization of bone, and these same tumors produce high levels of E-type prostaglandins (Bennett *et al.,* 1976, 1977).

VI. *In Vitro* Models of Tumor Cell Implantation and Invasion

A. Correlations and Quantitation

In this section we will describe the requirements for correlating data from studies of tumor cell implantation and invasion *in vitro* with data obtained *in vivo,* and the techniques and methods currently available for examining these phenomena. We have stressed repeatedly that any experimental attempt to correlate specific cellular properties with any behavioral trait such as implantation, invasion, and metastasis must fulfill certain basic criteria. Although many of these have been discussed in previous sections, these requirements demand that the tumor cells being studied be invasive *in vivo;* that any heterogeneity in the expression of these traits by cells within the same tumor cell population can be identified, and, if necessary, that the cells can be separated for further examination; and that the stabilities of specific cell phenotypes be known during serial passaging *in vitro* or *in vivo.* When these criteria are met, a combination of *in vitro* and *in vivo* techniques can be used to study the properties of implantation and invasion. Since each approach has its own limitations, these will be discussed along with newer methods for examining traits of malignant cells.

1. *Correlations of Data Obtained* in Vitro *and* in Vivo

The major source of experimental material used in studies on tumor implantation and invasion are tumor cells growing *in vitro.* As mentioned previously (Section IV,B), when cells are grown under controlled conditions, they can be relatively stable and large numbers can be obtained for detailed examination. The relative simplicity of *in vitro* tissue culture systems provides researchers with opportunities to study directly the cellular and subcellular changes induced by environment, cellular interactions, or exposure of cells to agents *in vitro* which modify their behavior *in vivo* (Poste, 1982b).

Provided that investigators are aware that phenotypic changes may be imposed on cells by cultivation *in vitro,* cell culture methods offer important opportunities for correlating specific cellular and subcellular properties

with implantation and invasion. It is thus paramount to pay close attention to the technical implications of cellular heterogeneity in malignant cell populations (Section IV) and to take appropriate steps to ensure that the cell populations being studied are as uniform as possible. Alternatively, if they are heterogeneous, the extent of cellular heterogeneity must be known in order to ensure that the properties under examination are expressed by those cells in the population which possess the relevant characteristics *in vivo.*

In order to correlate data on implantation and invasion obtained *in vitro* and *in vivo,* parallel studies must be conducted. Thus, in seeking to correlate properties detected in cultured cells with implantation and invasion *in vivo,* it is necessary to show that the same properties are still expressed by the malignant cells *in vivo* and are not subject to phenotypic modulation during growth *in vitro.* For example, Miner *et al.* (1982) examined the relationship between the expression of a 90,000 molecular weight cell surface glycoprotein (gp90) and the ability of B16 melanoma cells to colonize brain meninges. Tumor cell clones were obtained from the highly metastatic brain-colonizing subline B16-B14b, cultured *in vitro,* and then examined in parallel for expression of the gp90 glycoprotein *in vitro* (Fig. 1) and for their biologic properties *in vivo* (Fig. 5). Since some of the clonal populations were unstable and lost their brain-colonizing capacities with time, if the gp90 glycoprotein were important in brain colonization, its expression should change in parallel with the biologic properties of the cells being examined. In these experiments, a good correlation was revealed between the loss of exposure of the gp90 glycoprotein and brain-colonization capacity (Miner *et al.,* 1982). Similarly, in clones of the 13762NF mammary adenocarcinoma, the expression of two cell surface glycoproteins correlates with spontaneous metastatic potential. One of these components, a ~680,000 molecular weight sialogalactoprotein, increases with metastatic potential, while the other, a ~80,000 molecular weight sialoglycoprotein, decreases with metastatic potential (Steck and Nicolson, 1983). In any given cell clone or subline, different properties may determine implantation, survival, and growth characteristics. Thus, changes in any one of these properties could result in the loss of biologic activity in the assays *in vivo.*

2. *Quantitation of Implantation, Invasion, and Metastasis*

One of the problems encountered in analyzing tumor and/or host properties important in implantation, invasion, and metastasis has been the lack of uniformly adopted, reproducible techniques for assaying these characteristics *in vivo.* In a previous section (Section III), we discussed two important experimental approaches for assaying the formation of metastases. Obviously, scoring in a binomial fashion whether metastasis occurs or does

not occur in a set of animals chosen for these assays is not highly accurate. For example, 80% of the animals in an experiment could have a heavy burden of metastatic growths at multiple sites in one assay, and 80% of the animals in another group could have only a few metastatic colonies at one site and be scored exactly the same. Clearly, *in vivo* assays that quantitate the number and location of metastatic growths will yield more accurate data, and in some cases investigators may want to estimate the total tumor burden at a given site by taking into account the numbers and volumes of malignant tumor colonies. In at least one system this has been accomplished. Welch *et al.* (1983b) have determined the number-volume relationships of spontaneous and experimental metastasis assays using cloned lines of the rat 13762 mammary carcinoma (Fig. 14 and Table IV). By calculating the total tumor burden in each animal, these authors could closely estimate the actual tissue mass of metastatic involvement.

Another problem frequently ignored in analyzing data obtained *in vivo* is reproducibility. Obviously, experimentations *in vivo,* such as tumor implantation, invasion, and formation of metastases in experimental animals, are fraught with a number of variables which may not be easily controlled. Therefore, it is extremely important that the reproducibility of any assay *in vivo* is determined by repeated experimentation. We have found it imperative that the animals used for such studies be carefully matched by age, sex, weight, and colony, and that they be in an excellent state of health, without evidence of viral, fungal, or other infestations that could alter biologic assays *in vivo*. These aspects are discussed in more detail by Fidler

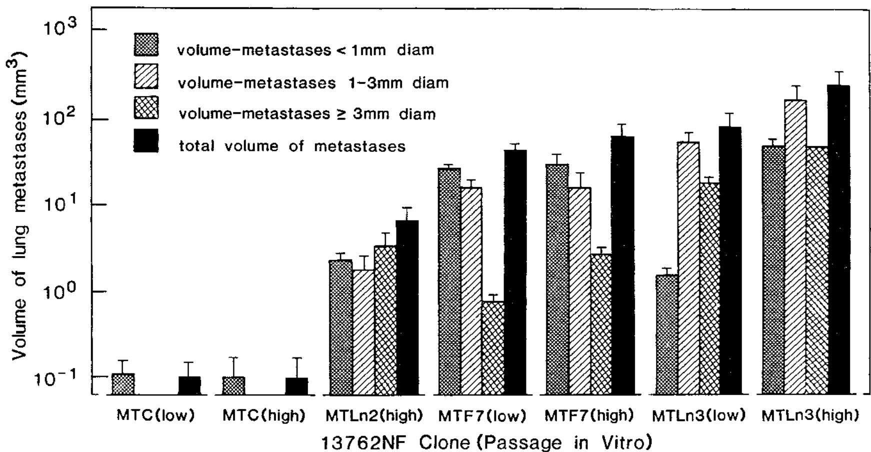

FIG. 14. Comparison of the average volumes of total tumor metastases and the average volumes of tumor metastases by size in lungs of Fischer 344 rats bearing subcutaneous 13762NF adenocarcinoma tumor clones. (Reproduced with permission from Welch *et al.,* 1983b.)

(1978a). We routinely screen for a variety of rodent viruses and contaminating microorganisms such as mycoplasmas. This is also true of the cells cultured *in vitro* to be used for animal experiments. Unfortunately, one of the main problems encountered in transferring tumor metastatic systems to other laboratories is that attention to these details is often taken lightly, resulting in frustrating losses of materials and time.

In order to quantify tumor implantation and invasion, radiolabeled tumor cells have been used (see review by Fidler, 1978a). Radiolabels used for this purpose include ^{51}Cr, ^{99m}TC, [^{3}H]thymidine, and [^{125}I]IUdR. The first two labels are incorporated into cells as nonspecifically bound ions, while the latter two are incorporated metabolically in the DNA of proliferating tumor cells. Only in the case of [^{125}I]IUdR is the radiolabel released after cell death from degraded DNA and not reutilized by surrounding cells. An accurate estimate of the number of viable tumor cells in studies of implantation and invasion can thus be made. In addition, the short half-lives of ^{99m}TC and ^{51}Cr preclude their use in long-term experiments, while [^{125}I]IUdR has a moderately long half-life and can be used for experiments of several weeks' duration. Use of rapidly released ions, such as ^{51}Cr ions, can result in high levels of spontaneous background release. For example, a release of 30-60% per day of ^{51}Cr has been reported by van Rooijen (1977).

While there are several advantages of using [^{125}I]IUdR as a tumor cell label, some problems are encountered in certain systems. In addition to the low rate of spontaneous leakage and lack of metabolic reutilization of released [^{125}I]IUdR, tumor cells can be labeled to high specific activities Fidler, 1976 , 1978a). Unfortunately, these high specific activities can also result in loss of cell viability due to radiopoisoning (Reading *et al.*, 1983). Thus, in certain systems, [^{3}H]thymidine or other labels that do not result in radiotoxicity must be used. An important control in such experiments is to ensure that the implantation, invasion, and metastasis properties of the tumor cells are not affected by the incorporation of the radiolabel. Fortunately, for many systems, optimum labeling can be achieved without altering the biological behavior of tumor cell populations (Fidler, 1975b, 1976b, 1978a; Fidler and Nicolson, 1976, 1977).

Radiolabeled tumor cells have been used in tumor invasion studies *in vitro,* although Mareel *et al.* (1977) found that the high background levels of spontaneous ^{51}Cr release can complicate quantitative interpretations of invasion. These authors noted that the high spontaneous release of ^{51}Cr from labeled embryonic chick heart tissue during invasion did not allow a correlation of invasion with host cell destruction. Hart and Fidler (1978) and Poste *et al.* (1980) have used [^{125}I]IUdR as a cellular label to quantitate the abilities of B16 melanoma sublines of differing invasive properties to invade tissue preparations of chick chorioallantoic membrane, mouse bladder membrane, and intact large veins.

B. Models for Tumor Cell Implantation and Invasion

A variety of experimental models of tumor implantation and invasion *in vitro* has been used to identify the factors that can influence these processes. Unfortunately, no single system can duplicate the situation *in vivo,* and all of the methods in current use appear only to mimic implantation and invasion. For example, the formation of metastases by spontaneous tumors may be considerably different from the majority of experimental systems, in which host tissues are typically challenged with large numbers of tumor cells and selected cell sublines. Elsewhere in this review (Section IV) we have stressed that the heterogeneity and phenotypic stabilities of tumor cell subpopulations must be taken into consideration. In addition, in many *in vitro* models host–tumor cell interactions are studied with cells from different species or strains, or with tissues that tumor cells would not ordinarily encounter during the normal pathogenesis of metastasis. Finally, all of the *in vitro* models in current use lack host defense reactions, such as those involving lymphocytes, macrophages, and other reticuloendothelial cells, as well as fibroblasts and other normal cells which can affect the process of invasion.

