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NATURE OF RECEPTOR-ACCEPTOR INTERACTIONS IN ORGAN SPECIFIC METASTASIS

by

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Date November 28, 1983

ABSTRACT

Using a modification of the *In Vitro* Frozen Section Adherence Assay (FSAA), it has been possible to demonstrate a role for receptor-acceptor interactions in a Marek's disease T-cell lymphoma. AL-2.1, selected for liver metastasis, adhered more strongly to frozen sections of liver than did AL-3.1 cells. AL-3.1 is an appropriate control for specific AL-2.1 adherence because it was selected from the same initial population as AL-2.1 but was selected for metastasis to the ovaries, and not the liver. The ratio of binding between AL-2.1 and AL-3.1 on liver in the FSAA was 2.0-5.0 to 1. Using a variety of other tissues such as lung or heart, the AL-2.1/AL-3.1 ratio was approximately 1 to 1. Since the nature of this assay requires the physical binding of tumour cells to the frozen section, this provides the first direct evidence for receptor-acceptor interactions between the tumour and its target organ.

To examine the nature of this receptor-acceptor interaction, a variety of carbohydrates, lectins, enzymes and monoclonal antibodies were used. Their effect on the interaction of AL-2.1 cells binding to liver sections has given some indication of the nature of the cell surface molecules involved. Significant inhibition of AL-2.1 foci formation *in vivo* or binding *in vitro* was achieved for *a*-L(-) fucose, p-nitrophenyl-*B*-D-fucopyranoside, lactulose, trypsin, and the lectins *Ulex europaeus* and *Tetragonolobus purpureas*. The inhibition obtained for *a*-L(-) fucose was approximately 50% of controls while the remaining compounds gave approximately 100% inhibition. The sugars for which no effect was observed included *a*-D(+) fucose, glucosylamine, galactosylamine, and a variety of nitrophenyl *a* and *B*, D and L, galacto- and gluco-pyranosides. Two monoclonal antibodies made against AL-2.1 were also inhibitory. However, these antibodies reacted with AL-3.1 cells and so the mode of inhibition may have been non-specific. Other sugars, lectins and monoclonal antibodies tested also failed to produce any inhibition.

The preponderance of fucose containing molecules that can inhibit this interaction suggested that this determinant may play a role in the receptor-acceptor interaction. Using enriched liver populations and the lectin *Ulex europaeus* (affinity for *a*-L(-) fucose), it is likely that liver endothelial cells possess fucose residues on their cell surface. Furthermore, AL-2.1 cells specifically bound ^{125}I-fucose-BSA, the binding being trypsin sensitive. This supports the existence of a lectin-like receptor on the tumour which interacts with the fucose determinants on the enriched liver endothelial population.

Attempts to produce monoclonal antibodies specific for AL-2.1 were unsuccessful. Furthermore, direct examination of the cell surface proteins and glycoproteins from AL-2.1 and AL-3.1 using polyacrylamide gel electophoresis failed to show any differences.

It is proposed that the receptor for fucose on the surface of AL-2.1 cells and the fucose containing cell surface determinant on enriched liver endothelial cells make up the receptor and acceptor molecules, respectively.

This data is examined in light of the mechanisms proposed for organ specific metastasis. The first mechanism, or "seed and soil", predicts that soluble molecules in the micro-environment of the organ provide the necessary milieu for certain tumour cells and thus affects the metastatic patterns observed. The second mechanism, or "receptor-acceptor", hypothesizes the existence of cell surface receptors on tumour cells that recognize acceptor molecules on the target organ. Through this interaction, the tumour specifically recognizes and subsequently colonizes its target organ.

PREFACE

Portions of the work described in this thesis have been published in *Cancer Metastasis Reviews.*

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Abbreviations

CAM	- chorioallantoic membrane
media	- RPMI 1640 + 10% FCS + Garamycin
MDV	- Marek's Disease Virus
DMSO	- dimethyl sulfoxide
DOC	- deoxycholate
Page gels	- polyacrylamide gel electrophoresis
SDS	- sodium dodecyl sulfate
NaN_3	- sodium azide
MAb	- monoclonal antibody
EDCI	- 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl
FCS	- fetal calf serum
GVH	- graft vs host disease
MW	- molecular weight
FSAA	- frozen section adherence assay
LMAA	- liver metastasis associated antigen
N.S.	- not-significant
S.E.	- standard error
Pd-C	- palladium charcoal
Con-A	- Concanavalin A
PNA	- Peanut agglutinin
MPA	- Maclura pomifera

I. LITERATURE REVIEW

The ability of a neoplasm to avoid elimination by both the immune system and the oncologist resides in the tumour's ability to i) evolve rapidly and ii) produce distant foci, i.e. metastases (reviewed by Fidler, 1978a; Fidler and Hart, 1981; Marx, 1982; Stangly and Sala, 1977; Fidler *et al.*, 1978; Fidler, 1978b; Sugarbaker, 1979; Roos and Dingemans, 1979; Poste and Fidler, 1980; Sugarbaker, 1981; Sugarbaker *et al.*, 1982; Nicolson, 1982; Hart, 1982; Schirrmacher *et al.*, 1983). The generation of variants within the primary tumour ensures that there is selection for more malignant phenotypes. Cells are able to break away from the primary tumour, move through the body and relocate, often in specific organs. While many potentially metastatic cells are unable to withstand their new environment, the heterogeneity of the primary tumour ensures a diverse array of variants, allowing some cells to evolve the necessary characteristics to survive and grow to form metastatic foci.

A. The Generation of Tumour Variants

The variability in phenotypes of tumour cells derived from the same population of cells has become one of the hallmarks of a developing neoplasm. The phenotypic variability of tumour populations is well documented for a number of cell properties including immunogenicity (Fugi and Mihich, 1975; Killion and Kollmorgen, 1976; Kerbel, 1979), invasion and metastasis (Fidler, 1978b; Kripke *et al.*, 1978; Dexter *et al.*, 1978; Suzuki *et al.*, 1978), drug sensitivities (Barranco *et al.*, 1972; Hakansson and Trope, 1973; Hakansson and Trope, 1974; Trope, 1975; Heppner *et al.*, 1978), and antigenicity (Prehn, 1965; Prehn, 1970; Fogel *et al.*, 1979; Shearman and Longenecker, 1981). Nowell's model (Nowell, 1976) provides a 'mechanism' for the observed heterogeneity in tumour cell populations. His model posits that variants within a tumour population arise as a result of genetic instability and are subjected to

environmental and immune selective pressures by the host. In this way, only those subclones that can successfully compete for nutrients and avoid elimination survive, resulting in an enhanced malignant phenotype. The primary tumour then acts as the raw material for metastases and the selection and evolution of the tumour ensure its refinement to the point where some cells can survive, migrate, and grow in their new environment. This process of the continuing evolution toward a more malignant tumour is referred to as "progression" (Nowell, 1976).

An examination of the property of tumour heterogeneity appears to confirm that tumours consist of a large array of clones that differ in their ability to metastasize (Fidler, 1978b; Fidler and Hart, 1981; Prehn, 1970). This heterogeneity can be generated from a cloned population of cells (Fidler and Hart, 1981), thus strongly supporting the hypothesis of Nowell (Nowell, 1976). While the characteristics of most tumours are heterogeneous, the 'original' tumour appears to have arisen from a single cell. This is inferred from studies based on i) the glucose-6-phosphate dehydrogenase enzyme in women (Sandberg and Hossfeld, 1970), ii) karyotype analysis (Fialkow, 1974) and, iii) immunoglobulin producing lymphoproliferative neoplasms (Milstein *et al.*, 1967). Not all tumours are found to be monoclonal in origin: the clonality of a tumour may be due to the way it is induced, as evidenced by chemically-induced neoplasms that are often polyclonal (Reddy and Fialkow, 1979).

The stability of tumour variants is difficult to assess *in vivo* because host selection is perpetually occurring; however, there is evidence that heterogeneity *in vivo* does occur, and may, in fact, be enhanced to that observed *in vitro* (Chow and Greenberg, 1980). *In vitro*, it appears that, even over short periods of time, significant changes in the malignant and cell surface properties of cells can occur; in some cases, these changes are reproducible, suggesting a genetic mechanism (Nicolson, 1982; Neri and Nicolson, 1981).

Furthermore, cells of high metastatic potential may be able to mutate more rapidly than their low metastatic counterparts as measured by their sensitivity to 6-thiopurine and/or ouabain (Cifone and Fidler, 1981).

In summary, many neoplasms appear to arise from single cells, multiply and rapidly produce genetic variants. These variants compete in the environment, where the less malignant phenotypes are selected against. Once in the circulation, the tumour cell must rapidly re-establish itself before the rigors of the circulatory and immune system destroy it.

B. **Organ Specific Metastasis (OSM)**

The preference of many metastasizing tumours for specific sites, both of natural (Sugarbaker, 1981) and experimental (Fidler and Nicolson, 1976; Shearman and Longenecker, 1980) origin, is now well-documented (reviewed by Nicolson, 1982; Hart, 1982). The precise mechanism by which tumours are able to selectively colonize a specific organ is more controversial. At present, three mechanisms stand out as possible explanations for the phenomenon observed. It is important to realize, however, that the mechanisms are not mutually exclusive: conceivably all three or a combination thereof, account for the organ specificity observed in a given system. The three mechanisms are refered to as the i) mechanical, ii) seed and soil, and iii) specific tumour cell adherence (STCA). While the third mechanism may be considered a special case of the seed and soil hypothesis, it will be regarded as separate in the context of this work (Hart, 1982).

C. Experimental Models of Organ Specific Metastasis

To study the diverse mechanisms involved in OSM, many investigators have selected metastatic variants and compared them either to the unselected parent line (Fidler and Nicolson, 1976) or to lines that were selected to colonize other organs (Shearman and Longenecker, 1980). This approach, coupled with data from lymphocyte homing and embryonic tissue organization experiments, has improved our understanding of the mechanisms of OSM.

A number of different cell lines selected for organ specific colonization have been compiled by Nicolson (Nicolson, 1982). Using these cell lines, factors such as sex (Shafie and Liotta, 1980; Thompson, 1976), age (Thompson, 1976), health (Fidler, 1978a), route of injection (Nicolson, 1982), and origin of tumour line (Nicolson, 1982) have all been shown to have profound effects on the types of metastatic spread obtained. The large number of available lines highlights the importance of the proper choice of an experimental animal model critical for the analysis of the phenomenon, since there are several factors that may affect the eventual outcome of the metastatic event (Poste and Fidler, 1980).

The development of tumour lines specifically enhanced for metastasis to a particular organ has had a great impact on our understanding of OSM. The method first developed by Fidler (Fidler, 1973a), has now been adapted by many laboratories for a variety of animal systems including mouse (Fidler, 1973a), rat (Talmadge *et al.*, 1981), guinea pig (Fidler, 1978a), chicken (Shearman and Longenecker, 1980), as well as for human cells in nude mice (Takahashi *et al.*, 1978). The basic procedure (Fidler, 1973a) is as follows: tumour cells (parental) are injected into an animal and metastases are allowed to form. Single foci are picked, grown up in culture and reinjected. This is repeated until a satisfactory increase in the number of organ specific metastatic

lesions occurs. While Fidler's original procedure called for intravenous (I.V.) injections, it is conceivable that tumour cells selected in this way may be unable to invade the circulation, an important step in the events leading to successful colonization. While it is not clear that this problem exists (Nicolson, 1982; Talmadge *et al.*, 1981), a new selection protocol can be used in which injections are given intraperitoneally (I.P.) or intramuscularly (I.M.) on each successive *in vivo* cycle of the selection procedure (Maguida *et al.*, 1980).

D. Mechanisms of OSM

1) The Mechanical Hypothesis:

The 'mechanical' hypothesis, first proposed by Ewing (Ewing, 1928), is composed of two parts: the first dealing with local tumour spread and the second with tumour formation by the trapping or sieving of tumour emboli.

Eaves (Eaves, 1973) proposed that tumours spread locally along the lines of least resistance, similar to the way in which plant roots move through the soil (Fidler *et al.*, 1978). As a result, those areas in direct line of the path of least resistance will become the target for local metastasis. For example, ovarian tumours often metastasize to the peritoneal cavity (Fidler, 1978a). One mechanism by which this physical extention of the tumour mass occurs may involve the copious secretion of enzymes by the tumour cells into the adjacent area (Strauch, 1972; Eisenstein *et al.*, 1975).

The second mechanical hypothesis involves the filtering (sieving) of individual tumour cells or emboli from lymph or blood. The ability of tumour cells to form emboli is well documented (Fidler *et al.*, 1978; Sugarbaker, 1981; Fidler, 1973b; Liotta *et al.*, 1976). Briefly, emboli can form as the

result of interactions between tumour cells (Liotta *et al.*, 1976), called homotypic interaction, or with blood components such as platelets (Gasic *et al.*, 1973) and lymphocytes (Fidler and Bucana, 1977), called heterotypic interactions. Emboli trapping usually occurs in the first capillary bed encountered; for tail vein injections, this organ is the lung (Fidler, 1973b; Liotta *et al.*, 1976). Tumor cells that are shed from the primary neoplasm often end up in the venous circulation which may account for the large number of lung metastases observed (Stangly and Sala, 1977). While the trapping of emboli is non-specific, in that an organ with an extensive capillary or sieve-like structure (lung, liver, lymph node) will stop any emboli, most tumour cells just upstream from the particular sieving structure can be trapped by that organ, producing an apparent organ specificity. However, many tumour cells may be able to escape this sieving as a result of their deformability (Zeidman and Buss, 1952; Zeidman, 1961; Wood, 1964; Sato and Suzuki, 1976).

There are many examples of OSM that cannot be explained by the mechanical hypothesis (Raz and Hart, 1980; Nicolson and Custead, 1982). Therefore, it is necessary to examine the role, if any, of the other two mechanisms mentioned above.

2) The Seed and Soil Hypothesis:

The second general mechanism is referred to as the "seed and soil" hypothesis, the name taken from the analogy drawn by Paget (Paget, 1889) to explain OSM. This hypothesis predicts the growth of tumour foci to be the direct result of the microenvironment provided by the specific target organ. When one considers the rigors of the blood circulatory and lymphatics systems, host defense mechanisms, and the need to lodge in the appropriate "soil", it might appear that cells shed from a primary tumour have little chance of surviving and, in fact, this is what occurs. Fidler (Fidler, 1970; Fidler, 1976)

and others (Reid and Gibbins, 1979) have found that 24 hours after the injection of tumour cells, only 1% of the cells were viable and that by the time foci were visable two weeks later, only 0.1% of the original cells remained. This may explain why so many cells need to be shed from the primary tumour in order to produce even a few metastatic lesions. Furthermore, specific interactions with an organ may aid in the rapid formation of stable associations between tumour and organ and allow more rapid escape of the tumour from the circulation.

Direct evidence for the seed and soil hypothesis has been difficult to obtain because of the problems in delineating the contributions of this mechanism from the specific tumour cell adherence (STCA) mechanism. It is extremely difficult to design an experiment that directly tests Paget's theory because disrupting the tissue environment can also alter tumour formation patterns (Agostino and Cliffton, 1965; Fisher *et al.*, 1967). Hart (1982) offers one plausible experimental approach: the injection of a single cell suspended in a small volume, which should not significantly disrupt the organ, so that 'normal' tumour development can be determined. The experiments that have addressed the "seed and soil" theory have centred on examining organ extracts for their ability to stimulate or suppress the growth of tumour cell lines (Hart, 1982). The data in this area remain equivocal, largely because no consistent finding between tumour cell line and organ extract has been found (Nicolson, 1982). For each enhancing factor found, there appears to be a suppressing factor (Klein *et al.*, 1979).

Experiments often used to support Paget's hypothesis (Sugarbaker, 1952) support the STCA hypothesis (see below) equally well. Such experiments (Kinsey, 1960; Hart and Fidler, 1980) have used organ grafts in subcutaneous sites. Tumor cells were injected I.V. into animals and found to home preferentially to the natural organ and the matched transplant. For example, a cell line specific

for lung metastasis would home to the lung and to the lung graft but not to grafts of different organ types, e.g. liver. Although these experiments support the existence of some mechanism for metastasis to specific organs, they do not differentiate between Paget's seed and soil hypothesis and the STCA hypothesis. Perhaps the best evidence in support of Paget's theory is the existence of factors capable of stimulating normal cell growth (Gospodarowicz *et al.*, 1978).

It appears therefore that the evidence in support of Paget's theory relies mostly on fragmentary data, although analogy with normal growth factors makes the theory very plausible. Direct evidence for the seed and soil hypothesis will have to await further experimentation.

While not wholly refuting Paget's theory, there is recent evidence that not all tumour metastases are due to adaptation of tumour cells to the organ microenvironment. In some experiments, brain or lung metastatic cells were targetted to their organs (injected directly into the brain or grown on beads that are trapped in the lungs). After repeated selection in this manner, these metastatic cells were no better able to colonize their respective organs than the original unselected line. Cells from the same original population injected I.V. and selected without the beads were more specifically metastatic after identical selections to those grown on beads (Nicolson and Custead, 1982). Nicolson and Custead (1982) concluded that the observed specific metastasis of the free cells was the result of selection of pre-existent metastatic cell types and was not due to adaptation of cells to their environment. The fact that cells targetted to the lungs on beads were able to grow into distinct foci, yet these same cells, injected in the absence of the beads, were unable to home to their 'selected organ', can be interpreted to mean that the environmental conditions within the organ were not necessary for the selection.

Further support stems from Brunson and Nicolson's (Brunson and Nicolson, 1980) report which showed that ten cycles of direct intracerebral injection of B16-F1 cells did not result in increased OSM to the brain following I.V. injection. Collectively, these experiments provide good evidence that the phenomenon of OSM is not solely due to alterations caused by adaptation of the tumour cells to a particular microenvironment.

3) The Specific Tumour Cell Adherence Hypothesis:

This leads to the third or STCA hypothesis which invokes the existence of molecules on tumour cell variants that specifically recognize the molecules on cells of the appropriate organ. Since cells in the circulation first come into contact with endothelial cells lining blood vessels, it is reasonable to propose that the initial recognition molecule is expressed on the endothelial cell surface. This does not preclude the existence of recognition molecules on the parenchymal cells involved in subsequent invasion of the organ parenchyma (Middelkoop *et al.*, 1982).

This recognition system can be envisioned easily by either of the following two mechanisms: i) Self-self interaction whereby the order and number of molecules at the cell surface is complementary to the same order and number of molecules on the other cell surface (Steinberg, 1963); or ii) a lock and key, receptor-acceptor type recognition whereby the recognition molecules may be receptors, lectins or enzymes (Hakomori, 1981). A common approach for looking at recognition systems has been to assay for rosettes or aggregrates between tumour and organ cells (Nicolson and Winkelhake, 1975; Kahan, 1979; Schirrmacher *et al.*, 1980). While these studies directly support the existence of recognition mechanisms, the organ cell suspensions were not purified or enriched for endothelial versus parenchymal cells, so the precise cell type to which the tumour was binding remains unclear. Roos has proposed that

tumour cells interact with endothelial cells by a receptor-acceptor interaction (Roos *et al.*, submitted). Tumour cell protrusions are extended through the endothelial cell and may be anchored to receptors at the extra-vascular site of the endothelium and make contact with the parenchymal cell surface. There, it establishes further stable interactions. In this way, the tumour cell is rapidly drawn out of the blood vessel and into the space between the endothelial and parenchymal cell, thus reducing the tumour cell's chances of being eliminated. Furthermore, Roos's group has produced a rabbit antiserum which inhibits the binding of the tumour cell to both endothelial (Roos *et al.*, submitted) and parenchymal cells (Middelkoop *et al.*, 1982). This data argues strongly in favour of specific recognition molecules being important in OSM.

