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OCULAR METASTASIS OF MURINE MELANOMA

by

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Abstract

Metastasis of Murine Ocular Melanoma

by

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The metastasis of murine ocular melanoma is characterized using the in vivo derived B16F10 melanoma cell line in C57BL/6J mice. Intraocular tumor readily metastasizes to the lungs and 100% of the animals die with extensive pulmonary metastasis 5-6 weeks post inoculation. In contrast, when cells passaged 5 times in tissue culture are inoculated intracamerally (ic), a marked decrease in the frequency and extent of metastasis, and an increase in survival is seen.

The efficacy of a new therapeutic immunomodulator, Linomide, is demonstrated against metastases from both ocular and flank tumors. Linomide was shown to significantly reduce metastases from either ocular or flank tumors when administered at a dosage of 160 mg/kg/day to mice. The combined treatment of Linomide and enucleation significantly reduced the number of pulmonary metastases, decreased the incidence of metastasis to the lung and lymph nodes, increased survival, and resulted in an apparent cure rate of 31%.

The role of natural killer (NK) cells in the regulation of metastasis of ocular tumors was also explored using animals with either depleted or enhanced NK activity. In C57BL/6J beige mice, with low NK activity, metastasis to the lungs was increased and survival decreased. In normal mice bearing intraocular tumors, treatment with PK136, an anti-NK cell monoclonal antibody, resulted in a significant increase in pulmonary metastases and an altered pattern of metastasis. Metastases were observed in spleen, liver, and adrenals.

In other experiments, the effect of a combined treatment protocol on metastasis and survival of tumor bearing mice was explored. In unenucleated mice, treatment with Linomide and cyclophosphamide (Cy) resulted in a significant reduction of pulmonary and lymph node metastases, and an increase in survival but no cures. The combined treatment of Linomide, Cy, and enucleation resulted in an apparent cure rate of 22%, a significant reduction of pulmonary and lymph node metastases, and an increase in survival.

Treatment protocols to reduce macrophage and T-cell activity in tumor-bearing mice were also utilized in this study and no effect on metastasis or survival was seen. In summary, the above work demonstrates the importance of the NK cell as a primary effector cell for the control of metastasis from in vivo derived B16F10 melanoma in this model of murine ocular melanoma.

This thesis is dedicated to Gail, Kaitlin, and my parents.

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Introduction

In the following chapters, the subject of immune regulation of murine ocular melanoma is explored in some detail using the B16F10 cell line. The experiments presented in the first chapter, lead to the establishment of a model for murine ocular melanoma in which metastases are routinely observed when cell suspensions or implants of solid *in vivo* derived tumor fragments are utilized (37). In addition, it is demonstrated that the differences observed in metastasis in other laboratories using cultured cells may be due to phenotypic drift of cultured B16F10 melanoma cells.

In the next chapter, we demonstrate a successful treatment for metastases arising from intraocular or subcutaneous inoculations of *in vivo* derived B16F10 melanoma (38), using the immunomodulatory drug Linomide (= LS2616, 49). The work described in the final chapter examines the possible roles played by macrophages, lymphocytes, and NK cells in growth and metastasis of murine ocular melanoma. This work demonstrates the importance of the NK cell in the regulation of metastasis from intraocular tumors of *in vivo* derived B16F10 melanoma (39). In contrast to results obtained by others using cultured cells, we demonstrate that the NK cell functions to regulate both the numbers of metastases and the pattern of metastasis when the primary tumor is intraocular.

THE PROCESS OF METASTASIS

The most difficult problem in the treatment of cancer patients is controlling or eliminating the dissemination of malignant cells from the primary growth site to distant organs and the subsequent growth of secondary metastases. Primary tumors contain extremely heterogeneous populations of tumor cells (27), and these cells may vary in metastatic potential, invasiveness, organ colonization preference, and response to drug therapy (90).

The process of metastasis is a sequence of interrelated steps (132). It is a very intricate and inefficient process, and very few cells survive to form secondary sites of growth (12). If disseminating tumor cells fail to complete any one of these intricate steps, metastasis will not occur. Metastasis begins with the growth of the primary tumor and invasion of the surrounding tissue. The process of intravasation may occur utilizing one of at least two methods; tumor cells may penetrate vessels directly (33) or angiogenic factors secreted by the primary tumor may direct the production of vessels or channels that become continuous with host vessels (29). In circulation, cells may extravasate by mechanical pressure (11) or by mechanisms similar to those responsible for intravasation. Sustained growth in the target organ requires the development of a vascular network (29) and continued evasion of the immune system.

MALIGNANT MELANOMA

Etiology and Histopathology

Malignant melanoma is the leading cause of death from diseases of the skin. Annually, approximately 22,000 people in the United States develop malignant melanoma and 5,500 die from this disease (31). If a lesion displays one of the following signals, it suggests a malignant change: 1) variegated color or a change in color from brown-black to shades of white, red, or blue, 2) the development of an irregular border, and 3) the presence of irregular surface elevations (134). Although there are 11 classes of extraocular melanoma, that is, melanoma originating outside of the uveal tract, only four classes are commonly seen: lentigo malignant melanoma (LMM), superficial spreading melanoma (SSM), nodular melanoma (NM), and acral lentiguous melanoma (ALM). Three of these types (ALM, SSM, LMM) can be clinically diagnosed at a very early stage due to the presence of an extended radial growth stage. This stage may last for years, and has little or no competence for metastasis (14). These lesions may be cured by simple surgical procedures. The acquisition of metastatic potential is associated with the ability to grow vertically and invade subcutaneous tissue. The extent of this second growth phase is the basis for the levels of invasion and tumor thickness tables utilized as prognostic indicators (13).

LMM is the most benign of the four most common melanoma types. It constitutes 10-15% of cutaneous melanoma. The median age of patients at the time of diagnosis is 70 years and the disease occurs in areas of the body heavily exposed to the sun (64). Radial growth occurs over decades

and is relatively innocuous while vertical growth results in metastasis in 25% of all cases.

SSM accounts for 70% of all extraocular melanoma cases, and is more invasive than LMM. The mean age at diagnosis is 55, and lesions are seen primarily on the legs and upper back. The radial growth phase in SSM is characterized by slight elevations in the lesion. The vertical growth phase develops rapidly (a few weeks to a couple of months) and tumors in this stage, depending upon the level of invasion, metastasize in 35-85% of all cases (64).

NM is the most malignant of the four classes and comprises 12% of all extraocular melanoma cases. NM has no radial growth phase and an accelerated vertical growth phase. The median age at diagnosis is 50 years. NM primary nodules grow and evolve quickly over the course of several months. The lack of a radial growth phase makes early diagnosis difficult. The rate of metastasis in NM depends on the level of invasion.

ALM occurs on the palms and soles. It is characterized by both a radial and a vertical growth phase. The radial growth phase of ALM typically has a duration of years. If left untreated, however, vertical growth will occur followed by metastasis.

Malignant melanoma is extremely invasive and early treatment is strongly correlated with greater survival. Breslow has demonstrated that metastases rarely developed in patients whose primary melanomas were less than 0.76 mm in thickness (8). In contrast, patients harboring tumors with a thickness greater than 3.0 mm had a ten year survival rate of only 48% (8). The pattern of metastasis is somewhat variable. The

lymphatics (17%), lung (18%), or liver (14%) are usually the initial sites of metastasis (4). The initial site of metastases may also occur in the central nervous system (12%), gastrointestinal tract (7%), and in multiple sites (20%). Mean survival time is greatest (19.9 months) when the initial site of metastasis is only a single regional lymph node.

Autopsy data and genetic information collected from melanoma patients have also been useful for describing the pathology of malignant melanoma. Zakka et al (132), using information taken from an extensive series of autopsies, have gathered useful data which correlates with the data presented above describing the distribution of primary tumor sites, the pattern of metastasis, and survival. Data obtained from melanoma patients and their families has been helpful in documenting the familial pattern of some forms of malignant melanoma. Greene (32) in a case study of 401 patients and their families, has demonstrated the importance of the dysplastic nevus syndrome (DNS) in the development of familial cutaneous melanoma. The probability of developing melanoma in patients with DNS and a history of familial melanoma was 56%. In a similar study, D'Arcy et al (19) analyzed environmental and genetic factors observed in melanoma patients and found that tanning ability, hair color, skin pigmentation, palpable nevi, family history, and ethnic origin were risk factors increasing the incidence of malignant melanoma.

Experimental Systems for the Study of Malignant Melanoma

A wide variety of experimental systems have been utilized in the literature for the study of growth and metastasis of malignant melanoma. Animal models have been extremely useful in elucidating specific steps of the metastatic cascade and growth rates of primary tumors. In the

mouse, the B16F10 melanoma was selectively cloned by Fidler (24) from the B16 parent line, a spontaneous pigmented melanoma syngeneic to C57BL/6 mice. This cell line metastasizes rapidly from a cutaneous site and colonizes the lungs exclusively. Other murine cell lines and clones have been carefully selected for variations in invasive potential (B16F10-B2, 92), variation in metastatic potential (K-1735b, 5), organ preference (B16-BL6, 118), and growth rate (G3.15, G3.5, 105). B16F10-B2, B16BL-6, G3.15, and G3.5 are subclones of the B16 parent melanoma cell line and colonize brain, bladder, and liver, respectively. The pathology of melanoma has also been explored in the hamster (100), the pig (23), the rabbit (58), and the cat (74).

Tissue culture has been used extensively for the study of melanoma cells. Studies range from the examination of cell surface properties (61, 94, 118, 119), antigenicity (60, 105) and invasive potential (110), to the examination of the effects of various aspects of the immune system on tumor growth and viability (21, 49, 86, 101, 103, 109, 117). These studies will be discussed in subsequent sections.

Some human melanomas taken from fresh tissue have been cultured <u>in vitro</u> or in the nude mouse. Though somewhat limited, these systems offer the advantage of studying the human melanoma cell. Using tissue cultured human melanoma cells, Itoh (46) has shown the suseptibility of these cells to lysis by lymphokine-activated killer (LAK) cells. A great deal of information has been learned about surface antigen expression through the use of tissue cultured cells, and this topic will be explored later. One of the major drawbacks to studying tissue cultured human melanoma cells has been demonstrated by Tsuchida et al (118). They have

demonstrated that the ganglioside composition of fresh human melanoma cells changes when these cells are grown in tissue culture. The pattern of ganglioside expression, observed in tissue culture, changes back to the original pattern when these cultured cells are grown in the nude mouse. Although most malignant human tumors grown in nude mice behave as benign tumors and do not metastasize, Kerbel et al (51) have developed a nude mouse model for studying metastasis of human melanoma cells. This group facilitated growth of MeWo melanoma cells by first incubating them with pieces of mouse lung tissue and then inoculating the tissue culture impregnated pieces subcutaneously (sc). Their results indicate that MeWo variants may be used as a human metastatic tumor analog of the B16 mouse melanoma line. In general, the nude mouse has been used to demonstrate the fact that the growth of human melanoma xenografts in the nude mouse may not correlate well with malignancy and growth in humans (134). The lung colonizing ability of xenografts taken from 4 melanoma patients was measured in nude CD-1 mice. Although some xenografts grew well in the mice and did metastasize, those that were most aggressive and metastatic in humans did not always give rise to lung colonies following tail vein injections in the mice. The site of inoculation in the nude mouse may determine the growth and metastatic pattern of allogeneic tumor. Kyriazis et al (57) have shown that subcutaneous inoculation of two human carcinoma cell lines resulted in a rapidly growing but highly necrotic primary tumor which did not metastasize with great frequency. The same cell lines inoculated intraperitoneally (ip) resulted in extensive metastasis in a shorter time frame with minimal necrosis. In later studies, this group (56) was showed histologic documentation of

these results. More recently, Koziowski et al (55) demonstrated that intrasplenic injection of tumor cells (melanoma and colon carcinoma) into nude mice allowed the most dramatic expression of metastatic capacity in these cell lines, resulting in frequent and large metastasis to the liver, lungs, and lymph nodes.

Antigenic Properties of Melanoma Cells

Surface antigens present on both human and murine melanoma cells under tissue culture and fresh explant conditions have been extensively studied. Gangliosides are glycosphingolipids containing sialic acids, and are found in the cell membranes of various tissues. The highest concentration of gangliosides is found in neuronal tissue (119). Malignant melanoma cells (which are derived from the embryonic neural crest) express high concentrations of gangliosides when compared to other neoplastic cells. Gangliosides are immunogenic in humans and have been intensively investigated. A vast number of monoclonal antibodies have been generated against many of the major gangliosides. Four major gangliosides have been found in many fresh and cloned melanoma cell lines. These have been abbreviated GM3, GM2, GD3, and GD2. In a recent study (119), using a total of 80 biopsied and cultured melanoma specimens, Tsuchida et al demonstrated that GM3 and GD3 appeared to be major ganglioside components while GM2 and GD2 were minor gangliosides in melanoma cells. This pattern of expression was seen in 95% of the biopsied specimens tested, and 54% of the cultured cells tested. Since the expression of GD2 and GM2 increased over time in cultured cells, they suggested elevated levels of these gangliosides might be a tissue culture artifact. Interestingly, GM3 and GD3 are major gangliosides in

many normal adult tissues. In normal melanocytes, for example, 90% of the gangliosides expressed are GM3 (31). Real et al (94) have shown that GD3 is present on newborn and adult cultured melanocytes, all cultured melanomas tested, fresh melanocytes, and 95% of all fresh melanoma specimens. Since melanoma cells have the highest expression of GD3 and GD3 is relatively specific for melanoma, antibodies to GD3 might be a useful therapy for metastases of human melanoma. This will be discussed in a later section.

The expression of gangliosides by melanoma cells can be altered when microenvironmental conditions change (118). It has been recently demonstrated that the pattern of ganglioside expression of fresh, biopsied specimens is altered when these cells are placed into tissue culture, and then altered again when these cells are placed in nude mice. Interestingly, the pattern of expression in the nude mouse resembles the pattern seen in fresh tumor (118). These findings are especially relevant to the study of immunogenicity of surface antigens and immunotherapy. Thurin et al (116) have correlated antibody-mediated cytotoxicity with the expression of surface antigens. They have developed an antibody, ME361, which detects both GD2 and GD3 antigens on a variety of melanoma cell lines. Although cell lysis occured in cell lines expressing GD2 alone or GD3 alone, this monoclonal was most effective in directing antibody mediated cytotoxicity in melanoma cell lines that expressed both antigens.

