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Growth and metastasis of 13762NF mammary adenocarcinoma

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GROWTH AND METASTASIS OF 13762NF MAMMARY ADENOCARCINOMA

by

HELEN SABZEVARI

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Chairman of Examining Committee
Prof. Jeanne Szalay, Queens College

1/27/90
date

Executive Officer
Prof. Peter C. Chabora

Prof. Jeanne Szalay

Prof. Elizabeth Boyland, Queens College

Prof. Vincent Merluzzi, Queens College

Dr. Kevin O'Boyle, Memorial Sloan Kettering Cancer Center

Dr. Gloria Koo, Merck, Sharp and Dohme Research Laboratories

Supervisory Committee

The City University of New York

Abstract

GROWTH AND METASTASIS OF 13762NF MAMMARY ADENOCARCINOMA

by

Helen Sabzevari

Advisor: Professor Jeanne Szalay

Using the transplantable mammary adenocarcinoma 13762NF, the growth and metastasis of intraocular tumors was examined following subcutaneous (SC) or intracameral (IC) tumor implantation. IC implantation of tumor lead to death after 7-9 weeks. In addition to extensive pulmonary metastasis, animals had metastasis to the cervical lymph nodes, body wall, heart, kidney, and liver. Animals receiving iridial implants with removal (enucleation) of tumor filled eyes prior to spread to the orbit remained healthy and the development of overt metastatic disease was prevented up to 2 years.When healthy enucleated rats were necropsied 0 days to 26 weeks post enucleation, small nodules were seen in the lung. SC tumors metastasize predominantly to lung and lymph node. Excision of small primary SC tumors did not prevent animals from dying after 7-8 weeks with overt pulmonary metastasis.

Examination of enucleated rats 3 months post-enucleation demonstrated the presence of small nodules in the lung, heart, or cervical

lymph node. Histological and immunocytochemical analysis demonstrated the presence of carcinoma in 3 of 4 sampled nodules. Cell suspensions from enucleated rat organs containing nodules were inoculated into sublethally irradiated rats and caused cachexia and death. Cachexia and death were not observed when cell suspensions from control rats were inoculated into sublethally irradiated rats. Examination of old enucleated rats 1-3 years after enucleation revealed the presence of viable transplatable metastatic adenocarcinoma in 23% of the animals.

The effect of treatment with a new therapeutic immunomodulator, Linomide (LS2616) was examined using the 13762NF tumor in rats that received SC or IC tumor implants or intravenous inoculation of tumor cells. Linomide was administered continuously in drinking water at a low dose (LD, 15 mg/kg) or high dose (HD, 60 mg/kg) beginning either one week before (pre-treatment) or 2 weeks after (post-treatment) tumor challenge. Post-treatment with Linomide in SC experiments resulted in a significant decrease in tumor size, partial or complete regression of tumors, and a low incidence of apparent cures. In rats with ocular tumors Linomide was shown to significantly reduce pulmonary as well as extrapulmonary metastasis. In an experimental metastasis assay, Linomide did not effect pulmonary metastasis. The specific effect of treatment with Linomide depended upon the time of initiation of treatment and route of administration of tumor. In conjunction with in vivo experiments, in vitro assays were performed in order to assess the effect of Linomide on NK and macrophage cytotoxicity and on lymphocyte proliferative responses in rats that received SC or IC implants or IV inoculation of tumor. Examination of the above mentioned group

revealed that Linomide was capable of enhancing macrophage activity and lymphocyte proliferation and of partially restoring depressed NK activity.

This thesis is dedicated to my family for their continuous support.

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INTRODUCTION

BREAST CANCER

Epidemiology, Etiology, and Prevention

Breast cancer next to lung cancer accounts for more deaths among American women than any other malignancy. It has been estimated that 1 out of 11 American women will develop breast cancer during her lifetime (122). Great variations in the incidence of breast cancer exist among different populations. The overall incidence of breast cancer is increasing 2% annually (106). Eastern European countries show a higher increase in incidence than Western European countries. The variation in mortality trends was not as great as in incidence rates. The incidence of breast cancer among Western and Northern European countries was substantially higher than in North America and Australia (106).

Risk Factors

The principal risk factors for breast cancer include menstrual and reproductive history, diet and family history. The menstrual factors basically point to the importance of the ovary. It has been shown that women with an earlier onset of menses have a significantly higher risk of developing breast cancer (71, 139). The hormonal events that surround menstruation are associated with progressive growth of the breast ductal epithelium and may on a long-term basis account for the steep rise in the incidence of breast cancer observed in women under the age of 40 (48, 139). A recent study shows that early onset of menstruation will produce a greater number of menstrual cycles and, therefore, a greater hormone exposure (48, 105). Oophorectomy

substantially reduces a woman's breast cancer risk (139). The 70% risk reduction in women who had oophorectomy before age 35 indicates an etiologic role for ovarian activity in at least two-thirds of breast cancer patients (139). Additional strong evidence for ovarian involvement in breast cancer is the lowered risk which follows menopause. It has also been shown that women with late natural menopause have increased breast cancer risk (77, 84, 108, 141, 163). It has been estimated that women with natural menopause at age of 55 or older had twice the risk of those whose menopause occurred naturally before age 45 (139).

Another important risk factor is the age at which a woman bears her first full-term child. Women pregnant before age 18 have about one-third the breast cancer risk of those whose first delivery is delayed until age 35 or older. The protective effect is essentially limited to the first birth and protection is exerted only by a full-term pregnancy (77, 84, 108, 141, 163). Women whose first birth is delayed to their mid- or late-30s have slightly higher risks compared to women who never have a child (163, 77, 84, 108). This phenomenon suggests that the first full-term pregnancy has a trigger effect, which either produces a permanent change in the factors responsible for the high risk, or changes the breast tissue and makes it less susceptible to malignant transformation. However, it is also possible that women with a late first birth are a group of sub-fertile women, and the hormonal imbalance which made them sub-fertile is also responsible for their increased risk of breast cancer (21, 82). An alternative explanation is that the stimulation of breast tissue that accompanies pregnancy promotes previously initiated tumor cells in older women (21, 82).

One important risk factor in breast cancer is the presence of the disease in an immediate family member. Women who have a first degree relative with breast cancer have a risk two or three times greater than that of the general population. This risk is substantially more if the relative was affected at an early age or had bilateral disease (76). It has been shown that women with a premenopausal relative with bilateral breast cancer have a nine-fold increased risk of developing the disease (2). It has been suggested that the breast cancer risk might be genetically related to variations in any of the several enzymes necessary for the synthesis of steroids and could thus produce altered levels of estrogens and other hormones (16, 48). It is therefore possible that these alterations in hormonal level can cause a predisposition to breast cancer, producing familial clusters.

Metastasis

The most urgent problem in breast cancer is the management of metastasis of tumor cells to distant organs. Breast carcinoma can metastasize to distant organs such as lung, liver, bone, spleen, and ovaries (40, 118, 144). The first sites of metastases are the regional lymph nodes (axillary, internal mammary, supraclavicular). The axillary lymph node is a major metastatic site and approximately 40% to 50% of patients with breast cancer have evidence of metastasis to these nodes (125). It seems that the involvement of axillary lymph node is directly related to the size of the tumor and not to its distance from the primary lesion. The second major drainage area for carcinoma of the breast is to the internal mammary lymph node (IMN). It seems that IMN involvement is not as extensive as axillary involvement

28% versus 42%, respectively (42). It also has been observed that involvement of the IMN is directly related to the size of the primary tumor (13). In primary tumors measuring less than 5 cm, IMN involvement was seen in 19% of the patients compared to 37% for primaries greater than 5 cm. Supraclavicular node involvement represents a late stage of axillary nodal involvement and is only found in patients with axillary lymph node involvement (13). Metastasis to supraclavicular lymph node apparently occurs through the axillary route (20).

Dormancy of Breast Cancer

Human breast cancer is one of the most common types of cancer capable of leading to a state of dormancy (quiescent state). One of the studies which has examined the long-term survival of patients with breast cancer, examined survival in 1141 patients (311 white, 830 black) diagnosed as having localized breast cancer. Survival from all causes was 62%, 43%, 33%, 25%, and 18% at 5, 10, 15, 20, and 30 years, respectively. Breast cancer specific survival was 76%, 65%, 63%, 61%, and 59% at 5, 10, 15, 20, and 30 years, respectively (50). Other studies have shown that the relative survival at 20 years was 31% for clinical stage I (axillary node not involved) and II (axillary nodes involved) patients, and 72% in patients with pathologic node negative disease (87, 160). By looking at the later statistic, we can observe that the delayed (5-20 years) appearance of metastases after adequate control of primary neoplasm is an established clinical phenomenon. Therefore, understanding the mechanisms involved in tumor dormancy should help us to create

therapeutic strategies capable of destroying the residual tumor cells left in the body after treatment of primary breast cancer.

Treatment of Breast Cancer in Humans

1. Surgical treatment and radiation

The object of breast cancer treatment is to eradicate all tumor cells in the breast region as well as in other sites throughout the body. Surgical procedures such as mastectomy can remove tumor from the breast, chest wall, and draining lymph node areas. Surgical operative techniques have changed tremendously through the years. Until the last decade, radical mastectomy (removal of the breast, major and minor pectoral muscles, axillary lymph nodes, and skin) was the prominent surgical procedure to remove the primary tumor. In the past few years, however, radical mastectomy has been replaced either with modified mastectomy (major pectoral muscle stays intact), partial mastectomy, or lumpectomy. While operative procedures may remove the primary tumor, they cannot eradicate residual tumor cells left locally or at distant sites. Therefore, postoperative procedures play an important role in breast cancer treatment. Radiation therapy has been used to eliminate local residual deposits of tumor. It has been shown that without postoperative radiation 20% to 25% of the patients with positive axillary lymph nodes will relapse in the supraclavicular area (140, 39). Using low dose radiation (3000-3500 rad in 4 weeks) will reduce the supraclavicular relapse to only 7%, and a moderate dose (4500-5000 rad in 4 weeks) will reduce the relapse rate to 1% (32). Even though postoperative radiation has a clear effect on the reduction of regional relapses, it does not

appear to prevent appearance of the distant metastases in breast cancer patients and therefore does not affect survival.

2. Estrogen therapy

About 50% of all primary tumors are estrogen receptor positive (ERP), with a slightly lower percent of positivity in metastatic lesions (48). The extent of positivity is related to the degree of differentiation and the histologic subtype. Approximately 90% of well differentiated ductal tumors and lobular carcinomas are ER positive. Premeneopausal patients have a lower incidence of ERP tumors (30%) compared to 60% in the postmenopausal period (48). Hormonal manipulation has been shown to be effective in breast cancer therapy. The basis for hormonal therapy is either to remove circulating estrogen or to block estrogen action. When estrogen is present in physiological amounts, it easily diffuses through the plasma membrane and binds reversibly to specific steroid-hormone-receptor proteins in the cytoplasm or nucleus. The binding of hormone activates the receptor, enabling it to bind with high affinity to specific DNA sequences that act as transcriptional enhancers. However, when estrogen is present in pharmacologic amounts, it binds to the ER and causes translocation of the complex to the nucleus followed by inhibition of cytoplasmic ER replenishment. In contrast physiological amounts of estrogen cause an increase in cytoplasmic ER levels (48).

Using 15 mg of diethylstilbesterol (DES), the overall estrogen treatment response in postmenopausal women is 29%.

3. Antiestrogen therapy

Antiestrogens bind to estrogen receptors and as a result competitively inhibit estrogen action. Antiestrogens not only compete with estradiol for the ER receptor but also appear to prevent the replenishment of the cytoplasmic receptor. The antiestrogen response is slow and affects skin, lymph nodes, breast, and bone metastasis. The mean response period is 12 months, and mean survival time is about 30 months for responders and 8 months for nonresponders (48). Antiestrogens include clomiphene citrate, nafoxidine, and tamoxifen (132). The first two drugs are rarely used because of toxicity problems. The overall response using tamoxifen in postmenopausal women is higher (38%) compared to premenopausal women (26%) (59). Patients with tumors that are positive for estrogen receptors have up to a 75% response rate. Soft tissue and osseous metastases are more likely to respond, sometimes requiring several weeks before their response becomes apparent (85).

4. Oophorectomy, Adrenalectomy, Hypophysectomy

Oophorectomy has been an important treatment of premenopausal patients with breast cancer. The overall response rate with oophorectomy is 29% in the premenopausal age group. The mean duration of response is 16 to 18 months. The metastatic sites likely to respond include bone, soft tissue, lymph nodes, and lung (119).

Adrenalectomy has been used to remove a secondary source of estrogen production. Adrenalectomy is useful therapy in patients with inactive ovaries. The overall rate of remission is 30% (123, 162). It has been shown that patients that are ER+ respond better to adrenalectomy than

patients that are ER- (49% compared to 12%) (123, 162). The metastatic sites that respond best to adrenalectomy are soft tissue, bone, and lung (162).

Hypophysectomy serves as a second line of hormonal manipulation. It removes ACTH in postmenopausal women, whereas in premenopausal women it removes gonadothrophic hormones and ACTH with subsequent adrenal and ovarian suppression. The overall response to hypophysectomy is 31% and the mean duration of the response is 15 months. Mean survival time is 24 months in responders and 5 months in nonresponders (80).

5. Chemotherapy of breast cancer

Chemotherapy has become an important postoperative treatment in breast cancer. In the past decade, one of the most exciting areas in the management of human breast cancer is the role of adjuvant therapy following local treatment. The percentage of patients who have metastatic breast cancer and respond to cytotoxic chemotherapy is almost twice as large as the percentage responding to endocrine therapy. Reports on the predictive value of the estrogen receptor for response to cytotoxic chemotherapy have been contradictory. Two series including 240 patients confirmed that hormone non-responders (which include a a high fraction of ERP patients) have an equivalent response to combination chemotherapy, although hormone responders tended to have a longer survival time from the initiation of chemotherapy (18-23 months versus 13 months) The response rate to combinations of drugs is higher than to single agents (52). It is important to keep in mind that chemotherapeutic agents basically affect DNA synthesis of tumor cells and are not effective against resting tumor cells or very differentiated tumor cells. The standard chemotherapeutic agents that

are usually used in breast cancer treatment either alone or in combination are cyclophosphamide (C), 5-fluorouracil (F), methotrexate (M), and doxorubicin (A) (22). Cyclophosphamide can be used either as a single agent or in combination with other drugs. The response rate obtained with cyclophosphamide is higher than with any other agent (49). This drug can be administered either orally or intravenously. 5-fluorouracil is usually administered intravenously and is typically used in combination with other therapeutic agents (3). Methotrexate has the same activity as 5-fluorouracil (143). In a recent study by Damron et al, 53 women with breast cancer were treated with a new 16 week dose intense, chemotherapy regimen. The chemotherapy regimen consisted of C, A, M, and 5-FU. At a median follow up of 17 months, there was 8 relapses in the 53 patients. The acturial 3-years disease free survival is 80%.

At present the most effective regimens available for general use are the CMF or CAF programs . In a recent study by Valagussa et al it was shown that in 165 women with breast cancer who were candidates for mastectomy because the largest diameter of the tumor was 3 cm or more, administration of CMF or CAF caused measurable tumor shrinkage in 157 patients and complete remission was documented in 7 patients (6). Also, responses to combination chemotherapy have been seen in all sites, including bone, liver, soft tissue, and viscera. The mean duration of response in most trials of combination chemotherapy varies from 12 months to 18 months (23, 74).

Immunotherapy

Immunotherapy in breast cancer patients usually is confined to the use of nonspecific immunostimulants combined with chemotherapy. It has been

shown that the use of 5-fluorouracil, doxorubicin, and cyclophosphamide in combination with Bacillus Calmette Guerin (BCG) is superior in terms of prolonging remission (51). In another study it has been shown that the administration of autologous irradiated tumor cells, mixed with BCG, in conjunction with 5-fluorouracil and radiotherapy for stage III breast cancer improves the prognosis (51, 48); however, this was shown in an uncontrolled study and should be confirmed in a properly randomized study.

Administration of IL-2 expanded tumor infiltrating lymphocytes (TIL) from resected surgical specimens is a new experimental approach of adoptive immunotherapy of cancer (115), and the effectiveness of this therapy against human breast tumors is currently under investigation.

13762NF Mammary Adenocarcinoma

The 13762NF mammary adenocarcinoma was originally induced by 7, 12-dimethylbenzanthracene and grows in the syngeneic Fischer 344 rat (120). This tumor line is a suitable model to study human breast cancer since the cells are weakly immunogenic, highly metastatic to other organs such as lung and lymph nodes and sensitive to chemotherapeutic agents (9, 37, 111, 8, 7), however, unlike most human tumors this tumor line is ER negative. Bogden has shown that excision of growing tumors on day 18 prolonged survival from 47 days in unexcised rats to 65.5 days in excised rats (7). In early studies examining the effect of hormones and chemotherapy, it was shown that a short non-immunosuppressive chemotherapy with 17-B estradiol (10 days) averaged 75% cures whereas the longer chemotherapeutic regimen (28 days) had an immunosuppressive effect and resulted in 25% cures (7, 28). In a series of in vivo studies it has been shown that perphenazine(which

increases the level of prolactin) increased the growth rate of 13762NF. Treatment of the hosts with estrogen prior to tumor implantation enhanced the effects of perphenazine on tumor growth (9).

13762NF Clones

One of the problems that oncologists have had to overcome in eradication of metastases is effective irradiation of tumor cell subpopulations with heterogenous sensitivities to various therapies. The 13762NF tumor contains rapidly diverging clones with different metastatic ability (89). Nicolson et al isolated and characterized five major subclones from 13762NF: MTA, MTF7, MTC, MTLn2, MTLn3, and MTPa. MTA and MTLn3 are highly metastatic, MTF7 has intermediate metastatic ability and MTC, MTLn2, and MTPa have very low metastatic ability (89, 149, 151). MTA has morphology of a giant cell sarcoma, MTF7 is a scirrous-like, well vascularized undifferentiated carcinoma. MTC has the morphology of an undifferentiated carcinoma. MTLn2 has the morphology of differentiated adenocarcinoma with defined luminal formation, whereas MTLn3 has a mixed cell population with no special arrangement (89). It has been shown that MTLn3 is capable of producing higher amounts of type IV collagenase than the clones with low metastatic ability (86).

The 13762NF clones have been examined for differences in metastases, enzyme production, sensitivity to radiation, and antigen expression (148, 150). Welch et al have shown that considerable clonal heterogeneity exists within the 13762 and its metastases with respect to responses to - radiation (148). MTA, MTLn2, and MTLn3 were incapable of accumulation or repair of

radiation damage, while clones MTC and MTF7 contained cells which had a high ability to accumulate and/or repair radiation damage (150).

A panel of monoclonal antibodies (MAbs) have been generated to react with different clones of 13762NF. The MAbs could be divided into three groups: those cross reactive with all 13762NF cells, those reactive with both clones MTLn3 and MTC cells, and those predominantly reactive with the highly metastatic MTLn3 cells (104). In a study by Steck et al (128), it has been shown that the presence of sialogalactoprotein (gp 580) correlates with the metastatic capabilities of the 13762NF clones. The gp 580 was expressed in increased amounts on the more highly metastatic clones. A different sialoglycoprotein (gp 80) was identified on the cell surface of these clones, but this molecule appeared to decrease in amount as the potentials of the cell clones to form metastatic lesions increased. Other investigators have grown 13762NF on collagen substrate and isolated more than 100 clones (111). They showed that all clones were epithelial and varied slightly in that some formed domes at high density and some tended to be more elongated. Six clones were tested for tumorigenicity by injecting 106 cells SC. It was shown that all were carcinomas with varying histopathology from organized adenocarcinoma to solid cords of carcinoma cells.

To investigate the possible role that the immune system plays in metastasis, subclones of 13762NF were implanted in rats pretreated with cyclophosphamide. Little or no effect on metastasis was seen (102). In another study the humoral response was investigated. Little or no antibody (Ab) was detected in rats bearing MTLn3, MTF7, MTLn2, and MTC subclones, and it was concluded that Ab seemed to play little or no role in metastasis of

subclones (102). Interestingly, it has been shown that when MTLn3 is implanted in nude rats, no metastasis was observed. It is possible that T-cell mediated immunity may play a role in the enhancement of spontaneous metastasis. It might also suggest that in nude rats 13762NF cells are susceptible to NK mediated cytolytic mechanisms. Alternatively, in immunocompetent hosts a T-cell mediated response (T suppressor) may enhance metastatic ability (102). North et al have shown that cells with low metastatic potential have the highest susceptibility to macrophage mediated cytolysis (103).

13762NF clones have the ability to undergo phenotypic drift. It has been shown that MTC fails to metastasize 23 days post injection SC but regains metastatic capability after several serial passage in vitro (149). Highly metastatic MTLn3 lost its metastatic ability with long-term cultures. Interestingly, the morphology of MTC changed as a result of serial passage in culture from epithelial-like cells to polyglonal and then to spindle-shaped with long cytoplasmic projections whereas the morphology of MTLn3 did not change (149, 151, 88).

Studies on heterogeneity and the hormonal responsiveness of clones derived from 13762NF have shown that clones also vary in their hormonal responsiveness (37). When the 13762NF was cultured in collagen gel matrix, different clones showed varying degree of positive responsiveness to hydrocortisone plus epidermal growth factor (EGF) (37). Progestrone and prolactin both synergized with EGF to promote cell growth, whereas 17b-estradiol alone or in combination with other hormones had no growth promoting effect (37).

13762A Mammary Adenocarcinoma

The 13762A mammary adenocarcinoma is an ascites form of 13762NF that has been used in examining immune responses in animals cured by resection and post surgical treatment. The most important qualities which make this tumor desirable for immunotherapeutic studies are its low immunogenicity and its high frequency of spontaneous metastasis (68). The growth and excision of 13762A did not lead to tumor rejection immunity. Initial injection of 1x106 tumor cells intradermally causes death within 40 days and extensive metastasis to lung and lymph nodes (68). Kreider et al (66) have shown that administration of 5x107 units of BCG intratumorally on day 7 of tumor growth, followed by tumor excision on day 20, decreased metastasis and increased the survival of hosts. BCG treated rats experienced prolonged survival and 20% were cured. It has been shown that the effect of BCG on tumor excised rats is due to activation of macrophages (66). Similar studies were done utilizing Corynebacterium parvum (CBP), administered at a dose of 1500 ug intratumorally on day 7 followed by primary excision on day 20, was more effective than BCG and produced 40% cures and prolonged survival (67). Studies in other systems have shown that CBP affects IFN induction, augments T-cell mediated immunity, and activates macrophages and NK cells (81). Howel et al (54) looked at the involvement of T-cells in CBP treatment and showed that rats depleted of T-cells by either neonatal thymectomy or by a combination of adult thymectomy, 900 rad irradiation and bone marrow reconstitution, did not show inhibition of tumor growth after treatment with CBP. However, when rats were restored with lymph node cells, treatment became effective again. It was shown that cells with the

T helper phenotype and pan T phenotype were responsible for the effect of CBP. Barlett et al (10, 65) have shown that tumor immunity in treated rats can be transferred to normal animals by peritoneal exudate T-cells from immune rats. Immunity by adoptively transferred T-cells was improved if recipient rats received cyclophosphamide (CY) before receiving donor cells. pre-treatment of recipients with CY enabled rats to reject higher numbers of tumor cells. Kreider et al (68) have also shown that 13762A contains different clones. They isolated a highly immunogenic clone (18A) from the parental tumor line and observed that this clone grew for 3 weeks in hosts, forming a 2-3 cm tumor and then regressed. These hosts were then immune to subsequent challenge by 13762A. In other experiments, pre-treatment of animals with 450 rad lead to the progression of inoculated tumor. Interestingly, tumor regression in immune rats was only observed when the clone was put SC, and it was shown that this regression was due to a mononuclear infiltration (65). Christensen et al (19) have shown that there are quantitative and qualitative differences in the in vitro host response to parental 13762A tumor and its clone 18A. When lymphocytes obtained from the spleen and peritoneum of rats immunized with 18A were tested for specific proliferation to parental 13762A and clone 18A, distinct regional differences were observed in T-cell activity or responses. Immune peritoneal exudate cells (PEC) proliferated strongly in response to clone 18A, but poorly to parental 13762A. In contrast, immune spleen cells proliferated strongly in response to parental 13762A but weakly to 18A and showed equal cytotoxicity to both 13762A and 18A. In a recent study, Christensen et al (19) examined the poor responses of immune PEC to 13762A in a greater detail and found that a

tumor-induced antiproliferative suppressor lymphokine (TISL) was produced from rat peritoneal T-cells in response to 13762A. The immune PEC response to clone 18A and responses of non-immune spleen cells was inhibited by TISL. In contrast the immune spleen cell response to 13762A was unaffected. These studies point to the heterogeneity that exists in tumor cell populations, as well as to a heterogeneity in regional immune response capability.

Dormancy

One aspect of metastasis that has not been sufficiently explored is the phenomenon of dormancy. An important problem in cancer therapy is to eliminate the few residual tumor cells remaining in the body following treatment with surgery, radiation, chemotherapy, or immunotherapy. In many patients with carcinoma of the breast, melanoma, or colon cancer, recurrence of tumors can occur anywhere between 2-40 years after treatment and/or removal of the primary tumor (153). Recurrence of tumor is believed to be a consequence of the growth of dormant metastases. The tumor dormant state has been defined as a state of suspended animation in which tumor cells remain in GO with little or no increase in cell number (157). Alternatively, proliferation of tumor cells may occur but may be accompanied by cytotoxic mechanisms capable of preventing a net increase in the number of tumor cells (157). Tumors can be restricted from growth either actively by means of humoral or cell mediated cytostasis or passively due to absence of a factor or factors required for proliferation (157). The use of animal models enables us to gain knowledge about mechanisms involved in dormancy. Three principally different mechanisms of tumor dormancy have been distinguished in animal models.

1. Avascularity and sequestration of tumor cells.
2. Constitutive dependency of tumor cells on growth factors.
3. Immunologic restraint.