Highly simplified models of tumor implantation and invasion *in vitro* lend themselves to specific approaches for determining which tumor and/or host cell characteristics are important in implantation and/or invasion. Detailed analyses of the role of specific cellular properties, such as cell adhesion, locomotion, degradation of stromal components, and others, could not be easily made *in vivo.*

Another invaluable characteristic of systems for analyzing tumor implantation and invasion *in vitro* is that tumor cells with particular properties can be recovered. For example, in tumor cell populations that display extensive cellular heterogeneity, cells with enhanced implantation properties or increased invasive capacities may represent only a fraction of the total cell population. It is thus sometimes important that these cell subpopulations be recovered in order to determine their cellular and biochemical characteristics in detail. Obtaining cells with altered implantation and/or invasive characteristics can be achieved by single-cell cloning or selection of cell subpopulations, by techniques similar to those used *in vivo* for the selection of metastatic variants (Section III,C). Some of these techniques will be described in more detail in the following sections.

1. *Cells in Suspension*

During the process of implantation, metastatic cells aggregate with a variety of host cells, such as platelets, lymphocytes, and possibly other normal blood cells. The interaction of tumor cells with platelets has been discussed

in Section V,A and B. Although these experiments appear straightforward and have been, in general, measured in platelet aggregometers under standard conditions, the platelet release assays probably do not duplicate events that occur *in vivo.* This is due to the fact that highly enriched platelet fractions are utilized in such assays, and their source is often a different animal species. The artificially high concentrations of tumor cells and platelets permit platelet aggregation and release to be monitored *in vitro,* with the reservation that such events may not accurately reflect platelet–tumor cell interactions *in vivo,* especially in the microcirculation. Similarly, although Fidler (1975b) has found that B16 melanoma cells with a higher ability to implant, invade, and survive in the lung aggregate better with syngeneic lymphocytes *in vitro,* the concentrations of these normal host cells are much higher in the experiments *in vitro* than those normally encountered in the circulation. Similarly, the measurement of the homotypic rates of aggregation of murine melanoma (Winkelhake and Nicolson, 1976; Nicolson *et al.,* 1976) and sarcoma (Nicolson, 1978b), utilizing an electronic particle counter to examine the loss of single cells as they aggregate during rotary gyration in small vessels, is highly artificial, even though such measurements often correlate with blood-borne implantation *in vivo.*

The adhesion of malignant cells to host organ cells has been used to study cellular interactions that could be involved in the distribution of tumor cells to various organs. Nicolson and Winkelhake (1975) used B16 melanoma lung-colonizing sublines and measured their abilities to adhere heterotypically to suspended organ cells. They found that B16 sublines of high lung-colonization ability attach at much faster rates to lung cells than to cells obtained from other organs that are not sites of metastasis. In similar experiments Phondke *et al.* (1981) examined the interactions of spleen-colonizing leukemia cells with spleen cell populations isolated from syngeneic animals. These authors found that the leukemia cells heterotypically adhered to the isolated spleen cells but failed to bind to suspended lung cells. Schirrmacher *et al.* (1980) observed that liver-colonizing lymphoma variants bound to hepatocytes in relation to their metastatic properties and suggested that these interactions might be important *in vivo.*

Monolayers of normal parenchymal cells have been used to show the selectivity of tumor–organ cell interactions. In an interesting tumor system developed by Kahan (1979), murine LT ovarian teratocarcinoma cells were removed from ovary and spleen metastases and cultured. Using monolayers of murine ovary cells or control monolayers of human fibroblasts, Kahan (1979) measured the kinetics of attachment of ovary- and spleen-colonizing LT tumor cells to the monolayers. The ovary-colonizing LT cells bound at faster rates than the spleen-colonizing cells to the ovary cell monolayers, while no such organ selectivity in attachment was found with the fibroblast monolayers. Not all investigations have shown this. For example, Hart (1982) could not demonstrate differential attachment of organ-colonizing

melanoma cells to target organ cell monolayers, although it is likely that these cultures contained mainly fibroblasts and a few endothelial cells.

One of the main difficulties in assessing the importance of the above observations is that malignant cells in the circulation interact first with vascular endothelial cells in capillaries and not with parenchymal cells in organs. Therefore, the interactions of tumor cells with normal cells randomly derived from various tissues may not be relevant to implantation at all; however, these interactions might be important in subsequent tumor cell invasion into the parenchyma after implantation. Nonetheless, there is some indirect evidence suggesting that the recognition structures on endothelial cells may also be expressed on parenchymal cells in the liver. Nicolson *et al.* (1982b) utilized Fab′ or $F(ab')_2$ fragments of antibodies against fetal liver parenchymal cells to block the colonization of the liver by adult lymphoma cells injected intravenously. These same reagents also blocked the adhesion of lymphoma cells to aggregates of embryonic liver cells (McGuire *et al.,* 1983) (Fig. 10).

2. *Monolayer and Multilayer Cultures*

Two-dimensional monolayer cultures have been used *in vitro* for studying malignant cell–host cell interactions (see reviews by Mareel, 1979, 1980, 1982; Mareel *et al.,* 1979). In surveying the literature, Mareel (1979) concluded that at least some of the properties of malignant cells, such as attachment to and locomotion and infiltration between normal cells, can be studied in monolayer systems. The experiments are usually performed by placing a suspension of tumor cells or tumor cell aggregates in contact with confluent monolayers of normal cells. In most, but not all, such experiments the malignant cells that bind to the monolayers do not exhibit contact inhibition of cell movement, being able to infiltrate adjacent cells in the monolayer. However, properties observed *in vitro* do not always compare with their *in vivo* correlates. For example, Mareel *et al.* (1979) found that mouse fibrosarcoma cells were unable to infiltrate or destroy two-dimensional monolayer cultures of chick heart cells but were invasive *in vivo,* and they also rapidly destroyed three-dimensional aggregate cultures of chick heart cells *in vitro.* In addition, a number of nonneoplastic cells are fully capable of infiltrating monolayers of normal cells *in vitro* (Armstrong and Lackie, 1975). Although the ability to infiltrate between normal cells in monolayer cultures is found more frequently in malignant than in normal cells, notable exceptions exist and the value of this approach in studying tumor invasion is questionable (see reviews by Mareel, 1979, 1980).

The use of parenchymal cells in monolayer culture systems as a model for tumor invasion must be questioned from the standpoint of several unresolved considerations (Poste, 1982b). (1) There are undoubtedly differences in the types of intercellular adhesive forces in two-dimensional as

compared to three-dimensional cell arrays. (2) Profound alterations in cell geometry imposed by two-dimensional cultivation may cause changes in cell surface properties that alter cellular responses to a variety of signals *in vivo*. (3) Disruption of three-dimensional tissue architecture and removal of extracellular stromal components and supporting basement membranes may predispose monolayers to invasion by cells that would normally not be invasive in the same tissues *in vivo*. (4) The role of the substratum in which the monolayer cells adhere in determining malignant cell–normal cell interactions is unclear. (5) Single tumor cells are often utilized in such monolayer models, whereas tumor invasion into tissues *in vivo* often involves multicellular masses of tumor cells (Willis, 1973).

A more appropriate use of two-dimensional cell monolayers is in the construction of an artificial vascular endothelium. Since the vascular endothelium is composed of a two-dimensional cell monolayer and underlying basal lamina or basement membrane, such *in vitro* models may not require the third dimension of underlying cells (Kramer and Nicolson, 1979; Nicolson, 1982c; Zamora *et al.,* 1980). Addition of malignant cells or malignant cell clumps to such vascular endothelial cell monolayers results first in attachment to the vascular endothelial cells and local breaking of endothelial cell junctions, and then in stimulation of endothelial cell retraction and exposure of the underlying basal lamina-like matrix (Fig. 15). Tumor cells quickly migrate to the basal lamina where they adhere firmly and spread on this matrix (Fig. 16) (Kramer and Nicolson, 1981). The net movement of metastatic cells to the basal lamina probably occurs because of the difference between the adhesive capacities of this structure and the apical surfaces of endothelial cells. This has been shown in experiments where the kinetics and the extent of adhesion during shear were examined in the endothelial monolayers in comparison to exposed basal lamina (Figs. 13 and 17) (Pearlstein and Hoffstein, 1981; Kramer *et al.,* 1980; Nicolson *et al.,* 1981; Nicolson, 1982c). Thus, the implanted malignant cells probably move to the basal lamina by following an adhesive haptotactic gradient (Carter, 1965). Eventually the malignant cells penetrate the endothelial basal lamina-like extracellular matrix and migrate through this structure to complete the invasive sequence (Fig. 18).