The influence of gangliosides on tumor growth and metastasis has also been reported. Alessandri et al (3) have shown that injections of a ganglioside mixture into tumor-bearing mice increased the growth rate of

the primary tumor and the number of metastases. The mixture of gangliosides was commercially prepared and contained GM1, GD1, GM2, and GD3 in varying concentrations. When added to the cell culture medium, the doubling time for the transformed cells was reduced to one-half the doubling time of the control tumor cells. Thus, a direct effect on proliferation seems likely. Interestingly, the same result was seen in cultures of capillary endothelial cells, suggesting an angiogenic function to the mixture. It was speculated that both events (increased cell proliferation and angiogenesis) were the mechanisms which may have been responsible for the increased *in vivo* growth rate of the primary tumor and increased numbers of metastases.

Many laboratories have demonstrated correlations between particular patterns of surface antigen expression (gangliosides and other components) and some phenotypic characteristics. Lehman et al (61) recently characterized 2 novel antigens, a glycoprotein of 113,000 m.w. and a protein of 76,000 m.w. found in a number of melanoma cell lines. Antibodies against these components were used to demonstrate the presence of these antigens on a large number of additional human melanoma cell lines (75 in total). Interestingly, the application of these monoclonal antibodies to benign lesions revealed the presence of the 76,000 protein in 5 of 14 dysplastic nevi and 3 of 4 Spitz nevi. Extensive examination of normal and malignant tissue indicated that expression of gp113 is correlated with the transformed state only, while p76 is also found on hepatocytes and gastric mucosal cells in addition to melanoma and normal pigmented cells.

Wang et al (123) have shown that SK Mel28 melanoma cells have the

ability to internalize surface antigen p97 (a glycoprotein) in response to treatment with p97-antibody. The expression of this antigen is greatest in melanoma cells, and is considered to be a likely target for antibody-dependent cytotoxicity therapy. The p97 glycoprotein appears structurally and functionally related to transferrin and lactoferrin. It is internalized, then re-expressed at a greater concentration when antibody treatment is repeated (even less than 24 hours later). This interesting finding has important implications for the diagnosis and therapy of human melanoma.

A highly restricted melanoma associated antigen has been found in the S91 mouse melanoma (60), and cross reactive tumor associated antigens have also be demonstrated in B16F10, K1735, JB/RH, and JB/MS (35). These cell surface antigens are capable of eliciting protective immunity against tumor challenge in these mouse systems, and provide the basis for the exploration of human vaccines against malignant melanoma.

Recently, Ono et al (87) have demonstrated that at least one melanoma antigen complex, in the mouse B16 system, was recognized by cytotoxic T lymphocytes and the addition of antibody to this antigen reduced CTL activity against the tumor cells. This antibody recognized some part of the GM3 antigen complex and effectively blocked lysis of tumor cells in vitro. In additional experiments, using pronase treated B16 cells, the blocking ability was greatly reduced but still present, indicating that the antigen is not GM3 itself but composed of a GM3/protein complex.

Immune Response to Melanoma

During the last fifteen years, animals models have been used

extensively for studying the immune response during the growth and metastasis of melanoma. Many workers employing different models with different cell lines have demonstrated the importance of various immune responses, both cellular and humoral, against the malignant cell. It is quite probable that numerous host defense responses act simultaneously or in sequence against an invading transformed cell. To elucidate the complexity of the immune response to tumor cells, most investigators have concentrated their efforts on one particular facet of that response. Some workers have studied antibody production by the host in response to tumor cell growth (76, 78, 106) while others have concentrated on the activity of one or more cytolytic effector cells (26, 28, 35, 49, 101, 109, 120). In addition, other investigators have studied chemotherapeutic agents that may function by direct lysis of tumor cells. Both immunomodulation and chemotherapeutic cytotoxicity have been shown to be effective in altering the growth of the primary tumor and/or metastases in many animal models of malignant melanoma.

Immunotherapy using recombinant human interleukin-2 (IL-2) was first developed in the mouse using B16 melanoma and other murine tumor lines. In earlier work by Mule and co-workers (68), IL-2 was added to the culture medium of lymphocytes taken from mice. After a period of 5-7 days in culture, these lymphocytes were transfered into tumor-bearing hosts and were shown to reduce tumor incidence and numbers of metastases. More recently, Rosenberg and colleagues (99) have shown that the systemic administration of IL-2 could also inhibit the growth of established tumors and metastases. Silagi and Schaefer (103) have recently demonstrated that 100% of 1 day B16 melanoma tumors and 87-91%

of 3 day tumors could be cured by a combination therapy of Cyclophosphamide and intralesional inoculation of IL-2. Without Cyclophosphamide, the cure rate was 64% for 1 day tumors and 67% for 3 day tumors. Several studies (22,86) have suggested that treatment with low doses of Cyclophosphamide facilitate immunotherapy, possibly by removal of immune suppressor cells.

Effector cells stimulated by IL-2 are known as lymphokine activated killer (LAK) cells, and may be similar to natural killer (NK) cells. Toshitani and co-workers (117) have shown that LAK cells lyse the same target cells susceptible to NK lysis, but also have the ability to lyse $H\text{-}2^{+}$ target cells. H-2 gene products (or MHC gene products in the human system) are composed of glycoproteins and maintain the integrity and recognition of self. The uniqueness of strain-specific H-2 or MHC gene products is thought to guide the T lymphocyte lytic process. NK cells may lyse tumor cells through a process that detects the absence of H-2 products or a recognition of foreign surface antigens. Both LAK and NK cells may consist of at least three different populations and some of these populations may overlap in phenotype, lytic ability, and responsiveness to IL-2 (52). The ability to increase the lytic activity of two different effector cell populations, as demonstrated by Toshitani et al (117), is important because solid tumors are extremely heterogeneous and may contain cells that are both $H\text{-}2^{+}$ and $H\text{-}2^{-}$.

NK cells are thought to give the host a spontaneous resistance against tumors and have been postulated to act <u>in vivo</u> as immune surveillance cells. When tissue culture derived tumor cells are inoculated into animals genetically deficient in NK activity (109) or

made NK deficient by treatment with monoclonal antibodies to NK cells (35, 49, 100) an increase in tumor growth rate and/or an increase in metastasis is seen when compared to normal or untreated controls. The beige mouse (95, 96) contains an autosomal mutation which results in decreased NK cell activity, and this model is widely used to study the role of NK cells. Monoclonal antibodies anti-GM1 (49) and anti-NK1.1 (47,52) have been used to selectively deplete NK cells in murine tumor systems. Alternately, enhancement of NK cell activity through the use of immunomodulators such as gamma interferon (109), and poly i:c (98) results in decreased tumor growth and a reduction in the number of tumor metastases in animal models of melanoma.

The role of the tumoricidal macrophage during tumor growth and metastasis has also been extensively explored. Fidler and colleagues (26,28) have compiled detailed studies demonstrating that macrophage activity could be enhanced in a mouse melanoma system and established metastases could be eradicated. The immunomodulatory agents in these experiments were muramyl dipeptide (MDP) and macrophage activating factor (MAF), and were administered in liposome encapsulated vesicles. The agents were administered either in vivo to tumor-bearing hosts or in vitro to macrophage cultures which were inoculated into tumor-bearing hosts. The increase in macrophage activity was confirmed both histologically and by specific depletion experiments.

The role of T lymphocytes in the regulation of melanoma growth and metastases appears to be limited. In aged (18-20 month) B16 melanoma-bearing mice, Tsuda et al (120) have shown that tumors grow significantly slower when compared to younger controls (6-8 week old).

Tumor growth was also slowed in young, thymectomized mice and young mice depleted of T cells by irradiation and reconstituted with aged lymphocytes. T cells (62) and macrophages (88) produce angiogenic factors essential to tumor growth, and it has been suggested that decreased T cell function in older mice may explain the reduction in growth rate of the primary tumor. Stackpole et al (106) have given direct evidence for T cell immunity to a variant B16 clone in C57BL/6 mice. The slow growing, non-metastatic and highly immunogenic clone produced metastases in mice made T cell deficient by sublethal irradiation. This evidence indicates that variants from the B16 parent line may be sensitive to lysis by classic CTL cells.

The importance of host antibody production against tumor cells in decreasing growth and metastasis has not extensively been explored. Although the presence of anti-tumor cell antibodies has been documented in animal models (76, 78, 125), antibody therapy has only recently been shown to decrease established melanoma metastases (20). Eisenthal et al (20) were able to decrease the numbers of hepatic metastases up to 90%, using an IgG2b anti-B16 antibody. The success of the treatment was shown to be dependent upon an asialo-GM1 sensitive effector population. In addition, the success of the therapy could be enhanced by combined treatment with IL-2. Antibody dependent cell mediated cytotoxicity appears to be an area in need of additional exploration.

A variety of newly-developed chemotherapeutic agents, whose anti-tumor activities cannot be easily explained in terms of our current knowledge of the immune system, have been recently developed using animal models of malignant melanoma. The perfluorochemical emulsion

Flusol-DA has been shown by Teicher et al (113) to be effective in reducing pulmonary metastases from intravenous B16 melanoma inocula. Flusol-DA was administered to mice intravenously and the number of pulmonary metastases were quantified at days 25 and 40 post-inoculation. The authors suggest that the action of this drug may be directly tumoricidal or may increase the activity of alveolar macrophages. The anti-tumor effect of methionine-enkephalin was recently demonstrated in B16-BL6 melanoma-bearing mice (69). The administration of this drug at a dosage of 50 ug/mouse/day in dorsal subcutaneous injections (far removed from the site of the primary tumor) resulted in a delay of growth of the primary tumor for 14 days. Once the primary tumor was established, however, the growth rate of tumor in the experimental animals was similar to that of controls. It was suggested that the drug may be enhancing NK cell activity, although this was not demonstrated in the report.

Treatment of Cutaneous Melanoma

The immune response to malignant melanoma in humans appears to have both cellular and humoral components and is similar in many ways to some of the immune response seen in the mouse. Therefore, courses of treatment have been developed that 1) enhance the cellular response to melanoma cells, 2) initiate or amplify antibody production against tumor cells, and 3) are cytotoxic to melanoma cells.

The success of interferon therapy in murine models of melanoma inaugurated the development of clinical trials for similar therapies in humans with stage 2 or 3 malignant melanoma. Creagan et al (15) have reported some short term success with interferon-alfa-2a in patients

with advanced malignant melanoma. 96 patients were treated three times, weekly, with 12 X $10^6 U/m^2$ and the observed response rate was 22%. Most regressions occurred within the first month but were transient in nature, and limited to soft tissues. Although the mean survival time of the entire group was 6 months, three patients showed long term regressions for up to 32-41 months.

Initial efforts to augment the humoral response to malignant melanoma utilized immunostimulants such as Bacillus Calmette-Guerin (BCG) or other bacterial products such as cell wall components. Recently, active immunization against tumor antigens was demonstrated in clinical trials using a melanoma vaccine (9). Bystryn et al demonstrated that the combination of antigens shed by four melanoma cell lines (HM31, HM34, HM49, SK Mel 28) into the medium during tissue culture could be concentrated and used as a vaccine to elicit antibody responses in melanoma patients. Biweekly immunization resulted in antibody production to melanoma antigens in 69% of stage 2 patients and 53% of stage 3 patients. Pretreatment with Cyclophosphamide augmented both the humoral response and the delayed type hypersensitivity response. The study was not designed to evaluate the effect of immunization on melanoma progression or survival.

Traditional chemotherapeutic strategies against malignant melanoma have achieved limited success. The standard chemotherapeutic drugs such as decarbazine, vinblastine, bleomycin, or cisplatin, demonstrate response rates from 14-21% (15). Recently, a new strategy employing anti-melanoma antibodies has been developed by two laboratories. Houghton et al (43) have developed a murine monoclonal antibody (R_{24})

directed against GD3, a prominent ganglioside expressed in high concentrations on human melanoma cells. R_{24} induced inflammatory reactions in metastatic nodules, lymphocyte infiltration, mast cell degranulation, and complement deposition. Major tumor regression occured in 3 of 12 patients. Employing a slightly different strategy, Irie and Morton (45) recently produced a human monoclonal antibody (L72) against the GD2 ganglioside by transforming blood lymphocytes from a melanoma patient into a human lymphoblastoid cell line using the Epstein-Barr virus. The antibody was shown to have a strong cytotoxic effect when assayed in vitro, and when administered intralesionally. Six of eight patients showed some form of response, from partial to complete. One patient had complete regression with no sign of recurrance 20 months after treatment. Histopathologic analysis showed: degeneration of tumor, fibrosis, free melanin, and some degree of lymphocyte or macrophage infiltration.

OCULAR MELANOMA

Etiology and Histopathology

Ocular melanoma is the general heading under which malignant melanomas of the choroid, iris, and ciliary body are grouped. Although cells of the pigmented epithelium of the iris, retina, and ciliary body can become neoplastic, this is an extremely rare occurence. Uveal melanocytes are the cells of origin for most intraocular neoplasms. The incidence of non-cutaneous melanoma in the United States is 0.7/100,000 (131), which is about 12% of all melanomas.

The etiology of ocular melanoma is unknown. The incidence does not appear to correlate with exposure to the sun. There does not appear to be a latitude gradient for uveal melanomas, as in the case of cutaneous melanomas. In addition, it is not certain that familial forms of ocular melanoma exist.

There are 5 major forms of malignant uveal melanoma (10). Spindle A melanomas contain elongated cohesive cells with ill-defined nucleoli and cell borders. These constitute approximately 5% of uveal melanomas. Spindle B melanomas are cohesive elongated cells with distinct nucleoli and cell borders. They constitute about 39% of ocular melanomas. Essentially all iridial tumors are of the spindle A or B type. Epitheloid tumors are poorly adhesive, round cells with prominent nucleoli. Tumors comprised of only epitheloid cells are extremely rare (3%). Mixed cell type melanomas contain both spindle B and epitheloid cells, and are the most common type of ocular melanoma comprising approximately 45%. Tumors so necrotic that the dominant cell type cannot

be determined are placed into a fifth category. They are called necrotic type melanomas and constitute 7% of uveal melanomas.