In further support of the STCA hypothesis is the work of Shearman and Longenecker (Shearman and Longenecker, 1981; Shearman and Longenecker, 1980; Shearman *et al.*, 1980), who were able to specifically inhibit the metastasis of a cell line selected for liver metastasis by pretreating the cell line with a monoclonal antibody directed against a specific cell surface determinant. Similar results have been obtained for normal lymphocyte homing (Stamper and Woodruff, 1976; Butcher *et al.*, 1979). In these experiments, specific subpopulations of lymphocytes or lymphomas were found to home to, and bind, the high endothelial venules of certain lymph nodes (Stamper and Woodruff, 1976; Butcher *et al.*, 1979) or brain (Kuttner and Woodruff, 1979). Gallatin *et al.*, (1983) have recently made a monoclonal antibody against the receptor on lymphomas that specifically inhibits the binding of these cells to their target organ. Characterization of the receptor is now in progress (see below). Stoolman and Rosen (1983) have just reported that this interaction can be specifically inhibited by a variety of fucose containing compounds *in vitro*. This work closely resembles the data presented here and provides strong support both for the approach taken and the results obtained. Further evidence for the

involvement of cell-surface molecules has come from the work of Poste and Nicolson (1980), who examined the requirement of the tumour cell membrane in organ specific metastasis. Briefly, they fused the membranes of a high metastatic cell line (B16-F10) to a low metastatic cell line (B16-F1) and found the modified B16-F1 cells behaved more like the high metastatic B16-F10 line. An improvement on this last experiment has been proposed by Nicolson (1982). The procedure requires the fusion of membrane vesicles from one highly metastatic cell line, say brain (Brunson *et al.*, 1978), with the surface of a second highly metastatic cell line, say liver (Tao *et al.*, 1979). Since both cell lines are highly metastatic, any change in metastatic pattern should be the result of surface molecules transferred from one to the other. If one of the cell lines is not highly selected for OSM (as in the experiment described above), a change in metastatic pattern could be attributed to the expression of cytoplasmic as well as surface molecules (Poste and Nicolson, 1980). The ability of labelled vesicles to home to specific organs could also provide good evidence for the STCA model.

In contradiction to the data presented above are studies that measured the ability of tumour cells to bind to monolayers of cultured organ cells. It was found that the binding did not correlate with the frequencies of tumours found in these organs *in vivo* (Hart, 1982) as the STCA model might predict. However, since purified cell populations were not used (ie., the cell monolayers presumably included fibroblasts, parenchymal and endothelial cells), no definite conclusions can be drawn with respect to the role of, for example, endothelial cells in STCA. In addition, it is known that endothelial cells may be shed *in vivo* exposing the basement membrane. Perhaps this structure, alone or in association with the endothelial cell, is important in the stablization of tumour cell-organ cell interactions (Hart, 1982).

In a recent review, Hart (1982) points out that there is a distinct lack of correlation between the initial arrest of tumour cells in a given organ and the subsequent development of metastasis in that organ. He argues that if cell adhesion plays a dominant role in determining metastatic patterns, distribution studies with labelled tumour cells should reveal that the initial pattern of arrest closely reflects patterns of metastatic development. However, few organ distribution studies using organ-specific metastatic variants have been reported. Hart (1982) describes an interesting series of experiments in which B16-F10 melanoma cells, selected for lung metastasis, were compared in organ distribution studies with M5076 reticulo-sarcoma cells, which show marked preference for the liver. Despite the differing patterns of metastasis of the two cell lines, the degrees of initial arrest in the lungs were almost identical, indicating that arrest of a tumour cell in an organ does not guarantee that it will form a metastatic lesion there.

In addition, caution should be exercised in the interpretation of organ distribution studies using radioisotopes since greater than 99% of injected tumour cells apparently die within 24 hours of injection, at least in some tumour systems. Indeed, the early studies of Green and Harvey (1964) demonstrated that viable radiolabelled tumour cells exhibited distinct patterns of tissue distribution and differing metastatic behaviour. One pattern was characterized by a limited distribution of tumour cells to organs that subsequently became the site of metastasis, whereas other cells demonstrated a widespread organ distribution without any apparent relationship to the eventual location of metastases. It is apparent that, while the retention of tumour cells at a particular site is an essential process in the establishment of metastasis, metastasis is not an inevitable consequence of tumour cell retention. Furthermore, these studies point out that generalizations about the behaviour of tumours based on studies with just a few tumour lines may not be universal.

With respect to normal cell types, the phenomenon of organ specific homing has been demonstrated for lymphocytes in the adult (Stamper and Woodruff, 1976), while in the embryo, cell migration and specific homing patterns are widespread and essential for normal development. Kahan's model for OSM is particularly fascinating in this regard (Kahan, 1979). She has observed that a number of unselected murine ovarian teratocarcinomas show metastatic growth that is restricted to the ovaries in the adult regardless of the route of injection of the tumour cells. This phenomenon resembles an important feature of normal embryonic cells, that is the, selective migration to the ovary by female germ cells, which in some species occurs via the blood stream, and their subsequent maintenance and growth in the gonads. In adhesion assays, specific adherence of the teratocarcinoma cells to embryonic ovary monolayers indicates that the specificity of cell surface recognition molecules may be of importance in the homing of the tumour cells to the ovary. However, there may be additional factors to consider, since in normal germ cell migration only those cells that successfully reach the gonad continue to survive and give rise to progeny. Thus "factors" that promote germ cell proliferation and survival would seem to be of equal importance to consider in the teratocarcinoma model. This re-emphasizes an important point: STCA and "seed and soil" are not mutually exclusive mechanisms of OSM and the teratocarcinoma model may be an excellent one for studying the relative roles and possible interactions involving these two mechanisms. Organ localization studies might be particularly informative in this model for determining whether or not there is an initial preference for arrest of the teratocarcinoma cells in the ovary. Another interesting possibility suggested by this model is that other types of tumour cells, some of which are known to re-express embryonic genes, might revert to other embryonic characteristics such as the capacity for organ specific colonization, perhaps by the re-expression of 'embryonic' cell surface receptors.

E. Detection of Cell Surface Antigens Associated with OSM

Our knowledge of the receptors on many cell surfaces will perhaps make the receptor-acceptor hypothesis easier to elucidate. Biochemical techniques for the analysis of cell surface determinants are fairly well advanced and have given many insights into the types of interactions, both protein and carbohydrate in nature, that make up cell-cell recognition systems (Feizi, 1981).

Of particular interest is the role of cell surface carbohydrates which are suggested to be involved in many aspects of cellular differentiation, proliferation, homing and invasion (Schirrmacher *et al.*, 1983; Simpson *et al.*, 1978). Many investigators have reported the presence of cell surface lectins on a variety of cell types where it is likely they function to mediate cellular interactions by binding with glycoproteins, glycolipids or glycosaminoglycans present on the surface of adjacent cells (Goldstein and Hayes, 1978; Hakomori, 1981; Yogeeswaran, 1983). Many lectin-resistant tumour variants demonstrate altered metastasizing capacities Kerbel *et al.*, 1982; Tao and Burger, 1982), suggesting that carbohydrates might have a functional role in cell recognition during metastasis. Raz and Lotan (1981) reported that several human and murine tumour cells have cell surface lectins specific for galactoside residues. Such lectins could presumably serve to mediate attachment of tumour cells to specific cell surface sugar residues and hence mediate the process of OSM. Conversely, lectins present on normal cells could mediate specific attachment to sugar residues on tumour cells.

A variety of results have been observed regarding the role of carbohydrates and metastasis. The gain or loss of metastatic capacity has depended on the tumour system used. Schlepper-Schafer (Schlepper-Schafer *et al.*, 1981) reported that certain rat tumour cells, but not normal blood cells, appeared to bind to normal liver cells via N-acetylgalactosaminyl or

galactosyl-specific lectins. Similar results were obtained with the ESb lymphoma which suggested that tumour-cell liver-cell rosetting is mediated by hepatocyte lectin receptors that can bind to asialoglycoproteins (Schirrmacher *et al.*, 1980; Schirrmacher *et al.*, 1983). Springer (Springer *et al.*, 1983) found that the binding of ESb cells to hepatocytes could be inhibited with the T antigen (*B*-Gal-*a* (1-3) GalNAc). This is compatible with hepatocyte lectin receptors (Ashwell and Morell, 1974) as being tumour cell "recognition" structures. The normal recirculation of T-lymphocytes to lymph nodes has also been inhibited by fucose determinants, again supporting the notion that carbohydrate-lectin like molecules do mediate cell-cell binding. Neuraminidase treated erythrocytes and lymphocytes adhere firmly to mammalian hepatocytes via a galactose-specific hepatic membrane receptor (Kolb *et al.*, 1978). The majority of neuraminidase-treated lymphocytes do not home to lymphoid organs but are trapped in the liver until normal membrane properties are regenerated (Freitas and de Sousa, 1976; Woodruff and Woodruff, 1974). The hepatic galactose specific lectin may therefore play a role in the trapping of desialylated cells. This suggests that tumour cell lines that have been selected for OSM to the liver should be examined to determine whether they have an affinity, which is inhibited by galactose, for hepatic parenchymal cells.

Many cell surface properties have been well characterized for differences between malignant and nonmalignant cell types (Nicolson, 1982; Hynes, 1976). The treatment of cells with a variety of different enzymes such as trypsin (Hagmar and Norrby, 1973) and neuraminidase (Sinha and Goldenberg, 1974) has shown that these can affect the pattern or outcome of metastases as well as the homing of lymphocytes to lymph nodes (Woodruff, 1974).

Recently, a great deal of evidence has been presented strongly suggesting that galactosyl and N-acetylgalactosaminyl residues in cell surface glycoproteins and/or glycolipids of tumour cells may be important in the

process of metastasis, not only to liver (see above), but to other organs as well. Irimura (Irimura *et al.*, 1981) has reported that tunicamycin treatment of B16 melanoma cells led to the reversible loss of the ability of these cells to produce lung metastases and this correlated with the loss of sialogalactoproteins from the tumour cell surface. Several investigators have reported that the degree of sialation of galactose containing cell surface glycoconjugates may be of special importance (Nicolson, 1982; Yogeeswaran *et al.*, 1978; Merritt *et al.*, 1978; Yogeeswaran and Stein, 1980; Yogeeswaran and Salk, 1981; Tao and Burger, 1977). For example, Tao and Burger (1977) reported that *in vitro* selected lectin-resistant B16 variants with decreased sialic acid content lost their metastatic properties. Yogeeswaran *et al.*, (1978) reported an increased sialic acid content on B16 sublines of high metastatic potential compared to sublines of low metastatic potential. Yogeeswaran and Salk (1981) recently proposed that the ability of a variety of murine tumour cells to metastasize from subcutaneous sites is directly proportional to their sialic acid content. Most interesting, however, was their data showing that the best correlation with metastatic capacity of a cell line was the degree of sialylation of galactosyl and N-acetylgalacetosaminyl cell surface residues. These investigators suggested that increased sialylation of cell surface galactoconjugates may contribute to increased metastatic capacity by increasing adhesiveness or decreasing the susceptibility of these cells to destruction by the immune system. The latter mechanism, mediated by pre-formed "natural" antibodies against asialogalactoconjugates, may play an especially important role in limiting metastasis (see below). It is important to realize that, while the data presented above suggests that lectin and cell surface carbohydrate interactions are important in OSM or their metastatic potential, there are other systems for which no such correlation can be made (Nicolson, 1982).

One method used to study the importance of cell surface molecules in metastasis has been to isolate a fraction of cells with a particular surface characteristic and compare that population to cells lacking this characteristic. This approach has been used many times for a variety of surface characteristics such as cell charge, density, and size (Turner *et al.*, 1980). Cell populations purified by surface charge alone have not yielded conclusive results (Klein and Klein, 1956; Weiss, 1979), although some investigators have been able to use combined cell surface properties (such as charge/sialic acid content) to enrich for cells with a higher metastatic phenotype (Miner *et al.*, 1981). This correlation does not necessarily mean that cell surface charge is responsible for metastasis since the molecular determinants involved in organ specific metastasis may be a group of interacting cell surface molecules. Surface charge, especially as a result of sialic acid, may only cover the 'real' molecule (compare to the theory of Yogeeswaran et al, 1978). This could explain why cell-surface charge can be important for selecting cells with enhanced metastatic phenotype, though removal of the negative charge by cleavage of the terminal sialic acid residues does not affect metastatic properties (Merritt *et al.*, 1978; Irimura and Nicolson, 1981).

Another approach to the characterization of the cell-surface molecules of metastatic cell lines has been lactoperoxidase-catalyzed ^{125}I-iodination and subsequent comparative autoradiography (Nicolson, 1982). The selection of RAW 117 lymphosarcoma for specific metastasis to the liver (Brunson and Nicolson, 1978) correlates with the loss of a 70K glycoprotein and the concomitant gain of a 135K molecule (Reading *et al.*, 1980). At present, however, it is unlikely that the aforementioned molecules, or similar molecules obtained from other systems (Goldstein and Hayes, 1978; Nicolson, 1981), are directly responsible for organ specific metastasis. They may be the result of the malignant transformation, or they may be molecules whose function is to stabilize the

relevant determinant on the cell surface, similar to the function of B_2-microglobulin for some class I MHC molecules. Furthermore, the cell lines used in this system appear to be macrophage dependent since the RAW 117P cell line does metastasize to the liver if macrophage activity is inhibited.

Fibronectin, a molecule known to be involved in cell-cell and cell-matrix interactions (Nicolson, 1982; Nicolson, 1981; Der and Stanbridge, 1980), has also been implicated for its role in metastasis (Smith *et al.*, 1979; Neri *et al.*, 1981). The wide distribution of this molecule on different cell types makes it an unlikely candidate for determining organ specific metastasis. More likely, it is involved in maintaining the attachment of the tumour cell to the target organ, perhaps in conjunction with the receptor-acceptor bond.

There has also been a concentrated effort to examine the role of enzymes in OSM, although most of it has centred on differences between malignant and nonmalignant cells and not on differences between cells of different organ specificity (Bosmann, 1972; Bosmann *et al.*, 1974). Correlations between highly malignant cells and their collagenase levels have been found (Liotta *et al.*, 1980) although this enzyme is more likely to be involved in the process of invasion than in organ recognition (Sage *et al.*, 1979). The role of plasminogen activator, a serine protease that converts serum plasminogen to plasmin (Unkeless *et al.*, 1974), does not appear to influence organ specific metastasis (Roblin, 1981). However, this enzyme may be involved in part of the malignant process (Chen and Buchanan, 1975). A number of other enzymes that degrade oligosaccharides (Dobrossy *et al.*, 1981; Chatterjee, 1979; Sloane *et al.*, 1981; Capel *et al.*, 1979) have also been correlated with malignant and metastatic potential. Again, it is unlikely that these enzymes play a role in the choice of organ; instead, they possibly assist in the invasion of the organ once the tumour cell has arrived (Nicolson, 1982).

Two problems have made it difficult to properly assess the data on enzymes in tumour systems (Nicolson, 1982). First, it is important to determine whether the enzymes under discussion are cytoplasmic or membrane-bound in origin. An understanding of this is necessary for a proper determination of their function. Second, the enzymes found in tumours can result from the contamination of host-immune or endothelial cells (Nicolson, 1982).

The involvement of glycolipids (Hakomori, 1981), especially the sialogangliosides GM_1, GM_2, and GM_3 have also been investigated recently (Raz *et al.*, 1980; Yogeeswaran and Stein, 1980; Yogeeswaran, 1983). Variations in the relative amounts of the different types of sialogangliosides, similar to the results obtained for glycoproteins, correlate with malignant capability (Yogeeswaran and Stein, 1980; Morre *et al.*, 1978). However, there is not enough information to extend significantly our knowledge of organ specific metastasis.

In summary, while many approaches have been used to isolate and characterize cell-surface determinants important for organ specific metastasis, the nature of these molecules remains elusive.

A more direct approach for the characterization of cell-surface determinants important in organ specific metastasis has been the production of monoclonal antibodies against organ specific cell lines. To study OSM, an avian model system was developed. AL-1 is a highly metastatic lymphoma cell line (Shearman and Longenecker, 1980) derived from a chicken with Marek's disease (MD). MD is a naturally occurring herpesvirus-induced T-cell lymphoma (Payne, 1972a; Nazerian, 1973). This model system was selected because of the pathological and etiological similarities between MD and Burkitt's lymphoma of man (Klein, 1972). One such similarity is the pattern of metastasis in which both malignancies give rise to a high incidence of ovarian and liver metastases (Payne, 1972a; Wright, 1972). It should be emphasized that the AL-1 cell line

shows the same pattern of metastasis as the natural disease. Therefore, an attempt was made to select OSM variants from this cell line that metastasize to those organs (liver and ovary) that show the highest frequency of metastatic lesions in the natural disease. This process was carried out on newly hatched chicks using standard protocols (Fidler, 1973a) and resulted in the isolation of two cell lines: AL-2, selected for liver metastasis, and AL-3 selected for ovarian metastasis. As expected, AL-2 cells were found to induce several hundred-fold more liver metastases than AL-3 cells (Shearman and Longenecker, 1981).

Interestingly, selection for ovarian preference of AL-3 may not have been successful (Shearman and Longenecker, 1981). It was suggested that this may be due to the status of the ovary as 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 because there would be no strong selective pressure in favor of specific tumour variants. A further obstacle would occur in situations, such as in the Marek's disease system, which have a high frequency of metastasis to the privileged site prior to selection. The involvement of the ovary as a common site of metastasis for MD lymphoma cells is interesting because females are known to be more susceptible than males to Marek's disease lymphoma and Burkitt's lymphoma (Payne, 1972a; Wright, 1972). Although the testes are seldom involved in Marek's disease, the rare testicular metastasis almost always occurs in the left testicle. This surprising and unexpected observation suggests that tumour cells can distinguish bilateral organs and, in this case, it may be related to the fact that the chicken ovary is unilateral and is derived from the left embryonic gonad, which also gives rise to the left testicle in the male. The right gonad degenerates during embryogenesis in females.

As stated previously, there is a large body of evidence suggesting that cell-surface molecules are involved in the process of OSM. In order to search for specific surface molecules on AL-2 cells important in OSM, CBA/J mice were immunized with these cells and a monoclonal antibody (MAb), called 1.20, which reacted specifically with AL-2 cells was produced (Shearman *et al.*, 1980). To test whether the antigen detected by 1.20 might be involved in liver specific metastasis, the capacity of 1.20 antibody to inhibit liver foci induced by AL-2 cells was determined. Only 1.20, and none of several other monoclonal antibodies of the same class also reacting with the cells, was found specifically to inhibit liver foci induced by AL-2 cells. Pre-incubation of the cells with the 1.20 antibody, followed by thorough washing prior to injection, was sufficient to partially inhibit liver metastasis; however, complete inhibition was never achieved. This is not surprising in view of the evidence that the expression of the antigen detected by 1.20 appears to be cell-cycle dependent (Shearman and Longenecker, 1981).

In order to confirm the importance of the antigen detected by 1.20 in liver metastasis, AL-2 clones were re-isolated and it was found that the number of liver foci formed by a given clone was directly proportional to the number of 1.20 antigen positive cells in that clone (Shearman and Longenecker, 1981). This strengthens the argument that the 1.20 antigen is involved in determining liver specific metastasis. These results also confirm the reports of several other investigators (Fidler, 1978a; Kripke *et al.*, 1978; Dexter *et al.*, 1978; Barranco *et al.*, 1972) concerning the clonal variation of metastatic properties in tumour cell lines and strongly suggests that this variation extends to the antigenicity of cloned metastatic variants (Shearman and Longenecker, 1981). The cells responsible for the production of liver metastasis are probably the same cells that express the 1.20 antigen, which is called the liver metastasis associated antigen or LMAA. LMAA may be functionally significant in the production of

liver metastasis, but the LMAA probably represents only one of the ways that a tumour cell may give rise to a liver metastasis: since other variants, which give relatively high liver metastasis and low LMAA expression can arise in culture (Shearman and Longenecker, 1981). This is not unexpected since the metastatic cascade is a complex series of events, only one of which might involve specific organ recognition. In this regard, it must be remembered that other mechanisms might be involved in this tumour model.