Avascularity and Sequestration of Tumor Cells

Tumor cells require nutrients, oxygen, and other essential elements to live and grow. Studies by Folkman and Hochberg (33) have shown that small tumor nodules that fail to develop a vascular network will be limited in size. In 1972 Gimborne introduced a new tumor dormancy model by implanting xenogeneic carcinoma in to the anterior chamber of rabbits (38). In this model tumor cells were unable to provoke capillary growth and failed to grow. These cells were considered to be dormant and survived for many months obtaining nutrients by simple diffusion.

In 1976 Brem and his colleagues implanted V2 carcinoma and mouse ependymoblastomas into the vitreous of rabbit or dog (12). The vitreous tumors failed to become vascularized. Tumor grew for weeks as small, unvascularized aggregates. However, it vascularization occurred when tumors reached the retinal surface. It seemed that the normal vitreous may act to inhibit capillary proliferation and neovascularization of tumor cells. Avascularization caused a delay of tumor growth. Studies by Folkman et al (33, 34) have shown that cells located at the surface of these aggregates received an abundant supply of nutrients whereas the cells that were located in the center died of malnutrition. The balance between replication and necrosis account for dormancy. As the result of these experiments, a concept of dormancy based on avascularization was put forward.

Constitutive Dependency of Tumor Cells on Growth Factors

This mechanism of tumor dormancy is characterized by a dependence of a tumor cell proliferation on exogenous growth factor or hormones. Nobel et al reported that adrenal, mammary, and other tumor cells implanted in animals remained dormant until stimulated by estrogen (100, 101). Kim et al (60, 61) showed that when rats bearing methylcholanthrene-induced mammary tumors were hypophysectomized, tumors regressed and animals stayed apparently tumor free. After a few months, they implanted mammotropin secretory pituitary tumors in the back of the rat's neck. This resulted in diminished dormancy and growth of original tumors. An in vitro model for hormonal dependent dormancy was established by Yuhas et al (159). They used human breast cancer cell line MDA-361 which requires insulin for growth and maintenance. Removal of insulin lead to the detachment of cells from plastic surface within 24 to 48 hours and formation of multicellular spheroids. Multicellular tumor spheroids enter the dormant state and remained dormant for 6 weeks.

Immunologic Restraint

One major mechanism for maintaining tumor dormancy, is immunological restraint. Different investigators have shown that tumor dormancy can be diminished by eliminating effector mechanisms capable of restraining or killing tumor cells. Eccles and Alexander proposed one of the first immunological restraint models (24). In their experiment hooded rats received the 3,4 benzopyrene induced sarcoma in the leg. Two weeks later tumor and the local draining lymph nodes were removed by amputation of the whole limb. Ten percent of the animals treated with the above

procedures died 55-100 days after the removal of the tumor; 90% of the animals stayed tumor free during an 18 month observation period. When these rats were subjected to whole body irradiation or 7 days of continual draining of the thoracic duct after the removal of the primary tumor, a very significant increase in the incidence of distant metastases was observed. Whole body irradiation and lymphocyte depletion caused the suppression or reduction of lymphocyte mediated immunity. Therefore, animals that appeared to be cured surgically carried dormant tumors that became manifest only after treatment which was immunosuppressive.

Another important model for immunological restraint of the dormant state was obtained using DBA/2 mice and Friend Leukemia Virus (FLV) classified as an oncovirus causing leukemia in DBA/2 mice (155). When DBA/2 mice are infected with FLV, animals develop erythroleukemia 21 days post infection. It seems that FLV affects lymphocytes and therefore results in rapid suppression of humoral and cellular immunity. The leukemogenecity of FLV is due to the immunosuppression of the host. Treatment with statolon (an extract from penicillum) caused the clearance of the virus from blood of the host, and animals became clinically tumor free. Even though apparently cured animals stayed tumor free for 2 years, some developed erythroleukemia after 2 years. It was shown that treatment with statolon caused a resumption of antibody production and implicated an involvement of antibodies in this dormancy model (15).

A third model established by Wheelock et al, used L5178Y, a methyl-cholanthrene induced lymphoma, in DBA/2 mice (146). To establish dormancy, DBA/2 mice received 1x106 viable L5178Y cells SC in the mid-

ventral surface of the abdomen. After 10 days the tumor was excised, and 7 days post excision animals were challenged ip with 5x104 viable L5178Y cells. It was shown that 4 days post challenge a strong CTL response was generated and gradually declined to background levels over several weeks. Accompanying the later event, there was an initial increase and then a decline in L5178Y tumor cell burden. Based on a series of studies by Wheelock et al (112, 113, 145, 146, 147, 156), it has been shown that the dormant state is mediated by a peritoneal cytolytic T-cell response, which destroys 99% of the L5178Y cells. Further studies on this model have shown that repeated stimulation of CTL activity in tumor dormant mice results in the elimination of L5178Y cells from a significant number of tumor dormant mice. During the course of the tumor dormant state, however, there is continual selection by CTLs for an emergent phenotype which preexists in the L5178Y cell population used to initiate the tumor dormant state. It has been shown that prior to termination of the tumor dormant state, immunosuppressive macrophages producing a PGE2-like factor appear in the peritoneal cavity, and inhibit the T-cell mediated cytolysis. In a recent study by Wheelock et al, it has been shown that the dormant state of L5178Y can be terminated by treatment with PGE2 (78). They have shown in vitro that tumor cell growth in PEC cultures from tumor dormant mice is enhanced by antibodies rMuIFN and MuTNF. This indicated that production of MuIFN and MuTNF may play a role in the restraining tumor growth (130). In a recent study by Wheelock et al (129), it has been shown that small concentrations of rMuINF- and rMuTNF synergize to induce antitumor activity in whole PC and in adherent and non-adherent subpopulations of PC.

The antitumor activity in the PC is the result of both cell-mediated cytotoxicity induced by synergy of these cytokines and by a diffusible inhibitor of tumor cell growth induced by rMuTNF alone.

Issues Addressed By Thesis Research

This thesis has used a 13762 mammary adenocarcinoma as a model for breast cancer in order to examine the following points:

1. Examination of parameters that affect metastasis.
2. Initiation and development of a dormancy model resembling metastases of human breast cancer.
3. Examination of the possible immunological pathways involved in metastasis and dormancy.
4. Examination of the effect of specific therapeutic regimens on metastasis and survival.

Chapter 1

Metastasis of Ocular and Flank Implants of the 13762NF Mammary Adenocarcinoma in Syngeneic Rats

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ABSTRACT

Solid fragments of 13762NF mammary adenocarcinoma, a syngeneic tumor of low immunogenicity were implanted on the anterior surface of the iris or posterior surface of the cornea in young Fischer 344 rats. Growth and metastasis of these implants were examined and compared with those occurring following SC tumor implantation in the flank. Corneal implants did not grow well or metastasize. Iridial implants grew well, and tumor typically filled the eye within a few weeks. If tumor-filled eyes were left intact, all rats died or became moribund 7-9 weeks later. Metastasis was seen in the lungs (11/11), cervical lymph nodes (7/11), body wall (5/11), heart (4/11), kidney (4/11), and liver (3/11). Removal (enucleation) of tumor-filled eyes prior to spread to the orbit prevented the development of overt metastatic disease for 1-2 years (11/11). When healthy enucleated rats were necropsied 0 days to 26 weeks post enucleation, small nodules were seen in the lung (9/13), liver (3/13), or spleen (1/13). Nodules were not seen in control rats that had been housed in our animal facility for 1-2 years (16/16), had eye surgery with tumor implanted on the posterior surface of the cornea (6/6), or had been subjected to the sham surgical procedures of corneal implantation and enucleation (3/3). When tumors were implanted SC, rats died after 6-7 weeks. Metastasis was seen in the lung (12/12), lymph nodes (12/12), spleen (1/12), and liver (1/12). Removal of very small flank tumors failed to prevent animals from dying 7-8 weeks later with pulmonary and lymph node metastasis (10/10). In summary, iridial implantation resulted in an altered pattern of metastasis. Animals that had iridial implants and

enucleation failed to develop overt metastatic disease and had small nodules in the lung, liver, or spleen.

INTRODUCTION

The 13762NF mammary adenocarcinoma in the syngeneic F344 rat is an extremely useful mammary tumor model. The highly aggressive nature of this tumor has been demonstrated following SC inoculation of cell suspensions into the footpad, flank, or fat pad of the mammary gland or IV (7, 9, 16, 89, 151). This tumor has a predictable incidence of lung and abdominal organ metastasis from SC implanted grafts into the flank; 18 days after implantation, 100% of the hosts have metastases to the lungs and/or internal organs (7). The 13762NF tumor possesses low immunogenicity, is sensitive to steroid alkylating agents, and has been used for chemotherapeutic studies designed to evaluate therapy combinations (7, 9, 152, 28, 11, 102, 103). When implanted SC in the flank or into the mammary gland fat pad, the 13762NF tumor shares the essential metastasizing and therapy response characteristics with many human breast cancers (7, 89, 102, 103). Clones of varying metastatic potential have been obtained from the parental tumor, and information is available concerning the effect of the immune status of the host on the spontaneous metastasis of these cell lines (151, 89, 102, 103). The early tumor spread by the lymphatic system has been demonstrated (16, 102).

We sought to develop and to utilize corneal and iridial ocular models for studying metastasis of solid fragments of in vivo derived syngeneic tumors of low immunogenicity in the rat. Ocular models were considered to be important for several reasons. The eyeball (with the exception of the conjunctiva) lacks lymphatic vessels. Thus appropriate ocular models offer

an opportunity for studying the initial stages of metastasis under conditions where tumor cells and/or antigens must gain access to the blood vascular system prior to being exposed to the lymphatic circulatory system. An additional advantage of an ocular model is the ability to work in vivo in a transparent chamber and to examine the initial stages of tumor growth and vascularization in situ. Since uveal (choroidal) metastasis of human breast cancer is common (83) and breast cancer is the most common primary tumor metastasizing to the ocular structures (14), we considered it likely that the 13762NF tumor would grow well on the iris.

In the present study we compare the growth and metastasis of corneal, iridial, and SC flank implants of in vivo-derived 13762NF mammary adenocarcinoma. We demonstrate that all animals with intact iridial or flank tumors die with extensive pulmonary metastasis, and the pattern of metastasis to other organs depends on the implantation site. Removal of small primary flank tumors has little or no effect on the development of overt metastatic disease, whereas removal of tumor-filled eyes prior to spread to the orbit markedly alters the progression of metastatic disease.

MATERIALS AND METHODS

The 13762NF mammary adenocarcinoma, obtained from the Breast Cancer Task Force Animal and Human Tumor Bank (Mason Research Institute, Worcester, MA), was maintained in our laboratory by SC implantations every 2 weeks of 1- to 2-mm3 tumor fragments into the flanks of 4- to 5-week-old F344 females (Charles River Breeding Laboratories, Wilmington, MA). Animals were fed Purina Rat Chow and maintained and

cared for according to National Institutes of Health guidelines on the care and use of laboratory animals.

Preparation and examination of ocular implants

Female rats 5-6 weeks old were anesthetized by means of Nembutal, and the iris was dilated with 1.0% Mydriacyl. The rat was placed on its side under an OPMI-1 dissecting microscope, and an incision was made in the center of the cornea with a microlancet. Iridectomy scissors were used to extend the corneal incision to a length of approximately 2.0 mm. One edge of the cut cornea was held with a jeweler's forceps, and a 0.5- to 1.0-mm3 tumor fragment was placed on the anterior surface of the iris immediately adjacent to the pupillary margin or on the posterior surface of the cornea 1-2 mm from the limbus. In the event that the lens was damaged or an iridial blood vessel was inadvertently broken, the eye was rejected for further experimentation.

At weekly intervals rats were lightly anesthesized with ether, and the ocular implant was examined and photographed. The width and length of the tumor were measured, and the degree of vascularization of the implant and/or cornea was noted. Eyes that had received iridial implants were routinely enucleated as soon as the eye became distended and the tumor could be seen filling the entire anterior chamber. At this stage, enucleated eyes were intact and turgid, and upon examination under the OPMI-1 microscope at 6x they were always solidly packed with tumor. The orbits were always checked at 6x and appeared to be free of tumor. Following enucleation, eyelids were sutured and animals followed for varying periods of time. When enucleation was performed as described above, a tumor did not subsequently appear in the orbit.

Implantation of extraocular tumor fragments

Tumor fragments (1-2 mm3) were injected SC into the left flank at the level of the fourth pair of mammary glands by means of a 16-gauge trocar. For determination of tumor size, the tumor was periodically palpated, and the width and length were measured with the use of a fine caliper.

Examination of animals for metastatic lesions

The internal organs of experimental animals and of control and sham-operated animals housed in our animal facility for varying periods of time were examined under the dissecting microscope at 6x. Tissue containing metastatic lesions or nodules and tissue from control animals were fixed either in 10% Formalin, Zenker's fixative, or in 2.5% glutaraldehyde-2.5% paraformaldehyde, or 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.3, processed, and embedded in paraffin.

RESULTS

Growth of Iridial Implants

The pattern and rate of growth of iridial implants were very variable. Most implants flattened out along the anterior surface of the iris and grew rapidly. In these animals, tumor growth was initially confined to the iris and the vascular bed derived from iridial vessels. By the end of the 1st week, the tumor filled approximately 20% of the anterior chamber and was often in contact with the cornea. Corneal vessels could then be seen growing inward from the limbus and over the region of the cornea contacting the tumor (Fig. 1A). The histologic appearance of a portion of a 1-week-old implant is shown in Figure 2A. During the 2d week the tumor grew rapidly and the cornea became heavily vascularized. By the 3d week most eyes became distended,

and the tumor could be seen throughout the anterior chamber. Progression to this stage was used as the basic criterion for enucleation. The histologic appearance of a portion of tumor from an enucleated eye is shown in Figure 2B.

In some eyes the tumor grew very slowly either, and it took several weeks for the eye to become distended with tumor and meet the criteria for enucleation. The above mentioned phenomenon could have been the result of different tumor population that made up the tumor fragment or due to how well the tumors were attached to iris and how fast they became vascularized. In a small number of implanted animals, the tumor appeared to be mostly free in the anterior chamber but was attached to the iris by a thin stalk of connective tissue and blood vessels. Such implants typically maintained their size for several weeks and then grew very rapidly.

Histologic and macroscopic examination showed that, regardless of the time required for tumor growth, enucleated eyes were always completely filled with tumor. Whatever the pattern of growth, enucleation at the time of initial distension of the globe prevented local recurrence.

Metastasis of Iridial Implants From Unenucleated Eyes and From Eyes Enucleated After Tumor Spread to the Orbit

To follow the course of metastasis from the iridial implant without subsequent enucleation, we gave 11 animals iridial implants and tumor-filled eyes were left intact. All animals became moribund and/or died 7-9 weeks post implantation. Tumor-filled eyes were very large, and the tumor had invaded the orbit. Metastases were present in the lung (11/11), cervical lymph nodes (7/11), body wall (5/11), kidney (4/11), heart (4/11), and liver

(3/11). In 10 rats the pulmonary metastasis was extensive, and entire lobes of the lung appeared as solid masses of tumor. In 1 animal only 2 small metastatic lesions were seen in the lungs, and the cause of morbidity was not apparent.

In another experiment we examined metastasis from ocular tumors when enucleation of tumor-filled eyes was delayed for 1 week after the time that this procedure was normally performed. Accordingly, eyes of 6 experimental animals were enucleated 1 week after becoming distended with tumor. In this experiment enucleation was unable to control local or distant disease. Animals died or became moribund 7-9 weeks after implantation was metastasis to the lungs (6/6), cervical lymph nodes (3/6), body wall (2/6), and heart (2/6), and large recurrent tumor masses were seen in the orbit (Table 1).

Examination of Enucleated Animals for Evidence of Metastasis

Twelve animals that received iridial implants were subjected to enucleation and then observed for long periods of time. Only 1 rat died with extensive pulmonary metastasis 9 weeks post enucleation, and in this animal only a large recurrent tumor had grown in the orbit. Eleven rats remained healthy and apparently free of metastatic disease for at least 1 year post enucleation.

We then sought to determine whether any evidence of metastasis would be present in animals that had been subjected to routine enucleation of tumor-filled eyes and maintained for several months. Accordingly, 6 apparently healthy rats were sacrificed and necropsied 16-26 weeks post enucleation. Nodules were seen in the lung (3/6) and liver (1/6) (Table 1).

Nodules were typically less than 0.5 mm in diameter, and fewer than 5 nodules were present in each organ.

To explore further the significance of the very small nodules seen in healthy rats sacrificed 16-26 weeks post enucleation, we studied several other groups of rats. To control for age, 16 rats maintained in our animal facility for 1-2 years were sacrificed and necropsied. Nodules were not seen in any internal organs (Table 1). Then, 7 rats that had received iridial implants were sacrificed on the day that enucleation would normally have occurred. Nodules were seen in the lung (6/7), liver (2/7), and spleen (1/7) (Table 1). Nodules resembled those seen in animals necropsied 16-26 weeks post enucleation and, with the exception of 1 pulmonary nodule that was 1.5 mm in diameter, were characteristically less than 0.5 mm in diameter.

An additional group of rats subjected to ocular surgical procedures was necropsied and examined for the presence of nodules. Six rats that had received corneal tumor implants were sacrificed and necropsied at periods of time up to 16 weeks post implantation, and no nodules were seen. Finally, 3 rats that had corneal implants and enucleation were sacrificed and necropsied, and no nodules were observed (Table 1).

A typical pulmonary nodule seen in rats given iridial implants and necropsied at the time that enucleation would normally be performed is shown in Figures 2C and 2D. Histologic examination of nodules at high magnifications revealed the presence of small clusters of large cells with lightly staining nucleoplasm and prominent nucleoli (Figs. 3A-3C). The large cells in these nodules resembled those seen in the 13762NF tumor (Fig. 3D).

Growth and Metastasis of Corneal Implants

Thirteen animals received corneal implants. The pattern of tumor growth was very variable. Two implants failed to grow. The remaining 11 grew very slowly over a period of weeks or months and then either remained unchanged or were resorbed. In all cases, limbal vessel invaded the cornea during the 1st week of tumor growth, and by the 2d week post implantation a dense and highly branched vascular bed was superimposed over the area containing the tumor (Fig. 1B). When implants were resorbed, the corneal vascular bed underwent a dimunition until only a few long-branched corneal vessels extended from the limbus to the area of the original implant. Corneal implants grew in two dimensions and did not grow larger than 5 mm long x 2 mm wide. Eyes with corneal implants never required enucleation.

Six animals whose corneal implants had grown were sacrificed and necropsied at periods of time ranging from 6 to 16 weeks post implantation. No nodules or evidence of metastasis was seen.

Growth and Metastasis of SC Implanted Tumor

The growth and metastasis of flank-implanted tumor are readily investigated by utilizing tumor fragments (1-2 mm3). The growth rate of this size fragment is depicted in text Figure 1. Six to 7 weeks after implantation of 1- to 2-mm3 fragments of tumor, rats either died or became moribund and were necropsied. It was not possible to quantitate pulmonary metastasis since in all rats extensive portions of the lungs were obscured by masses of tumor. Lymph node metastases were present. Metastases were also seen in the liver (1/12) and spleen (1/12) (Table 1).

In examining the growth and metastasis of flank tumor, an attempt was made to more closely approximate the conditions used in our ocular

implant experiments by injecting 0.5- to 1.0-mm3 tumor fragments into the flanks of 10 rats. However, the injection of these small implants proved to be unreliable, and tumor often failed to grow. The possibility that this size tumor fragment may not have been expelled from the 16-gauge trocar cannot be ruled out. The size and rate of growth of 4 successful implants are shown in text-Figure 1. The rate of growth of the smaller tumor implants was slower for the first 4 weeks than that seen with the use of 1- to 2-mm3 fragments. Seven weeks post implantation, hosts that had received the small implants were sacrificed and necropsied. All animals had pulmonary metastases, and nodules ranged in size from 0.1 to 6.0 mm. The average number of metastases per rat was 28.3 + 11.8 mm (SE). The extensive areas of lung obscured by tumor seen in rats that had received 1- to 2-mm3 tumor fragments were not observed.

To assess the effect of the removal of the primary tumor burden on metastasis and to more closely approximate conditions encountered during and after enucleation of tumor-bearing eyes, we gave 10 rats injections of 1- to 2-mm3 flank implants and palpated them daily; the tumor was removed as soon as it became palpable. The tumor was excised along with a wide margin of connective tissue. The geometric mean diameter of the palpated tumor was 4.3 + 0.3 mm (SE), roughly half the diameter of a normal rat eye. Nonetheless, 1 week later, the tumor could again be palpated in the flanks of all 10 rats and grew progressively. Animals either died or were sacrificed when moribund 6-7 weeks post excision, and all showed extensive lung metastases; entire portion of lobes of the lung were obliterated by solid masses of tumor.

DISCUSSION

Deviant immune responses affecting growth and metastasis have been described in experiments utilizing the inoculation of suspensions of syngeneic tumor cells into the anterior chamber of the mouse eye (98, 95, 97, 161, 93, 92, 91). This work has been done with the use of cultured tumor cells. Very little information is available concerning the growth and metastasis of ocular implants of in vivo passaged syngeneic tumors of low immunogenicity (46).

Several interesting findings emerged from the present studies. In unenucleated animals with iridial implants, the pattern of metastasis differed from that seen following tumor implantation in the flank. Metastasis was not always observed in the lymph nodes of animals with iridial implants, but metastases were present in the body wall (55%), kidney and heart (36%), and liver (27%). The cause of this altered pattern of metastasis is not known. Possible explanations include: a) changes in the phenotype of the tumor growing in the milieu of the anterior chamber, b) changes associated with metastasis from the eye by a primarily hematogenous route, and c) selective pressure for evolution of tumor cells with different colonization potential as a result of the presence or absence of specific immune responses occurring in the eye. Since an altered pattern of ocular metastasis was not observed when comparable experiments were performed with the use of B16F10 melanoma in mice (46), species and tumor differences must also be considered.

In spite of the aggressive nature of the 13762NF tumor, enucleation of relatively large ocular tumors completely prevented the development of metastatic disease for at least 1 year. More surprising, however, was the

finding that nodules were typically present in the lungs and/or liver at the time of enucleation and in healthy enucleated animals necropsied 16-26 weeks post enucleation. Since these nodules were not observed in control animals housed in our animal facility or in corneal implanted (sham-operated) animals, they appear to be specifically associated with the growth of iridial tumors. Considering the histologic finding of clusters of very large cells within these nodules, and the resemblance of these cells to those seen in primary 13762NF tumors, it is our working hypothesis that these nodules contain dormant tumor cells.

We have evidence that dormant micrometastases are present throughout the body of the enucleated rat and that immunologic mechanisms operate in these animals to prevent the growth of an extraocular tumor (Sabzevari, H., Szalay J.: Manuscript in preparation). The nature of the immune response(s) is under investigation. Again, in experiments examining the ocular metastasis of in vivo derived B16F10 tumor cells, enucleation of tumor-filled eyes failed to alter the progression of metastatic disease, and all mice died with extensive pulmonary metastases 5-6 weeks post enucleation (46). Thus the ability to obtain dormant metastases under our experimental conditions is also species and/or tumor dependent.

In the syngeneic mammary adenocarcinoma studies presented in this paper, unenucleated rats became moribund or died with extensive pulmonary metastases 7-9 week post implantation, whereas SC implanted rats died 6-7 weeks post implantation. The apparently longer survival of rats with ocular tumors could be attributed to the slower growth and metastasis of the smaller-sized tumor fragments used in the ocular studies. This receives

support from our experiments with the use of 0.5-mm3 flank implants. In these experiments the primary tumor grew more slowly for 4 weeks, and the tumor burden in the lung at 7 weeks was markedly less than that observed in animals that had received 1- to 2-mm3 implants. In similar experiments examining the ocular metastasis of B16F10 melanoma in mice, we were able to use smaller needles and place 0.5-mm3 tumor fragments in the flank and on the iris. In these experiments no difference was observed in the rate of metastasis from these two locations (46).

Iridial implants that were largely free in the anterior chamber and attached to the iris by a vascular stalk grew very slowly. Recent work by others has shown the importance of specific matrix and extracellular molecules in affecting tumor cell adhesion, migration, and metastasis in vitro (135, 136). The difference in growth characteristics between implants that attach and spread along the iris and those that become vascularized but do not spread on the anterior surface of the iris may be in vivo documentation of the importance of different matrix molecules on growth and metastasis.

Although corneal tumors typically grew slowly and spread along the posterior surface of the cornea, they failed to undergo rapid and/or sustained growth or to metastasize from the eye. We have found that tumor cells do not appear to cross Descemet's membrane and enter the cornea and that blood vessels present in the area of the cornea containing tumor do not appear to cross Descemet's membrane and enter the tumor (unpublished data). Failure to become vascularized may thus be limiting the growth and/or metastasis of corneal tumors. This interpretation is consistent with studies performed by

others, demonstrating the importance of the vascularization of tumors to growth and metastases (38).