Although the components of the vascular endothelial cells that are most important in tumor cell adhesion are not known, the components of the basal lamina that are most likely to be involved in binding malignant cells are the glycoproteins fibronectin (Kramer *et al.,* 1980; Nicolson *et al.,* 1981; Pearlstein and Hoffstein, 1981) and laminin (Terranova *et al.,* 1980; Gospodarowicz *et al.,* 1981; Vlodavsky and Gospodarowicz, 1981), type IV collagen (Murray *et al.,* 1980; Liotta *et al.,* 1980a), and perhaps sulfated proteoglycans (Irimura *et al.,* 1983b). The role of these matrix components in the binding of tumor cells to the basal lamina has been approached by

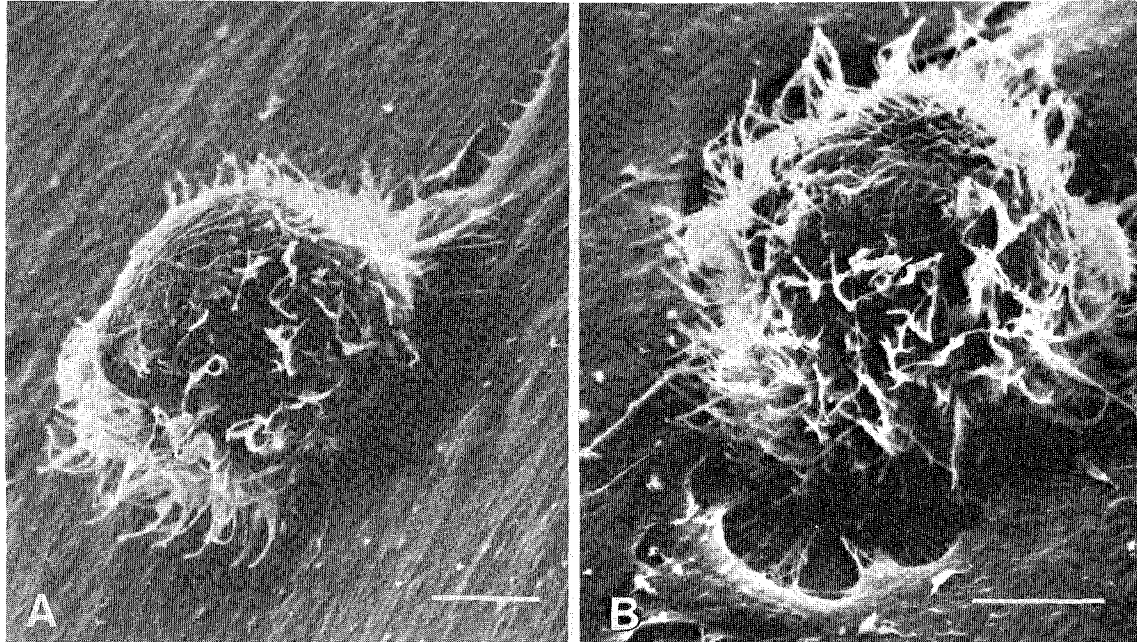

FIG. 15. Attachment of human breast cancer cells to, and invasion of, an human endothelial cell monolayer. (A) Tumor cell binds to the endothelial cells in the region of an endothelial cell–endothelial junction; (B) tumor cell-induced endothelial cell retraction has exposed the underlying basal lamina-like matrix, and tumor cell processes are being inserted under adjacent endothelial cells (1.5 hours). Bars = 5 μm. (Reproduced with permission from Kramer and Nicolson, 1981.)

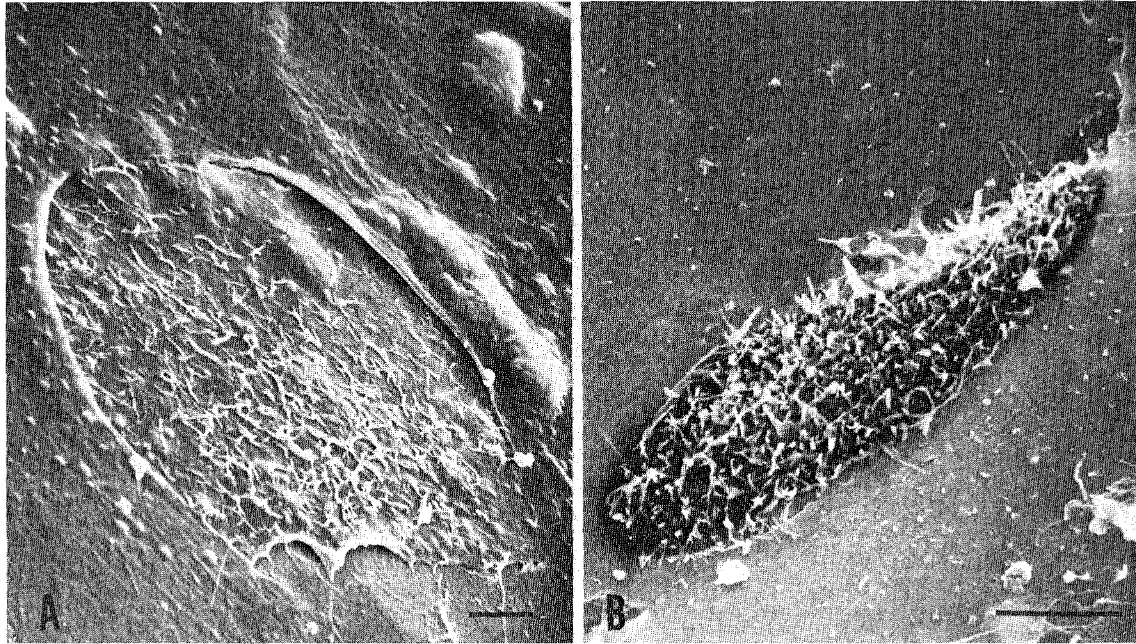

FIG. 16. Invasion of human endothelial cell monolayers by human melanoma cells. (A) A tumor cell has induced extensive endothelial cell retraction and has spread on the underlying basal lamina-like matrix. Tumor cell underlapping of adjacent endothelial cells is evident at 4 hours; (B) after tumor cell spreading and underlapping of adjacent endothelial cells at 4 hours, the latter begin to move back over the spread tumor cell and reform their endothelial cell junctions. Bars = 5 μm. (Reproduced with permission from Kramer and Nicolson, 1981.)

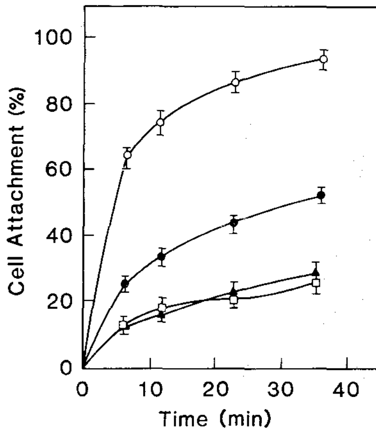

FIG. 17. Kinetics of adhesion of B16 melanoma sublines to endothelial cell monolayers or their subendothelial matrix. (▲) B16-F1 melanoma cell attachment to bovine aortic endothelial cell monolayer; (□) B16-F1 melanoma cell attachment to murine brain capillary endothelial cell monolayer; (●) B16-B14b (brain-selected) melanoma cell attachment to murine brain capillary endothelial cell monolayer; (○) B16-F1 melanoma cell attachment to bovine aortic basal lamina-like subendothelial matrix. (Reproduced with permission from Nicolson, 1982c.)

blocking certain matrix components with antibodies (Nicolson *et al.*, 1981) (Fig. 13), or, alternatively, the purified matrix components have been immobilized and tested for their ability to bind malignant tumor cells (Murray *et al.*, 1980; Liotta *et al.*, 1980a; Kramer *et al.*, 1980).

The importance of tumor cell surface components in the adhesive interactions with vascular endothelial cells and their underlying basal lamina has also been investigated. Irimura *et al.* (1981) used the antibiotic tunicamycin to block glycosylation of tumor cell surface glycoconjugates. Treatment with tunicamycin eliminated blood-borne implantation of B16 melanoma cells, and inhibited the ability of these cells to bind to vascular endothelial cell monolayers and to the underlying subendothelial basal lamina (Fig. 13). From the kinetics of loss of tumor cell adhesion to endothelial cell monolayers and the basal lamina, a specific class of tumor cell surface components was implicated in these processes. Irimura *et al.* (1981) and Irimura and Nicolson (1981) concluded that the most reasonable targets for tumor cell interactions with vascular endothelial cells are the high-molecular-weight sialogalactoproteins. These components could function as specific receptor sites for adhesion, or one or more of these components might be the actual adhesive molecule(s). Tumor cell surface adhesion molecules specific for carbohydrates (or lectins) have been found on several human and rodent tumor cells by Raz and Lotan (1981). These tumor cell lectins were analyzed and appear to be specific for galactosyl- or asialogalactosyl-containing glycoproteins. In other studies, Schlepper-Shäfer *et al.* (1981) found that Walker carcinoma BD10 lymphoma and Ushida hepatoma cells possess receptors for binding to normal rat hepatocytes, and inhibition studies indicated that *N*-acetylgalactosaminyl- or galactosyl-specific lectins on the liver cells might be involved in these interactions since hepatocytes can bind to sialoglycoproteins via a specific membrane lectin (Kolb *et al.*, 1978).

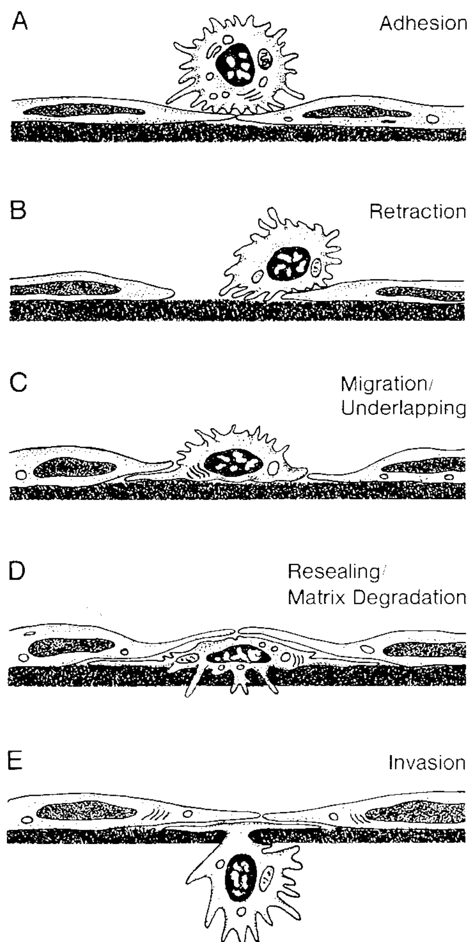

FIG. 18. Sequence of events during metastatic tumor cell attachment and invasion of vascular endothelial cell monolayers and their underlying basal lamina-like extracellular matrix. (A) Tumor cell attachment to endothelial cells; (B) endothelial cell retraction; (C) tumor cell migration and attachment to the underlying extracellular matrix, and underlapping of adjacent endothelial cells; (D) destruction of the endothelial basal lamina-like matrix and reformation of endothelial cell junctions; and (E) invasion of the malignant cell into surrounding tissue parenchyma. (Reproduced with permission from Nicolson, 1982a.)

Difficulties in ascertaining organ specificity in tumor–endothelial cell interactions have been overcome by recent progress in culturing vascular endothelial cells from different organs (for example, see Joseph *et al.,* 1983). When B16 melanoma sublines were tested in cell attachment assays with murine brain vascular endothelial cell monolayers, the brain-colonizing B16

cells attached at faster rates than the lung-colonizing B16 cells (Nicolson, 1982c) (Fig. 17). This may explain, in part, the organ preference of brain-colonizing tumor cells. Obviously, more extensive work with other organ-derived endothelial cells will be needed to confirm these findings before this hypothesis can be fully evaluated.