Two hypothesis have been considered as possible explanations for the role of LMAA in liver specific metastasis (Shearman and Longenecker, 1981). The LMAA may act as a receptor which recognizes the liver specific acceptor on the surface of hepatic blood vessel endothelial cells. An interaction between receptor and acceptor would arrest and trigger the metastasizing tumour cell to invade the liver. An alternative hypothesis is that the LMAA may not be involved in the specific recognition of liver endothelium, but might, in some other way, play a role in the successful colonization of the liver by the tumour cells. More in keeping with the "seed and soil" hypothesis, the LMAA may act as a cellular interaction structure (CIS) involved in the positional control of the tumour cells in the liver. The cells expressing this determinant 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 migration of embryonic cells (Lilien *et al.*, 1979). Maslow and coworkers (1980) have shown that metastatic variant cells, or conditioned medium from these cells, can inhibit embryonic cell aggregation and adhesion assays. Recently, Nicolson (1982) found that antisera directed against an embryonic antigen involved in fetal liver cell aggregation could inhibit liver metastasis by RAW 117 lymphosarcoma cells. If the LMAA is a CIS, then it might be predicted that the LMAA should inhibit the aggregation of liver cells or liver cells with AL-2 cells. Unfortunately, the low affinity of this 1.20 antibody has made immunoprecipitation of the LMAA very

difficult. However, recent data on the specificity of the 1.20 antibody show that it reacts with a synthetic trisaccharide (Shearman, personal communication) blood group-like antigen; this suggests that carbohydrate structures on AL-2 cells may be important determinants for OSM.

The cell-surface interactions involved in lymphocyte homing (Stamper and Woodruff, 1976; Butcher *et al.*, 1979) have provided the framework for an *in vitro* procedure to detect the adherence of lymphocytes to high endothelial venules (HEV) in frozen sections. Using this assay, the presence of subpopulations of lymphocytes with specific receptors for HEV of various lymph nodes has been shown (Stamper and Woodruff, 1976; Butcher *et al.*, 1979). Furthermore, these investigators suggest that this is the manner by which specific lymphocyte subpopulations migrate to specific lymph nodes. The incorporation of an internally labelled fluorochrome standard population of cells (erythrocytes or fixed lymphocytes) serves to control for random, non-specific association of the test population with HEV and makes the assay an extremely sensitive and reproducible one. The advantages of the frozen section adherence assay applied to the study of metastasis over the *in vivo* approach are numerous: i) The assay requires only a couple of hours while *in vivo* metastatic assays require many days or weeks. ii) A variety of organs that do not produce easily visible foci (e.g. ovary) can be assayed for the adherence of tumour cells. iii) The technique easily lends itself to 'panning' in which organ specific cell lines can be selected from unselected lines without the lengthy and laborious methods of *in vivo* selection (Fidler and Nicolson, 1976). iv) This system can be used to examine cellular interactions in human systems and in allogeneic and xenogeneic combinations without the complications of immune rejection. v) Finally, the assay is amenable to the testing of various compounds, including specific monoclonal antibodies, lectins, and sugars that might inhibit adherence and thus allow for a better definition of the types and specificities

of cell surface molecules involved in adherence. The application of this technique to the study of human metastasis may be of great benefit and reduce our dependence on data from complicated clinical investigations or from xenogeneic model systems. Similar approaches using cultured endothelial cells from specific organs or their membrane sonicates can complement the frozen section assay and provide a more convenient method for selecting OSM variants *in vitro*. Nicolson (personal communication) has now developed long-term endothelial cell lines from many organs which will make these kinds of experiments possible.

F. The Role of the Liver Metastasis Associated Antigen (LMAA)

While the LMAA is an interesting and exciting marker, it is unlikely that it evolved solely so that lymphoma cells could metastasize to the liver. More likely, LMAA is a molecule that is important for specific lymphocyte homing to the liver (recall that the tumour arose from a T lymphocyte). Cell surface [redacted]hat enable lymphocyte subpopulations to migrate to specific lymph [redacted] been described (see previous section). Recently, Gallatin *et al.*, [redacted]e derived a monoclonal antibody that apparently blocks the HEV [redacted]eceptor on normal lymphocytes and lymphoma variants. Using the f[redacted]section adherence assay described above, they found that their antibody in[redacted] the binding of normal lymphocytes and lymphoma cells to high en[redacted]lial venules in specific lymph nodes. These investigators have also used th[redacted]ntibody to purify and characterize the receptor. Interestingly, the apparent M[redacted] of the lymphoma receptor (=92,000 daltons) differs from the apparent M[redacted] of the normal lymphocyte receptor (=80,000 daltons). There is little ev[redacted]ce for the existence of specific lymphocyte receptors for organs other than lymp[redacted] nodes. The existence of such receptors on normal lymphocytes would provide a rational basis for OSM of lymphomas. If lymphomas use

normal homing-recognition mechanisms to achieve OSM, then what mechanisms do non-lymphoid malignancies employ? Many of the clinical observations, as well as much experimental work on the phenomenon of OSM, have involved non-lymphoid malignancies whose normal cell counterparts are not highly migratory, except perhaps during embryogenesis. The determination of the nature of putative receptor molecules on non-lymphoid malignancies, and their possible relationship to lymphoid receptors, must await the development of specific probes.

G. The Chicken Embryo as a Model For the Study of Metastasis

Most experimental data on the metastatic process have come from murine tumour systems. In these systems, one is usually limited to the testing of syngeneic tumour cells. Nude mice may allow the testing of some xenogeneic tumours but metastasis in this system is difficult to maintain. An alternative is to use an embryo, in which the immune system is not sufficiently developed to actively reject xenografts. The chicken embryo offers several advantages in this respect. First, it is inexpensive and easy to handle and maintain. Second, the highly vascular chorioallantoic membrane (CAM), which surrounds the embryo, readily accepts transplants from a variety of xenogeneic tissues. Furthermore, a variety of tumours readily metastasize to the CAM and form easily counted metastatic lesions. Third, the chick embryo lacks a competent complement system, thus allowing one to examine the blocking effect of antibodies on cell surface receptors without having to worry about complement-mediated cytotoxicity. Fourth, the chick embryo can be manipulated over a wide temperature range, providing an opportunity for testing temperature-sensitive tumour cells for properties thought to be involved in the metastatic process. Finally, a variety of mammalian tumours will form extensive metastases following I.V. injection or even CAM implantation (Leighton, 1964).

The major disadvantage to using the chick embryo is the time limitation imposed by the 21-day gestation period, although sensitive assays such as the one developed by Chambers *et al.*, (1982), can be used to identify submacroscopic metastases for slow-growing tumours.

Leighton has reported that a variety of human tumours grow on the CAM (Leighton, 1964) including some tumour material freshly isolated from surgical specimens (Leighton *et al.*, 1972). Although his results are the best reported thus far, only about half of the tumours could be grafted successfully. Easty and colleagues (1969a; 1969b) found that most rodent tumours fell into two well-defined groups with respect to their capacity to metastasize following I.V. injection into 11-day old embryos. In the first group, tumours that did not grow well in chick embryos were those that were specific for the inbred strain of origin of the tumour. Most of these did not grow in any embryonic organs except the brain, even with the maximum tolerated inoculum. In the second group, tumours that grew and metastasized extensively in chick embryos were those that had lost their strain specificity and grew in many different inbred strains of rodents, even in the face of histo-incompatibility. Further resistance to tumour growth occured with increased embryonic development, and the period in which resistance increased most coincided with the appearance of maternal antibodies from the yolk sac in the embryonic circulation. The speed with which older embryos eliminate many strain-specific tumours (Easty *et al.*, 1969a), as well as the resistance of embryos to treatments known to inhibit an active immune response (Easty *et al.*, 1969b), strongly suggest that maternal "natural" antibodies are responsible for tumour resistance in this system.

The chick embryo system has been used to study the relationship between the initial organ distribution of radiolabelled mammalian tumour cells and the subsequent organ distribution of metastases (Easty *et al.*, 1969b). There is

an excellent direct correlation in most tumours between the extent of subsequent growth and the initial trapping efficiency of the organ. There are two interesting exceptions, however. The brain traps relatively few tumour cells, although it often supports vigorous growth (Easty *et al.*, 1969b; Clarkson *et al.*, 1964), possibly because it is an immunologically privileged site; the spleen often traps many cells but is a poor site for metastatic growth, possibly because of high reticular endothelial system (R.E.S.) activity (Easty *et al.*, 1969b).

The metastatic properties of HEp-3, a human epidermoid carcinoma, have been studied extensively using the chick embryo model (Leighton, 1964; Ossowski and Reich, 1980a; Skipski *et al.*, 1980). HEp-3 is a highly metastatic and invasive tumour in the chick embryo. Following transplantation of tissue fragments of the tumour to the CAM, the tumour grows locally and disseminates widely, with metastasis to the lung and heart being especially prominent. The tumour secretes copious amounts of plasminogen activator, which serves as the basis for sensitive assays of tumour growth and metastasis. The tumour retains the capacity for progressive growth and metastasis under allogeneic conditions in human volunteers after having been passaged for several years in chick embryos. In this sense, the tumour is similar to the non-strain specific murine tumours described by Easty and colleagues (1969a, 1969b) (see above). Upon serial *in vitro* passage of HEp-3 cells, a rapid loss of metastatic potential was noted while tumourigenicity declined less rapidly but nevertheless, progressively (Ossowski and Reich, 1980b). Loss of metastatic ability and tumourgenicity was accompanied by a loss of production of plasminogen activator, an enzyme associated with malignancy and transformation. Anchorage and serum-independence, which are generally correlated with the transformed phenotype, were inversely correlated with metastatic capacity in the chick embryo. The existence of a correlation between the level of gangliosides in

HEp-3 cells and the ability of these cells to metastasize has also been found in this system (Skipski *et al.*, 1980).

In studies of OSM with lymphoma cells, two quantitative assays were developed utilizing the chick embryo and intraveneous injection of the lymphoma variants (Shearman and Longenecker, 1980). One assay quantitates liver specific metastasis by the enumeration of tumour foci on the embyonic liver. In the chick embryo liver, tumour cells do not pass through pre-existing channels, but instead have to transverse the endothelium to reach the parenchyma (Skipski *et al.*, 1980). As expected, AL-2 cells induced hundreds of embryonic liver foci while AL-3 cells formed only a few (Shearman and Longenecker, 1981). These observations strongly suggest that those host processes or properties that determine the capacity of tumour variants to metastasize to the adult liver, have already developed in the chick embryo. Furthermore, the observation of the phenomenon of OSM in the embryo argues that the immune system has little or no role in determining this process.

The second assay, CAM metastatic focus formation, correlates with the virulence of the intraveneously - injected lymphoma variants (Shearman and Longenecker, 1980). In order to form lesions, cells injected I.V. into the embryo must penetrate the vascular endothelium, invade the CAM mesoderm, proliferate and acquire a blood supply. Others have reported that highly metastatic tumour cells can penetrate the CAM more readily than tumours of low metastatic potential (Easty and Easty, 1974; Hart and Fidler, 1978). These assays are done *in vitro* and probably measure the invasiveness of the tumour cells by their capacity to penetrate through an epithelial surface and enter the mesoderm of the CAM. A major difference between the *in vitro* and the *in ovo* assays is that the latter requires that tumour cells demonstrate the properties of invasiveness, proliferation and angiogenesis while the former assay is simply a measure of invasiveness.

A major criticism of the use of heterologous tumour systems in the chick embryo may be that species-specific mechanisms will restrict the usefulness of such systems to study a process such as OSM. However, several observations suggest that important cellular recognition structures may be highly conserved in evolution. A great deal of classical work in embryology serves to illustrate this principle (Curtis, 1979). Most embryological systems study homotypic aggregation phenomena, while the process of homing and OSM in vertebrates must involve heterotypic recognition. Nevertheless, Butcher and his colleagues (Butcher *et al.*, 1979) have presented data that suggest that the HEV recognition mechanism (see above) is conserved to some degree because lymphocytes from a variety of mammalian and even avian species show significant binding to murine HEV. Recently, lung and liver metastatic variants have been selected from the spontaneous murine mammary carcinoma cell line, TA3-HA in A strain mice. Following I.V. injection of the liver selected line into chick embryos, numerous liver metastases formed seven days later. In contrast, only a few CAM lesions formed in the same embryos. More work will be required to establish unequivocally the organ specificity in this system, but, in the absence of selection, most tumours form far more CAM than liver metastatic foci. In addition, using the frozen section assay described above, it has been observed that AL-2 cells adhere more avidly to mouse liver than do AL-3 cells. This suggests the existence of a conserved recognition system (see Results).

H. **Role of Natural Antibody in Tumour Metastasis**

Several investigators have reported that metastases can be inhibited by natural antibodies (Lewis *et al.*, 1977; Vaage and Agrawal, 1976; Greenberg *et al.*, 1980; Wolosin and Greenberg, 1979; Vaage, 1978). Greenberg (Greenberg *et al.*, 1980; Wolosin and Greenberg, 1979) have reported the existence of

natural anti-tumour antibodies in mouse sera and have suggested that these antibodies may play a crucial role in anti-tumour immunity. Vaage (1978) found that normal serum components can lyse murine mammary carcinoma cells in the circulation of mice. Galton and coworkers (Galton *et al.*, 1982) recently reported that, following injection of transplantable fibrosarcomas, bursectomized, agammaglobulinemic chickens showed much more metastatic involvement of organs than did normal recipients of the same age.

In order to investigate the expression of certain asialoglycoconjugates on tumour cells, an attempt was made to generate monoclonal antibodies (MAb) against neuraminidase treated human red blood cells (NE-RBC) (Rahman and Longenecker, 1982). NE-RBC's were used because a variety of human adenocarcinomas, (especially breast carcinomas) express a normally cryptic carbohydrate antigen (called T-antigen) which is exposed following neuraminidase treatment of human RBC's (Springer *et al.*, 1979). One MAb, called 49H.8 (Longenecker *et al.*, submitted) was found to react with neuraminidase-treated lymphocytes of both murine and human origin but not with untreated lymphocytes. The fine specificity of this MAb was determined by sugar inhibition studies using synthetic blood group like antigens (Rahman and Longenecker, 1982). 49H.8 reacted with both T-antigen (*B*Gal (1-3)*a* GalNAc) and the immunodominant determinant of Asialo GM_1 (*B*Gal (1-3) *B*GalNAc) but not with several other closely related compounds. Most interestingly, 49H.8 was found to react strongly with a variety of murine lymphomas as well as with the spontaneous murine mammary carcinoma TA3-Ha, but not with a subline of TA3-Ha called TA3-St. Since all normal sera have been reported to contain "natural" antibodies against T-antigen (*B*Gal (1-3) *a*GalNAc) (Springer *et al.*, 1979), both human and murine sera were examined for the presence of natural antibodies against the 49H.8 determinant. It was found that both types of sera contain antibodies that block the binding of 49H.8 to NE-RBC's. Thus it is

postulated that natural antibodies to this determinant might be important in clearing potentially metastasizing cells from the blood stream of animals injected with TA3-Ha cells bearing the 49H.8 determinant. In contrast, one might predict that TA3-St cells, which lack the 49H.8 determinant, might be cleared less rapidly. This, in fact, is the case: ^{131}IUDR-labelled TA3-Ha cells are cleared much more rapidly than are TA3-St cells.

Finally, the RAW 117 lymphosarcoma cell line was examined for 49H.8 expression and compared to the RAW 117-H10 subline, which was selected for liver metastasis (Brunson and Nicolson, 1978). Although the parental line expresses the 49H.8 antigen, little or no antigen expression was detected on the highly virulent RAW 117-H10 line. When the H10 line was treated with neuraminidase, the 49H.8 antigen was revealed, thus demonstrating that it is cryptic in this line and "covered" by sialic acid. This finding suggests that selection of the RAW 117-H10 line resulted in variants with increased sialic acid that can mask the 49H.8 antigen. This is in keeping with the aformentioned evidence for the importance of the degree of sialylation of cell surface Gal and GalNAc. Taken together, the available evidence indicates that natural antibodies may be important in the inhibition of blood-borne metastatic cells expressing asialogalactoconjugates.

I. Summary

Much of the available evidence indicates an important role for surface carbohydrate molecules as specific recognition structures in OSM. It is apparent that organ specific metastasis is a complicated process encompassing a variety of mechanisms, all of which may play a role in the eventual outcome of the metastatic cascade.

II. MATERIAL AND METHODS

Experimental Design.

The experiments outlined in this report are presented as comparisons between AL-2.1 and AL-3.1 cells. All of the work reported has been repeated and the unpublished results obtained are similar to those presented below.

A. Cells

The chicken T-cell lymphoma cell lines AL-2 and AL-3 were originally selected by Dr. P. Shearman (Shearman and Longenecker, 1980). These lines were derived from the MDV transformed, non-producer MDCC-RP1 cell line obtained from Dr. K. Nazerian (Regional Poultry Research Laboratory, East Lansing, Michigan). These cell lines were maintained in this laboratory 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). The cells were later grown in a mixture of 5% FCS and 5% chicken serum (Gibco) and for the last three years, have been maintained in RPMI-1640 + 10% FCS (Gibco) with 0.06 mg/ml garamycin (Johns Scientific, Toronto, Ontario). The cells were passaged twice weekly by diluting 1:20 to 1:40 in media. Each passage is considered one *in vitro* passage. No cells were used after the fifteenth passage unless otherwise noted.

B. Eggs

Random bred fertile eggs were supplied by the Poultry Division, Department of Animal Science, University of Alberta. Eggs were incubated for 20-21 days in a Robbins Hatchomatic Incubator (37-38°C, 95% humidity, and periodic rotation; Robbins Incubator Co., Denver, Colorado).

C. **Re-selection of AL-2 (Liver) and AL-3 (Ovary) Cell Lines**

5×10^4 AL-2 or AL-3 cells were injected I.V. into the CAM vein of 11 day chick embryos. The eggs were returned to the incubator for 6-7 days before opening. All CAMs were examined for the presence of foci as a control for successful injections.

AL-2 was re-selected by dissecting the livers of injected embryos and removing surface foci. These were placed in media and allowed to grow. Once sufficient numbers of cells had been obtained, 15 ampules were frozen and used as a stock (2×10^6 cells in 10% DMSO (Fisher, Edmonton, Alberta), 15% FCS (Gibco), 75% RPMI-1640 (Gibco) in liquid nitrogen). One ampule was thawed and re-injected over a large dose range into the CAM vein of 11 day chick embryos. The livers were removed 6 days later and the number of surface foci determined. Only those cell lines which gave large numbers of liver foci were maintained. A similar approach was used for AL-3 cells however, since these cells do not produce discrete foci on the ovaries (no liver foci are seen), the entire ovary was removed and tumour cells allowed to grow out. The cells obtained in this manner had a morphology similar to the original AL-3 cell line, and continued to grow as non-adherent cells. The cell lines re-selected in this manner have been designated AL-2.1 and AL-3.1 according to the rules for nomenclature recommended in the report of the Ad hoc committee on

Avian cell lines and transplantable tumour nomenclature, 1980.

D. **AL-2.1 and AL-3.1 Growth Kinetics**

The growth kinetics of AL-2.1 and AL-3.1 were determined by seeding 1, 5, or 10 x 10^5 cells into a petri dish with 20 mls of media and measuring their growth every 24 hours by trypan blue dye exclusion.

For carbohydrate studies, 0.5 to 2.5mg sugar were added either i) directly to 5 x 10^5 cells, incubated at 22°C for 10 minutes, washed and placed in 20 mls media, or ii) added directly to the media along with 5 x 10^5 cells. The kinetic analysis was performed as above.

E. **AL-2.1 and AL-3.1 Heterogeneity In Vitro**

After 10 *in vitro* passages, AL-2.1 and AL-3.1 cells were subcultured in 96 well plates by limiting dilution (2-5 cells/well) in media supplemented with 1% murine RBC's. Those wells which had cells growing were picked and expanded in culture. 5 x 10^4 cells were injected into day 11 chick embryos and their metastatic capacity measured as before. All wells that contained two or more clones were discarded.

F. **Enrichment of Liver Parenchymal and Endothelial Cells**

The livers of day 15-19 chick embryos were removed and cut into small pieces. They were pressed through a wire screen to obtain a single cell suspension and then washed twice in media. The parenchymal and endothelial cells were separated using the method of Roos *et al.*, (submitted) in which the

suspension was repeatedly spun at 100g for 30 seconds. The parenchymal cells pellet to the bottom while the endothelial cells remain suspended. This was repeated 4 times for both cell populations to further enhance their purity. The purity of the two cell types was determined by examination using electron microscopy performed by Dr. E. Sanders, Dept. of Physiology, University of Alberta. The parenchymal cells were easily identified by their shape and cellular structure while the endothelial cells were identified by their characteristic cytoplasmic fenestrations (Roos *et al.*, in press).

