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# A Cell Surface Antigen Functionally Associated with the Metastasis of Cancer

by

© Peter J. Shearman

A thesis

submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Department of Immunology

Edmonton, Alberta

Fall, 1980

The University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A Cell Surface Antigen Functionally Associated with the Metastasis of Cancer submitted by Peter J. Shearman in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Abstract

The Marek's disease virus transformed, non-producer, lymphoma cell line, MDCC-RP1, was selected by sequential transplantation to produce a highly malignant variant, MDCC-AL1. This is evidenced by an 18-fold decrease in $LD_{50}$. The new cell line has an increased ability to form metastatic lesions in a distribution which mimics the natural disease. Further selections for organ specific metastasis were undertaken with the isolation of two new cell lines, MDCC-AL2, selected for liver metastasis, and MDCC-AL3, selected for ovary metastasis. In vivo studies show that the selection was unsuccessful in the case of the ovary but successful in the case of the liver. Two assays were developed utilizing the chick embryo and intravenous injection of lymphoma variant cells. One assay measures liver specific metastasis by the enumeration of tumour foci on the embryonic liver. The second assay, chorio-allanoic membrane focus formation, correlates with the virulence of the injected lymphoma cells. The liver selected tumour variant cells form more liver foci than any other tumour variant cell line. The genetic background of the embryo used in the assay does not affect liver focus formation by metastatic variant cells. Resistance to chorioallantoic membrane focus formation by unselected cell lines correlates with major histocompatability complex associated resistance to Marek's disease. Monoclonal antibodies were used to probe the cell surface

of the metastatic variant cells. A liver specific metastasis associated antigen (LMAA) is defined by the reaction of a monoclonal antibody with the liver metastatic selected variant AL2. The anti-LMAA antibodies specifically inhibit liver metastasis of AL2. There is a correlated, clonal variation in LMAA expression and liver metastasis in both the AL2 and AL3 cell lines. The variation in liver metastatic ability and LMAA expression is thought to represent clonal progression of the tumour cell lines. The LMAA probably represents only one of the many ways that a tumour cell may give rise to a liver metastasis. Two hypotheses are presented utilizing the LMAA in a functional role in the homing of metastatic tumour cells to the liver or the colonization of the liver by metastatic tumour cells.

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| | |
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| ADRA | antibody directed rosette forming cell assay |
| CAM | chorioallantoic membrane |
| CAM-FFA | chorioallantoic membrane focus forming assay |
| CIS | cellular interaction structure |
| CMCA | complement mediated cytotoxicity assay |
| CRBC | chicken red blood cells |
| CWBC | chicken white blood cells |
| EBV | Epstein-Barr virus |
| EL-FFA | embryonic liver focu forming assay |
| FCS | fetal calf serum |
| $LD_{50}$ | lethal dose 50 (i.e. 50% of animals at risk die) |
| LMAA | liver metastasis associated antigen |
| MATSA | Marek's disease associated tumour specific antigen |
| MD | Marek's disease |
| MDV | Marek's disease virus |
| MHC | major histocompatability complex |
| NK | natural killer |
| RB | random bred |

I. Introduction: The Natural Acquisition of Cancer and Metastasis

A. Causes and Characteristics of Cancer

The three known causes of cancer have the same target of action, a cell. In essence, cancer can be viewed as a disease of cells as opposed to a disease of organisms or a disease of organs. Things that cause cancer are called carcinogens, which were defined by Clayson (1962) as agents or processes which increase the yield of neoplasms in a population. Neoplasms are the first evidence of cancer, giving rise to a tumour in some cases. What may be a carcinogen under one set of conditions or in a particular individual may be apparently harmless under a different set of conditions or in another individual. As reviewed by Bingham, Niemeier and Reid (1976), all carcinogens may have varying levels of effects depending on the circumstances of action and the environment of the host. In general, the three known causes of cancer are chemicals (Fishbein, 1979), radiation (Upton, 1975; Storer, 1975) and viruses (Rous, 1911; de-Thé et al, 1978).

It is thought that carcinogens act by transforming cells in such a way that the body can no longer control them. This may occur via the carcinogen's ability to induce mutations in the genetic material contained in a cell (Ames and McCann, 1976). A carcinogenic agent may appear to induce a particular type of cancer as a one step

process. Analysis of the carcinogenic event has shown that at least two steps are necessary for most tumours to be induced, initiation and promotion (Berenblum and Shubik, 1948). Initiation predisposes a tissue to transformation while promotion completes the process. Incomplete carcinogens can perform one of these functions, which must occur in a defined sequence to result in cancer. Complete carcinogens can perform both the initiating and the promoting functions. Cocarcinogens (Berenblum, 1969) must both be present at the same time for a neoplasm to result while with incomplete carcinogens the initiator must precede the promoter for cancer to follow. The initiator and promoter need not be present at the same time. Chemical carcinogens can be complete, incomplete or cocarcinogens. Following irradiation, initiating effects predominate though there is some promotion at intermediate doses. High dose irradiation causes enough other tissue damage for cancer not to be a problem for the irradiated organism. Viruses may be complete or incomplete carcinogens. Cancer cells induced by chemical or radiation methods are known to produce viruses not detectable in corresponding normal cells. A retroviral genome, however, has been found in many normal cells. Whether these viruses are activated by the transformation process and whether they have a role in it are open questions. They may be

contaminants of the cells brought about by the methods used to study cancer.

Many techniques have been developed for the study of cancer. Experimentation in whole animals or the study of naturally occurring tumours is the most relevant but the most difficult approach. Studies undertaken *in vitro* can yield valuable information but may result in artifacts. The advantage here is the ease of repetition and the speed of data accumulation. Oncogenesis *in vitro* has often resulted in transformed cells which do not grow in the appropriate host animal. Likewise, no one criterion of transformation *in vitro* is characteristic of cancer cells in general (Sanford, 1974). In the final analysis, to determine what causes cancer, one must look at what causes cancer *in vivo*. Another problem in the search for what causes cancer is latency. In most human studies, exposure to a carcinogen does not result in cancer until up to 20 years later. By that time it can be uncertain as to which one or a combination of potential carcinogens that an individual has been exposed to is at fault (Selikoff, 1968). Thus, animal studies where a large proportion of the lifespan of the experimental animal is observed in a short time are extremely valuable as exposure to carcinogens can be controlled as well.

Ewing (1941) defines malignancy as the property that differentiates normal from cancer cells. This is reflected in his list of characteristics of a

malignant tumour:

1) infiltrative growth
2) local destructive properties
3) recurrence after removal
4) formation of metastases
5) local interference with function
6) toxic action of absorbed tumour products.

In all cases, the tumour has escaped the control mechanisms by which the body maintains its integrity. Willis (1967) uses the term innosence as the opposite of malignancy to describe the difference between normal and cancer cells. He states that the terms innosence and malignancy are at extreme opposite ends of a spectrum of properties such that there is no clear division between the two. Generally speaking, cancer cells have a higher growth rate than their normal counterparts and there is an inverse relationship between growth rate and degree of differentiation of the tumour cell (Gray and Pierce, 1964).

The transformation event can take place in one or a few cells in the same site which give rise to many daughter cells forming the tumour. Initial studies indicated that tumours have a clonal origin, i.e. arose from one cell (Linder and Gartler, 1965; Fialkow et al, 1970) but work with chemically induced tumours has shown that a multicellular origin is more likely (Reddy and Fialkow, 1979). Earlier studies may have examined only the dominant clone of cells in the tumour as the material examined had been in situ for quite a while. In chronic

granulocytic leukemia, a specific chromosomal aberration is associated with the disease, the Philadelphia chromosome. This abnormality occurs in all cells of the stem line of the myeloid class in dicating that a particular stem cell was probably transformed (Nowell, 1975). In a lymphoid cancer, myeloma or plasmacytoma, a paricular antibody producing plasma cell is probably transformed (Potter, 1967; 1977) as the incidence of double myelomas is lower than expected. The monoclonal origin of this tumour is indicated by the copious quantities of a single species of antibody that are present in the serum of affected individuals.

The cells which make up a tumour have the capacity to respond to their environment as a population. This property has long been explained as the developmental acquisition of characteristics, usually malignant, in the

tumour (Greene, 1951) or likened to embryonal epigenesis (Foulds, 1954). The best examples are seen in the cases of transplantable tumours. It is possible to convert some tumours to growth in an unnatural site indicating a response or adaptation to enviroment (Klein and Klein, 1956). As transplantation is continued, a faster growth rate is observed, and the cells become correspondingly less differentiated. In this way, transplantation selects for the fastest growing cells (Gray and Pierce, 1964). As one is usually not transplanting single cells, the transplants are not clonal, but are a population of cells responding to their enviroment. Variation within the cells of a tumour can now be shown in many different respects, not only amongst well established, long transplanted tumours (Prehn, 1970; Fidler, 1978), but in tumours of recent origin (Dexter et al, 1978; Kripkie et al, 1978). Such evidence supports Nowell's theory of the clonal evolution of tumour cell populations (Nowell, 1976). As the lifespan of a tumour increases, genetic changes accumulate in the cells of the tumour resulting in subtle variations in tumour cell properties. This results in the acquisition of increasing degrees of malignancy which is of selective advantage for the survival of the tumour. This phenomenon has been known historically as tumour progression. Thus, one can view a tumour as a rapidly growing group of cells, derived from a single ancestor cell, continually amassing genetic instabilities, leading to faster growth rates,

less differentiated properties, and more malignant properties. It has been reported by Chow and Greenberg (1980) that tumour progression occurs at a faster rate in the *in vivo* state than *in vitro*. This is to be expected as the selective pressure on cells *in vivo* and *in vitro* will be very different. Also, one must keep in mind that various transforming agents, even those that are closely related, and the variation in their target cell of action can lead to large differences in tumorigenic and metastatic properties of the resultant cancer (Yogeeswaran *et al*, 1980).

## 2. General Remarks Regarding Metastasis

Willis (1967) considers invasiveness to be the fundamental and distinguishing attribute of malignancy made possible by other tumour cell properties such as:

1) progressive growth
2) increased motility
3) loss of adhesiveness
4) phagocytotic activity
5) the elaboration of toxins.

All these characteristics, plus the ability to induce new blood vessel formation or angiogenesis, could be responsible for the metastasis of cancer. Progressive growth allows a tumour to acquire the properties enabling it to survive in the host enviroment. Transformed cells in a tumour do not stay in synchronous cell cycle much

beyond the 8-16 cell stage. This could be due to altered cellular regulation of the individual tumour cells as influenced by each cell's environment. Cell kinetic studies have shown that for growth to occur in a differentiated tumour, production of cells must be greater than loss of cell out of the dividing differentiated state to the terminal differentiated state. Undifferentiated tumours, on the other hand, have cell loss only to death and necrosis (Lightdale and Lipkin, 1975). Thus, undifferentiated tumours amass more cells in a shorter period of time than do differentiated tumours eventhough they may have the same mitotic rates. Angiogenesis allows the tumour to acquire a blood supply from the surrounding normal tissue and grow as a multilayered mass, avoiding central necrosis. Generally, once a tumour has vascularized, its growth rate increases markedly, and hense, its progression. It has been shown that some tumours elaborate an angiogenesis factor which can induce capillary endothelial cells to divide and directs the growing capillary in the direction of the tumour (Folkman, 1975). It has been estimated that for each new capillary endothelial cell in a tumour, 50 new tumour cells may be supplied.

The invaisiveness of a tumour controls the extent to which it will metastasize. Invasion of normal tissue by a tumour is thought to occur by the ways listed below (Fidler et al, 1978):

1) mechanical pressure

2) destruction of normal tissue

3) Lack of adhesiveness and increase in the motility of tumour cells.

Mechanical pressure by itself cannot explain all the clinical instances of invasion, but it must account for some of the factors involved in metastasis by direct extension.

As a tumour enlarges, it encounters anatomical structures which are more or less resistant to the growth of the tumour (Zeidman, 1957). Hard tissues such as bone, cartilage, tendons and ligaments provide more of a barrier than do soft tissues such as fat, parenchymous organs and fluid-filled cavities. Elaborate models have been developed using gels or fluids which show that under pressure, the gel will spread along anatomical planes of cleavage (Eaves, 1973). It is proposed that tumour cells also follow the line of least resistance when spreading by direct extension. It has been observed that while tumours will grow around solid anatomical structures, the soft tissues are often replaced by tumour cells (Leighton et al, 1960). At the leading edge of an invading tumour there is a considerable amount of cell death. Degradative enzymes have been isolated from this area of growth, indicating that the tumour may have the capacity to destroy normal tissue (Quigley, 1979). An alternative hypothesis is that tumour cells generally have a higher metabolic rate than do normal cells and may successfully compete for nutrients and flood the normal cells with wastes. The enzymes found above could be normal cell

components or debris from dead cells (Weiss, 1977). Regardless of which hypothesis is correct, these enzymes can lead to decreased cellular adhesiveness allowing motile tumour cells to invade normal tissue with greater facility (Coman, 1947; Zeidman, 1957). Tumour cell motility may account by itself for invasion as isolated tumour cells can penetrate various types of normal tissues (Easty and Easty, 1974; Hart and Fidler, 1978, De Bruyn and Cho, 1979; Poste et al, 1980). Indeed, a primary tumour surrounded by satellite metastases is a common occurence.

Distant metastasis, i.e. not connected with the primary tumour, has been linked to a cascade of events (Fidler et al, 1978):

1) entry into the circulation
2) circulation
3) lodging at a distant site
4) exit from the circulation
5) growth.

In both cases where a tumour cell must cross a vessel wall, entry and exit from the circulation, the property of invasion is a prerequisite. An effective barrier to entry into the circulation is provided by the dense elastic and muscular tissue in arterial walls (Zeidman, 1957). Thus, nearly all metastasizing tumour cells enter the circulation via the venous or lymphatic circulation. It becomes obvious that in a highly vascularized tumour there will be more opportunities for access to these vessels

and more cells are released. De Bruyn and Cho (1979) have visualized the process of tumour cells entering the lumen of vessels via a migration pore in the endothelial cell luminal sheet. No destruction of normal tissue is apparent but extracellular enzymes could loosen the cells surrounding a vessel's patent lumen allowing the tumour cell to crawl through. Trauma of vessel walls may allow noninvasive tumour cells to gain access to the circulation. Indeed, the tissue pressure of tumours is higher than normal tissues and blood favouring the release of tumour cells should no barrier be present under traumatized conditions (Young et al, 1950; Young and Griffiths, 1950).

Most experimental models of metastasis bypass the initial entry into the circulation of metastatic tumour cells by the intravenous injection of such cells. This mimics the situation of a traumatized tumour where a sudden shower of tumour cells flood into the blood. As the length of time with a tumour in situ increases, the number of cells released into the circulation per unit time also increases (Martinez et al, 1956; Romsdahl et al, 1961). There is a clear separation in kinetics in the release in the experimental as opposed to the clinical situation. This can be avoided by using tumours transplanted to discrete sites. How severe this kinetic critisism is is dubious as studies have shown that only in cases where tumours are heterotransplantable (Watanabe, 1954) or have metastasized spontaneously (Wallace, 1956), i.e.

highly invasive, is intravenous transplantation possible. Still, the number of intravenous cells administered correlates well with the number of metastases formed, mimicing the clinical situation (Zeidman et al, 1950).

Tumour cells entering the lymphatic system will end up in the venous blood as the lymphatic drainage enters the vena cava via the thoracic duct. The only major barrier to tumour cell traffic in the lymphatic circulation are the lymph nodes. It has been shown that the majority of tumour cells in the afferent lymph traverse the node and appear in the efferent lymph and venous blood (Fisher and Fisher, 1966a; 1967a). This indicates that some antigen in the form of metastasizing tumour cells is sequestered in the node allowing the possibility of an immune response. From the point of view of metastasis, however, enough cells can pass the node for its barrier function to be insignificant.

Having gained access to the venous circulation directly or via the lymphatic circulation, tumour cells must face the rigours of blood flow and interaction with blood elements. Many studies have shown that the majority of tumour cells in the circulation are destroyed (Fidler, 1970; Reid and Gibbons, 1979). The mode of destruction can be due to many factors such as the natural death of cells, host immune or para-immune mechanisms (Ioachim et al, 1976; Fidler, 1977; Fidler et al, 1977; Carlson et al, 1980). Of all the blood elements,

however, the major interaction is between circulating tumour cells and the coagulation system. In fact, the formation of emboli of tumour cells, platelets and fibrin can be a significant occurrence (Baserga and Sffiotti, 1955; Wood, 1958; Gasic et al, 1973; Warren, 1973) and treatment with anticoagulants may reduce the formation of metastases (Wood et al, 1956; Fisher and Fisher, 1961a; Gasic et al, 1968). This leads to the view that only tumour cell emboli can form metastases (Saphir, 1947; Fidler, 1973a).

Extensive human studies have shown that cancer cells in the blood have no clinical significance with regards to recurrence or prognosis (Engell, 1955; 1959; Roberts et al, 1961). Also, tumour cells have been shown to pass capillary beds (Zeidman and Buss, 1952; Zeidman et al, 1956; Fisher and Fisher, 1967c). This is significant as most metastasizing cells in the venous blood would be expected to lodge in the first capillary bed encountered if metastasis was a purely mechanical process. This does not appear to be the case as some tumours show metastatic distributions not explainable on an emboli frequency basis (Sugarbaker, 1952), though the opposite view has equally as fervently been espoused (Coman et al, 1949; Coman, 1953). Still, the retention of tumour cells in a capillary bed does not necessarily correlate with the ability to form metastases (Karpas and Silversmith, 1963; Greene and Harvey, 1964) and embolization

may not be important. One would expect tumour emboli to be trapped when a blood vessel narrows to a size too small for it while single cells have shown an amazing plasticity that determines passage through capillary beds (Zeidman, 1961a). Coupled with the property of invasiveness, it has been postulated that metastasizing cancer cells traffic through the body in much the same way that lymphocytes do (Fisher and Fisher, 1966b; Weiss, 1980).

Entry into the tissues from a blood vessel where a metastasizing tumour cell has come to rest is essentially the reverse process to entry into the circulation (Wood, 1958). This need not occur as the tumour cell or emboli may grow and occlude the blood vessel or die and be subject to encapsulation and necrosis. The establishment of a distant metastasis is much the same as the establishment of a primary tumour from transformed cells but a higher level of malignancy is evident from the beginning. There can be fundamental differences between a primary tumour and its metastases in terms of cell surface antigens (Fogel et al, 1979; Gorelik et al, 1979; Schirrmacher et al, 1979; 1980) and suceptibility to host defense mechanisms (Gorelik et al, 1979; Carlson et al, 1980). A metastatic growth undergoes the same processes of clonal succession as does the primary tumour and may in turn produce metastases as well.

3. The Organ Specificity of Metastasis

One of the striking observations of the clinical oncologists is the variable propensity for different tumours to invade and metastasize to various tissues. For example, cancer of the prostate and thyroid metastasize almost exclusively to the bones while Burkitt's lymphoma will invade the ovaries in female patients. Willis (1973) has surveyed the clinical literature on this subject and has come to the conclusion that the evidence for Paget's seed/soil hypothesis is overwhelming. Briefly, Paget (1889) used the metaphor of a fertile seed falling upon the correctly prepared soil to describe the interactions that a metastasizing tumour cell has with its host organ. Willis (1973) cites evidence from diverse sources. This is listed below:

1) the discrepencies between the relative blood supplies and the relative incidences of metastases in various organs

2) the disproportionate frequency of metastases in certain organs from particular primary tumours

3) the multiplicity and bilaterality of metastatic tumours in certain organs

4) the dissociations and associations of the sites of metastases (dissociation; the intestine but not the liver: association; intestine, pancreas, stomach and gall bladder)

5) individual peculiarities of metastatic distribution

6) the situation of metastases in pathologic areas

7) the fact that tumour embolism is not metastasis

8) differences in mitotic activity of metastatic growths in different situations

9) tissue culture studies (chemical enviroment for the survival of certain cells)

10) experimental oncology (of which more will be dealt with below).

Willis speculates that the explanation for the evidence in favour of Paget's theory over a strictly mechanical hypothesis is based on biochemical and nutritional factors. In other words, the internal milieu of certain tissues may be incompatable with the growth of certain types of tumours such that essential nutrients cannot be supplied in sufficient quantity to the metastasis or certain nutrients removed efficiently. Willis, however, does not discount the roles played by unknown factors in the distribution of metastases.