Shammas and Blodi (102) identified 9 factors that significantly influenced prognosis:

1) Age of the patient at the time of enucleation - older patients tend to have larger tumors and a poorer prognosis.
2) Location of the tumor and its anterior border - tumors located on the iris have a better prognosis than posterior tumors.
3) Largest tumor diameter in contact with the sclera - a greater area of contact with the sclera results in a poor prognosis.
4) Scleral infiltration by tumor cells - invasion of the sclera results in a poor prognosis.
5) Height of the tumor - large height equals poor prognosis.
6) Integrity of Bruch's membrane - invasion of the membrane means a poor prognosis.
7) Cell type - spindle A or B types are much less aggressive than epitheloid type cells.
8) Pigmentation - increasing pigmentation is associated with increased mortality.
9) Largest tumor diameter - this is the single most important clinical and prognostic factor. Prognosis is good when the largest tumor diameter is less than 10 mm., and poor when it is greater than 10 mm.

In contrast to the pathology of cutaneous malignant melanoma,

Zimmerman (133) has demonstrated that 1) uveal melanomas rarely, if ever, spread via the lymphatics, 2) hematogenous dissemination is responsible for virtually all tumor deaths, 3) tumor deaths, while uncommon until 6 months after enucleation, are concentrated mostly during the first 5 years after enucleation, 4) a small percentage of surviving patients may show a delay in the onset of metastatic disease for as long as 25 years after diagnosis of uveal melanoma. The most controversial aspect of uveal melanoma pathology is the observation that enucleation appears to increase the number of deaths from metastasis. Zimmerman offers a variety of possible explanations for this phenomenon including the traumatic effects of the operation itself which may include the release of large numbers of tumor cells. Additional variables include the immune status of the patient, or increased virulence of the tumor developing concomitently with the onset of symptoms.

Metastasis, although a rare event in the absence of enucleation, does occur in ocular melanoma. When metastasis does occur, it is usually to the liver and almost never to the regional lymph nodes, setting ocular melanoma distinctly apart from cutaneous melanoma. Utilizing data from 38 autopsies, Jensen (47) has shown that when metastasis did occur, the liver alone was affected in 38% of the cases while the liver and some other organ were involved in 63% of the cases. Rarely (3%) was the liver spared from metastasis. Small ocular melanomas, however, may remain dormant over a period of years, and metastasis from a small tumor is exceedingly rare.

Treatment of Ocular Melanoma

Treatment of uveal melanoma depends largely upon the size of the tumor. Small (less than 10 mm.) tumors may not require immediate removal, but it is generally held that larger tumors with diffuse involvement of the iris, angle of the anterior chamber, or sclera or tumors that do not respond to therapy should be removed. Their size and location determines whether enucleation is neccessary. In small to medium size tumors, when enucleation is not performed, a variety of irradiation techniques exist. Wilkes and Gragoudas (127) have shown that proton beam radiation can be accurately aimed at lesions within the eye. Patients were given small doses of irradiation over 5-8 days. Of 44 tumors treated, 7 lesions disappeared, 33 decreased in size, and 4 remained unchanged during a follow-up period of 22 months. ^{60}Co radiation plaques continue to be used successfully (107). Stallard implanted ^{60}Co radiation plaques in 100 patients, and in 69 eyes the tumor completely disappeared. Only 6 deaths occured in this group after 5 years of observation. Treatment of metastases arising from intraocular melanomas is identical to therapies for metastases arising from cutaneous melanomas. Various strategies concerning immunotherapy and chemotherapy of melanoma metastases have been discussed earlier.

Animal models of ocular malignant melanoma are extremely limited in number. Therapies for the treatment of metastasis of ocular melanoma in animals is also very limited. Among the first groups to explore treatment for metastases of ocular melanoma was Yokoyama et al (130) whose work was discussed earlier. In their model of murine ocular melanoma, pulmonary metastases were significantly reduced by the

administration of a single intraperitoneal injection of recombinant human interferon-alpha A/D at a dosage of 2 X 10^5 U. This treatment resulted in a significant decrease of pulmonary metastases in experimental animals when compared with controls (1.2±0.8 and 8.1±7.6 lung colonies, respectively). NK activity, as measured in a standard ^{51}Cr-release assay, was significantly increased in interferon-treated animals, and it was suggested that the NK cell plays an important role in the regulation of pulmonary metastasis in this model of murine melanoma. Recently, Niederkorn et al (84), using less aggressive tissue culture derived B16F10 melanoma cells, immune compromised mice, and traumatic enucleation techniques, have demonstrated the effectiveness of difluoromethylornithine (DFMO) in decreasing pulmonary metastases from ocular murine melanoma. A slight increase in survival was also observed.

Antigenic Properties of Uveal Melanoma Cells

The study of cell surface antigen expression in uveal melanoma is extremely limited. The difficulty of obtaining fresh tumor explants and limited cell culture techniques for the growth of these cells are two factors contributing to curtailing research in this area. Damato et al (18), however, have recently presented evidence regarding the characterization of some cell surface antigens on uveal melanoma cells. Twelve uveal melanoma samples taken from surgical procedures were tested by enzyme-linked immunosorbant assay (ELISA) with monoclonal antibodies raised against uveal antigens. Five monoclonal antibodies of the IgM class detecting surface antigens on uveal melanoma cells were created (4A3, 1B1, 4B4, 1B4, and 1C4). All 5 antibodies recognized some component of each of the twelve tumor explants. The reactions were quite

heterogeneic, however, and tumors contained cells that expressed more or less of each of the antigens. Monoclonal antibody 4A3 reacted significantly against all twelve tumors and against none of the control tissues. 4B4 and 1B4 reacted preferentially with tumor tissue, while 1B1 and 1C4 were nonspecific in that they reacted to an equal degree with both tumor and control tissue. The antigen detected by monoclonal 4A3 is a 55,000 m.w. protein doublet. It was not detected by any of the widely used melanoma antibodies to cutaneous melanoma and is currently under investigation.

Animal Models of Ocular Melanoma

The biology of ocular melanoma in animals has been studied for a number of years. Using the Green melanoma cell line in hamsters, Fraunfelder explored the use of enucleation as a therapy for ocular melanoma in the late 1970's (77). Later, Niederkorn, Shadduck, and Albert (74) presented data concerning the development of ocular melanoma in cats. In 1982, while studying mouse models for the development of ocular melanoma, Niederkorn and Streilein (75) elucidated mechanisms involved in anterior chamber-associated immune deviation (ACAID). Using allogeneic P815 mastocytoma inoculated intracamerally (ic) into BALB/c mice, they reported a subversion of the host's immune system. This deviation depended upon the degree of disparity of MHC antigens. However, in general ACAID manifested itself in three ways: the allogeneic tumor cells grew progressively in the anterior chamber of the eye, allogeneic skin grafts were accepted indefinitely, and subcutaneously (sc) inoculated P815 tumor cells grew into flank tumors in mice harboring intraocular P815 tumors. Examination of the immune

system revealed that ACAID is characterized by suppression of delayed type hypersensitivity (DTH), normal cytotoxic T-lymphocyte (CTL) activity, and normal or elevated levels of antibody production (75). Removal of the spleen abrogated all three parameters of ACAID. It was suggested that an antigenic signal may originate within the eye and may be transmitted to the spleen where it results in the suppression of classic delayed type hypersensitivity responses. Since tumor cells were not found systemically, the signal was not in the form of viable tumor cells. Similar results were obtained when B16F10 melanoma cells were inoculated ic into LP/J mice (75). In other studies (78), Niederkorn has demonstrated that ACAID is also induced by the ic presentation of syngeneic tumor cells. When B16F10 melanoma cells containing melanoma associated antigens (MAA) were inoculated into the anterior chamber of syngeneic C57BL/6J mice, hosts produced significant quantities of antibodies against MAA, yet failed to develop DTH reactivity. Adoptive transfer experiments demonstrated that ic presentation of MAA induced the development of T suppressor cells, suggesting a mechanism for the DTH suppression. Interestingly, ic presentation of irradiated syngeneic tumor cells also evokes protective immunity from subsequent challenge of viable cells, but Niederkorn's experiments indicate that neither antibody production nor DTH reactivity is directly involved in this protection.

As described previously, Zimmerman (133) has shown that enucleation as a therapy for ocular melanoma may increase metastasis. The mouse model of ocular melanoma developed by Niederkorn (79) utilized cultured B16F10 tumor cells. Thus, cultured cells were utilized to study the

efficacy of enucleation in tumor bearing animals. It was demonstrated that neither enucleation or mechanical manipulation of the melanoma-containing eye promoted a significant increase in the incidence of metastasis. However, enucleation or traumatic manipulation of the tumor bearing in concert with systemic immune impairment (T cell deficiency induced by sublethal gamma irradiation) produced a sharp increase in the incidence and number of pulmonary metastases in intraocular-bearing mice. In contrast, atraumatic removal of rapidly frozen eyes prevented metastasis in similar hosts. Collectively, these experiments demonstrated that induction of distant metastases in hosts harboring intraocular melanomas requires two simultaneous processes: 1) mechanical manipulation of the melanoma containing eye, and 2) concomitant impairment of T cell dependent immune processes. Recent experiments by Niederkorn (83) have been useful in elucidating the role of T cells in his model of ocular metastasis. In these experiments, mice were rendered T cell deficient by thymectomy and lethal whole body irradiation. Thymectomized and bone marrow restored mice were selectively reconstituted with immune lymph node cell suspensions that were depleted of specific T cell subsets. The selectively reconstituted hosts were used to evaluate the role of specific T cell subsets in controlling the metastatic spread of intraocular melanomas. It was demonstrated that the effector cells responsible for protection against metastasis demonstrated a surface phenotype characteristic of cytotoxic T lymphocytes: Thy-1+, Lyt-1+, and Lyt-2+.

Models for ocular melanoma were also developed in other laboratories. Yokoyama et al (130) presented evidence that NK cell

activity played an important role in the control of metastasis from murine ocular melanoma. Using cultured B16 melanoma, they demonstrated an increase in the number of pulmonary metastases when anti-asialo GM1 antibody was administered to mice harboring ocular melanomas. In contrast, a significant decrease in the numbers of pulmonary metastases was observed when NK cell activity was augmented by alpha interferon, a known NK cell enhancer. In this model, consistant metastases were observed without the need for traumatic enucleation and immune depression, in contrast to the work of Niederkorn. An additional conflict exists because earlier work by Niederkorn (78) and more recent work by Niederkorn et al (83) suggest that NK cells do not play a role in the regulation of metastasis of murine ocular melanoma.

Issues Addressed By Thesis Research

This thesis has attempted to resolve some important problems observed in animal models of ocular melanoma, explore therapeutic strategies, and elucidate cell mediated cytotoxic responses in our model. We have: 1) established a new model of ocular metastasis, 2) demonstrated a successful therapy regimen for decreasing metastasis and increasing survival in this model, and 3) demonstrated the NK cell as the primary effector cell for the regulation of metastasis from ocular murine melanoma.

Chapter 1

Ocular Metastasis of *In Vivo* and *In Vitro* Derived Syngeneic Murine Melanoma

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Abstract

We examine ocular metastasis of syngeneic murine melanoma in C57BL/6J mice and compare the metastatic capability of B16F10 tumor cells maintained *in vivo* with those maintained in culture. We demonstrate that as long as the tumor cells are derived from an *in vivo* source, intraocular tumor readily metastasizes to the lungs. When *in vivo* derived tumor is introduced as an intracameral (ic) cell suspension or as a solid fragment on the iris, 100% of the animals die with extensive pulmonary metastasis 5-6 weeks later. In contrast, when B16F10 cells are passaged five times in culture and inoculated ic, a marked decrease in the frequency and extent of metastasis, and an increase in survival is seen. These studies demonstrate an alteration in the ability of cultured B16F10 cells to metastasize from the eye. When metastasis of *in vivo* derived tumor from the eye was compared with metastasis from an extraocular location, the extent and frequency of pulmonary metastasis and survival of the hosts was the same. The effect of enucleation on the metastasis of B16F10 from the eye has only been previously examined using cultured cells. In this paper, we demonstrate that the efficacy of enucleation depends upon whether B16F10 melanoma cells have been passaged *in vivo* or *in vitro*.

Syngeneic tumors of low immunogenicity grow progressively in the anterior chamber of the eye (75,79,130). Experiments designed to examine the metastatic fate of syngeneic murine melanoma cells introduced into the eye have yielded conflicting results. Following intracameral (ic) inoculations of suspensions of cultured B16F10, a highly metastatic subclone of B16, into C57BL/6J mice, little or no metastasis was observed and animals died from unknown causes 30 days post inoculation (75,79). Examination of the effect of enucleation on metastasis of cultured B16F10 inoculated ic showed that enucleation alone had no effect on metastasis, but enucleation in consort with immunosuppression promoted metastasis (79). In other studies (130), B16 was cultured in Eagle's minimal essential medium (EMEM) as modified by Yokoyama et al. Following ic inoculation into syngeneic mice, pulmonary metastasis was seen in 6/7 mice examined 40 days later. In the above experiments using cultured B16F10 or B16 melanoma cells, metastasis from the eye was not compared with that occuring from an extraocular location.

The major purpose of our investigation was to determine whether *in vivo* as opposed to *in vitro* derived B16F10 melanoma cells would readily metastasize from the eye. In addition, we used *in vivo* derived tumor to compare metastasis from the eye to that occuring from an extraocular location, and to examine the effect of enucleation on ocular metastasis. We demonstrate that: 1) *in vivo* derived B16F10 cells metastasize readily from the eye, 2) the extent of metastasis from ocular and flank tumors is the same, and 3) the efficacy of enucleation depends upon whether tumor is derived from an *in vivo* or *in vitro* source.

MATERIALS AND METHODS

Experimental Animals

Five to seven week old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) of both sexes were used as experimental subjects and to maintain tumor lines *in vivo*. The present investigations conform to the ARVO Resolution on the Use of Animals in Research. Animals were maintained according to the recommendations outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council. Tumor bearing animals were examined and handled daily. Animals were judged as healthy if their physical appearance (with the exception of the presence of a primary tumor) and responses to handling were normal. Animals not judged to be healthy were classified as moribund.