G. Lectin Binding to Cells

3 x 10^6 cells were washed twice in media, and spun in a conical tube. Fluorescent lectins were purchased from E-Y Laboratories, San Mateo, CA. To the cell pellet, 30*ul* of a solution containing 50*ug* lectin in media was added. The cells were allowed to incubate at 4°C for 15-20 minutes after which 1 ml of a 2% formaldehyde (Fisher)/PBS solution was added. One ml of FCS was underlayed and the tube spun at 500g for 5 minutes at room temperature. The supernatant was removed and the pellet resuspended in 1 ml of the 2% gluteraldehyde /PBS solution. The cells were analyzed on a FACS IV by Dr. T. Zipf, Oncology Research Group, University of Calgary.

H. Production of Anti-AL-2.1 Monoclonal Antibodies (MAb's)

In an attempt to produce MAbs specific for AL-2.1, a variety of priming regimens were employed. These included small, medium, and large doses of cells over a wide range of injection schedules. The fusion and ascites methods have been previously published (Mosmann *et al.*, 1980). Potential MAb's were tested in the ELISA assay using an enzyme peroxidase kit (Kirkegaard & Perry

Lab., Maryland).

I. In Vivo Inhibition of Metastasis

In vivo inhibition of metastasis was performed by preparing the cells and embryos as before. Just prior to injection, the cells were spun, resuspended in 0.5-5 mg of the specific sugar (Sigma, St. Louis, MO), 10*u*g of the specific lectin (EY Labs), or 100*u*l of a 1:10 dilution of anti-chicken T-cell monoclonal antibodies and immediately injected I.V. into the CAM vein. Other groups of cells were treated with tunicamycin (15*u*g/ml, 6 hours, 37°C; Sigma), trypsin (0.02 mg/ml, 30 minutes, 37°C; Sigma), or neuraminidase (25 units, 20 minutes, 37°C; Sigma), washed, resuspended in PBS and injected. The eggs were opened 6 days later and the organs weighed and the foci counted.

To test for non-specific toxic effects of the inhibitory reagents, $1x10^3$ AL-2.1 cells were treated as above and injected I.V.. Using this cell dose, accurate estimates of CAM foci were made.

J. In Vitro Frozen Section Adherence Assay (FSAA)

Day 15-19 embryonic chicken liver, lung, heart, ovary, and kidney were removed from the eggs just prior to preparing the sections. 2-7mm square chunks of tissue were frozen in OCT Compound (Lab-Tek, Naperville, Illinois) and allowed to sit at -20°C for 30 minutes. 10*u* Sections were cut on a cryostat (Lab-Tek) and serial sections placed next to each other on glass slides (Corning, Corning, N.Y.). Each slide was allowed to air dry for 2-4 hours.

^{51}Cr labelled AL-2.1 and AL-3.1 cells were thoroughly washed and counted prior to use. The cells were used only if the two cell lines were

labelled to the same extent. Each section was encircled with a wide grease pencil to prevent the cells from running off the section. The slides were placed on an orbital shaker (Bellco Glass, Vineland, N.J.) at 7°C and 0.2 mls of the radiolabelled cell suspension containing $0.5\text{-}1 \times 10^5$ cells (1.5-2 cpm/cell) added. After 1 hour of rotation at 50 rpm, the slides were removed, rinsed in PBS + 2% gluteraldehyde, and the tissue section removed with the end of a cotton swab. The cotton swabs were placed into tubes and counted on a gamma counter (LKB).

K. In Vitro Inhibition of Adherence

The frozen section adherence assay was employed to determine the ability of different compounds to specifically inhibit OSM. The assay was performed as above except in the step prior to injection, the cells were resuspended in media containing 0.5-5 mg of the specific sugar (Sigma), 10*ug* of the specific lectin (EY Labs) or 100*ul* of a 1:10 dilution of anti-chicken T-cell MAb's. The assay was then completed as above.

L. Radiolabelling of Sugar Compounds

25 mgs of p-nitrophenyl-*B*-D-fucopyranoside was converted to the amino form by Pd-C hydrogenation. Reactants and products were compared on TLC using ether:methanol 8:2 as a solvent. Staining of the plates was performed with ninhydrin or sulfuric acid. This amino-phenyl derivative, and others that were purchased (Sigma), were linked to BSA (Sigma) in a 20:1 molar ratio using 0.1mg in 100*ul* EDCI as the coupling reagent in mannitol/saline. These product or those purchased from E-Y Laboratories were then radiolabelled with ^{125}I by the chloramine T method (Barton *et al.*, 1977) and dialyzed to remove free ^{125}I.

1×10^5 [illegible] cells were added to 1×10^5 counts for 1 hour at 4°C. The cells were [illegible] the number of counts determined. The technical assistance and a[illegible] B. Singh and Dr. E. Fraga is gratefully appreciated.

III. RESULTS

A. AL-2.1 and AL-3.1 Re-selection

The organ specificity of AL-2.1 and AL-3.1 is known to change with time, *in vitro*. For this reason, it was necessary to re-select them from the original lines. As previously stated, it was not possible to determine the successfulness of the AL-3.1 re-selection because discrete foci are not visable at the intended site of metastasis. AL-2.1 on the other hand, produced a large number of liver foci (its selected organ) over a wide range of injected inoculum. At the optimal inoculum of 5×10^4 cells/embryo, AL-2.1 produced approximatly 100 times as many liver foci as AL-3.1 ($p < 0.001$). A comparable dose of AL-3.1, considered a good control for AL-2.1 since it was selected from the same inital population but not for the liver, produced few liver foci (Figure 1). Both cell lines had comparable levels of CAM foci. Similar patterns were observed for the effect of AL-2.1 and AL-3.1 on the weight of the liver.

B. Growth Kinetics

The growth kinetics of AL-2.1 and AL-3.1 were examined over a five day period. They displayed very similar growth curves with doubling times of 11.9 hours for AL-2.1 and 12.0 hours for AL-3.1 (Figure 2). The similarities of these growth properties suggest differences in the metastatic behaviour of the two cell lines is not a result of different growth potentials.

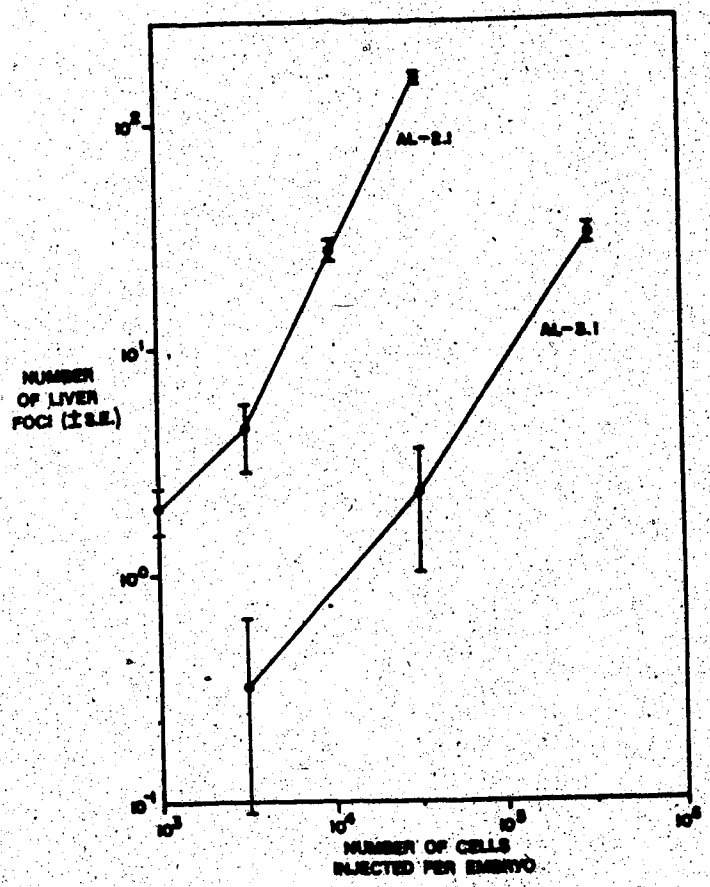

Figure 1.

Number of liver foci *in vivo* as a function of the number of I.V. injected lymphoma cells.

AL-2.1 and AL-3.1 cells were reselected from their respective organs, plated and grown *in vitro*. The cells (in graded numbers) were injected iv into the CAM membranes of day 11 chick embryos (8 eggs/group). Six days later, the eggs were opened and the liver foci enumerated.

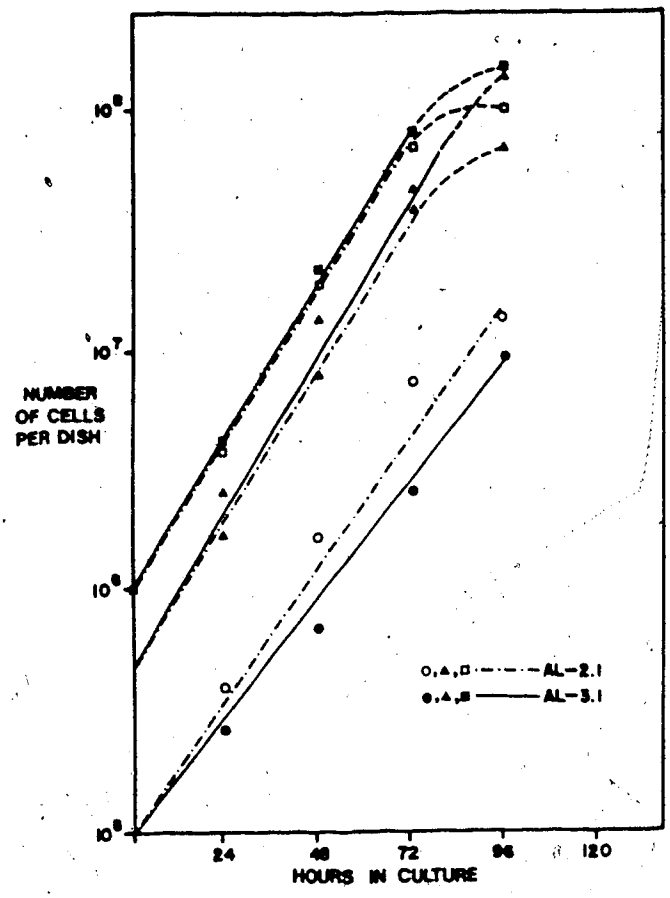

Figure 2.

Growth Kinetics of AL-2.1 and AL-3.1 *in vitro*.

Representative doubling times were calculated from the upper-most lines (1×10^6 cells/dish). Points obtained after 72-96 hrs were not included in the analysis because by this time, the cultures had overgrown.

C. **Heterogeneity of AL-2.1 and AL-3.1**

Each cell line was plated by limiting dilution as previously described in the materials and methods. The sublines were expanded and injected into the CAM vein of day 11 embryos. Foci formation and total liver weight differed between the lines supporting the notion of heterogeneity within the original cell lines (see above). This was most easily observed with AL-2.1 where large differences were observed. For example, AL-2.1 subline 3 had no difference in weight or foci number to un-injected controls. Subline 19 on the other hand, was 34.9 mgs heavier (twice the normal weight). Smaller differences were observed for AL-3.1 sublines. For example, AL-3.1 subline 3 and subline 17 were slightly different in mean liver weight although these differences were not statistically significant (Table 1). With increase passaging, these differences might become more substantial. The choice of the word "subline" has been used in place of "clone" because 2-5 cells/well were plated and therefore it is not possible to know if the cells obtained contained progeny from one or more of the original cells. The number of CAM foci appeared similar among all the sublines although crowding occured which made accurate counting difficult and so small differences might have existed and gone undetected.

D. **Tumour and Lectin Cell Surface Binding**

To identify cell surface carbohydrates on AL-2.1 and AL-3.1 as well as on liver parenchymal and endothelial cells (Figure 3), fluorescent labelled lectins were used (Table 2 and Figures 4, 5 and 6). The lectin *Ulex europaeus*, specific for L-fucose, was the only lectin tested that showed a significant difference in its binding to AL-2.1 and AL-3.1. At the maximal sensitivity of the FACS, no binding of this lectin was observed on AL-2.1 (mean=59) while significant binding of the lectin was detected on AL-3.1 (mean=406). Two

Table 1

HETEROGENEITY OF AL-2.1 AND AL-3.1 SUBLINES IN VIVO

Cell Line[3]	Subline #	Change in Liver Weight ± S.E.[1] (mgs)	# Liver Foci
AL-2.1	2	0.2 ± 2.1	1.6 ± 0.6
	3	0.0 ± 1.9	0.9 ± 0.3
	14[2]	12.1 ± 3.0	7.4 ± 3.2
	15	0.3 ± 2.3	0.8 ± 0.3
	16	22.8 ± 3.3	46.6 ± 8.9
	18	37.3 ± 3.9	60.8 ± 12.1
	19	34.9 ± 5.5	76.6 ± 12.8
AL-3.1	2	3.7 ± 3.1	0.0 ± 0.0[4]
	3	0.0 ± 4.3	0.0 ± 0.0
	12	0.2 ± 3.4	0.0 ± 0.0
	14	0.2 ± 2.6	0.0 ± 0.0
	15	7.3 ± 3.4	0.0 ± 0.0
	17	7.7 ± 3.1	0.0 ± 0.0
	22	1.6 ± 2.7	0.0 ± 0.0

[1] Liver weight calculated by subtracting un-injected control liver weight.
[2] $p < 0.01$ compared to subline 2 and 19.
[3] 5×10^4 viable cells were injected I.V. into each egg.
[4] No liver foci observed on visual inspection.

Figure 3.

Electron micrographs of enriched parenchymal and endothelial liver cells.

A) Enriched parenchymal (P) cells.

B) Enriched endothelial (E) cells. Note small size and characteristic fenestrations (f) of endothelial cells. Nucleus, n; RBC, r.

Table 2

FLUORESCENT LECTIN BINDING TO TUMOUR OR LIVER CELLS IN VITRO

	AL-2.1		AL-3.1	
Lectin Source	Mean[1]	Voltage[2]	Mean	Voltage
PNA	191	1.0	179	1.0
CON-A	345	0.03125	305	0.03125
WHEAT GERM	206	0.03125	304	0.03125
SOY BEAN	466	0.25	299	0.25
ULEX[3]	59	1.0	406	1.0
RICINUS	178	0.0625	157	0.0625
TETRA	27	1.0	26	1.0
	ENRICHED PARENCHYMAL		ENRICHED ENDOTHELIAL	
Lectin Source	Mean	Voltage	Mean	Voltage
PNA	130	1.0	324	1.0
CON-A	155	0.0625	387	0.0625
WHEAT GERM	415	0.0625	202	0.0625
SOY BEAN[3]	81	0.5	478	0.5
ULEX[3]	147	0.5	478	0.5
RICINUS	323	0.0625	406	0.0625
TETRA[3]	32	1.0	117	1.0

[1] Mean represents the average fluorescent intensity at a given voltage.

[2] For direct comparison of mean fluorescent intensity, voltage differences between AL-2.1 and AL-3.1 were normalized. No comparison between lectins was performed.

[3] Significance to $p < 0.01$ level.

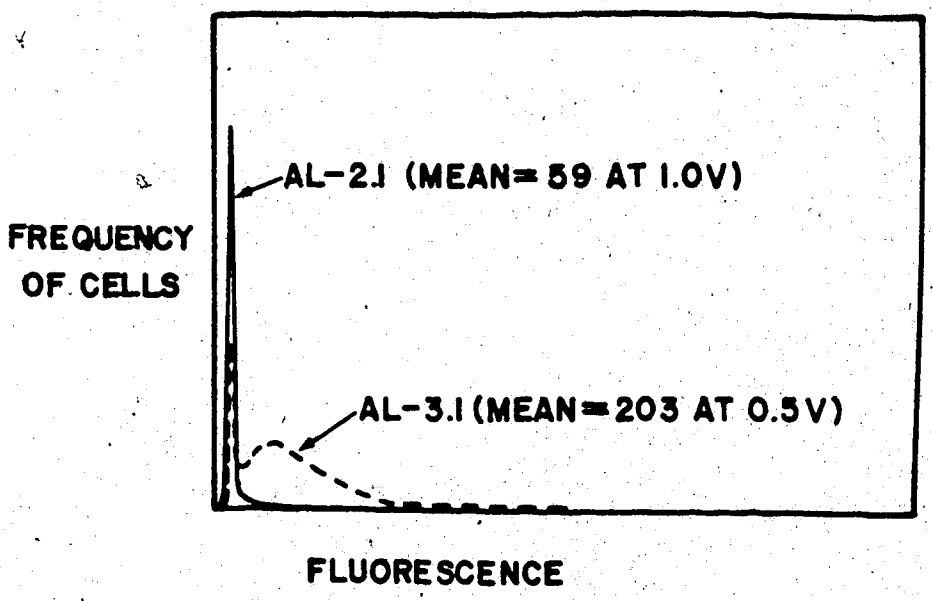

Figure 4.

AL-2.1 and AL-3.1 labelling with fluorescent *Ulex europaeus* lectin.

Fluorescent profiles for AL-2.1 and AL-3.1. Conversion of the AL-3.1 value to correspond to the voltage used for AL-2.1 will enhance the difference (move the AL-3.1 line to the right), $p < 0.01$.

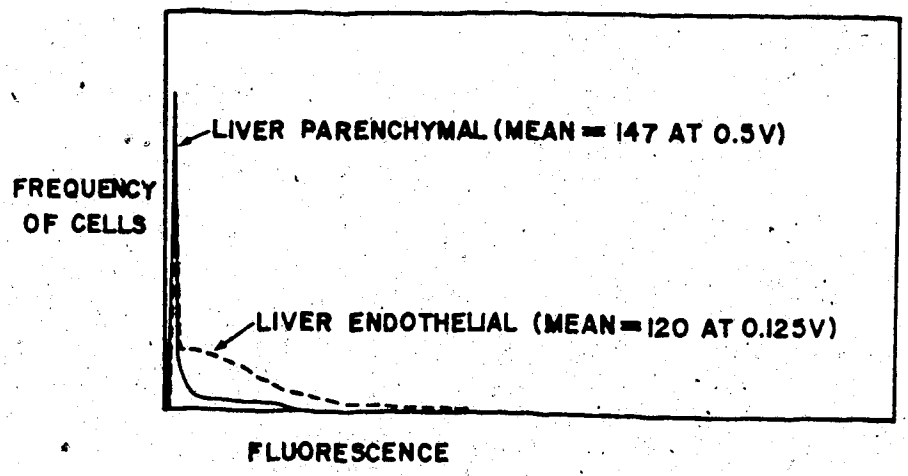

Figure 5.

Labelling of liver parenchymal and endothelial cells with fluorescent *Ulex europaeus* lectin.

Fluorescent profiles for enriched liver parenchymal and endothelium. Conversion of the endothelial value to correspond to the voltage used for parenchymal cells will enhance the difference (move the endothelial line to the right) $p < 0.01$.

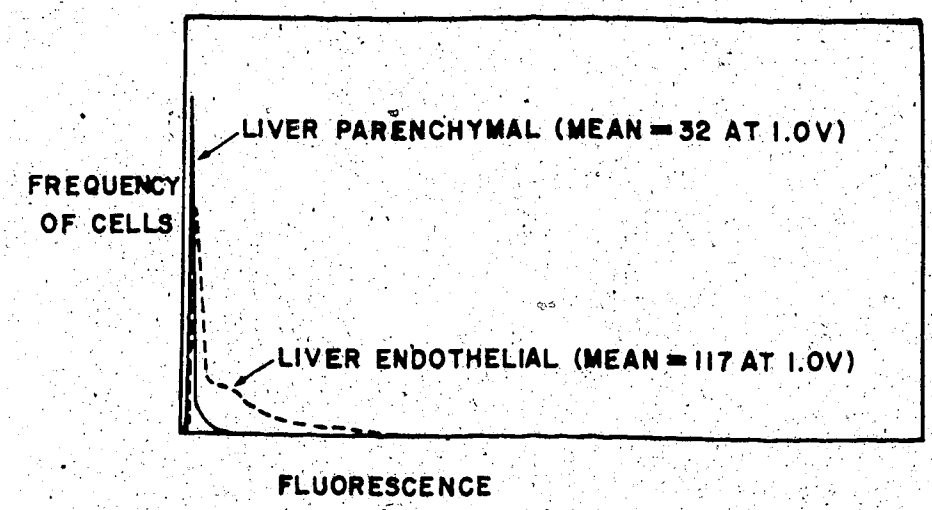

Figure 6.