In experimental oncology, the organ specificity of tumours or preferential organ metastasis has been observed for some time. The earliest studies were complicated by the fact that inbred strains of animals or immunologically impaired animals were not used. Still, there were a number of papers whose fundamental observation was the organ specificity of metastasis (Levin and Sittenfield, 1910; Tyzzer, 1913). Lucké's studies in the leopard frog provided a firm experimental basis (Lucké, 1934; 1938) though his model system supplied too few animals to study as metastases occurred rarely. However, the observation is clear that liver metastases occur more

frequently than any other gross metastatic lesion. Kinsey (1960) showed that the organophilic tendencies of a tumour do not depend on the anatomical site of the organ. Ectopically transplanted lung was able to attract as many lung seeking tumour cells as the normally situated lung in the mouse. This is important as a number of studies appearing later showed that retention in a capillary bed does not correlate with the ability to form tumours (Karpas and Silversmith, 1963; Greene and Harvey, 1964). Greene and Harvey (1964) postulated an endothelial bond between the tumour cell and the vascular endothelium that may account for their observations. That is, only in specific locations where a favourable interaction occurs between a particular endothelium and a tumour cell can a metastasis occur. Indeed, Pilgrim (1969) surveyed the literature for reticuloendothelial cell tumours and concluded that they have organophilic tendencies to metastasize to lymphoid organs.

An important breakthrough in the study of organ specific metastasis was the selection of organ specific metastatic variants by Fidler (Fidler, 1973b, Fidler and Nicolson, 1977). By successively passaging the spontaneously originating B16 melanoma through the lungs of mice, Fidler obtained a tumour cell line with enhanced metastatic preference for the lungs. This may have merely resulted from an increased capacity of the cells to live in mice as it had been previously reported that the B16 melanoma does not give any metastases outside

of the lungs (Fidler, 1970). Regardless, it has since been shown that the same tumour can be selected for preferential metastasis to a number of organs (Nicolson and Brunson, 1978; Tao et al, 1979). That the procedure of successive selection for organophilic tendencies works for a number of organs and more than one tumour (Brunson and Nicolson, 1978) implies a basic mechanism to the phenomenon of organ specific metastasis.

## 4. Some Mechanisms of Metastasis

The mechanisms of metastasis have been mentioned above but have not been explored to any great depth. There are three theoretical mechanisms of metastasis that have been evoked to explain why a tumour will form a metastasis at a particular site. These are:

1) the mechanical hypothesis
2) Paget's seed soil hypothesis
3) the variant selection hypothesis.

Metastasis by direct extension is purely a mechanical process, as mentioned earlier, once the necessary invasive prerequisites have been met by the tumour. The succeeding discussion will center around distant metastasis.

On a purely mechanical basis, the cells shed into the circulation from a tumour will come to rest in the first capillary bed where the lumen of the blood vessel has a smaller diameter than the tumour cell or

embolus (Coman et al, 1949; Coman, 1953). As most cells are shed into the venous side of the circulation, this will occur in the lungs. Indeed, the lung is the most common site for metastasis (Willis, 1973). Anatomical sites which have a relationship through a portal circulation will show a high incidence of metastasis in the downstream site from a primary tumour upstream (e.g. the liver downstream from the small intestine; Fisher and Fisher, 1965). All this can be explained by using the metaphor that the first capillary bed encountered by the metastasizing tumour cell or embolus acts like sieve, filtering out of the blood tumour cells which will then result in metastases. Still, many patterns of metastasis cannot be accounted for by invoking a simple sieving action of capillary beds (Sugarbaker, 1952; Willis, 1973; Fidler et al, 1978; Poste and Fidler, 1980). Anatomical studies have shown the presence of arterio-venous shunts in many organs (Prinzmetal et al, 1948) possibly allowing the passage of tumour cells. Also, tumour cells can pass through organs (Zeidman and Buss, 1952; Zeidman et al, 1956; Fisher and Fisher, 1967c). This could explain many cases of what would seem to be aberant metastases under a strictly mechanical interpretation. A mechanical explanation cannot explain the metastasis of certain tumours to one or a few highly specific sites. Thus, eventhough mechanical factors undoubtably play a role in the determination of the distribution of metastatic cancer cells,

a stricly mechanical interpretation cannot account for all the observed distributions of metastases.

Other factors that would modify the mechanics of metastasis are the variables within the metastasizing cell and the organ in which the metastasis will be formed. This concept was first introduced by Paget (1889) and has come to be known as the seed/soil hypothesis of metastasis due to the analogy used by him. As in the parable of the sower (Matt. 14: 3-9), only when the correct seed falls upon fertile soil will a plant be germinated. Likewise, a metastatic cell must have the capacity to respond to its environment and the environment must be of a type that can send signals to the cell. One can see that the outcome of the interaction between a tumour cell and a distant site will determine the success or failure of a metastasis. As mentioned earlier, tumour progression allows the incorporation of variations in malignant properties in a tumour cell population (Green, 1951; Foulds, 1954; Nowell, 1976). In this way, the potentialities of a tumour cell may be limited but those of the population of tumour cells can be endless. Indeed, variations between cloned populations of tumour cells with regards to drug resistance (Barranco et al, 1972; 1973; Hakansson and Tropé, 1974), invasiveness and metastasis (Dexter et al, 1978; Fidler, 1978; Kripkie et al, 1978:; Suzuki et al, 1978) appears to be the normal situation. Thus, a tumour can sow many different types of seeds.

It is evident that the local environment of different organs will differ in many ways as well. Some of these are mechanical pressure, blood flow, amount and types of connective tissue, etc.. Paget's hypothesis (Paget, 1889) then reduces to matching the cell with a certain potential to the site where this potential may be realized. This would seem to be a very inefficient process from the standpoint of the survival of the tumour cell. A large number of cells would have to be released by a tumour for one cell to evade host defense mechanisms, survive the mechanical rigours of blood flow, lodge in a favorable site for that particular cell, extravasate and proliferate to form a metastasis. Clinical studies, as mentioned above, have shown that cancer cells in the blood have nothing whatever to do with prognosis (Engell, 1955; 1959; Roberts et al, 1961). This could be due to the sheer numbers of cells that must circulate for one cell to have an effect. Circulating cells will differ in their malignant potential depending on how far the primary tumour has progressed. Thus, comparing many individuals with many different stages and types of tumours will not yield consistent results. Most animal models of metastasis require relatively large numbers of cells to observe relatively few metastases. Studies have shown that 99% of the cells injected intravenously do not survive 24 hours (Fidler, 1970; Reid and Gibbons, 1979). This result will depend on the individual tumour but it is important to note that tumour embolism is not metastasis.

An analogy for this situation can be found in the maturation of T lymphocytes. The prethymic T cell metastasizes from the bone marrow to the thymus where it undergoes proliferation and maturation. It is thought that this is the site where the T lymphocytes acquire their functional differentiation. Mature T lymphocytes are released into the circulation and enter the blood stream. They are found in the lymph a short time later (Gowans and Knight, 1964). The T lymphocytes have left the circulation at a specific anatomical structure in the post-capillary venule of the lymph node which has a high endothelium (Gutman and Weissman, 1973). Not only do the T cells show a preference for this anatomical structure through which they extravasate, but peripheral node lymphocytes recirculate preferentially through peripheral nodes as do mesenteric node lympgocytes recirculate preferentially through mesenteric nodes (Cahill et al, 1977).

An extension of Paget's seed/soil hypothesis is suggested by the recent literature (Fidler et al, 1978; Weiss, 1979; Poste and Fidler, 1980). This will be refered to as the variant selection hypothesis of cancer metastasis. The basic premise is the production of a myriad of tumour variants by the tumour, resulting in tumour progression, perhaps in the manner suggested by Nowell (1976). Succession of variant tumour cell clones for dominance of

of the tumour cell mass will produce the gradual increase in malignancy of the cancer. When the necessary invasive qualities have been acquired by the tumour cells and access to the circulation has been gained, metastatic tumour cells are released into the circulation. Whichever cells butt onto the access to the blood will be released. These may not necessarily be from the dominant clone in the tumour at that time. Cells released will have individual potentialities to metastasize. Thus, as in Paget's hypothesis, where the metastasizing tumour cell can realize its potentialities will be where that cell will produce a metastasis. As the cells circulate, each micro-enviroment in the host selects those cells, or the enviroment is selected by the cells, which can produce a metastasis in their milieu. This process would parallel that of the lymph node specific recirculation of lymphocytes (Cahill et al, 1977). Membrane mediated events, possibly involving specific receptors, have been implicated in this phenomenon (Ford, 1975; Woodruff et al, 1977). A similiar process may be operative in the arrest of blood-borne tumour cells. Membrane mediated events have been shown to be important in the organ selective metastasis of Fidler's B16 melanoma variants (Poste and Nicolson, 1980).

## 5. Factors that Affect the Metastatic Process

In the study of experimental metastasis, many investigators have treated their systems in different ways so as to perturb the metastatic cascade. There is no clinical treatment specific for metastasis and many of the manipulations that patients undergo may actually increase the likelihood of metastasis. The interferences most often applied to experimental systems of metastasis are surgery, radiation, chemotherapy and immunotherapy. These are the same options of treatment open to a cancer patient with or without metastatic disease.

It has been mentioned that cancer cells in the circulation do not have any correlation with prognosis nor does traumatization of the tumour during surgery for its removal (Engell, 1955; Moore et al, 1957; Engell, 1959; Roberts et al, 1957; 1961). Still, tumour cells that have already metastasized may be revived from a dormant state by surgical trauma (Fisher and Fisher, 1959). Thus, if surgery is to be successful in curing the patient of his tumour, then the surgeon must remove the tumour before it has metastasized or remove the metastases with the primary. Localization of tumours and metastases has always been a major problem in cancer treatment. Removal of a primary tumour usually has little effect on the metastases that are well established, they are autonomous entities. Removal of the primary may delay the onset of metastasis

from the residual tumour as it may once again have to progress to sufficient size and malignancy to metastasize (Ketcham and Sugarbaker, 1977). In keeping with the postulate that metastases must undergo all the stages of tumour progression that the primary does is the observation that metastases do not metastasize until they are sufficiently established with a blood supply. This will also cause some delay in the further spread of a cancer after the removal of the primary tumour.

The local, lethal irradiation of certain tumours has provided an additional way of controlling cancer with or without prior surgery. Irradiation of the tumour is only successful with reference to metastasis if the tumour has not metastasized to a distant site outside the area of irradiation. For this reason, total body irradiation was instituted but the total dose must be reduced, and hense, the effectiveness of the treatment course has been impaired. Significant advances in radiosensitizers have been made enabling the radiation to be administered at a lower overall dose but at a higher effective dose within the tumour (Andrews, 1978). The effect of whole body irradiation in a mouse is to increase the number of metastases (Kaplan and Murphy, 1949; von Essen and Kaplan, 1952; Fidler and Zeidman, 1972). Von Essen and Kaplan (1952) speculated that the radiation altered the local host/tumour interaction while Fidler and Zeidman (1972), writing after Greene and Harvey (1964) who propsed

the endothelial bond theory of metastasis, postulated a difference between the endothelial cell stickiness pre- and post-irradiation. None of these studies surveyed the dissemination of metastases in the animal so whether or not an altered distribution is effected by the whole body irradiation is open to question.

As for surgery and radiation treatments of cancer, there is no chemotherapeutic modality specifically designed for use against metastasis (Pratt and Ruddon, 1979). Like whole body irradiation, chemotherapy is a systemic treatment of cancer and can reach metastases as they occur over the course of treatment. Metastases are once again controlled best, as for surgery and radiation therapy, when the treatment is started before the metastases occur. Combinational therapy of reducing the patient's tumour burden by surgery or irradiation followed by chemotherapy can be used successfully to control metastasis. Most chemotherapeutic modalities, however, center around the selective poisoning of the tumour in hopes that the host will not be too deliteriously affected. The stickiness of the vascular endothelium is thought to be important again here, as for the radiation induced enhansement of metastasis. Prior treatment of mice with steroids (cortisone has been used in most studies) greatly augments the arrest of tumour cells in capillary beds after intravenous injection (Zeidman, 1961b; Fidler and Leiber, 1972). This activity has been directly correlated with the glucocorticoid

activity of the individual steroid involved (Albert and Zeidman, 1962). It has been claimed that only tumour cell clumps form metastases (Saphir, 1947; Fidler, 1973a) and the number of metastases has been correlated with the number of tumour cell clumps or additional cells added (living or dead) for tumour cells to form clumps with (Fidler, 1973a). In fact, it has been shown that thromboplastic emboli are more likely to stick to vascular endothelia (Warren, 1973). Heparin, plasmin, (Fisher and Fisher, 1961a) neuraminidase or anti-platelet antiserum (Gasic et al, 1968) reduces the number of metastases. Gasic et al (1973), in a later study, postulated that th[illegible] arrest is not the important factor in determi[illegible]location of a metastasis but that retention in [illegible] is better if platelets are aggregated in a tumour cell [illegible]lus. Indeed, factors active in the coagulation path[illegible] are produced by tumour cells (Stringfellow and Fitzp[illegible]rick, 1979; Fitzpatrick and Stringfellow, 1979; Wang et al, 1980). The ability to activate the host coagulation system in a local way may be one of the phenotypes necessary to give rise to a successful metastasis.

Immunotherapeutic modalities specific for metastasis are in their infancy. It was shown that reticuloendothelial blockade or stimulation increases the number of metastases (Fisher and Fisher, 1961b; 1962). The reason for this may be purely mechanical. Agents which stimulate or block the reticuloendothelial system also

swell the liver Kupffer cells to occlude the sinusoids thereby trapping more cells within the liver. In a series of experiments, Fidler demonstrated that tumour sensitized lymphocytes (Fidler, 1974a) or activated macrophages (Fidler, 1974b) reduced metastasis. Ioachim et al (1976), working in a different system, concluded that immune mechanisms do not play a significant role in the metastatic pattern of a tumour. Fidler et al (1977), in their original system, could not find a correlation between metastasis and tumour rejection but concluded that tumour cell arrest could be influenced by immune factors. In another system, Fidler et al (1979) demonstrated that the effects of immunity and immunosuppression on a tumour's pattern of metastasis is idiosyncratic. The immunogenicity of the tumour probably plays a role here. As Fidler has done much of this work in a mouse melanoma model, a highly responsive type of tumour to immunotherapy in man as well as mouse, it is not surprising that discrepancies exist in the effects of immunity on metastasis. It has been reported that cytotoxic lymphocytes can distinguish antigenic differences on tumour cells that have variable propensities to metastasize (Fogel et al, 1979; Schirrmacher et al, 1979). Fogel et al (1979) postulate that the highly metastatic subline of Fidler's melanoma tumour model has lost some of the antigenicity of the parental marginally metastatic cell line. Schirrmacher et al (1979), working with a lymphoid tumour, denote an antigenic change between

highly and marginally metastatic tumour cell lines. Para-immune mechanisms have been implicated as factors in the host resistance to metastasis (Gorelik et al, 1979; Carlson et al, 1980). Gorelik et al (1979), working with Fidler's system, report that local tumour cells are killed more easily than are their counterparts in a metastasis in a natural killer (NK) cell assay. They postulate a loss in the NK cell recognition structure on the metastatic tumour cells as compared to the local tumour cells. Another interpretation is that the metastatic tumour cells are more resistant to killing than the local tumour cells.

One approach to the specific therapy of metastasis is a combined chemotherapy and immunotherapy (Hurwitz et al, 1979). A drug is coupled to an immuno-globulin which specifically recognizes the tumour and its metastases. The conjugate is more effective than the uncoupled drug and antibody administered together or alone. This approach is only practical if the tumour in question has a characteristic antigen that is immunogenic in some host. Also, a similiar approach could be useful as a diagnostic tool for metastasis. Antibodies could be coupled to tracers which, because of the specificity of the antibody, would localize in metastases with the tumour antigen. This would make conventional radiation or surgical treatment of cancer more effective (Ballou et al, 1979).

## 6. Cell Surface Structures Implicated in Metastasis

The first interaction of a metastasizing cell when it lodges in a distant site is that of the tumour cell membrane coming in contact with the cell membrane of the vascular endothelial cells. A number of workers have addressed this interaction as being fundamental to the metastatic process. Indeed, cell membrane vesicles from highly metastatic cells can confer the metastatic phenotype on marginally metastatic cells if the vesicles are fused to the latter (Poste and Nicolson, 1980). Generally, treatment with proteolytic or glycosidic enzymes alters the metastatic distribution of tumour cells. Numerous workers have reported that neuraminidase or trypsin treatment of the tumour cells increases metastases (Hagmar and Norrby, 1973; Sinha and Goldenberg, 1974; Weiss et al, 1974). An early dissenting report (Gasic and Gasic, 1962) states that one has to treat the vascular endothelium by prior injection of neuraminidase to have any effect at the reduction of metastasis while the neuraminidase treatment of the tumour cells does not have any effect. Schirrmacher et al (1980) report that the neuraminidase treatment of the tumour cells increases binding to hepatocytes, not hepatic epithelium, which can be inhibited by anti-major histocompatability complex (MHC) antibody. This inhibition by antibody is thought to be acting via a steric mechanism where the antibody blocks the neuraminidase exposed sites

by virtue of their position. It has been shown that neuraminidase treatment of lymphocytes results in their retention in the liver (Woodruff and Woodruff, 1974; Ford, 1975) and this is thought to occur via a hepatic cell membrane protein that specifically binds asialoglycoproteins (Pricer and Ashwell, 1971; Kawasaki and Ashwell, 1976; Kolb et al, 1978). Schirrmacher et al (1980) postulate that they are observing a similiar phenomenon.

As any enzymatic treatment of a cell's surface may also lead to a change in surface charge, the issue is more complex than it first appears. Hagmar and Norrby (1970) report that the pretreatment of tumour cells with polyanions or polycations which bind to the cell surface markedly alter the surface charge and the distribution of metastases. There is not a simple correlation with surface charge as dextran, which increases cell surface negativity, can have the same effect on metastasis as DEAE-dextran, which reduces the surface negativity (Hagmar, 1972). Raz et al (1980) show that it is the distribution of anionic sites that is important when one is considering the relationship between cell surface charge and metastasis; the more metastatic cells have a more clustered array of anionic sites. This is in keeping with the chemical studies of sialic acid and fucose content of the cell membranes of tumour cells which show that there is no difference in the terminal saccharides in highly or marginally metastatic cell lines (Warren et al, 1975; Yogeeswaran et al,

1978; 1979). These terminal monosaccharides on the carbohydrate chains of glycoproteins account for most of the cell surface negative charge.

Agglutinability with lectins, which bind to specific sugars on the tumour cell surface, has been used to select non-metastasizing variants from metastasizing melanoma cells without any apparent change in the cell surface (Tao and Burger, 1977). The metastatic phenotype is thought to be due, once again, to the arrangement of the lectin receptors on the cell surface (Wright et al, 1978). Drugs that disrupt the cytoskeleton, such as colchicine or cytochalasin B, alter the metastatic pattern of the tumour cells (Hagmar and Ryd, 1977) and also reduce adhesion, migration, homeotypic aggregation, and agglutination by lectins (Hart et al, 1980), indicating that an intact cytoskeleton may be important if a cell is to metastasize successfully.

Simple aggregation of metastatic cells has been shown to alter the metastatic pattern of a tumour cell line (Hagamr and Norrby, 1978) or its malignancy (Parks, 1975). Cells surviving interactions with immune or paraimmune defense mechanisms might be considered better able to give rise to a successful metastasis though highly metastatic cell lines tend to form more clumps with lymphocytes than marginally metastatic ones (Fidler, 1975). Cell lines selected in vitro for their ability to resist lymphocyte mediated cytolysis actually show themselves

to be less metastatic than their parent cell line (Fidler et al, 1976). The ability to form heterotypic cell clumps appears to be a general property of metastatic cell lines (Winkelhake and Nicolson, 1976) and is related to the organ preference of the tumour cell line (Nicolson and Winkelhake, 1975). Metastatic tumour cells or their culture supernatants can alter the properties of adhesion of normal cells (Maslow and Weiss, 1979; Maslow et al, 1980). One postulate arising out of this work is that metastatic or invading malignant tissue disrupts the normal cell to cell adhesion by releasing large amounts of competing aggregation factors, which occupy the receptors on the normal cells causing the normal tissue to become disorganized (Balsamo and Lilien, 1974; Kramer and Nicolson, 1979; Lilien et al, 1979). One other aspect to tumour cell agglutination in the metastatic process is the basement membrane that a tumour cell must cross to invade an organ. Glycoproteins from basement membrane have been shown to agglutinate normal cells and it is thought that this helps maintain the cells' differentiated state (Gerfaux et al 1979). Metastatic tumour cells preferentially attach to type IV collagen, that found in large amounts in the basement membrane of vascular endothelium, over all other types of collagen (Murray et al, 1980).