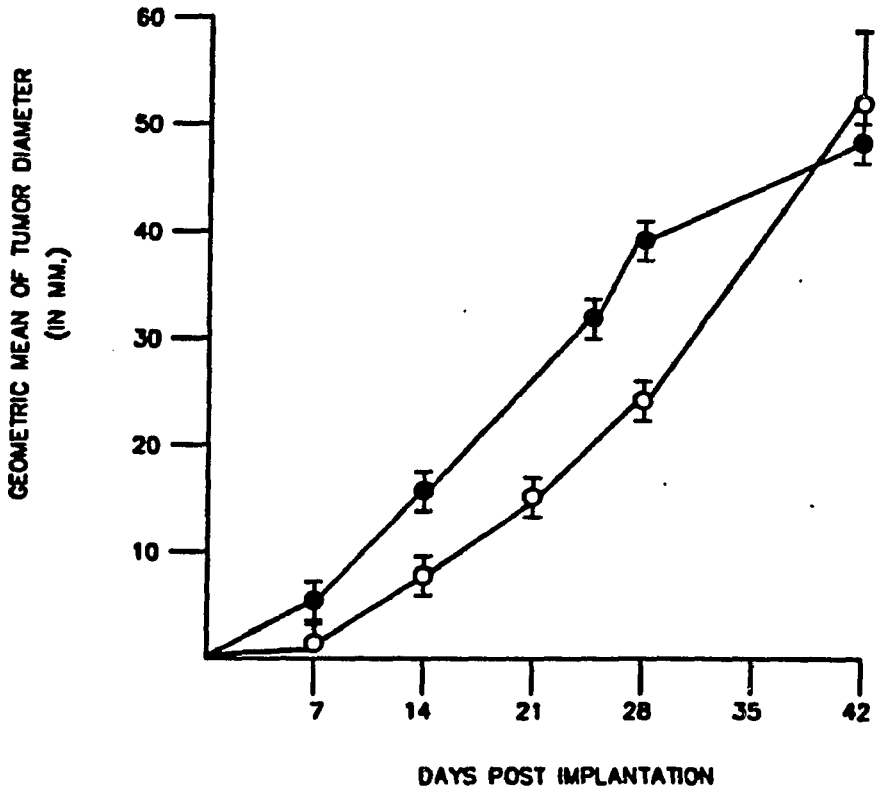
In summary, we have developed and utilized new ocular models for studying the growth and metastasis of in vivo derived implants of a syngeneic mammary adenocarcinoma of low immunogenicity in the rat. In comparing the growth and metastasis of intact iridial and flank tumors, we have demonstrated that iridial implantation results in an altered pattern of metastasis. In comparing the effect of removal of the primary tumor on metastasis to distant sites, we find that iridial tumors differ from flank tumors in that removal of the primary tumor prior to spread to the orbit prevents the development of overt metastatic disease and may be conducive to the formation of dormant metastases. It is not yet known whether these findings may relate to the lack of a lymphatic circulation in the eye or to the presence of deviant immune responses associated with tumor growth in the anterior chamber. These questions are under investigation in our laboratory.

TABLE 1.– *Metastasis and nodule formation from ocular and flank tumor implants*[a]

Primary tumor implants	Incidence of metastasis or nodule formation in internal organs						
	Lung	Lymph node	Body wall	Kidney	Heart	Liver	Spleen
Flank							
Intact	12/12	12/12	0/12	0/12	0/12	1/12	1/12
Removal of 4.0-mm primary	10/10	10/10	0/10	0/10	0/10	0/10	0/10
Iridial							
Intact (unenucleated)	11/11	7/11	5/11	4/11	4/11	3/11	0/11
Enucleation delayed 1 wk	6/6	3/6	2/6	0/6	2/6	0/6	0/6
Routine enucleation with:							
Necropsy on same day	(6/7)[b]	(0/7)	(0/7)	(0/7)	(0/7)	(2/7)	(1/7)
Necropsy 16–26 wk later	(3/6)	(0/6)	(0/6)	(0/6)	(0/6)	(1/6)	(0/6)
Total incidence of nodules	(9/13)	(0/13)	(0/13)	(0/13)	(0/13)	(3/13)	(1/13)
Controls							
No tumor, 1– to 2-year-old rats	(0/16)	(0/16)	(0/16)	(0/16)	(0/16)	(0/16)	(0/16)
Corneal implant with necropsy 6–16 wk later	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)	(0/6)
Corneal implant and enucleation	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)

[a]*Numbers without parentheses* represent the incidences of metastasis. *Numbers in parentheses* represent the incidences of nodule formation with all nodules <0.5 mm in diameter and <5 nodules present/organ.

[b]Nodule formation with 1 nodule=1.5 mm and the remainder of the nodules <0.5 mm; <5 nodules present/organ.



TEXT-FIGURE 1.–Graph depicting the growth of 0.5-mm^3 and 1- to 2-mm^3 flank implants.
O–O , the mean geometric tumor diameter of 0.5-mm^3 implants ($n = 4$).
●–● , the mean geometric tumor diameter of 1- to 2-mm^3 fragments ($n = 12$).
Vertical brackets denote standard error of the mean.

FIGURE 1A.—In vivo photograph of a 1-wk-old iridial implant. Tumor is vascularized from below by iridial vessels (area denoted by arrowheads). The pupil is very constricted, and iridial vessels are slightly dilated. Corneal vessels (arrows) extend from the limbus to the region of the cornea above the tumor. × 30

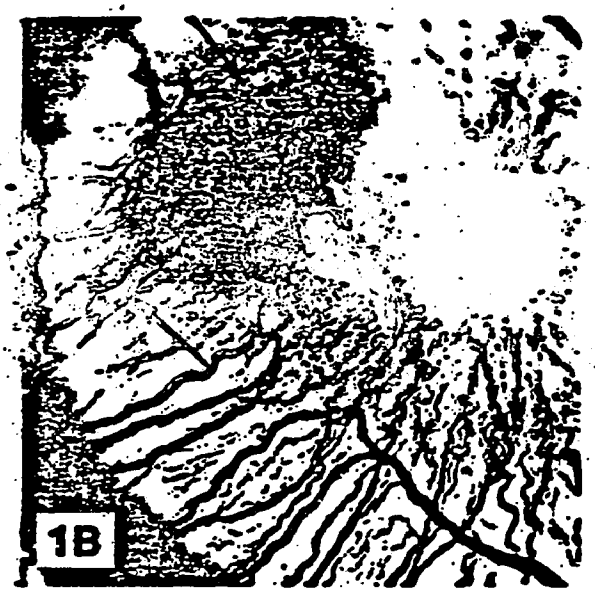


FIGURE 1B.—In vivo photograph of a 1-wk-old corneal implant. Corneal vessels are seen extending from the limbus to the region of the cornea above the tumor (arrows). × 22

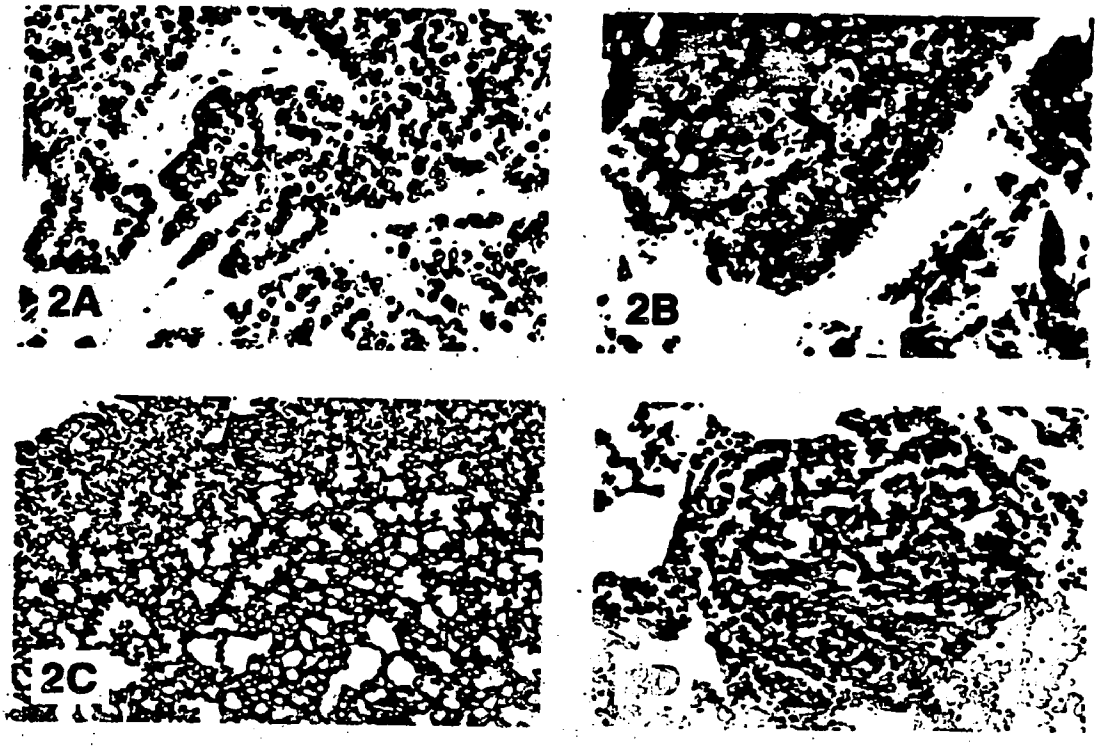


FIGURE 2.—2A) 1-wk-old tumor from an iridial implant. × 280. 2B) Tumor from an eye enucleated prior to spread to the orbit. Cells are very closely packed, and some areas of necrosis are present. × 280. 2C) 1 nodule is shown in a region of the lung taken from the same animal whose tumor is shown in 2B. × 45. 2D) Higher magnification of the nodule shown in 2C. × 240

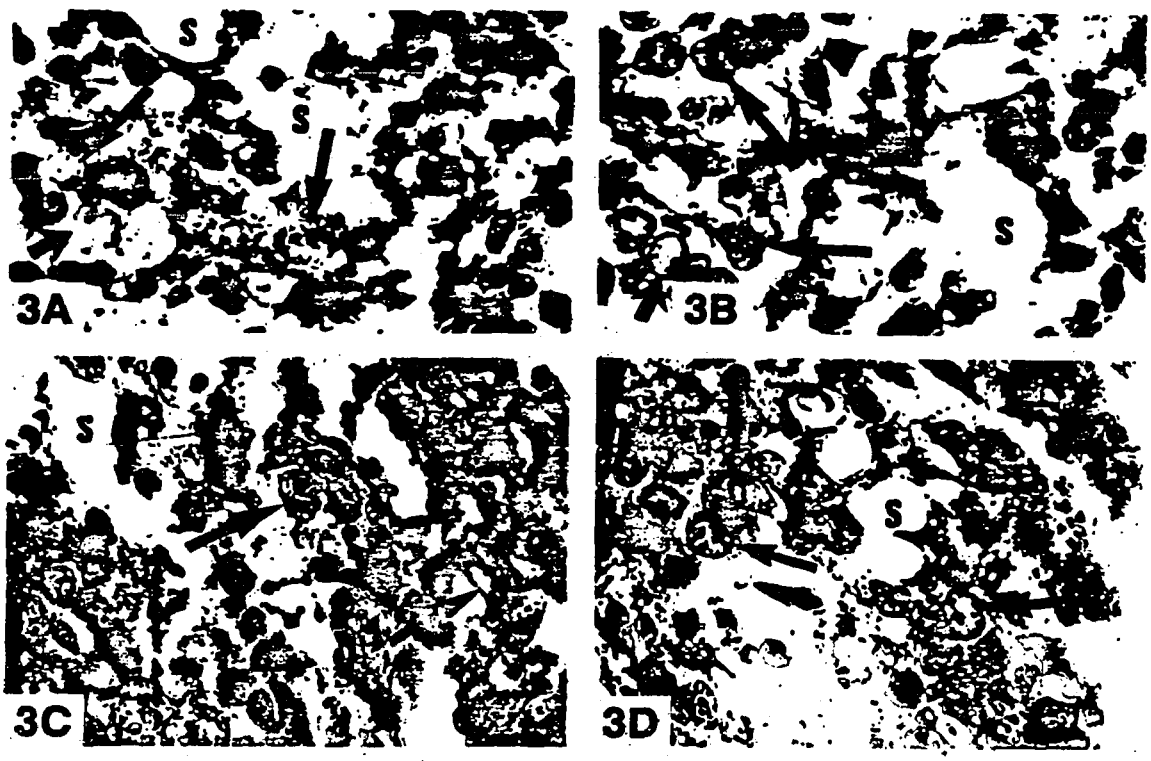


FIGURE 3.—Oil-immersion photographs: 3A-3B) The central region of the same nodule shown in fig. 2D. Cells with very large pale-staining nuclei (*arrows*) are shown, and in some of these cells prominent nucleoli are included in the focal plane. Spaces (S), similar to those characteristically seen in advanced stages of growth of primary tumors (fig. 3D), are present. 3C) The central region of a nodule from another animal necropsied at the time of enucleation. *Note* large nuclei with prominent nucleoli and spaces (S) (*arrows*). 3D) From the ocular tumor shown in fig. 2B. Cells with large nuclei and prominent nucleoli are seen (*arrows*), as well as large spaces (S) in the connective tissue. 3A-3D, × 1,120

Chapter 2

Dormant Metastases of the 13762NF Mammary Tumor in Fischer 344 Rat

ABSTRACT

We have previously shown that rats with intact iridial implants of the 13762NF tumor die within 7-9 weeks with pulmonary metastasis and extrapulmonary metastasis to the cervical lymph node, liver, heart, kidney, or body wall. If tumor-bearing eyes are removed (enucleated) rats remain healthy for 1-3 years and contain occasional small nodules in the lung. In the present study, we begin to develop a model for dormancy of an adenocarcinoma and examine enucleated rats (ER), for the presence of dormant viable tumor. Others have shown that formalinized tumor cells (FTC) potentiate the growth of dormant metastases. In the present study, ER were injected with 5x105 FTC 2 days after enucleation. Seventy-eight percent of the inoculated ER became moribund with widespread metastatic disease 2-3 months later and had metastases to the lung (77%), kidney (55%), liver (33%), ovary (33%), body wall (22%), thymus (11%), and pericardium (11%). Control ER and ER inoculated with formalinized liver cells did not develop metastatic disease, and normal animals challenged with FTC did not develop tumor. In other experiments, ER were examined 3 months post-enucleation and 40-70% contained nodules in the lungs, heart, or cervical lymph node. Histological and immunocytochemical analysis permitted definitive diagnosis of carcinoma in 3 of 4 nodules sampled. When cell suspensions from ER organs that contained nodules were inoculated into sublethally irradiated rats (SIR), hosts became cachexic and died within 5 days. Cachexia and death were not observed when CS from organs lacking visible nodules or from control rats were inoculated into SIR. Examination of old ER 1-3 years after enucleation revealed the presence of viable metastatic adenocarcinoma

in 23% (3/13) of the rats. Adenocarcinoma was not present in 11 age-matched controls. Collectively, our data support the existence of dormant viable metastatic carcinoma in ER.

INTRODUCTION

The 13762NF mammary adenocarcinoma is a highly aggressive transplantable syngeneic tumor of low immunogenicity in the F344 rat and is useful as a model for studying breast cancer (8, 9, 37, 111, 120). The 13762NF tumor and various subclones have been used in studies designed to evaluate the effect of immune factors, therapy combinations, and dietary regulation on tumor growth and metastasis (7, 28, 58, 102, 103, 152). In a previous study, we showed that implantation of tumor on the iris leads to death after 7-9 weeks. In addition to extensive pulmonary metastasis, animals had metastasis to the cervical lymph nodes, body wall, heart, kidney, and liver. If animals received iridial implants with removal (enucleation) of tumor-filled eyes prior to spread to the orbit, animals remained healthy and the development of overt metastatic disease was prevented. When healthy ER were necropsied 0 days to 26 weeks post-enucleation, small nodules were seen in the lung. The identity and/or tumorigenicity of cells from these nodules was not determined (131).

The phenomenon of dormancy is an extremely important aspect of metastasis. In patients, tumor-dormant states are manifested by the recurrence of tumor at distant sites, years after removal of the primary tumor. Dormancy is most frequently seen in patients with breast carcinomas and malignant melanomas (41).

Experimental models for tumor dormancy or dormant metastases (24-27, 35, 60, 78, 110, 124, 120, 130, 142, 154), predominantly involve lymphomas and sarcomas. Very few animal models are available to study the phenomenon of tumor dormancy most typically seen clinically. The use of an ocular model appears to have fortuitously provided us with an opportunity to begin to develop a model for studying dormancy of metastases of a mammary adenocarcinoma.

The present study was designed to determine whether ER harbor dormant viable metastatic carcinoma. In one experiment we examine the effect of formalinized tumor cells (FTC) on putative metastases in ER. It has been shown by others that soluble tumor extracts (STE) or FTC administered subcutaneously (SC) 2-4 days after excision of a small primary tumor can potentiate metastasis and suppress tumor-induced immune responses (62, 63, 137, 138). In addition to studying the effect of FTC on metastasis shortly after enucleation, we examine the morphology and tumorigenic capability of putative dormant cells in ER 3 months after enucleation. The results of a 1-3 year follow-up of untreated ER are also presented.

MATERIALS AND METHODS

Maintenance of tumor

The 13762NF mammary adenocarcinoma, obtained from the Breast Cancer Task Force Animal and Human Tumor Bank (Mason Research Institute, Worester, MA) is maintained in our laboratory by SC implantation every 2 weeks of 1-2mm3 tumor fragments into the flanks of 5-8 week old F344 viral-free females (Charles River Breeding Laboratories, Wilmington, MA). Tumor was free of murine viruses and mycoplasma, as analyzed by a

standard RAP test (Anmed, Biosafe Inc., Rockville, MD). Animals were fed Purina rodent chow and maintained and cared for according to National Institutes of Health guidelines on the care and use of laboratory animals.

Preparation and examination of tumor implants

Iridial implants: The general procedure for preparing iridial implants has been described in detail previously (131). In the present study, female rats 5-8 weeks old were anesthesized, the iris dilated, an incision made in the center of the cornea, and a 1.0 mm3 fragment of tumor placed on the iris immediately adjacent to the pupillary margin. In one protocol eyes were removed (enucleated) 10 days post-implantation and rats examined 3 months post-enucleation (3PE rats). In another protocol, tumor-filled eyes were removed and old enucleated rats (OER rats) examined when moribund. In either protocol animals with recurrent growth in the orbit were excluded from the experiment.

Floating implants: Implants placed in the anterior chamber of the eye are maintained for long periods of time, but do not grow significantly. The procedure for preparation is the same as for iridial implants except that tumor is placed in the anterior chamber of the eye and not upon the iris.

SC implants: To obtain SC implants or to transfer solid tissue suspected of containing tumor, 1-2 mm3 fragments of tissue were injected ventrally SC into the third mammary gland using a 16-gauge trocar.

Inoculation of cell suspensions from enucleated rats into irradiated and non-irradiated hosts

Cell suspensions (CS) were prepared from organs previously shown to contain metastatic lesions in animals with unenucleated iridial implants.

Accordingly, lung and heart cell suspensions were made from organs of healthy 3PE rats, or from moribund OER 14-33 months post-enucleation. Cell suspensions were inoculated into control, or sublethally irradiated (420RAD 137Cs, one day prior to the experiment) 5-8 week old F344 females.

Preparation of cell suspensions

Cell suspensions were obtained by mincing the organs and passing the tissues through a 250 uM Nitex filter in 10 ml of a-minimum essential medium; (a-MEM; Gibco, Grand Island, NY), and centrifugation (1000 rpm, 5 min). Pellets were resuspended in 0.5 ml of a-MEM. Hosts received 0.5 ml of a lung cell suspension (LCS) SC into the third mammary gland on the right side, and 0.5 ml of a heart cell suspension (HCS) into the third mammary gland on the left side.

Formalinized tumor cells

Formalinized tumor cells (FTC) were prepared as described by Ross, et al (116). Briefly, single cell suspensions of tumor were resuspended in 25 volumes of formalin and left at room temperature for at least 12 hours. The cells were washed 3 times in saline and stored at 40C for up to 1 month.

Separation of adherent cells from non-adherent

To obtain adherent and non-adherent populations of cells from lungs, cell suspensions were incubated for 2 hours in 5% CO2. Non-adherent cells were washed off and adherent cells were gently removed by pipetting. Both cell populations were washed in 10% MEM and inoculated into sublethally irradiated hosts.

Examination of animals for metastatic lesions

The internal organs of experimental and control animals were examined under the dissecting microscope at 6x. Organs containing metastatic lesions or nodules, and tissue from control animals, were fixed in Bouin's fixative, processed, and embedded in paraffin. Tumor volume in the lung was determined using the calculation reported by others (151).

Immunocytochemistry

Immuno-labeling of all specimens was performed utilizing the Avidin-Biotin-Chromagen (ABC) method from Vector Laboratories, Burlingame, CA. Incubation times for deparaffinized, rehydrated 4 um sections were: 10 minutes in universal blocking agent, 1 hr in primary antibody, 30 minutes in secondary (bridging) antibody, 1 hr with the peroxidase complex, and 10 minutes in 3,3' -diaminobenzidine tetrahydrochloride (Sigma Chem. Co., MO). Sections were counterstained in dilute hematoxylin, dehydrated, and mounted with non-aqueous mounting media. Anti- LCA, CEA, desmin, and myoglobin antibodies and chromogranin were obtained from Lipshaw, Detroit, MI, and polyclonal anti-keratin antibody was purchased from Dako, Copenhagen, DK. Analysis of pathological and immunocytochemical data was done in blind fashion.

RESULTS

Enhancement of metastasis in enucleated rats by FTC

In order to determine whether FTC could potentiate metastasis in enucleated rats, 9 ER were inoculated SC with 5x105 FTC 2 days after enucleation. To control for the effect of injecting ER with syngeneic formalinized cells, 5 ER received 5x105 formalinized liver cells (FLC) 2 days after enucleation. Additional control ER received no formalinized cells

(N=18). All ER injected with FTC became moribund 2-3 months after enucleation and were sacrificed and necropsied. Metastasis was seen in the lung (77%), kidney (55%), liver (33%), ovary (33%), body wall (22%), thymus (11%), and pericardium (11%), Table 1. This metastasis was very extensive, and exceeded that observed in any of our experiments to date with rats bearing unenucleated ocular tumors. All enucleated controls, and ER inoculated with FLC remained healthy and were sacrificed either 16, 80, or 96 weeks after enucleation, necropsied, and found to be free of metastic disease.

Examination of rats 3 months post-enucleation (3PE) for the presence of tumor cells

The object of this experiment was to examine the tumorigenicity and identity of putative tumor cells from organs containing visible nodules. The general protocol was to necropsy rats and examine the morphology and viability of tumor cells in 3PE organs. If an organ contained more than one visible nodule, a nodule was removed for light microscopic examination. Cell suspensions were then prepared from the remainder of organs containing nodules and injected into young sub-lethally irradiated hosts (SIR). SIR were chosen as hosts, as it was felt that the use of these immune-compromised animals might facilitate growth and/or metastasis of dormant tumor cells.

Accordingly, 10 healthy 3PE rats were sacrificed and necropsied. Six of these rats contained 1-3 pulmonary nodules approximately 0.5 mm in diameter. One of the 6 rats also contained an enlarged cervical lymph node, and a seventh rat contained a heart nodule. One nodule was removed from each of 3 lungs, along with a portion of the enlarged cervical lymph node, and

processed for histological or immunocytochemical analysis. Cell suspensions were prepared from the remainder of these organs and from the lungs and hearts of the 3 ER lacking visible metastases, and inoculated into the mammary gland of SIR. The protocol followed, and a summary of pertinent results are summarized in Fig. 1.

Histological analysis revealed that 3 of the 4 ER specimens sampled contained carcinoma. The cervical lymph node contained readily identifiable adenocarcinoma (Fig. 2a). Analysis of lung nodules showed that in 1 rat, tumor cells were arranged in small clusters, had a moderate nucleocytoplasmic ratio, and reacted positive for keratin (2+), Fig. 2b and positive for CEA. The specimen was diagnosed as undifferentiated carcinoma. A control section not treated with primary antibody is shown in Fig. 2c. In the lung of another 3PE, there was a small nodule containing approximately 50 cells with large nuclei, prominent nucleoli, and an increased nucleocytoplasmic ratio. A few mitotic figures were present. The section was diagnosed as metastatic poorly differentiated carcinoma (Fig. 2d). Sections obtained from the same block for immunocytochemical reaction no longer contained the nodule. The lung parenchyma surrounding the nodules of undifferentiated carcinoma in the 3PE rats was congested. While the general architecture was preserved, there were areas with dilatation of alveolar spaces and blood vessel congestion.

In SIR inoculated with CS from 3PE, a palpable mass developed at the site that had been inoculated with lung or heart CS from animals whose lungs (N=6) or heart (N=1) had contained visible nodules. These rats became markedly cachetic and died 3-5 days after SC inoculation. Histological analysis

of the SC masses showed that the masses resembled abscesses and contained areas that were very fibrous, Fig. 3a, as well as areas that were highly cellular, Fig. 3b. In some abscesses a necrotic center lined by fragmented polymorphonuclear leukocytes was present. In general, immunocytochemistry for keratin and CEA did not disclose the presence of positive cells. However, in one mass a small, perfectly encapsulated nodule of neoplastic cells with numerous mitoses was present, Fig. 3c. Immunocytochemistry for keratin did not disclose the presence of positive cells. Lymphocytes and leukocytes intermingled with these cells. This mass was derived from the lung CS prepared from the lung that had contained the nodule shown in Fig. 2b.

The host that received a portion of the cervical lymph node shown in Fig. 2a did not become cachetic. A SC mass grew very slowly and first became palpable after 1 month. The animal became moribund and was sacrificed 3 months after SC inoculation. The lung contained readily identifiable adenocarcinoma.

The three irradiated hosts that received CS from donors whose lungs and heart had not contained visible nodules remained healthy and did not develop SC masses or become cachetic.

In order to investiagate the cachexia further,lung cell suspensions from five 3PE rats were made and adherent and non-adherent cell populations were seperated as described previously. The adherent and non-adherent cells were injected to 5 SIR. Interestingly, non of these animals became cachexic or died. This observation suggests the necessecity of both adherent and non-adherent for the cachexia phenomenon.

Controls: Three SIR were inoculated with lung CS and heart CS from control rats and were sacrificed 7 months later. Eight SIR received lung and heart CS from sham-implanted and enucleated animals. Five were sacrificed after 3 months, and 3 were sacrificed after 14 months. All hosts receiving CS from control or sham-operated rats were healthy when sacrificed and were free of SC masses, tumor, or nodules. Three rats were sacrificed 3 weeks after receiving iridial implants. Lungs from these animals contained small metastatic lessions. Lung cell suspensions were prepared and inoculated into 3 SIR. Hosts did not become cachexic.