Labelling of liver parenchymal and endothelial cells with fluorescent *Tetragonolobus purpureas* lectin.

Fluorescent profiles for enriched liver parenchymal and endothelium. Conversion of the endothelial value to correspond to the voltage used for parenchymal cells will enhance the difference (move the endothelial line to the right) $p < 0.01$.

lectins showed differential binding to purified populations of liver (parenchymal and endothelial) cells. Glycine max (soy bean), which recognizes *a*-D-GalNac and Gal, was detected on endothelial but not parenchymal cells. A difference in *Ulex europaeus* lectin binding was also observed, again the binding being prominent on endothelial cells. The small amount of binding to parenchymal cells may be due to residual endothelial cells in this population. Similar results were also later obtained with the lectin *Tetragonolobus purpureas* which is also *a*-L(-) fucose specific (Figure 6).

E. AL-2.1 and AL-3.1 Carbohydrate Interactions

The ability of AL-2.1 to specifically bind the fucose moiety was examined using fucose-BSA conjugates as outlined in the materials and methods. The results are presented in Table 3 and show that there is a significant increase in binding of ^{125}I-fucose-BSA to AL-2.1 compared to AL-3.1 ($p < 0.05$). This differential binding was lost when the cells were trypsinized but returned when AL-2.1 cells were given an opportunity to re-express their receptors ($P < 0.001$). ^{125}I-galactose-BSA on the other hand, bound equally well to both AL-2.1 and AL-3.1.

F. In Vivo Inhibition of OSM

A further approach to the elucidation of some aspects of the cell surface was the treatment of cells and embryos with a variety of sugars, lectins, and enzymes *in vivo*. While this *in vivo* approach did not allow for an estimate of which surface the treatment had affected, these experiments have given some insight into cell surface structures important in organ specific metastasis (Table 4). For example, AL-2.1 cells incubated with *a*-D(+) fucose,

a-L(-) fucose, p-nitrophenyl-*B*-D-fucopyranoside, o-nitrophenyl-*B*-D-gluco- pyranoside and lactulose had dramatically different effects. The percentage of liver foci observed with AL-2.1 and lactulose or p-nitrophenyl-*B*-D-fuco- pyranoside compared to AL-2.1 cells alone was 13 and 23 respectively. A similar reduction was also obtained when liver weights were compared (Table 4). For example, lactulose was able to inhibit the liver enlargement associated with injection of AL-2.1 cells (17 ± 24% of normal AL-2.1 liver weight) and yet this sugar had no such effect on spleen weight (Table 4). To control for toxic effects of the sugars on the cells, the CAM membranes were visually inspected using cell doses that allowed for proper CAM foci determination. No such toxic effects were noted (data not shown). The differential effect of AL-2.1 or AL-3.1 and the sugars on spleen weights were minimal. This might be expected if the increased spleen weight was the result of some sort of non-specific response. One further explanation is that subpopulations of tumour cells (both AL-2.1 and AL-3.1) home specifically to the spleen, perhaps as a remnant of their T-cell lineage. While the data presented does not differentiate between these two explanations, it remains consistent with liver metastasis being the result of organ-specific mechanisms that are specifically sensitive to particular sugars. A further test of the effects of the sugars on the tumour cells was performed by growth kinetics as described in the material and methods. No difference was observed in the growth kinetics of cells growing either in the presence of sugar or after the sugar had been washed out except for p-nitrophenyl-*B*-D-fucopyranoside. The effect of this sugar was observed in culture only and was associated with a drastic change in media colour (Figure 7). The absence of this effect *in vivo* might be due to removal of, or modification of the sugar metabolites by the embryonic liver, a process not possible in culture.

Table 3

BINDING OF ^{125}I-SUGAR-BSA CONJUGATE TO AL-2.1 AND AL-3.1

Expt #	AL-2.1[1] (cpm ± S.E.)	AL-3.1[1] (cpm ± S.E.)	Treatment	Ratio	p Value
1	96 ± 26	36 ± 10	^{125}I-fucose-BSA	2.67	0.05
2	427 ± 100	128 ± 36	^{125}I-fucose-BSA	3.34	0.05
	229 ± 44	175 ± 13	^{125}I-fucose-BSA/Trypsin	1.31	-
	373 ± 45	143 ± 12	^{125}I-fucose-BSA/Trypsin + 5hr Incubation-37°C	2.61	0.001
Control	101 ± 17	77 ± 12	^{125}I-galactose-BSA	1.30	--

[1] $1x10^5$ tumour cells were incubated with $5x10^5$ counts of the radiolabelled sugar-BSA conjugate for 1 hour at 4°C. Specific counts (background of machine subtracted).

Table 3

BINDING OF ^{125}I-SUGAR-BSA CONJUGATE TO AL-2.1 AND AL-3.1

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	373 ± 45	143 ± 12	^{125}I-fucose-BSA / Trypsin + 5hr Incubation 37°C	2.61	0.001
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[1] 1×10^5 tumour cells were incubated with 5×10^5 counts of the radiolabelled sugar-BSA conjugate for 1 hour at 4°C. Specific counts (background of machine subtracted).

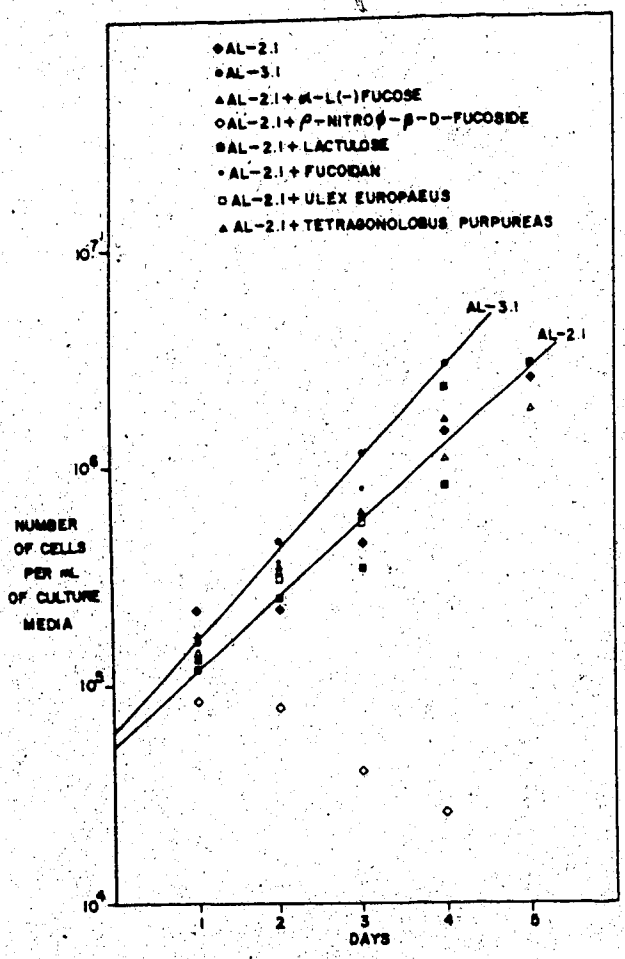

Figure 7.

Growth kinetics of AL-2.1 and AL-3.1 cells incubated with carbohydrates. Plates contained 20 mls medium, 5×10^4 cells/ml and 5mg sugar. Every 24 hrs, the cells were counted using trypan blue. A significant difference in growth kinetics was observed for p-nitrophenyl-β-D-fucopyranoside.

Substantial effects were also observed with trypsin, as this enzyme completely inhibited the metastasis of AL-2.1 to the liver (Table 5). When the cells were re-incubated at 37°C for 3-6 hours in medium prior to injection, the resultant inhibition was decreased. This effect is consistent with the presence of cell surface determinants important in organ specific metastasis which are either protein or glycoprotein in nature. The results for the sugar and enzyme *in vivo* inhibition are summarized in Table 4 and 5 respectively. The effect of the lectin *Ulex europaeus* on *in vivo* metastasis was also pronounced. This lectin, which recognizes fucose determinants, was able to inhibit OSM (Table 5). The effect of sugars on *in vivo* CAM foci formation is presented in Table 6.

Significant inhibition of OSM was also observed for 24C.8 and 24C.35 (Table 7). The mean liver weights of eggs injected with AL-2.1 + MAb compared to AL-2.1 alone were 56 and 46% for 24C.8 and 24C.35 respectively. These two MAbs were prepared against AL-2.1 cells although they also recognized AL-3.1 as measured by fluorescent binding to the two cell types. They were tested to see if they recognized a conserved region of the receptor common to both cell lines. One further antibody tested, an anti-murine lymphosarcoma antisera kindly provided by Dr. E. Roos, produced no inhibition of OSM.

G. In Vitro Frozen Section Adherence Assay

The ability of certain tumour cell lines (such as AL-2.1) to adhere specifically to frozen sections of particular organs was demonstrated by a modification of the frozen section assay of Stamper and Woodruff (1976) and Butcher *et al.*, (1979). The modified assay is diagrammatically represented in Figure 8. The assay has been maximized for a number of components and the resultant maximum conditions are presented in Table 8. The maximum conditions

Table 4

IN VIVO OSM CARBOHYDRATE INHIBITION

#	CELL LINE	SUGAR[1]	% LIVER FOCI INCREASE[2]	% LIVER WEIGHT INCREASE[2]	% SPLEEN WEIGHT INCREASE[2]
1	-	-	0	0	0
2	AL-2.1	none	100 ± 31	100 ± 10	100 ± 19
3	AL-3.1	none	1 ± 1[4]	25 ± 18[4]	83 ± 11
4	AL-2.1	*a*-D(+) FUCOSE	56 ± 12	51 ± 12[3]	83 ± 11
5		*a*-L(-) FUCOSE	25 ± 15[3]	28 ± 14[5]	49 ± 8[6]
6		*a*-LACTOSE	50 ± 21	130 ± 22	96 ± 20
7		D(+) MANNOSE	91 ± 28	55 ± 20	95 ± 21
8		D-MANNITOL	55 ± 25	93 ± 34	108 ± 15
9		L(-) MANNOSE	136 ± 33	85 ± 22	64 ± 10
10		D(+) GALACTOSE	75 ± 13	71 ± 21	74 ± 17
11		L-FUCOSYLAMINE	25 ± 8[3]	29 ± 27[3]	85 ± 20
12		D-MANNOSAMINE	99 ± 21	75 ± 25	96 ± 23
13		*a*-METHYL-D-MANNOSIDE	111 ± 24	58 ± 36	71 ± 11
14		*B*-D(-) FRUCTOSE	28 ± 12[3]	54 ± 17[3]	89 ± 12
15		1-o-METHYL-*a*-D-GALACTOPYRANOSIDE	105 ± 26	81 ± 29	130 ± 48
16		1-o-METHYL-*a*-D-GLUCOPYRANOSIDE	50 ± 17	59 ± 23	73 ± 14
17		o-NITROPHENYL-*a*-D-GALACTOPYRANOSIDE(2.5)	102 ± 41	95 ± 16	120 ± 27
18		o-NITROPHENYL-*B*-D-GLUCOPYRANOSIDE(1.0)	76 ± 34	106 ± 8	155 ± 22
19		p-NITROPHENYL-*B*-D-FUCOPYRANOSIDE	23 ± 7[3]	6 ± 11[5]	77 ± 17
20		CHONDROITIN SULFATE(1.0)	41 ± 23	76 ± 31	71 ± 10
21		LACTULOSE	13 ± 6[3]	17 ± 24[4]	144 ± 64

[1] Values in brackets represent mgs of sugar added/embryo ± S.E. All others received 5 mg sugar each.
[2] Calculated as AL-2.1 = 100% and -ve as 0%.
[3] $p < 0.05$, [4] $p < 0.01$, [5] $p < 0.001$ compared to AL-2.1 without sugar.
[6] $p < 0.05$, This reduction in spleen weight was not usually observed.
Values are represented as ± S.E. Each group consists of eight embryos.

Table 5

IN VIVO OSM ENZYME/LECTIN INHIBITION

#	CELL LINE	ENZYME	# LIV FOCI[1]	CHANGE IN SPL WT (mg)[1]
1	AL-2.1	none	10 ± 3	12 ± 2
2	AL-3.1	none	0 ± 0	4 ± 2
3	AL-2.1	trypsin	0 ± 0	5 ± 1
4	AL-3.1	trypsin	0 + 0	10 ± 3
5	AL-2.1	trypsin + 3-6 hr incubation	4 ± 1	8 ± 1
6	AL-3.1	trypsin + 3-6 hr incubation	0 ± 0	11 ± 3
7	AL-2.1	none	14 ± 2	12 ± 4
8	AL-3.1	none	0 ± 0	25 ± 7
9	AL-2.1	Ulex europaeus(0.5mg)	4 ± 1	11 ± 1
10	AL-2.1	Tetragonolobus purpureas(0.5mg)	2 ± 1	15 ± 3
11	AL-2.1	fucoidan	3 ± 1	17 ± 3

[1] (± S.E.)

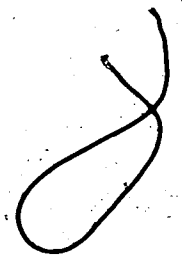

Table 7

MONOCLONAL ANTIBODY INHIBITION OF OSM

#	Cell Line	MAb[4]	% Liv Wt	% Spl Wt
1	AL-2.1	none	100 ± 14	100 ± 15
2	AL-3.1	none	7 ± 6[3]	34 ± 15[1]
3	AL-2.1	24C.8	56 ± 6[3]	68 ± 17
4	AL-2.1	24C.30	89 ± 11	79 ± 20
5	AL-2.1	24C.31	86 ± 12	132 ± 31
6	AL-2.1	24C.35	46 ± 5[2]	64 ± 15

[1] $p < 0.05$, See Table 4 for explanation. [2] $p < 0.01$, [3] $p < 0.001$ compared to AL-2.1 without MAb.
[4] These MAb's were raised against AL-2.1 cells but were found to bind both AL-2.1 and AL-3.1. 100*u*l ascites fluid was injected with 5×10^4 tumour cells.

were those that produced the greatest difference in binding between AL-2.1 and AL-3.1 cells on liver frozen sections. All of the assays performed were done at the conditions stated in Table 8 unless otherwise noted. Figure 9 represents the results of nine different experiments expressed as the ratio of AL-2.1/AL-3.1 on either liver or control tissues such as heart, ovary or kidney. The distribution of these ratios confirms there is no overlap between the ratio of AL-2.1/AL-3.1 cells on liver and control tissues ($p < 0.001$). Figures 10 and 11 show the individual results from two such experiments. Ratios of 2:1 to 5:1 for AL-2,1/AL-3.1 binding to liver sections were common although an occasional lower or higher ratio was obtained. Assays which did not show a ratio significantly higher than 1.0 could usually be accounted for by technical reasons or failures and accounted for only a very small proportion of the assays performed. An attempt to find an appropriate measure of background binding of cells to the sections has yet to be successful. Once a cell type is available whose non-specific binding gives a measure of background, its value can be subtracted from these results, and the relative differences between the ratio of AL-2.1/AL-3.1 on liver compared to other tissues should increase dramatically. As such, the results presented here represent the minimal differences that occur in this system.

The effect of time and passage number of the cell line on tumour cell foci formation was profound. The *in vitro* frozen section adherence assay was used to determine the capacity of different passages of AL-2.1 and AL-3.1 cells to bind to selected frozen sections. A rapid reduction in the specific binding ratio was consistently observed past the fifteenth passage (Figure 12). This effect is probably the result of increasing heterogeneity within the cell line.

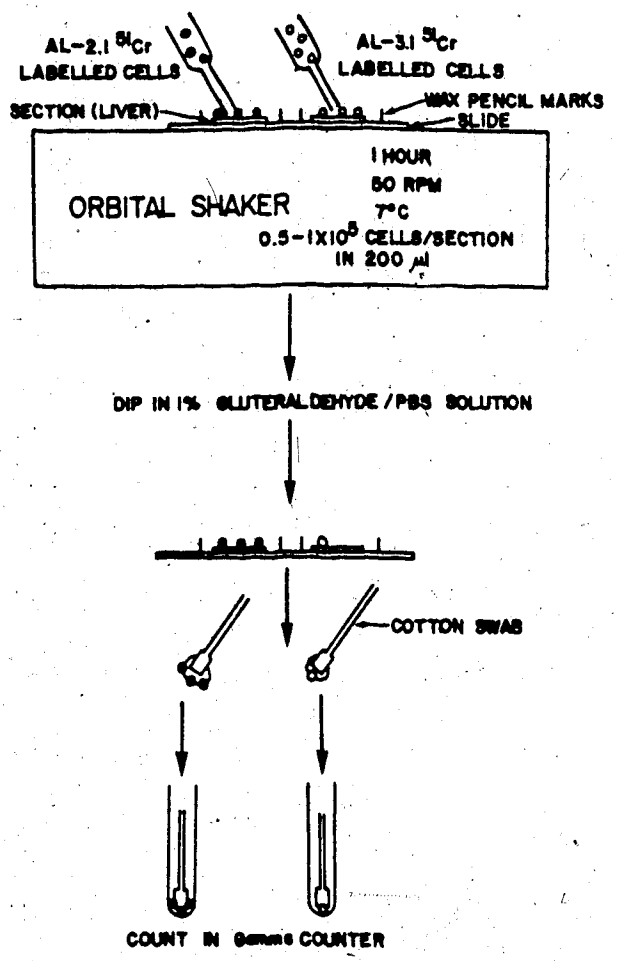

Figure 8.

Diagramatic representation of *in vitro* frozen section adherence assay.

Table 8

IN VITRO FROZEN SECTION ADHERENCE ASSAY

Condition	Maximum	Range Tested
Cell Dose	0.5-1x10^5 cells/well	1x10^3 - 1x10^7
Rotations	50 rpm	0-100 rpm
Temperature	7°C	4-37°C
Section Radius	none	2-7mm
Thickness	10μ	8-50μ
Serum Concentration	10% FCS	0-10% FCS
Cell Passage	1-15	1-30
Age of Animal	Day 15-19 embryos	day 15 embryo - 1 year

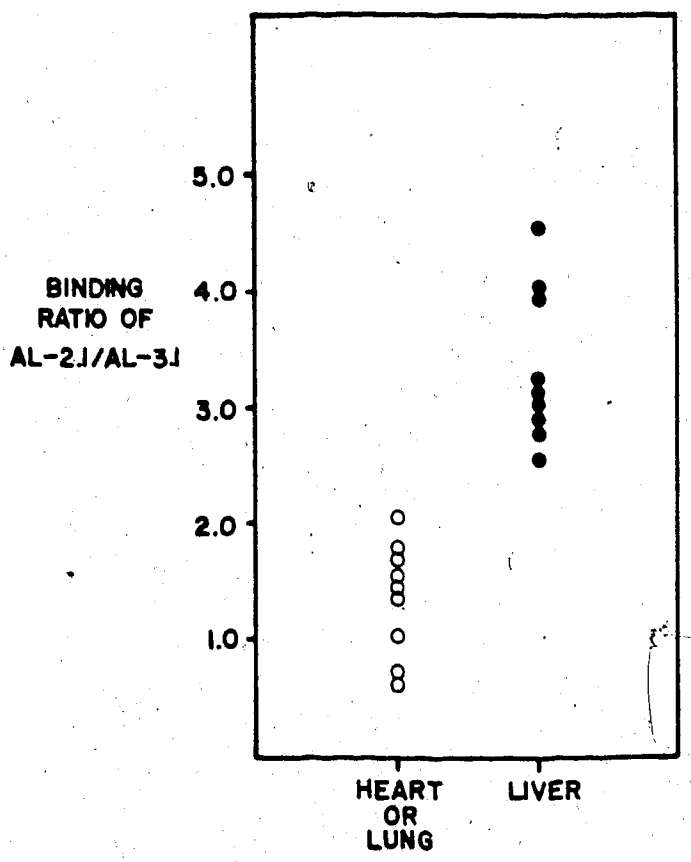

Figure 9.