Metastases can be antigenically distinct from their primary (Fogel et al, 1979; Gorelik et al, 1979; Schirrmacher et al, 1979). Also, the quantitative

amount of a tumour associated antigen has been correlated with the metastatic phenotype (Ghosh et al, 1979). Thus far, it is not known how these antigens are functionally related to the metastatic cascade.

## 7. Marek's Disease as an Experimental Model of Malignancy

Marek's disease (MD) is a natually occurring, herpesvirus induced, T lymphocyte malignancy of chickens (Payne, 1972; Nazerian, 1973). The ubiqitous Marek's disease virus (MDV) infects all types of chickens and free virus is shed from the feather follicles, resulting in horizontal transmission of the disease (Nazerian and Witter, 1970). There are two major pathological syndromes caused by MDV, a neurologic pathology leading to paralysis and a malignant pathology leading to lymphoma development. One or both of these pathologies is associated with infection by various strains of the virus (Biggs and Milne, 1972). Marek's disease is the only naturally occurring cancer to which resistance can be induced by vaccination (Biggs, 1975). Protection is only against the pathologic attributes of the disease and not against infection by the virus. Much work has been done in recent years on the genetics of natural resistance to MD lymphoma development. One form of genetically determined resistance is associated with the $B^{21}$ allele (Longenecker et al, 1976) of the chicken MHC, which may involve active rejection of prolif-

erating lymphoma cells (Longenecker et al, 1977a; Longenecker and Gallatin, 1978). Another non-MHC linked locus determines the susceptibility of the target cell for transformation, the T lymphocyte (Gallatin and Longenecker, 1979). There are pathological and etiological similiarities between MD and Burkitt's lymphoma (Klein, 1972). One of these similiarities is the pattern of metastasis in which both malignancies give rise to a high incidence of ovarian and liver lesions (Payne, 1972; Wright, 1972).

Other herpesvirus induced malignancies or malignancies in which herpesviruses have been implicated as a causative agent are the Lucké carcinoma of the frog (Mizell, 1969), Burkitt's lymphoma in man (de-Thé et al, 1978) and a malignant lymphoma of lower primates associated with infection by either Herpesvirus saimiri or Herpesvirus ateles (Melendez et al, 1972). It is interesting to note that only herpesviruses associated with lymphoid cells cause or have been strongly implicated in the cause of malignancy, with the exception of the Lucké herpesvirus. In the cases of Herpesvirus saimiri, MDV, and Epstein-Barr virus (EBV; Burkitt's lymphoma), the virus does not integrate into the host genome as a provirus in the transformed cells yet the cells replicate the viral genome (Sugden et al 1979). Very speculative associations have been made between EBV and nasopharyngeal carcinoma (Ho, 1972) and Herpes simplex I and cervical carcinoma (Rawls et al, 1968). Outside of that mentioned above for MD and Burkitt's lymphoma,

nasopharyngeal carcinoma exhibits preferential metastasis to bone, liver and lung (Shanmugaratnam, 1972) while the Lucké carcinoma has a strong preference to metastasize to the liver (Lucké, 1934; 1938).

Recently, the study of MD has been facilitated by the establishment of lymphoblastoid cell lines from MDV induced lymphomas (Powell et al, 1974; Kato and Aikyama, 1975; Nazerian et al, 1977; Calnek et al, 1978; Hahn et al, 1978). All the cell lines are T cell in origin, have T cell antigens and have the Marek's disease associated tumour specific antigen (MATSA; Witter et al, 1975). One cell line, MDCC-RP1 (old nomenclature: RPL-1; Nazerian et al, 1977) does not produce virus. RP1 cannot be induced to produce virus yet it carries the MDV genome (Nazerian and Payne, 1978). This cell line was isolated from a transplantable MD lymphoma (JMV-1; Sevoian et al, 1964) and the lymphoma itself is a nonproducer of virus (Stephens et al, 1976). RP1 carries the $B^1$ allele of the chicken MHC and was probably derived from a line S chicken (Longenecker et al, 1977a). Birds receiving a transplant of RP1 show the same pattern of metastasis as birds who have been naturally infected with MDV and have developed lymphomas (Longenecker and Gallatin, 1978).

# II. Materials and Methods

## 1. Cells

The MDV transformed, non-producer, continuous cell line MDCC-RP1 was kindly supplied by Dr. K. Nazerian (Regional Poultry Research Laboratory, East Lansing, Michigan) and was cultivated in this laboratory for over two years in RPMI-1640 (Gibco, Calgary, Alberta) supplemented with 20% fetal calf serum (FCS; Gibco) and antibiotics (penicillin/streptomycin, 50 units/ml each or gentamicin, 0.5 mg/ml; Microbiological Associates, Walkersville, Maryland). Later, it was discovered that better cell growth could be obtained in a serum mix of 5% FCS and 5% chicken serum (Gibco), which became the standard culture conditions for chicken cells lines in this laboratory. As much as possible, antibiotics were omitted during the course of the experiments reported here. The chicken cell lines MDCC-RP1, MDCC-AL1, MDCC-AL2, MDCC-AL3, were split twice a week at a dilution of 1:30, each subcultivation denoted as one *in vitro* passage. The cells normally have a doubling time of 10-12 hours in log phase culture which can occur for the first three days of culture. Therefore, 6-8 generations of cells can be said to be produced per passage.

The myeloma cell line 315.43 was obtained from Dr. T.R. Mosmann (Dept. of Immunology, University

of Alberta). This line was maintained in RPMI-1640 supplemented with 10% FCS. Hybridoma cell lines were similiarly maintained once established.

2. Eggs

Random bred (RB), fertile hen's eggs were supplied by the Poultry Division, Dept. of Animal Science, University of Alberta. Line SC, fertile hen's eggs were obtained from Hyline International, Dallas Center, Iowa. Lines N, P, S, and 15, fertile hen's eggs were supplied by the Biosciences Animal Center, University of Alberta. Eggs were incubated in a Robbins Hatchomatic Incubator (constant temperature, humidity, periodic rotation; Robbins Incubator Co., Denver, Colorado).

3. Selection for Virulence

The transplantable cell line MDCC-RP1 was injected intraperitoneally into newly hatched, line N chicks at a dose of $10^5$ cells per bird. Ten to 14 days later, moribund birds were sacrificed and their spleens removed. A cell suspension of each spleen was made by mincing the tissue with scissors and repeated pipetting. The cells were examined microscopically for viability and resemblance to RP1. The original cell line RP1 has a typical lymphoblastoid morphology with prominent nucleoli

A dose of $10^5$ RP1-like cells was administered intraperitoneally to a new group of line N chicks. After the fifth transplantation, the dosage to transfer the tumour was reduced to $10^4$ cells per bird. The spleens from the tenth passage birds were cultivated *in vitro* in Hahn's media (Hahn *et al*, 1978) and, after sufficient growth of one of the cultures, it was designated MDCC-AL1 according to the new nomenclature convention for avian cell lines (Report of the *Ad hoc* Commitee on Avian Cell Line and Transplantable Tumour Nomenclature, 1980).

4. *In vivo* Mortality

Day old, line SC chicks were inoculated intraperitoneally with tumour variant cells MDCC-RP1 or AL1. Mortality was monitored daily with the surviving birds terminated after 30 days.

5. Selection for Preferential Organ Metastasis

Line SC chicks were injected intraperitoneally with $10^4$ MDCC-AL1 tumour cells. Moribund birds were sacrificed 10-14 days post-inoculation and examined for metastases. Individual metastatic lesions were dissected free from the surrounding normal tissue and cultivated *in vitro* in Hahn' media (Hahn *et al*, 1978). When a sufficient number of cells had grown up in a culture, $10^4$ of these

cells per bird were injected as before into a new group of chicks. After five such transplantations, two new variant cell lines were isolated *in vitro*, MDCC-AL2, passaged five times through the liver, and MDCC-AL3, passaged five times through the ovary.

6. *In vivo* Metastasis

Day old, line SC chicks were inoculated intraperitoneally with tumour variant cells MDCC-AL2 or AL3. Ten days later, the birds were sacrificed and examined for metastases.

7. Embryonic Liver Focus Forming Assay (EL-FFA)

Fertile hen's eggs were incubated for 11 days and candled to locate a vein in the chorioallantoic membrane (CAM). A window was sawed in the shell and the cell suspension was injected intravenously into the chick embryo. Six days later, after further incubation, the eggs were opened and the embryonic livers harvested into Bouin's solution. After a few days fixation, white foci on the surface of the yellow livers were counted.

8. Chorioallantoic Membrane Focus Forming Assay (CAM-FFA)

This assay was performed as for the EL-FFA but the CAM's are harvested into saline and stored at $4^{\circ}C$.

9. Pro[redacted]f Hybridoma Cell Lines

For the generation of anti-tumour monoclonal ant[redacted]odie[redacted] five CBA/J mice were injected intravenously with $10^6$ [redacted]DCC-AL2 cells each. Three days later, the spleen cells[redacted]n the mice were pooled and fused with 315.43 cells as pr[redacted]ously described (Longenecker et al, 1979). The fusion [redacted]atio was 10 spleen cells to one myeloma cell. Cells were pl[redacted]ed at a density of $10^5$ myeloma cells/ml in flat bottome[redacted]microculture plates (Flow Laboratories Inc., Mississa[redacted]a, Ontario) with $10^7$ mouse red blood cells per ml as feeders. Clones appearing within three weeks of fusion we[redacted]tested for agglutination activity in their supernat[redacted]s against AL2 cells. Clones with positively reacting supernatants were grown for further analysis.

10. Agglutination Assay

Diluent used for tumour cells in this assay was RPMI-1640 with 1% FCS and 10mM Hepes buffer (Flow Labs.). For other cell types, phosphate buffered saline was used. The pH of the diluent was adjusted to 7.4 before use. Mid-log phase indicator tumour cells were washed once in diluent and adjusted to the appropriate concentration. Chicken white blood cells (CWBC) were harvested from adult birds by collecting blood from the wing vein and separating the red and white blood cells by low speed (700g) centri-

fugation. The resultant CWBC rich plasma was washed three times in diluent before use. Chicken red blood cells were harvested as for CWBC and washed three times before used.

Assays were performed in V-bottomed microtiter plates (Flow Labs.), usually combining 100 $\mu$l of indicator cells with 100 $\mu$l of test reagent. Direct agglutination assays were done using 5 x $10^5$ cells/test, incubating at 4° C for 60 minutes before the results were scored. For indirect assays, cells from the direct tests were washed three times in diluent and 100 $\mu$l of a 1:400 dilution of goat anti-mouse immunoglobulin antiserum (Cappel Laboratories Inc., Cochranville, Pennsylvania) in diluent was added. After incubation for 90 minutes at 4° C, the results were scored.

11. Antibody Directed Rosette Forming Cell Assay (ADRA)

Tumour cells were obtained as for the agglutination assays. The ADRA was performed as described by Mosmann et al (1980) and in a similiar manner to the agglutination assays but 1 x $10^5$ cells/test were used. Briefly, indicator cells were incubated for 60 minutes at 4° C with the test reagent. After washing three times with diluent, CRBC's with goat anti-mouse immunoglobulin antiserum covalently coupled to their surfaces were added at a ratio of 10 CRBC's to one tumour cell. After incubating at 4° C

for 180 minutes, rosette forming cells were counted microscopically. Specific rosette forming cells were calculated by subtracting background rosettes formed in the presence of diluent alone. Typical spontaneous rosette values vary from day to day and range from 1-5%.

12. Complement Mediated Cytotoxicity Assay (CMCA)

Tumour cells were obtained as indicator cells as for the agglutination assays. The CMCA was accomplished in a manner similiar to the agglutination assays but 1 x $10^5$ cells/test were used. Indicator cells were incubated for 60 minutes at $4^\circ$ C with the test reagent and washed three times in diluent before adding the previously determined appropriate dilution of guinea pig complement for 45 minutes at $37^\circ$ C. Indicator cells were then washed once in diluent and viability determined by trypan blue dye exclusion. Specific cytotoxicity was calculated by subtracting cytotoxicity due to complement alone. Typical spontaneous lysis values vary from batch to batch of complement and range from 10-20%.

13. Antibody Mediated Inhibition of the Embryonic Focus Forming Assays

Tumour cells were obtained as for the agglutination assays. Prior to injection into the eggs, cells

were left untreated or incubated with monoclonal antibodies for 30 minutes at $4^{\circ}$ C. For complement dependent antibody mediated inhibition of the focus forming assays, tumour cells were treated as for the complement mediated cytotoxicity assay.

14. Growth Inhibition Assay

For growth inhibition assays, mid-log phase tumour cells were seeded of $2 \times 10^5$ cells/ml in microculture plates (Flow Labs.). Growth was considered to be inhibited at the drug concentration tested by the failure of the cells to undergo more than one doubling in 48 hours.

15. Statistical Tests

To compare means, a simple t test was used. Linear regression analysis was performed by the method of least squares using, in some cases, the University of Alberta, Computer Services program. Analysis of variance was performed using the Computer Services program.

## III. Results

### 1. The Effect on Tumour Virulence of Sequential Allotransplantation

The MDV transformed, transplantable cell line MDCC-RP1 was serially passaged in newly hatched chicks in order to increase its virulence. After the tenth serial passage of splenic brei from tumourous spleens, the $LD_{50}$ of the cell line was reduced to $2 \times 10^3$ cells from approximately $3.6 \times 10^4$ cells in the original RP1 line (Figure 1). This new cell line, called MDCC-AL1, is more rapidly lethal than RP1, with the first mortality observable at 9 days rather than 12 days post-transplantation in the original RP1 line. In addition, there was an increase in the type and number of metastatic lesions with increased in vivo passage. Splenomegaly and numerous metastatic lesions in the ovary, liver, kidney and skeletal muscle were observed.

### 2. The Selection of Metastatic Variants by Sequential Allotransplantation

Having obtained a highly virulent, metastatic tumour cell line, selection for organ specific metastasis was attempted. In contrast to the preceding selection for virulence, a period of cell culture was interceded between each in vivo selection. This allowed the rapidly

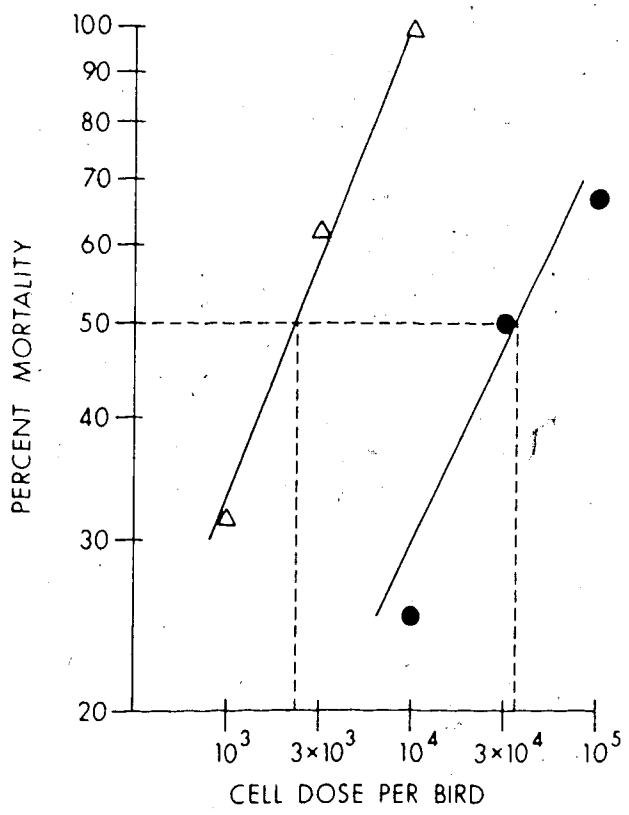


Figure 1. Mortality Due to Tumour Transplants.

Cells of tumour variant MDCC-RP1 (●) or AL1 (△) were injected intraperitoneally into day old, line SC, white leghorn chicks. Mortality was monitored daily and the surviving chicks terminated after 30 days. The $LD_{50}$ for RP1 was calculated to be $3.6 \times 10^4$ cells per bird and for AL1 it was $2 \times 10^3$ cells per bird.

growing tumour variant cells to outgrow the normal cells which were transfered to culture along with the metastatic lesion. As shown above, transplantation alone will increase the virulence and metastasizability of this cell lineage. In order to be assured that organ specific metastasis was being obtained by selection, two variants were derived after five selections in parallel for metastasis to their respective organs. Inoculation of tumcur variant cells MDCC-AL2, which was selected for liver metastasis, led to a higher frequency of birds with liver lesions than those birds that were inoculated with MDCC-AL3 ($p<0.05$), which was selected for ovary metastasis (Figure 2). In contrast, both AL2 and AL3 induce similiar frequencies of ovarian lesions at the cell dosages tested. Thus, selection for increased ovarian metastasis was unsuccessful and selection for increased liver metastasis was successful despite the fact that less than a two fold increase in the frquency of birds with liver lesions was observed.

3. Embryonic Liver Focus Forming Assay: A Quantitative Assay for Liver Metastasis

Further selections for liver metastasis would not be expected to increase appreciably the frequency of birds with liver lesions because of the high proportion of birds with these lesions, yet a frequency analysis appeared to underestimate the differential capacities

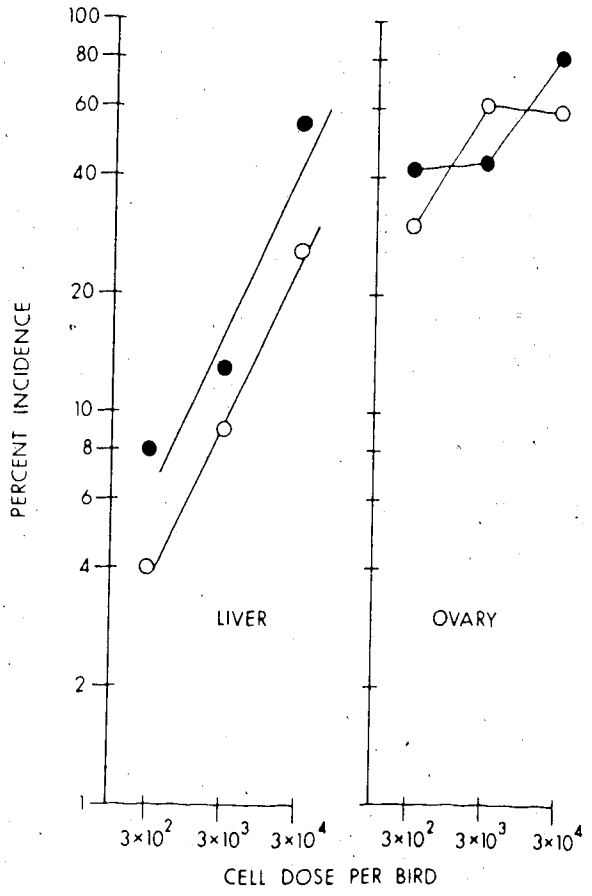


Figure 2. Metastasis of Organ Selected Tumour Variants.

Cells of tumour variant MDCC-AL2 (●), selected for liver metastasis, and AL3, (Δ) selected for ovary metastasis, were injected intraperitoneally into day old, line SC chicks. Ten days later, the birds were sacrificed and examined for gross metastases. The left hand panel shows that AL2 produces significantly more liver metastases than AL3 ($p<0.05$, calculated from linear regression lines). The right hand panel shows that AL2 and AL3 do not differ in their abilities to produce ovary metastases.

of AL2 and AL3 cells to produce focal lesions in the liver. To attempt to develop a quantitative assay for liver metastasis, AL2 cells were injected intravenously into 11 day old chick embryos and the embryonic livers were harvested six days later. Following fixation of these livers in Bouin's solution, foci on the livers became prominant and easy to count. The slope of the dose response curve is 1.05, which does not differ significantly from the ideal slope of 1.00 for such assays (Figure 3). These liver foci are comprised of cells which are morphologically identical to AL2 cells. Furthermore, preirradiation of AL2 cells with 1500 rads completely inhibited the formation of liver foci indicating that liver foci depend on the proliferation of AL2 cells (data not ahown).