Artificial endothelium has also been constructed with endothelial cell monolayers and their underlying matrix deposited on top of collagen gels (Zamora *et al.,* 1980) or multilayers of smooth muscle cells (Jones and deClerck, 1980; Jones *et al.,* 1981). In the experiments of Zamora *et al.* (1980), mammary tumor cell spheroids were added to endothelial monolayers on collagen. As found in earlier studies (Kramer and Nicolson, 1979, 1981), the attachment of tumor cells or emboli in this system occurred most often near intercellular junctions between endothelial cells, and the endothelial cells near the attached spheroids retracted. Cells from the spheroids were able to underlap adjacent endothelial cells, and were observed to invade the endothelial basal lamina and the collagen gel. They also extended as cords of cells on the endothelial cell apical surface. Jones (1979) has constructed an artificial blood vessel wall with cultured endothelial and smooth muscle cells. The usefulness of this model is that it permits the stromal components under the basal lamina to be varied; thus, a variety of invasive substrates can be tested in parallel.

Metastatic tumor cells must invade the subendothelial basal lamina in order to extravasate and gain entry into the surrounding tissues. Several investigators have utilized endothelial cell monolayer systems or endothelial cell monolayer on muscle cell multilayers to study the degradation of the endothelial cell basement membrane-like matrix during invasion. Highly metastatic cells appear to be capable of degrading or solubilizing all of the major components of the endothelial basal lamina, including glycoproteins (Kramer *et al.,* 1982; Jones and deClerck, 1980), collagens (Liotta *et al.,* 1977, 1980b), and sulfated proteoglycans (Kramer *et al.,* 1982; Irimura *et al.,* 1983a,b; Nakajima *et al.,* 1983a,b). When malignant cell sublines of varying metastatic potentials were examined for their ability to solubilize matrix glycoproteins (Nakajima *et al.,* 1983), sulfated proteoglycans (Irimura *et al.,* 1983a,b; Nakajima *et al.,* 1983a,b), or type IV collagen (Liotta *et al.,* 1980a,b), the more metastatic cells degraded these components at higher rates (Fig. 19).

In some cases, unique fragments of subendothelial matrix components have been produced by the action of tumor cells. For example, Kramer *et al.* (1982) found that metastatic B16 melanoma cells solubilized sulfated proteoglycans and released fragments of their glycosaminoglycan side chains. The released fragments turned out to be almost entirely heparan sulfate, and the size of the fragments (approximately 10,000 molecular weight) indicated that they constitute approximately one-third of the native

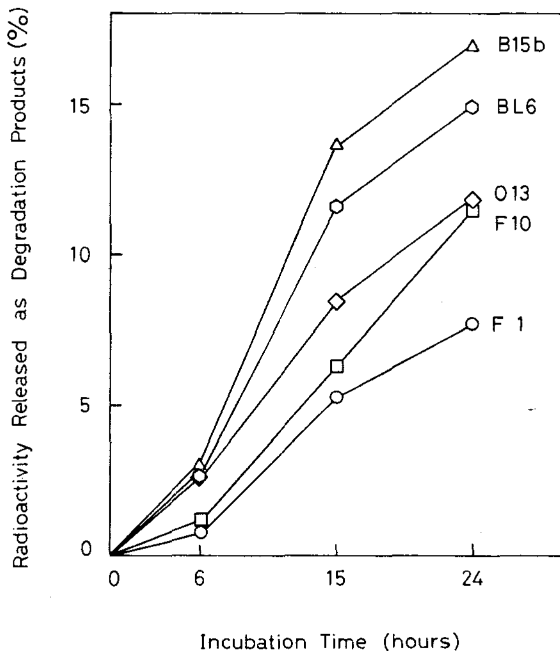

FIG. 19. Solubilization of sulfated glycosaminoglycans from basal lamina-like subendothelial matrix by B16 melanoma sublines. Endothelial cell cultures were labeled metabolically with sodium [^{35}S]sulfate (25 μCi/ml) in sulfate-depleted medium for 1 week, and subendothelial matrix was prepared according to Kramer *et al.* (1982). B16 melanoma cells were grown and harvested, and 2 ml each of cell suspensions were plated on matrix at a concentration of 1.5 $\times$ 10^5 cells/ml. At various times during the incubation (37°C) aliquots of the media were removed and centrifuged at 40,000 *g* for 30 minutes, and their radioactivities were then determined. Each symbol represents the average of triplicate samples (SD $<1\%$ of data); controls contained medium only. (Reproduced with permission from Nakajima *et al.*, 1983a).

glycosaminoglycan side chains on the heparan sulfate proteoglycans. Irimura *et al.* (1983a) have utilized high-speed gel permeation chromatography to separate and isolate glycosaminoglycan fragments produced by tumor cell glycosidases. These studies indicated that B16 melanoma cells produce both endo- and exoglycosidases active against heparan sulfate-type glycosaminoglycans, and Nakajima *et al.* (1983b) found that only B16 melanoma heparan sulfate endoglycosidase activity correlated with experimental metastasis. The tissue-degradative activities of metastasizing cells are discussed in more detail elsewhere (Nicolson, 1982a).

There are limitations in the use of endothelial cell monolayers as models for tumor invasion and implantation, and the same limitations apply to this system with an underlayer of smooth muscle cells or collagen. First, cellular

interactions are often examined by means of cells from different species. For example, the most popular type of vascular endothelial cell used in such experiments is the bovine aortic endothelial cell, because of its relative simplicity in terms of growth and culture requirements. This has been made more complicated by the construction of an artificial vessel wall involving bovine endothelial cells and rat smooth muscle cells (Jones, 1979). Another problem concerns the source of the endothelial cells. Most investigators have utilized endothelial cells derived from large vessels, but the use of endothelial cells from small vessels obviously better approximates events *in vivo* in which invasion and extravasation occur almost exclusively in vessels of the microcirculation of very small diameter. Although cultured endothelial cells appear normal and apparently produce a rather normal basal lamina-like matrix, the latter structure is not identical to native basement membrane, probably because of dissimilar growth microenvironments. In addition, there may be differences in the formation and maintenance of intercellular junctions and of other structures in endothelial cells grown *in vitro*. Nonetheless, such systems have been proven to be quite valuable in defining some of the activities necessary for tumor–endothelial cell binding and invasion.

3. *Organ Cell Cultures*

The use of organ cell cultures to study aspects of invasion *in vitro* has been reviewed by Mareel (1980, 1982). Among the first to use multicell aggregates for studies of invasion were Leighton and his collaborators (1960). They utilized a sponge matrix culture containing normal fibroblasts, and then introduced multicell clumps of human HeLa carcinoma cells into the sponge matrix, which was then examined histologically at various times. One of the first to utilize multicell aggregates for invasive studies was Wolff (1970), who placed fragments of embryonic chicken organs on agar and studied the abilities of tumor cell suspensions or fragments of animal and human tumors to invade these normal tissues in organ cultures. Easty and Easty (1963) expanded this method and used mammalian organs as substrates for tumor invasion.

More recent modifications of organ invasion assay techniques have utilized tissue fragments maintained in fluid media, usually in culture vessels on a gyratory or rotary shaker. When malignant cells are added in the form of single cells or cell aggregates into such an assay, a number of events can be measured. These include attachment of tumor cells to the aggregates, their subsequent movement into the aggregates, and heterotypic intermixing of cells in the aggregates (Gershman, 1982). The normal cell aggregates can be formed from single cells grown in tissue culture or from tissue fragments cut directly from normal embryonic or adult tissue. It is well known that

cell intermixing occurs in cell aggregates and tissue fragments, especially when embryonic tissues are used (Armstrong and Armstrong, 1978). In these types of experiments, usually one cell type or a portion of the same cells is labeled with [^{3}H]thymidine, and autoradiography is used to identify the labeled cells in the tissue sections. It is interesting that embryonic cells and adult normal cells can yield different results in such assays, possibly because the adult cells tend not to intermix rapidly in the aggregates (Gershman *et al.,* 1979).

The mobilities of tumor cells within normal cell aggregates have been examined by Gershman and Drumm (1975). In these studies untransformed 3T3 cells intermixed at low rates by comparison to the Simian virus 40 (SV40)-transformed derivatives of 3T3. While the authors freely admit that their "normal" cells probably represent poor models of actual normal tissues, their assays are nonetheless interesting and they might be used to study differences between cells of different invasive potentials. For such studies, most researchers have chosen to examine the interactions of malignant cells with heterotypic tissues, either in suspension or attached to an agar base.

One of the more popular strategies for examining interactions between malignant cells and normal tissues *in vitro* has been to incubate tumor cells or their aggregates with fragments of normal tissues maintained in organ or tissue culture. Most studies, such as those of Wolff and Schneider (1957), who studied murine sarcoma 180 cell binding and invasion into various chick embryo tissues, have utilized fragments of embryonic or adult tissue. Elaborating on these experiments, Easty and Easty (1963) used an agar base, or floating raft, for tissue support in their experiments on the invasion of normal tissues by tumor cells. Other investigators, such as Yarnell and Ambrose (1969a,b), used fragments of fetal mouse heart as targets for binding and invasion of untransformed and virus-transformed hamster BHK cells. Although pioneering, these early studies are open to arguments that the tumor models examined may be irrelevant to invasion and metastasis *in vivo* (see Section III), and also that the use of embryonic tissues as models for adult tissues may not accurately reflect conditions *in vivo.*

Improvements in organ block models of invasion *in vitro* have been made by Mareel and his collaborators (1977, 1979). Unfortunately, in these studies 7-day-old chick embryonic tissue fragments were used, and it has been shown by Gershman *et al.* (1979) that embryonic tissues of this age undergo rapid cell intermixing. This could complicate interpretations of invasion that depend on the movements of tumor cells into tissues that are not undergoing rapid intermixing. It should be noted that invasion of tumor cells into embryonic chick heart tissue appears to require an intact cytoskeletal system. This was shown in studies where Mareel and de Brabander (1978) examined the effects of microtubule inhibitors on tumor cell invasion.