Ratio of binding of AL-2.1/AL-3.1 to different tissues.

A total of 9 representative experiments performed over a two month period. The order of data points in the two conditions are not necessarily matched.

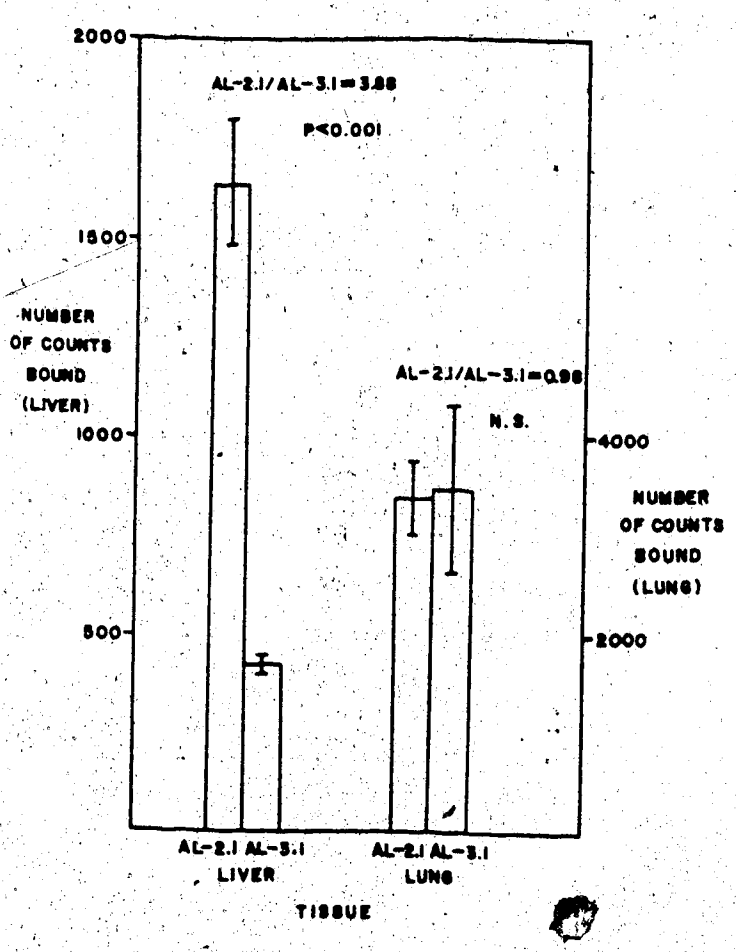

Figure 10.

Representative experiment of the ratio of AL-2.1/AL-3.1 on liver and lung tissue.

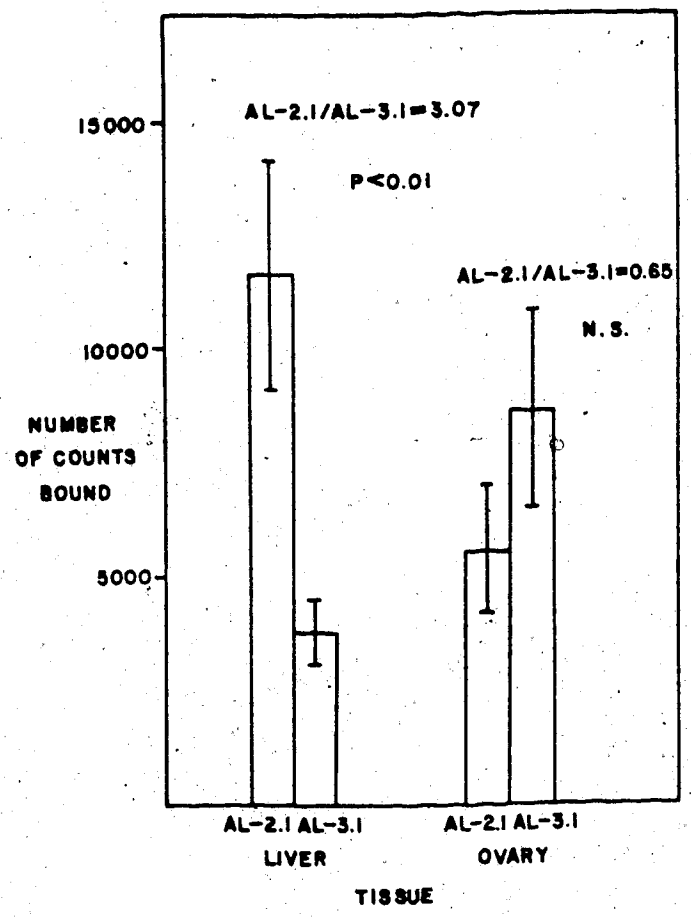

Figure 11.

Representative experiment of the ratio of AL-2.1/AL-3.1 on liver and ovary tissue.

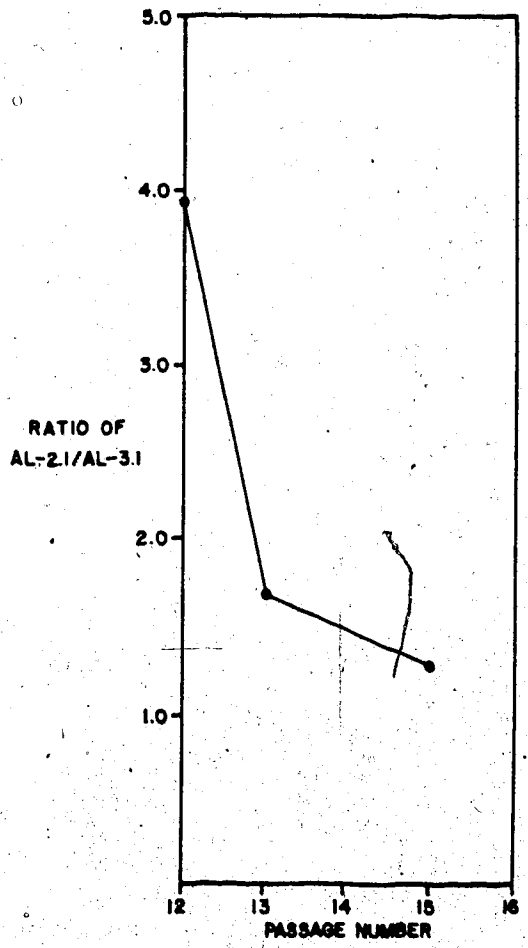

Figure 12

* Effect of AL-2.1 Passage # *in vitro* on the frozen section adherence assay.

The age of the chick embryos or hatched chickens also had an effect on the adherence capacity of AL-2.1. The livers of adult chickens did not display any specific adherence of AL-2.1 cells while embryonic or newly hatched chicks did (Figure 13). In this context, it is interesting that adults are not susceptible to Marek's Disease.

H. **In Vitro Carbohydrate Inhibition**

The ability of certain carbohydrates to inhibit binding of AL-2.1 cells to liver sections was measured (Figures 14,15,16). The results obtained were in good agreement with those obtained *in vivo*. While no inhibition was observed for *a*-D(+) fucose, partial inhibition was obtained for *a*-L(-) fucose and complete inhibition was observed for p-nitrophenyl-*B*-D-fucopyranoside, fucoidan and lactulose (Figure 14,15). The rather high counts observed for AL-2.1 and AL-3.1 on liver with the sugar fucoidan (Figure 14) were perhaps the result of enhanced non-specific binding. By comparing the binding of AL-2.1 and AL-3.1, significant differences were obtained only in the first two groups (AL-2.1/AL-3.1 alone or with *a*-D(+) fucose). The addition of *a*-L(-) fucose partially yet significantly reduced the difference between the two cell lines and the remainder of the compounds completely inhibited AL-2.1 so that the AL-2.1/AL-3.1 ratio became approximately 1:1. The inhibition observed was not the result of any toxic effect on AL-2.1 cells by the sugars since cells treated with sugars and tested for viability one hour later with trypan blue showed no cell death (data not shown). Inhibition of adherence was also observed with lectins specific for fucose but not those specific for other carbohydrate determinants (Figure 16).

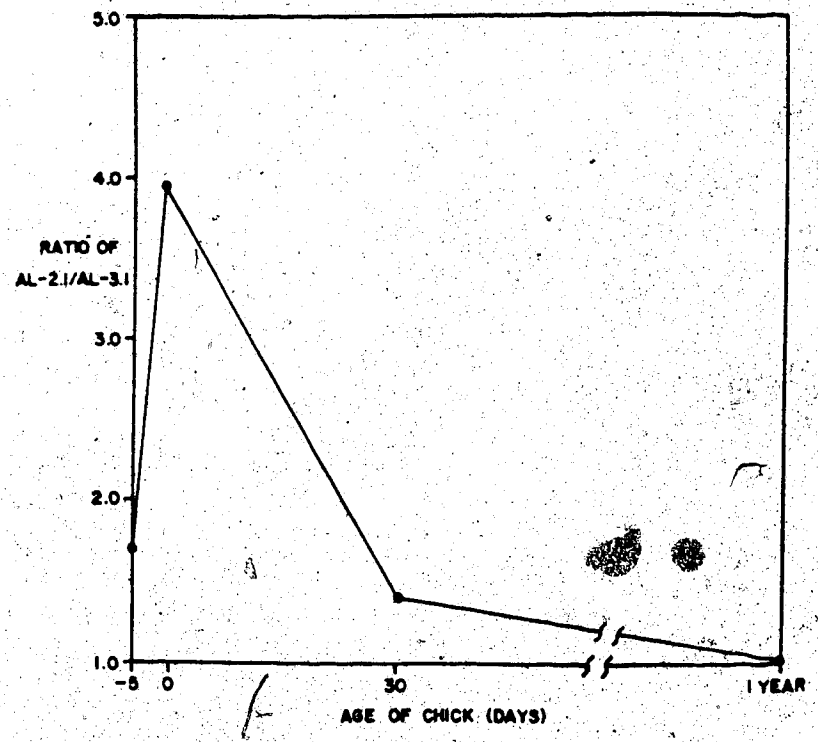

Figure 13

Effect of age of tissue on the frozen section adherence assay.

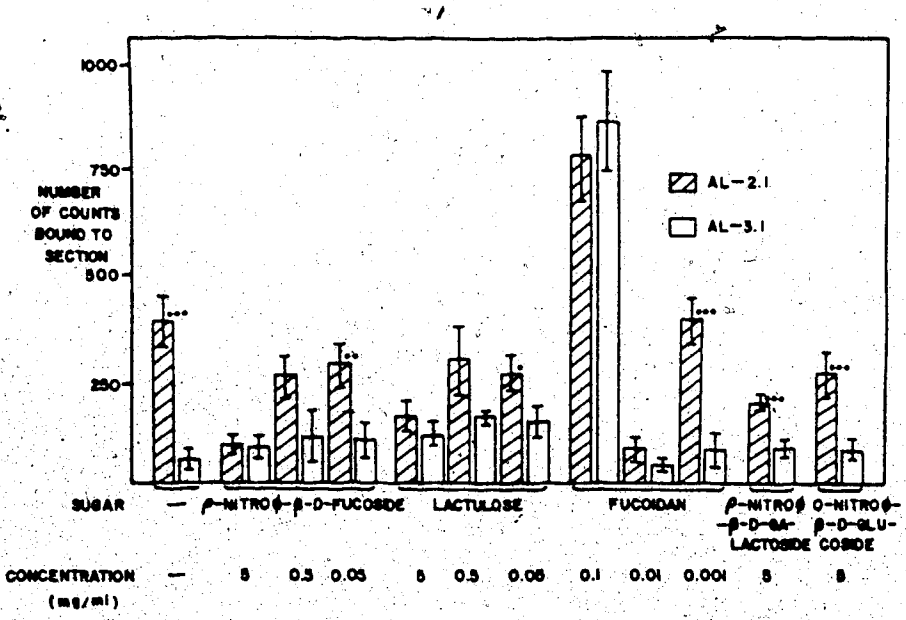

Figure 14

Inhibition of in vitro frozen section adherence assay with carbohydrate.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Data for fucoidan at 0.1 and 0.01 mgs/ml have been reduced by a factor of 10 to fit on scale.

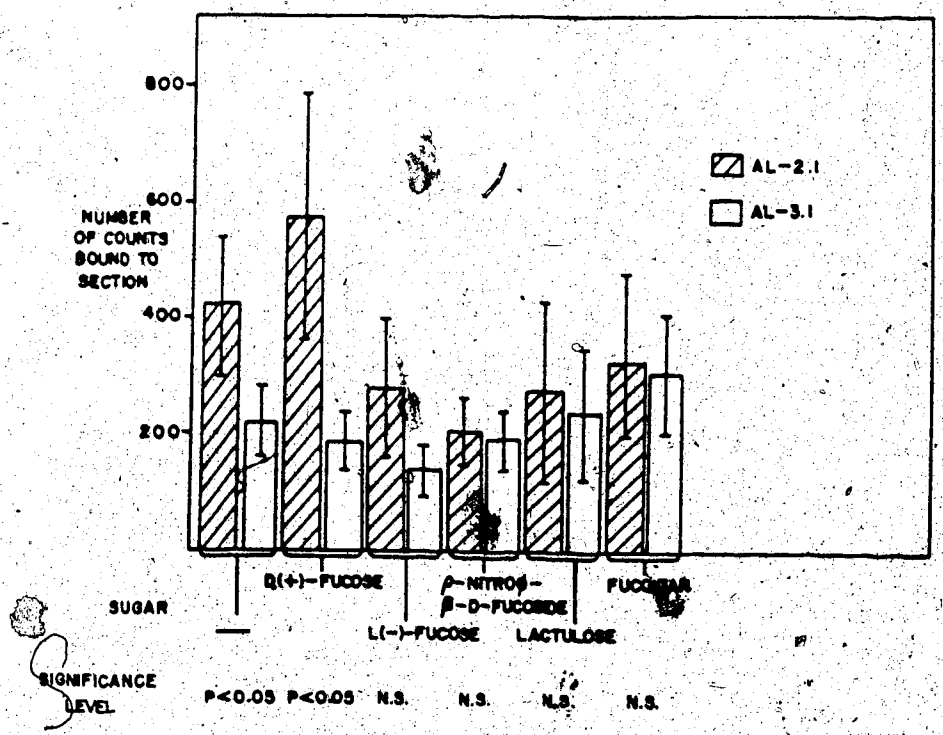

Figure 15

Inhibition of in vitro frozen section adherence assay with carbohydrate.

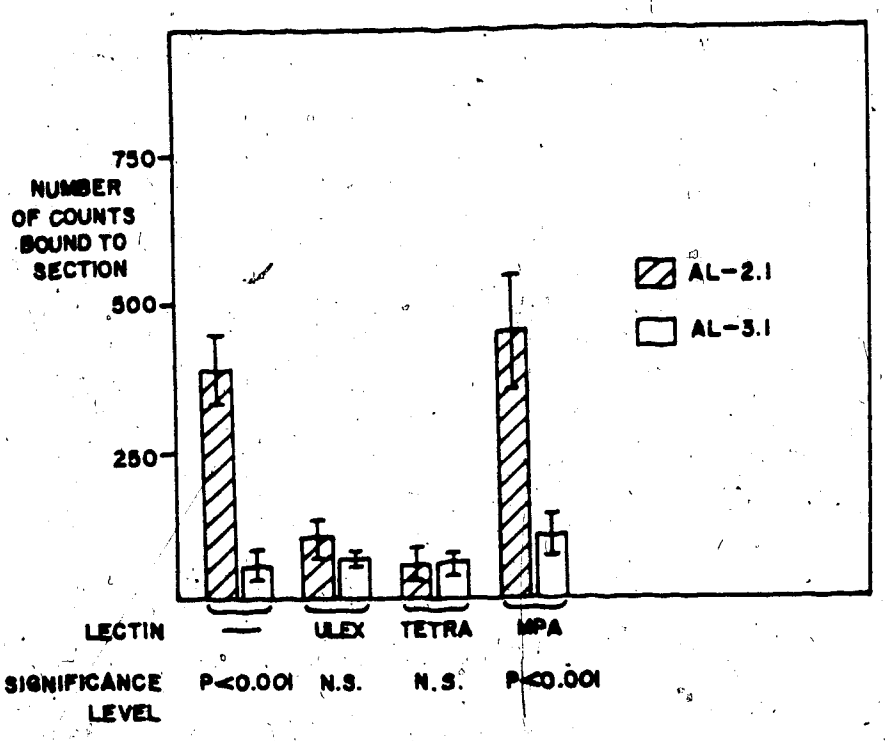

Figure 16

Inhibition of in vitro frozen section adherence assay with lectins. The lectin MPA, which is specific for *β*-Gal-*α* GalNAc was used as a control lectin.

DISCUSSION

A. MODELS OF ORGAN SPECIFIC METASTASIS (OSM)

The phenomenon of OSM has been observed for over a century yet little is known of the precise mechanisms involved. In particular, the seed-soil and receptor-acceptor hypotheses have been put forward to explain the phenomenon yet neither one has been fully supported or refuted. The problem lies in the similarities of both mechanisms since both suggest OSM is mediated by cell surface receptors on tumour cells. The mechanisms differ chiefly in the nature of the molecules on the target cells being recognized. The seed and soil hypothesis predicts that the molecules recognized by the tumour cells are factors, nutrients or signal molecules that perhaps induce proliferation or invasion. These molecules are present in the microenvironment of each organ and presumably each organ will have varying forms or quantities of these molecules. On the other hand, the receptor-acceptor hypothesis predicts that cell surface determinants on the organ are recognized by the tumour cell. These determinants (or acceptor molecules) may be organ specific cell surface molecules that exist in a particular orientation peculiar to each organ. These two models do not need to be mutually exclusive and some evidence in favour of this view has recently been obtained from this laboratory.

To differentiate and help characterize one of these hypotheses (receptor-acceptor), a modification to the frozen section adherence assay of Stamper and Woodruff, (1976), and Butcher *et al.*, (1979) is reported. The specific adherence of liver specific AL-2.1 tumour cells to frozen sections of liver directly supports this hypothesis. Furthermore, this specific adherence could be inhibited with a number of carbohydrates and lectins, especially those for

fucose.

B. AL-2.1 AND AL-3.1 TUMOUR MODEL

The tumour system chosen was a naturally occuring Marek's Disease induced T-cell lymphoma of chickens. The cell lines were reselected from originals for the liver (AL-2.1) and ovaries (AL-3.1) (Shearman and Longenecker, 1980). These tissues were chosen because they represent the common sites of involvement in the natural etiology of the disease (Payne, 1972b). The successfulness of the selections were measured by *in vivo* foci formation. While this assay was useful for liver foci (Figure 1), no discrete foci were formed in ovaries and therefore this *in vivo* assay does not measure all tumour-tissue interactions even when metastasis to this site is known to occur. In contrast to the inability of the *in vivo* assay to measure foci on the ovaries, the *in vitro* assay did show slight differences of AL-3.1 binding to ovary tissue compared to AL-2.1 (Figure 11). Immunological complications as a result of using allogeneic tumour cells do not appear to play an important role in this model system since chick embryos which are immunologically immature were used. The absence of immunolgical considerations per se in these sorts of cell-cell interactions has been supported in the homing of T-cells to HEV (Butcher and Weissman, 1980).

C. The Frozen Section Adherence Assay

This assay was chosen to study OSM because it directly measures the binding of cells to frozen tissue sections. In this way, the participation of receptor-acceptor interactions in the binding of organ specific tumour cell lines to particular frozen sections was shown. The advantages of using the *in vitro* frozen section adherence assay were twofold. First, as stated above, it allowed for a direct measurement of the role of receptor-acceptor interactions in OSM

by quantifying the adherence of tumour cells to target and non-target tissues. Second, the assay allowed us to monitor specific interactions between the receptor and acceptor molecules by comparing the abilities of different reagents to inhibit adherence.