This new assay was used to compare the capacities of RP1, AL1, AL2 and AL3 cells to induce liver foci in different types of embryos. AL3 cells are the appropriate control cells for transplantation effects when compared to AL2 cells (Table I). The liver selected variant, AL2, forms over five times as many liver foci as AL3 or AL1 ($p<0.001$). This difference was consistently maintained despite the genotype of the host embryo used for the assay. The original RP1 cell line did not induce liver foci at the cell dosage tested.

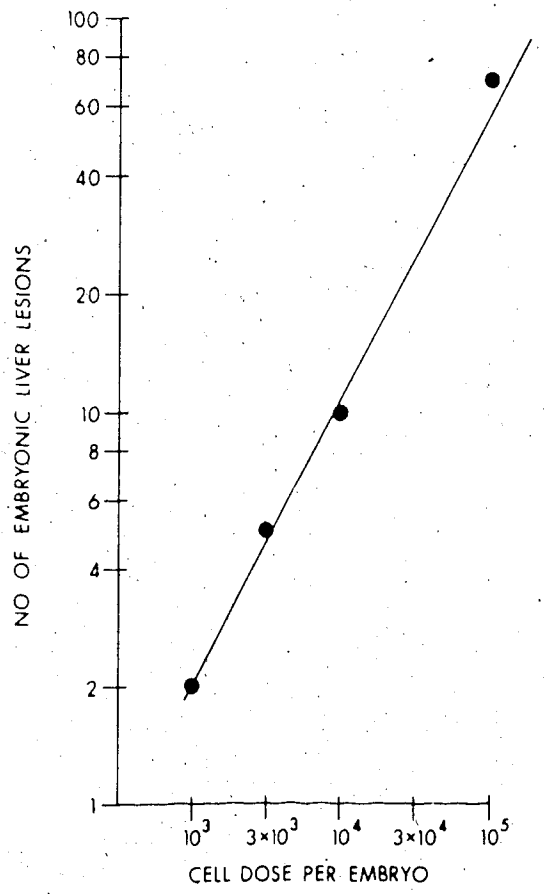


Figure 3. Cell Dose Dependent Formation of Embryonic Liver Foci

Cells of the liver metastasis selected tumour variant MDCC-AL2 were injected into the CAM veins of 11 day old, random bred, chick embryos. Six days later, the embryonic livers were harvested and fixed in Bouin's solution. The dose response of liver focus formation on the number of cells injected conforms to a linear regression with a slope of 1.05, not significantly different from a slope of 1.00.

Table I. The Effect of Selection for Liver Metastasis on the Capacity of a Lymphoma Cell Line to Form Embryonic Liver Foci.

| Tumour Variant | Embryo Type | | | | | | Relative Efficiency[2] |
|---|---|---|---|---|---|---|---|
| | N | SC | 15 | RB | P | S | |
| RP1 | - | - | - | 0±0(8)[1] | - | - | - |
| AL1 | - | - | - | 16±8(6) | - | - | - |
| AL2 | 67±35(3) | 71±7(2) | 84±33(5) | 70±29(8) | 67±31(4) | 71±7(2) | 5.24 |
| AL3 | 17±9(4) | 14±2(2) | 16±8(7) | 13±7(9) | 11±9(5) | 14±2(2) | 1.00 |
| p[3] < | 0.05 | 0.01 | 0.01 | 0.001 | 0.01 | 0.01 | 0.001 |

1) Day 11 chick embryos were injected with $10^5$ tumour cells via a CAM vein. Six days later, the embryonic livers were harvested into Bouin's solution. The mean ± the standard deviation of liver foci formed is reported with the number of embryos per group in parentheses.

2) arithmetic ratio

3) Compares AL2 with AL3 for each genotype and the relative efficiency.

4. The Formation of Proliferative Chorioallantoic Membrane Foci Correlates with the Increased Virulence of a Lymphoma Cell Line

Following intravenous injection of lymphoma cell lines, discrete foci form in the CAM. These foci may be of two types, virally induced or proliferative (Longenecker et al, 1975; 1977b). Productively infected cells may induce both types of foci while nonproductively infected cells induce only proliferative foci. Since proliferative foci represent colonies of dividing donor cells, they can be completely inhibited following lethal preirradiation of the lymphoma cells. The cell lines used in this study are nonproductively infected so it is no surprise that preirradiation with 1500 rads completely inhibited the cells' ability to induce CAM foci (data not shown). The capacities of the variant lymphoma cell lines to induce proliferative CAM foci were compared (Figure 4). None of the cell dose response curves differed significantly from the ideal slope of 1.00 and the slopes do not differ from each other. The calculated relative efficiencies of each variant to form CAM foci is RP1:AL1:AL2:AL3::1.00:1.61:3.25:4.07. These values are significantly different from one another at the $p<0.001$ level.

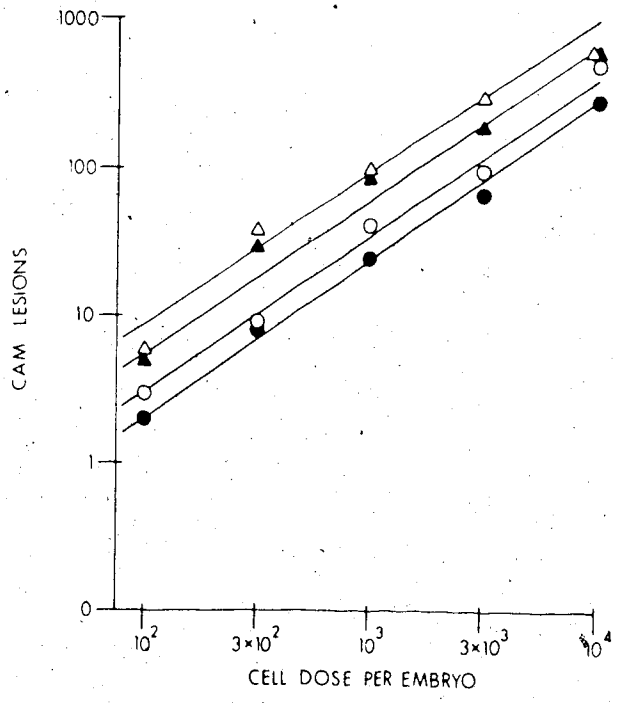


Figure 4. Cell Dose Dependent Formation of CAM Foci.

Cells of tumour variant MDCC-RP1 (●), the original tumour, AL1 (O), selected for virulence, AL2 (▲), selected for liver metastasis, AL3 (Δ), selected for ovary metastasis, were injected into the CAM veins of 11 day old, random bred, chick embryos. Six days later, the membranes were harvested and foci counted. In every case, the dose response of CAM focus formation on the number of cells injected conforms to a linear regression with slopes of RP1:AL1: AL2:AL3::1.03:1.14:1.01:1.04, none significantly different from a slope of 1.00. The relative efficiencies of focus formation of the tumour variants are RP1:AL1:AL2:AL3::1.00: 1.61:3.25:4.07, all significantly different from each other ($p<0.001$).

5. Genetic Variation in the Permissiveness to Chorioallantoic Membrane Focus Formation

Since chickens of various genetic backgrounds differ in susceptibility to MD, four lines of chick embryos were compared for their permissiveness to CAM focus formation induced by the variant cell lines of RP1 (Table II). As in the previous figure 4, the relative efficiencies of CAM focu formation by the variant tumour cell lines correlated well with the virulence of the cell line. Furthermore, an analysis of variance revealed significant variation due to the genetic background of the embryo in the numbers of CAM foci formed by RP1 and AL1 cells. Line N is the least permissive while RB, a random bred population, is the most permissive to CAM focus formation by these two cell lines. The comparison of lines N and P is the most interesting and valid since these lines were derived from the same flock. Line N was selected for resistance to MD while line P was selected for susceptibility to MD (Cole et al, 1968). Fewer foci were formed in line N embryos than in line P embryos by RP1 or AL1 cells (p 0.001). Metastatic variant cell lines, AL2 and AL3, however, do not differ in their ability to form CAM foci in the embryos tested. This may indicate that highly virulent cell lines, as evidenced for AL2 and AL3 by their high relative efficiencies of CAM focus formation, may overcome genetic mechanisms of resistance.

II. Genetic Variation in Permissiveness to CAM Focus Formation

| | Embryo Type | | | | Relative Efficiency[2] |
|---|---|---|---|---|---|
| | N | SC | P | RB | |
| RP1 | 28±14(7)[1] | 39±18(5) | 51±14(8) | 75±27(9) | 1.00 |
| AL1 | 50±18(8) | 67±12(5) | 64±37(8) | 76±25(10) | 1.44 |
| AL2 | 128±22(7) | 122±10(4) | 132±34(7) | 178±43(10) | 3.13 |
| AL3 | 157±53(7) | 143±19(4) | 177±54(9) | 130±33(8) | 3.34 |
| Relative Permissiveness[2] (a) | 1.00[3,4] | 1.39 | 1.55[3] | 2.10 | |
| (b) | 1.00 | 0.93 | 1.08 | 1.10 | |

1) Day 11 chick embryos were injected with $3 \times 10^3$ tumour cells via a CAM vein. Six days later, the membranes were harvested into normal saline. The mean ± standard deviation is given with the number of embryos per group in parentheses.

2) arithmetic ratio (a) RP1 and AL1 pooled (b) AL2 and AL3 pooled

3) $p<0.001$

4) $p<0.001$

## 6. Antigenic Analysis of Virulent and Metastatic Variant Lymphoma Cell Lines with Monoclonal Antibodies

Hybrid clones appearing within three weeks of fusion of AL2 immunized spleen cells with 315.43 myeloma cells were screened for direct and indirect agglutination activity in their supernatants against AL2 cells. Positive clones were picked and grown for further analysis. Sixteen clones proved to be stable antibody producers. Table III lists the agglutination reactivity of antibodies from these clones against a panel of variant tumour cells (RP1, AL1, AL2, AL3), another MDV transformed cell line MDCC-MSB1 (Kato and Aikyama, 1974) and normal chicken blood cells from seven different MHC (B allele) genotypes of birds. Antibody from one clone, 1.3, is reactive with all cells tested.

RP1 and its variants AL1, AL2 and AL3 express the chicken MHC $B^1$ antigen on their surfaces (Longenecker et al, 1977a; data not shown). The progenitor transplantable lymphoma to the variants, JMV-1 (Sevoian et al, 1964), was probably derived from a line S chicken (Longenecker et al, 1977a). Line S has a high frquency of the $B^1$ allele (Pazderka et al, 1975). Thus, line S birds were typed for the presence of the $B^1$ allele and positive birds were used as sources of $B^1$ cells. The other B allele genotypes used in this study are maintained as homozygous breeding flocks. It is possible, therefore, to identify 10 of the 16 clones as producers of anti-B locus or another polymorphic system

Table III. Reactivity of Monoclonal Antibodies to Malignant and Normal Cells.

| Target Cell | Monoclonal Antibody[1] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1.1 | 1.3 | 1.5 | 1.7 | 1.8 | 1.9 | 1.11 | 1.14 | 1.15 | 1.17 | 1.18 | 1.20 | 1.21 | 1.24 | 1.26 | 1.27 |
| MDCC-RP1 | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + |
| Al1 | + | + | + | + | + | + | + | + | - | + | + | - | + | + | + | + |
| AL2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| AL3 | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + |
| MDCC-MSB1 | - | + | + | + | - | + | + | + | - | + | + | - | - | + | + | + |
| CRBC | - | a | b | - | - | - | - | - | - | - | - | - | - | - | c | - |
| CWBC | - | a | d | - | - | a | e | d | - | f | d | - | d | e | d | d |

1) reactivity indicated: (+) positive; (-) negative; (a) reacts with B alleles 1, 2, 13, 14, 15, 19, 21; (b) 1, 2, 14, 15, 19; (c) 1, 13, 14; (d) 1; (e) 1, 14; (f) 1, 2, 14.

antibodies (1.5, 1.9, 1.11, 1.14, 1.17, 1.18, 1.21, 1.24, 1.26, 1.27). Four of these react exclusively with CWBC from line S chickens (1.14, 1.18, 1.21, 1.27) and may be monospecific for $B^1$ on CWBC. Two antibodies (1.5, 1.26) cross-react with various genotypes of CRBC. The remaining four putative anti-B locus antibodies (1.9, 1.11, 1.17, 1.24) react with more than one B allele type on CWBC.

The target cell for MDV transformation is the T lymphocyte (Payne, 1972). RP1 has been shown to carry chicken T cell antigens (Nazerian et al, 1977). Therefore, the appropriate control to demonstrate tumour-associated antigens is the T lympgocyte. None of our monoclonal antibodies react with thymus cells in a fashion not explainable by cross reactivity with B locus or other lymphocyte antigens (data not shown). The source of thymus, bursa and spleen cells used in this experiment was a $B^2/B^2$ chicken. Once again an agglutination assay was used. The antibodies reacting with $B^2$ thymus cells (1.3, 1.24) also react with other genotypes of cells. None of the monoclonal antibodies save those from clone 1.3 reacted with bursa or spleen cells.

The remaining five of the 16 antibody producing clones react with MDV transformed cell lines but not with any normal blood cell tested. Clone 1.7 antibodies react with both RP1 and its variants and MSB1. Four antibody producing clones are specific for RP1 and its variants. These show various patterns of reactivity. Antibodies from clones 1.1 and 1.8 react with all the variants. Clone 1.15

antibodies do not react in an agglutination assay with AL1, selected for virulence, but do react with AL2 and AL3, both more virulent than AL1. In the more sensitive CMCA, clone 1.15 antibodies kill approximately 20-20% of all of the RP1 variants. This indicates that AL1 expresses the antigen in question equally well as the other variants. AL1 cells may therefore be more difficult to agglutinate.

Clone 1.20 antibodies have the most interesting pattern of reactivity. These antibofies detect in agglutination assays an antigen present on AL2 cells exclusively. In the more sensitive CMCA, approximately 20-25% of AL2 cells are killed by clone 1.20 antibodies. Figure 5 shows the reactivity of clone 1.20 ascites antibodies in a CMCA against AL2 and AL3 cells. As cell lines AL2 and AL3 were selected in parallel for liver and ovary metastasis respecitively, they are the appropriate controls for each other. It is seen that while AL2 cells have a peak reactivity with the clone 1.20 antibodies of 25% specific cytotoxicity, the AL3 cells are not killed above a level of 6%, a marginally positive reaction. Thus, there may be some cells in the AL3 culture which react with the 1.20 antibodies. Similiarly, RP1 and AL1 exhibit a specific lysis that is lower than that of AL3, usually about 3% (data not shown). The antigen defined by the clone 1.20 antibodies is named the liver metastasis associated antigen.

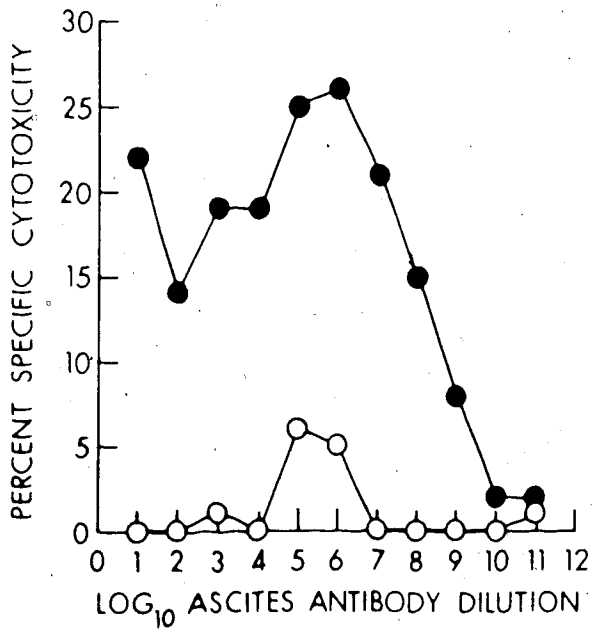


Figure 5. Complement Mediated Cytotoxicity Assay Titration of Anti-Liver Specific Metastasis Associated Antigen Monoclonal Antibodies.

MDCC-AL2 (●) or AL3 (○) cells were incubated with various dilutions of 1.20 ascites antibody for 60 minutes at 4° C then at 37° C for 45 minutes with guinea pig complement. Cytotoxicity is plotted as an average of duplicate tests expressed as a percentage. Spontaneous lysis subtracted.

## 7. Clonal Variation in Liver Specific Metastasis

Fidler and others (Fidler, 1978; Dexter et al, 1978; Kripkie et al, 1978; Suzuki et al, 1978) have shown that tumour cell lines are composed of subpopulations which vary in their ability to invade and metastasize. Clones derived from invasive and metastatic cell lines have a characteristic expression of these properties which appears to be random. Nowell (1976) has postulated that variant clones succeed each other in a tumour cell population, each variant with ever increasing malignant properties, to bring about tumour progression. Such clones as shown by Fidler and others above may represent samples of these variant subpopulations. By limiting dilution, the metastatic variant cell line AL2 and AL3 were cloned and tested in the EL-FFA and the CAM-FFA. It is seen in Table IV that the clones have a randomly differing capacity to metastasize to the liver while the ability of the clones to invade the CAM is similiar. The values obtained in the EL-FFA and the CAM-FFA do not correlate ($r=0.20$, $p>0.20$), indicating that liver specific metastasis and invasion of the CAM are independent events. The values obtained in the CAM-FFA show that the clones have as high a level of invasion of the CAM as their parental lines.

Previously, a five fold difference in liver focus forming ability of AL2 over AL3 was observed (Table I). In the present experiments (Table IV), this difference

Table IV. Clonal Variation in Liver Specific Metastasis.

| Variant Clone | CAM-FFA[1] | EL-FFA[2] Expt. 1 | EL-FFA[2] Expt. 2 |
|---|---|---|---|
| AL2 | 218±72(8) | 116±28(10)[3] | 67±20(8)[5] |
| AL2.1 | 242±105(7) | 70±23(9) | 56±17(9) |
| AL2.2 | 212±87(9) | 44±9(10) | 29±10(9) |
| AL2.3 | 216±64(9) | 87±16(10) | 51±32(7) |
| AL2.4 | 256±61(6) | 131±39(10) | 77±27(8) |
| AL2.5 | 323±136(8) | 180±39(9) | 147±45(8) |
| AL2.6 | 201±72(9) | 220±50(10) | 122±18(9) |
| AL3 | 216±235(8) | 52±13(10)[3] | 38±17(10)[5] |
| AL3.1 | 225±91(7) | 117±40(9) | 92±32(9) |
| AL3.2 | 294±68(8) | 69±28(9) | 64±27(7) |
| AL3.3 | 264±116(8) | n.d.[4] | 41±19(8) |
| AL3.4 | 207±92(10) | n.d. | 66±21(8) |

1) CAM foci reported as mean ± standard deviation with the number of embryos per group in parentheses. $3 \times 10^3$ cells per embryo were injected.

2) Liver foci reported as mean ± standard deviation with the number of embryos per group in paretheses. $10^5$ cells per embryo were injected.

3) $p<0.001$

4) n.d. = not done.