Tumor Maintenance and Preparation

B16F10 melanoma: The B16F10 melanoma cell line was obtained in 1984 from Dr.Artemio Ovejera (N.I.H., Bethesda, MD). Tumor cells were maintained *in vivo* by subcutaneous (sc) implantations of 0.5 mm^3 fragments of tumor taken from the periphery of flank tumors carried for 3 weeks. Transfers were made by injecting tumor fragments suspended in 0.2 ml of Hank's Balanced Salt Solution (HBSS, Sigma Chemical Co., St.Louis, MO) into the flank using a 16 g needle.

Suspensions of cells from 3 week old flank primary tumors were made by forcing 1.0 cm^3 pieces of tumor through a 60 mesh stainless steel tissue grid. Cell suspensions were washed twice in HBSS. B16F10 cells were grown as monolayers in Falcon tissue culture flasks and passaged

five times before inoculation. Cultures were grown at 37°C and 5.0% CO_2 in DMEM with 10% fetal bovine serum and 0.05 mg/ml gentamycin (Gibco Laboratories, Grand Island, NY) as described by Niederkorn (79). Cells were harvested using a 2 minute incubation with 0.25% trypsin in HBSS at 37°C and 5.0% CO_2. The viablility of tissue culture cells and cells from primary tumors was assayed by a trypan blue exclusion test. Suspensions containing greater than 90% viable cells were used in all experiments.

JBRH melanoma: The JBRH tumor line was obtained in 1984 from E.Natoli with permission of Dr.J.Berkelhammer (Memorial Sloan Kettering, New York, NY). This tumor arose by application of croton oil to neonatal C57BL/6J mice. Although the JBRH tumor is amelanotic, the cells contain melanosomes and premelanosomes (5,6). The serology of JBRH has been examined. Several major surface antigens have been characterized, and a monoclonal antibody against the predominant ganglioside located on the cell surface (70). In vivo lines were maintained in our laboratory by sc implantations of 0.5 mm^3 fragments every 4 weeks.

Establishment of Primary Tumors

Iridial implants: Mice were anesthetized by intraperitoneal (ip) injection with 0.06 mg/gm of Nembutol (Abbott Laboratories, North Chicago, IL) and the pupils dilated with one drop of 1.0% mydriacil (Alcon Laboratories, Fort Worth, TX). Using the Zeiss OPMI-1 dissecting microscope (X25), a 0.5 mm incision was made with a microlancet and enlarged with iridectomy scissors to approximately 1.5 mm. With a jeweler's forceps, a 0.5 mm^3 solid fragment of either B16F10 or JBRH melanoma was inserted onto the iris approximately 2.0 mm from the pupillary margin. Immediately after transplantation, the cut edges of

the cornea came together.

Intracameral inoculations: Mice were anesthetized and the pupils dilated as above. Five microliters of cell suspensions containing 10^5 B16F10 cells in HBSS were inoculated into the anterior chamber of mice using the technique described by Niederkorn et al (78). The cells originated either from tissue culture or from primary tumor as described above.

In the event of damage to lens or iris, or bleeding into the anterior chamber, experimental animals receiving intraocular tumors were excluded from the protocol.

Tumor growth and morphological changes were observed daily in unanesthetized mice. In all mice that had received tumor, a faint corneal opacity and engorgement of limbal vessels could be seen on day 2. On days 3-9, corneal opacification and vascularization had progressed such that tumor size could not be estimated. All experimental eyes were dissected and examined for tumor growth following enucleation or termination of the experiment.

Enucleation

Mice were anesthetized, tumor bearing eyes were enucleated 4, 7, or 10 days after ic inoculations or iridial implantation, and a suture was used to close the eyelids. The enucleations were clean; eyes came out as intact structures, and tumor did not subsequently appear in the area of the incision. Enucleated eyes were dissected and examined under the OPMI-1 microscope for evidence of tumor within the globe.

Subcutaneous Tumor Growth and Metastasis

A 0.5 mm^3 fragment of B16F10 or JBRH tumor was implanted sc into the flank. The two largest diameters of the primary tumor were measured

at weekly intervals in unanesthetized mice using a vernier caliper.

Assay for Metastasis

In most experiments, mice either died or became moribund and were necropsied. In a few protocols noted below, healthy animals were sacrificed at 6 weeeks and necropsied. The lungs, spleen, liver, intestine, lymph nodes, brain, heart, and kidneys were removed from the animal and examined at X 10 for evidence of metastasis. The number of metastases was determined during necropsy using a previously described method (25). In addition, nodule size was quantified and the number of nodules 0.1-0.5 mm, 0.6-1.0 mm, and greater than 1.0 mm recorded.

Statistical Analysis

Differences in the mean number of nodules and in primary tumor size were analyzed using Duncan's Multiple Range Test (S.A.S.Institute, NC).

RESULTS

Metastasis of B16F10 Cells From Primary Tumor Passaged In Vivo

These experiments were designed to examine the metastatic potential of *in vivo* derived cells placed intraocularly. Accordingly, ten mice received iridial implants and 12 received ic inoculations of tumor that had been passaged in the flank. All experimental animals became moribund or died at 5-6 weeks with large ocular tumors 9.2±1.6 mm (SD) and 8.6±1.4 mm (SD) in diameter for cell inoculated and implanted animals, respectively. All animals had extensive pulmonary metastases. Metastases were seen only in the lungs. The survival, frequency and extent of metastasis was similar in animals that had received cell suspensions and solid implants of this *in vivo* derived tumor (Table 1).

Metastasis of B16F10 Cells Derived From Tissue Culture

Experiments were designed to determine whether culturing of B16F10 cells would affect ocular metastasis. Nine mice were given ic inoculations of tumor cells (10^5) passaged five times in DMEM. All mice developed large ocular tumors and the mean tumor diameter was 9.9±2.5 mm (SD). Only 33% (3/9) of these mice became moribund 6 weeks post inoculation and developed pulmonary nodules (Table 1). Experimental animals had a mean number of only 11±7 (SEM) pulmonary nodules, all less than 0.5 mm in diameter. Metastasis was not observed at other sites. The 6/9 mice that were healthy when sacrificed 6 weeks post inoculation did not develop pulmonary metastases. A comparison of these results with those obtained using *in vivo* derived cells shows that B16F10 cells cultured for only five passages exhibit a decrease in ability to

metastasize from the eye. Survival is increased, and the extent and incidence of metastasis is significantly less (P less than 0.05) than that seen using flank derived tumor (Table 1). Examination of experimental eyes of all animals at 6 weeks revealed the presence of large ocular tumors.

In order to determine whether trypsin used prior to passaging the tumor cells in vitro had affected the metastatic potential of cultured cells, cell suspensions obtained from in vivo flank primary tumors were treated with trypsin, washed and incolulated ic into a group of seven mice. No change in the metastatic potential of the inoculum was observed compared to cell suspensions that had not been trypsinized.

The Effect of Enucleation on the Metastasis of In Vivo and In Vitro Derived Primary Ocular Tumors

In order to determine the effect of enucleation on animals that had received in vivo derived cells, panels of mice that had received iridial implants were enucleated on day 4 (N=10), day 7 (N=11), or on day 10 (N=12). Only 30% of the animals enucleated at day 4 became moribund or died at 5-6 weeks. These animals had significanly fewer nodules than animals that had not been enucleated (Table 1). The remaining 70% were sacrificed and necropsied at 6 weeks post implantation and found to be free of metastatic disease. Enucleation on day 7 or 10 had no notable effect on metastasis and all animals died or became moribund at 5-6 weeks with extensive pulmonary metastasis. The mean number of pulmonary nodules and the survival of the animals did not differ from that observed in unenucleated mice (Table 1). When animals (N=9) were inoculated with cells suspensions derived from flank tumor, enucleation

at day 10 did not improve survival or lower the frequency or extent of metastasis (Table 1). Examination of all enucleated eyes revealed the presence of tumor. By 10 days, tumor completely filled the globe. Thus, when in vivo derived B16F10 cells are introduced into the eye, only enucleation at 4 days was even partially effective in preventing the development of metastatic disease.

In order to determine the efficacy of early enucleation on the metastasis of ic inoculated cultured cells, panels of seven mice were inoculated with cells passaged five times and enucleated either 4 or 7 days later. All enucleated mice were healthy when sacrificed 6 weeks post inoculation. Upon necropsy, no pulmonary metastases were seen (Table 1). The presence of tumor was confirmed in all enucleated eyes. Thus, early enucleation appeared to be completely effective in preventing metastasis of ic inoculations of cultured cells.

Growth and Metastasis of SC B16F10 Melanoma

These experiments were designed in order to compare the metastasis of cell suspensions and solid implants of in vivo derived tumor placed in the flank with metastasis from an intraocular location. One group of seven mice received sc implants, and eight animals received sc inoculations of in vivo derived cells in suspension. The growth of solid implants of B16F10 melanoma is depicted in Figure 1. All animals became moribund or died at 5-6 weeks. The frequency and extent of metastasis was the same in both groups and did not differ significantly from the frequency and extent of metastasis seen following introduction of implants or in vivo derived cell suspensions into the eye (Table 1).

Growth and Metastasis of JBRH Melanoma

In order to determine whether this tumor would readily metastasize from the eye, and to compare such metastasis with that occuring from an extraocular location, solid fragments of tumor were placed sc or on the iris. Tumor failed to grow in a small percentage of animals that had received iridial or sc implants of JBRH. The sc growth of 11 successful 0.5 mm^3 implants of JBRH melanoma is shown in Figure 1. The primary tumor grew at a significantly slower rate (P less than 0.05) than the B16F10 tumor. Growth of iridial implants was observed in ten animals. All animals that had received ocular and sc implants became moribund or died with extensive pulmonary metastases at 8 weeks post implantation. Results were similar to those obtained using the B16F10 melanoma in that there was no significant difference between the metastasis or survival of hosts when JBRH was implanted sc or on the iris.

DISCUSSION

Several investigators have shown that cultured populations of tumor cell lines change phenotypically with time to produce cells with altered malignant potential (40,71,72,89,92,93,105,121,128). This is referred to as phenotypic drift. Phenotypic drift has also been observed following either short term tissue culture or short term incubation of cultured cells. In these experiments serum and connective tissue components have been shown to influence the metastatic potential in many tumor lines (6,114,115).

It is clear from our experiments that B16F10 cells passaged five times in culture using DMEM supplemented with 10% FBS and 0.05 mg/ml gentamycin, exhibit a phenotypic drift which results in a decrease in metastatic potential of cells inoculated ic. Since phenotypic drift can affect cell adhesion, motility and enzyme activity, as well as immunogenicity and antigenicity (40,71,72,89,92,93,105,121,128), the explanation for the decreased ocular metastasis of cultured B16F10 cells awaits further elucidation.

In our experiments, the size of the primary ocular tumors resulting from the inoculation of cultured or in vivo derived cells was not significantly different 5-6 weeks post inoculation. However, the size of the primary ocular tumors was significantly less than that of primary flank tumors. While the explanation for this difference cannot be determined from our experiments, a decreased vascular supply and an increased compression of ocular tumors may be contributing factors.

In Niederkorn's experiments, all mice became moribund or died 35 days post inoculation of cultured cells, and no metastases were seen.

Animals were reported to have died from progressively growing intraocular tumors (78). In our experiments using cultured cells, only animals with evidence of metastasis died or became moribund. In spite of the presence at 6 weeks of large ocular tumors approximately 10 mm in diameter, the remainder of the animals appeared healthy and on necropsy were found to be free of metastatic nodules. Thus, even at 6 weeks primary tumor burden from these ocular tumors does not kill the host. In fact, even in our experiments utilizing sc implants and inoculations of *in vivo* derived tumor, mean diameters of flank tumors of approximately 10 mm were seen 2 weeks post inoculation and animals again appeared healthy and in fact lived an additional 3-4 weeks.

It has been shown that phenotypic changes occurring in cultured tumor cells are non-random and reproducibly occur at specific passage numbers *in vitro* (40,71,72). We used cells passaged five times in culture. The number of passages of B16F10 in the work of Niederkorn et al (75,79,130) was not specificed but may have been much greater than five. Thus, changes in some cellular property (production of some hydrolytic enzymes or factors capable of promoting intravascular coagulation, changes in immunogenicity or antigenicity, etc.) may have caused the death of the host in their experiments.

From our data, the question of enucleation for the prevention of ocular metastasis would appear to depend on the metastatic potential or phenotype of the tumor growing in the anterior chamber. Enucleation on day 4 or 7 was completely effective in preventing metastasis when cultured cells were inoculated ic. However, when the more highly metastatic *in vivo* derived melanoma cells were used, a decrease in the

frequency of metastasis and an increase in survival was noted only in animals enucleated 4 days post-implantation.

As a general statement concerning the efficacy of enucleation, it can be seen from our experiments this procedure resulted in either a decrease in the number of pulmonary metastases, a decrease in the frequency of metastasis, an increase in survival, or no observable effect. Enucleation never resulted in an observable increase in metastasis or decrease in survival. These findings are important since the only other work done on enucleation of tumor bearing eyes involved cultured cells of low metastatic potential. Thus, in the work of Niederkorn, enucleation alone had little or no effect on promoting metastasis, but enucleation in consort with immunological impairment promoted metastasis of intraocular melanoma (79). The ability of enucleation to prevent metastasis could not be studied since metastasis rarely occurred even in unenucleated animals.

The immunogenicity of JBRH seems to fall between that of the B16F10 cell line and tumors with potent tumor-specific transplantation antigens (TSTA). The immunogenicity appears to be greater than that of B16F10 melanoma in that immunization can be obtained by the sc inoculation of a single dose of 5×10^6 UV-irradiated tumor cells (6). However, this tumor behaves differently from syngeneic tumors with potent TSTA in that the latter fail to grow in the flank and only grow transiently in the anterior chamber(7,25,75,81,82). Nonetheless, qualitatively similar results to those obtained with B16F10 were obtained using <u>in vivo</u> derived JBRH; metastasis and survival was the same whether the tumor had been implanted in the flank or in the eye.