In his review on the use of cell culture model systems for invasion and their correlation with malignant cell invasion *in vivo,* Mareel (1979) concluded that most cells of known malignancy can actively invade normal tissues *in vitro.* Many of the studies reported in his review utilized chick embryonic tissues as the substrate for invasion, with the apparent problem of rapid, random cell movements. In addition, xenogeneic differences existed between most of the tumor systems analyzed, and the utilization of cell lines of unknown metastatic potential in their syngeneic hosts resulted in few actual definitive correlations between invasion *in vitro* and *in vivo.*

Mareel (1982) has outlined the case for using embryonic tissues rather than adult host tissue in organ tissue invasion assays. One of the most important reasons is that the maintenance of embryonic organ tissue *in vitro* is much simpler, and the embryonic cells adapt to cultivation *in vitro* much more easily than do adult cells. Adult cells also appear to be much more susceptible to conditions of hypoxia, and this is considered a major problem in using blocks of organized tissues for studies *in vitro* of invasion. Also, Mareel (1980) indicates that the invasion of malignant cells in organ culture has been demonstrated with both embryonic and adult tissues. Since embryonic organ fragments maintained *in vitro* resemble their tissue of origin and their behavior *in vivo* more closely than comparable adult tissues, embryonic tissues have been the choice of many investigators for assays of tumor cell invasion *in vitro* (Mareel, 1980).

We have performed tissue invasion experiments *in vitro* using adult syngeneic organ tissues as substrates for the binding and invasion of B16 melanoma sublines which differ in their patterns of organ colonization (Nicolson, 1982a). Very small (0.1–1.0 mm^3) pieces of organ tissue from newborn C57BL/6 mice can be maintained after a 24-hour preincubation period in culture for up to 48 additional hours without evidence of necrosis or tissue degeneration. Three sources of tissue were used for these studies: lung, ovary, and heart muscle. Although these tissues basically maintain their normal histiotypic organization during the culture period, other tissues from newborn mice, such as brain tissue, undergo dramatic rearrangements during culture *in vitro* (Wang and Nicolson, 1983). B16 melanoma sublines selected for their abilities to colonize lung, ovary, or brain, and a series of B16 melanoma sublines selected for enhanced tissue invasiveness, were used to examine the ability of tumor cell suspensions to attach to and invade syngeneic host tissues within 48 hours. Depending on the gyration conditions in the culture, the concentration of tumor cells in suspension, and the number and size of the organ tissue fragments, we found differential attachment and invasion of the various melanoma sublines. For example, high lung-colonizing B16-F10 cells attached more rapidly to lung tissue and invaded lung tissue *in vitro* more rapidly than any of the other B16 melanoma

TABLE V
In Vitro ATTACHMENT AND INVASION OF B16 MELANOMA SUBLINES TO ORGAN TISSUES *in Vitro*[a]

		Time of first attachment (hours)			Time of first invasion (hours)		
Subline	Selection	Lung	Ovary	Heart	Lung	Ovary	Heart
B16–F1	1 × lung	1	3	0.5	18–24	—	—
B16–F10	10 × lung	0.5	1	1	6–12	18	—
B16–010	10 × ovary	1	0.5	3	12–24	12	—
B16–B14b	14 × brain	1	3	1	12–24	48	—
B16–BL6	6 × invasion	0.5	1	0.5	18–24	36–48	12

[a] From Nicolson (1982a).

sublines (see Table V). In general, the specificity of tumor cell attachment and subsequent invasion into different syngeneic tissues mimicked the preferences of organ colonization *in vivo* (Table V) (Nicolson, 1982a).

In similar studies Schirrmacher *et al.* (1979) used small fragments of lung to monitor the binding and invasion of nonmetastatic and highly metastatic murine lymphoma cell lines. They found that the metastatic lymphoma cells attached more quickly to and invaded tissue fragments in culture, while under similar conditions the nonmetastatic lymphoma cells failed to do so. However, liver tissue was not used in this study, although Schirrmacher et al. (1980) have found that the metastatic ESb lymphoma cells attach more readily to and invade liver cell monolayers *in vitro,* again mimicking their biologic properties *in vivo.* Lohmann-Matthes *et al.* (1980) have also examined the ability of murine tumors to bind to and invade lung tissue pieces *in vitro.* These authors found that metastatic murine tumors, but not nonmetastatic tumors, were capable of invading pieces of tissue *in vitro.* Once problems in maintaining certain tissues, such as brain, in culture can be overcome, these techniques may prove to be very useful in analyzing the abilities of cancer cells or tumor biopsies to invade human tissues *in vitro,* although the source of such tissues remains problematic. After the experimental details have been established and the assays have been found to be reproducible, nonhuman tissues may provide an accurate source for studies of invasion of organs.

4. *Multicellular Membranes*

Assays of tumor cell invasion have used multicellular membranes or fragments of multicellular membranes as substrates for invasion. These biological membranes are, in general, multilayered complex structures which often

contain basal lamina or basement membrane zones. Examples of such membranes are the chick chorioallantoic membrane (CAM), mouse urinary bladder, human amnion membrane, and human decidua graviditatis (reviewed in Nicolson, 1982a; Poste, 1982b). The first such biological membrane utilized for studies of invasion was the chick chorioallantoic membrane. Easty and Easty (1974) used the CAM to study the invasive properties of a variety of normal and tumor cells. They found that normal cells and cells of low tumorigenicity only rarely penetrated the CAM, while highly tumorigenic cells or highly malignant tumors rapidly invaded the CAM *in vitro.* We have utilized this assay to examine the abilities of B16 melanoma sublines to invade CAM *in vitro,* and have found that highly metastatic sublines, such as B16-F10, invade CAM faster than B16 melanoma cells of lower metastatic potential (Nicolson *et al.,* 1977). Hart and Fidler (1978) have quantified the penetration of CAM by B16 melanoma cells with [^{125}I]IUdR-labeled tumor cells. By removing the melanoma cells that penetrated through the CAM, these authors determined quantitatively that the more metastatic B16 cells are more able to invade the CAM than are the B16 sublines of low metastatic potential.

Certain deficiencies of the CAM invasion assay have been overcome by providing a mechanical support so that radiolabeled tumor cells can be applied to the ectodermal epithelium. In such an apparatus (Fig. 20), a two-chamber system is used in which tumor cells traversing the CAM can be recovered in a layer of agar underlying the CAM, or can be recovered from the CAM tissue itself (Poste *et al.,* 1980). The invasive cells can then be harvested, grown, and characterized in order to determine the properties

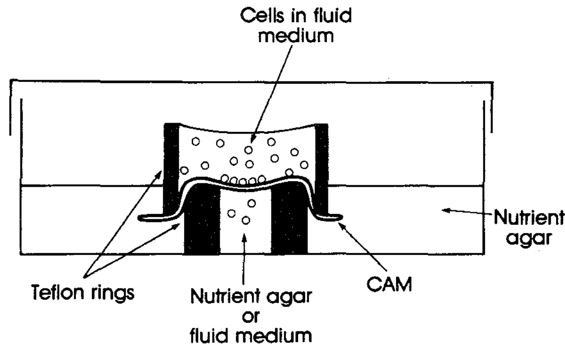

FIG. 20. Schematic illustration of a chamber for quantitative measurement of the ability of tumor cells to invade the chick chorioallantoic membrane (CAM) *in vitro.* Tumor cells (○) are added to the surface of the CAM, and cells that can successfully invade and penetrate the full thickness of the CAM are recovered in the lower chamber. (Reproduced with permission from Poste *et al.,* 1980.)

important in invasive behavior. An advantage of using such biologic membranes is that tumor cells are placed onto an intact, nontraumatized epithelial cell surface, and penetration of tumor cells into the subepithelial regions requires that they breach the integrity of the epithelium and its associated, intact basal lamina.

The CAM invasion technique has been used by Hart (1979) and Poste *et al.* (1980) to select B16 melanoma variants with altered invasive properties. B16 melanoma sublines were subjected to sequential selections for increased ability to invade the CAM by procedures similar to those discussed in Section III,C. After the sixth *in vitro* selection for CAM invasion, the B16 sublines were examined *in vivo* for their blood-borne implantation, survival, and growth properties. Unfortunately, selections for CAM invasion, although successful in obtaining B16 cells with high CAM invasive abilities, failed to produce any change in metastatic properties *in vivo*, indicating that the CAM system may not be a relevant substrate for analyzing the invasion of mammalian tumors.

There are both advantages and disadvantages to the CAM invasion system. An obvious advantage is that eggs are inexpensive and readily available and that tumor formation on the CAM *in situ* can be observed directly via a window created in the shell. In certain cases, invasion by the CAM *in situ* is accompanied by the penetration of tumor cells into the chick vascular system and the formation of metastases in various organs of the developing embryo (see Chambers *et al.,* 1982). Detracting from the CAM assay are the variabilities in thickness of different regions of the CAM, the fact that minor changes in the incubation conditions can produce extensive changes in the CAM structure, and the fact that the CAM may be less demanding, in terms of an invasive matrix, compared to other multicell membranes and may not duplicate the events required for the invasion of mammalian tissue (see Poste, 1982b).

Another epithelial membrane, the bladder wall, appears to be much more appropriate for the selection of invasive variants of mammalian tumor cell populations, because variants selected for increased invasiveness also exhibit enhanced metastatic capacities *in vivo* (Hart, 1979; Poste *et al.,* 1980). This method has the obvious advantage that syngeneic tissue can be used. The assay is performed by placing a tumor cell suspension into an aseptic, syngeneic bladder and incubating the organ for various times in culture. Cells that are capable of invading through the bladder wall can be recovered and grown in tissue culture, and the process is then repeated to obtain invasive variant sublines.

The decidual membrane of human pregnancy, or the decidua graviditatis, has been used in analogous fashion to measure the invasive potential of human tumor cells. Schleich *et al.* (1974, 1976) have examined the invasive potentials of human tumor cells using the decidual membrane in an invasion

assay. These authors found a good correlation between the degree of invasion and destruction of the decidua by malignant human tumor cells and their behavior *in vivo*. For example, human tumor cells obtained from metastatic lesions showed rapid invasion, proliferation, and destruction of the decidual tissue, while freshly explanted fetal or normal adult cells failed to invade and destroy the decidual tissue during parallel incubations.

Another human membrane that shows promise in tumor invasion studies is the amniotic membrane (Liotta *et al.*, 1980c; Tchao *et al.*, 1980; Russo *et al.*, 1982). Tchao *et al.* (1980) found that human HeLa cells and a colon adenocarcinoma line were capable of invading the amniotic membrane, while normal human fibroblasts were unable to invade, even after 7 days in culture. Similar observations by Liotta *et al.* (1980c) indicate that highly malignant human tumor cells are capable of invading the amnion and can be recovered from its external side after penetration through the epithelial cell layer and underlying connective tissue. The advantage of the amniotic membrane for studies of invasion is that it consists of a monolayer of epithelial cells with an underlying, intact basal lamina and collagenous connective tissues. In addition, it is available in adequate amounts from most hospitals.