5) $p<0.005$

is reduced to two fold but is still significant. This reduction in discrimination of the EL-FFA could be due to the possibility that the cell lines are changing in culture. It has been known for some time that cultured cell lines loose virulence with time (Earle et al, 1950). When the five fold difference in liver focus forming ability between AL2 and AL3 was demonstrated, the cells were in their 18th passage in vitro. In the present experiments, the cells were in their 28th to 33rd in vitro passages. The limiting dilution clones were produced at passage 10 and tested at similar total in vitro passages as AL2 and AL3. To test if the cell lines had indeed changed during their period in culture, various passage levels of AL2 and AL3 were tested after recovery from storage in liquid nitrogen for their liver focus forming ability. Figure 6 shows that there is a dramatic increase in the liver focus forming ability of AL3 while there is only a slight decrease in the ability of AL2 to form liver foci. A 100 fold difference in liver focus forming ability of AL2 verses AL3 at passages 5-10 becomes two fold at passages 25-30. In this assay, the EL-FFA was performed with a relatively large number of cells to allow maximum precision of the assay on cell lines which may have shown low levels of liver focus forming ability. The increase in liver focus forming ability of AL3 may also explain the observation that all the clones derived from AL3 are equal to or greater than AL3

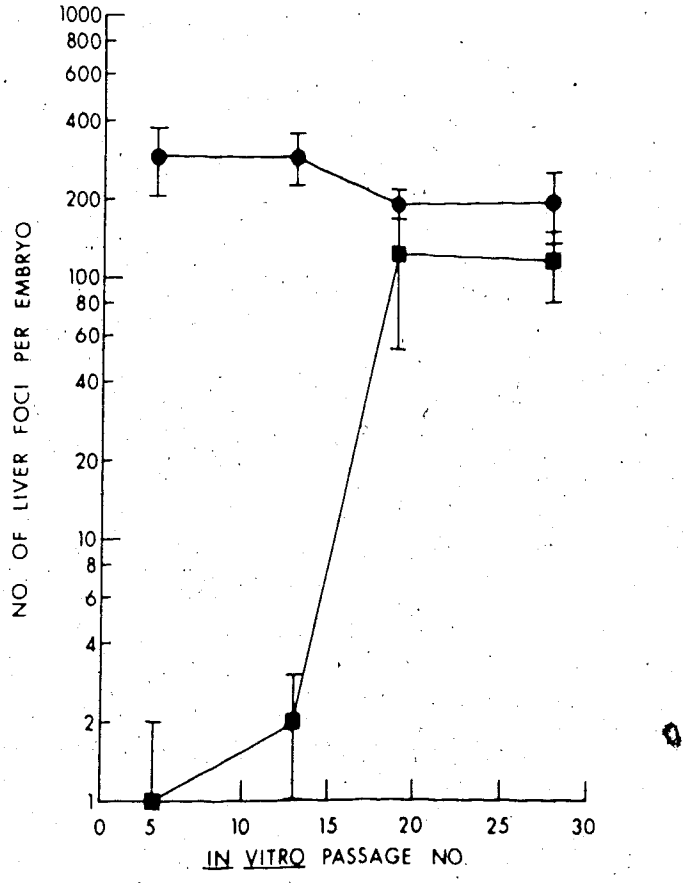


Figure 6. Variation of Liver Metastasis with In Vitro Culture.

Various in vitro passage levels of metastatic cell lines MDCC-AL2 (●) and AL3 (O) were tested in the EL-FFA at a relatively high cell dose of $5 \times 10^5$ cells per embryo in order that passages with a low capacity to metastasize to the liver could be measured.

in their liver focus forming ability. The clones were produced at a point where the in vitro changes in metastatic ability of AL3 were possibly underway. Given the doubling times and number of passages involved, it is possible for a cell to give rise to a dominant population in the AL3 culture. Starting at a ratio of $10^6$ marginal liver metastatic cells to one high metastatic cell at passage 5 and postulating a two hour difference in doubling time favouring the high metastatic cell, by passage 20 the ratio is reversed to become one marginally liver metastatic cell to $10^3$ high liver metastatic cells.

8. Clonal Variation in the Expression of a Liver Specific Metastasis Associated Antigen

As mentioned above, random variation in invasive and malignant properties can be an explanation for tumour progression. It has been shown that tumours in situ may vary in their antigenicity (Prehn, 1970). The cloned metastatic variant cell lines were tested with anti-LMAA monoclonal antibodies in two types of assays, the CMCA and the ADRA. From Table V it is seen that there is clonal variation in the expression of the LMAA. The ADRA is more sensitive that the CMCA by a factor of nearly two in assays done on the same day (CMCA and ADRA #1). The assays correlate to a high degree ($r=0.85$, $p<0.001$) indicating that the assays are detecting the same parameter.

Table V. Clonal Variation in the Expression of a Liver Specific Metastasis Associated Antigen.

| Variant Clone | CMCA[1] | ADRA[2] Expt. 1 | ADRA[2] Expt. 2 |
|---|---|---|---|
| AL2 | 21 | 33 | 17 |
| AL2.1 | 13 | 19 | 9 |
| AL2.2 | 34 | 63 | 20 |
| AL2.3 | 20 | 54 | 8 |
| AL2.4 | 30 | 42 | 21 |
| AL2.5 | 34 | 48 | 34 |
| AL2.6 | 21 | 76 | 40 |
| AL3 | 1 | 3 | 2 |
| AL3.1 | 13 | 41 | 38 |
| AL3.2 | 2 | 1 | 4 |
| AL3.3 | 1 | 7 | 3 |
| AL3.4 | 0 | 10 | 15 |

1) Complement mediated cytotoxicity assay expressed as an average of duplicate tests. Spontaneous lysis subtracted.

2) Antibody directed rosette forming cell assay expressed as an average of duplicate tests. Spontaneous rosettes subtracted.

Successive testing of the clones at *in vitro* passages 27 and 32 shows a decrease in reactivity to the anti-LMAA antibodies of the clones, ADRA #1 being 1.9 times as sensitive as ADRA #2, but a high degree of correlation is again shown between the two experiments ($r=0.73$, $p<0.001$). This is interesting as the EL-FFA clonal variation tests reproted earlier (Table IV) showed the same loss of sensitivity from the 28th to the 33rd passage but these measurements remain highly correlated as well ($r=0.91$, $p<0.001$). Thus, eventhough the sensitivity of the ADRA and the EL-FFA may change from day to day, the reliability of the tests is good.

When the various passage levels of the tumour variant cell lines AL2 and AL3 are tested for anti-LMAA reactivity in the CMCA (Figure 7), the expression of the LMAA is seen to be relatively constant. If the LMAA is a marker for metastasis, then the AL3 cell line may have acquired the capacity to metastasize to the liver independently of the LMAA. This is not unexpected as the selective forces on a cell population are very different *in vivo* as *in vitro*.

## 9. Correlation of Liver Specific Metastasis with a Liver Specific Metastasis Associated Antigen Expression

Clonal variation can be detected in metastatic ability and surface antigen expression (Tables IV and V).

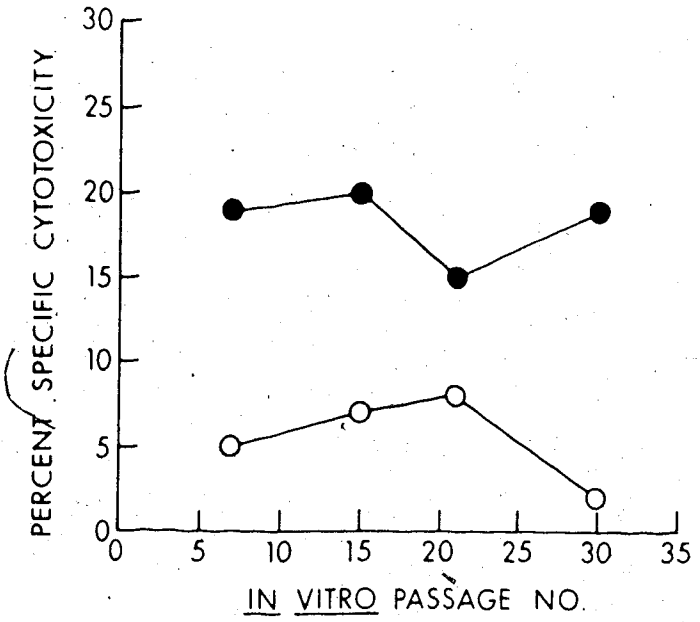


Figure 7. Variation of a Liver Specific Metastasis Associated Antigen Expression with In Vitro Culture

Various passage levels of metastatic variant cell lines MDCC-AL2 (●) and MDCC-AL3 (○) were tested in a CMCA for LMAA expression. Ascites antibody of clone 1.20 was used at a dilution of 1:100. Average of duplicate tests plotted.

The EL-FFA and the ADRA were done on cells one passage apart for each experiment and the two experiments were done five passages apart. Thus, it would be interesting to determine what correlation there is between metastatic ability and cell surface antigen expression of the clones in the two experiments. Figure 8 shows the liver foci produced by the clonal variants plotted as a function of LMAA expression. It is evident that these parameters are highly correlated. For experiment #1 (Figure 8A), the correlation coefficient is 0.58 ($p=0.033$) and for experiment #2, $r=0.80$ ($p<0.005$). Taken together, the correlation coefficient for the two experiments is 0.70 ($p<0.001$). CAM foci, on the other hand, do not correlate with LMAA expression ($r=0.008$, $p>0.20$). This demonstrates the specificity of association of liver specific metastasis with LMAA expression. The clones were derived at passage 10 before AL3 had acquired detectable levels of the higher metastatic phenotype. Therefore, late passage AL3 cells probably will not fit into the correlation of liver metastasis with LMAA expression. All the AL3 clones, however, have as high or higher liver metastasis than their parent. The high liver metastatic phenotype may have been selected for by the cloning procedure.

Metastatic variant clone AL3.1 which shows high liver metastasis and high LMAA expression, unlike its parent or sister clones, is important. In order to test whether this clone is a contaminant of AL3 by AL2,

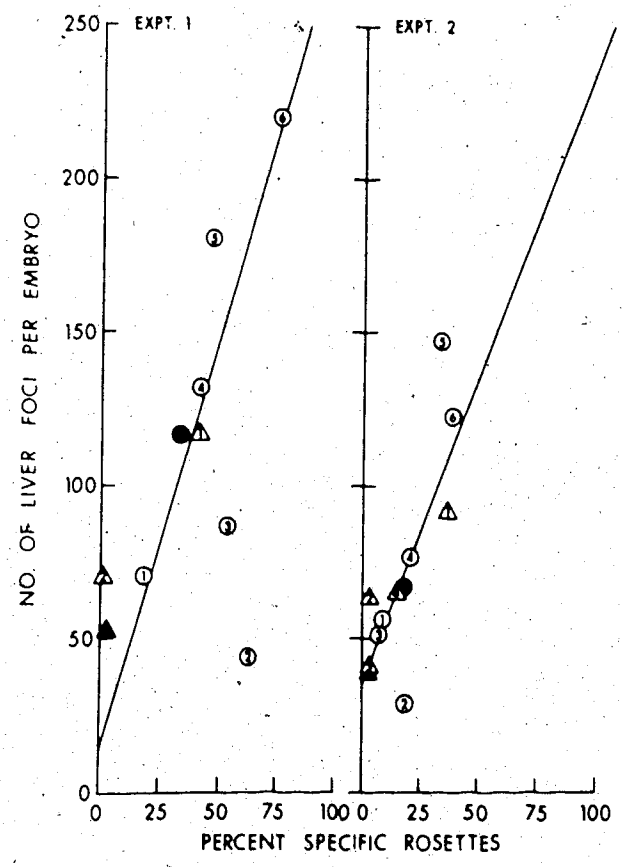


Figure 8. Correlation of Liver Specific Metastasis with the Expression of a Liver Specific Metastasis Associated Antigen.

The cell lines MDCC-AL2 (●) plus six limiting dilution clones (numbers in circles) and AL3 (▲) plus four clones (numbers in triangles) were tested for LMAA expression by the ADRA and for liver metastatic ability in the EL-FFA on two occasions. Experiment 1 is plotted in the left hand panel where a correlation coefficient of 0.58 ($p=0.033$) is obtained. Experiment 2 is plotted in the right hand panel where a correlation coefficient of 0.80 is obtained ($p<0.005$). Combination of these experiments results in a correlation coefficient of 0.70 ($p<0.001$).

a natural difference in drug sensitivities between AL2 and AL3 was exploited. AL2 is resistant to the drugs 6-thioguanine and 8-azaguanine up to a dose of 2.5 $\mu$g/ml of each in culture while AL3 is less resistant, up to a level of 0.6 $\mu$g/ml. All the clones including AL3.1 tested as for the parental type in a growth inhibition test (Table VI). Thus, the possibility that clone AL3.1 is a contaminant of AL3 by AL2 can be excluded. In another growth inhibition test on various passage levels of AL2 and AL3 (data not shown), it can be excluded that the dramatic change in metastatic character of AL3 between passages 10 and 20 is due to contamination of AL3 by AL2. Clone AL3.1 may represent a subpopulation of cells which reacts with anti-LMAA antibodies and gives rise to liver foci in the AL3 tumour cell line.

Originally, clone AL2.2 had high LMAA expression but low liver metastasis. On subsequent testing, it was observed that the LMAA expression decreased dramatically while the liver focus forming ability of the clone remained constant. The original LMAA measurement may have been spurious. This is borne out by the CAM-FFA results (Table IV) which show that AL2.2 falls within the expected range of values. Removing this point from the aggregate correlation calculation improves the coefficient to 0.82 ($p<0.001$).

Table VI. Drug Resistance of Metastatic Variant Clones to 6-Thioguanine and 8-Azaguanine.

| Variant Clone | Drug Concentration[1] 2.5 μg/ml | 0.6 μg/ml | 0.15 μg/ml |
|---|---|---|---|
| AL2 | - | + | + |
| AL2.2 | +/- | + | + |
| AL2.3 | - | + | + |
| AL2.4 | - | + | + |
| AL2.5 | - | + | + |
| AL2.6 | - | + | + |
| AL2.1 | - | + | + |
| AL3 | - | - | + |
| AL3.1 | - | - | + |
| AL3.2 | - | - | + |
| AL3.3 | - | - | + |
| AL3.4 | - | - | + |

1) Cells were cultured for 48 hours in drug containing media then scored for growth. (+) normal growth; (-) growth inhibited.

## 10. Functional Interaction of the Liver Specific Metastasis Associated Antigen and Liver Metastasis

In order to test whether the LMAA might be functionally associated with liver specific metastasis, the capacity of anti-LMAA monoclonal antibodies to inhibit liver foci in the EL-FFA produced by AL2 cells was tested. It was found that preincubation of AL2 cells with anti-LMAA antibodies (Table VII) specifically blocks the ability of AL2 cells to form liver foci while not affecting the ability of the same cells to produce CAM foci. In contrast, CH-4, a monoclonal antibody which detects a chicken MHC antigen not found on AL2 cells (Longenecker et al, 1979; data not shown), or 1,24 monoclonal antibodies which detect a polymorphic antigen found on AL2 cells (Table III), inhibited neither CAM nor liver foci. To test another monoclonal antibody binding to AL2 cells, the capacity of anti-LMAA antibodies to inhibit liver foci was compared with that of 1.5 monoclonal antibodies, similiar in specificity to 1.24 monoclonal antibodies. This test was done with or without the prior complement mediated killing of AL2 cells. As for the first test, equal numbers of viable cells were injected per embryo so the results in the second test underestimate the effects of the antibody by a factor of three. Anti-LMAA but not 1.5 monoclonal antibodies significantly inhibited the development of liver foci in the presence of complement (Table VIII). It is

Table VII. Specific Inhibition of Liver Metastasis by Monoclonal Antibody

| Monoclonal Antibody[1] | CAM-FFA[2] | EL-FFA[3] |
|---|---|---|
| - | 60±17(8) | 63±11(8) |
| CH-4 | 59±16(8) | 78±21(5) |
| 1.20 | 61±11(7) | 32±10(9) |
| 1.24 | 68±17(10) | 56±6(8) |

1) Prior to injection, cells were left untreated or incubated for 30 minutes with ascites antibodies from hybridomas CH-4, 1.20 or 1.24 at a final dilution of 1:100.

2) CAM foci expressed as mean ± standard deviation with the number of embryos per group in parentheses. $10^3$ cells were injected per embryo.

3) Liver foci expressed as mean ± standard deviation with the number of embryos per group in parentheses. $10^5$ cells were injected per embryo. Statistically significant differences were obtained in comparing results for 1.20 with control ($p<0.001$); 1.20 with CH-4 ($p<0.01$); and 1.20 with 1.24 ($p<0.001$).

Table VIII. Specific Inhibition of Liver Metastasis by Monoclonal Antibody plus Complement.

| Monoclonal Antibody[1] | CAM-FFA[2] | EL-FFA[3] |
|---|---|---|
| - | 70±51(5) | 88±14(6) |
| C' | 60±38(7) | 75±11(6) |
| 1.20 | 115±33(7) | 62±23(7) |
| 1.20 + C' | 93±15(7) | 34±12(8) |
| 1.5 | 184±58(7) | 76±11(8) |
| 1.5 + C' | 86±29(6) | 63±9(8) |

1) Prior to injection, cells were incubated at 37° C for 45 minutes with diluent or the culture supernatants (full strength) from hybridomas 1.20 or 1.5 in the presence or absence of guinea pig complement (C'). The cells were washed and the concentrations of viable cells adjusted to be equivalent.

2) CAM foci expressed as mean ± standard deviation with the number of embryos per group in parentheses. Statistically significant differences were obtained in comparing results for control or 1.5 + C' with 1.5 alone ($p<0.01$). $10^3$ cells per embryo were injected.

3) Liver foci reported as mean ± standard deviation with the number of embryos per group in parentheses. Statistically significant differences were obtained in comparing results from C' or 1.5 + C' with 1.20 + C' ($p<0.001$); 1.20 and 1.20 + C' ($p<0.02$); and control with 1.20 ($p<0.03$). $10^5$ cells per embryo were injected.

noteworthy that 1.5 antibodies, which directly agglutinate AL2 cells, caused a significant enhansement in the number of CAM foci produced by AL2 cells, suggesting that large emboli of tumour cells might be non-specifically trapped in the CAM. Under the same conditions, however, 1.5 antibodies did not cause a significant reduction in liver foci which is consistent with the previous observations. These results suggest that the homing or colonization of the liver by AL2 cells is a specific process and not just the non-specific trapping of tumour cell emboli. The fact that 1.5 monoclonal antibodies caused a slight, but not significant reduction in the number of liver foci suggests that at least a few of the liver focus forming cells may have been trapped within cell aggregates. As different classes of mouse antibody may affect the formation of liver foci differently, the class of antibody used has been standardized. All the monoclonal antibodies used in these studies are IgM as determined by electrophoresis in denaturing gels with known IgM antibodies as standards (data not shown).

To test if LMAA expression or liver metastatic ability could regenerate in culture over two days after treatment with anti-LMAA antibodies plus complement, the following experiment was performed on passage 35 cells. Metastatic variant cell lines AL2 and AL3 were either left untreated, treated with complement alone or treated with anti-LMAA antibody plus complement. The same pro-

cedure as for the CMCA was used. It is seen (Table IX) that only AL2 cells had a significant level of anti-LMAA specific cytotoxicity. The untreated cells, from which the treated cells were drawn, were tested for anti-LMAA ADRA before culture (Day 0). Both cell lines AL2 and AL3 tested within their normal range of values. All groups were tested after two days of culture in the ADRA, CAM-FFA and EL-FFA. On Day 2, the anti-LMAA values for AL2 were markedly depressed when the cells were treated on Day 0 with anti-LMAA antibody plus complement. Complement alone had no significant effect on the expression of the LMAA. Another antigen, detected by 1.24 monoclonal antibodies, was not affected by any of the treatments. Thus, the LMAA positive cells do not regenerate over two days in culture after removal by specific antibody plus complement. When tested in the EL-FFA, it is seen that liver specific metastasis is reduced only in the group of AL2 cells treated with anti-LMAA antibodies plus complement. This may indicate that the LMAA positive population of cells is identical to the population of cells that gives rise to liver metastases. The fact that not all of the liver metastases are removed strongly suggests that there is more than one mechanism by which AL2 cells may give rise to liver metastases and the LMAA is a functional marker for only one of these. The rise in liver metastatic ability of AL3 with no change in LMAA expression supports this hypothesis. Treatment of AL3

Table IX. Concommitant Inhibition of Liver Specific Metastasis and Liver Specific Metastasis Associated Antigen Positive Cells.

| Metastatic Variant | Treatment[1] | CMCA[2] | ADRA[2] Day 0 1.20 | ADRA[2] Day 0 1.24 | ADRA[2] Day 2 1.20 | ADRA[2] Day 2 1.24 | CAM-FFA(Day 2)[3] | EL-FFA(Day 2)[3] |
|---|---|---|---|---|---|---|---|---|
| AL2 | - | - | 18 | 99 | 23 | 98 | 366±132(9) | 34±16(9)[4] |
| | C' | - | - | - | 20 | 97 | >500(9) | 45±21(9)[5] |
| | 1.20 + C' | 35 | - | - | 1 | 99 | >500(9) | 18±13(9)[4,5] |
| AL3 | - | - | 6 | 99 | 1 | 99 | 299±119(10) | 10±4(10) |
| | C' | - | - | - | 1 | 98 | >500(9) | 19±14(9) |
| | 1.20 + C' | 0 | - | - | 1 | 99 | >500(9) | 9±4(9) |

1) Prior to culture, cells were treated with diluent or a 1:100 dilution of 1.20 ascites antibody at 4° C for 60 minutes, washed and incubated with or without guinea pig complement (C') as indicated for 45 minutes at 37° C.