The growth and metastasis of syngeneic ocular tumors derived from cultured cells has been studied by others (73,75,77-79,81,82). As a result of this work deviant immune responses affecting both tumor growth and metastasis have been characterized. The growth of intraocular syngeneic tumors with potent TSTA is transient and is accompanied by a transient down-regulation of delayed-type hypersensitivity (DTH). Reappearance of DTH is associated with destruction of the ocular tumor by ischemic necrosis, and the destruction of distant lung metastasis is associated with Lyt 1^+, 2^+ lymphocytes (81,82). Less information is available concerning the growth and metastasis of syngeneic tumors of low immunogenicity. Cultured cells have been used to show that these tumors grow progressively in the anterior chamber of the eye and that intraocular tumor growth is associated with a long term suppression of DTH (78,130). In our work with in vivo derived B16F10 and JBRH melanoma, little or no difference in metastasis from ocular and extraocular locations is observed. However, using in vivo derived implants of a syngeneic mammary adenocarcinoma of low immunogenicity in rats, we have recently found differences between the metastasis of iridial and flank implants. The relevance of anterior chamber associated deviant immune responses to the growth and metastasis of in vivo derived syngeneic ocular tumors of low immunogenicity remains unclear.

Table 1. Pulmonary Metastasis of B16F10 Melanoma from Ocular and Subcutaneous Sites

Tumor Origin	Survival ‡	Pulmonary Metastases *				
				Size Distribution of Nodules †		
		Frequency §	Mean+SEM(Range)	0.1-0.5 mm	0.6-1.0 mm	1.0 mm
In Vivo Derived:						
Subcutaneous implant	0/7	7/7	195±19 (99-316)	140	40	15
Subcutaneous cell suspension	0/8	8/8	253±53 (69-459)	199	38	16
Iridial implant	0/10	10/10	227±89 (11-885)	158	57	12
Iridial implant with enucleation at day 4	7/10	3/10	88±57 (0-523)	64	19	5
Iridial implant with enucleation at day 7	0/11	11/11	304±16 (88-652)	229	48	27
Iridial implant with enucleation at day 10	0/12	12/12	92±25 (19-281)	70	15	7
Intracameral cell suspension	0/12	12/12	172±72 (5-851)	110	47	15
Intracameral cell suspension with enucleation at day 10	0/9	9/9	168±47 (6-347)	140	22	6
Tissue Culture Derived:						
Intracameral cell suspension	6/9	3/9	11±7 (0-60) ¶	11	0	0
Intracameral cell suspension with enucleation at day 4	7/7	0/7	0±0 (0) ¶	0	0	0
Intracameral cell suspension with enucleation at day 7	7/7	0/7	0±0 (0) ¶	0	0	0

* Pulmonary metastases determined when moribund or sacrificed at week 6 as described in Materials and Methods.

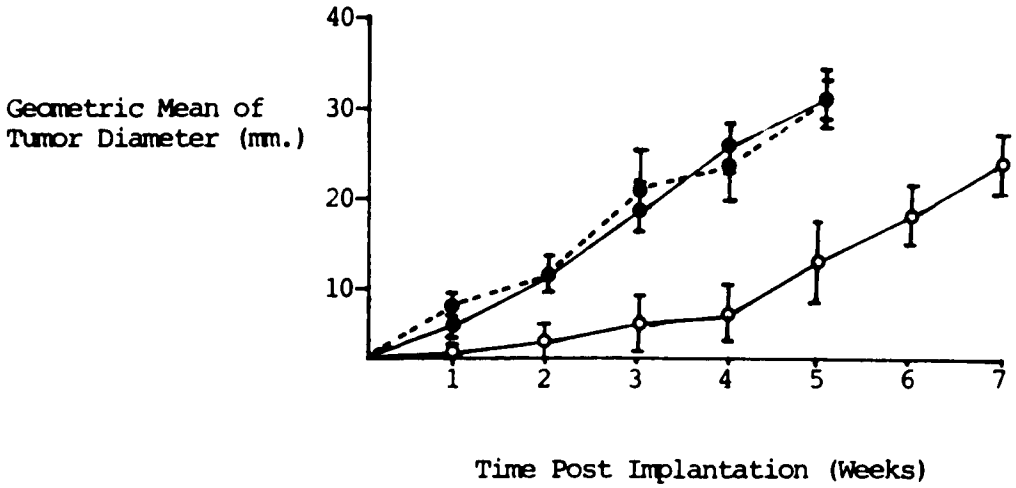
† Nodule size determined at X25; expressed as the mean in each of three categories.

‡ Survival = number of mice alive and healthy at 6 weeks post inoculation or implantation per number of mice examined.

§ Frequency = number of mice with one or more nodules per number of mice examined.

¶ Significantly different (P less than 0.05) when compared with animals receiving ic cell suspensions of *in vivo* derived tumor.

Figure 1. Subcutaneous flank growth rates of B16F10 and JBRH melanoma cell lines in C57BL/6J mice.



B16F10: Mice received either a 0.5 mm^3 fragment (—●—) N=7, or a cell suspension (- -●- -) N=8.
JBRH: Mice received 0.5 mm^3 fragments (—o—) N=11. Growth of the primary tumor was measured weekly. Vertical bars= ±SEM.

Chapter 2

A Treatment For Metastasis Of Murine Ocular Melanoma

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Abstract

Using cultured cells, LS2616 has been shown to decrease growth of primary tumors and pulmonary metastasis of murine melanoma (49). In the present study, we examine the efficacy of LS2616 for the prophylactic and therapeutic treatment of metastases from ocular and flank inoculations of the highly aggressive in vivo derived B16F10 melanoma in C57BL/6J mice. Experimental animals were treated with 160mg/kg/ day of this drug in drinking water, until they became moribund or died. When mice were pretreated for seven days and inoculated subcutaneously (sc) or intracamerally (ic) with 10^5 in vivo derived B16F10 tumor cells, the mean number of pulmonary metastases was significantly reduced, and the incidence of pulmonary metastases decreased. In ocular experiments, when pretreatment with drug was combined with enucleation at day 7, the mean number of lung nodules was significantly reduced, the incidence of metastasis to the lung and lymph nodes decreased, and survival increased. An apparent cure rate of 31% was observed. Treatment beginning on the day of enucleation (day 7) resulted in a reduction of pulmonary metastases, a decrease in metastasis to the lungs and lymph nodes, and no change in survival. LS2616 did not alter tumorigenicity of either sc or ic inoculations. In an in vivo neutralization assay, spleen cells of mice treated for 7 days with LS2616 demonstrated an increase in cytostatic or cytotoxic activity when incubated with B16F10 melanoma cells. Thus, the present study demonstrates that LS2616 is very effective in decreasing metastases of both ocular and extraocular tumors of the highly aggressive in vivo derived B16F10 melanoma and appears to possess immunostimulatory properties in this system. The potential

usefulness of LS2616 as a therapeutic agent for treating metastases of melanoma is discussed.

In a recent report, we described the ocular metastasis of in vivo derived B16F10 melanoma cells in syngeneic C57BL/6J mice, and found that melanoma cells passaged in culture exhibit a decrease in metastatic potential compared with that of melanoma cells passaged in vivo. In vivo derived B16F10 tumor cells metastasize extensively to the lungs from flank or ocular sites, and animals die of metastatic disease 6 weeks after receiving tumor. In contrast, metastasis from an ocular site is only observed in 33% of mice inoculated intracamerally with cultured cells (37).

LS2616, a quinoline 3-carboxamide (48), is a recently discovered immunomodulator with little or no toxic side effects, that is currently being used in clinical trials with patients in Sweden. It has been examined experimentally using cultured B16F10 tumor cells and shown to decrease primary and secondary tumor growth and metastasis (49). This drug enhances natural killer (NK) cell activity, the delayed type hypersensitivity reaction to bacterial antigens (104), and proliferative T cell responses (59), and provides a theraputic treatment in some murine autoimmune syndromes (111,112).

In the present study, we examine the effectivness of LS2616 in controlling the growth and metastasis of ocular and flank tumors of the highly metastatic in vivo derived B16F10 tumor. In animals treated with LS2616, we report: 1) a significant decrease in the number of pulmonary metastases from ocular and flank tumors, 2) a decrease in metastases, and an increased survival and cure in enucleated animals, 3) increased activity of cytolytic or cytostatic effector cells in the spleen, and 4) little or no effect on the growth of the primary tumor.

MATERIALS AND METHODS

Experimental Animals

Five to seven week old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) of both sexes were used as experimental subjects and to maintain the tumor line *in vivo*. Tumor bearing animals were examined and handled daily. Animals were judged as healthy if their physical appearance (with the exception of the presence of a primary tumor) and responses to handling were normal. Animals not judged to be healthy were classified as moribund. The present investigations conform to the ARVO Resolution on the Use of Animals in Research. Animals were maintained according to the recommendations outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

Drug Treatment

LS2616 was graciously provided by Dr.T.Stalhandske (AB Leo, Helsingborg, Sweden) and was administered to mice in drinking water at a dosage of 160mg/kg/day (49). When compared to control mice receiving drinking water only, this concentration does not affect total daily water intake.

Tumor Maintenance and Preparation

The B16F10 melanoma cell line was obtained in 1984 from Dr.Artemio Ovejera (N.I.H., Bethesda, MD). Tumor cells were maintained *in vivo* by subcutaneous (sc) implantations of $0.5mm^3$ fragments of tumor taken from the periphery of flank tumors carried for 3 weeks. Transfers were made by injecting tumor fragments suspended in 0.2ml of Hank's Balanced Salt

Solutions (HBSS, Sigma Chemical Co., St.Louis, MO) into the flank using a 16G needle.

Tumor cell suspensions were prepared by forcing $1.0cm^3$ pieces of 3 week old flank primary tumor through a 60 mesh stainless steel tissue grid. Cell suspensions were washed twice in HBSS.

Establishment of Primary Tumors

Intracameral (ic) and subcutaneous inoculations: Tumor inoculations were carried out as described previously (37). Briefly, mice were anesthetized by intraperitoneal (ip) injection with 0.06mg/g of Nembutal (Abbott Laboratories, North Chicago, IL) and the pupil dilated with one drop of 1.0% mydriacil (Alcon Laboratories, Fort Worth, TX). Using the Zeiss OPMI-1 dissecting microscope (25X), five microliters of a cell suspension containing 10^5 B16F10 cells in HBSS was inoculated into the anterior chamber. Flank inoculations were made as described previously (2).

Flank tumor growth was measured weekly using a vernier caliper.

Enucleation

Anesthetized mice were enucleated 7 days after ic inoculation of a tumor cell suspension and a suture used to close the eyelids. Enucleated eyes were dissected and examined as described previously (37). When using this protocol, enucleated eyes were always filled with tumor and recurrent growth of tumor in the orbit did not occur.

Assay for Metastasis

In most experiments, mice either died or became moribund and were necropsied. In one protocol noted below, healthy animals were sacrificed at 10 weeks and necropsied. In all experiments, the lungs, spleen,

liver, intestine, lymph nodes, brain, heart, and kidneys were removed from the animal and examined at 25X for evidence of metastasis. The number of lung metastases was determined as described previously (25). Nodule size was quantified and the diameter of nodules 0.1-0.5mm, 0.6-1.0mm, and greater than 1.0mm recorded.

In Vivo Neutralization Assay

A Winn-type neutralization assay modified by Merluzzi et.al (65) was used to assess the cytostatic or cytolytic activity of LS2616 activated splenocytes. Suspensions of 10^5 in vivo derived B16F10 melanoma cells were mixed with spleen cells from animals treated with LS2616 or with splenocytes from untreated animals at an effector/target ratio of 100:1. The suspension containing tumor cells and spleen cells was centrifuged at 30g for 5 min, resuspended in DMEM with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY) and incubated at 37°C and 5.0% CO_2 for five hours. The cell mixtures were washed three times in HBSS, resuspended to contain 10^5 tumor cells and 10^7 effector cells and injected sc into the flanks of mice.

Statistical Analysis

Differences in the mean number of nodules were analyzed using Duncan's Multiple Range Test (S.A.S. Institute, NC).

RESULTS

The effect of pretreatment with LS2616 on spontaneous metastasis from an ocular site in unenucleated mice

In order to examine the effect of LS2616 on metastasis of ocular tumor, eleven animals pretreated with 160mg/kg/day of LS2616 for seven days were then inoculated with 10^5 in vivo derived B16F10 melanoma cells, and given daily treatment with LS2616 for the duration of the experiment. Twelve animals were inoculated with tumor and received no drug treatment. All animals died or became moribund during the sixth week of the experiment. Thus, survival of animals treated with LS2616 (0%, 0/11) was similar to that of the controls (0%, 0/12, Table 1). The effect of drug treatment on the frequency and extent of metastasis was also examined. All animals in the untreated group had extensive pulmonary metastasis and tumor in the ipsilateral submandibular lymph nodes (SMLN). The frequency of pulmonary metastases decreased from 100% (12/12) in the untreated group to 73% (8/11) in the treated group. Whereas the frequency of SMLN metastasis remained unchanged. The mean number of pulmonary metastases in the drug treated group (21 $\pm$ 15.8 nodules/lung) was significantly less (P less than 0.01) than that seen in controls (172 $\pm$ 71.2 nodules/lung), Table 1, Fig.1.

The effect of pretreatment with LS2616 on spontaneous metastasis in enucleated mice

To determine the combined effect of enucleation and drug treatment, 13 animals that began treatment with LS2616 at day -7, were inoculated ic with 10^5 B16F10 tumor 7 days later and enucleated 7 days

post-inoculation (day +7). Drug was administered for the duration of the experiment. Control animals received no drug, and enucleation on day 7. Of the 13 drug-treated enucleated mice, 9 died 5 weeks post inoculation. The remaining 4 animals were healthy. These healthy mice were sacrificed 10 weeks post inoculation and showed no evidence of metastasis, and no recurrence of the primary ocular tumor. Thus, survival was increased and 31% of the mice appear to have been cured. In addition, the incidence of pulmonary metastases was reduced from 100% (7/7) in mice that received enucleation and no treatment to 54% (7/13) in mice that received enucleation and drug treatment. The incidence of metastasis to the ipsilateral SMLN was reduced from 100% (7/7), to 0% (0/13) in drug treated mice. The mean number of pulmonary metastases in the enucleation and drug-treated group was significantly less (P less than 0.01) than in the group that received enucleation but no drug (8 $\pm$ 3.9 nodules/lung compared to 191 $\pm$ 15.4 nodules, respectively), Table 1.