Because of the unusual finding of precipitous cachexia and morbidity in SIR inoculated with CS from 3PE lungs that had shown nodules, the above experiment was repeated. In addition, in order to determine whether implantation of tumor in the eye of donor rats may have contributed to the cachexia and death in SIR hosts, an additional control group was added, consisting of rats that contained floating implants of the 13762NF tumor in the anterior chamber of the eye, with enucleation 10 days later. Accordingly, 12 animals received iridial implants and enucleation, and 5 animals received floating implants and enucleation. Five animals were sham-implanted and enucleated. Three months after enucleation lung and heart CS from the 12 iridial implanted animals were inoculated into SIR. Pulmonary nodules had been observed in 5/12 of these animals. Once again, all hosts receiving CS from organs containing visible nodules became cachetic and died within 3-5 days. None of the SIR that received CS from sham-operated rats or from rats that had contained floating implants in the anterior chamber of the eye developed cachexia, or became moribund. These results confirm the finding

that only lung or heart CS with visible nodules from 3PE cause precipitous cachexia and death in SIR, and further show that the presence of tumor in the anterior chamber is not responsible for the sequence of events associated with the cachexia-inducing capability of CS derived from ER organs.

Growth and metastasis of tumor cell suspensions in irradiated and non-irradiated rats

The extensive metastasis seen in ER treated with FTC suggested that widespread microscopic deposits of tumor cells might be present throughout the lungs and other metastatic sites of ER. Thus, one possible explanation for the pronounced cachexia in irradiated hosts only 3-5 days after SC inoculation of cell suspensions from 3PE is that very high concentrations of tumor cells present in donor organs might induce cachexia when inoculated into irradiated hosts. In order to assess the effect of varying concentrations of 13762NF tumor cells on young irradiated hosts, irradiated and non-irradiated hosts were inoculated SC with either 5x104, 5x106, or 5x108 tumor cells. After inoculation, SC masses were measured on a weekly basis for 4 weeks. At the end of 4 weeks all rats were sacrificed, and the mean volume of pulmonary metastases determined.

Analysis of the growth of the SC tumors revealed that the size of the primary tumor was unaffected by prior irradiation of the host. At the end of 4 weeks, none of the hosts showed signs of cachexia. Analysis of the mean volume of tumor in the lung and of the incidence of metastasis to the axillary lymph nodes showed that there was a significant increase in the incidence of lymph node metastasis in SIR following inoculation with 5x106 tumor cells, and an increase in the extent of pulmonary metastasis in irradiated hosts

receiving 5x106 or 5x108 tumor cells when compared with results obtained using non-irradiated hosts, Table 2.

Long-term follow-up of old enucleated rats (OER) for evidence of tumor dormancy

Thirteen OER were maintained for prolonged periods of time. Ten/13 OER became moribund or died 14-33 months post-enucleation, and 3 were apparently healthy when sacrificed 30 months after enucleation. All OER were necropsied and tissue suspected of containing tumor cells was passaged in young hosts. As it was not always possible to obtain SIR when an OER died, control non-irradiated hosts were also used. In general, the availability of hosts at the time of unexpected morbidity or death of an OER, and the number and size of nodules found upon necropsy, dictated that sample size and nature of the host population.

The first OER became moribund 14 months after enucleation. A SC mass was present in the flank, and a few small nodules less than 1 mm diameter were seen in the lung, Table 3. Solid fragments of tissue from the mass were passaged SC in 5 non-irradiated hosts. After a lag of 10-12 months, tumor grew SC and metastasized to the lung in all hosts, Table 4. A sample pulmonary nodule consisted of a circumscribed nodular mass made up of poorly differentiated neoplastic cells and was diagnosed as metastatic adenocarcinoma, Fig. 4a.

OER 2 became moribund 19 months post-enucleation, and a few small nodules were present in the lung and liver, Table 2. Extensive necropsy of the brain and other internal organs failed to reveal any primary carcinoma. Solid fragments of lung nodules were inoculated SC into 2 non-irradiated

hosts. The hosts became moribund 4 and 9 months later. Tumor had grown SC (2/2) and metastasized to the lung (2/2), ovary (1/2), kidney (1/2), and lymph node (2/2), Table 4. Analysis of sections from the lung showed alveolar septa that were thickened and infiltrated by medium size cells with oval or slightly convoluted nuclei, and moderate amounts of clear cytoplasm which reacted positively with keratin and CEA, Fig. 4d-e. The tumor was diagnosed as adenocarcinoma. Additionally, OER 2 had contained tumor in the atrium of the heart. A non-irradiated host received fragments from this tumor. The host became moribund 3 months later. Tumor had grown SC and metastasized to the lung and lymph node, Table 4. The SC mass contained medium-sized cells with small round or oval nuclei and scanty cytoplasm. Immunocytochemical reaction for LCA was positive and negative for keratin. The diagnosis was compatible with lymphoma. Combined, these results show that OER 2 had a few small pulmonary nodules of metastatic adenocarcinoma and lymphoma.

A third OER developed a large mass in the axilla 24 months after enucleation, and a few small nodules were seen in the lung, Table 2. The axillary tumor was observed histologically to be a well differentiated fibroadenoma. Extensive necropsy revealed no other tumor. Fragments of the axillary mass were passaged SC in one non-irradiated host and in one irradiated host and no SC masses appeared, Table 4. Lung and heart CS from OER 3 were passaged in one non-irradiated and one irradiated host. The non-irradiated host receiving these CS was sacrificed after one year with no evidence of any tumor. The irradiated host that received CS died after 4.5 months with a SC mass in the region that had been inoculated with lung CS

and poorly differentiated adenocarcinoma was present in the lung, Fig. 4b. Thus, OER 3 had metastatic adenocarcinoma in the lung and spontaneous fibroadenoma.

Of the 10 remaining OER that became moribund or died after enucleation, 4 contained tumors. These tumors included angioleiomyoma of the uterus in a 26 month old rat, rhabdomyosarcoma (large and small cells of the tumor were positive for myoglobin and desmin) in a 33 month old rat, and fibroadenoma in a 17 and a 33 month old animal. Eleven age-matched control animals were housed for 2.5 to 3 years in our animal facility. One developed lymphoma and 2 had fibroadenoma.

DISCUSSION

Using a syngeneic mammary tumor of low immunogenicity in mice, Klein, et al (62) concluded that tumor plasma cell membranes or the products released by them play an important role in the growth of dormant metastases. Soluble tumor extracts or FTC administered SC 2-4 days after excision of a small primary tumor potentiated metastasis and suppressed tumor-induced responses (62, 63, 137, 138). In our experiments, enucleated rats challenged with FTC developed extensive pulmonary and extrapulmonary metastases. The pattern of metastasis in FTC challenged animals was similar to that seen in moribund rats with intact iridial implants (131), except for the additional presence of metastases in the ovary and thymus gland in FTC treated animals. It is important to note that even rats inoculated IV with 13762NF tumor cells fail to develop widespread extrapulmonary metastases despite the presence of extremely heavy tumor burdens in the lung. The pattern of metastasis in FTC-treated ER demonstrates that 13762NF tumor cells capable of

colonization are widely distributed through the body of these rats. The potentiation of metastasis in ER by FTC may result from an FTC-induced suppression of tumor immunity (62). The failure of FLC to affect metastasis in the ER supports the general hypothesis (63) that specific tumor antigens may be required for this potentiation. In preliminary experiments we have found that FTC are capable of affecting splenic macrophage cytostatic activity in ER. We are currently examining the effect of FTC on the immune system in the ER model.

Our morphological analysis of nodules and/or presumptive tumor in 3PE enable us to definitively identify carcinoma or adenocarcinoma in 75% of the tissues sampled. While it would have been desirable to obtain specimens for immunocytochemistry from all organs with nodules, a major aim of this experiment was to determine whether viable tumor cells were present in select organs from enucleated rats, and it was considered important that the CS inoculated into hosts contain cells from at least one macroscopically identifiable nodule/donor organ. Consequently, a nodule could only be spared from the lungs of 3 rats, and a portion of a cervical lymph node from a fourth animal. Interestingly, the varying morphology of tumor cells present in dormant nodules from the tissues analyzed indicates a heterogeneity in phenotype Fig. 2.

In our physiological experiments involving the inoculation of cell suspensions from 3PE tissues suspected of harboring dormant nodules or tumor into SIR, we had anticipated that dormant tumor cells might slowly grow and metastasize. Unfortunately, the rapid onset of morbidity and death in the SIR host did not allow us to adequately test this hypothesis. Only the

SIR inoculated with cells from a portion of a cervical lymph node failed to develop cachexia and adenocarcinoma grew and metastasized in this host. Neoplastic cells were also identified within one abscess present in a SIR that had received LCS from another donor ER (Fig. 3d). The neoplastic cells did not react with anti-keratin antibody, and definitive identification of this mass could not be made. The highly cellular regions from other abscesses also failed to react with keratin. Lack of reactivity with antibody should be interpreted with caution, however, especially in the case of the 13762 tumor. Using a panel of antibodies against keratin, it has been shown by others (75) that various lines and clones of 13762NF differ qualitatively and quantitatively in the expression of cytokeratin and that expression also depends upon the environment.

The unusual development of rapid cachexia and death in SIR hosts inoculated with CS from 3PE is interesting and at present inexplicable. Only SIR inoculated with lung and heart CS prepared from tissues containing visible nodules became cachexic. SIR inoculated with tumor cells along or with lung and heart CS from controls did not develop precipitous cachexia. It is currently our hypothesis that immune effector cells combined with cytokines and/or tumor cells from 3PE donor organs, combine to elicit reactions resulting in cachexia and death. This hypothesis is consistent with the observations of an inflammatory response in the parenchyma of the lung surrounding the nodules examined histologically. It is interesting to note that in a model for dormancy of a murine lymphoma, adherent and non-adherent peritoneal exudate cells, PGE2, MuTNF, and Mug-INF appear to be capable of interacting to maintain dormancy. In this model ongoing immune

responses balanced by immunosuppressive mechanisms appear to enable viable tumor cells to live for prolonged periods of time in mice harboring dormant tumor (78, 129, 130). The data in Table 2 of the present study suggests the involvement of T-cells in the host response to 13762NF since there is a significant increase in the volume of metastasis in SIR than normal rats.. We have preliminary data suggesting an involvement of macrophages as well.

The results obtained in the long-term follow-up of enucleated rats demonstrate that 23% of the OER harbored viable metastatic adenocarcinoma, and in 2 of these animals no primary carcinoma was present. It is possible that the SC flank tumor seen in OER 1 was spontaneous adenocarcinoma and that the nodules seen in the lung were metastases derived from this tumor; however, in a study of 1754 aging female Fischer 344 rats, the incidence of mammary adenocarcinoma was 2.2% and these tumors were rarely metastatic (126). In view of these statistics and of the similarity in size and number of the pulmonary nodules seen in OER 1 with those seen in 3PE and OER's 2 and 3, it seems likely that the tumor present in this rat originated from 13762NF.

It is interesting to note that in 3PE and OER, overt metastatic disease has not been observed to occur. Furthermore, the incidence of pulmonary nodules present in ER would seem to slowly decrease with time (from a 42% - 70% incidence in 3PE, to a 23% incidence in the OER). This may suggest that ongoing immune or host defense mechanisms present in the ER may act to maintain or slowly eliminate small metastatic deposits.

In summary, the data from experiments on FTC-treated ER and on ER 3 months and 1-3 years after enucleation, collectively support the hypothesis that dormant metastatic viable tumor cells are present in enucleated rats. We are currently investigating immunological mechanisms that may act to restrain or prevent growth of metastatic tumor in the ER. Considering the suitability of the 13762NF tumor in the syngeneic Fischer 344 rat as a model for human breast cancer, it would be important to develop a model for dormancy of this tumor. Development of such a model could increase our understanding of the phenomenon of dormancy and lead to experiments that could increase our ability to design therapeutic strategies for dealing with the problem of dormancy seen clinically.

FIGURE LEGENDS

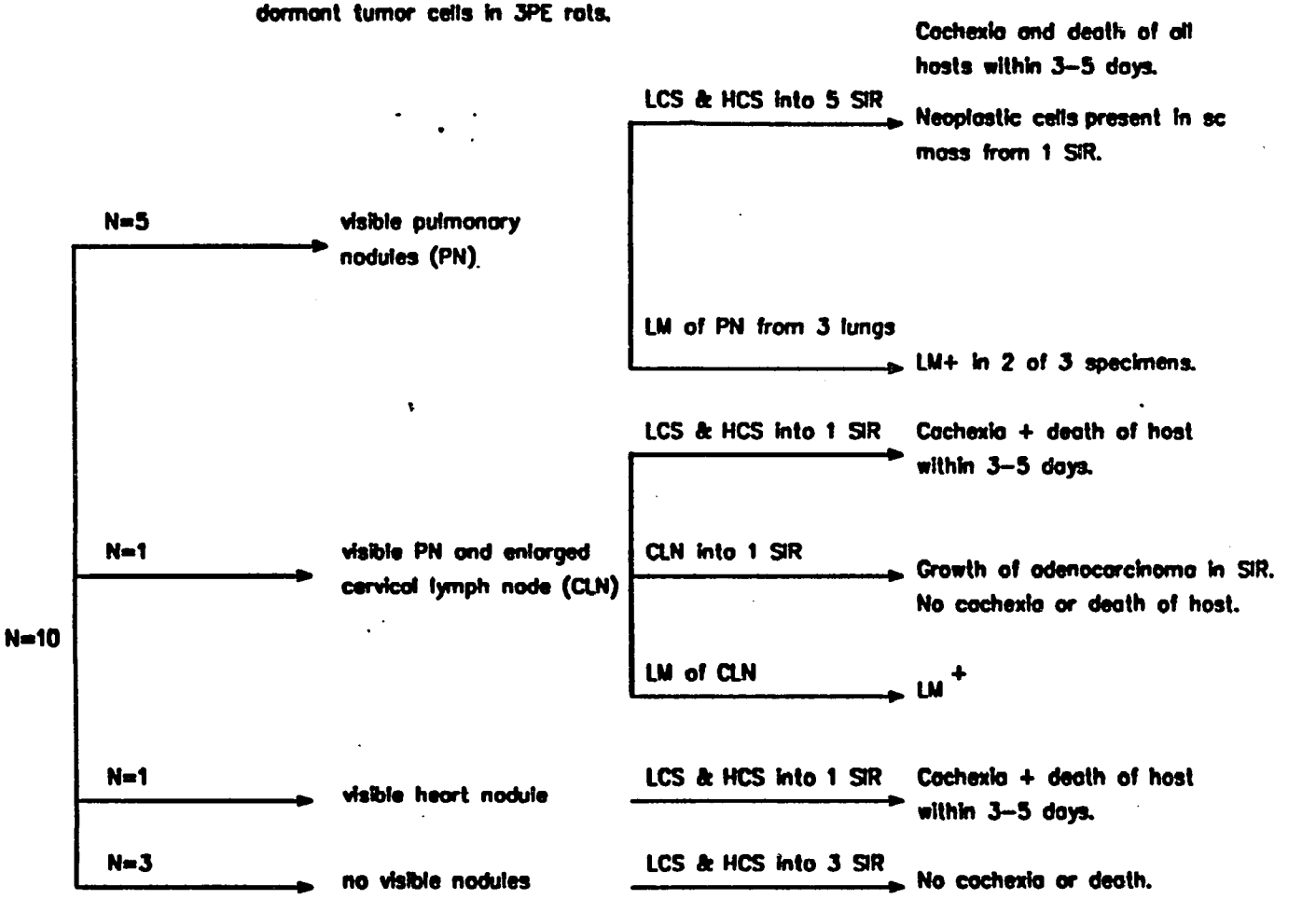
Fig. 1. Flow chart summarizing the protocol and results obtained in examining dormant tumor nodules in 3PE rats.

Fig. 2. Light microscopic examination of tumor from 3 PE rats. 2a is adenocarcinoma from a cervical lymph node, 435X. 2b is a pulmonary nodule diagnosed as poorly differentiated carcinoma, showing positive staining with anti-keratin antibody, 480X. 2c is a negative control from an adjacent area that did not receive primary antibody, 480X. 2d is a pulmonary nodule containing a small cluster of cells (arrows) diagnosed as poorly differentiated carcinoma, 435X.

Fig. 3. Micrographs from SC masses that developed in SIR's inoculated with CS from 3PE rats. 3a and 3b are typical areas seen in all abscesses: 3a is from a fibrous region, 435X, and 3b from a cellular region, 435X. 3c and 3d are from a mass containing encapasulated undifferentiated neoplastic cells, 270X and 435X, respectively.

Fig. 4. 4a is anodule containing a circumscribed mass of poorly differentiated metastatic adenocarcinoma in the lung of a host inoculated with fragments of an SC mass from OER1, 210X. 4b poorly differentiated adenocarcinoma in the lung of a SIR host inoculated with CS from the lungs of OER3, 270X. 4c-e are from section of a lung nodule diagnosed as metastatic adenocarcinoma from OER2. 4c is a negative control for immunocytochemistry in an adjacent area of the specimen not treated with primary antibody, 480X. 4d, cells reacting positively with anti-CEA antibody, 4e, cells reacting positively with anti-keratin antibody were medium sized and contained slightly convoluted nuclei and moderate amounts of clear cytoplasm, 480X 4d, adenocarcinoma from adjacent section.

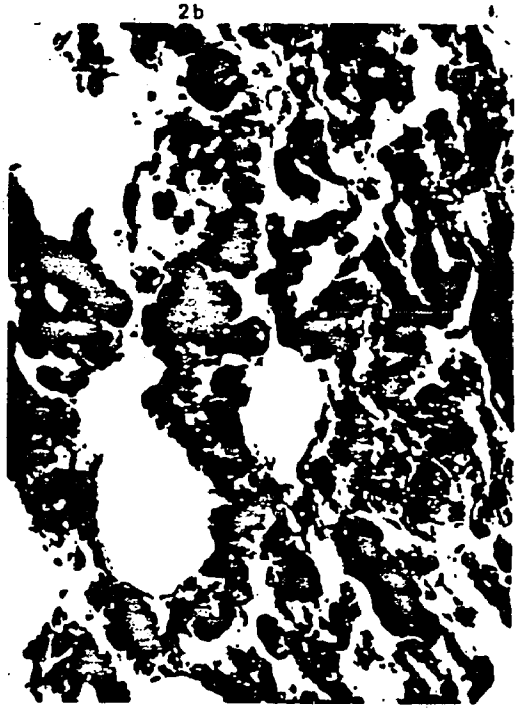
Fig. 1. Flow chart showing the experimental protocol for examining dormant tumor cells in 3PE rats.



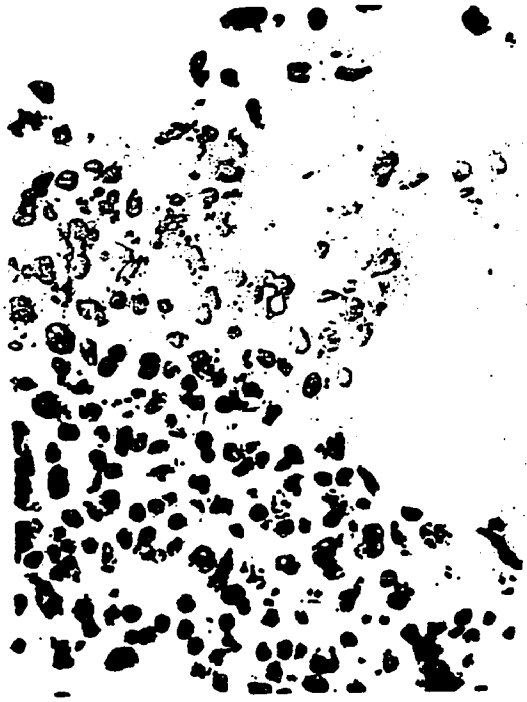
N = Number of 3PE HCS = Heart Cell Suspension CLN = Cervical Lymph Node
PN = Pulmonary Nodule SIR = Sublethally Irradiated Rat
LCS = Lung Cell Suspension LM+ = Tissue examined by light microscopy and found to be + for adenocarcinoma

2a

2b

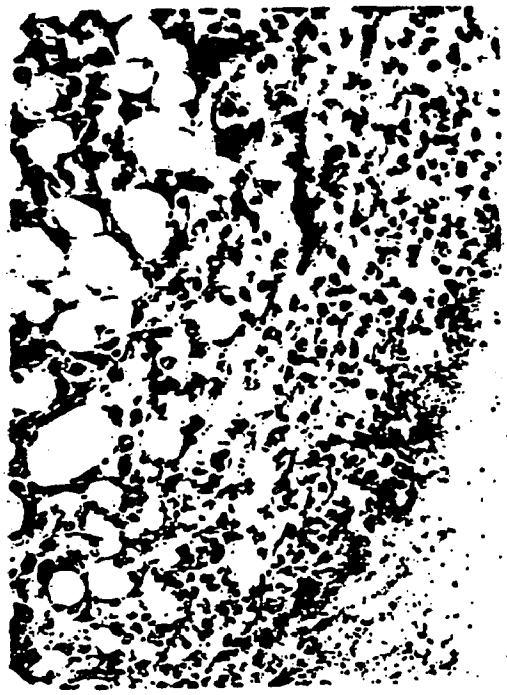


2c



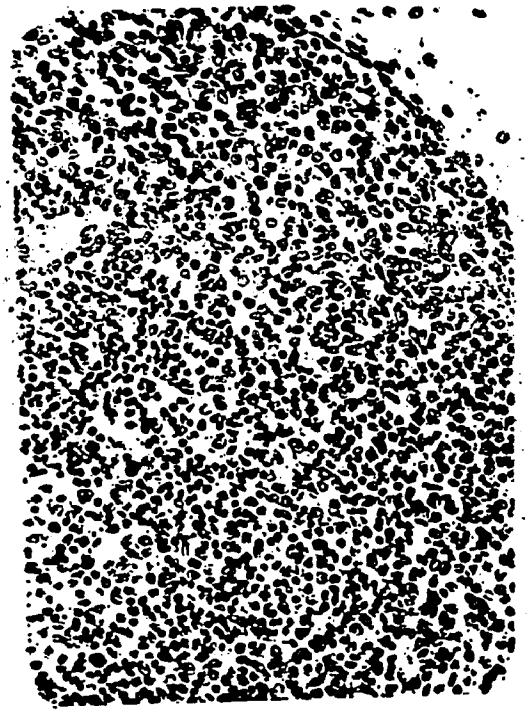
2d

3a

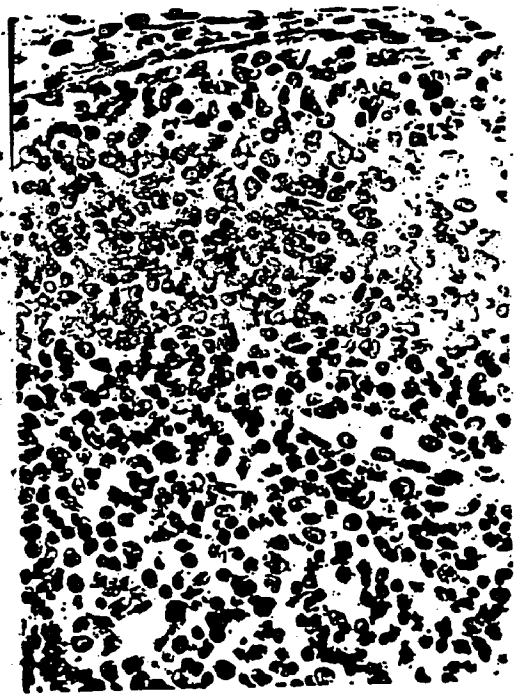


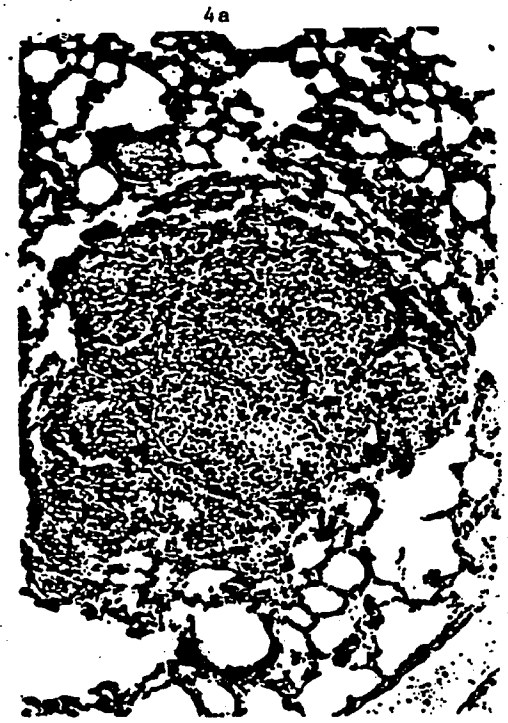
3b

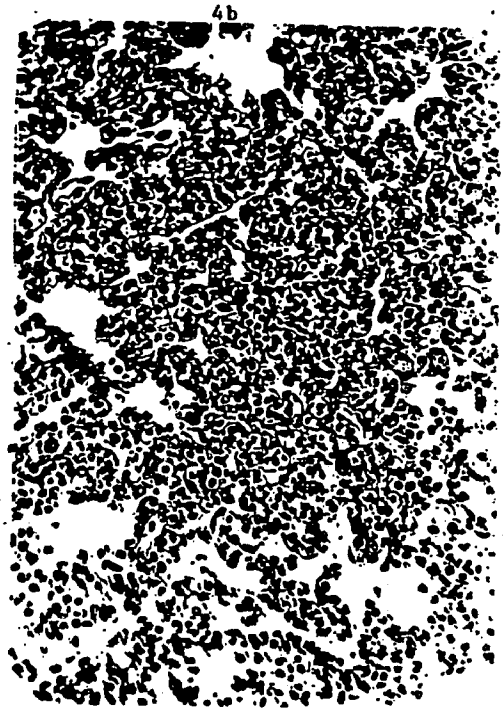
3c



3d




4a


4b


4c


4d


4e

Table 1. Potentiation of metastasis in enucleated rats treated with formalinized tumor cells.