2) Complement mediated cytotoxicity assay and antibody directed rosette forming cell assay expessed as a percentage. Spontaneous lysis or rosettes subtracted. Average of duplicate tests.

3) CAM foci and liver foci reported as mean ± standard deviation with the number of embryos per group in parentheses. $10^4$ cell per embryo were injected for the CAM-FFA and EL-FFA.

4) $p<0.03$

5) $p<0.005$

cells resulted in no significant change in any parameter tested. CAM foci showed an increase over the untreated group in all cases with AL2 and AL3 cells where complement was used. The CAM's had over 500 foci each which leads to significant crowding effects in the membrane, making accurate foci counts impossible. Indeed, this effect has been seen before when AL2 cells were injected with a monoclonal antibody that has excellent agglutination properties. Care was taken to inject a single cell suspension but micro-aggregates may have formed and been trapped in the highly vascular CAM. It has been observed on many occasions in the performance of the CMCA that guinea pig complement enhances the agglutination of monoclonal antibody treated cells. Also, there may be natural anti-chicken antibody in the guinea pig serum which was used as the complement source, which could enhance CAM foci. The important point here is, however, that the anti-LMAA monoclonal antibodies which decrease liver foci do not have an effect on CAM foci.

## IV. Discussion

In this thesis, the derivation of three new MD lymphoma cell lines from the parent MDCC-RP1 line is reported. RP1 was originally derived (Nazerian *et al*, 1977) from the *in vivo* transplantable JMV-1 MD lymphoma of Sevoian (Sevoian *et al*, 1964; Stephens *et al*, 1976) and it was adapted for growth *in vitro* as well as *in vivo*. Its *in vivo* virulence was reestablished by 10 serial passages in newly hatched chicks and this new cell line is called MDCC-AL1. AL1 is highly metastatic, forming the same number and type of lesions often seen during the evolution of the natural disease following exposure to MDV. Since ovarian and liver lesions are the most common forms of metastatic lesion in MD (Payne, 1972), metastatic variants with increased propensity for metastasis to these organs were sought by selection.

After only five selections for organ specific metastasis, two cell lines, MDCC-AL2, selected for liver preference, and MDCC-AL3, selected for ovary preference, were derived. Selection for ovarian preference did not appear to be successful since both AL2 and AL3 produce the same frequency of ovarian tumours following injection into newly hatched chicks. This could be due to the fact that the ovary is an immunologically privileged site (Cock, 1962; Barker and Billingham, 1977). If the immune response is important in metastasis, then selection for specific

metastasis to a privileged site may be unsuccessful as there would be no strong selective pressure in favour of specific tumour variants. A further hindrance would be in a situation, like the MD system, where a high frequency of metastasis to the privileged site is observed prior to selection. There is no quantitative assay for ovary specific metastasis in the embryo, itself relatively immunologically inert, so it cannot be tested in ovo whether selection for ovarian preference has been successful. On the other hand, selection for liver preference was successful and a sensitive and convenient assay for liver specific metastasis was developed using the chick embryo. Following intravenous injection of AL2 cells, liver foci which form on the outer surface of the liver are counted. The number of liver foci is directly proportional to the number of cells injected and the slope of the dose response curve did not differ from the ideal slope of 1.00. This indicates that each liver focus is derived from a single injected cell or preformed unit. The number of surface liver foci underestimates by at least an order of magnitude the total number of liver foci. This assay is similiar to that of Chevalier and Frindel (1972) for the mouse but differs in that the metastasizing properties of the injected tumour cells are not confounded by the immune response. These results strongly suggest that the same process or property which determines the capacity of tumour variants to metastasize to the mature liver has developed

by late in the second trimester of chick embryo development. At this stage, the major aspects of organogenesis are complete and the embryonic liver is a functional and metabolically active organ (Romanof, 1960). The fact that one can detect organ specific metastasis in the embryo, which is relatively immunologically inert, strongly argues that the immune response has little or no role in determining liver specific metastasis.

Recently, several investigators have made successful attempts to select organ specific metastatic variants of transplantable tumour cell lines. The most popular system for selection has been the B16 melanoma, a spontaneous tumour of mice. Using this tumour system, lung, brain and liver colonizing variants have been established (Fidler, 1973; Nicolson and Brunson, 1978; Tao et al, 1979). The best studied series of B16 variants are those selected for lung metastasis. Some controversy exists, however, as to the specificity of these lung variants since recent results of Tao et al (1979) are at variance with those of Fidler and Nicolson (1977). Fidler and Nicolson (1977) reported an increased number of lung tumours with increased number of selections and that the number of tumour nodules formed did not depend on the route of injection. Tao et al (1979), by contrast, found a route of injection dependence of the lung selected cell line but did report the successful selection of a liver colonizing variant of the B16 melanoma which showed preferenee

for growth in the liver regardless of the route of injection. The lines selected for brain metastasis reported by Nicolson and Brunson (1978) further show that other organ selected variants of the B16 melanoma can be obtained. The results presented here show that herpesvirus induced lymphoma cells can be selected to have preference for growth in the chicken liver. It is important to note that the cells which form liver foci in the chick embryo are not simply forming tumours where they encounter the first major capillary network. Cells which are injected into a CAM vein are carried back to the chick embryo heart and are immediately distributed to the general circulation of the embryo and extraembryonic membranes (Romanof, 1960). A large number of foci form in the CAM, an extraembryonic membrane which serves the same function for the embryo as the adult lung. It is possible that, like the mammalian lung, the chick embryo CAM simply sieves or nonspecifically traps injected tumour cells which grow and form CAM foci. Great care was taken to inject a single cell suspension of lymphoma cells, which grow in vitro as single cells, but it is possible that emboli of tumour cells form within the embryo following injection. Further studies will be required to clarify this issue but it is noteworthy that the relative efficiencies of CAM focus formation by the tumour variants is correlated with their virulence.

To demonstrate that a tumour is virulent, the tumour is required to grow and kill a host animal.

During this process, the properties of uncontrolled cell division, invasiveness and angiogenesis must occur to allow the tumour to be successful. The CAM-FFA may require some or all of these same properties of the tumour cells. Cells injected into the embryo, in order to form foci, must penetrate the vascular endothelium, invade the CAM mesoderm, proliferate and acquire a blood supply. Others have reported that highly metastatic tumour cells penetrate the CAM more readily than tumours of marginal metastatic potential (Easty and Easty, 1974; Hart and Fidler, 1978) and highly invasive tumour variants have been selected in a mouse bladder system (Poste et al, 1980). These assays are done in vitro and probably measure just the ability of the tumour cells to penetrate an epithelial surface. A major difference between these assays and the in ovo assay is that the CAM-FFA requires that the tumour cells illustrate the properties of a virulent tumour and not just the capacity to penetrate an epithelial surface.

Using the CAM-FFA it has been shown that embryos of different genotypes differ with respect to their capacity to support CAM foci produced by RP1 and AL1. Line N, which was selected for resistance to MD, is the least permissive to CAM focus formation and line P, which was selected for susceptibility from the same ancestral flock as line N (Cole et al, 1968), was significantly more permissive than line N. The resistance of line N to MD can be accounted for by the fact that

this line is uniformly homozygous for the $B^{21}$ allele of the chicken MHC (Longenecker et al, 1976). This allele is associated with resistance to MD as well as the inhibition of growth of RP1 cells (Longenecker and Gallatin, 1978) and the JMV-1 lymphoma (Longenecker et al, 1977a) from which RP1 and its variants were ultimately derived. Therefore, these results suggest that the mechanism by which $B^{21}$ bearing birds inhibit the growth of MD tumour cells may already be detectable between day 11 and day 15 of embryogenesis. It is important to note that the lymphoma cell lines used in this study are allogeneic to the embryos used for the assays. It has been demonstrated previously that the presence of shared histocompatability antigens cannot account for the fact that $B^{21}$ bearing birds restrict the growth of JMV-1 or RP1 cells (Longenecker et al, 1977a; Longenecker and Gallatin, 1978). Sharma and Coulson (1979) have used allogeneic, MD derived, lymphoma cell lines to detect the presence of NK cells in the chicken. It is possible that the inhibition of growth of JMV-1 derived cell lines in newly hatched chicks and embryos of genetically resistant chickens measures a primitive form of resistance to tumour growth in the chicken similar to that described by Carlson for the mouse (Carlson and Wegmann, 1977; Carlson et al, 1980). This genetic resistance, however, appears to be overcome by highly virulent cell lines, such as AL2 and AL3, indicating that whatever the mechanism, the genetic resistance observed in ovo is weak. By contrast, $B^{21}$ bearing birds are almost totally resistant to lymphoma development. Thus, genetic

resistance to MD lymphoma development may be effective only in the early phases of tumour growth, before the tumour has acquired significant resistance.

The fact that allogeneic selections for metastasis in newly hatched chicks can be measured in the embryo, a relatively immunologically inert environment, indicates that MHC incompatabilities between the tumour and the host were not a factor affecting the organ specific metastasis observed in this study. In fact, in the EL-FFA, the same difference between the two metastatic variants AL2 and AL3 was seen in line S as was seen in any other type of embryo. Line S is the probable strain of origin of JMV-1, the parental tumour of RP1 (Sevoian et al, 1964; Nazerian et al, 1977). All RP1 derived cell lines react equally well with anti-$B^1$ antiserum, indicating the preservation of cell surface MHC antigens during the selections for virulence and metastasis. Invasion of allogeneic tissue by tumours has been reported (Lohmann-Matthes et al, 1980) as well as xenogeneic combinations (Leighton, 1960). Butcher and Weissman (1980) have shown that the T lymphocyte recirculation specificity is controlled by a non-MHC linked locus in the mouse. In fact, allogeneic combinations of lymphocytes and high endothelial venules show as much binding as sysngeneic combinations in their in vitro assay. Crossing species barriers, however, results in the gradual loss of recognition between the lymphocytes and the venules as greater phylogenetic distance is achieved. Thus, the spread and specific homing of cells in the body may be controlled by primitive recognition mechanisms.

In order to probe the cell surface of our tumour cell variants, monoclonal antibodies were made against the liver metastatic variant AL2 by the cell fusion technique. Sixteen hybridoma clones proved to be stable, antibody producers. Of these, 10 clones produced antibody which appeared to detect B locus or another polymorphic system antigens. Only one clone produced antibody which reacted with all types of chicken cells tested. Such clones have been shown on further testing to produce antibody detecting a few non-B locus antigens of low polymorphism (Longenecker and Mosmann, unpublished results). Five clones produced antibody detecting antigens on tumour cells alone. One of these, which reacts with more than one MDV transformed cell isolate, is a candidate anti-MATSA monoclonal antibody. MATSA is an antigen present on the surface of all MD tumours, original or transplantable, and cell lines (Witter et al, 1975). It is not a viral antigen. Three clones produced antibody which reacted with all RP1 derived cell lines. One clone produced antibody which reacted exclusively with AL2 cells. This antibody defines the liver metastasis associated antigen (LMAA). The range of specificities exhibited by these monoclonal antibodies is in keeping with current work from this department where it has been shown that the mouse preferentially responds to polymorphic antigens on cells (longenecker et al, 1979; Mosmann and Longenecker, unpublished results; Mackie and Longenecker, unpublished results). These include transplantation antigens, blood group antigens, as well as bacterial serotype antigens. This observation can now be extended to tumour cells. Tumour specific,

tumour isolate specific and tumour cell variant specific antigens are revealed by analysis of monoclonal antibody producing cell clones. Other workers have produced monoclonal antibodies to previously defined human tumour antigens (Ritz et al, 1980) and have used the monoclonal antibody technique to define novel human tumour antigens (Barnstable et al, 1978; Koprowski et al, 1978).

It has become generally accepted that one explanation for tumour progression is the production of variant clones of cells in a tumour that vary in their malignant phenotype (Nowell, 1976; Fidler et al, 1978; Poste and Fidler, 1980). Those clones with a survival advantage will become dominant in a tumour but will, in turn, give rise to more variant clones. This hypothesis is supported by the data of Fidler and others (Fidler, 1978; Dexter et al, 1978; Kripkie et al, 1978; Suzuki et al, 1978). Weiss (1979), however, warns of accepting a hypothesis based on subpopulations too hastily and introduces the concept of a transient metastatic compartment in a primary tumour. This is similiar to stating that metastatic subpopulations are not stable. Whatever the stability to these subpopulations, the results presented here show that there is clonal variation in liver specific metastasis. As it is possible to discriminate two locations of metastasis in this system, it is also possible to show that while there may be variation in the ability of the clones to produce liver specific metastasis, their invasive

ability into the CAM is remarkably similar. This indicates that invasion and metastasis may be under separate cellular control. Intuitively, invasive ability is a necessary prerequisite for metastasis. Every clone that was isolated has a high level of invasion into the CAM compared with parental values and those expected for less invasive cell lines. In addition, the expression of an organ specific metastasis associated antigen, the LMAA, is variable amongst the cloned tumour variant cell lines. Both these parameters, LMAA expression and liver metastasis, vary in culture, but remain highly correlated over short intervals in vitro. LMAA expression does not correlate with invasion as measured in the CAM-FFA. The fact that liver specific metastasis is observed in the absence of LMAA expression may be due to the differential sensitivities of the ADRA and the EL-FFA or there may be variation in the linkage between LMAA expression and liver specific metastasis for a given cell line. This is not unexpected since metastasis is a complex series of events only one of which might involve specific organ recognition. This result is predicted from Weiss's (1979) concept of the transient metastatic compartment.

Kerbel (1979) has reported that random cloning produces unstable metastatic variants in his system in contrast to the work of Fidler (Fidler et al, 1978). Stable metastatic variants were produced by Kerbel only after treatment with mutagens and lectins (Kerbel, 1979).

Tao and Burger (1977), however, reported that lectins produced marginally metastatic variants in Fidler's system. Kerbel's result implies that a genetic change is necessary to produce a stable metastatic variant. The metastatic variants described here show a decrease in the difference between their abilities to form embryonic liver foci that appears to correlate with time in vitro. The major contribution to this is the dramatic rise in liver metastatic capacity of tumour variant cell line AL3. The speed and magnitude of this rise indicates that a fast growing, highly virulent clone may have come to dominate the AL3 culture. Studies with drug resistance traits of the cell lines show that the change in AL3 is not due to contamination from AL2. Also, AL2 and AL3 maintain their relative frequencies of LMAA positive cells. This indicates that AL3 has acquired liver metastatic ability utilizing a mechanism for which the LMAA is not a marker. A stable, genetic change may have been induced by the selections for liver metastatic ability such that the AL2 cell line has become highly metastatic for the liver. Tumour variant AL3, on the other hand, was not negatively selected for liver metastasis but was positively selected for ovarian metastasis. Therefore, the instability in relation to liver metastatic phenotype of AL3 is not unexpected especially if one invokes Weiss's hypothesis (Weiss, 1979). Chow and Greenberg (1980) report that they were unable to detect the production of tumour heterogeneity

*in vitro* as compared to *in vivo*. The selection for organ specific metastasis described here occured over 5 passages *in vivo*. After 5 passages *in vitro*, the AL3 line still retained its character of marginal liver metastasis. Thus, in agreement with Chow and Greenberg (1980), the metastatic variant cell lines may have a higher rate of generation of heterogeneity *in vivo* than *in vitro*. Still, the results presented in this study indicate that profound heterogeneity can be generated *in vitro*.

The treatment of AL2 cells with anti-LMAA antibodies with or without the prior to injection complement mediated killing of the cells reduces the amount of liver metastasis in the EL-FFA. CAM focus formation is not affected by similiar treatment. Thus, anti-LMAA monoclonal antibodies specifically inhibit the formation of liver metastases. No other monoclonal antibody tested had this ability. LMAA positive cells cannot regenerate in culture over two days and liver specific metastasis is similiarly affected. Taken together with the correlated clonal variation of the LMAA and liver specific metastasis, these experiments support the conclusion that liver metastases are produced by the same population of cells that are LMAA positive. This conclusion is in keeping with the theoretical work of Nowell (1976) and Weiss (1979) and the work of Fidler *et al* (1978). Recently, Poste and Nicolson (1980) have reported that membrane vesicles will alter the metastatic properties of tumour cells. This is

in agreement with the results presented here implying a role for cell surface structures in the metastatic distribution of tumour cells. The antibody inhibition experiments strongly suggest a functional role for the LMAA in at least one mechanism of liver specific metastasis.

The fact that liver metastases were never completely inhibited by anti-LMAA antibody indicates that there could be more than one mechanism for liver specific metastasis. This is supported by the observation that when the AL3 cell line acquired a high liver metastatic phenotype it did not acquire the LMAA in a higher frequency. Another interpretation for the failure of specific antibody treatment to completely inhibit liver specific metastasis is that the LMAA could be present in significant concentration only on cells in a certain stage of the cell cycle. This is doubtful as it is shown that liver metastatic ability and LMAA expression cannot regenerate in culture over two days (approximately four doublings). More likely, there is a distribution of antigen density on the tumour cells such that only the cells with the highest antigen density are detected by the assays used due to a threshold effect. This would explain why only an apparent subpopulation of AL2 cells have the LMAA. The clones may vary in their LMAA density and therefore would vary in their frquencies of antigen positive cells. Likewise, the antibody inhibition experiments, having a threshold, would only detect and remove the high density LMAA positive cells.

The mechanisms by which liver specific metastasis may occur are many, but two hypotheses are worthy of note. It is conceivable that the LMAA acts as a receptor which recognizes a liver specific acceptor on the surface of hepatic blood vessel endothelial cells. The interaction of receptor and acceptor could arrest and trigger the metastasizing tumour cell to invade the liver. The presence of a population of receptor positive cells within a tumour would result in metastasis to the organ with the specific acceptor. A receptor positive population may have been selected for in the passages that ultimately resulted in the derivation of AL2. In this case, the receptor/acceptor combination results in increased liver metastasis. AL3, selected for ovarian metastasis, would not have been enriched for this receptor positive population. Masking the receptor with antibody would reduce the amount of liver metastasis as is shown. One would predict that if the LMAA is a receptor, then flooding the chick embryo with purified LMAA would reduce liver metastasis. The alternative hypothesis is that the LMAA may not be involved in the specific trapping and successful growth of metastasizing tumour cells in the liver, but it might be involved in the successful colonization of the liver

by the tumour cells. Tumour cells have been shown to recognize and fuse with the normal constituents of an epithelium, forming all types of intercellular junctions (Pitelka et al, 1980a; 1980b). The LMAA may act as a cellular interaction structure (CIS) involved in the positional growth control of the tumour cells in the liver. A population of CIS positive cells in a tumour, once they have metastasized to the liver, would be able to receive the appropriate positional and growth signals from the surrounding normal liver cells. CIS's have been implicated in the selective sorting and ligration of embryonic cells (Lilien et al, 1979). Maslow et al (1980) have shown that metastatic variant cells or conditioned media from these cells can inhibit embryonic cell aggregation and adhesion assays. This is important as these cells have previously been shown to aggregate preferentially with cells from the organ to which they metastasize (Nicolson and Winkelhake, 1975). If the LMAA is a CIS, then the LMAA would inhibit the aggregation of liver cells or liver and tumour cells. Schirrmacher et al (1980) have shown that hepatocytes and highly metastatic tumour cells will form rosettes. Only by neuraminidase treatment will marginally metastatic tumour cells also exhibit this property. Interestingly, the rosettes are inhibitable in a nonspecific, steric blocking fashion by anti-MHC antibody. As neuraminidase

removes sialic acid from glycoproteins and glycolipids on the cell surface, Schirrmacher postulates that the rosette formation is due to a lectin-like receptor for asialoglycoproteins of the hepatocyte (Kolb et al, 1978). If the liver specific metastasis observed in the system presented in this thesis is operative by a mechanism similiar to Schirrmacher's hypothesis, the the important CIS on metastasizing tumour cells would be an asialoglycoprotein. This indicates that the carbohydrate modifications of cell surface structures can be significant in the pathogenesis of cancer.

References

Albert. D., and I. Zeidman. Relation of glucocorticoid activity of steroids to the number of metastases. Cancer Res. 22, 1297-1300 (1962).