In an effort to employ the frozen section adherence assay, many modifications of the system were required (Table 8). Using AL-2.1, the liver selected cell line as the positive, and AL-3.1, which was ovary selected as the negative cell line, a high degree of specificity as measured by the ratio of these lines on liver frozen sections was obtained. AL-3.1 is an appropriate control in this case because it was selected from the same initial population of cells but for a different organ specificity (Shearman and Longenecker, 1980). Although the binding between AL-2.1 and AL-3.1 is variable, it remains reproducible within experiments. For example, ratios of AL-2.1 to AL-3.1 on liver ranged from 2 to 1 to greater than 5 to 1. This specific binding always remained greater than the 1 to 1 ratios observed for AL-2.1/AL-3.1 on tissues other than liver, such as lung or heart. It should be mentioned that this assay was attempted initially with fluorescently labelled cells with the expectation it might be possible to visualize the liver structure with which the tumour cells were binding (Gallatin *et al.*, 1983). While these assays were successful in terms of the differential binding ratio obtained, it was not possible to determine which liver cell type was being recognized by the tumour cells. This problem has since been overcome using a different approach (see below).

In summary then, the results presented for the specificity of binding of AL-2.1 cells to liver sections is consistant with what is expected for the receptor-acceptor hypothesis. The nature of this assay, that is, the direct binding of cells to sections, provides direct evidence for receptor-acceptor interactions in this T-cell lymphoma system.

D. **Inhibition of Adherence In Vitro and Metastasis In Vivo**

Once specific adherence between tumour cells and target tissue could be demonstrated, it was necessary to examine the nature of the cell surface determinants responsible for this interaction. This was done using three approaches. First, evidence existed that carbohydrates in association with either protein or lipid were involved in cell-cell interactions suggesting that these molecules could be involved in this system (Table 4). Second, metabolic inhibitors or enzymes were utilized to determine their effect on the ability of AL-2.1 to metastasize or adhere. The three reagents chosen were tunicamycin (an inhibitor of N-linked glycosylation; Duksin and Bornstein, 1977), trypsin (a protease), and neuraminidase (which cleaves N-acetyl-neuraminic acid). The third and final approach was to use lectin and monoclonal antibodies to examine how they might affect adherence in the frozen section assay or liver foci formation *in vivo*. Since both *in vitro* and *in vivo* assays were monitored, a clearer understanding of the importance of the results could be ascertained.

A number of carbohydrates were identified that inhibited metastasis albeit to varying degrees. Perhaps the most surprising result was that the inhibitory sugars generally had a fucose molecule in their structure. Moreover, the simple fucose molecules had to be in a specific conformation since *a*-D(+) fucose gave no consistant inhibition while *a*-L(-) fucose consistently showed approximately 50% inhibition (Table 4).

The second approach again revealed some interesting information because trypsin pre-treatment of cells produced 100% inhibition *in vivo*. Furthermore, this inhibition was reversible since tumour cell metastasis regain their potential to metastasize towards their normal levels if incubated at 37°C for 3-6 hours (Table 5).

The involvement of carbohydrates was further implicated by the ability of certain lectins to inhibit OSM *in vivo* and adherence *in vitro*. These lectins were derived from *Ulex europaeus*, and from *Tetragonolobus purpureas* which have affinities for L(-) fucose. As shown in Figure 4, 16 and Table 5, *Ulex* binding is completely negative on AL-2.1 at the maximum sensitivity of the fluorescent activated cell sorter but is positive on the enriched endothelial population of liver cells. The lack of binding to AL-2.1 rules out the possibility that the fucose moiety bound by the lectin resides on this cell. Lectins specific for other carbohydrate moieties had no effect (Figure 16).

To rule out any effect of the inhibition *in vivo* or *in vitro* by non-specific cell death, a variety of approaches were undertaken. The CAM membrane of the chicken acts as the organ for gaseous exchange in the embryo and is highly supplied with vessels and capillaries which serve this purpose. Since a large portion of the embryo's blood supply is devoted to this area, the extensive involvement of tumour cells injected I.V. is not too surprising. Enumerating these foci serves as a good control for successful injections and tumour cell viability. Because the number of cells required to observe effects on the liver produces too high a density of CAM foci to accurately count, smaller inocula were required. In all the data obtained, no such reduction in CAM foci was observed between treated and non-treated controls (Table 6). The same result was observed by examining the growth kinetics of the tumour cell lines after treating them with sugars with and without washing. Again, no change in growth kinetics nor tumour cell toxicity as measured by trypan blue exclusion was observed except for p-nitrophenyl-*B*-D-fucopyranoside. The toxicity in this case was observed only *in vitro* and over long time periods and was probably the result of toxic metabolites produced from the sugar collecting in the culture medium. Based on these [illegible] it is reasonable, although not proved, that the effects observed [illegible] and

specific. Other investigators have also examined these potential effects and found similar results (see below).

Thus, the specific adherence of the liver selected AL-2.1 T-cell lymphoma cell line to liver frozen sections could be specifically inhibited by a number of different reagents. The recurring structure of these molecules (i.e. fucose specificity) provides the first molecular data regarding this receptor-acceptor interaction in this tumour model. This is further supported by the data which shows differential binding of fucose (^{125}I-fucose-BSA) by AL-2.1 but not AL-3.1 cells (Table 3). Furthermore, the endothelial enriched population contain cells that bind the fucose-specific lectin which suggests this may be the surface acceptor molecule. Finally, this receptor-acceptor interaction could be inhibited by incubating AL-2.1 cells with the enzyme trypsin. This preliminary analysis suggests that the lectin-like receptor is a cell surface protein. Since AL-2.1 cells do not bear the fucose determinant recognized by the lectins, this supports the proposal that the fucose containing molecule is on the endothelial cell and not the tumour cells.

E. THE ROLE OF FUCOSE IN CELLULAR INTERACTIONS

The importance of fucose containing molecules in cell-cell interactions is similarly observed in other systems. Using the frozen section adherence assay, Stoolman and Rosen (1983) have reported a group of compounds containing fucose or to a lesser degree mannose that are inhibitory in the homing of lymphocytes. While mannose was not inhibitory in the present system, fucose was. Furthermore, this inhibitory effect was stereospecific in that a partial effect was observed for *a*-L(-) fucose but not *a*-D(+) fucose (Table 4).

These authors (Stoolman and Rosen, 1983) have excluded non-specific effects by the sugars. For example, they found that while salt concentrations

were very important for binding, the osmolarity of sugars was not. It was also noted that sugars do not work by agglutinating the cells, a process which might lower the "effective" concentration and thus cause reduced cell binding. However, one different characteristic noted was the inhibitory effect their sugars had on both cell division and DNA synthesis. This effect was not observed with the tumour cells in this system except for p-nitrophenyl-*B*-D-fucopyranoside. Perhaps tumour cells are less sensitive to these compounds, or it may be that the concentrations used were sufficiently low in the present assay not to have these effects. For example, *a*-L(-) fucose was used at 150mM in their system while it was used at 30mM herein.

Other investigators have also found fucose associated with endothelial cell surfaces (Holthofer *et al.*, 1982). This particular group has reported that all human endothelial cells are positive for *Ulex* lectin binding. However, they do point out that another fucose specific lectin (*Tetragonolobus purpureas*) does not detect these fucose determinants on human endothelial cells. If this finding is extrapolated to other species, this could be interpreted as meaning that while all endothelial cells are fucose positive, specific endothelial cells (liver in this case) have a different fucose containing cell surface determinant which is responsible for OSM. Both of these lectins were capable of inhibiting OSM *in vivo* in this system. These results raise some interesting questions that will require further investigation. If fucose residues are located on all endothelial cells, and these ubiquitous determinants turn out to be the acceptor molecule, then how could the tumour cell use this determinant in an organ specific manner? There are at least two possible explanations: i) perhaps tumour cells use fucose binding to take themselves out of the circulation and then use seed-soil or other receptor-acceptor interactions to complete the metastatic cascade. Alternately, ii) the OSM receptor might recognize a more complex moiety containing fucose. Perhaps two forms of fucose reside on the endothelium, one form of which is

liver specific.

F. Models For Receptor-Acceptor Interactions

The evidence presented above regarding the nature of the receptor and acceptor cell surface determinants has shed some light onto the biochemical nature of these molecules. For example, the role of fucose in the interaction makes it possible that the receptor may be lectin-like in nature. This would certainly be consistent with some other reports of lectin-like activity on tumour cells or on their normal cell counterparts. For example, Schlepper-Schafer *et al.*, (1981) has isolated a lectin on the hepatocyte that specifically interacts with a galactosyl residue on the tumour cell. While this is the reverse of the situation presented here, it does provide a nice conceptual bases for the data presented. Other examples, such as red blood cell scavenging, is known to operate by lectin molecules on liver cells that recognize cryptic antigens, which become exposed on aging or dying cells (Kolb *et al.*, 1978). These lectin-like activities may be more comparable to those seen in embryonic cell migration but again are consistant with these results.

Regarding the origin of these cellular interactions, other aspects also need to be examined. As pointed out earlier, the system used in this study was a T-cell lymphoma. In light of the work of Stamper and Woodruff (1976) and Butcher *et al.*, (1979), the use of a T-cell lymphoma in this system may have been fortuitous. Perhaps leukemias and lymphomas have modified normal recognition functions into ones that allow for OSM. Since this specific homing mechanism does not apply to adherent adult cells, solid tumours may have evolved quite unique mechanisms. A true comparison can be attempted only when the cell surface determinants involved in solid tumour metastasis are elucidated.

In this regard, the information presented in this thesis has provided two important steps in the elucidation of the complex mechanisms of organ specific metastasis. While evidence in favour of receptor mediated OSM had been provided by the work of Shearman and Longenecker (1981), this data did not provide the exact mechanisms of tumour cell-target tissue interactions. With the development of the frozen section adherence assay, it has now been shown that i) receptor-acceptor interactions are involved in OSM in the MD-derived T-cell lymphoma model system used. Furthermore, ii) the assay has provided the means to examine some aspects of the nature of this receptor-acceptor interaction, particularly the role of fucose. With the aid of fucose or lectin columns, it should be possible to purfiy and characterize the integral cell surface molecules involved.

G. The Evolution Of OSM

In this study, there was no attempt to examine the underlying mechanisms of OSM, that is, <u>why</u> certain tumours reproducibly colonize the same organs. Whether OSM occurs by the seed and soil or receptor-acceptor hypothesis, no clear understanding presently exists for the mechanism by which the receptor for a specific organ is often "induced" or "expressed" in one tumour cell type and yet a different target organ receptor is generated for another tumour cell type. Is there something about the mechanism of the malignant transformation that reproducibly triggers the expression of a particular receptor? Is it possible that certain malignant cell types have cell surface determinants that closely resemble the receptor required to recognize an organ that the non-malignant counterpart would not normally encounter? Perhaps the recent explosion in the literature on the association of oncogenes with malignant transformation will provide some insight into these questions.

H. SUMMARY

A T-cell lymphoma with organ specific metastasis *in vivo* to the liver was selected. Using a modified *in vitro* frozen section adherence assay, this cell line (AL-2.1) bound preferentially to liver frozen sections compared to a non-liver selected cell line (AL-3.1). This specific adherence of AL-2.1 provides direct evidence for receptor-acceptor interactions in this model system. Furthermore, a number of reagents, including L(-) fucose, p-Nitrophenyl -*B*-D-fucopyranoside, lactulose and fucoidan could inhibit both OSM *in vivo* and adherence *in vitro*. A study of the cell surfaces of enriched liver parenchymal and endothelial cells using lectins has shown the existance of fucose or fucose-like determinants on the endothelial enriched cell surface. Further to this, ^{125}I-fucose-BSA was found to bind AL-2.1 cells (putative receptor) greater than AL-3.1 (putative acceptor) and this differential binding was trypsin sensitive.

REFERENCES

Agostino, D., Cliffton, E.E.: Organ localization and the effect of trauma on the fate of circulating cancer cells. Cancer Res. 25: 1728-1732, 1965.

Ashwell, G., Morell, A.G.: The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. Adv. Enzymol. 41: 99-128, 1974.

Barker, C.F., Billingham, R.E.: Immunologically privileged sites. Advanc. Immunol. 25: 1-54, 1977.

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

Barton, M.A., Singh, B., Fraga, E.: Synthetic polypeptide antigens of defined geometry. J. Am. Chem. Soc. 99: 8491-8496, 1977.

Bosmann, H.B.: Elevated glycosidases and proteolytic enzymes in cells transformed by RNA tumor virus. Biochem. Biophys. Acta. 264: 339-343, 1972.

Bosmann, H.B., Lockwood, T., Morgan, H.R.: Surface biochemical changes accompanying primary infection with Rous sarcoma virus: II. Proteolytic and glycosidase activity and sublethal autolysis. Exp. Cell Res. 83: 25-30, 1974.

Brunson, K.W., Beattie, G., Nicolson, G.L.: Selection and altered properties of brain-colonising metastatic melanoma. Nature 272: 543-545, 1978.

Brunson, K.W., Nicolson, G.L.: Selection and biologic properties of malignant variants of a murine lymphosarcoma. J. Natl. Cancer Inst. 61: 1499-1503, 1978.

Brunson, K.W., Nicolson, G.L.: In: Brain Metastasis, Weiss L, Gilbert H, Posner J.B., (ed), Hall, Boston, 50, 1980.

Butcher, E.C., Scollay, R.G., Weissman, I.L.: Lymphocyte adherence to high endothelial venules: Characterization of a modified *in vitro* assay, and examination of the binding of syngeneic and allogeneic lymphocyte populations. J. Immunol. 123: 1996-2003, 1979.

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

Capel, I.D., Jenner, M., Pinnock, M.H., Dorrell, H.M., Payne, D.C., Williams, D.C.: Correlation between tumour size, metastatic spread and galactosyl transferase activity in cyclophosphamide-treated mice bearing the lewis lung carcinoma. Oncology 36: 242-244, 1979.

Chambers, A.F., Shafir, R., Ling, V.: A model system for studying metastasis using the chick embryo. Cancer Res. 42: 4018-4025, 1982.

Chatterjee, S.K.: Glycosyltransferases in metastasizing and non-metastasizing rat mammary tumors and the release of these enzymes in the host serum. Europ. J. Cancer 15: 1351-1356, 1979.

Chen, L.B., Buchanan, J.M.: Plasminogen-independent fibrinolysis by proteases produced by transformed chick embryo fibroblasts. Proc. Natl. Acad. Sci. 72: 1132-1136, 1975.

Chow, D.A., Greenberg, A.H.: The generation of tumor heterogeneity *in vivo*. Int. J. Cancer 25: 261-265, 1980.

Cifone, M.A., Fidler, I.J.: Increasing metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms. Proc. Natl. Acad. Sci. 78: 6949-6952, 1981.

Clarkson, B., Katz, A., Dann, M.A., Karnofsky, D.A.: Behavior of P388 mouse leukemia in the chick embryo and hatched chick. J. Natl. Cancer Inst. 32: 471-505, 1964.

Cock, A.G.: The immunological status of ovarian homografts in fowls. Immunol. 5:169-180, 1962.

Curtis, A.S.G.: Histocompatibility systems, recognition and cell positioning. Develop. Compar. Immunol. 3: 379-387, 1979.

Der, C.J., Stanbridge, E.J.: Alterations in the extracellular matrix organization associated with the reexpression of tumorigenicity in human cell hybrids. Int. J. Cancer 26: 451-459, 1980.

Dexter, D.L., Kowalski, H.M., Blazer, B.A., Fligiel, Z., Vogel, R., Heppner, G.H.: Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res. 38: 3174-3181, 1978.

Dobrossy, L., Pavelic, Z.P., Bernacki, R.J.: A correlation between cell surface sialyltransferase, sialic acid, and glycosidase activities and the implantability of B16 murine melanoma. Cancer Res. 41: 2262-2266, 1981.

Duksin, D., Bornstein, P.: Changes in surface properties of normal and transformed cells caused by tunicamycin, an inhibitor of protein glycosylation. Proc. Natl. Acad. Sci. 74: 3433-3437, 1977.

Easty, D.M., Easty, G.C.: Measurement of the ability of cells to infiltrate normal tissues *in vitro*. Br. J. Cancer 29: 36-49, 1974.

Easty, G.C., Easty, D.M., Tchao, R.: The growth of heterologous tumor cells in chick embryos. Europ. J. Cancer 5: 287-295, 1969a.

Easty, G.C., Easty, D.M., Tchao, R.: The distribution of heterologous tumor cells in chick embryos following intravenous injection. Europ. J. Cancer 5: 297-305, 1969b.

Eaves, G.: The invasive growth of malignant tumours as a purely mechanical process. J. Path. 109: 233-237, 1973.

Eisenstein, R., Kuettner, K.E., Neapolitan, C., Soble, L.W., Sorgente, N.: The resistance of certain tissues to invasion. III. Cartilage extracts inhibit the growth of fibroblasts and endothelial cells in culture. Am. J. Pathol. 81: 337-348, 1975.

Ewing, J.: In Neoplastic Diseases, a treatise on tumors, 3rd ed., Sanders, Philadelphia, Pennsylavania, 1928.

Feizi, T.: Carbohydrate differentiation antigens. Trends Biol. Sci. 5: 333-335, 1981.

Fialkow, P.J.: The origin and development of human tumors studied with cell markers. New Engl. J. Med. 291: 26-35, 1974.

Fidler, I.J. Metastasis: Quantitative analysis of distribution and fate of tumor emboli labeled with ^{125}I-5-Iodo-2' deoxyuridine. J. Natl. Cancer Inst. 45: 773-782, 1970.

Fidler, I.J: Selection of successive tumor lines for metastasis. Nature 242: 148-149, 1973a.

Fidler, I.J.: The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. Europ. J. Cancer 9: 223-227, 1973b.

Fidler, I.J.: In: fundamental aspects of metastasis., Patterns of tumor cell arrest and development, Chapt 14, Weiss L. (ed), North-Holland, Amsterdam, 275-289, 1976.

Fidler, I.J.: General considerations for studies of experimental cancer metastasis. IN: Busch H (ed) Methods in Cancer Research, Vol. XV. Academic Press, New York, 1978a.

Fidler, I.J.: Tumor heterogeneity and the biology of cancer invasion and metastasis. Cancer Res. 38: 2651-2660, 1978b.

Fidler, I.J., Bucana, C.: Mechanism of tumor cell resistance to lysis by syngeneic lymphocytes. Cancer Res. 37: 3945-3956, 1977.

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

Fidler, I.J., Hart, I.R.: The orgin of metastatic heterogeneity in tumors. Eur. J. Cancer 17: 487-494, 1981.

Fidler, I.J., Nicolson, G.L.: Organ selectivity for implantation survival and growth of B16 melanoma variant tumor lines. J. Natl. Cancer Inst. 57: 1199-1202, 1976.

Fisher, B., Fisher, E.R., Feduska, N.: Trauma and the localization of tumor cells. Cancer 20: 23-30, 1967.

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

Freitas, A.A., de Sousa, M.: The role of cell [redacted]ns in the control of lymphocyte traffic. Cell. Immunol. 22: 345-35[redacted].

Fuji, H., Mihich, E.: Selection for high immunogenicity in drug-resistant sublines of murine lymphomas demonstrated by plaque assay. Cancer Res. 35: 946-952, 1975.

Gallatin, W.M., Weissman, I.L., Butcher, E.C.: A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 304: 30-34, 1983.

Galton, J.E., Xue, B., Hochwald, G.M., Thorbecke, G.J.: Derivation of transplantable, dimethylbenzanthracene-induced chicken fibrosarcoma lines with differences in metastasizing properties and organ specificity. J. Natl. Cancer Inst. 64: 535-541, 1982.

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

Goldstein, I.J., Hayes, C.E.: The lectins: Carbohydrate-binding proteins of plants and animals. Adv. Carbohyd. Chem. Biochem. 35: 127-340, 1978.

Gospodarowicz, D., Greenburg, G., Bialecki, H., Zetter, B.R.: Factors involved in the modulation of cell proliferation *in vivo* and *in vitro*: The role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. In Vitro 14: 85-118, 1978.