The most reasonable system yet devised to quantify tumor invasion through a relevant tissue matrix and simultaneously to achieve recovery of the invasive cells may be the blood vessel perfusion–invasion chamber system developed by Poste *et al.* (1980) (Fig. 21). In this system tumor cells interact with segments of vein maintained in a perfusion apparatus. This allows tumor cells to interact with either the outer adventitial elements of the vessel or the luminal endothelial surface, in the latter case by inversion of the vessel before the insertion of cells into the chamber. Thus, it is possible to study both intravasation and extravasation with the same apparatus. Another important feature of this apparatus is that malignant cells that can invade and cross the wall of the vein and be recovered in the internal perfusion circuit can later be compared with the noninvasive cells harvested from the outer injection chamber. The blood vessel perfusion–invasion system (Fig. 21) offers new opportunities for studying the events involved in the initial penetration of tumor cells into vessels, as well as for studying the invasion of implanted cells into the extravascular tissues. In addition, the perfusion–invasion apparatus can be used with mixtures of host cells, such as leukocytes, platelets, and lymphocytes, along with metastatic or nonmetastatic tumor cells, to determine the roles that these normal host blood cells play in the implantation and extravasation of blood-borne tumor cells.

The one drawback of the perfusion–invasion apparatus in its current design is that only vessels or veins of large diameter can be used. The small size and delicate structure of capillary networks precludes their use in this

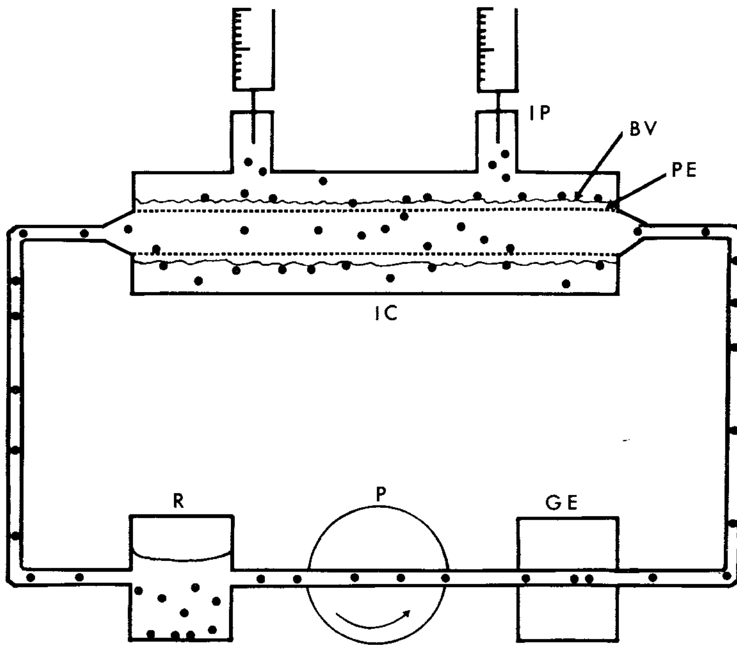

FIG. 21. Schematic illustration of a perfusion chamber system to study the ability of tumor cells to invade segments of vein. A segment of vein (BV) is fitted over a central tube of porous, ultrahigh-molecular-weight polyethylene (PE) containing 20-μm-diameter pores. Tumor cells are introduced into the invasion chamber (IC) via injection ports (IP). Any cells that invade and cross the vessel than pass through pores in the PE tuve to enter the internal perfusion circuit where they can be harvested from a reservoir (R). A peristaltic pump (P) and a gas exchanger (GE) are used to ensure the flow of culture medium through the chamber at the correct CO_2 content and pH. The various components of the unit are not drawn to scale. (Reproduced with permission from Poste *et al.*, 1980.)

apparatus, although normally blood-borne implantation and invasion takes place in the microcirculation and rarely occur in vessels of large diameter of the kind used in the apparatus. It is not known whether miniaturization of the perfusion system can be used to eliminate this deficiency and gather information on the interactions of malignant cells with endothelium in small vessels.

VII. Host Responses and Metastasis

Tumor metastasis is influenced by host factors and the microenvironment at the metastatic site. Some of the host characteristics important in tumor metastasis were discussed in Section V.

A. Nonimmune Responses

Host tissues appear to be capable of responding nonspecifically to the presence of proliferating and invading tumors. For example, some tissues, such as the connective tissues, contain inhibitors which can block tumor-released proteases (Eisenstein *et al.,* 1975). Normal structures, such as platelets, may also affect tumor cells, both by the release of tumor growth factors as well as by eliciting tumor cell aggregation (Section V,B). Platelets are known to have abundant stores of platelet growth factor (Antoniades *et al.,* 1979), and this, and other substances, could have profound effects on tumor growth at secondary sites.

One of the most common host responses to tumors involves stromal cell proliferation. In benign tumors, stromal responses are quite dramatic, resulting in the encapsulation of tumors in a fibroblastic and/or myoepithelial cell sheath. In malignant tumors, the presence of such cellular encapsulation is variable, and in many cases nonexistent. At distant secondary sites there is less evidence for extensive stromal responses, such as the kind present in many primary benign tumors or certain malignant tumors at their primary sites. In addition, malignant cells usually infiltrate the host's stroma, and in some cases host immunocytes may invade the stromal layer as well as the tumor (see next section).

The presence of a surrounding stromal layer or fibroblastic capsule may limit certain tumors from exhibiting their maximum malignant potentials. In an interesting study by De Vore *et al.* (1980), highly malignant human small cell carcinoma implants were found to stimulate host stromal responses at subcutaneous sites in nude mice. The stromal cells appeared to limit the invasive capacities of the human tumors by releasing factors that blocked tumor-released degradative enzymes such as collagenases. Cells of the stroma can thus modulate to a certain degree the malignant characteristics of tumor cells, although in metastases this may be less apparent.

The role of normal cells in regulating the survival and growth of malignant tumor cells has been studied in an interesting mammary tumor model by Slemmer (1979). He studied the role of normal stromal cells in regulating the malignant potential of murine mammary tumors by cotransplanting tumor cells together with normal mammary parenchymal cells such as myoepithelial, aveolar epithelial, or ductal epithelial cells. This approach was based on the observation that during neoplastic progression tumor cells were observed to retain their association with normal cells. When transplanted into the brains of mice in the absence of normal mammary cells, the tumors lost their growth potential and eventually aged and died. However, addition of normal mammary cells to such neoplastic cells resulted in rejuvenation of tumor growth. Histologic examination of the mixed neoplastic and non-neoplastic tumors [termed *mosaic-dependent* (M-D) tumors] revealed the

presence of normal epithelial and myoepithelial cells within the tumors. When lung metastases formed by the M-D-type mammary tumors were transplanted subcutaneously into compatible recipients, outgrowth of normal lactating gland occurred, indicating the presence of normal mammary cells in the lung metastasis. However, when this experiment was repeated with recipients that were histoincompatible with the normal stromal cells, not only did mammary outgrowth not occur, but the tumor cells also failed to grow, even though they were syngeneic and compatible with the recipient. Presumably, the tumor cells required some factor(s) from the normal mammary cells for survival and growth. Although Slemmer did not examine the normal mammary cells further to see if they released substances, such as growth factors, that can affect the survival and growth of neoplastic cells, these studies amply demonstrate that at least certain tumors require, and are regulated by, normal cells from their original environment.

An important host response during the growth of primary or metastatic lesions is neovascularization or angiogenesis (reviewed in Folkman, 1974, 1975; Folkman and Cotran, 1976). If a tumor colony is small in size (usually less than 2 mm), additional blood flow via new vascular channels is unnecessary. However, once a tumor achieves a diameter of greater than approximately 2 mm, tumor growth will slow drastically unless neovascularization occurs. Algire and Chalkley (1945) were among the first to establish that substances released by tissue wounding or by the presence of neoplastic transplants can induce neovascularization. Using plastic chambers containing rat mammary carcinoma or sarcoma cells implanted into the ears of rats or into hamster cheek pouches, they noted that soluble substances were released from the chambers which induced neovascularization in the implant area. In similar experiments, Folkman *et al.* (1971) placed neoplastic or normal tissues into Millipore chambers that were implanted into rat skin. They found that only the neoplastic cells secreted diffusible material that stimulated neovascularization. They called this material "tumor angiogenesis factor" (TAF).

The production of TAF from widely different histologic classes of tumors (see reviews just mentioned) has been documented *in vitro* as well as *in vivo*. When Folkman *et al.* (1971) attempted to purify TAF, their early isolations yielded a high-molecular-weight protein (approximately 100,000 MW) that possessed potent angiogenesis activity. In these studies TAF was purified from Walker 256 carcinoma cell lysates. Tumor angiogenesis factor activity was found in different cellular fractions, and one rich source was found to be the nuclear fraction, which yielded, upon partial purification, a TAF-active nuclear nonhistone protein (Taun *et al.,* 1973). The high-molecular-weight TAF preparation obtained by Folkman has not been fully characterized, even though it was initially isolated some 10 years ago. Adding to the confusion, TAF has been variously identified as a high-molecular-weight

protein containing carbohydrate, RNA, and possibly other substances, or as a low-molecular-weight nonprotein component. It is interesting that Weiss *et al.* (1979) and McAuslan and Hoffman (1979) started with Walker 256 carcinoma cells and purified a low-molecular-weight (approximately 200 MW; Fenselau *et al.*, 1981) nonprotein component that was highly active in picogram quantities in stimulating angiogenesis in CAM *in vitro* or endothelial cell hyperthrophy and enlargement of vascular beds *in vivo*.

Isolation and purification of TAF molecules has proved to be demanding because of the multifactorial nature of the vascular response. Angiogenesis actually comprises several biologic processes: endothelial cell mitogenesis, endothelial cell chemotaxis, and endothelial cell invasion (reviewed by Nicolson, 1982a). Ideally, these processes should be studied independently with separate assays, such as stimulation of vascular endothelial cell proliferation, directed endothelial cell chemotaxis, endothelial cell destruction of basal lamina, and endothelial cell invasion into parenchymal tissues.