Ames, B.N., and J. McCann. Carcinogens are mutagens: A simple test system. In: R. Montesano, H. Bartsch and L. Tomatis (ed). Screeening Tests in Chemical Carcinogenesis. pp. 493-501. IARC, Lyon. (1976).

Andrews, J.R.. The Radiobiology of Human Cancer Radiotherapy. 2nd Ed. University Park Press, Baltimore. (1978).

Ballou, B., G. Levine, T.R. Hakala, and D. Solter. Tumour location detected with radioactively labelled monoclonal antibody and external scintigraphy. Science 206, 844-847 (1979).

Balsamo, J., and J. Lilien. Embryonic cell aggregation: Kinetics and specificity of binding of enhancing factors. Proc. U.S. Natl. Acad. Sci. 71, 727-731 (1974).

Barker, C.F., and R.E. Billingham. Immunologically privileged sites. Adv. Immunol. 25, 1-54 (1977).

Barnstable, C.J., W.F. Bodmer, G. Brown, G. Galfre, C. Milstein, A.F. Williams and A. Zeigle. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antige - New tools for genetic analysis. Cell 14, 9-20 (1978).

Barranco, S.C., D.H.W. Ho, B. Drewinko, M.M. Romsdahl and R.M. Humphrey. Differential sensitivities of human melanoma cells grown in vitro to arabinosylcytosine. Cancer Res. 32, 2733-2736 (1972).

Barranco, S.C., B. Drewinko and R.M. Humphrey. Differential response by human melanoma cells to 1,3-bis-(2-chloroethyl)-1-nitrosourea and bleomycin. Mutation Res. 19, 277-280 (1972).

Baserga, R. and U. Saffiotti. Experimental studies on histiogenesis of blood-borne metastasis. Arch. Pathol. 59, 26-34 (1955).

Berenblum I., and P. Shubik. A new quantitative approach to the study of the stages of chemical carcinogenesis in the mouses' skin. Brit. J. Cancer 1, 383-391 (1947).

Berenblum, I.. A re-evaluation of the concept of cocarcinogenesis. Prog. exptl. Tumour Res. 11, 21-30 (1969).

Biggs, P.M., and B.S. Milne. Biological properties of a number of Marek's disease virus isolates. In: P.M. Biggs, G. de-Thé and L.N. Payne (ed). Oncogenesis and Herpesviruses. pp. 88-94. IARC, Lyon. (1972).

Biggs, P.M.. Vaccination against oncogenic herpesviruses. In: G. de-Thé, M.A. Epstein and H. zur Hausen (ed). Oncogenesis and Herpesviruses II. pp. 317-327. IARC, Lyon. (1975).

Bingham, E., R.W. Niemeier and J.B. Reid. Multiple factors in carcinogenesis. Ann. N.Y. Acad. Sci. 271, 14-21 (1976).

Brunson, K.W., and G.L. Nicolson. Selection and biologic properties of malignant variants of a murine fibrosarcoma. J. U.S. Natl. Cancer Inst. 61, 1499-1503 (1978).

Butcher, E.C., and I.L. Weissman. Cellular, genetic and evolutionary aspects of lymphocyte interactions with high endothelial venules. Ciba Found. Symp. 71, 265-286 (1980).

Cahill, R.N.P., D.C. Poskitt, H. Frost and Z. Trnka. Two distinct pools of recirculating T lymphocytes: Migratory characteristics of nodal and intestinal T lymphocytes. J. exptl. Med. 145, 420-428 (1977).

Calnek, B.W., K.K. Murthy and K.A. Schat. Establishment of Marek's disease lymphoblastoid cell lines from transplantable versus primary lymphoma. Int. J. Cancer 21, 100-107 (1978).

Carlson, G.A., and T.G. Wegmann. Rapid in vivo destruction of semi-syngeneic and allogeneic cells by nonimmunized mice as a consequence of nonidentity at H-2. J. Immunol. 118, 2130-2137 (1977).

Carlson, G.A., D. Melnychuk and M.J. Meeker. H-2 associated resistance to leukemia transplantation: Natural killing in vivo. Int. J. Cancer 25, 111-122 (1980).

Chevalier, C., and E. Frindel. Une nouvelle technique permittant de déterminer le nombre de cellules L1210 clonogènes. Bull. Cancer 59, 339-344 (1972).

Chow, D.A., and A.H. Greenberg. The generation of tumour heterogeneity in vivo. Int. J. Cancer 25, 261-266 (1980).

Clayson, D.B.. Chemical Carcinogenesis. J. & A. Churchill Ltd., London. 1962.

Cock, A.G.. The immunological status of ovarian homografts in fowls. Immunology 5, 169-180 (1962).

Cole, R.K.. Studies on genetic resistance to Marek's disease. Avian Dis. 12, 9-28 (1968).

Coman, D.R., Mechanisms of invasiveness of cancer. Science 105, 347-348 (1947).

Coman, D.R., R.B. Eisenberg and M. McCutcheon. Factors affecting the distribution of tumour metastasis. Experiments with V2 carcinoma of rabbits. Cancer Res. 9, 649-660 (1949).

Coman, D.R.. Mechanisms responsible for the origin and distribution of blood-borne tumour metastasis: A review. Cancer Res. 13, 397-404 (1953).

De Bruyn, P.P.H., and Y. Cho. Entry of metastatic malignant cells into the circulation from a subcutaneously growing myelogenous tumour. J. U.S. Natl. Cancer Inst. 62, 1221-1223 (1979).

Dexter, D.L., H.M.Kowalski, B.A. Blazer, Z. Fligiel, R. Vogel and G.H. Heppner. Heterogeneity of tumour cells from a single mouse mammary tumour. Cancer Res. 38, 3174-3179 (1978).

Earle, W.R., E. Shelton and E.L. Schilling. Production of malignancy in vitro. XI. Further results from reinjection of in vitro cell strains into strain C3H mice. J. U.S. Natl. Cancer Inst. 10, 1105-1112 (1950).

Easty, D.M., and G.C. Easty. Measurment of the ability of cells to infiltrate normal tissues in vitro. Brit. J. Cancer 29, 36-49 (1974).

Eaves, G.. The invasive growth of malignant tumours is a purely mechanical process. J. Pathol. 109, 233-237 (1973).

Engell, H.C.. Cancer cells in the circulating blood. Acta Chir. Scand. (suppl.) v. 201 (1955).

Engell, H.C.. Cancer cells in the blood. Ann. Surg. 149, 457-461 (1959).

von Essen, C.F., and H.S. Kaplan. Further studies on metastasis of a transplantable mouse mammary carcinoma after roentgen irradiation. J. U.S. Natl. Cancer Inst. 12, 883-892 (1952).

Ewing, J.. Neoplastic Diseases. 4th Ed. W.B. Saunders Co., Philadelphia. (1941).

Failkow, P.J., G. Klein, S.M. Gartler and P. Clifford. Clonal origin for individual Burkitt's tumours. Lancet i, 384-386 (1970).

Fidler, I.J. Metastasis: Quantitative analysis of distribution and fate of tumour emboli labeled with $^{125}$I-5-iodo-2'-deoxyuridine. J. U.S. Natl. Cancer Inst. 45, 773-782 (1970).

Fidler, I.J., and S. Leiber. Quantitative analysis of the mechanism of glucocorticoid enhansement of experimental metastasis. Res. Comm. Chem. Pathol. Pharmacol. 4, 607-613 (1972).

Fidler, I.J., and I. Zeidman. Enhansement of experimental metastasis by X-ray: A possible mechanism? J. Med. 3, 172-177 (1972).

Fidler, I.J.. The relationship of embolic heterogeneity, number, size and viability to the incidence of experimental metastasis. Eur. J. Cancer 9, 223-227 (1973a).

Fidler, I.J.. Selection of successive tumour lines for metastasis. Nature New Biol. 242, 148-149 (1973b).

Fidler, I.J.. Immune stimulation - Inhibition of experimental cancer metastasis. Cancer Res. 34, 491-497 (1974a).

Fidler, I.J.. Inhibition of pulmonary metastasis by intravenous injection of specifically activated macrophages. Cancer Res. 34, 1074-1078 (1974b).

Fidler, I.J.. Biological behavior of malignant melanoma cells correlated to their survival in vivo. Cancer Res. 35, 218-224 (1975).

Fidler, I.J., D.M. Gersten and M.B. Budmen. Characterization in vivo and in vitro of tumour cells selected for resistance to syngeneic lymphocyte mediated cytotoxicity. Cancer Res. 36, 3160-3165 (1976).

Fidler, I.J.. Host-tumour interaction in the pathogenesis of metastasis. Gann Mono. 20, 121-126 (1977).

Fidler, I.J., D.M. Gersten and C.W. Riggs. Relationship of host immune status to tumour cell arrest, distribution and survival in experimental metastasis. Cancer 40, 46-55 (1977).

Fidler, I.J., and G. Nicolson. Organ selectivity for implantation , survival and growth of B16 melanoma variant tumour lines. J. U.S. Natl. Cancer Inst. 57, 1199-1202 (1977).

Fidler, I.J.. Tumour heterogeneity and the biology of Cancer invasion and metastasis. Cancer Res. 38, 2651-2660 (1978).

Fidler, I.J., D.M. Gersten and I.R. Hart. The biology of cancer invasion and metastasis. Adv. Cancer Res. 28, 149-250 (1978).

Fidler, I.J., D.M. Gersten and M.L. Kripke. Influence of host immune status on the metastasis of three murine fibrosarcomas of different immunogenicities. Cancer Res. 39, 3816-3821 (1979).

Fishbein, L.. Potential Industrial Carcinogens and Mutagens. Elsevier Scientific Publ. Co., Amsterdam. (1979).

Fisher, B., and E.R. Fisher. Experimental evidence in support of the dormant tumour cell. Science 130, 918-919 (1959).

Fisher, B., and E.R. Fisher. Experimental studies of factors which influence hepatic metastases. VIII. Effect of anticoagulants. Surg. 50, 240-247 (1961a).

Fisher, E.R., and B. Fisher. Experimental studies of factors influencing hepatic metastases. VII. Effect of reticuloendothelial interference. Cancer Res. 21, 275-280 (1961b).

Fisher, E.R., and B. Fisher. Experimental studies of factors influencing hepatic metastases. X. Effect of reticuloendothelial stimulation. Cancer Res. 22, 478-483 (1962).

Fisher, E.R., and B. Fisher. Experimental study of factors influencing development of hepatic metastases from circulating tumour cells. Acta Cytol. (Balt.) 9, 145-159 (1965).

Fisher, B., and E.R. Fisher. Transmigration of lymph nodes by tumour cells. Science 152, 1397-1398 (1966a).

Fisher, B., and E.R. Fisher. The interrelationship of hematogenous metastasis and lymphatic cell dissemination. Surg. Gynec. Obstet. 122, 791-798 (1966b).

Fisher, B., and E.R. Fisher. Barrier function of lymph node to tumour cells. I. Normal nodes. Cancer 20, 1909-1913 (1967a).

Fisher, B., and E.R. Fisher. Barrier function of lymph node to tumour cells and erythrocytes. II. Effect of X-ray, inflammation, sensitization and tumour growth. Cancer 20, 1914-1919 (1967b).

Fisher, B., and E.R. Fisher. The organ distribution of disseminated $^{51}$Cr-labeled tumour cells. Cancer Res. 27, 412-420 (1967c).

Fitzpatrick, F.A., and D.A. Stringfellow. Prostaglandin $D_2$ formation by malignant melanoma cells correlates inversely with cellular metastatic potential. Proc. U.S. Natl. Acad. Sci. 76, 1765-1769 (1979).

Fogel, M., E. Gorelik, S. Segal and M. Feldman. Differences in cell surface antigens of tumour metastases and those of the local tumour. J. U.S. Natl. Cancer Inst. 62, 585-588 (1979).

Folkman, J.. Tumour angiogenesis. In: F.F. Becker (ed). Cancer: A Comprehensive Treatise. v. 3. pp. 355-388. Plenum Press, New York. (1975).

Ford, W.L.. Lymphocyte migration and immune responses. Prog. Allergy 19, 1-59 (1975).

Foulds, L.. The experimental study of tumour progression. Cancer Res. 14, 327-339 (1954).

Gallatin, W.M., and B.M. Longenecker. Expression of genetic resistance to an oncogeneic herpesvirus at the target cell level. Nature (Lond.) 280, 587-589 (1979).

Gasic, G., and T. Gasic. Removal of sialic acid from the cell coat in tumour cells and vascular endothelium, and its effects on metastasis. Proc. U.S. Natl. Acad. Sci. 48, 1172-1177 (1962).

Gasic, G.J., T. Gasic and C.C. Stewart. Antimetastatic effects associated with platelet reduction. Proc. U.S. Natl. Acad. Sci. 61, 46-52 (1968).

Gasic, G.J., T.B. Gasic, N. Galanti, T. Johnson and S. Murphy. Platelet-tumour cell interactions in mice. The role of platelets in the spread of malignant disease. Int. J. Cancer 11, 704-718 (1973).

Gerfaux, J., F. Chany-Fournier, P. Bardos, J-P. Muh and C. Chany. Lectin-like activity of components extracted from human glomerular basement membrane. Proc. U.S. Natl. Acad. Sci. 76, 5129-5133 (1979).

Ghosh, S.K., A.L. Grossberg, U. Kim and D. Pressman. A tumour-associated organ-specific antigen characteristic of spontaneouly metastatic rat mammary carcinoma. J. U.S. Natl. Cancer Inst. 62, 1229-1233 (1979).

Gorelik, E., M. Fogel, M. Feldman and S. Segal. Differences in resistance of metastatic tumour cells and cells from a local tumour growth to cytotoxicity of natural killer cells. J. U.S. Natl. Cancer Inst. 63, 1397-1409 (1979).

Gowans, J.L., and E.J. Knight. The route of re-circulation of lymphocytes in the rat. Proc. R. Soc. Lond. B Biol. Sci. 159, 257-282 (1964).

Gray, J.M., and G.B. Pierce. Relationship between growth rate and differentiation of melanoma. J. U.S. Natl. Cancer Inst. 32, 1201-1211 (1964).

Greene, H.S.N.. A conception of tumour autonomy based on transplantation studies: A review. Cancer Res. 11, 899-903 (1951).

Greene, H.S.N., and E.K. Harvey. The relationship between the dissemination of tumour cells and the distribution of metastases. Cancer Res. 24, 799-811 (1964).

Gutman, G.A., and I.L. Weissman. Homing properties of thymus-independent follicular lymphocytes. Transplant. 16, 621-629 (1973).

Hakansson, L., and C. Tropé. On the presence within tumours of clones that differ in sensitivity to cytostatic drugs. Acta. Pathol. Microbiol. Scand. Section A. 82, 35-40 (1974).

Hagmar, B., and K. Norrby. Evidence for effects of heparin on cell surfaces influencing experimental metastases. Int. J. Cancer 5, 72-84 (1970).

Hagmar, B.. Cell surface charge and metastasis formation. Acta. Pathol. Microbiol. Scand. Section A. 80, 357-366 (1972).

Hagmar, B., and K. Norrby. Influence of cultivation, trypsinization and aggregation on the transplantability of melanoma B16 cells. Int. J. Cancer 11, 663-675 (1973).

Hagmar, B., and W. Ryd. Tumour cell locomotion - A factor in metastasis formation? Influence of cytochalasin B on a tumour dissemination pattern. Int. J. Cancer 19, 576-580 (1977).

Hahn, E.C., R.M. Jakowski, L. Ramos and A.J. Kenyon. Lymphoproliferative diseases of fowl: Characterization of transplantable G-B1 Marek's disease tumour cells in culture. Avian Dis. 22, 409-421 (1978).

Hart, I.R., and I.J. Fidler. An in vitro quantitative assay for tumour cell invasion. Cancer Res. 38, 3218-3224 (1978).

Hart, I.R., A. Raz and I.J. Fidler. Effect of cytoskeleton disrupting agents on the metastatic behavior of melanoma cells. Cancer Res. 64, 891-900 (1980).

Ho, H.C.. Current knowledge of the epidemiology of nasopharyngeal carcinoma - A review. In: P.M. Biggs, G. de-Thé and L.N. Payne (ed). Oncogenesis and Herpesviruses. pp. 357-366. IARC, Lyons. (1972).

Hurwitz, E., B. Schechter, R. Arnon and M. Sela. Binding of anti-tumour immunoglobulins and their daunomycin conjugates to the tumour and its metastases. In vitro and in vivo studies with Lewis lung carcinoma. Int. J. Cancer 24, 461-470 (1979).

Ioachim, H.L., A. Pearse and S.E. Keller. Role of immunologic mechanisms in metastatic patterns of hemopoeitic tumours in rats. Cancer Res. 36, 2854-2862 (1976).

Kaplan, H.S., and E.D. Murphy. The effect of local roentgen irradiation on the biological behaviour of a transplantable mouse mammary carcinoma. I. Increased frequency of pulmonary metastasis. J. U.S. Natl. Cancer Inst, 9, 407-413 (1949).

Karpas, C.M., and N. Silversmith. Biological filtration of tumour cells by various capillary beds. Proc. Am. Ass. Cancer Res. 4, 33 (1963).

Kato, S., and Y. Akiyama. Lymphoid cell lines from lyphomas of Marek's disease. In: G. de-Thé, M.A. Epstein and H. zur Hausen (ed). Oncogenesis and Herpesviruses II. pp. 101-108. IARC, Lyons. (1975).

Kawasaki, T., and G. Ashwell. Chemical and physical properties of an hepatic membrane protein that specifically binds asialoglycoproteins. J. Biol. Chem. 251, 1296-1302 (1976).

Kerbel, R.S.. Immunologic studies of membrane mutants of a highly metastatic murine tumour. Am. J. Pathol. 97, 609-622 (1979).

Ketcham, A.S., and E.V. Sugarbaker. The relation of surgery to the metastatic potential of neoplastic disease. Gann Mono. 20, 173-180 (1977).

Kinsey, D.L. An experimental study of preferential metastasis. Cancer 13, 674-676 (1960).

Klein, G.. A summing up. In: P.M. Biggs, G. de-Thé and L.N. Payne (ed). Oncogenesis and Herpesviruses. pp. 501-515. IARC, Lyons. (1972).

Klein, G., and E. Klein. Conversion of solid neoplasms into ascites tumours. Ann. N.Y. Acad. Sci. 63, 640-661 (1956).

Kolb, H., A. Kriese, V. Kolb-Bachofen and H-A. Kolb. Possible mechanism of entrapment of neuraminidase treated lymphocytes in the liver. Cell Immunol. 40, 457-462 (1978).

Koprowski, H., Z. Stephlewski, D. Herlyn and M. Herlyn. Study of antibodies against human melanoma produced by somatic cell hybrids. Proc. U.S. Natl. Acad. Sci. 75, 3405-3409 (1978).

Kramer, R.H., and G.L. Nicolson. Interactions of tumour cells with vascular endothelial cell monolayers: A model for metastatic invasion. Proc. U.S. Natl. Acad. Sci. 76, 5704-5708 (1979).

Kripkie, M.L., E. Gruys and I.J. Fidler. Metastatic heterogeneity of cells from an ultraviolet light induced murine fibrosarcoma of recent origin. Cancer Res. 38, 2962-2967 (1978).

Leighton, J., D.A. Dreyer and M.J. Mahoney. Transplantation and malignancy in a companion-host system on the chorioallantoic membrane. Science 132, 1763-1765 (1960).

Levin, I., and M.J. Sittenfield. The formation of metastases after an intravascular injection of tumour emulsions. Proc. Soc. exptl. Biol. Med. 8, 114-117 (1910).

Lightdale, C., and M. Lipkin. Cell division and tumour growth. In: F.F. Becker (ed). Cancer: A Comprehensive Treatise. v. 3. pp. 201-215. Plenum Press, New York. (1975).

Lilien, J., J. Balsamo, J. McDonnough, J. Hermolin, J. Cook and R. Rutz. Adhesive specificity among embryonic cells. In: R.O. Hynes (ed). Surfaces of Normal and Malignant Cells. pp. 389-419. J. Wiley and Sons, Chicester. (1979).

Linder, D., and S.M. Gartler. Glucose-6-phosphate dehydrogenase mosaicism: Utilization as a cell marker in the study of leimyomas. Science 150, 67-69 (1965).

Lohmann-Matthes, M-L., A. Schleich, G. Schantz and V Schirrmacher. Tumour metastases and cell-mediated immunity in a model system in DBA/2 mice. VII. Interaction of metastasizing and nonmetastasizing tumours with normal tissues in vitro. J. U.S. Natl. Cancer Inst. 64, 1413-1425 (1980).