Greenberg, A.H., Chow, D.A., Wolosin, L.B.: Natural surveillance of NK-resistant tumors. The role of natural anti-tumor antibody (NAb) and macrophages (mph). In: Genetic Control of Natural Resistance to Infection and Malignancy. Skamene, E., Kongshavn, P., Landy, M., (ed), Academic Press, New York, 455-465, 1980.

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

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

Hakansson, L., Trope, C.: An *in vitro* study of the effect of cytostatic drugs on DNA synthesis in methylcholanthrene induced mouse sarcomas and in rat walker 256 tumours. Acta. Path. Microbiol. Scand. 81: 552-558, 1973.

Hakansson, L., Trope, C.: On the presence within tumours of clones that differ in sensitivity to cytostatic drugs. Acta. Path. Microbiol. Scand. 82: 35-40, 1974.

Hakomori, S-I.: Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Ann. Rev. Biochem. 50: 733-764, 1981.

Hart, I.R.: "Seed and Soil" revisited: Mechanisms of site-specific metastasis. Cancer Metastasis Review 1: 5-16, 1982.

Hart, I.R., Fidler, I.J.: An *in vitro* quantitative assay for tumor cell invasion. Cancer Res. 38: 3218-3224, 1978.

Hart, I.R., Fidler, I.J.: Role of organ selectivity in the determination of metastatic patterns of B16 melanoma. Cancer Res. 40: 2281-2287, 1980.

Heppner, G.H., Dexter, D.L., DeNucci, T., Miller, F.R., Calabresi, P.: Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. Cancer Res. 38: 3758-3763, 1978.

Holthofer, H., Virtanen, I., Kariniemi, A.L., Hormia, M., Linder, E., Miettinen, A.: Ulex europaeus I lectin as a marker for vascular endothelium in human tissues. Lab. Invest. 47: 60-66, 1982.

Hynes, R.O.: Cell surface proteins and malignant transformation. Biochem.

Biophys. Acta. 458: 73-107, 1976.

Irimura, T., Gonzàlez, R., Nicolson, G.L.: Effects of tunicamycin on B16 metastatic melanoma cell surface glycoproteins and blood-borne arrest and survival properties. Cancer Res. 41: 3411-3418, 1981.

Irimura, T., Nicolson, G.L.: Affinity isolation of cell surface sialoglycoproteins from metastatic B16 melanoma cells and their interaction with endothelial cell basal lamina. J. Cell Biol. 91: 118a, 1981.

Kahan, B.: Ovarian localization by embryonal teratacarcinoma cells derived from female germ cells. Somatic Cell Genet. 5: 763-780, 1979.

Kerbel, R.S.: Implications of immunological heterogeneity of tumours. Nature 280: 358-360, 1979.

Kerbel, R.S., Dennis, J.W., Largarde, A.E., Frost, P.: Tumor progression in metastasis: An experimental approach using lectin resistant tumor variants. Cancer Metast. Rev. 1: 99-140, 1982.

Killion, J.J., Kollmorgen, G.M.: Isolation of immunogenic tumour cells by cell-affinity chromatography. Nature 259: 674-676, 1976.

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

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

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

Klein, K., Coetzee, M.L., Madhav, R., Ove, P.: Inhibition of tritiated thymidine incorporation in cultured cells by rat kidney extract. J. Natl. Cancer Inst. 62: 1557-1564, 1979.

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

Kolb, H., Schudt, C., Kolb-Bachofen, V., Kolb, H-A.: Cellular recognition by rat liver cells of neuraminidase-treated erythrocytes. Exp. Cell Res. 113: 319-325, 1978.

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

Kuttner, B.J., Woodruff, J.J.: Selective adherence of lymphocytes to myelinated areas of rat brain. J. Immunol. 122: 1666-1671, 1979.

Leighton, J.: Invasion and metastasis of heterologous tumors in the chick embryo. Progr. Exp. Tumor Res. 4: 98-125, 1964.

Leighton, J., Mansukhani, S., Fetterman, G.H.: Clinical and experimental tumors of the kidney in tissue culture and in the chick embryo. Europ. J. Cancer 8: 281-285, 1972.

Lewis, M.G., Phillips, T.M., Rowden, G., Jerry, L.M.: Biological mechanisms and therapy. In: Cancer invasion and metastasis., Day, S.B., Laird, W.P., Stansly, P., Garattini, S., Lewis, M.G., (ed), Raven Press, New York, 245-258, 1977.

Lilien, J., Balsamo, J., McDonnough, J., Hermolin, J., Cook, J., Rutz, R.: Adhesive specificity among embryonic cells. In: Hynes R.O., (ed), Surfaces of normal and malignant cells., J. Wiley and Sons, Chichester, 389-419, 1979.

Liotta, L.A., Kleinerman, J., Saidel, G.M.: The significance of hematogenous tumor cell clumps in the metastatic process. Cancer Res. 36: 889-894, 1976.

Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I.R., Foltz, C.M., Shafie, S.: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284: 67-68, 1980.

Maguida, M., Shur, P., Lockwood, J., Boston, J., Williams, D.C.: In Metastasis: clinical and experimental aspects. Hellman K, Hilgard P, Eccles S (ed), 179-183, Martinus Nijhoff, The Hague, 1980.

Marx, J.C.: Tumors: A mixed bag of cells. Science 215: 275-277, 1982.

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

Merritt, W.D., Richardson, C.L., Keenan, T.W., Morre, D.J.: Gangliosides of liver tumors induced by N-2-Fluorenylacetamide. I. Ganglioside alterations in liver tumorigenesis and normal development. J. Natl. Cancer Inst. 60: 1313-1327, 1978.

Middelkoop, O.P., Roos, E., Van de Pavert, I.V.: Infiltration of lymphosarcoma cells into hepatocyte cultures: Inhibition by univalent antibodies against liver plasma membranes and lymphosarcoma cells. J. Cell Sci. 56: 461-470, 1982.

Milstein, C.B., Frangioni, B., Pink, J.: Studies on the variability of immunoglobulin sequence. Cold Spring Harbour Symp. Quant. Biol. 32: 31-36, 1967.

Miner, K.M., Walter, H., Nicolson, G.L.: Subfractionation of malignant variants of metastatic murine lymphosarcoma cells by countercurrent distribution in two-polymer aqueous phases. Biochem. 20: 6244-6250, 1981.

Morre, D.J., Kloppel, T.M., Merritt, W.D., Keenan, T.W.: Glycolipids as indicators of tumorigenesis. J. Supramol. Struct. 9: 157-177, 1978.

Mosmann, T.R., Gallatin, M., Longenecker, B.M.: Alteration of apparent specificity of monoclonal (hybridoma) antibodies recognizing polymorphic histocompatibility and blood group determinants. J. Immunol. 125: 1152-1156, 1980.

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

Neri, A., Nicolson, G.L.: Phenotypic drift of metastatic and cell-surface properties of mammary adenocarcinoma cell clones during growth *in vitro*. Int. J. Cancer 28: 731-738, 1981.

Nicolson, G.L.: Trans-membrane control of the receptors on normal and tumor cells. II. Surface changes associated with transformation and malignancy.

Biochem. Biophys. Acta. 458: 1-72, 1976.

Nicolson, G.L.: In: Cancer invasion and metastasis., Liotta, L.A., Hart, I.R., (ed), Martinus Nijhoff, U.S.A., In press.

Nicolson, G.L.: Cancer metastasis: Organ colonization and the cell surface properties of malignant cells. Biochem. Biophys. Acta. 695: 113-176, 1982.

Nicolson, G.L., Custead, S.E.: Tumor metastasis is not due to adaptation of cells to a new organ environment. Science 215: 176-178, 1982.

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

Nowell, P.C.: The clonal evolution of tumor cell populations. Science 194: 23-28, 1976.

Ossowski, L., Reich, E.: Experimental model for quantitive study of metastasis. Cancer Res. 40: 2300-2309, 1980a.

Ossowski, L., Reich, E.: Loss of malignancy during serial passage of human carcinoma in culture and discordance between malignancy and transformation parameters. Cancer Res. 40: 2310-2315, 1980b.

Paget, S.: The distribution of secondary growths in cancer of the breast. Lancet 1: 571-573, 1889.

Payne, L.N.: Pathogenesis of Marek's disease - a review. In: Biggs P.M., de-The G., Payne L.N., (ed), Oncogenesis and herpes viruses. IARC, Lyons, 21-37, 1972a.

Payne, L.N.: Pathogenesis of Marek's disease - a review. In: Biggs P.M., de-The G., Payne L.N., (ed), Oncogenesis and herpes viruses. IARC, Lyons, 217-229, 1972b.

Posté, G., Fidler, I.J.: The pathogenesis of cancer metastasis. Nature 283: 139-146, 1980.

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

Prehn, R.T.: Cancer antigens in tumors induced by chemicals. Fed. Proc. 24: 1018-1022, 1965.

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

Rahman, A.F.R., Longenecker, B.M.: A monoclonal antibody for the Thomsen-Freidenreich cryptic T-antigen. J. Immunol., 129: 2021-2024, 1982.

Raz, A., Hart, I.R.: Murine melanoma: A model for intracranial metastasis. Br. J. Cancer 42: 331-341, 1980.

Raz, A., Lotan, R.: Lectin-like activities associated with human and murine neoplastic cells. Cancer Res. 41: 3642-3647, 1981.

Raz, A., Mclellan, W.L., Hart, I.R., Bucana, C.D., Hoyer, L.C., Sela, B-A., Dragsten, P., Fidler, I.J.: Cell surface properties of B16 melanoma variants with differing metastatic potential. Cancer Res. 40: 1645-1651, 1980.

Reading, C.L., Brunson, K.W., Torrianni, M., Nicolson, G.L.: Malignancies of metastatic murine lymphosarcoma cell lines and clones correlate with decreased cell surface display of RNA tumor virus envelope glycoprotein gp70. Proc. Natl. Acad. Sci. 77: 5943-5947, 1980.

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

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

Roblin, R.: Cancer Biol. Rev. 2: Contributions of secreted tumor cell products to metastasis. 59-94, 1981.

Roos, E.: Cellular interactions during the formation of liver metastases. Ph.D. Thesis, 1981.

Roos, E., Dingemans, K.P.: Mechanisms of metastasis. Biochem. Biophys. Acta 560: 135-166, 1979.

Roos, E., Tulp, A., Middelkoop, O.P., van de Pavert, I.V.: Interaction between liver-metastasizing lymphoid tumor cells and hepatic sinusoidal endothelial cells. Submitted.

Roos, E., Van de Pavert, I.V., Middelkoop, O.P.: Infiltration of tumour into cultures of isolated hepatocytes. J. Cell Sci. 47: 385-397, 1981.

Sage, H., Crouch, E., Bornstein, P.: Collagen synthesis by bovine aortic endothelial cells in culture. Biochem. 18: 5433-5442, 1979.

Sandberg, A.A., Hossfeld, D.K.: Chromosomal abnormalities in human neoplasma. Ann. Rev. Med. 21: 379-408, 1970.

Sato, H., Suzuki, M.: Deformability and viability of tumour cells by transcapillary passage with reference to organ affinity of metastasis in cancer. In: Weiss L. (ed), Fundamental Aspects of Metastasis, North-Holland, Amsterdam, 311-317, 1976.

Schirrmacher, V., Altevogt, P., Fogel, M., Dennis, J., Waller, C.A., Barz, D., Schwartz, R., Cheingsong-Popov, R., Springer, G., Robinson, P.J., Nebe, T., Brossmer, W., Vlodavsky, I., Paweletz, N., Zimmermann, H.P., Uhlenbruck, G.: Importance of cell surface carbohydrates in cancer cell adhesion, invasion and metastasis. Invasion and Metastasis 2: 313-360, 1983.

Schirrmacher, V., Cheingsong-Popov, R., Arnheiter, H.: Hepatocyte-tumor cell interaction *in vitro*. J. Exp. Med. 151: 984-989, 1980.

Schlepper-schafer, J., Friedrich, E., Kolb, H.: Galactosyl specific receptor on liver cells: Binding site for tumor cells. Eur. J. Cell Biol. 25: 95-102, 1981.

Shafie, S.M., Liotta, L.A.: Formation of metastasis by human breast carcinoma cells (MCF-7) in nude mice. Cancer Lett. 11: 81-87, 1980.

Shearman, P.J., Gallatin, W.M., Longenecker, B.M.: Detection of a cell-surface antigen correlated with organ-specific metastasis. Nature 286: 267-269, 1980.

Shearman, P.J., Longenecker, B.M.: Selection for virulence and organ-specific metastasis of herpesvirus-transformed lymphoma cells. Int. J. Cancer 25: 363-369, 1980.

Shearman, P.J., Longenecker, B.M.: Clonal variation and functional correlation of organ-specific metastasis and an organ-specific metastasis-associated antigen. Int. J. Cancer 27: 387-395, 1981.

Simpson, D.L., Thorne, D.R., Loh, H.H.: Lectins: endogenous carbohydrate-binding proteins from vertebrate tissues: Functional role in recognition processes. Life Sci. 22: 727-748, 1978.

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

Skipski, V.P., Gitterman, C.O., Prendergast, J.S., Betit-Yen, K., Lee, G., Luell, S., Stock, C.C.: Possible relationship between glycoshingolipids and the formation of metastasis in certain human experimental tumors. J. Natl. Cancer Inst. 65: 249-256, 1980.

Sloane, B.F., Dunn, J.R., Honn, K.V.: Lysosomal catheprin B: Correlation with metastatic potential. Science 212: 1151-1153, 1981.

Smith, H.S., Riggs, J.L., Mosesson, M.W.: Production of fibronectin by human epithelial cells in culture. Cancer Res. 39: 4138-4144, 1979.

Springer, G.F., Cheingsong-Popov, R., Schirrmacher, V., Desai, P.R., Tegtmeyer, H.: Proposed molecular basis of murine tumor cell-hepatocyte interaction. J Biol. Chem. 258: 5702-5706, 1983.

Springer, G.F., Desai, P.R., Murthy, M.S., Tegtmeyer, H., Scanlon, E.F.: Human Carcinoma-associated precursor antigens of blood group MN system and the host's immune response to them. Prog. Allergy 26:42-73, 1979.

Stamper, H.B., Woodruff, J.J.: Lymphocyte homing into lymph nodes: *In vitro* demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. J. Exp. Med. 144: 828-833, 1976.

Stangly, P.G., Sala, H.: Cancer metastasis, approaches to the mechanism, prevention and treatment. GANN Monograph on Cancer Research No. 20, University Park Press, Tokyo, 1977.

Steinberg, M.S.: Reconstruction of tissues by dissociated cells. Science 141: 401-408, 1963.

Stoolman, L.M., Rosen, S.D.: Possible role for cell-surface carbohydrate-binding molecules in lymphocyte recirculation. J. Cell Biol. 96: 722-729, 1983.

Strauch, L.: The role of collagenases in tumour invasion. In: Tarin (ed). Tissue interactions in carcinogenesis. Academic Press, New York, 399-434, 1972.

Sugarbaker, E.V.: The organ selectivity of experimentally induced metastases in rats. Cancer 5: 606-612, 1952.

Sugarbaker, E.V.: Cancer metastasis: A product of tumor-host interactions. Metastasis. Curr. Prob. Cancer 3: 1-59, 1979.

Sugarbaker, E.V.: Patterns of metastasis in human malignancies. In: Cancer Biology Reviews, Volume 2, J.J. Marchalonis, M.G. Hanna, and I.J. Fidler (ed), Marcel Dekker, Inc., New York, pp 235-278, 1981.

Sugarbaker, E.V., Weingrad, D.N., Roseman, J.M.: Observations on cancer metastasis in man. In: Tumor Invasion And Metastasis, L.A. Liotta and I.R. Hart (ed), Martinus Nijhoff Publishers, The Hague, pp 427-465, 1982.

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

Takahashi, S., Konishi, Y., Nakatani, K., Inui, S., Kojima, K., Shiratori, T.: Coversion of a poorly differentiated human adenocarcinoma to ascites form with invasion and metastasis in nude mice. J. Natl. Cancer Inst. 60: 925-929, 1978.

Talmadge, J.E., Starkey, J.R., Stanford, D.R.: *In vitro* characteristics of metastatic variant subclones of restricted genetic origin. J. Supramol. Struct. 15: 139-151, 1981.

Tao, T-W., Burger, M.M.: Non-metastasising variants selected from metastasising melanoma cells. Nature 270: 437-43[illegible] 1977.

Tao, T-W., Burger, M.M.: Lectin-resistant variants of mouse melanoma cells. I. Altered metastasizing capacity and tumorigenicity. Int. J. Cancer 29: 425-430, 1982.

Tao, T-W., Matter, A., Volgel, K., Burger, M.M.: Liver-colonizing melanoma cells selected from B-16 melanoma. Int. J. Cancer 23: 854-857, 1979.

Thompson, S.C.: Effect of age and sex on lung-colony-forming efficiency of injected mouse tumor cells. Br. J. Cancer 34: 566-570, 1976.

Trope, C.: Different sensitivity to cytostatic drugs of primary tumor and metastasis of the lewis carcinoma. Neoplasma 22:171-180, 1975.

Turner, G.A., Sherbet, G.V., Wildridge, M.: A comparison of the electrokinetic properties of metastasizing and non-metastasizing forms of a hamster lymphosarcoma. Exp. Cell Biol. 48: 385-392, 1980.

Unkeless, J., Dano, K., Kellerman, G.M., Reich, E.: Fibrinolsis associated with oncogenic transformation. J. Biol. Chem. 249: 4295-4305, 1974.

Vaage, J.: *In vivo* and *in vitro* lysis of mouse cancer cells by antimetastatic effectors in normal plasma. Cancer Immunol. Immunother. 4: 257-261, 1978.

Vaage, J., Agrawal, S.: Stimulation or inhibition of immune resistance against metastatic or local growth of a C3H Mammary carcinoma. Cancer Res. 36: 1831-1836, 1976.

Weiss, L.: Membrane dynamics and the metastasis of cancer. Cell Biophys. 1: 331-343, 1979.

Wolosin, L.B., Greenberg, A.H.: Murine natural anti-tumor antibodies. I. Rapid *in*

vivo binding of natural antibody by tumor cells in syngeneic mice. Int. J. Cancer 23: 519-527, 1979.

Wood, S.: Experimental studies of the intravascular dissemenation of ascitic V2 carcinoma cells in the rabbit, with special reference to fibrinogen and fibrinolytic agents. Bull. Schweig. Akad. Med. Wiss. 20: 92-121, 1964.

Woodruff, J.J.: Role of lymphocyte surface determinants in lymph node homing. Cell. Immunol. 13: 378-384, 1974.

Woodruff, J.J., Woodruff, J.F.: Virus-induced alterations of lymphoid tissue: IV. The effect of Newcastle disease virus on the fate of transfused thoracic duct lymphocytes. Cell. Immunol. 10: 78-85, 1974.

Wright, D.H.: The pathology of Burkitt's Lymphoma - a review. In: Biggs P.M., de-The G., Payne L.N., (ed). Oncogenesis and herpes viruses. IARC, Lyons, 217-229, 1972.

Yogeeswaran, G.: Cell surface glycolipids and glycoproteins in malignant transformation. Adv. Cancer Res. 38: 289-350, 1983.

Yogeeswaran, G., Salk, P.L.: Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. Science 212: 1514-1516, 1981.

Yogeeswaran, G., Stein, B.S.: Glycoshingolipids of metastatic variant RNA virus-transformed nonproducer Balb/3T3 cell lines: Altered metabolism and cell surface exposure. J. Natl. Cancer Inst. 65: 967-976, 1980.

Yogeeswaran, G., Stein, B.S., Sebastian, H.: Altered cell surface organization of gangliosides and sialylglycoproteins of mouse metastatic variant lines selected *in vivo* for enhanced lung implantation. Cancer Res. 38: 1336-1344, 1978.

Zeidman, I.: The fate of circulating tumor cells. I. Passage of cells through capillaries. Cancer Res. 21: 38-39, 1961.

Zeidman, I., Buss, J.M.: Transpulmonary passage of tumor cell emboli. Cancer Res. 12: 731-733, 1952.