Tumor cell-associated and released products that stimulate endothelial cell proliferation have been identified as hormones or growth factors (Fenselau and Mellow, 1976; Birdwell *et al.*, 1977; McAuslan and Hoffman, 1979; Fenselau *et al.*, 1981). An example is the sarcoma growth factor isolated from murine sarcoma virus-transformed cells (De Larco and Todaro, 1978). Tumor angiogenesis factors that separately stimulate endothelial cell movement (Zetter, 1980) and the formation of capillary structures (Folkman and Haudenschild, 1980) have not been identified. However, some enzymes of endothelial cells that degrade basal lamina have been found, such as collagenases (Gross *et al.*, 1981) and plasminogen activators (Tökés and Sorgente, 1976). How these factors and enzymes are stimulated in and released from endothelial cells should be important in elucidating the neovascularization process.

We have often stated that angiogenesis is not a tumor-specific process (Nicolson, 1982a; Nicolson and Poste, 1982). It is a normal physiologic response that occurs during tissue wounding, inflammation, and other normal events. The evidence that angiogenesis occurs as a normal tissue repair and rearrangement process is supported by the finding that lymphocytes (Sidky and Auerbach, 1975), macrophages (Polverini *et al.*, 1977a), monocytes (Polverini *et al.*, 1977b), and other normal tissues (Glaser *et al.*, 1980) release TAF-like molecules. Other normal processes, such as the growth and involution of blood vessels in the capillary wreaths that surround the ovarian follicle at the time of ovulation, also appear to involve TAF-like activities, because the ovarian corpus luteum can produce TAF-like factors that stimulate the proliferation of vascular endothelial cells *in vitro* and produce neovascularization *in vivo* (Gospodarowicz and Thakral, 1978). Brown *et al.* (1980) have found that TAF-like molecules are produced in osteoarthritis and ankylosing spondylitis, and similar molecules can be obtained

from the synovial fluid of patients suffering from these diseases. Angiogenesis factors are thus not tumor specific, and they might be more appropriately viewed as normal molecules which are released by tumor cells and evoke normal tissue responses (Nicolson, 1982a; Nicolson and Poste, 1982).

B. Immune Responses

Immune responses against primary and secondary tumors are generally thought to be an important determinant of tumor behavior. However, Hewitt (1976, 1978) has challenged this concept and has argued that immune surveillance and host reactions to tumor cell antigens are of little importance in human cancer. Although the importance of host antitumor immunity in metastasis has been documented in a number of different animal tumor systems (see reviews by Fidler and Kripke, 1980; Fidler and Nicolson, 1981; Nicolson, 1982a; Stutman, 1975), immune responses against metastasizing tumors in animals can either increase or decrease in the incidence of metastasis depending on the tumor being studied. Thus it is virtually impossible to generalize about the role of specific immune responses in metastatic disease, because in some animal tumor models suppression of antitumor immune responses increases both spontaneous and experimental metastasis formation, while in other tumor systems suppression of antitumor immune responses decreases or even abolishes metastasis. Finally, in many animal tumor systems, particularly slowly growing spontaneous tumors, antitumor immune responses appear to have no significant effect on the frequency or kinetics of metastasis.

The question of whether host antitumor immune responses are effective against metastasizing spontaneous tumors cannot be answered easily. Because of experimental limitations, most investigators have utilized highly antigenic, usually virally or chemically induced, transplantable tumors for immunologic experiments. The use of weakly antigenic, slowly growing, spontaneous tumors rather than highly antigenic, rapidly growing tumors, and the differing responses of host immune systems to these neoplasms, have been discussed in detail by Prehn (1972). Prehn hypothesized that a weak, stimulatory, antitumor response can occur at the early stages of tumor development or in weakly antigenic tumors, but that at the later stages of tumor development, or in instances where tumors are highly antigenic, a strong inhibitory antitumor response should develop.

There appears to be some support for the unique nature of immune responsiveness to different tumors. For example, when Umeil and Trainin (1974) transferred syngeneic spleen cells from tumor-bearing mice to recipients containing subcutaneous transplants of 3LL carcinoma cells, the

transferred spleen cells enhanced tumor growth at the subcutaneous sites and in lung metastases. These authors showed that specific antitumor immunity was involved by demonstrating that the growth enhancement was not produced by spleen cells from tumor-free donors. Spleen cell populations from tumor-bearing animals were then separated into fractions containing either tumor-"enhancing" or-"inhibiting" cell subpopulations (Small and Trainin, 1976). Further support of Prehn's hypothesis that the host response can either inhibit or stimulate ("immune inhibition-stimulation theory") metastasis is provided by the results of Fidler (1974a). By varying the input ratios of tumor-bearing donor lymphocytes to B16 melanoma cells, Fidler showed that the experimental formation of metastases could be either inhibited or enhanced over controls. Simultaneous intravenous injection of B16 cells with low numbers of lymphocytes (approximately 100:1, lymphocytes:B16 cells) resulted in enhancement of experimental lung metastases in X-irradiated, thymectomized recipients by comparison to animals injected with B16 melanoma cells alone. However, when the experiment was repeated with high ratios of lymphocytes to B16 cells (approximately 5000:1), inhibition of experimental metastasis compared to controls was found.

In other studies the effects of immune status on experimental metastasis have been studied in unimmunized normal and immune-manipulated recipients. Fidler *et al.* (1977) and Fidler and Nicolson (1978) found that abrogation of host antitumor immune responses affected the rates of implantation, survival, and growth of lung-colonizing B16 melanoma cells. Interestingly, immunosuppression was found to reduce, rather than to increase, the number of experimental lung colonies formed, a result that suggests that in this tumor system immune enhancement may aid in the metastatic process.

The role of host immunity in the spontaneous metastasis of UV-induced fibrosarcomas has been studied in their syngeneic hosts. In this system, different fibrosarcoma cell lines of varying immunogenicities were used to show that weakly immunogenic fibrosarcoma cells can grow and metastasize more readily in immune-competent hosts than in immune-incompetent hosts. However, the use of highly antigenic fibrosarcoma sublines resulted in inhibition of growth and metastasis in immune-competent recipients (Fidler *et al.*, 1979). The conclusions from these studies are that antitumor host immunity can vary, even within the same tumor system, and, by extrapolation, antihost tumor immunity may be different for individual subpopulations within the same tumor. Fidler and his collaborators concluded that the optimum level of host antitumor immune response was probably intermediate between the extremes found in their studies. Although these particular observations support Prehn's (1972) hypothesis, the wide range of results obtained with different tumors suggests that generalizations about

the outcome of metastasis with respect to host antitumor immunity may be meaningless.

The immunologic properties of tumor cells also differ between the primary site and the secondary metastatic lesions (see Nicolson, 1982a, for review). It has been known for some time that tumor cells in metastases can be distinguished antigenically from cells in primary tumors (Sugarbaker and Cohen, 1972). In this case, highly antigenic, chemically-induced sarcomas from primary or spontaneous metastatic sites were transplanted subcutaneously in syngeneic, immunized mice. Sugarbaker and Cohen found that the metastases expressed types or amounts of antigens that differed from those in tumors in assays which measured tumor rejection by an immune host.

In addition to differences between metastases and their primaries, phenotypic heterogeneity in immunologic properties also exists within the same tumor. Pimm and Baldwin (1977) and Pimm *et al.* (1980) found that tumor cell sublines established from different portions of the same tumor (or from renal and pulmonary metastatic sites) were often immunologically distinct. Analogous findings with chemically induced mammary carcinomas have been noted by Kim (1970). In the latter case, the most metastatic mammary carcinomas were found to be less immunogenic than similar (but not identical) nonmetastatic tumor lines. Only the weakly immunogenic or nonimmunogenic mammary tumors were capable of metastasizing. However, when Kim immunized rats with radiation-killed tumor cells in order to prevent their spontaneous metastasis, he found that the immunization procedure prevented the growth of nonmetastasizing tumor cells but enhanced the growth of the metastatic carcinomas. Again, these data are consistent with Prehn's immune stimulation–inhibition hypothesis.

Phenotypic differences between 3LL Lewis lung carcinoma cells growing locally or in lung metastases have been demonstrated by lymphocyte-mediated cytotoxicity. Fogel *et al.* (1979) found that syngeneic lymphocytes sensitized *in vitro* against 3LL tumor cells growing locally were less cytotoxic toward 3LL cells obtained from lung metastases. They discovered the converse to be true; lymphocytes sensitized against metastatic 3LL cells were less cytotoxic against tumor cells obtained from the locally growing tumor than those obtained from lung metastases. They also found that lymphocytes sensitized against metastatic 3LL cells could inhibit spontaneous metastases to the lung when the 3LL cells were injected (subcutaneously) simultaneously with lymphocytes. Thus, tumor cells in metastases can differ in their immunologic phenotypes from cells present in primary tumors.

Metastatic tumor cells could be immunologic variants that exist with minute frequency in the primary tumor, or, alternatively, they could arise by phenotypic diversification while at the metastatic site (Section IV,B). Thus, phenotypic variability in tumor antigens and tumor cell immunogenicity

could be the result of spontaneous events, or they could be due to selective, antitumor responses by the host. Support for the role of spontaneous mechanisms in generating immunologic variants has come from cell-cloning experiments which clearly indicate that antigenic variants exist in primary tumors or tumor cell populations before they metastasize (Kripke *et al.*, 1978; Reading *et al.*, 1980b; Shearman and Longenecker, 1981; Nicolson, 1982e). Selection of cells that have lost antigens for metastasis has been noted in a murine large cell lymphoma system. Reading *et al.* (1980b) have analyzed a number of cell clones in this system for the presence of cell surface viral antigens in the parent tumor and in sublines selected either *in vitro* or *in vivo,* and have found variants with altered metastatic potentials. Loss of viral antigens, such as the murine RNA tumor virus envelope glycoprotein gp70, correlated ($r = 0.93$) with the potential for metastasis to the liver (Reading *et al.*, 1980b; Nicolson *et al.*, 1982e). In this system, metastatic colonization of the liver may require escape from host antitumor responses, probably by selection of variant cells that had lost antigens. The gp70 antigens on these large lymphoma cells may be of a recombinant type with altered immunologic properties (Nicolson, 1982c). In other murine tumor systems that express an endogenous gp70, such as B16 melanoma (presumably not a gp70 recombinant), no relationship between gp70 expression and metastatic potential was evident (Fidler and Nicolson, 1981).