The effect of therapeutic treatment with LS2616 administered at the time of enucleation

In examining the effectiveness of drug treatment in controlling metastasis, when administered after enucleation, thirteen animals were inoculated ic with tumor, enucleated on day 7, and given daily drug treatment starting on day 7. Treatment and enucleation resulted in a decrease in pulmonary metastasis (58 $\pm$ 15.2 nodules/lung) when compared to controls (191 $\pm$ 15.4 nodules/lung). However, this decrease was not statistically significant. The incidence of pulmonary metastasis was reduced from 100% (7/7) to 85% (11/13), and metastasis to the ipsilateral SMLN was reduced from 100% (7/7) to 15% (2/13). Two

experimental animals died with no evidence of metastasis, Table 1.

The effect of pretreatment of LS2616 on spontaneous metastasis from a subcutaneous site

In order to determine the effectiveness of LS2616 on metastasis from an extraocular location, ten mice were pretreated on day -7 with LS2616 (160mg/kg/day), received sc inoculations of 10^5 B16F10 tumor cells on day 0, and continued to receive drug treatment daily throughout the remainder of the experiment. Eight control mice received sc tumor inoculation but no drug. Treatment of sc inoculated mice resulted in a significant decrease (P less than 0.01) in pulmonary metastases (22 $\pm$ 7.2 nodules/lung) when compared to sc inoculated animals that had received no drug treatment (195 $\pm$ 19.1 nodules/lung). The incidence of pulmonary metastasis decreased from 100% (8/8) in the untreated group to 50% (5/10) in the sc inoculated and treated mice.

The effect of pretreatment of LS2616 on the growth of primary tumors

In order to determine whether treatment of hosts with LS2616 would affect the growth of the primary tumor, animals were pretreated for 7 days with drug, inoculated sc with tumor, and given drug for the duration of the experiment. The primary tumor was measured weekly. Tumor grew in all animals and no differences in the geometric mean tumor diameters were seen between control (N=8) and experimental (N=8) animals (Fig.2). In order to determine if drug treatment could decrease tumor take at a lower tumor burden, 6 animals were pretreated with drug and inoculated sc with 10^4 tumor cells. Tumorigenicity in the group was again 100%. Furthermore, in all of the ic inoculated drug-treated groups shown in Table 1, tumorigenicity was always 100% (N=56).

In Vitro Neutralization Assay

In order to determine whether spleen cells from drug-treated animals could effect the tumorigenicity of B16F10 cells, a Winn assay as modified by Merluzzi et. al (65) was performed. Animals were treated with drug for 7 days. Control animals received no drug. Splenocytes from either drug treated animals (N=8) or untreated mice (N=8) were incubated with tumor cells for 5 hours and injected sc into the flank of recipient mice. In addition, tumor cells in DMEM or DMEM alone were also inoculated into groups (N=8, each group) of recipient mice. None of the animals (0/8) inoculated with tumor cells incubated with splenocytes from drug treated animals had palpable tumor at day 21 post-inoculation. In contrast, all recipients inoculated with tumor cells incubated alone in DMEM (8/8), or with splenocytes from untreated mice (8/8), had palpable tumors 21 days after inoculation (Table 2).

DISCUSSION

Our results demonstrate the effectiveness of LS2616 against lymph node and pulmonary metastasis of *in vivo* derived B16F10 melanoma. When mice pretreated with drug were inoculated with tumor intraocularly or subcutaneously and the primary tumor left intact, there was no effect on the primary tumor. However, a significant decrease in the number of pulmonary metastases was seen. The incidence of metastasis also declined in both groups. Experimental mice, however, showed no increase in survival and eventually died. It is possible that deleterious effects of very large primary tumors contributed to morbidity.

When mice with ocular tumors were enucleated and treatment with LS2616 initiated the day of enucleation, the incidence of pulmonary and lymph node metastasis was reduced. A decrease in the number of pulmonary metastases was seen, but was not statistically significant, and survival was unaffected. In contrast, the combination of pretreatment with LS2616 and enucleation, resulted in an apparent cure in 31% of the mice. These mice were healthy and appeared to be completely free of metastatic growth when sacrificed and necropsied 10 weeks post inoculation. In the group as a whole, the extent of pulmonary or SMLN metastasis was markedly decreased in the drug-treated enucleated mice.

In unenucleated mice, pretreatment with LS2616 did not affect the incidence of metastasis to the ipsilateral SMLN. In enucleated mice pretreatment or treatment at the time of enucleation markedly decreased or prevented metastasis to the ipsilateral SMLN and only partially affected the incidence of pulmonary metastasis. This demonstrates varying degrees of susceptibility of different organ metastases to

treatment with LS2616 and suggests that the effectiveness of this drug may be dependent upon the tumor burden of the host.

It is interesting to note that in earlier work, the only metastases we observed from primary tumors of B16F10 were in the lungs. In more recent studies, however, we have also been observing metastasis to the ipsilateral submandibular region of both enucleated and unenucleated mice. It would appear that there has been an increase in the metastatic capability of this in vivo passaged line.

Previous work by others has demonstrated that LS2616 treatment in C57Bl/6 mice results in an increase in NK activity and an immunostimulatory affect on macrophages which indirectly facilitates polyclonal and antigen specific T cell responses (59,104). Our modified Winn assay confirms the fact that a drug stimulated spleen cell population when incubated with B16F10 melanoma cells at a ratio of 100:1 effector to target cells, is capable of causing a decrease in tumorigenicity.

The importance of NK cell activity in preventing metastasis of murine ocular melanoma remains unclear. Yokoyama et.al (130) reported a significant increase in pulmonary metastases of B16 melanoma when NK cell activity was inhibited with anti-GM1 antibodies and a significant decrease in pulmonary metastases when NK cell activity was stimulated with interferon gamma. Niederkorn, in earlier reports (79,80) suggested NK cell activity did not play a significant role in increasing pulmonary metastases of in vitro cultured B16F10 melanoma. In his system there is evidence that T-cell effectors play a critical role in preventing metastasis. Recently, selective T-cell depletion experiments continue to

support Niederkorn's earlier findings (83).

In our experiments, pretreatment with LS2616 did not affect tumor take in animals receiving 10^4 or 10^5 tumor cells sc or 10^5 tumor cells intracamerally. Kalland (49) reported a decrease in tumorigenicity in LS2616 pretreated animals. The reason for the discrepancy between our results and that of Kalland's is not known, however a major difference in our experiments was our use of in vivo derived as opposed to cultured tumor cells. It is possible that the lack of effectiveness of LS2616 in reducing tumor take in our experiments is due to the greater tumorigenicity of in vivo passaged tumor.

Until recently, there have been no chemotherapeutic agents shown to be effective in treating metastases arising from intraocular melanoma. In humans, current chemotherapeutic strategies range in response rates from 17% to 21% (22,34,67) while the response rates of immunotherapeutic strategies vary from 22% to 25% (16,43). Niederkorn, using cultured B16F10 tumor cells, has shown that administration of difluoromethylornithine (DFMO) decreases the number of pulmonary metastases in enucleated mice (84). Little increase in survival was noted in these experiments. It is difficult to compare the usefulness of DFMO with that of LS2616 as a therapy for treating metastases of murine intraocular melanoma, as there are major differences between the experimental systems used by Niederkorn and co-workers and our laboratory. One major difference between our protocols is the use of cultured versus in vivo derived cells. A second major difference is that in order to obtain baseline or control metastasis of cultured cells, Niederkorn's group renders control mice immunoincompetent by sublethal

irradiation and subjects them to traumatic enucleation involving squeezing the globe 10 times before its removal (79,80,84). It is important to note that even these procedures result in fewer pulmonary metastases in control animals, 39 nodules/lung, compared to 191 nodules/lung in our work with immunocompetent mice and in vivo derived B16F10 melanoma (84). Using in vivo derived tumor obtained from our laboratory, Niederkorn (personal communication) has recently confirmed our earlier observations (37) and found that this line spontaneously metastasizes from the eye of immune competent C57BL/6 hosts. In contrast, cultured cells, including those recently obtained from the Frederick Cancer Center do not metastasize from the eye unless hosts are immunosuppressed or the eyes subjected to trauma.

In summary, LS2616 effectively decreases metastasis from subcutaneous and intraocular primary tumors of in vivo derived B16F10 melanoma. The ability to achieve apparent cures when enucleated mice are pretreated with this drug is promising. It would seem that a combination of LS2616 treatment with other therapeutic strategies may prove to be an effective means of decreasing metastases and increasing survival after enucleation. We are currently examining the effectiveness of LS2616 combined with other immunomodulatory strategies in an attempt to increase survival and effect cures when treatment is begun immediately prior to or at the time of enucleation. Although LS2616 has been shown to have potent immunostimulatory effects, and it is effective against metastases from ocular and flank tumors in mice, its usefulness in humans with uveal melanoma is only speculative at this time.

Acknowledgments

We gratefully acknowledge the interest and advice of Dr. Vincent J. Merluzzi and the technical assistance of N. Gelernter.

TABLE 1. The effect of LS2616 treatment on tumor growth and metastasis of B16F10 melanoma inoculated subcutaneously and intraocularly.

Experimental Protocol	Tumorigenicity‡ %	Survival§ %	Number of Pulmonary Metastasis* Mean ±SEM(Range)	Size Distribution of nodules† 0.1-0.5mm	0.6-1.0mm	1.0mm	Incidence* %	SMLN Incidence¶ %
SC Inoculations								
No LS2616	100(7/7)	0(0/7)	195±19.1(99-316)	140	40	15	100(7/7)	0(0/7)
LS2616	100(10/10)	0(0/10)	22±12.1(0-102)**	12	4	6	50(5/10)	0(0/10)
IC Inoculations								
No LS2616, no enuc.	100(12/12)	0(0/12)	172±71.2(21-289)	102	59	11	100(12/12)	83(10/12)
LS2616, no enuc.	100(11/11)	0(0/11)	21±15.8(0-182)**	16	4	1	73(8/11)	100(11/11)
No LS2616, enuc.	100(7/7)	0(0/7)	191±15.4(142-256)	132	34	25	100(7/7)	100(7/7)
LS2616, enuc.	100(13/13)	31(4/13)	8±3.9(0-46)**	6	1	1	69(9/13)	0(0/13)
Enuc., LS2616	100(13/13)	0(0/13)	58±15.2(0-178)	39	11	8	85(11/13)	15(2/13)

*Pulmonary metastases determined when moribund or sacrificed at week 10 post inoculation as described in Materials and Methods.
† Nodule size determined at 25X; expressed as the mean ± SEM (range) in each of three categories.
‡ Tumorigenicity = percent, and number of mice with palpable tumors at day 21 (sc) or number of mice with tumor in the anterior chamber at day 7 per number of mice examined.
§ Survival = percent, and number of mice alive and healthy at 6 weeks post inoculation per number of mice examined
* Incidence = percent, and number of mice with one or more nodules per number of mice examined.
¶ SMLN Incidence = percent, and number of mice with metastasis to the ipsilateral submandibular lymph nodes per number of mice examined.
**Significantly different (P less than 0.01) when compared to controls.

Table 2. In Vivo neutralization of B16F10 inocula by LS2616-activated cytotoxic cells.

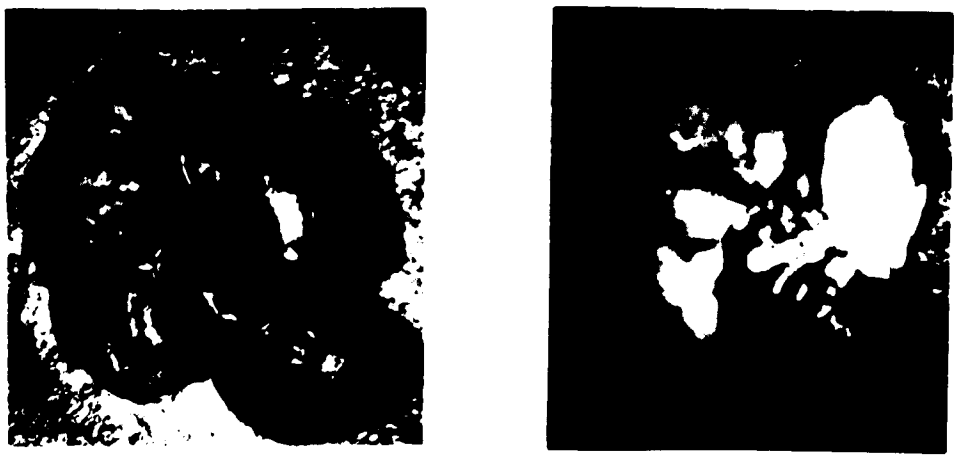
Transferred Cells[1]		
10^5 B16F10 melanoma cells[2]	10^7 effector cells[3]	Tumor Incidence[4]
+	-	8/8
+	control spleen cells	8/8
+	drug-stim. spleen cells	0/8

[1] Effector/target cell ratio = 100:1; sc flank injection.

[2] B16F10 melanoma cell suspension was derived from *in vivo* passaged tumor in C57Bl/6J host.

[3] Effector cells were taken from normal C57Bl/6J mice or from mice treated for 7 days with 160mg/kg of body weight of LS2616. Effector and target cells were incubated for 5 hours in DMEM before inoculation at a concentration of 10^5 melanoma/10^7 effector cells into the flank of recipient mice.

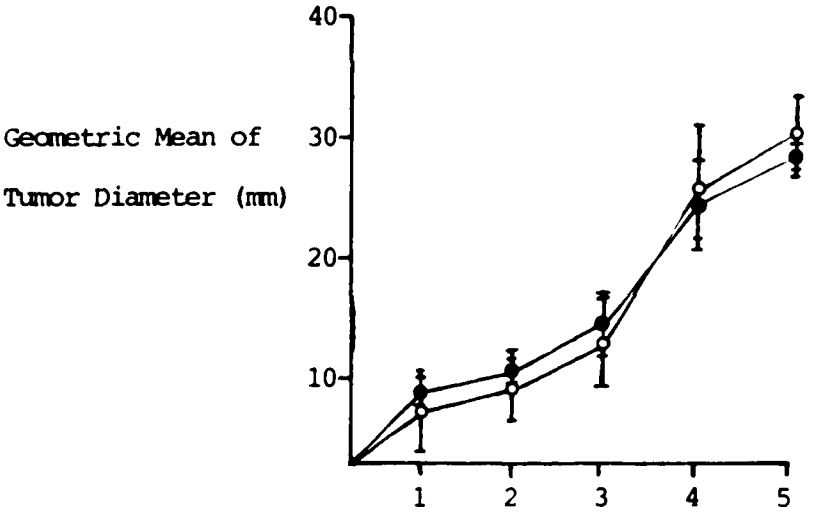
[4] Tumor incidence = number of mice with palpable tumor at day 21 per number of mice examined.