Treatment Group	Mean Survival Time	Organs Containing Extensive Metastases						
		Lung	Liver	Kidney	Ovary	Body wall	Pericardium	Thymus
Enucleated, FTC[a]	7 weeks	7/9	3/9	5/9	3/9	2/9	1/9	1/9
Enucleated, FLC[b]	96 weeks [c]	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Enucleated	16 weeks [c]	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Enucleated	80 weeks [c]	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Normal[d]	12 weeks [c]	0/6	0/6	0/6	0/6	0/6	0/6	0/6

[a] = Enucleated rats received 5×10^5 formalinized tumor cells

[b] = Enucleated rats received 5×10^5 formalinized liver cells

[c] = Healthy rats were sacrificed

[d] = Rats received 5×10^5 formalinized tumor cells

Table 2. Metastasis of cell suspensions of the 13762NF tumor in irradiated and non-irradiated hosts.

Cell suspension	Group size		Percentage of rats with metastases		Volume of pulmonary metastases $(mm)^3$	
	irrad.	non-irrad.	irrad.	non-irrad.	irrad.	non-irrad.
5×10^4	7	8	100% lung 43% l.node	100% lung 13% l.node	39.88 ± 55.77	2.47 ± 2.11
5×10^6	6	6	100% lung 100% l.node	100% lung 0% l.node	218.8 ± 182.0	7.2 ± 5.06*
5×10^8	7	6	100% lung 100% l.node	100% lung 83% l.node	86.73 + 52.77	20.39 + 11.52*

* = Statistically significant at $P \leq 0.01$ when compared with volume of metastases in irradiated hosts

Table 3. The formation of nodules and large masses in OER 1–3

Rat	Survival Time (months post–enucleation)	Presence of nodules		Presence of a large growth		
		Lung	Liver	Sc	Heart	Mammary gland
1	14	+	–	+[a]	–	–
2	19	+[a]	+	–	+[a]	–
3	24	+[b,c]	–	–	–	+[b,c]

[a] = Tumor or nodules passaged in normal hosts
[b] = Cell suspension passaged in normal hosts
[c] = Cell suspensions passaged in irradiated hosts

Table 4. Growth and metastasis of tissues from OER in irradiated and non-irradiated hosts

Donor OER	Tissue passaged from OER	Incidence of Growth of sc primary	Presence of metastase				Mean survival for host (months)
			Lung	Kidney	Heart	l.node	
1[a]	sc tumor	5/5	5/5	0/5	0/5	0/5	12
2[a]	lung nodules	2/2	2/2	1/2	1/2	2/2	6.5
2[a]	mass from heart	1/1	1/1	0/1	0/1	1/1	3
3[b]	lung & heart	0/1	0/1	0/1	0/1	0/1	14[d]
3[b]	axillary tumor	0/1	0/1	0/1	0/1	0/1	14[d]
3[c]	lung & heart	0/1	1/1	0/1	0/1	0/1	4.5
3[c]	axillary tumor	0/1	0/1	0/1	0/1	0/1	14[d]
CONTROLS							
Normal rat[b]	lung & heart	0/3	0/3	0/3	0/3	0/3	7[d]
Sham operated[c]	lung & heart	0/3	0/3	0/3	0/3	0/3	14[d]
Sham operated[c]	lung & heart	0/5	0/5	0/5	0/5	0/5	3[d]

[a] = Tumor or nodules passaged in non-irradiated hosts
[b] = Cell suspensions passaged in non-irradiated hosts
[c] = Cell suspensions passaged in irradiated hosts
[d] = Healthy animals sacrificed and necropsied

Chapter 3

The Effect of LS2616 on Growth and Metastasis of a Rat Mammary Adenocarcinoma

ABSTRACT

Linomide (LS2616) is a carboxamide quinoline immunomodulator capable of modulating natural killer cell activity, macrophage function, and T lymphocyte responses. In earlier work Linomide was shown to be effective in treating murine melanoma. In the present study we examine the effect of Linomide on growth and metastasis of a syngeneic mammary adenocarcinoma (13762NF) in rats given subcutaneous (SC) or intracameral (IC) tumor implants, or intravenous (IV) inoculation of tumor cell suspensions. Linomide was administered continuously in drinking water at a low dose (LD, 15 mg/kg) or high dose (HD, 60 mg/kg) beginning either one week before (pre-treatment) or two weeks after (post-treatment) tumor challenge. When tumor was implanted SC, all treatment protocols resulted in a significant decrease in tumor size. However, only post-treatment with drug resulted in partial or complete regression of primary tumors and in apparent cures. Post-treatment with LD drug resulted in partial regression (30%; 3/10), complete regression (20%; 2/10), and one apparent cure, while post-treatment with HD drug resulted in partial regression (36%; 4/11), complete regression (9%; 1/11) and one apparent cure. Post-treatment had little or no effect on metastasis, but pre-treatment with HD drug significantly increased pulmonary metastasis. In rats with ocular tumors, a beneficial affect of Linomide on metastasis was observed. Administration of drug to rats with well established ocular tumors resulted in a significant decrease in the incidence of metastasis to the kidney, rib cage, and lymph node. Post-treatment with drug also resulted in a statistically significant decrease in the extent of pulmonary metastasis. In an experimental metastasis assay a clear

effect of drug on pulmonary metastasis could not be demonstrated. Collectively, our work demonstrates that Linomide can effectively inhibit tumor growth, cause tumor regression, decrease pulmonary and extrapulmonary metastasis, and affect apparent cures when administered to rats with well established mammary adenocarcinoma. The specific effect of treatment with Linomide depends upon the time of initiation of treatment and the location of the primary tumor.

INTRODUCTION

Linomide (LS2616), a quinoline 3 carboximide, is an immunomodulator that can be effectively administered in drinking water and has little or no toxic side effects. Linomide has been shown to be effective in treating murine melanoma (43-45, 55). Kalland demonstrated that drug treatment decreased growth and metastasis of cultured B16F10 melanoma cells and showed that antitumor activity was mediated by NK cells and non-NK cell host defense mechanisms. We demonstrated the effectiveness of linomide in decreasing metastasis from intracameral (IC) and subcutaneous (SC) tumors derived from highly metastatic in vivo passaged B16F10 melanoma and also showed the importance of NK (natural killer) cell modulation in this system (43-45). Linomide has been shown to enhance NK cell activity and proliferative T-cell responses and to increase the MHC class 11 expression in areas of inflammation (56, 72, 127). More recently it has been shown that Linomide is capable of increasing bone marrow NK progenitor cells (57). Linomide is currently being used in clinical trials with melanoma patients in Sweden.

Little or no information is available concerning the possible usefulness of Linomide in controlling growth or metastasis of carcinomas. The 13762NF tumor is a highly aggressive transplantable syngeneic mammary adenocarcinoma of low immunogenecity (7, 9, 16, 89, 102, 151) and is useful as a model for studying human breast cancer. We have previously shown that in rats with SC tumors, metastasis is predominantly to the lungs and lymph node, whereas in rats with IC tumors frequent metastasis is observed to additional organs including the rib cage, heart, kidney, and liver (131).

In the present paper we examine the effect of Linomide on tumor growth and metastasis in animals bearing intact SC or IC tumors and in hosts inoculated intravenously (IV) with a tumor cell suspension and demonstrate the ability of this drug to control growth and metastasis of an aggressive mammary adenocarcinoma

MATERIALS AND METHODS

Animals

Five to seven week old F344 rats were obtained from The Frederick Cancer Research Center, maintained according to the recommendations outlined by the National Research Counsel and housed in a pathogen free facility. Routine serology (Charles Rivers Lab, MA) confirmed the absence of indigenous rat viruses.

Tumor Maintenance & Administration

Maintenance: The 13762NF mammary adenocarcinoma was maintained in vivo by serial SC implantation every 2 to 3 weeks of 1-2 mm3 fragments of tumor into 5 to 7 week old F344 female rats. Tumor was free of

mycoplasma and of indigenous rat viruses when assessed in a standard RAP test (Anmed Biosafe, Inc., MD).

SC implants: A 1 mm3 fragment of tumor was inoculated SC in the region of the third mammary gland using a 16-gauge trocar. SC tumors were palpated weekly and the length and width measured with the use of a fine caliper.

IC implants: The procedure for obtaining IC implants has been described previously (131). Briefly, 5 to 7 week old rats were anesthetized with Nembutal, the iris dilated using mydriacyl, an incision made in the center of the cornea, and a 0.5 to 1.0 mm3 fragment of tumor placed on the iris immediately adjacent to the pupillary margin.

Experimental metastasis

Animals were inoculated in the tail vein with 5 x 104 tumor cell

Tumor cell suspensions

In vivo passaged tumor was minced and then trypsinized (0.25% trypsin in DMEM) at room temperature for 5 minutes. Cell suspensions were passed through a nitex filter (150), washed 3x in DMEM (1000 rpm for 10 minutes) and counted in a hemocytometer. Viability was assessed using the trypan blue exclusion technique and was routinely greater than 95%.

Drug Treatment

Linomide, provided by Dr. T. Stalhandske (Pharmacia, AB Leo, Helsingborg, Sweden) was administered in drinking water. In preliminary experiments rats were given 15, 60, 120, or 160 mg/kg Linomide in drinking water. Daily water consumption was inhibited in rats given 120 or 160 mg/kg. Accordingly, 15 and 60 mg/kg referred to as a low dose (LD), or high dose

(HD), respectively, were used in the present study. Drug treatment began either one week prior to tumor implantation or inoculation or 2 weeks after administration of tumor and continued for the duration of the experiment.

Examination of rats for metastatic lesions

Animals were sacrificed when moribund and internal organs examined under the dissecting microscope at 6x.

Survival time

Survival time was calculated as the number of days between administration of tumor and morbidity or from the time of tumor administration to the time that apparently healthy animals were sacrificed. Survival data is presented as median survival time.

Statistical analysis

Analysis of statistical differences between mean lung weights was performed using Duncans's Multiple Range Test. Median survival times were compared using the Mann Whitney U test, and statistical analysis of the incidence of metastasis was performed using Fischer's exact test.

RESULTS

The effect of Linomide on growth and metastasis of SC tumors

In order to examine the efficacy of Linomide in controlling growth and metastasis of SC tumors, panels of rats received drinking water only, or a HD or LD of drug beginning prior to or after tumor implantation. Primary tumor growth was measured weekly for 5 weeks, and animals were sacrificed and necropsied when moribund.

In animals pre-treated with Linomide, tumor grew progressively. Rats receiving drug had a significantly lower geometric mean tumor diameter

during weeks 2 through 5 than controls, Fig. 1a. Drug treatment appeared to predominantly affect initial tumor growth; LD (N=11) and HD (N=12) were equally effective. All controls (N=7) and experimental rats became moribund or died and the median survival time was 42 (controls), 48.5 (LD), and 54 (HD) days. Extensive metastasis to the lungs was observed in all groups and was significantly greater in animals receiving pre-treatment with HD drug than in untreated tumor bearing rats (Fig. 2). The incidence of extrapulmonary metastasis to lymph node and kidney was similar in all groups, Table I.

When administration of drug was initiated two weeks after tumor implantation, well established tumors were already present. Nonetheless, significant differences in geometric mean tumor diameter at weeks 3, 4, and 5 were seen between controls (N=7), and rats post-treated either with a LD (N=10) or a HD (N=11) of drug, Fig. 1b. In addition, partial and complete regression of primary tumor was observed. Partial regression occurred in 30% (3/10) of the rats receiving a LD, and in 36% (4/11) of the rats receiving a HD of drug, Table II. Complete tumor regression was observed in 20% (2/10) of the rats receiving LD drug and in 9% (1/11) of the rats on HD drug. In one rat receiving LD drug, primary tumor appeared to be eradicated for 10 weeks and then recurrent growth occurred. The remaining 2 rats with complete regression of primary tumor were healthy and showed no evidence of tumor or metastasis when sacrificed 20 weeks after implantation. The survival of experimental and control rats is depicted in Table I. The median survival time for rats post-treated with LD and HD of linomide was 53 and 51 days respectively. All moribund rats contained primary tumors. No significant

differences in metastasis were observed between experimental and control rats, Table I and Fig. 2.

The effect of Linomide on metastasis of IC tumors

In order to examine the effect of Linomide on metastasis of ocular tumors, animals were given either a LD or HD of drug beginning one week before or 2 weeks after tumor implantation. Control animals received drinking water only. All rats possessed primary tumors and became moribund. Pre-treatment with Linomide had no effect on the incidence of pulmonary metastasis but decreased the incidence of extrapulmonary metastasis. However, this was not statistically significant at $P < 0.05$, (Table II). Post-treatment with drug resulted in a non-significant decrease in the incidence of metastasis to the lungs. Pulmonary metastasis was observed in 81% of the rats receiving LD drug and 62% of the rats receiving HD drug. Animals lacking pulmonary metastasis also did not show extrapulmonary metastasis. Post-treatment with LD drug caused a significant decrease in the incidence of extrapulmonary metastasis to the lymph node, kidney, and rib cage ($P < 0.05$), and post-treatment with HD drug caused a significant decrease in the incidence of metastasis to the kidney and rib cage ($P< 0.02$), and resulted in a significant decrease in tumor burden in the lungs, Table II, Fig. 3. Linomide treatment did not affect survival, Table II. Although post-treatment with HD drug appeared to completely inhibit metastasis in 38% of the rats, animals still contained primary tumors and median survival time was not affected.

The effect of Linomide on experimental metastasis

In order to examine the effect of Linomide on metastasis in an experimental metastasis assay, rats received a LD or HD of drug before or after IV inoculation of $5x10^4$ tumor cells. Control rats received tumor but no drug. All rats became moribund and were sacrificed 4 weeks after tumor cell inoculation and had extensive pulmonary metastasis. A low incidence of extrapulmonary metastasis was seen in animals receiving no drug (rib cage, 11%) and in rats pre-treated with the drug. Nonetheless, treatment with Linomide did not significantly affect the incidence or extend of pulmonary metastasis, or the incidence of extrapulmonary metastasis and no difference was seen in mean survival time (Table III, Fig. 4.).

DISCUSSION

While considerable progress has been made in the treatment of lymphomas and leukemias, the development of effective therapy for most solid tumors (lung, colon, breast, pancreas, and melanoma) has not met with commensurate success (NIH guide, 1990). Modest progress has been made with techniques designed to modify or boost host natural defense mechanisms and/or immune responses utilizing lymphokines, cytokines, and immunomodulators.

Experiments utilizing immunomodulators have focused on the use of bacterial extracts and products, low doses of cyclophosphamide, or synthetic compounds like muramyl di- and tri-peptides and levamisole (LMS) (7, 107, 69, 18, 19, 29, 31). LMS has proved effective either alone or in combined therapy in animals and in humans with colon cancer (73, 121, 53, 158, 47). However, several experimental and clinical studies have found LMS to be

ineffective or even counterproductive against some carcinomas and melanomas (107, 30, 6, 4).

Linomide is a novel immunomodulator capable of enhancing natural killer cell production, macrophage function, and proliferative T-cell responses (55, 57, 72, 127). Its potential usefulness in cancer therapy has been demonstrated with murine melanoma. Using in vitro passaged B16F10 melanoma cells, Kalland has shown that Linomide (160 mg/kg) decreased tumorigenicity and pulmonary metastasis (55). Using the same dose of drug and the more aggressive in vivo passaged B16F10 line, we found little or no effect of this drug on tumor growth. However, administration of Linomide prior to inoculation of tumor cells IC or SC resulted in a decrease in pulmonary metastasis (43-45). In both melanoma studies, the importance of the natural killer (NK) cell was demonstrated.

In view of the effects of Linomide on growth and metastasis of murine melanoma and of encouraging preliminary data obtained from clinical trials with melanoma patients in Sweden (personal communication, Dr. T. Stalhandske), we chose to examine the effectiveness of Linomide against an aggressively metastastic mammary adenocarcinoma. In these experiments we found that doses comparable to those used in mice inhibited water consumption, while 15 and 60 mg/kg were well tolerated. These lower doses were therefore examined for prophylactic and therapeutic anti-tumor activity using both ocular and extraocular tumor implants and an experimental metastasis assay.

The anterior chamber of the eye is an immune privileged site, and others have shown that foreign antigens as well as syngeneic or allogeneic

tumors placed in this location result in a phenomenon called anterior chamber associated immune deviation (ACAID). ACAID is characterized by a suppression of delayed type hypersensitivity (DTH), a normal or slightly elevated humoral response, and the appearance of primed but inactive cytotoxic T-cell precursors (94, 96, 98, 99). Thus, we anticipated that a comparison of treatment with Linomide in rats harboring primary tumors in IC or SC compartments might afford a unique opportunity to examine the effect of immunomodulation under conditions where tumor may evoke different immune responses from the host. In addition, since we had already demonstrated a more varied pattern of metastasis from IC tumors, the use of animals with ocular implants would enable us to test the effectiveness of Linomide in controlling extrapulmonary metastasis.

In animals with SC tumors, pretreatment with drug significantly decreased tumor growth, but HD drug appeared to potentiate pulmonary metastasis. In contrast, when Linomide was administered to rats with well established SC tumors a decrease in tumor growth, partial or complete tumor regression, and apparent cures were observed.

In our IC experiments, primary tumors in animals treated with Linomide were approximately half the size of tumors in the no-treatment groups, but weekly measurements were not obtained. It seems likely that here too, growth of primary tumor was inhibited by Linomide. In contrast to the results obtained with SC and IV inoculation of tumor, Linomide significantly inhibited metastasis when applied in a therapeutic regimen to animals with established ocular tumors (Table II, Fig. 3). In fact despite the presence of primary tumors, 26% of the rats receiving post-treatment (2/11

LD and 3/8 HD) showed no incidence of metastatic disease and statistically significant decreases were seen in the extent of pulmonary metastasis (HD) and in the incidence of extrapulmonary metastasis (LD + HD).

Our experiments utilizing IV inoculations of tumor cell suspensions show that treatment with Linomide had little or no effect on metastasis or survival. In contrast Kalland found that Linomide inhibited metastasis of SC and of IV injected B16F10 melanoma in mice (55). In this mouse system NK modulation appeared to play a major role. We have found that SC and IV inoculation of 13762 does not significantly affect NK activity, and Linomide treatment does not significantly boost NK activity above normal levels in tumor bearing animals (117). Consistent with our findings of a differential effect of Linomide treatment in rats receiving SC and IV tumor, is data obtained by Aslakson & Starkey (5) demonstrating a differential effect of immunomodulation on spontaneous and experimental metastasis. These investigators found that Levamisole decreased spontaneous metastasis following SC inoculation of tumor cells but increased metastasis following IV tumor inoculation.

In summary, the present study demonstrates that Linomide is capable of affecting primary tumor growth as well as pulmonary and extrapulmonary metastasis of an aggressive mammary adenocarcinoma in rats. In animals with primary tumors drug treatment is more effective when given after tumor inoculation than when administered prior to the presentation of the tumor, in addition our results suggest that the specific therapeutic efficacy of treatment with this immunopotentiator may depend upon the location of the primary tumor and/or the immune status of the tumor bearing host and on

the dose and time of drug administration. In a subsequent paper, we describe the effects of Linomide on the immune system of tumor bearing animals and attempt to correlate the in vivo responses presented here, with an in vitro analysis of cellular host immune/defense responses.

TABLE I: The effect of Linomide on metastasis and survival in rats with SC tumors.

Treatment	Incidence of metastasis to internal organs			Incidence of apparent cures	Median survival time in days
	Lung	LN	Kidney		
No treatment, NT	100%(8/8)	62%(5/8)	12%(1/8)	0/8	42
Pre-treatment, LD	100%(8/8)	37%(3/8)	37%(3/8)	0/8	48.5
Pre-treatment, HD	100%(7/7)	42%(3/7)	14%(1/7)	0/7	54
Post-treatment, LD	87%(7/8)	25%(2/8)	0%(0/8)	1/8	53
Post-treatment, HD	83%(5/6)	33%(2/6)	0%(0/6)	1/6	51

LD= Low dose of Linomide.
HD= High dose of Linomide.

Table II: The effect of Linomide on metastasis and survival in rats with IC tumors.

Treatment	Incidence of metastasis in internal organs						Median survival time in days
	Lung	L.N	Pericardium	Kidney	Rib cage	Diaphragm	
No treatment, NT	100%(10/10)	70%(7/10)	10%(1/10)	50%(5/10)	50%(5/10)	40%(4/10)	47.5
Pre-treatment, LD	100%(9/9)	66%(6/9)	0% (0/9)	22%(2/9)	11%(1/9)	11%(1/9)	49
Pre-treatment, HD	100%(8/8)	63%(5/8)	0% (0/8)	12.5%(1/8)	12.5%(1/8)	25%(2/8)	47
Post-treatment, LD	81%(9/11)	27%(3/11)	0% (0/11)	9%(1/11)[2]	9%(1/11)[2]	9%(1/11)	47
Post-treatment, HD	62%(5/8)	38%(3/8)	0% (0/8)	0%(0/8)[1]	0%(0/8)[1]	0%(0/8)[1]	46.5

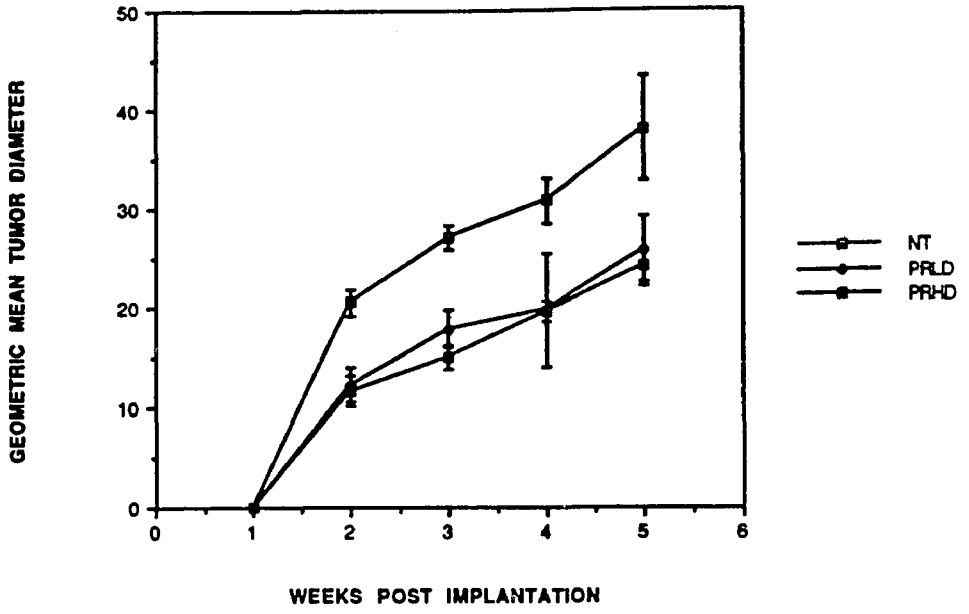
LD= low dose of Linomide.
HD= High dose of Linomide.
1= Significantly different from control, $P < 0.02$
2= Significantly different from control, $P < 0.05$

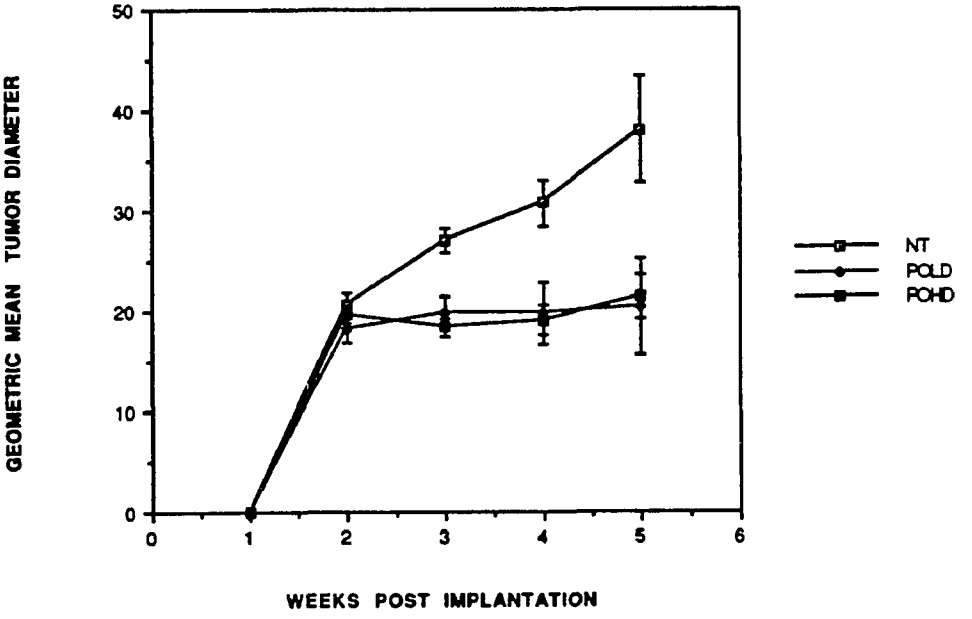
Table III: Incidence of metastasis in rats given intravenous tumor and treated with Linomide.

Treatment	Incidence of metastasis to internal organs		
	Lung	Kidney	Rib cage
No treatment, NT	100%(9/9)	0%(0/9)	11%(1/9)
Pre-treatment, LD	100%(9/9)	22%(2/9)	11%(1/9)
Pret-reatment, HD	100%(8/8)	25%(2/8)	0%(0/8)
Post-treatment, LD	100%(7/7)	0%(0/7)	0%(0/7)
Post-treatment, HD	100%(6/6)	0%(0/6)	0%(0/6)

LD= Low dose of Linomide.
HD= High dose of Linomide.