Longenecker, B.M., F. Pazderka, H.S. Stone, J.S. Gavora and R.F. Ruth. In ovo assay for Marek's disease virus and turkey herpesvirus. Infect. Immun. 11, 922-931 (1975).

Longenecker, B.M., F. Pazderka, J.S. Gavora, J.L. Spencer and R.F. Ruth. Lymphoma induced by herpesvirus: Resistance associated with a major histocompatability gene. Immunogen. 3, 401-407 (1976).

Longenecker, B.M., F. Pazderka, J.S. Gavora, J.L. Spencer, E.A. Stephens, R.L. Witter and R.F. Ruth. Role of the major histocompatability complex in resistance to Marek's disease: Restriction of growth of JMV-MD tumour cells in genetically resistant birds. In: A.A. Benedict (ed). Avian Immunology. pp. 297-298. Plenum Publ. Corp., New York. (1977a).

Longenecker, B.M., J. Menezes, E.J. Sanders, F. Pazderka and R.F. Ruth. A new class of infectious agents detectable by the production of chorioallantoic membrane lesions by human lymphoblastoid cell lines and their culture supernatants. J. U.S. Natl. Cancer Inst. 58, 853-861 (1977b).

Longenecker, B.M., and W.M. Gallatin. Genetic control of resistance to Marek's disease. In: G. de-Thé,

W. Henle and F. Rapp (ed). Oncogenesis and Herpesviruses III. pp. 845-850. IARC, Lyons. (1978).

Longenecker, B.M., T.R. Mosmann and C. Shiozawa. A strong, preferential response of mice to polymorphic antigenic determinants of the chicken MHC, analyzed with mouse hybridoma (monoclonal) antibodies. Immunogen. 9, 137-147 (1979).

Lucké, B.. A neoplastic disease of the kidney of the frog, Rana pipiens. II. On the occurrence of Metastasis. Am. J. Cancer 22, 326-334 (1934)

Lucké, B.. Caricinoma of the kidney of the leopard frog: The occurrence and significance of metastasis. Am. J. Cancer 34, 15-30 (1938).

Lyon, M.. X-chromosome inactivation. Adv. Teratol. 1, 25-54 (1966).

Martinez, C., G. Miraff and J.J. Bittner. Effect of size and/or rate of growth of a transplantable mouse adenocarcinoma on lung metastasis production. Cancer Res. 16, 313-315 (1956).

Maslow, D.E., and L. Weiss. Contributions of macrophages to interactions between tumour cells and other tissues assessed in an in vitro system. Int. J. Cancer 24, 450-454 (1979)

Maslow, D.E., E. Mayhew and J. Feldman. Differential inhibition of in vitro malignant cell assays by B16 melanoma variants. Cancer Res. 64, 635-638 (1980).

Melendez, L.V., M.D. Daniel, R.D. Hunt, C.E.O. Fraser, F.G. Garcia, N.W. King and M.E. Williamson. Herpesvirus saimiri. V. Further evidence to consider this as the etiological agent of a lethal disease in primates which resembles a malignant lymphoma. J. U.S. Natl. Cancer Inst. 44, 1175-1181 (1970).

Mizell, M.. State of the art: Lucké renal adenocarcinoma. In: M. Mizell (ed). Recent Results in Cancer Research Supplement: Biology of Amphibian Tumours. pp. 1-25. Springer-Verlag, New York. (1969).

Mosmann, T.R., W.M. Gallatin and B.M. Longenecker. Alteration of apparent specificity of monoclonal (hybridoma) antibodies recognising polymorphic histocompatability and blood group determinants. J. Immunol. in press. Sept. (1980).

Moore, G.E., A. Sandberg, J.R. Schubarg. Clinical and experimental observations of the occurence and fate of tumour cells in the blood stream. Ann. Surg. 146, 580-587 (1957).

Murray, J.C., L.Liotta, S.I. Rennard and G.R. Martin. Adhesion characteristics of murine metastatic and nonmetastatic tumour cells in vitro. Cancer Res. 40, 347-351 (1980).

Nazerian, K., and R.L. Witter. Cell-free transmission and in vivo replication of Marek's disease virus. J. Virol. 5, 388-397 (1970).

Nazerian, K.. Marek's disease: A neoplastic disease of chickens caused by a herpesvirus. Adv. Cancer Res. 17, 279-315 (1973).

Nazérian, K., E.A. Stephens, J.M. Sharma, L.F. Lee, M. Gailitis and R.L. Witter. A nonproducer T lymphoblastoid cell line from Marek's disease transplantable tumour (JMV). Avian Dis. 21, 69-76 (1977).

Nazerian, K., and W.S. Payne. Expression of Marek's disease virus (MDV) genome in producer and nonproducer cell lines. In: G. de-Thé, W. Henle and F. Rapp (ed). Oncogenesis and Herpesvirus III: pp. 635-638. IARC, Lyon. (1978).

Nicolson, G.L., and J.L. Winkelhake. Organ specificity of blood-borne tumour metastasis determined by cell adhesion? Nature (Lond.) 255, 230-232 (1975).

Nicolson, G.L., and K.W. Brunson. Organ selectivity of malignant B16 melanomas: In vivo selection for organ preference of blood-borne metastasis. Gann Mono. 20, 15-24 (1977).

Nowell, P.C.. Cytogenetics. In: F.F. Becker (ed). Cancer: A Comprehensive Treatise. v. 1. pp. 3-31. Plenum Press, New York. (1975).

Nowell, P.C.. The clonal evolution of tumour cell populations. Science 194, 23-28 (1976).

Paget, S.. The distribution of secondary growths in cancer of the breast. Lancet i, 571-573 (1889).

Parks, R.C., Increased tumour metastasis after in vitro alteration of the cell surface. J. U.S. Natl. Cancer Inst. 54, 1473-1474 (1975).

Payne, L.N.. Pathogenesis of Marek's disease - A review. In: P.M. Biggs, G. de-Thé and L.N. Payne (ed). Oncogenesis and Herpesviruses. pp. 21-37. IARC, Lyons. (1972).

Pazderka, F., B.M. Longenecker, G.R.J. Law, H.A. Stone and R.F. Ruth. Histocompatability of chicken populations selected for resistance to Marek's disease. Immunogen. 2, 93-100 (1975).

Pilgrim, H.I.. The kinetics of the organ-specific metastasis of a transplantable reticuloendothelial tumour. Cancer Res. 29, 1200-1205 (1969).

Pitelka, D.R., S.T. Hamamoto and B.N. Taggart. Epithelial cell junctions in primary and metastatic tumours of mice. Cancer Res. 40, 1588-1599 (1980a).

Pitelka, D.R., S.T. Hamamoto and B.N. Taggart. Basal lamina and tissue recognition in malignant mammary tumours. Cancer Res. 40, 1600-1611 (1980b).

Poste, G., J. Doll, I.R. Hart and I.J. Fidler. In vitro selection of murine B16 melanoma variants with enhansed tissue invasive properties. Cancer Res. 40, 1636-1644 (1980).

Poste, G., and I.J. Fidler. The pathogenesis of cancer metastasis. Nature (Lond.) 283, 139-146 (1980).

Poste, G., and G.L. Nicolson. Arrest and metastasis of blood-borne tumour cells are modified by fusion of plasma membrane vesicles from highly metastatic cells. Proc. U.S. Natl. Acad. Sci. 77, 399-403 (1980).

Potter, M.. The plasma cell tumours and myeloma proteins of mice. Meth. Cancer Res. 2, 102-157 (1967).

Potter, M.. Antigen-binding myeloma proteins of mice. Adv. Immunol. 25, 141-211 (1977).

Powell, P.C., L.N. Payne, J.A. Frazier and M. Rennie. T-lymphoblastoid cell lines from Marek's disease lymphomas. Nature (Lond.) 251, 79-80 (1974).

Pratt, W.B., and R.W. Ruddon. The Anticancer Drugs. Oxford University Press, New York. (1979).

Prehn, R.T.. Analysis of antigenic heterogeneity within individual 3-methylcholanthrene induced mouse sarcomas. J. U.S. Natl. Cancer Inst. 45, 1039-1045 (1970).

Pricer, W.E., and G. Ashwell. The binding of desialylated glycoproteins by plasma membranes of rat liver. J. Biol. Chem. 246, 4825-4833 (1971).

Prinzmetal, M., E.M. Ornitz, B. Simkin and H.C. Bergman. Arterio-venous anastomoses in the liver, spleen and lungs. Am. J. Physiol. 152, 48-52 (1948).

Quigley, J.P.. Proteolytic enzymes of normal and malignant cells. In: R.O. Hynes (ed). Surfaces of Normal and Malignant Cells. pp. 247-285. J. Wiley & Sons, Chicester. (1979).

Rawls, W.E., W.A.F. Tomkins, M.E. Figueroa and J.L. Melnick. Herpesvirus type 2: Association with carcinoma of the cervix. Science 161, 1255-1256 (1968).

Raz, A., C. Bucana, W. McLellan and I.J. Fidler. Distribution of membrane anionic sites on B16 melanoma variants with differing lung colonising potential. Nature (Lond.) 284, 363-364 (1980).

Reddy, A.L., and P.J. Fialkow. Multicellular origin of fibrosarcomas in mice induced by the chemical carcinogen 3-methylcholanthrene. J. exptl. Med. 150, 878-887 (1979).

Reid, G.H., and J.R. Gibbons. Fate of cultured cells after injection into the circulation of syngeneic animals. Cancer Res. 39, 4724-4731 (1979).

Report of the Ad hoc Committee on Avian Cell Line and Transplantable Tumour Nomenclature. R.L. Witter, Chairman. A proposed method for designating avian cell lines and transplantable tumours. Avian Pathol. 8, 487-498 (1979).

Ritz, J., J.M. Pesando, J. Notis-McConarty, H. Lazarus and S.F. Schlossman. A monoclonal antibody to human acute lymphoblastic leukemia antigen. Nature (Lond.) 283, 583-585 (1980).

Roberts, S.S., A.L. Watne, E.A. McGrew, R.G. McGrath, S. Nanos and W.H. Cole. Clinical significance of cancer cells in the circulating blood. Surg. Forum 8, 146-151 (1957).

Roberts, S., O. Jonasson, L.Long, R. McGrath, E.A. McGrew and W.H. Cole. Cancer cells in the circulating blood. Ann. Surg. 154, 362-371 (1961).

Romanof, A.L.. The Avian Embryo, Structural and Functional Development. MacMillan, New York. (1960).

Romsdahl, M.D., E.W. Chu, R. Hume and R.R. Smith. The time of release of circulating tumour cells as determined in an experimental system. Cancer 14, 883-888 (1961).

Rous, P.. A sarcoma of the fowl transmissable by an agent separable from the tumour cells. J. exptl. Med. 13, 397-411 (1911).

Sanford, K.K.. Biologic manifestations of oncogenesis in vitro: A critique. J. U.S. Natl. Cancer Inst. 53, 1481-1485 (1974).

St. Matthew, 14: 3-9. The Holy Bible. Revised Standard Version. The World Publ. Co., Cleveland. (1962).

Saphir, O.. The fate of carcinoma emboli in the lung. Am. J. Pathol. 23, 245-249 (1947).

Schirrmacher, V., K. Bosslet, G. Shantz, K. Clauer and D. Hubsch. Tumour metastasis and cell-mediated immunity in a model system in DBA/2 mice. IV. Antigenic differences between a metastasizing variant and the parental tumour line revealed by cytotoxic T lymphocytes. Int. J. Cancer 23, 245-252 (1979).

Schirrmacher, V., R. Cheinsong-Popov and H. Arheiter. Hepatocyte-tumour cell interaction in vitro. I. Conditions for rosette formation and inhibition by anti-H-2 antibody. J. exptl. Med. 151, 984-989 (1980).

Selikoff, I.J., C. Hammond and J. Churg. Asbestos exposure, smoking and neoplasia. Am. Med. Ass. J. 204, 106-112 (1968).

Sevoian, M., R.N. Larose and D.M. Chamberlain. Avian lymphomatosis: Incresed pathogenicity of JM virus. Proc. 101st Ann. Mtg. Am. Vet. Med. Ass. pp. 342. (1964).

Shanmugaratnam, K.. The pathology of nasopharyngeal carcioma - A review. In: P.M. Biggs, G. de-Thé and L.N. Payne (ed). Oncogenesis and Herpesviruses. pp. 239-248. IARC, Lyons. (1972).

Sharma, J.M., and B.D. Coulson. Presence of natural killer cells in specific pathogen free chickens. J. U.S. Natl. Cancer Inst. 63, 527-531 (1979).

Sinha, B.K., and G.J. Goldenberg. The effect of trypsin and neuraminidase on the circulation and organ distribution of tumour cells. Cancer 34, 1956-1961 (1974).

Stephens, E.A., R.L. Witter, L.F. Lee, J.M. Sharma, K. Nazerian and B.M. Longenecker. Characteristics of JMV Marek's disease tumour: A nonproductively infected transplantable cell lacking in rescuable virus. J. U.S. Natl. Cancer Inst. 57, 865-874 (1976).

Stringfellow, D.A., and F.A. Fitzpatrick. Protaglandin $D_2$ controls pulmonary metastasis of malignant melanoma cells. Nature (Lond.) 282, 76-78 (1979).

Storer, J.B.. Radiation carcinogenesis. In: F.F. Becker (ed). Cancer: A Comprehensive Treatise. v. 1. pp. 453-483. Plenum Press, New York. (1975).

Sugarbaker, E.D.. The organ selectivity of experimentally induced metastasis in rats. Cancer 5, 606-612 (1952).

Sugden, B., C.R. Kintner and W. Mark. The molecular biology of lymphotrophic herpesviruses. Adv. Cancer Res. 30, 239-278 (1979).

Suzuki, N., H.R. Withers and M.W. Koehler. Heterogeneity and variability of artificial lung colony forming ability among clones from mouse fibrosarcoma. Cancer Res. 38, 3349-3351 (1978).

Tao, T., and M.M. Burger. Non-metastasizing variants selected from metastasizing melanoma cells. Nature (Lond.) 270, 437-438 (1977).

Tao, T., A. Matter, K. Vogel and M.M. Burger. Liver colonizing melanoma cells selected from B-16 melanoma. Int. J. Cancer 23, 854-857 (1979).

de-Thé, G., A. Geser, N.E. Day, P.M. Turkei, E.H. Williams, D.P. Beri, P.S. Smith, A.G. Dean, G.W. Bornkamm, P. Feorino and W. Henle. Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. Nature (Lond.) 274, 756-761 (1978).

Tyzzer, E.E.. Factors in the production and growth of tumour metastasis. J. Med. Res. 28, 309-332 (1913).

Upton, A.C.. Physical carcinogenesis: Radiation - history and sources. In: F.F. Becker (ed). Cancer : A Comprehensive Treatise. v. 1. pp. 387-403. Plenum Press, New York. (1975).

Wallace, A.C.. The occurence of metastasis in a group of related rat tumours. Brit. J. Cancer 10, 724-732 (1956).

Wang, B.S., G.A. McLoughlin, J. Richie and J.A. Mannick. Correlation of the production of plasminogen activator with tumour metastasis in B16 mouse melanoma cell lines. Cancer Res. 40, 288-292 (1980).

Watanabe, S.. The metastasizability of tumour cells. Cancer 7, 215-223 (1954).

Warren, B.A.. Enviroment of the blood-borne tumour embolus adherent to vessel wall. J. Med. 4, 150-177 (1973).

Warren, L., I. Zeidman and C.A. Buck. The surface glycoproteins of a mouse melanoma growing in culture and as a solid tumour in vivo. Cancer Res. 35, 2186-2190 (1975).

Weiss, L., D. Glaves and D.A. Waite. The influence of host immunity on the arrest of circulating cancer cells and its modification by neuraminidase. Int. J. Cancer 13, 850-862 (1974).

Weiss, L.. Tumour necrosis and cell detachment. Int. J. Cancer 20, 87-92 (1977).

Weiss, L.. Dynamic aspects of cancer cell populations in metastasis. Am. J. Pathol. 97, 601-608 (1979).

Weiss, L.. Cancer cell traffic from the lungs to the liver: An example of metastatic inefficiency. Int. J. Cancer 25, 385-392 (1980).

Willis, R.A.. Pathology of Tumours. 4th Ed. Butterworths, London. (1967).

Willis, R.A.. The Spread of Tumours in the Human Body. 3rd Ed. Butterworths, London. (1973).

Winkelhake, J.L., and G.L. Nicolson. Determination of adhesive properties of variant metastatic melanoma cells to Balb/3T3 cells and their virus transformed derivatives by a monolayer attachment assay. J. U.S. Natl. Cancer Inst. 56, 285-291 (1976).

Witter, R.L., E.A. Stephens, J.M. Sharma and K. Nazerian. Demonstration of a tumour associated surface antigen in Marek's disease. J. Immunol. 115, 177-183 (1975).

Wood, J.S., E.D. Holyoke and J.H. Yardley. An experimental study of the influence of adrenal steroids, growth hormone and anticoagulants on pulmonary metastasis formation in mice. Proc. Am. Ass. Cancer Res. 2, 157 (1956).

Wood, S.. Pathogenesis of metastasis formation observed in vivo in the rabbit ear chamber. Arch. Pathol. 66, 550-568 (1958).

Woodruff, J.J., and J.F. Woodruff. Virus induced alterarions of lymphoid tissues. IV. The effect of Newcastle disease virus on the fate of transfused thoriacic duct lymphocytes. Cell Immunol. 10, 78-85 (1974).

Woodruff, J.J., I.M. Katz, L.E. Lucas and H.B. Stamper. An in vitro model of lymphocyte homing. II. Membrane and cytoplasmic events involved in lymphocyte adherence to specialized high endothelial venules of lymph nodes. J. Immunol. 119, 1603-1610 (1977).

Wright, D.H.. The pathology of Burkitt's lymphoma - A review. In: P.M. Biggs, G. de-Thé and L.N. Payne (ed). Oncogenesis and Herpesviruses. pp. 217-229. IARC, Lyons. (1972).

Wright, T.C., T.E. Ukena and M.J. Karnovsky. Relation of lectin induced and spontaneous adhesion to tumourigenecity. Prog. exptl. Tumour Res. 22, 1-27 (1978).

Yogeeswaran, G., B.S. Stein and H. Sebastian. Altered cell surface organization of gangliosides and sialylgycoproteins of mouse metastatic melanoma variant lines selected in vivo for enhansed lung implantation. Cancer Res. 38, 1336-1344 (1978).

Yogeeswaran, G., H. Sebastian and B.S. Stein. Cell surface sialylation of glycoproteins and glycospingolipids in cultured metastatic variant RNA virus transformed non-producer Balb/c 3T3 cell lines. Int. J. Cancer 24, 193-202 (1979).

Yogeeswaran, G., B.S. Stein and H. Sebastian. Characterization of tumourigenic and metastatic properties of murine sarcoma virus transformed nonproducer Balb/c 3T3 cell lines. J. U.S. Natl. Cancer Inst. 64, 951-957 (1980).

Young, J.S., and H.D. Griffiths. The dynamics of parenchymatous embolism in relation to the dissemination of malignant tumours. J. Pathol. Bact. 62, 293-311 (1950).

Young, J.S., C.E. Lumsden and A.L. Stalker. The significance of the "tissue pressure" of normal testicular and neoplastic (Brown-Pierce carcinoma) tissue in the rabbit. J. Pathol. Bact. 62, 313-333 (1950).

Zeidman, I., M. McCutcheon and D.R. Coman. Factors affecting the number of tumour metastasis: Experiments with a transplantable mouse tumour. Cancer Res. 10, 357-359 (1950).

Zeidman, I., and J.M. Buss. Transpulmonary passage of tumour emboli. Cancer Res. 12, 731-733 (1952).

Zeidman, I., W.J. Gamble and W.L. Clovis. Immediate passage of tumour cell emboli through the liver and kidney. Cancer Res. 16, 814-815 (1956).

Zeidman, I.. Metastasis: A review of recent advances. Cancer Res. 17, 157-162 (1957).

Zeidman, I.. The fate of circulating tumour cells. I. Passage of cells through capillaries. Cancer Res. 21, 38-39 (1961a).

Zeidman, I.. A mechanism of cortisone action in increasing numbers of metastasis. Proc. Am. Ass. Cancer Res. 3, 281 (1961b).