1A. 1B.

Fig.1. Pulmonary metastasis of in vivo derived B16F10 melanoma. 1A. Typical control lung (ic inoculated, unenucleated animal) at 6 weeks post inoculation. 1B. Typical LS2616-treated lung (ic inoculated, unenucleated animal) at 6 weeks post inoculation.

Figure 2. Subcutaneous growth of B16F10 primary tumor in control and LS2616 treated mice.



Mice received 10^5 *in vivo* derived B16F10 melanoma cells sc and normal drinking water (–●–) or daily treatment with 160mg/kg body weight of LS2616 in drinking water (–○–).

Growth of the primary tumor was measured weekly. Vertical bars = ± SEM.

Chapter 3

Regulation of the Metastasis of Murine Ocular Melanoma by Natural Killer Cells

This chapter has been submitted for publication in Investigative Ophthalmology and Visual Science.

ABSTRACT

In the present study we examine parameters affecting the metastasis of ocular tumors of in vivo derived B16F10 melanoma. In C57BL/6J beige (bg/bg) mice, with low NK activity, metastasis to the lungs was increased and survival time decreased. In C57BL/6J normal (+/+) mice treatment with PK136, a highly specific monoclonal anti-NK antibody (Ab), caused a depletion of NK cytotoxic activity as demonstrated using a standard ^{51}Cr release assay. In animals bearing ocular tumors, treatment with PK136 Ab resulted in significantly increased pulmonary metastasis and an altered pattern of metastasis. The effect of combined treatment protocols using LS2616 and cyclophosphamide (Cy) was examined in enucleated and unenucleated animals. Treatment with LS2616 and Cy resulted in a significant decrease in mean pulmonary metastases (MPM), a decreased frequency of metastasis to the submandibular lymph nodes, and an increase in mean survival time. In enucleated mice this combined treatment protocol resulted in apparent cures, the lowest MPM and the longest survival time observed. When tumor bearing mice were treated with either silica, carrageenan, or sublethal gammma irradiation, no effect on metastasis or survival was observed.The present study demonstrates the importance of the NK cell as a primary effector cell for the control of metastasis from in vivo derived ocular B16F10 melanoma.

The role of cellular immunity in the regulation of metastasis from ocular murine melanoma has been examined by others using cultured B16F10 cells. In these experiments, the importance of cytotoxic T cells in regulating metastatic growth of in vitro derived ocular B16F10 melanoma in immune-compromised mice was demonstrated. NK cells did not appear to play a role in the regulation of metastasis in this system (79,83). In contrast, Yokoyama et.al (130) have shown that enhancement of NK activity with alpha-interferon resulted in significantly fewer pulmonary metastases from in vitro derived ocular B16 melanoma in normal C57BL/6 mice. Therefore, it is still not clear if NK cells regulate metastasis in these systems.

LS2616, a newly discovered quinoline 3-carboxamide, has been shown by others to increase NK cytotoxic activity (48) and reduce pulmonary metastases arising from subcutaneous (sc) and intravenous (iv) inoculations of in vitro derived B16F10 melanoma cells (49). More recently, we demonstrated the effectiveness of LS2616 (Linomide) in inhibiting metastasis of ocular and subcutaneous tumors of highly aggressive in vivo derived B16F10 tumor cells (38). In vivo derived cells differ from cultured B16F10 cells in spontaneously metastasizing from the eye of immune competent C57BL/6J hosts (37). When combined with enucleation 7 days after intracameral (ic) inoculation of tumor cells, LS2616 therapy resulted in an apparent cure in some animals. In these experiments we noted that LS2616-enhanced splenic effector cells were capable of causing a significant reduction of pulmonary metastasis from ic and sc inoculations of in vivo derived B16F10 melanoma. The identity of the effector cell was not determined.

Monoclonal Ab PK136 detects NK1.1 antigen on NK cells and has been found to be useful in eliminating greater than 95% of splenic NK activity in C57BL/6J mice (52,53). PK136 Ab treatment does not alter other lymphocyte populations in the spleen, and does not effect T cell cytotoxicity or humoral immunity (109). This Ab has been used to demonstrate the role of NK cells in the regulation of experimental metastasis and survival in mice inoculated iv with in vitro derived B16F10 melanoma cells (109).

In the present experiments we examine parameters affecting metastasis of in vivo derived B16F10 ocular tumors and further explore the role of NK cells in the system.

MATERIALS AND METHODS

Experimental Animals

Five to seven week old C57BL/6J normal (+/+), and beige (bg/bg) mice (Jackson Laboratories, Bar Harbor, ME) of both sexes were used as experimental subjects. Normal C57BL/6J mice were used to maintain the tumor line *in vivo*. Tumor bearing animals were examined as described earlier (38). Mice were maintained according to the recommendations outlined in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council. The present investigations conform to the ARVO Resolution on the Use of Animals in Research.

Tumor Maintenance and Preparation

The B16F10 melanoma cell line was obtained in 1984 from Dr.Artemio Ovejera (N.I.H., Bethesda, MD). Tumor cells were maintained *in vivo* as described previously (38).

Tumor cell suspensions were prepared by gentle trypsinization (0.25% trypsin in DMEM, at 37^{o}C in 5.0% CO_2 for 3 minutes) of 1cm^3 pieces of primary tumor grown in the flank. Cell suspensions were washed 2X in DMEM.

Establishment and Resection of Primary Tumors

Tumor inoculations and enucleations were carried out as described previously (37) except that lower concentrations of tumor cells were used in the present experiments. Briefly, mice were anesthetized by intraperitoneal (ip) injection with Nembutal (Abbot Laboratories, North Chicago, IL) and a 5 microliter cell suspension of 10^2 B16F10 tumor cells in

DMEM was inoculated into the anterior chamber. Anesthetized mice were enucleated 10 days later (E10) and a suture used to close the eyelids. Enucleated eyes were always filled with tumor and recurrent growth of tumor in the orbit did not occur.

Drug Treatment and Irradiation

LS2616 was graciously provided by Dr.T.Stalhandske (AB Leo, Helsingborg, Sweden) and was administered to mice in drinking water at a dosage of 160mg/kg/day as described previously (38). LS2616 treatment began 7 days prior to inoculation of tumor cells and continued for the duration of the experiment. Cyclophosphamide (Cy; Sigma Chemical Co., St.Louis, MO) was dissolved in sterile saline and administered ip weekly at a dose of 25mg/kg. Treatment began one day after inoculation of tumor cells. Ip injections of either silica or carrageenan (Sigma Chemical Co.,St.Louis, MO) at a dosage of 1mg/mouse were administered weekly (138). Treatment began one day after inoculation of tumor cells. Mice were irradiated (500 rads, ^{137}Cs) at a dosage of 117 rads/minute.

Antibody Treatment

The derivation of monoclonal Ab PK136 has been described previously (53). Administration of PK136 Ab can be maintained for 20-24 weeks (52). A mouse IgG2a monoclonal Ab, H16-L10-4, was used in some experiments as a specificity control. This Ab recognizes influenza A virus nucleoprotein and is not known to react with mouse antigens (137). PK136 and H16-L10-4 were diluted 1:10 in sterile saline and 0.5ml of diluted antibody was injected ip into mice. Mice were injected with Ab every 7-10 days, beginning one week before inoculation of tumor cells.

Assays

In Vitro NK cytotoxicity: Natural killer cell activity in spleen cells was assessed against YAC-1 tumor cells by using a 4 hour ^{51}Cr-release assay (52).

Metastasis: In most experiments, mice either died or became moribund and were necropsied. However, in some protocols noted below, healthy animals were sacrificed and necropsied. In all experiments, the lungs, spleen, liver, intestine, lymph nodes, brain, heart, kidneys, and adrenals were removed and examined at 25X for evidence of metastasis. The number of metastases was determined as described previously (25).

Mean Survival Time: Mean survival time was calculated as the average number of days between tumor cell inoculation and either morbidity or the sacrifice and necropsy of apparently healthy mice at the end of 11 weeks. Frequency of survival was the number of mice alive and apparently healthy at the end of 11 weeks/ number of mice in the group.

Statistical Analysis

Differences in the mean number of nodules or mean survival time were analyzed using Duncan's Multiple Range Test (S.A.S.Institute, NC).

RESULTS

Survival of bg/bg and control (+/+) mice bearing intracameral tumors.

The bg/bg mutation in the C57BL/6J mouse is an autosomal recessive mutation that results in severely depressed NK cell activity, decreased T cell function and decreased macrophage cytotoxicity (103,104). In the present experiments, bg/bg mice received ic inoculations of B16F10 tumor. Tumorigenicity and survival of hosts was compared to that observed in control (+/+) mice. In examining the effect of varying concentrations of tumor cell inocula on growth and metastasis in C57BL/6J (+/+) mice, we found that 10^2 tumor cells inoculated ic results in 100% tumorigenicity and pulmonary metastasis, and death within 5-6 weeks. We now routinely inoculate 10^2 tumor cells in most of our ic experiments. When nine bg/bg mice received ic inoculations of 10^2 melanoma cells, mean survival was 16.6±1.8 days. In contrast, nine control (+/+) mice receiving 10^2 tumor cells had a survival of 31.1±1.6 days. When eight bg/bg mice received 10^3 tumor cells, the survival time was 22.3±2.2 days. Control (+/+) animals receiving 10^3 cells ic survived 32.3±1.7 days. At both tumor cell concentrations, survival was significantly decreased in bg/bg mice, Table 1. The mean survival of bg/bg mice inoculated ic with 10^2 tumor cells was not statistically different from that of bg/bg mice receiving 10^3 tumor cells. Tumorigenicity was 100% in all experimental and control groups.

Metastasis of intracameral tumors in bg/bg and control (+/+) mice.

To determine the effect of the beige mutation on pulmonary metastasis in mice harboring ic tumors derived from *in vivo* derived B16F10 melanoma, bg/bg mice were inoculated ic with tumor cells and

sacrificed when moribund. Control (+/+) mice received ic tumor cells and were sacrificed at the time that experimental animals had become moribund. Nine bg/bg mice received ic inoculations of 10^2 tumor cells and survived an average of 16.6±1.8 days. Seven of nine had pulmonary metastases and the mean number of pulmonary metastases (MPM) was 30.5±11.3. Six control (+/+) mice that received 10^2 tumor cells were sacrificed on day 17 and the MPM was 1.3±0.7 (P less than .01), Table 2.

Eight bg/bg mice received ic inoculations of 10^3 melanoma cells and their survival was 22.3±2.2 days. All eight animals had pulmonary metastases and the MPM was 120.0±23.2. In contrast, only three of six control mice receiving 10^3 tumor cells ic and sacrificed at day 23 had pulmonary metastases and the MPM was only 2.4±2.5 (P less than .01).

The effect of LS2616 and cyclophosphamide on survival and metastasis in enucleated or unenucleated tumor bearing mice.

The present experiments were designed to examine the effect of LS2616 and Cy on survival and tumor metastasis in mice harboring ic tumors. Treatment of unenucleated mice with LS2616 alone, decreased both MPM and the incidence of SMLN, but had no effect on survival (Table 3). Cy alone had no effect on survival or metastasis. Combined treatment with LS2616 and Cy decreased the MPM and frequency of SMLN metastasis and increased survival. The decrease in pulmonary metastasis was significantly greater than that seen using LS2616 alone.

The effect of drugs was also examined in animals whose tumor-filled eyes were enucleated 10 days post inoculation of tumor cells. Enucleation alone had no effect on MPM or frequency of SMLN metastasis (Table 3). Enucleation plus LS2616 treatment resulted in apparent cures.

Two of seven (29%) mice remained healthy and were sacrificed and necropsied 11 weeks after enucleation. These mice showed no evidence of metastasis. The group of enucleated and LS2616 treated mice, as a whole, showed a decrease in MPM and frequency of SMLN metastasis and an increase in mean survival time. When enucleated animals treated with LS2616 and Cy combined were examined, the apparent cure rate was (22%), and the group as a whole showed a significant increase in mean survival time. In fact, survival time was the greatest observed in any treatment regimen. MPM was low, and SMLN metastasis was completely prevented (Table 3).

Metastasis of intracameral melanoma in PK136 Ab treated mice.

To study the effect of marked NK cell depletion on growth and metastasis of ocular tumors, PK136 Ab was administered, as described, to mice inoculated ic with in vivo derived B16F10 melanoma cells. PK136 Ab treatment resulted in the greatest value for MPM ever observed using a 10^2 tumor cell inoculum (Table 4).

Interestingly, treatment with PK136 resulted in the appearance of extra-pulmonary metastases never previously observed in our model of B16F10 murine ocular melanoma (Table 4). Metastasis was observed in adrenal glands (4/10), spleen (4/10), and liver (2/10). In order to examine the effect of PK136 Ab treatment on the LS2616-induced decrease in metastasis from ic tumors, animals were treated with PK136 Ab, given LS2616 as described, and inoculated ic with 10^2 tumor cells. PK136 Ab completely abrogated the decrease in both the MPM and frequency of metastasis to the SMLN induced by LS2616. Moreover, these mice had the same MPM as control untreated mice (Table 4).

Sustained enhancement or depletion of NK cell activity with LS2616 or PK136 Antibody.