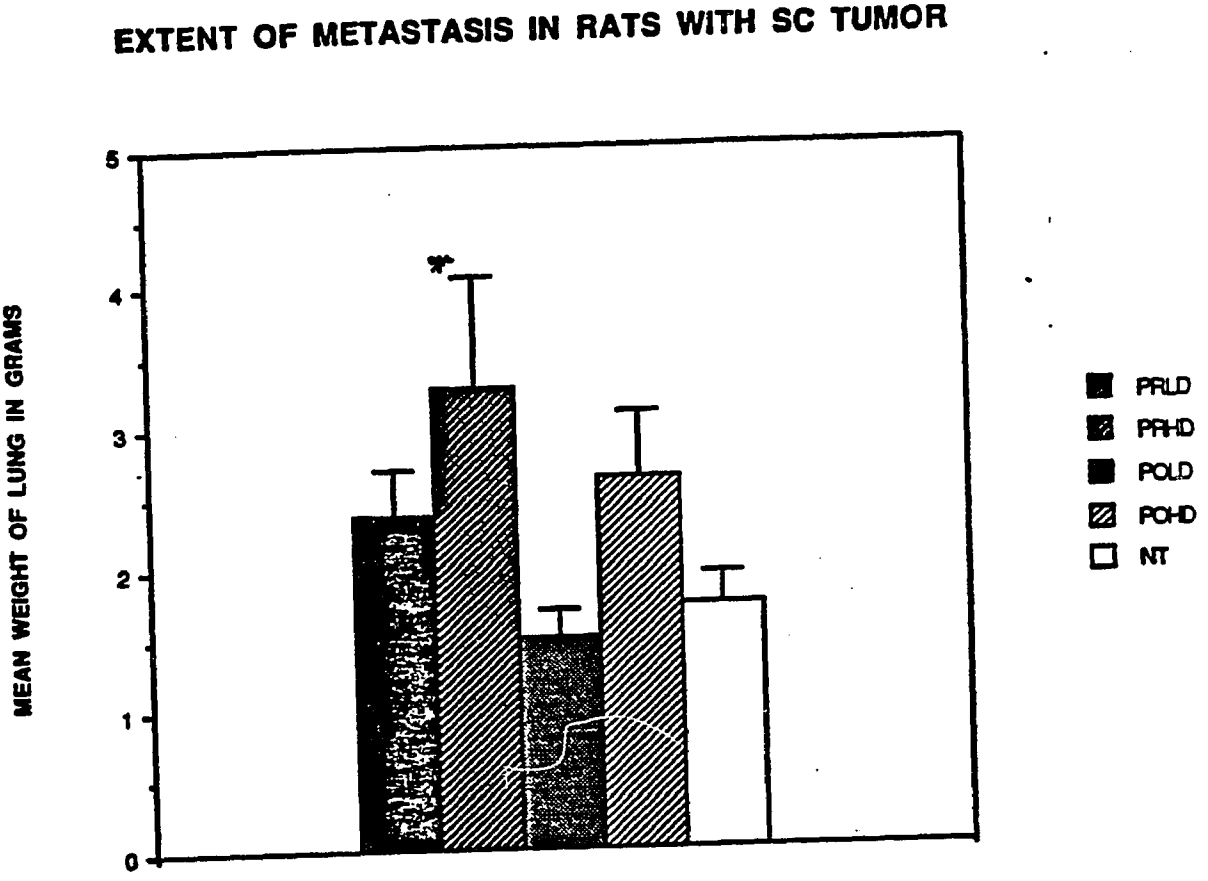
EFFECT OF LINOMIDE ON SC GROWTH OF 13762NF





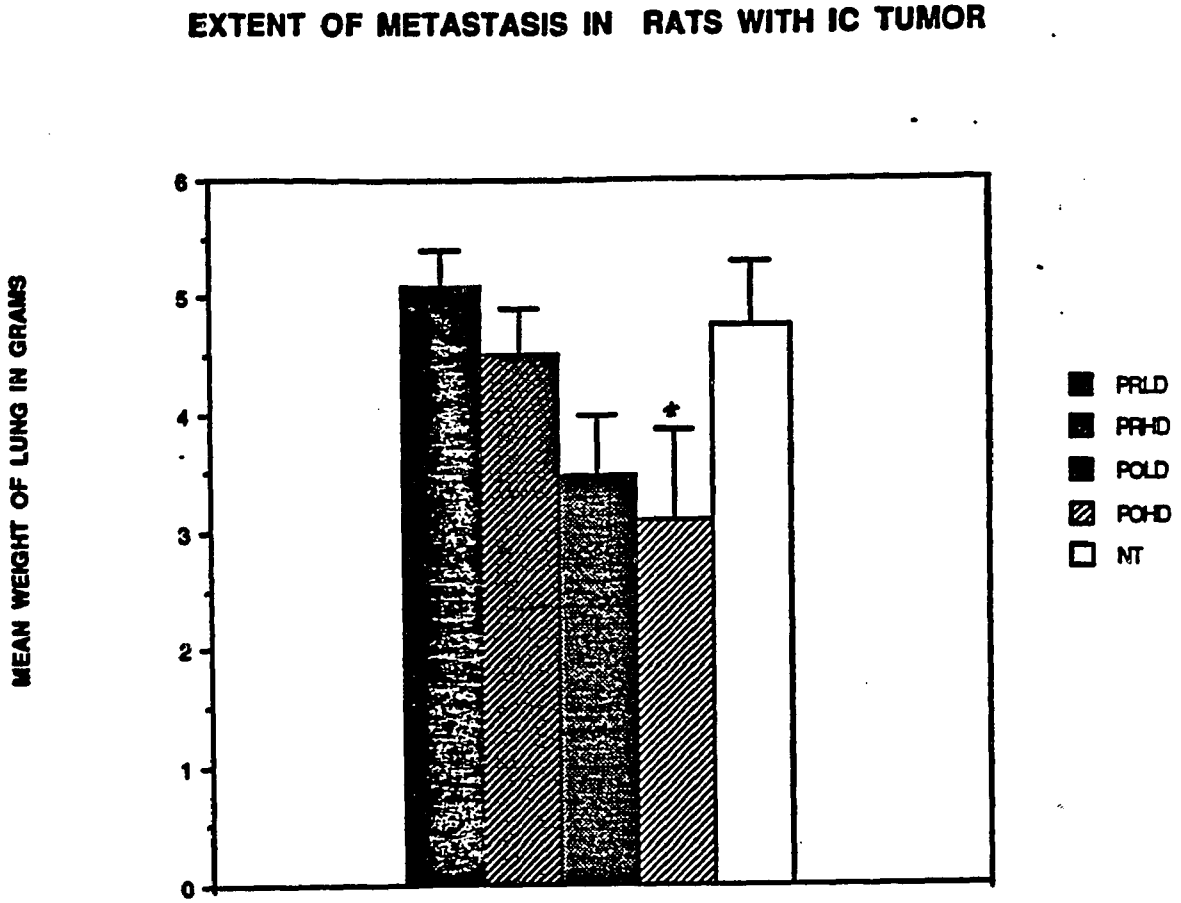
PRLD= Pre-treatment with low dose of Linomide.
PRHD= Pre-treatment with high dose of Linomide.
POLD= Post-treatment with low dose of Linomide.
POHD= Post-treatment with low dose of Linomide.
NT= No treatment.
Vertical Bar= Denotes standard error of the mean.

FIGURE 2



PRLD= Pre-treatment with low dose of LS 2616.
PRHD= Pre-treatment with high dose of LS 2616.
POLD= Post-treatment with low dose of LS 2616.
POHD= Post-treatment with low dose of LS 2616.
NT= No treatment.
Vertical Bar= Denotes standard error of the mean.
*= Significantly different from NT, $P< 0.05$

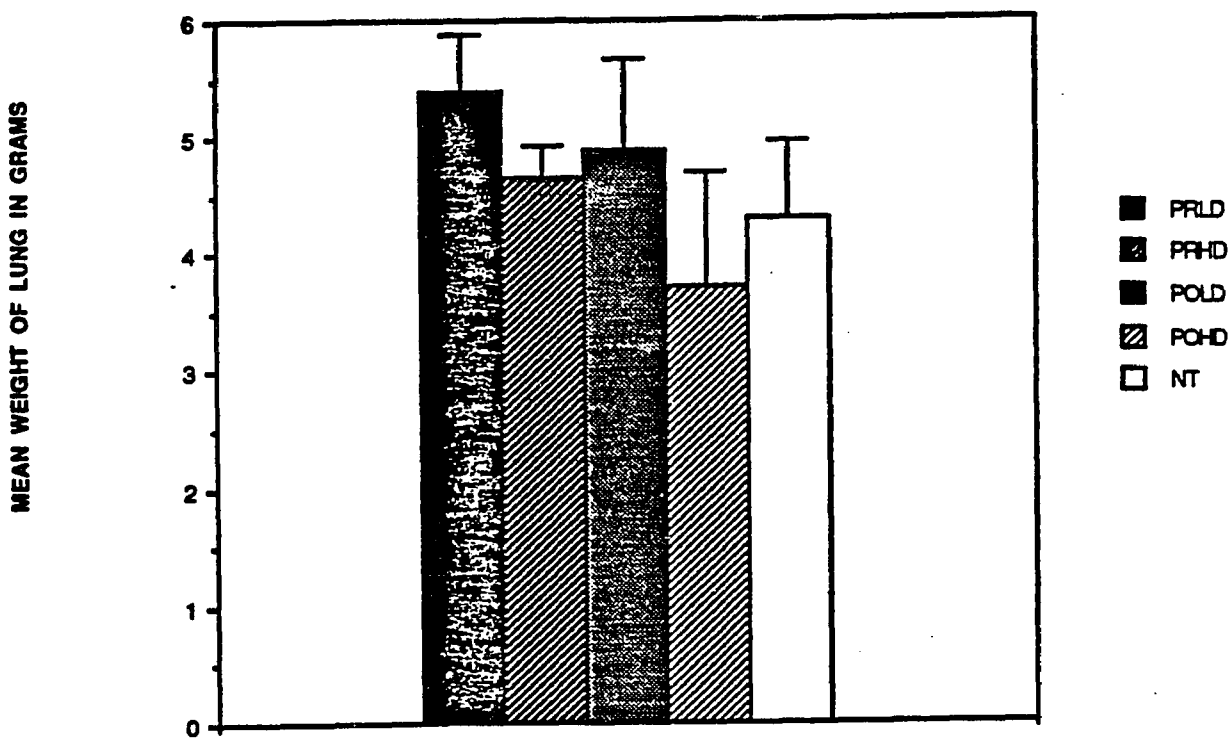
FIGURE 3



PRLD= Pre-treatment with low dose of LS 2616.
PRHD= Pre-treatment with high dose of LS 2616.
POLD= Post-treatment with low dose of LS 2616.
POHD= Post-treatment with low dose of LS 2616.
NT= No treatment.
Vertical Bar= Denotes standard error of the mean.
*= Significantly different from NT, $P< 0.05$

FIGURE 4

EXTENT OF METASTASIS IN RATS RECEIVING IV INOCULATION OF TUMOR



PRLD= Pre-treatment with low dose of LS 2616.
PRHD= Pre-treatment with high dose of LS 2616.
POLD= Post-treatment with low dose of LS 2616.
POHD= Post-treatment with low dose of LS 2616.
NT= No treatment.
Vertical Bar= Denotes standard error of the mean.
*= Significantly different from NT, $P< 0.05$

Chapter 4

The Effect of LS2616 on Different Immune Pathways Involved in Regulation of Growth and Metastasis of a Rat Mammary Adenocarcinoma

ABSTRACT

In a previous study we demonstrated that the immunomodulator Linomide (LS2616) was capable of inhibiting primary tumor growth and metastasis of the 13762NF mammary adenocarcinoma in F344 rats. The specific effects of treatment depended upon the route of tumor inoculation, drug dose, and the time of drug administration.

In the present study, we examine the effect of Linomide on natural killer (NK) activity, macrophage cytotoxicity, and lymphocyte proliferation using effector cells obtained from rats given subcutaneous (SC), intracameral (IC), or intravenous (IV) tumor. Cytotoxicity is examined at weeks 3 and 5 after tumor implantation, and lymphocyte proliferation at 5 weeks. Panels of rats received pre- or post-treatment with a low dose (LD) or high dose (HD) of drug, tumor with no drug treatment (NT), or no tumor and no drug treatment (N). Results show that SC tumor did not significantly affect macrophage or NK cytotoxicity, or lymphocyte proliferation. All drug treatments elevated macrophage cytotoxicity and lymphocyte proliferation, but pre-treatment with HD drug depressed NK activity compared with N or NT groups. Ocular tumor did not significantly affect macrophage cytotoxicity or lymphocyte proliferation, but by week 5 caused a depression of NK activity compared with N. Post-treatment with drug partially restored this depression. Pre-treatment with HD drug depressed NK activity compared with N. Some drug regimens resulted in elevated macrophage cytotoxicity compared with N or NT groups and some resulted in increased lymphocyte proliferation when compared with NT. In rats given IV tumor, macrophage cytotoxicity was depressed. However, at week 3 all drug regimens resulted in

elevated macrophage cytotoxicity, and in rats post-treated with drug this elevation was still present at week 5. Pre-treatment with drug tended to depress NK activity. Lymphocyte proliferation was increased in all animals receiving drug. In normal rats Linomide caused a prolonged enhancement of macrophage cytotoxicity and lymphocyte proliferation compared with untreated rats, but NK activity was decreased by prolonged treatment with HD drug. Collectively, our work demonstrates that the effect of tumor on host effector cell activity depends upon the route of tumor administration. Linomide can cause enhanced macrophage cytotoxicity and lymphocyte proliferation in normal and tumor bearing rats, and restore depressed NK activity in tumor bearing rats. Specific effects depend upon the route of tumor administration, drug dose, and time of drug administration. The relevance of the present findings to tumor growth and metastasis is discussed.

INTRODUCTION

In recent years, considerable effort has been expended in an attempt to understand immune pathways that may affect tumor growth and metastasis. Immunomodulators have enhanced our knowledge concerning the antitumor potential of the immune system. Linomide (LS2616) a quinoline 3 carboximide, is an immunomodulator with little or no toxicity that is currently being used in clinical trials with melanoma patients in Sweden. Using in vitro passaged B16F10 melanoma cells, Linomide was shown to affect tumor growth and metastasis in syngeneic mice by NK cell dependent as well as independent mechanisms (55). Our laboratory has shown the effectiveness of this drug in decreasing metastasis of both subcutaneous (SC) and intracameral (IC) inoculations of the more aggressive in vivo derived

B16F10 melanoma and demonstrated the importance of NK cell modulation in this system (43-45). Recently it has been shown that Linomide significantly accelerates the maturation of NK cells from bone marrow precursors (57). Linomide has shown promising properties as a therapeutic agent by enhancing NK cell activity in an IFN-independent manner and by increasing lymphocyte proliferative responses (55,72). It has also been demonstrated in animals that LS2616 is effective in treatment of autoimmune diseases such as systemic lupus erythematosus (133,134).

We recently demonstrated that treatment with Linomide can result in a low incidence of apparent cures in F344 rats harboring 13762NF mammary adenocarcinoma. Drug treatment was capable of causing partial as well as complete tumor regression in approximately 40% of treated rats and of decreasing pulmonary as well as extrapulmonary metastasis (117). In these studies the specific therapeutic response observed in vivo depended upon time of initiation of drug treatment, drug dosage, and route of administration of tumor. In order to attempt to understand immunological mechanisms that might be affected by Linomide treatment, in vitro analysis of host effector cell activity was carried out in parallel with the in vivo studies. The result of these in vitro studies are presented here.

MATERIALS AND METHODS

Animals: 5 to 7 week old F344 rats were obtained from the Frederick Cancer Research Center, maintained according to the recommendations outlined by the National Research Counsul and housed in a special pathogen free facility. Routine serology (Charles Rivers, MA) showed that animals were pathogen free.

Maintenance: The 13762NF mammary adenocarcinoma was maintained in vivo by serial SC implantation every 2 to 3 weeks of 1-2 mm^3 fragments of tumor into 5 to 7 week old F344 female rats.

SC implants: A 1 mm fragment of tumor was inoculated SC in the region of the third mammary gland using a 16-gauge trocar. SC tumors were palpated weekly and the length and width measured with the use of a fine caliper.

IC implants: The procedure for obtaining IC implants has been described previously (131). Briefly, 5 to 7 week old rats were anesthetized with nembutal, the iris dilated using mydriacyl, an incision made in the center of the cornea, and a 0.5 to 1.0 mm fragment of tumor placed on the iris and immediately adjacent to the pupillary margin.

Tumor cell suspension: In vivo passaged tumor was minced and then trypsinized using 0.25% trypsin in DMEM at room temperature for 5 minutes. Cell suspensions were washed 3x in DMEM at 1000 rpm for 10 minutes and counted in a hemocytometer. Viability was assessed using the trypan blue exclusion technique and was routinely greater than 95%. In experimental metastasis studies, animals were inoculated in the tail vein with $5x10^4$ tumor cells.

Drug treatment: Linomide provided by Dr. Stalhanske (Pharmacia, AB Leo, Helsingborg, Sweden) was administered. In preliminary experiments rats were given 15, 60, 120, or 160 mg/kg Linomide in drinking water. Daily water consumption was inhibited in rats given 120 or 160 mg/kg. Accordingly, 15 and 60 mg/kg were used and referred to as a low dose (LD), or high dose (HD) in the present study. Drug treatment began either one week prior to tumor

implantation or inoculation (pretreatment) or 2 weeks after administration of tumor (post-treatment) and continued for the duration of the experiment.

Target Cells: The target for the NK and macrophages assays were YAC-1, a Moloney virus induced T-cell lymphoma from A/J mice and P815, a mastocytoma from DBA/2 mice, respectively.

Complete medium: Culture medium for growing YAC and P815 was RPMI 1640 supplemented with 10% FBS, 1% penicillin, 1% streptomycin, 1% L-glutamine, and 1.4% HEPES (Gibco, Grand Island, NY). Medium used for culturing spleen cells consisted of complete medium supplemented with 5% rat T-cell polyclone and $5x10^{-5}$ M 2-mercaptoethanol (Sigma).

Spleen cell suspension: Spleens were aseptically removed and cells were teased from the capsule in complete media. The spleen cell suspension was passed through a 250 u nitex filter and cells were washed 3x in complete media for 10 minutes (1000 rpm) at 4^0C.

Peritoneal macrophages: Cells were obtained from the peritoneal cavity after lavaging with 20-ml of cold RPMI-1% Heparin, washed twice in complete media at 4^oC and plated from $1x10^7$ cells/ml into round bottom costar 96 well plates for 2 hr in 5% CO2. Adherent cells were washed 3x with complete media.

Natural Killer Cell Assay: Natural Killer cell activity in the spleen was assessed against YAC-1 tumor cells, in a 4 hr ^{51}Cr release assay using a stock of $5x10^7$/ml target cells (64). All assays were run at effector: target ratios of 200:1, 100:1, 50:1, and 25:1. The data are presented as percentage of specific cytotoxicity which was determined by:

% cytotoxicity =

$$\frac{\text{Experimental release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} \times 100$$

Macrophage-Mediated Cytotoxicity Assay: Peritoneal exudate macrophages were collected from 3 rats and pooled for each experiment. The macrophage cytotoxicity assay was performed as described previously (109). Adherent cells were incubated for 18 hr with ^{51}Cr labelled P815 cells ($5x10^4$ cells/ml) and the percent cytotoxicity assessed as mentioned before. All assays were run at effector to target ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1, 3.1:1, 1.5:1. Lytic units were calculated by using 4 fit parameter in VHS program.

Lymphocyte Proliferation Assay: $1x10^6$ spleen cells were cultured in complete media plus 2ME in 96-well round bottom microtiter plates. Cultures were assayed for proliferation 7 days later. 1uCi of [H3] thymidine was added to each well and cells were harvested 24 hrs. later.

Cytotoxic T Lymphocyte assay: $2x10^7$ spleen cells were cocultured with either $5x10^6$ irradiated 13762NF, YAC, or P815 tumor cells in complete media plus 2ME and 5% rat polyclone for 1 week or 2 weeks.After a week of culturing, lymphocytes were washed and plated at $1x10^6$/well in complete media at effector to target ratio of 100:1, 50:1, 25:1, and 12.5:1. targets were ^{51}Cr labelled 13762NF, YAC or P815 tumor cells. A standard ^{51}Cr cytotoxicity assay as described previously was performed (64).

Statistical analysis: Analysis of statistical differences between NK cell cytotoxicity, or of lymphocyte proliferation was performed using Duncan's Multiple Range Test.

RESULTS

The effect of Linomide on NK and macrophage activity and on lymphocyte proliferation in rats implanted with SC tumor:

In order to assess in vitro NK and macrophage activity and lymphocyte proliferation and to ascertain the effects of sustained drug treatment on host immune/defense functions, in vitro NK and macrophage immune assays were performed 3 or 5 weeks after tumor implantation on normal rats (N), untreated tumor bearing rats (NT), and on tumor bearing rats pre- or post-treated with LD or HD drug. Panels of 3 randomly selected rats/group were used for all assays. Lymphocyte proliferation was assessed 5 weeks after tumor implantation.

Analysis of peritoneal exudate macrophage activity at 3 and 5 weeks showed that there was no difference in cytotoxic activity in normal rats compared with tumor bearing rats receiving no drug treatment (Table 1). In all treatment groups lytic activity of macrophages was enhanced in comparison to normal animals (N) or to tumor bearing rats receiving no treatment (NT). At 3 weeks a pronounced elevation of macrophage activity was observed in all treatment groups except for rats pre-treated with HD Linomide; however, at 5 weeks all treatments produced an equivalent stimulatory effect.

Analysis of spleen NK activity at 3 and 5 weeks revealed no differences between normal rats and tumor bearing rats receiving no treatment (Table 2).

Linomide had little or no effect on NK cytotoxicity. At both time intervals, the NK activity of all groups was similar except for a decrease in cytotoxicity in rats pre-treated with HD drug (Table 2).

The effect of Linomide on proliferation of lymphocytes from the spleen was examined in rats bearing 5 weeks old tumor. Results show that lymphocyte proliferation was the same in normal rats as in the untreated tumor bearing animals but was significantly increased in all rats treated with Linomide (Figure 1).

The effect of Linomide on NK and macrophage activity and on lymphocyte proliferation in rats implanted with IC tumor:

Analysis of macrophage activity showed no difference between normal rats and the no treatment group. HD Linomide significantly elevated macrophage activity at 3 weeks. By 5 weeks all treatment protocols brought about an increase in lytic activity with the marked stimulation observed in rats receiving pre-treatment with drug (Table 1).

In vitro analysis of spleen NK cell activity at 3 weeks showed that the NK activity from normal rats was the same as the NK activity from untreated tumor bearing animals, Table 2. A significant decrease in NK activity was seen as a result of either pre-treatment or post-treatment of experimental rats with HD drug. When NK activity was assayed 5 weeks after tumor implantation, there was a significant decrease in cytotoxicity in untreated tumor-bearing rats when compared with normal animals. In treated rats, HD Linomide caused a decrease in NK cytotoxicity at week 3 when compared with N or NT. At 5 weeks rats post-treated with HD or LD Linomide showed an increased NK activity compared to the no treatment group; however, the NK

activity in these groups was not completely restored to the same level as seen in normal rats.

Analysis of lymphocyte proliferation 5 weeks after tumor implantation showed decreased 3H thymidine incorporation in the no treatment group compared with normal rats, but this was not statistically significant. pre-treatment with LD and post-treatment with HD elevated lymphocyte proliferation above values observed in the no treatment group (Figure 2).

The effect of Linomide on NK and macrophage cytotoxicity and lymphocyte proliferation in rats inoculated IV with a tumor cell suspension.

Analysis of PEC macrophage activity 3 and 5 weeks after tumor implantation showed that cytotoxicity was decreased in untreated tumor bearing rats compared with normal rats. At 3 weeks all Linomide regimens increased macrophage activity to levels above those observed in normal and NT rats, at 5 weeks only rats receiving post-treatment showed a significant elevation of cytotoxicity compared with normal rats and the NT group (Table 1).

In vitro assays of NK activity at 3 and 5 weeks showed no difference between normal and untreated rats. At 3 weeks, pre-treatment with HD Linomide decreased cytotoxic activity, and cytotoxicity observed at 5 week was decreased by pre-treatment with LD drug but this was not statistically significant (Table 2).

Analysis of lymphocyte proliferation showed that proliferation in untreated tumor bearing rats was less than that observed in normal rats, but this was not statistically significant. Treatment of tumor bearing rats with Linomide increased proliferation of lymphocytes when compared with

untreated tumor bearing rats, and the stimulation was independent of dosage and the time of administration of the drug (Figure 3).

The effect of Linomide on CTL activity in rats with SC, IC tumors or inoculated IV with a tumor cell suspension.

Spleen cells from rats inoculated SC, IC or IV were assayed for cytotoxic activity after 7 days in culture with irradiated 13762NF, YAC-1 or P815 tumor cells. No lysis of any of the tumors was observed in 4-hr ^{51}Cr release assays. Similar observations were made utilizing an 18-h ^{51}Cr release assay. In an another experiment CTLs were cultured with the above mentioned irradiated tumors for 2 weeks and assayed for 4-h as well as 18-h ^{51}Cr release assay and no lysis of any of the tumors were observed. The above mentioned experiments showed a lack of generation of CTL activity within 1-2 weeks post stimulation in culture.

The effect of Linomide on NK and macrophage activity, and on lymphocyte proliferation in normal rats receiving prolonged treatment with Linomide.

In order to determine the effect of long-term treatment with Linomide on cytotoxicity and proliferation, panels of rats received either LD drug, HD drug, or no drug and were sacrificed 1, 3, 4, or 6 weeks after initiation of drug treatment. Examination of macrophage activity showed that Linomide was capable of sustaining enhanced macrophage cytotoxicity for at least 6 weeks (Table 3).

Examination of NK activity showed that for the first 3 weeks Linomide treatment had little or no effect on NK activity of normal (non-tumor bearing) rats (Table 3). However, by week 4 HD Linomide caused a non-

significant decrease in NK activity, and the decrease observed at week 6 was statistically significant.