Although selection of "antigen-loss variants" is probably not a universal characteristic of highly metastatic cells, it can apparently occur under circumstances where strong antigens elicit effective antitumor immune responses. Thus, strong selection pressures may exist at the primary or secondary sites and result in the survival of subpopulations of tumor cells that lack the antigen(s) against which the host response has been mounted. Dennis *et al.* (1981) have examined the frequency with which antigen-loss tumor cell variants were generated during the metastatic process in the MDAY-D2 tumor. When they examined the tumor-associated transplantation antigens of locally growing and spontaneous metastatic lesions, they found that 1 out of 15 animals had tumors which had lost tumor-associated transplantation antigens. All of the metastases from this one animal yielded cells that were resistant to cytotoxic T cell killing. In contrast, tumor cells established from the original transplant site retained their surface antigens and were killed in a T cell-dependent cytolytic assay. Phenotypic instability of malignant cells may result in modifications of antigen expression that could be important or unimportant to the metastatic processes. During continuing selection in the host the eventual emergence of stable subpopulations with variant antigens may be a relatively common feature of slow-growing neoplasms (Nicolson, 1982a).

Evidence exists that increased metastatic potential can also correlate with increases in particular antigens. Shearman and Longenecker (1981) detected

an antigen on chick MDCC-AL2 lymphoma cells by means of a monoclonal antibody and found that the expression of this antigen correlated with an increased potential to colonize liver. Treatment of the lymphoma cells with a monoclonal antibody against this antigen partially inhibited blood-borne liver colonization (Shearman *et al.,* 1980). Similar experiments have been conducted by us with a murine lymphoma using Fab′ or F(ab′)$_2$ fragments against fetal liver antigens, as described in Section V,B.

Environmental factors appear to be capable of eliciting phenotypic changes in cell surface components. When Bosslet and Schirrmacher (1981) studied murine lymphoma variants that arose *in vivo* at high frequency in metastatic foci of the spleen, the variant cells obtained from spleen metastases were completely resistant to cytolytic T cells obtained from immunized animals, while tumor cells obtained from lung or liver metastases or from subcutaneous sites were killed by the cytolytic T cells. The variants found in the spleen were not generally resistant to T cell-mediated immunity and remained phenotypically stable for over 100 generations. Although the authors claim that these cells did not preexist in the parental tumor cell population, their cloning techniques (30 clones) were not exhaustive. Since genetic marker studies confirmed that the variant cell population was derived from the original tumor, Schirrmacher (1980) speculates that the spleen-colonizing variants were generated by a process of induction rather than selection. Unfortunately, direct inoculation of the parental population into the spleen was not performed. Such an experiment could have revealed whether organ trophic effects can result in "induction" of phenotypic variants.

As discussed in Section V,B, natural host immunity is important in metastatic phenomena, particularly blood-borne metastasis. This has been reviewed elsewhere by Herberman and Holden (1978), Kiessling and Wigzell (1979), and Hanna (1982). The role of NK cells in host surveillance against metastasizing tumors may not be universal, because many animal tumor systems are not readily susceptible to this arm of the host defense system (Schirrmacher, 1981; Reading *et al.,* 1983). Natural killer cells or perhaps NC cells seem to be most effective against tumor cells while in the circulation (Hanna, 1980; Hanna and Fidler, 1980) (see also Section V,B). Therefore the role of NK cells in blood-borne metastasis has been examined by selecting sublines of malignant cells that are resistant to NK cell cytolytic mechanisms. Gorelik *et al.* (1982a,b) have selected NK cell-resistant sublines of 3LL carcinoma. After eight selections for resistance, these authors found that the selected cells were refractory to NK cell-mediated cytolysis and exhibited enhanced abilities to metastasize spontaneously. Similar results have been obtained by Hanna and Fidler (1981), who found that NK cell-resistant tumor sublines were highly metastatic in adult nude mice, in contrast to the parental tumor cell line which failed to metastasize in adult

nude mice, presumably because of the development of high NK cell activities.

Although there is good evidence that NK cells appear to be involved in inhibiting blood-borne metastasis in animal models, clinical evidence for the role of such systems in inhibiting malignant disease has been less impressive. In some patients, NK cell activities apparently decline or are depressed in situations of large tumor burden (Pross and Baines, 1976; Takasugi *et al.,* 1977). Direct evidence, such as the modulation of NK cell activities by treatments which augment host immunity against metastatic cancer (Golub, 1981), suggest, but do not prove, that NK mechanisms operate against human malignancies.

One of the most important host defense systems against cancer is mediated by tumoricidal macrophages. Historically, macrophages have been found associated with, and their numbers even correlated with, the ability of a host to respond successfully against a growing tumor (see reviews by Fidler *et al.,* 1978; Fidler and Poste, 1982; Nicolson, 1982a). Although not universally accepted, it is clear that macrophages can be activated to kill tumor cells nonspecifically and to prevent tumor growth and metastasis (see reviews by Alexander, 1976a,b, 1977; Fidler, 1978c, 1980, 1982). It is well documented that activated macrophages can inhibit the growth of tumors at primary sites (see references just mentioned), and intravenous injection of activated macrophages can result in destruction of primary (Den Otter *et al.,* 1977) and metastatic (Fidler, 1974b, 1978c, 1980; Sone and Fidler, 1980, 1981) tumors. Similarly, administration of liposomes containing macrophage-activating molecules, such as lymphokines or muramyl dipeptide, was used to activate tumoricidal macrophages without causing nonspecific host toxicity (Hart *et al.,* 1981). The use of liposome-encapsulated muramyl dipeptide as a macrophage activator appears effective in eliminating even the most highly metastatic B16 melanomas growing at subcutaneous sites (Fidler, 1980; Fidler *et al.,* 1981, 1982; Poste and Fidler, 1981, 1982).

The activation of tumoricidal macrophages and their ability to obliterate established metastases may result in the development of an important new clinical approach to anticancer therapy. It is thought that macrophages can kill tumor cells independent of the tumor cell growth capacity, invasiveness, and metastatic potential (Fidler, 1978c; Fogler *et al.,* 1980; Fidler and Poste, 1982). The ability of activated macrophages to recognize virtually all cells in a malignant tumor cell population has been suggested by experiments in which the selection of macrophage-resistant sublines has not been successful (reviewed in Hart and Fidler, 1981). In contrast, universal sensitivity of tumors to activated macrophages has not been seen, nor has it been seen in highly metastatic tumor subpopulations. Miner and Nicolson (1983) found that highly metastatic, murine large cell lymphoma sublines and

clones selected *in vitro* or *in vivo* were more resistant to the cytolytic and cytostatic effects of activated macrophages *in vitro* than less metastatic sublines and clones. In this system, suppression of macrophage function *in vivo* by a variety of different procedures enhanced only the abilities of the less metastatic sublines and clones to metastasize, while the same procedures had essentially no effect on the highly metastatic sublines (Reading *et al.*, 1983). Although Fidler (1978c) has proposed that B16 melanoma cells are uniform in their sensitivities to activated macrophage-mediated cytolysis, Miner *et al.* (1983) obtained different results by using lower macrophage–tumor cell ratios. These latter conditions are considered to reflect events *in vivo* more accurately. Miner *et al.* (1983) found that high brain-colonizing and lung-colonizing B16 variants were less susceptible to activated macrophage-mediated cytolysis and cytostasis than were low metastatic B16 sublines *in vitro*. The highly metastatic B16 cells were also less sensitive to macrophage-released cytokines and cytokines released from the monocytic tumor cell line J774.

Highly metastatic tumor cells may modulate antitumor immune responses by the host. For example, it is well known that malignant tumors are capable of suppressing host immune responses (Ioachim *et al.*, 1974). The induction of tolerance (or stimulation of immune suppressor cells) appeared to be triggered by the administration of antigen at birth, and resulted in tolerance by the adult immune system of the normally recognized tumor antigen. The induction of "suppressor" cells which inhibit antitumor immune responses has also been seen by Kripke (1977). She found that chronic exposure of mice to ultraviolet light can produce skin tumors, but these were often highly antigenic and were rejected after transplantation into syngeneic hosts unless the animals were immunologically modulated by exposure to intermittent doses of ultraviolet light (Kripke and Fisher, 1976). This effect of UV light was subsequently shown to be due to an imbalance in suppressor lymphocytes, which are apparently capable of preventing the destruction of the fibrosarcomas by host antitumor immunity (Fisher and Kripke, 1977, 1978). Obviously, the development of suppressor cells and their inhibition of host antitumor responses could pose problems during immunotherapy of cancer.

In cancer patients, as well as in many animal tumor systems, impaired antitumor host responses may be related to an imbalance in suppressor cells (Broder and Megson, 1981; Serrou *et al.*, 1981). Immune suppression has been implicated in impaired host immunity against certain fungal, microbacterial, and parasitic infections (Serrou *et al.*, 1981), so one must consider that immune suppression could play a role in antitumor activity against malignant cells.

Considering the number of diverse ways in which antitumor host re-

sponses could enhance, inhibit, or have no effect on the metastatic process, generalizations or extrapolations from one system to another may not be useful. One of the biggest challenges facing clinical immunology is to determine which patients will respond favorably and which will respond unfavorably (or not respond at all to any given approach to the biological modification of response.

VIII. Conclusions

Although metastasis could be a random process in which any tumor cell in a malignant cell population might participate, the available evidence suggests that metastasis is a nonrandom, selective process. Since the individual tumor cells in a malignant lesion possess widely differing characteristics, including those relating to metastasis, tumor models which fail to take this into account are of questionable value. Indeed, tumor models or cell lines containing multiple, heterogeneous subpopulations are less than ideal when one attempts to study the process of metastasis and to relate specific events to particular tumor and/or host properties. This is especially true for animal models in which cell subpopulations capable of metastasis represent a minute fraction of all tumor cells.

Selection and/or cloning procedures designed to obtain variant metastatic cells have been particularly useful for elucidating tumor and/or host properties associated with metastasis. However, careful attention must be paid to the stabilities of such cell subpopulations. The inherent instabilities of clonal and even polyclonal tumor cell subpopulations have made investigations more difficult and time consuming. However, this can be overcome by proper attention to the conditions under which the tumor cells are grown and to their biologic properties *in vivo*.

The mechanisms responsible for the rapid generation of phenotypic diversity in tumor cell subpopulations and the regulation of subpopulation diversity during progressive growth of primary and metastatic lesions are extremely important, and their elucidation will require intensive efforts by researchers from a wide variety of disciplines. In tumor-bearing hosts, the generation of subpopulations of malignant cells with altered phenotypes could be one of the most important factors contributing to the survival, progression, and metastasis of tumors. In concert with our other reviews on the properties of malignant cells (Nicolson, 1982a,b; Nicolson and Poste, 1982) and their clinical ramifications (Poste, 1982a; Nicolson and Poste, 1983), we have attempted to survey some of the achievements and problems that face researchers studying the pathogenesis of cancer metastasis and developing more effective modalities for its treatment.

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