Examination of lymphocyte proliferation at 4 weeks showed that both LD and HD stimulated lymphocyte proliferation significantly above that seen in normal rats (Figure 4).

DISCUSSION

Linomide has shown promising potential as a therapeutic agent in experimental tumor models. It's antitumor efficacy has been attributed to its immunomodulating properties. Previous studies with B16F10 melanoma have clearly demonstrated that Linomide enhances NK activity in an IFN-independent manner (55) and affects progenitor NK cells (57). It has also been shown that this drug is capable of enhancing the delayed type hypersensitivity response and of increasing IL2 production and T-cell proliferation in response to antigen.(72, 127).

In a separate study (117), we examined the effect of Linomide on tumor growth and metastasis and demonstrated that pre-treatment with LD Linomide inhibited SC tumor growth but had little or no effect on metastasis while pre-treatment with HD inhibited SC tumor growth and appeared to stimulate metastasis. In contrast, post-treatment with LD or HD Linomide resulted in partial or complete regression of primary tumor and a low percent of apparent cures. In order to investigate the effect of Linomide on the immune system of F344 rats bearing 13762NF SC tumors, in vitro assays were run in parallel with the above in vivo studies. In rats with SC tumors, NK activity of all groups was similar at both 3 and 5 weeks except for a decrease in cytotoxicity in rats pre-treated with HD Linomide. It is interesting to point

out that in our in vivo study rats pre-treated with HD Linomide showed a significant increase in the extent of pulmonary metastasis compared with all the other groups. This may suggest that suppression of NK activity may play a role in controlling metastasis in rats with SC tumors. Analysis of macrophage activity demonstrated an enhancement in cytotoxicity at 3 and 5 weeks. Lymphocyte proliferation was significantly increased in all treated animals compared with the no treatment group or normal rats.

In vivo experiments run in parallel with the experiments presented here, demonstrated that administration of Linomide to rats with well established ocular tumors resulted in a decrease in metastasis (117). Post-treatment was most effective and resulted in a significant decrease in the extent of pulmonary metastasis and in the incidence of extrapulmonary metastasis. While primary tumors in all treatment groups had been observed to be approximately half the size of tumors in the no treatment group, weekly measurements were not obtained.

In our present study of rats with ocular tumors no clear cut correlation existed between physiological response and cytotoxicity or lymphocyte proliferation when rats were treated with Linomide. Post-treatment with Linomide was significantly more effective than pre-treatment in partially restoring the tumor induced depression of NK activity. In conjunction with SC experiments, this points to a role for NK cells in regulating metastasis of 13762NF tumor. Studies by Welch et al have demonstrated that 13762NF contains a variety of clones that are not susceptible to NK killing (1). One may speculate that lymphokine secretion by NK cells may contribute to antitumor activity.

Our results demonstrate a differential effect of Linomide on primary tumor growth in IC vs SC experiments in that complete regression of ocular tumors was not observed. One can speculate that the lack of complete regression of ocular tumors might be associated with a phenomenon referred to as anterior chamber associated immune deviation (ACAID). ACAID is evoked by foreign antigens or growth of tumor in the anterior chamber of the eye and is characterized by a suppression of DTH and the appearance of primed but inactive CTL precursor cells (94, 96, 98, 99). It has been shown that DTH often plays an important role in combating primary tumor growth in several tumors including the 13762A mammary adenocarcinoma (70). Others have shown that treatment with Linomide enhances the DTH response in rats with suppressed cell-mediated immunity (127). One may only speculate that the DTH activity of rats bearing ocular tumor is increased by Linomide, and is greater in rats with SC tumors, than in animals bearing ocular tumor.

In our current study, IV inoculation of tumor caused a depression of macrophage cytotoxicity. In vivo examination of the effect of Linomide on metastasis and survival in rats inoculated with tumor IV had demonstrated that treatment with Linomide had little or no effect (117). In our current study post-treatment with Linomide increased macrophage activity substantially 3 weeks and 5 weeks after tumor implantation. In addition, lymphocyte proliferation was also enhanced by Linomide treatment. It would appear that macrophage cytotoxicity and lymphocyte proliferation are not effective in inhibiting experimental metastases of the 13762 tumor. While Linomide treatment had a beneficial effect on rats bearing SC or IC tumors, It had little or no beneficial effect in rats inoculated IV with tumor cells. We

have demonstrated elsewhere (chapter 2) that sublethal irradiation of hosts had no effect on growth of SC tumors but increased metastasis, suggesting the T cell involvement in the latter process. In contrast, macrophage activity may play an important role in controlling primary tumor growth. Based on the above observations, identification and characterization of specific lymphocyte subsets in in rats that received IV inoculation of tumor and treatment with Linomide would be important.

In summary, the 13762NF mammary adenocarcinoma has differential effects on the immune system depending upon route of tumor administration. SC tumor has little or no effect on cytotoxicity or lymphocyte proliferation. Ocular tumor depresses NK activity and IV tumor depresses macrophage cytotoxicity. In examining the effect of Linomide on the immune system of normal rats, Linomide was capable of enhancing macrophage cytotoxicity and lymphocyte proliferation and decreasing NK activity at high doses after prolonged treatment. In tumor bearing animals it was observed that Linomide is capable of effectively enhancing macrophage cytotoxicity and lymphocyte proliferation to levels above those seen in normal and untreated rats and of restoring depressed NK cytotoxicity. The time of administration of Linomide, drug dosage, and route of tumor administration play an important role in determining the efficacy of drug treatment in regulating host effector cell activity.

TABLE I: Macrophage cytotoxic activity in rats with SC or IC tumors, or inoculated IV with $5x10^4$ tumor cells.

TREATMENT	LYTIC UNITS	
SC tumor	WEEK 3	WEEK 5
N	6.7	4.4
NT	8.3	3.4
PRLD	58.0	13.3
PRHD	13.9	16.9
POLD	40.2	12.6
POHD	31.3	11.4
IC tumor		
N	1.2	12.4
NT	4.2	14.9
PRLD	6.4	77.7
PRHD	21.0	100.0
POLD	5.0	28.5
POHD	14.6	20.4
IV tumor		
N	6.7	14.8
NT	3.1	8.1
PRLD	11.2	5.5
PRHD	29.3	3.4
POLD	36.2	66.2
POHD	76.1	60.1

N= Normal rats.
NT= No treatment (tumor bearing rats).
PRLD= Pretreatment with low dose of linomide.
PRHD= Pretreatment with high dose of linomide.
POLD= Post-treatment with low dose of linomide
POHD= Post-treatment with high dose of linomide.

TABLE II: NK activity in rats with SC or IC tumors, or inoculated IV with $5x10^4$ tumor cells.

TREATMENT	WEEK3	WEEK5
SC tumor	100:1	100:1
N	21 +/- 1.1	28 +/- 2.3
NT	18 +/- 5.1	21 +/- 15.5
PRLD	19 +/- 5.4	28 +/- 13.0
PRHD	14 +/- 3.3[1]	13 +/- 2.6
POLD	17 +/- 10.0	25 +/- 9.2
POHD	30 +/- 7.7	24 +/- 1.4
IC tumor		
N	34 +/- 3.8	28 +/- 5.1
NT	31 +/- 2.9	10 +/- 3.4[1]
PRLD	35 +/- 7.5	18 +/- 6.1[1]
PRHD	19 +/- 1.5[3]	11 +/- 4.9[1]
POLD	35 +/- 3.8	16 +/- 1.6[1,2]
POHD	21 +/- 2.9[3]	15 +/- 0.2[1,2]
IV tumor		
N	28 +/- 7.7	40 +/- 3.0
NT	22 +/- 2.9	36 +/- 15.0
PRLD	24 +/- 2.9	24 +/- 5.7
PRHD	16 +/- 1.4[4]	26 +/- 11.0
POLD	26 +/- 1.1	38 +/- 18.0
POHD	30 +/- 6.1	32 +/- 12.0

N= Normal rats.
NT= No treatment (tumor bearing rats).
PRLD= pretreatment with low dose of linomide.
PRHD= Pretreatment with high dose of linomide.
POLD= post-treatment with low dose of linomide.
POHD= post-treatment with high dose of linomide.
+/- = Standard Deviation of samples.
1= Significantly lower than N. $P \leq 0.05$
2= Significantly higher than NT. $P < 0.05$
3= Significantly lower than N, NT, PRLD, POLD. $P < 0.05$
4= Significantly lower than all the other groups.

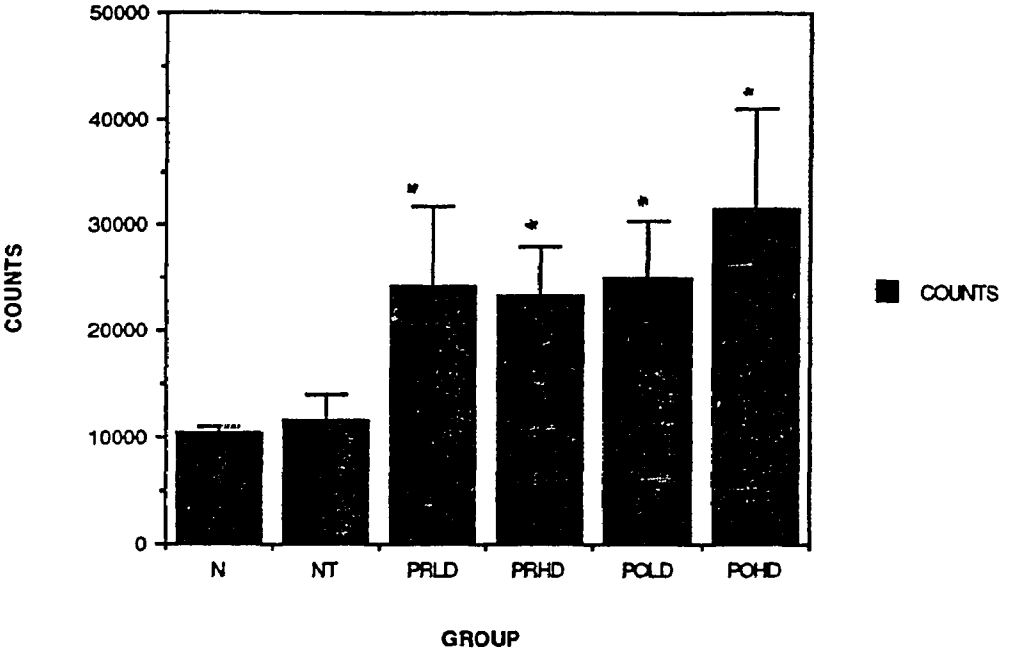
TABLE III. NK AND MACROPHAGE ACTIVITY IN NORMAL RATS AND IN RATS TREATED WITH LS 2616 FOR 1, 3, 4, AND 6 WEEKS.

TREATMENT	%CYTOTOXICITY YAC-1 100:1	LYTIC UNIT P815
ONE WEEK		
N	12 +/- 3.2	2.8
LD	13 +/- 8.3	6.0
HD	17 +/- 2.7	5.9
THREE WEEKS		
N	16 +/- 3.1	2.56
LD	13 +/- 0.8	9.94
HD	12 +/- 2.6	9.77
FOUR WEEKS		
N	35 +/- 5.0	14.2
LD	33 +/- 4.3	61.9
HD	23 +/- 12.0	ND
SIX WEEKS		
N	32 +/- 4.4	6.8
LD	26 +/- 2.5	29.3
HD	19 +/- 3.2*	34.1

+/-= Standard deviation.
N= Normal
LD= Low dose of Linomide.
HD= High dose of Linomide.
*= Denotes significant differece from N and LD, $P \leq 0.05$.

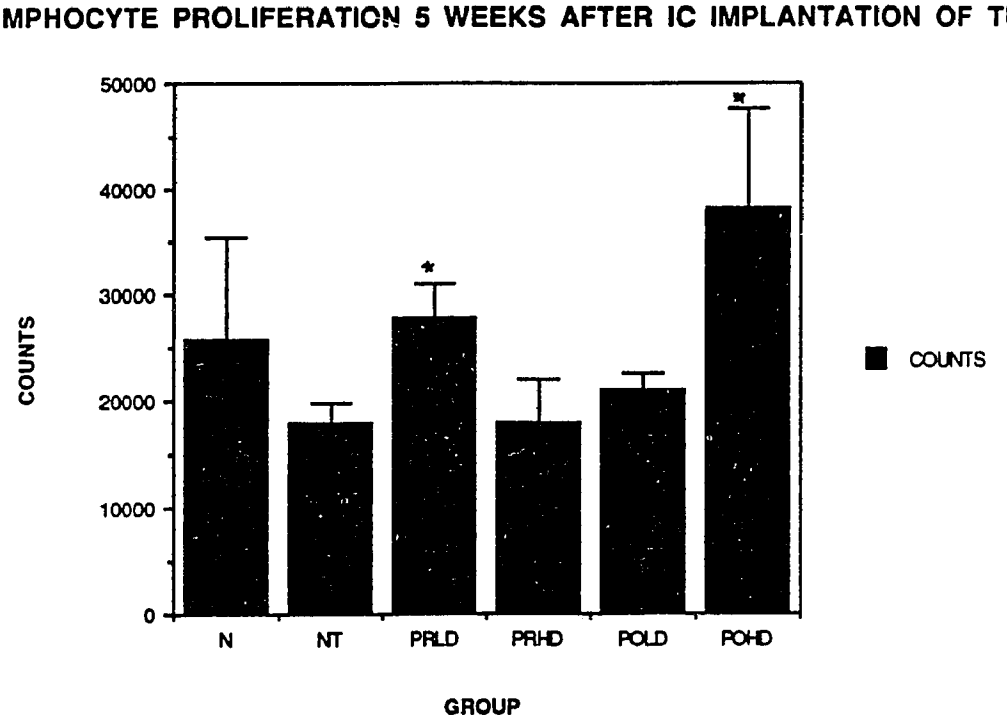
Figure 1

LYMPHOCYTE PROLIFERATION 5 WEEKS AFTER SC IMPLANTATION OF TUMOR



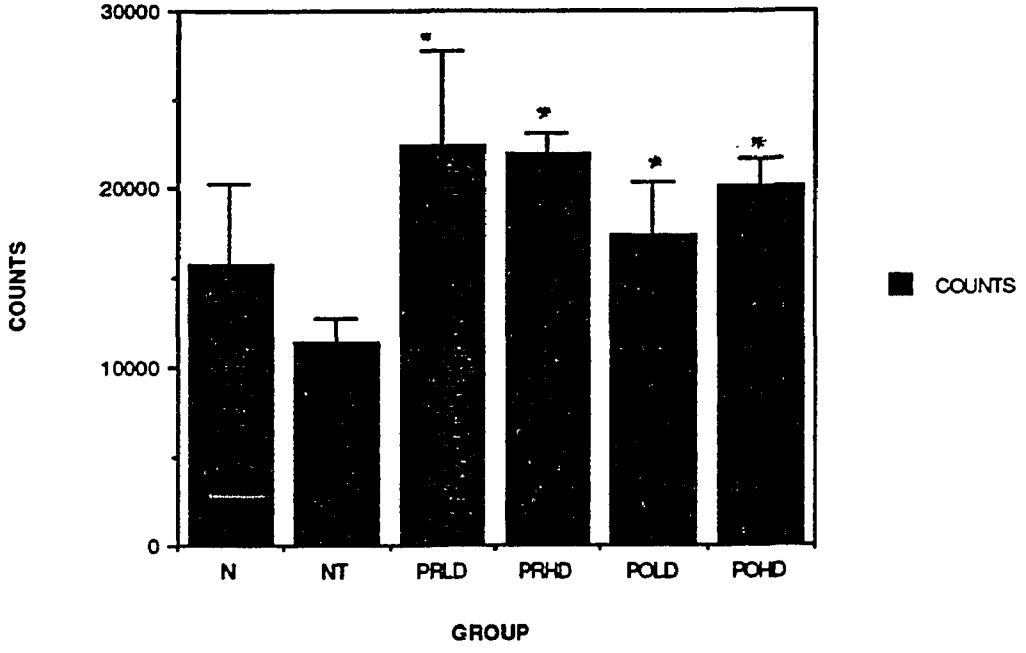
N= Normal rats.
NT= No treatment
PRLD= Pretreatment with low dose of Linomide.
PRHD= Pretreated with high dose of Linomide.
POLD= Post-treatment with low dose of Linomide.
POHD= Post-treatment with high dose of Linomide.
Vertical Bar= Denotes standard deviation.
*= Significantly different than NT, $P \leq 0.05$.

Figure 2



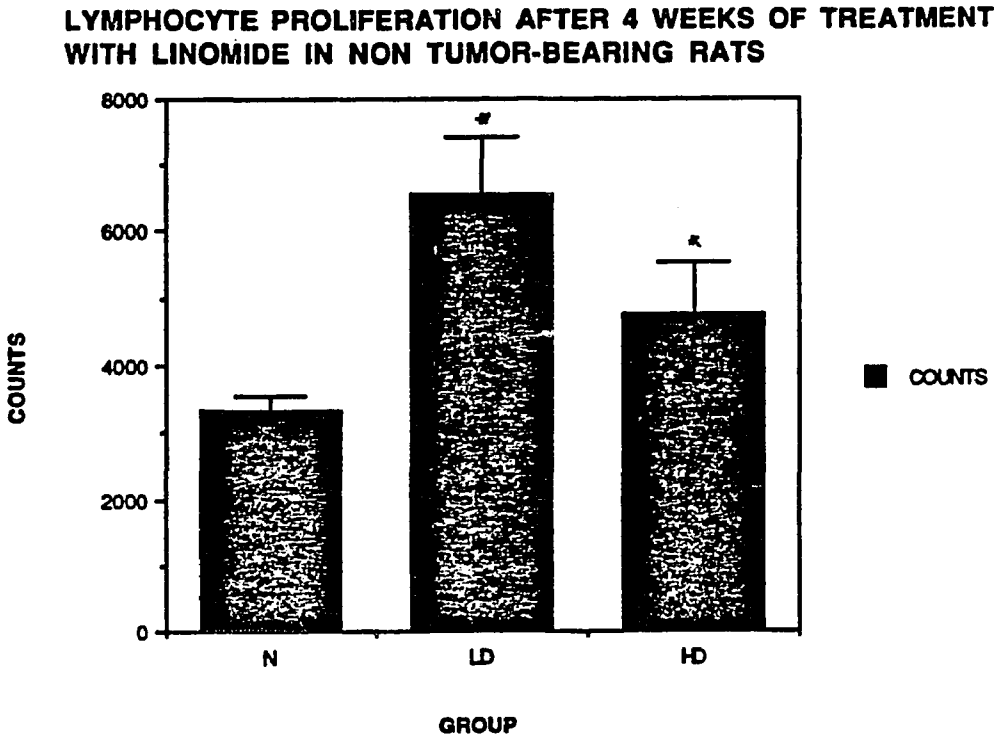
N= Normal rats.
NT= No treatment
PRLD= Pretreatment with low dose of Linomide.
PRHD= Pretreated with high dose of Linomide.
POLD= Post-treatment with low dose of Linomide.
POHD= Post-treatment with high dose of Linomide.
Vertical Bar= Denotes standard deviation.
*= Significantly different than NT, $P \leq 0.05$.

LYMPHOCYTE PROLIFERATION 5 WEEKS AFTER IV INOCULATION OF TUMOR



N= Normal rats.
NT= No treatment
PRLD= Pretreatment with low dose of Linomide.
PRHD= Pretreated with high dose of Linomide.
POLD= Post-treatment with low dose of Linomide.
POHD= Post-treatment with high dose of Linomide.
Vertical Bar= Denotes standard deviation.
*= Significantly different than NT, $P \leq 0.05$.

Figure 4



N= Normal rats
LD= Low dose of Linomide.
HD= High dose of Linomide.
Vertical Bar= Denotes standard deviation.
*= *Significantly different than N, P< 0.05.*

Bibliography

1. Aeed, P. A., Welch, D. R.: Sensitivity of locally recurrent rat mammary tumor cell lines to syngeneic polymorphonuclear cell, macrophage, natural killer cell cytolysis. British J. Cancer, 58(6):746-52, 1988.

2. Anderson, D. E.: A genetic study of human breast caner. JNCI 48:1029, 1972.

3. Ansfield, F. J., Ramirez, G., Mackman, S., et al: A ten-year study of 5 fluorouracil in disseminated breast cancer with clinical results and survival times. Cancer Res. 29:1062-1066, 1969.

4. Arnaud, J. P., Buyse, M., Nordlinger, B., et al: Adjuvant therapy of poor prognosis colon cancer with levamisole: results of an EORTC double-blind randomized clinical trial. Br. J. Surg. 76:284-9, 1989.

5. Aslakson, C. J., Starkey, J. R. The nature of tumor presentation in the animal changes the effect of Levamisole treatment on metastasis. Immunol. Invest. 16 50:399-411.

6. Bonadonna, G. et al: Primary chemotherapy to avoid mastectomy in tumors with diameters of the three centimeters or more. JNCI. 82(19): 1990.

7. Bogden, A. E., Esber, H. J., Taylor, D. J., Gray, J. H.: Comparative study on the effect of surgery, chemotherapy, and immunotherapy, alone and in combination, on metastases of the 13762 mammary adenocarcinoma. Cancer Res. 34:1627-1631, 1974.

8. Bogden, A. E., Taylor, D. J.: Predictive mammary tumor test systems for experimental chemotherapy. In: Heuson J. C., Mattheien W., Rozensweig, M., eds. Breast Cancer: Trends in research and treatment. New York: Raven Press, 95-110.

9. Bogden A.E., Taylor, D. J., Kuo, E. Y., et al: The effect of perphenazine-induced serum prolactin response on estrogen-primed mammary tumor-host systems; 13762 and R-35 mammary adenocarcinomas. Cancer Res. 34:3018-3025, 1974.

10. Boyer, C. B., Kreider, G. W., Barlett, G. L. Systemic adoptive transfer of immunity to 13762A rat mammary adenocarcinoma. Cancer Res., 41:2394-2400, 1981.

11. Braunschweiger, P. G., Schiffer, L. M.: Growth kinetics of mammary tumor 13762 in rats previously cured by chemotherapy. JNCI 64:671-674, 1980.

12. Brem, S., Brem, H., Folkman, J., Finkelstein, D., Patz, A.: Prolonged tumor dormancy by prevention of neovascularization in the vitreous.

13. Bucaloss P. Veronesi, D., Zingo, L., et al: Enlarged mastectomy for breast cancer; review of 1213 cases. Am. J. Roentgen Rad Ther. Nucl. Med. 111-119, 1971.

14. Bullock, J. D., Yanes B.: Ophthalmic manifestations of metastatic breast cancer. Ophthalmology 87:961-973, 1980.

15. Callahan, R. M., Marx, P. A., Wheelock, E. F.: Group-specific cytolytic antibody directed against the major glycoprotein (gp 70) of murine leukemia viruses in serum of mice with dormant F/V Infections of Virol 97:55-67, 1979.

16. Carr, I., Carr, J., Dreher B.: Lymphatic metastatis of mammary adenocarcinoma. An experimental study in the rat with a brief review of the literature. Invasion Metastasis 1:34-53, 1981.

17. Chlebowski, R. J., Zrwin, L. E., Pugh, R. P., et al: Survival of patients with metastatic breast cancer treated with either combination or sequential chemotherapy. Cancer Res. 39:4503-4506, 1979.

18. Christensen, N. D., Kreider, J. W., Bartlett, J. L.: Distinct T-cell proliferative responses to 13762A rat mammary adenocarcinoma and derived clones. Cell Immunol. 97:433, 1986.

19. Christensen, N. D., Kreider, J. W., Horetsky, R. L.: Suppressor lymphokine produced by rat T-cells in response to syngeneic mammary adenocarcinoma 13762A. Cancer Res. 48:943 1988.

20. Dahl-Iversen, E.: Recherches sur les metastases microscopiques des cancers du sein dans les ganglions lymphatiques parasternaux et susclavicularies. Mem. Acad. de chin 78:651, 1952.

21. Dao, T. L. Suderland, J.: Mammary carcinogenesis by 3-methylcholantheune hormonal aspects of tumor incidence and growth. J. Natl Cancer Inst. 23: 567-585, 1959.

22. Davis, H. L., Jr., Ramirez, G., Ellerby, R. A.: Five drug therapy in advance breast cancer. Factors influencing toxicity and response.

23. Decker, D. A., Ahmann, D. L., Bisel, H. L., et al: Complete responders to chemotherapy in metastatic breast cancer, characterization and analysis. JAMA 242:2075-2079, 1979.

24. Eccles, S. A., Alexander, P.: Immunologically-mediated restraint of latent tumor metastases. Nature 257:52, 1975.

25. Eccles, S. A., Alexander, P.: Macrophage content of tumors in relation to metastatic spread and host immune reaction. Nature 250:667, 1974.

26. Eccles, S. A., Heckford, S. E., Alexander, P.: Effect of cyclosporin A on the growth and spontaneous metastasis of syngeneic animal tumors. Br. J. Cancer 42:252, 1980.

27. Eccles, S. A.: Host factors in metastasis. In tumor progression and markers, Kugler, Amsterdam, 1982.

28. Esber, H. J., Menninger, F. F., Taylor, D. J., Bodgen, A. E.: Non-specific stimulation of tumor-associated immunity by methanol-soluble fraction of Mycobacterium butyricum. Cancer Res. 32:795-803, 1972.

29. Fidler, I. J., Sone, S., Fogler, W. E., et al: Erradication of spontaneous metastases and activation of alveolar macrophages by iv injection of liposomes containing muramyl dipeptide. PNAS 78:1680, 1981.

30. Fidler, I. J., Spitler, L. E.: Effects of levamisole on in vivo and in vitro murine host response to syngeneic transplantable tumor. JNCI 55:1107, 1975.

31. Fischer, B., Rubin, H., Saffer, E., et al: The effect of Cornybacterium Parvum in combination with 5-fluorouracil, L-phenylalanine mustard, or methotrexate on the inhibition of tumor growth. Cancer Res. 36:2714, 1975.

32. Fletcher, G. H.: Local results of irradiation in the primary management of localized breast cancer. Cancer 29:545-552, 1972.

33. Folkman, J., Hochberg, M. Self-regulation of growth in 3 dimensions of Exp. Med, 138:745-753, 1973.

34. Folkman, J.: Tumor angiogenesis. A possible control point in tumor growth. Ann. Internal Med., 82:96-100, 1975.

35. Gavilando, J., Fernandez, A., Amador-Perez, J. F.: Pattern of organ colonization of metastasizing mouse L929-MM4 cells and the stimulation of lung tumor formation by cyclophosphamide. Clin. Exp. Metastasis 3:241-50, 1984.

36. Gavilando, J., Fernandez, A., Castillo, R., Lage, A.: Neoplastic progression evidenced in the L929 cell system. I. Selection of tumorigenic and metastasizing cell varients. Neoplasma 28:269-279, 1982.

37. Geradts, J., Richards, J., Edery, M., Pang, K., Larson, L., Nandi, S.: Heterogeneity in the hormonal responsiveness of clones derived from the 13762 rat mammary tumor. Cancer Res. 46:1920-1927, 1986.

38. Gimbrone, M. A., Jr., Leapman, S. B., Folkman, J.: Tumor dormancy in vivo by prevention of neovascularization: An experimental model using the rabbit cornea. J. Natl. Cancer Inst. 52:413-427, 1974.

39. Haagensen, C. D.: Diseases of the breast, 2nd ed., p. 426, Philadelphia, W. B. Saunders, 1971.

40. Haagensen, C. D.: Treatment of curable carcinoma of the breast. Int. J. Rad. Oncol. Biol. Phys. 2:975-980, 1977.

41. Hadfeld, G.: The dormant cancer cel. Br. Med. J. 2:607, 1954.

42. Handley, R. S.: Carcinoma of the breast. Ann R. Coll Surg. 57:59-66, 1975.

43. Harning R., Szalay, J.: A treatment for metastasis of murine ocular melanoma. Invest Ophthalmol. Vis. Sci. 29:1505, 1988.

44. Harning R., Koo, G. C., Szalay, J.: Immune regulation of metastasis from in vivo derived syngeneic murine melanoma. Regional Immunol. in press.

45. Harning R., Koo, G. C., Szalay, J.: Regulation of the metastasis of murine ocular melanoma by natural killer cells. Inves. Ophthalmol. Vis. Sci. 30:1909, 1989.

46. Harning, R., Szalay, J.: Ocular metastasis of in vivo and in vitro derived syngeneic murine melanoma. Invest. Ophthalmol. Vis. Sci. In press.

47. Hattori, T., Hamai, Y., Ikeda T., et al: Inhibitory effects of immunopotentiators on the enhancement of lung metastases induced by operative stress in rats. Gann. 73:132-35, 1982.

48. Hoogstraten, B., Macdivitt, R. W.: Breast Cancer, CRC press, 1981.

49. Hoogstraten, B., Fabian, C.: A reappraisal of single drugs in advanced breast cancer. Cancer Clin. Trials 2:101-109, 1979.

50. Horm, J. W., Asinl, A. J., Young, J. L.: Seer Program: Cancer incidence and mortality in the United States. 1973-81, Bethesda, MD. National Cancer Institute, 1984.

51. Hortobagy, G. N., Smith, T. L., Leha, S. S., et al: Multivariate analysis of prognostic factors in metastatic breast cancer treated with combination chemotherapy. Cancer 46:438-445, 1980.

52. Hortobogy, G. N., Gutterman, J. V., Blumenshein, G. R., et al: Combined chemoimmunotherapy for advanced breast cancer. A comparison of BCG and levamisole. Cancer 43:1112-1122, 1979.

53. House, A. K., Maley, M. A. L.: Clinical and in vivo response following surgery or surgery plus adjuvant chemotherapy or immunotherapy for colorectal carcinoma in a rat model. J. Royal Soc. Med. 76:833-40, 1983.

54. Howell, L. E., Kreider, G. W., Bartlett G.: Role of T-cell subsets in the destruction of established metastases of 13762A rat mammary adenocarcinoma. JNCI, 77:187-192, 1986.

55. Kalland T.: Effects of the immunomodulator LS2616 on growth and metastasis of murine B16F10 melanoma. Cancer Res. 46:3018, 1986.

56. Kalland, T., Alm, G., Stalhandske, T.: Augmentation of natural killer activity by LS2616 a new immunomodulator. J. Immunol. 134:3956, 1985.

57. Kalland, T.: Regulation of natural killer progenitors. J. Immunol. 144:4472, 1990

58. Katz, E., Boylan, E.: Stimulatory effect of high polyunsaturated fat diet on lung metastasis from the 13762 mammary adenocarcinoma in female retired breeder rats. JNCI 351-358, 1987.

59. Kiang, D. T., Kennedy, B. J.: Tamoxifen (anti estrogen) therapy in advance breast cancer. Ann Intern Med. 87:687-690, 1977.

60. Kim, Su., Furth, J.: Vitam. Horm. NY, p. 34:107-136, 1976.

61. Kim, U., Depowski, M. J., Progression from hormone dependence to autonomy in mammary tumors as an in vitro manifestation of sequential clonal selection. Cancer Res. 35:2068-2077, 1975.

62. Klein, S., Bonaparte, Y. P., D'Elia, I.: Enhancement of the incidence of metastasis in tumor-resected mice. Influence of soluble tumor extract and splenectomy. Invasion Metastasis 5:309-316, 1985.

63. Klein, S., Colombo, L., D'Elia, I., Bonaparte, Y. P.: Exaltaction de metastasis por antigeno especifico en tumores de mama murino. Medicina 41:675-676, 1981.

64. Koo, G. C., Dumont, F. J., Tutt, M., Hackett, J., Kumar, V.: The NK1.1 mouse: A model to study differentiation of murine NK cells. J. Immunol. 137:3742, 1986.

65. Kreider, G. W., Bartlett, G. L., Christensen, N. D., Welsh, P.: Systemic adoptive transfer of immunity and low dose irradiation eradicate metastases of 13762A rat mammary adenocarcinoma. Int. J. Cancer, 3b:217-224, 1985.

66. Kreider, G. W., Bartlett, G. L., Purnell, D. M.: Suitability or rat mammary adenocarcinoma 13762 as a model for BCG immunotherapy. JNCI, 56:797-802, 1976.

67. Kreider, G. W., Bartlett, G. L., Purnell, D. M.: Immunotherapy of an established rat mammary adenocarcinoma (13762A) with intratumor injection of cornybacterium parvum. Cancer Res., 38:689-692, 1978.

68. Kreider, G. W., Bartlett, G. L.: Increased immunogenicity of a spontaneous varient clone of the 13762A rat mammary adenocarcinoma. JNCI, 75:141-149, 1985.

69. Kreider, J. W., Bartlett, J. L. Purnell, D. M., et al: Destruction of regional lymph node metastasis of rat mammary adenocarcinoma 13762A by treatment with Cornybacterium Parvum. Cancer Res. 38:4522, 1978.

70. Kreider, J. W., Howell, L. E., Bartlett, G. L.: Characteristics of T-cells involved in the expression of delayed hypersensitivity and tumor rejection

immunity to 13762A rat mammary adenocarcinoma. Cellular Immunology, 93:26-37, 1985.

71. Krale, J., Heuch, I.: Menstrual factors and breast cancer risk.

72. Larson, E. L., Joki, A., Stalhandske, T.: Mechanism of action of the new immunomodulator LS2616 on T-cell responses. Int. J. Immunopharmacol. in press.

73. Laurie, J. A., Moetral, C. G., Fleming, T. R., et al: Surgical adjuvant therapy of large bowel carcinoma: An evaluation of levamisole and the combination of levamisole and fluorouracil. J. Clin. Oncol. 7:1447, 1989.

74. Lefha, S. S., Buzda, A. V., Smith, T. L., et al: Complete remissions in metastatic breast cancer treated with combination drug therapy. Ann Intern Med. 91:847-852, 1979.

75. Lichtner, R. B., Julian, J. A., Glasser, S. R., Nicolson, G. L.: Characterization of cytokeratins expressed in metastatic rat mammary adenocarcinoma cells. Cancer Res. 49:104-111, 1989.

76. Lilienfeld, A. M.: The epidemiology of breast cancer. Cancer Res. 23:1503-1513, 1963.

77. Lin, T. M. Chen, K. P., MacMahon B.: Epidemiologic characteristics of cancer of the breast in Taiwan. Cancer 27:1497-1504, 1971.

78. Liu, C-M., Okayusu, P., Goldman, Y., Suzuki, K., Wheelock, E. F.: Immune regulation of the L5178Y murine tumor-dormant state. I. In vivo and in vitro effects of prostaglandin E2 and indomethacin on tumor cell growth. J. Exp. Med. 164:1259, 1986.

79. MacMahon, B., Cole, P., Lin, T. M., Lowe, C. R., et al: Age of first birth and breast cancer risk. Bull WHO 43:209-221, 1970.

80. Manni, A., Pearson, O. H., Brodkey, J.: Trans-sphenoidal hypophysectomy in breast cancer. Cancer 44:2330-2337, 1979.

81. Mazurek, C., Chalvet, H., Stiffel, C.: Mechanism of corynebacterium parvum antitumor activity. Int. J. Cancer, 20:532-534, 1977.

82. McCormick, G. M., Moon, R. C.: Effect of pregnancy and lactation on growth of mammary tumors induced by 7,12 dimethylbenzanthracene (DMBA). Br. J. Cancer 24:160-166, 1963.

83. Mewis, L., Young, S. E.: Breast carcinoma metastatic to the choroid. Ophthalmology 89:147-151, 1982.

84. Mirra, A. P., Cole, P., MacMahon B.: Breast cancer in the area of high parity; Sao Paulo, Brazil. Cancer Res. 31:77-83, 1971.

85. Morgan, L. R., Jr., Schein, P. S., Woolley, P. V., et al: Therapeutic use of tamoxifen in advanced breast cancer. Cancer Treat. Rep. 60:1437-1443, 1976.

86. Nakagima, M., Welch, D. R., Belloni, P. N., Nicolson, G. L.: Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. Cancer Res. 47:4869-76, 1987.

87. National Cancer Institute. Annual cancer statistics review for the national advisory board. Bethesda, MD. National Cancer Institute, Dec. 2, 1985.

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

89. Neri, A., Welch, D., Kawaguchi, T., et al: Development and biologic properties of malignant cell sublines and clones of a spontaneously metastasizing rat mammary adenocarcinoma. JNCI 68:507-517, 1982.

90. Neiderkorn, J. Y., Knisely, T. L., Mayhew E.: Immune rejection of metastasis arising from intraocular tumors in mice. Invest. Ophthalmol. Vis. Sci. 27:1355-1361, 1986.

91. Niederkorn, J. Y., Meunier, P. C.: Spontaneous immune rejection of intraocular tumors in mice. Invest. Ophthalmol. Vis. Sci. 26:877-884, 1985.

92. Niederkorn, J. Y., Streilein, J. W., Kripke, M. L.: Promotion of syngeneic intraocular tumor growth in mice by anterior chamber-associated immune deviation. JNCI 71:193-199, 1983.

93. Niederkorn, J. Y., Streilein, J. W., Shadduck, J. A.: Deviant immune responses to allogeneic tumors injected intracamerally and subcutaneously in mice. Invest. Ophthalmol. Vis. Sci. 20:355-363, 1981.

94. Niederkorn, J. Y., Streilein, J. W.: Analysis of antibody production induced by allogeneic tumor cells inoculated into the anterior chamber of the eye. Transplantation 33:573-77, 1982.

95. Niederkorn, J. Y., Streilein, J. W.: Induction of anterior chamber-associated immune deviation (ACAID) by allogeneic tumors does not require splenic metastasis. J. Immunol. 28:2470-2474, 1982.

96. Niederkorn, J. Y., Streilein, W.: Alloantigen placed in anterior chamber of the eye induce specific suppression of delayed type hypersensitivity but normal cytotoxic T lymphocyte and helper T lymphocyte responses. J. Immunol. 131:2670, 1983.

97. Niederkorn, J. Y.: Enucleation in consort with immunological impairment promotes metastasis of intraocular melanomas in mice. Invest. Ophthalmol. Vis. Sci. 25:1080-1086, 1984.

98. Niederkorn, J. Y.: Suppressed cell immunity in mice harboring intraocular melanomas. Inves. Ophthalmol. & Vis. Sci. 25:447, 1984.

99. Niederkorn, J. Y.: T-cell subsets involved in the rejection of metastases arising from intraocular melanomas in mice. Inves. Ophthalmol. & Vis. Sci. 28:1397-1403, 1987.

100. Noble, R. L., Hoover, L.: Classification of transplantable tumors in the Nb rats controlled by estrogen from dormancy to autonomy. Cancer Res. 35:2935-2941, 1975.

101. Noble, R. L.: Production of Nb rat carcinoma of the dorsal prostate and response of estrogen-dependent transplants to sex hormones and tamoxifen. Cancer Res., 40:3574-3550, 1980.

102. North, S. M., Nicolson, G. L.: Effect of host immune status on the spontaneous metastasis of cloned cell lines of the 13762NF rat mammary adenocarcinoma. Br. J. Cancer 52:747-755, 1985.

103. North, S. M., Nicolson, G. L.: Heterogeneity in the sensitivities of the 13762NF mammary adenocarcinoma cell clones to cytolysis mediated by extra- and intra-tumoral macrophages. Cancer Res 45:1453-1460, 1985.

104. North, S. M., Steck, P. A., Nicolson, G. L.: Monoclonal antibodies against cell surface antigens of the metastatic rat 13762NF mammary adenocarcinoma and their cross-reactivity with human breast carcinoma. Cancer Res. 46:6393-6399, 1986.

105. Pattenburger, R. S., Kampert, J. B., Change, H. G.: Characteristic that predict risk of breast cancer before and after the menopause. Am. J. Epidemiol, 112:258, 1980.

106. Perkin, D. M., Stjernsward, J., Mair, C. S.: Estimates of the world wide frequency of twelve major cancers. Bull WHO 62:163, 1984.

107. Purnell, D. M., Bartlett, J. L., Kreider, J. W., et al: Comparative antitumor effects of Cornybacterium Parvum, Bordetella Pertussis, Bacillus Calmette-Guerin, and levamisole alone or in combination with cyclophosphamide in the CaD2 murine mammary adenocarcinoma system. Cancer Res. 39:4838, 1975.

108. Ravnihar, B., MacMahon, B., Lindtner, J.: Epidemiologic features of breast cancer in slovenia, 1965-67, Eur. J. Cancer, 7:295-306, 1971.

109. Raz, A., Fogler, W. E., Fidler, J.: The effect of experimental conditions on the expression of in vitro tumor cytotoxicity mediated by murine macrophages. Cancer Innumol. Immunother. 7:157-163, 1979.

110. Reeve, J. G., Twentyman, P. R.: Clonal variation in the arrest, survival and growth of RH-1 mouse sarcoma cells in the lungs of C3H mice. Br. J. Cancer 47:833-410, 1983

111. Richards, J. E., Edery, M., Osborn, R. C., Larson, L. N., Nandi, S.: Effect of hormones and epidermal growth factor on the growth of the hormone-responsive 13762NF rat mammary tumor in collagen gel culture. JNCI 76:669-679, 1986.

112. Robinson, M. K., Truitt, G. A., Takeshi, O., Wheelock, E. F.: Enhanced suppressor macrophage activity associated with termination of the L5178Y cell tumor-dormant state in DBA/2mice. Cancer Res. 43:5831-5836, 1983.

113. Robinson, M. K., Wheelock, E. F. Synergistic cytolytic activity by combined populations of peritoneal T lymphocytes and macrophages during the L5178Y cell tumor dormant state in DBA/2 mice. Cell. Immunol. 73:230-246, 1982.

114. Robinson, M. K., Wheelock, E. F.: Identification of macrophage-mediated cytolytic activity as a tumor suppressive mechanisms during maintenance of the L5178Y-tumor dormant state in DBA/2 mice. J. Immunol. 126:673-679, 1981.

115. Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P. Lotze, M. T., Yang, J. C., Seipp, C. A., Simpson, C.: Use of tumor infiltrating lymphocytes and IL2 in the immunotherapy of patients with metastatic melanoma. N. Engl. J. Med. 319:1676-1680, 1988.

116. Ross, C. E., Cochran, A. J., Hoyle, D. E., Grant, R. M. Mackie, R. M.: Formalinized tumor cells in the leukocyte migration inhibition test. Clin. Expl. Immunol. 22:126-30, 1975.

117. Sabzevari, H., Koo, G. C., Szalay, J.: The effect of LS2616 on growth and metastasis of a rat mammary adenocarcinoma. Manuscript in prep.

118. Saphillo, O., Parker, M. L.: Metastases of primary carcinoma of the breast with special reference to spleen, adrenal glands, and ovaries. Arch. Surg. 42:1003, 1941.

119. Schweitzer, R. J.: Oophorectomy/adrenalectomy. Cancer 46:1061-1065, 1980.

120. Segaloff, A.: Hormones in breast cancer. Recent Progr. Hormone Res. 22:351-374, 1966.

121. Shiraishi, M., Himeno, K., Nomoto, K.: Restoration by levamisole of immune responses suppressed in tumor bearing mice. Cancer Immnol. Immunother. 15:121, 1983.

122. Silverberg, E., Lubera, J.: Cancer statistics, 38:2-19, 1988.

123. Silverstein, M. J., Byron, R. L., Yonemoto, R. H., et al: Bilateral adrenalectomy for advanced breast cancer: A 21-year experience. Surgery 77:825-831, 1975.

124. Siu, H. Vitetta, E. S., May, R. D., Uhr, J. W.: Tumor dormancy I. Regression of BCL1 tumor and induction of dormant tumor state in mice chimeric at the major histocompatibility complex. J. Immunol. 137:1376-1382, 1986.

125. Smith, J. A., Gamez-Araujo, J. J., Gallagher, H. S., et al: Carcinoma of the breast: Analysis of total lymph node involvement versus level of metastasis. Cancer 39:527, 1977.

126. Solleveld, H. A., Haseman, J. K., McConnell, E. E.: Natural history of body weight gain, survival, and neoplasia in the F344 rat. JNCI 72:929-939, 1984.

127. Stalhandske T., Kalland, T.: Effects of novel immunomodulator LS2616 on the delayed-type hypersensitivity reactions to Bordetella Pertussis in the rat. Immunopharmacol. 11:87, 1985.

128. Steck, P. A., North, S. M. Nicolson, G. L.: Purification and partial characterization of a tumor metastasis associated high glycoprotein from rat 13762NF mammary adenocarcinoma cells. Biochem. J., 242:779-87, 1987.

129. Suzuki, Y., Chen, L., Liu, C-M., Morita, T., Wheelock, E. F.: Immune regulation of the L5178Y murine tumor-dormant state: Induction of cell- and soluble factor-mediated inhibition of tumor cell growth by tumor necrosis factor and interferon. Cancer Res. 49:1899-2198, 1989.

130. Suzuki, Y., Liu, C-M., Chen, L., Ben-Nathan, Wheelock, E. F.: Immune regulation of the L5178Y murine tumor-dormant state. II. Interferon-requires tumor necrosis factor to restrain tumor cell growth in peritoneal cell cultures from tumor-dormant mice. J. Immunol. 139:3146-3152, 1987.

131. Szalay, J., Sabzevari, H.: Metastasis of ocular and flank implants of the 13762NF mammary adenocarcinoma in syngeneic rats. JNCI 79:837, 1987.

132. Tagnon, H. J.: Antiestrogens in treatment of breast cancer. Cancer 39: 2959-2964, 1977.

133. Tarkowski, A., Gunnarsson, K., Nilsson, L. A., Lindholm, L., Stalhandske, T.: Successful treatment of autoimmunity in MRL/1 mice with LS2616, a new immunomodulator. Arthritis. Rheum., 29(11):1405-9, 1986.

134. Tarkowski, A., Gunnarsson, K., Stalhandske, T.: Effects of LS2616 administration upon the autoimmune disease of (NZBXNZW) F1 hybrid mice. Immunology, 59:589-594, 1986.

135. Terranova, V. P., Liotta, L. A., Russo R. G., et al: Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res. 42:2265-2269, 1982.

136. Terranova, V. P., Williams, J. E., Liotta, L. A., et al: Modulation of the metastatic activity of melanoma cells by laminin and fibronectin. Science 226:982-985, 1984.

137. Thomson, D. M. P., Eccles, S., Alexander, P.: Antibodies and soluble tumor antigens in blood and lymph of rats with chemically induced sarcomata. Br. J. Cancer 28:6-15, 1973.

138. Thomson, D. M. P., Proctor, J. W., Grosser, N., Sutherland, M.: Impairment of systemic concomitant anti-tumor immunity by excess soluble tumor antigen. Cancer Immunol. Immunother. 8:127-132, 1980.

139. Trichopoulos, D., MacMahon, B., Cole, P.: Menopause and breast cancer risk. J. Natl. Cancer Inst. 48:605, 1972.

140. Valagussa, P., Bonadonna, O.: Patterns of relapse and survival following radical mastectomy. Cancer 41:1170-1178, 1978.

141. Valaras, V. G., MacMahon, B., Trichopoulos, D., et al: Lactation and reproductive historics of breast cancer patients in greater Athens., 1965-67, Int. J. Cancer 4:350-363, 1969.

142. Varani, J., Lovett, E. J.,. Lundy, J.: A model of tumor cell dormancy: effects of anesthesia and surgery. J. Surg. Oncol. 17:9-14, 1981.

143. Vogler, W. R., Furtado, V. P., Huguley, C. M., Jr.: Methotrexate for advanced cancer of the breast. Cancer 21:26-30, 1968.

144. Warren, S., Witman, E. M.: Studies on tumor metastasis: The distribution of metastases in cancer of the breast. Surg. Gynecol. obstet 57:81, 1937.

145. Weinhold, K. G., Goldstein, L. T., Wheelock, E. F.: The tumor dormant state - quantitation of L5178Y cells and host immune responses during the establishment and course of dormancy in syngeneic DBA/2 mice. J. Exp. Med. 149:732-744, 1979.

146. Weinhold, K. G., Goldstein, L. T., Wheelock, E. F.: Tumor dormant states established with L5178Y lymphoma cells in immunized syngeneic murine hosts. Nature 270:59-61, 1977.

147. Weinhold, K. G., Miller, D. A., Wheelock, E. F.: The tumor dormant state. Comparison of L5178Y cell s used to establish dormancy with those that emerge after its termination. J. Exp. Med. 149:745-757, 1979.

148. Welch, D. R., Evans, D. P., Tomasovic, S. P., Milas, L., Nicolson, G. L.: Multiple phenotypic divergence of mammary adenocarcinoma cell clones II. sensitivity to radiation, hyperthermia and Fudr. Clin. Expl. Metastasis 2:357-371, 1984.

149. Welch, D. R., Krizman, D., Nicolson, G. L.: Multiple phenotypic divergence of mammary adenocarcinoma cell clones. I. In vitro and in vivo properties. Clinical and Experimental Metastasis 2:333-355, 1984.

150. Welch, D. R., Milas, L., Tomasovic, S. P., Nicolson, G. L.: Heterogeneous response and clonal drift of sensitivities of metastatic 13762NF mammary adenocarcinoma clones to g-radiation in vitro. Cancer Res. 43:6-10, 1983.

151. Welch, D. R., Neri, A., Nicolson, G. L.: Comparison of spontaneous and experimental metastasis using the rat 13762 mammary adenocarcinoma metastatic cell lines. Invasion Metastasis 3:65, 1983.

152. Welch, D. R., Nicolson, G. L.: Phenotypic drift and heterogeneity in response of metastatic mammary adenocarcinoma cell clones to Adriamycin, 5-40-2'deoxyuridine and methotrexate treatment in vitro. Clin. Exp. Metastasis 1:317, 1983.

153. Wheelock, E. F., Brodovsky, H. S.: In prolonged arrest of cancer, edited by B. A. Stoll, John Wiley, New York, p. 87, 1982.

154. Wheelock, E. F., Brodovsky, H. S.: Do metastases arise from pre-existing subpopulation of cancer cells? Br. J. Cancer 47:81, 1982.

155. Wheelock, E. F., Toy, S. T., Weislow, O. S., Levy, M. H.: Restored immune and non-immune functions in Friend virus leukemic mice treated with statolon. Prog. Exp. Tumor Res. 19:369-389, 1974.

156. Wheelock, E. F., Weinhold, K. G., Ingenito, G. G., Goldstein, L. T.: In vivo lysis of L5178Y cells in the establishment of the tumor dormant state in DBA/2 mice. J. Immunol 124:1642-1647, 1980.

157. Wheelock, E. F., Weinhold, K. G., Levich, J.: The tumor dormant state. Advances in Cancer Res. 34: 107-140, 1981.

158. Windle, R., Bell, P. R. F., Shaw, D.: Five year results of a randomized trial of adjuvant 5-fluorouracil and levamisole in colorectal cancer. Br. J. Surg. 74:569, 1987.

159. Yahas, J. M., Tarleton, A. E.: Dormancy and spontaneous recurrence of human breast cancer in vitro. Cancer Res., 38:3584-1589, 1978.

160. Yancik, R., Ries, L. G., Yates, G. W.: Breast cancer in aging women. A population based study of contrasts in stage, surgery, and survival. Cancer 63:976-981, 1989.

161. Yokoyama, T., Yoshie, O., Aso, H., et al: Role of natural killer cells in intraocular melanoma metastasis. Invest. Ophthalmol. Vis. Sci. 27:516-518, 1986.

162. Yonemoto, R. H., Tan, M. S. C., Byron, R. L., et al: Randomized sequential hormonal therapy vs. adrenalectomy for metastatic breast carcinoma. Cancer 39:547-555, 1977.

163. Yuasa, S., MacMahon, B.: Lactation and reproductive histories of breast cancer patients in Tokyo, Japan. Bull WHO 42:192-204, 1970.