In the present experiments NK cell activity was assayed in order to ascertain the effect of sustained treatment with either PK136 Ab, or LS2616. Panels of three mice were treated with either saline, H16-L10-4 Ab, PK136, or LS2616, sacrificed 7 or 42 days after the start of treatment, and in vitro lysis of ^{51}Cr-labeled YAC-1 target cells measured (Table 5). Mice treated with PK136 Ab and sacrificed at day 7 showed a significant reduction in NK lysis of YAC-1 target cells when compared with saline or H16-L10-4 treated animals (P less than .01, Table 5). Mice treated daily for 7 days with 160 mg/kg of LS2616 in drinking water and sacrificed at day 7 showed significantly enhanced NK cell activity when compared to saline treated controls (P less than .01, Table 5). Mice treated with PK136 Ab and sacrificed at day 42 continued to show a significant decrease (P less than .01) in lytic activity while mice treated with LS2616 and sacrificed at day 42 continued to show a significant increase (P less than .01) in NK activity when compared with controls (Table 5).

The effects of treatment with silica, carrageenan, or gamma irradiation on metastasis of intracameral tumors.

Silica or carrageenan treatment has been shown by others to be a useful treatment for the reduction of macrophage activity (15,16,17). In mice inoculated ic with 10^2 B16F10 tumor cells, weekly injections of either silica or carrageenan resulted in MPM of 155.0±62.5/lungs and 191.3±56.3/lungs, respectively. MPM in experimental mice was not significantly different from that observed in controls receiving ic tumor but no drug treatment. Similarly, drug treatment did not appear to

affect metastasis to the SMLN. The frequency of metastasis to the SMLN was 8/9 in the controls and 4/6 and 5/6 in the silica and carrageenan groups, respectively (Table 6).

Sublethal gamma irradiation reduces T cell immune responses in the C57BL/6J mouse (1,2,18). In the present study, panels of mice were inoculated ic with tumor cells and irradiated on day 0 (N=8), day 7 (N=7), or day 21 (N=7) post inoculation. No statistically significant differences were seen in the MPM or SMLN when control mice were compared with mice that had received irradiation (Table 6).

DISCUSSION

Natural killer cells are large granular lymphocytes with cytolytic activity against a variety of tumor and virus infected cells (22,23,24). NK deficient mice and rats have been shown to have an increased incidence of spontaneous lymphomas (50) and enhanced growth of metastases from different tumor cell lines (85,109). In addition, NK cells may play a role in the regulation of growth and metastasis of human neoplasms (42,46,54).

In the present experiments, bg/bg mice and PK136 Ab treated +/+ mice were used to examine the role played by NK cells in metastasis of ocular tumors of in vivo derived B16F10 melanoma. Monoclonal Ab PK136 is specific for NK1.1 antigen in C57BL/6J mice, and can be used repeatedly in vivo for long term depletion of NK cells without altering T cell cytotoxicity or humoral immunity (101). In contrast, anti-asialo GM1 Ab, utilized in many NK depletion studies, recognizes some macrophage (1,2) and T cell populations (116) and may induce serum sickness in long term studies (52).

Treatment with PK136 Ab resulted in greater than 95% depletion of NK activity (Table 5). In mice bearing ocular tumors, pulmonary metastasis was increased and metastasis was seen in the spleen, adrenal glands, and liver (Table 4). This altered pattern of metastasis was striking, as we have only previously observed pulmonary and lymph node metastasis with the B16F10 melanoma cell line. These results suggest that NK cell immune surveillance may play a critical role in the regulation of the pattern of metastasis. In comparable experiments, animals harboring flank tumors and depleted of NK cells with PK136 Ab,

show occasional extrapulmonary metastasis, but do not demonstrate the pronounced alteration in metastatic pattern observed in mice harboring ocular tumors (manuscript in preparation). We speculate that NK cells in consert with some feature of anterior chamber-associated immune deviation (ACAID), may determine the pattern of metastasis of ocular B16F10 melanoma.

Examination of metastasis in bg/bg mice showed that these animals had an increase in pulmonary metastasis, and a decrease in survival when compared to control +/+ mice. In contrast, survival was not affected in PK136 Ab NK-depleted tumor bearing mice with ic tumors, even though these animals had a much greater tumor burden in the lungs. In addition, bg/bg mice did not show an altered pattern of metastasis. These differences may be due to the fact that bg/bg mice have decreased T cell functions and decreased macrophage cytotoxicity (13,14) in addition to having decreased NK activity.

LS2616 treatment activates NK cell activity in C57BL/6J mice (Table 5). In mice bearing ocular tumors, this drug resulted in a decrease in pulmonary and SMLN metastasis, but did not increase survival (Table 3, ref.38). Combined treatment of PK136 Ab with LS2616, gave results indistinguishable from those observed in saline treated control tumor bearing animals suggesting that the effect of LS2616 is dependent on the presence of NK cells, as previously implicated (48). In contrast, when LS2616 was used in conjunction with Cy therapy, a decrease in pulmonary and SMLN metastasis was seen and an increase in survival was obtained. This is interesting since Cy alone was incapable of affecting survival time or metastasis in our system, and LS2616 therapy alone, had no

effect on survival. When LS2616 and Cy were combined with enucleation at day 10, an apparent cure rate of 22% was observed, and the mean survival time of the group as a whole was the greatest observed in any experimental group. With this drug combination, very low values were obtained for pulmonary metastasis, and metastasis to the SMLN was completely abolished.

Low doses of Cy have been shown to enhance T cell functions by removal of T suppressor cell activity (86,97). Our results suggest the possibility that a decrease in suppressor activity in conjunction with stimulation of NK cell activity may act to reduce metastatic growth and increase survival. These effects would appear to be maximized under conditions of reduced tumor burden (i.e. enucleation).

Silica or carrageenan treatment of mice harboring ic tumors had no effect on the numbers of pulmonary metastases or survival in tumor bearing animals. These results are consistent with those obtained by Yokoyama et. al who reported no increase in pulmonary metastasis using similar protocols (138) and cultured cells of the B16 melanoma cell line.

Gamma irradiation at doses known to reduce T cell function in mice had no effect on metastasis to the lungs or SMLN, or on survival in our model. Merluzzi (66) has demonstrated that NK cells are resistant to sublethal doses of gamma irradiation (500 rads), while cytotoxic T lymphocytes are sensitive and can be depleted. Therefore, our findings again suggest that NK cells are important in this system. Our results obtained using gamma irradiation appear to be in conflict with data obtained by Niederkorn (83), who demonstrated the importance of Thy-1$^+$,

Lyt-1^+, Lyt-2^+ lymphocytes in controling ocular metastasis arising from ic inoculations of B16F10 tumor cells. However, our experiments are not directly comparable. One major difference in our systems is the use of in vivo as opposed to in vitro derived B16F10 tumor cells (38). In addition, use of cultured cells necessitated the utilization of irradiated hosts as well as traumatic enucleation of both control and experimental mice in order to obtain pulmonary metastases (83).

In summary, our experiments clearly demonstrate the importance of the NK cell in regulating metastasis from ocular tumors produced by ic inoculation of in vivo derived B16F10 melanoma cells. In experimental protocols examining the effect of drug therapy on ocular metastasis in tumor bearing mice, treatment with the NK-enhancer LS2616 in conjunction with low doses of Cy, was shown to be effective in decreasing metastasis and increasing survival. An apparent cure rate of 22%, and the greatest control of metastasis and increase in survival, was seen in enucleated mice receiving both Cy and LS2616 treatment.

Table 1. Survival of bg/bg and control mice inoculated intracamerally with in vivo derived B16F10 melanoma cells

	Survival		
Genotype/Inoculum[1]	Frequency	Mean Days (±SEM)[2]	Tumorigenicity[3]
Control/IC 10^2	0/9	31.1 (±1.6)	9/9
beige /IC 10^2	0/9	16.6 (±1.8)[4]	9/9
Control/IC 10^3	0/8	22.3 (±1.7)	8/8
beige /IC 10^3	0/8	22.3 (±2.2)[4]	8/8

[1] Inoculum = control (+/+) or beige (bg/bg) mice received intracameral inoculations of either 10^2 or 10^3 B16F10 melanoma cells.

[2] Mean days = mean survival time as described in Materials and Methods.

[3] Tumorigenicity = number of mice that developed ocular tumors / number of mice per group.

[4] P less than .01

Table 2. Pulmonary metastasis of in vivo derived B16F10 melanoma cells inoculated intracamerally in bg/bg and control mice

	Survival	Pulmonary Metastases	
Genotype/Inoculum[1]	Mean Days (±SEM)[2]	Frequency[3]	Mean (±SEM)[4]
Control/IC 10^2	17.0[5]	3/6	1.3 (±0.7)
beige /IC 10^2	16.6 (±1.8)	7/9	30.5 (±11.3)[6]
Control/IC 10^3	23.0[5]	3/6	2.4 (±2.5)
beige /IC 10^3	22.3 (±2.2)	8/8	120.0 (±23.2)[6]

[1] Inoculum = control (+/+) or beige (bg/bg) mice received intracameral inoculations of either 10^2 or 10^3 B16F10 melanoma cells.

[2] Mean days = mean survival time as described in Materials and Methods.

[3] Frequency = number of mice with one or more nodule / number of mice per group.

[4] Mean number of nodules per mouse.

[5] Apparently healthy tumor bearing mice were sacrificed.

[6] P less than .01

Table 3. Effect of LS 2616 and Cyclophosphamide treatment on survival and metastases in C57BL/6J mice inoculated intracamerally with 10^2 in vivo derived B16F10 melanoma cells.

	Survival		Pulmonary Metastases		SMLN
Treatment	Mean Days (±SEM)	Frequency	Mean (±SEM)	Frequency	Frequency
Unenucleated mice					
-	31.1 (±1.6)	0/9	105.6 (±28.6)	9/9	8/9
LS2616	35.9 (±1.3)	0/11	51.8 (±15.0)[1]	11/11	1/11
Cy	28.9 (±2.0)	0/6	110.9 (±15.1)	6/6	6/6
LS2616+Cy	41.5 (±1.8)[2]	0/9	26.6 (±7.6)[3]	8/9	2/9
Enucleated mice					
E10[4]	33.4 (±2.3)	0/8	122.2 (±25.8)	8/8	8/8
E10+LS2616	44.2 (±2.2)[5]	2/7	30.1 (±11.9)[5]	5/7	1/7
E10+Cy	30.8 (±3.1)	0/9	115.8 (±20.4)	9/9	9/9
E10+LS2616+Cy	62.2 (±2.1)[6]	2/9	14.0 (±5.1)[6]	6/9	0/9

[1] P less than .05, when compared with untreated controls or Cy alone.

[2] P less than .01, when compared with untreated controls, LS2616 alone, or Cy alone.

[3] P less than .01, when compared with untreated controls, or Cy alone; P less than .05 when compared to LS2616 alone.

[4] E10 = enucleation 10 days post inoculation of tumor cells.

[5] P less than .05, when compared with E10 or E10+Cy.

[6] P less than .01, when compared with E10 or E10+Cy; P less than .05 when compared with E10+LS2616.

Table 4. Metastasis of intracesseral inoculations of 10^2 B16F10 melanoma cells in NK cell-depleted or NK cell enhanced mice.

	Survival		Pulmonary Metastases		SPLN	Adrenal Metastases		Spleen Metastases		Liver Metastases	
Treatment	Mean Days (±SEM)	Frequency	Mean (±SEM)	Frequency	Frequency	Mean (±SEM)	Frequency	Mean (±SEM)	Frequency	Mean (±SEM)	Frequency
-	32.9(±2.6)	0/12	113.7(±28.1)	12/12	12/12	0.0(±0.0)	0/12	0.0(±0.0)	0/12	0.0(±0.0)	0/12
H16-L10-4	33.3(±2.0)	0/7	93.4(±35.4)	7/7	7/7	0.0(±0.0)	0/7	0.0(±0.0)	0/7	0.0(±0.0)	0/7
PK136	30.4(±1.9)	0/10	307.2(±54.0)[1]	10/10	10/10	8.0(±0.1)[2]	4/10	8.0(±4.2)[2]	4/10	2.6(±1.8)	2/10
-	38.0(±1.9)	0/10	125.6(±20.5)	10/10	9/10	0.0(±0.0)	0/10	0.0(±0.0)	0/10	0.0(±0.0)	0/10
LS2616	37.4(±3.0)	0/8	64.6(±10.9)	8/8	2/8	0.0(±0.0)	0/8	0.0(±0.0)	0/8	0.0(±0.0)	0/8
LS + PK136	34.6(±2.0)	0/11	150.6(±40.8)	11/11	9/11	0.0(±0.0)	0/11	0.0(±0.0)	0/11	0.0(±0.0)	0/11

[1] P less than .01 when compared with untreated controls or H16-L10-4.

[2] P less than .01 when compared with untreated controls or LS+PK136.

Table 5. Sustained enhancement or depletion of NK cell activity with LS2616 or PK136 Ab treatment.

		% Lysis (±SEM)[1] Spleen Cell to Target Cell Ratio		
Day	Treatment	100:1	50:1	25:1
7	Saline	14.3(±1.9)	9.0(±0.7)	6.3(±1.7)
	PK136	1.1(±0.9)[2]	.8(±0.3)[2]	.6(±0.4)[2]
	LS2616	38.1(±5.3)[3]	24.0(±4.5)[3]	10.1(±1.5)[3]
42	Saline	17.4(±1.3)	10.3(±0.9)	4.1(±1.1)
	PK136	1.1(±0.5)[2]	.8(±0.3)[2]	.3(±0.1)[2]
	LS2616	33.2(±3.4)[3]	18.4(±2.6)[3]	12.1(±2.1)[3]

[1] Values indicate percent cytotoxicity obtained by killing of YAC-1 target cells in a ^{51}Cr-release assay. Results are the mean for three mice ±SEM.

[2] P less than .01 when compared with saline or LS2616.

[3] P less than .01 when compared with saline or PK136.

Table 6.The effects of silica, carrageenan, and gamma irradiation treatment on metastasis of intracameral inoculations of 10^2 in vivo derived B16F10 melanoma cells in mice.

	Survival		Pulmonary Metastases		SMLN
Treatment	Mean Days (±SEM)	Frequency	Mean (±SEM)	Frequency	Frequency
-	31.3 (±1.6)	0/9	105.6 (±29.0)	9/9	8/9
silica[1]	34.0 (±3.6)	0/6	155.0 (±62.5)	6/6	4/6
carrageenan[2]	37.0 (±2.9)	0/6	191.3 (±56.3)	6/6	5/6
gamma irrad[3]	33.9 (±4.1)	0/7	141.5 (±58.9)	7/7	6/7
gamma irrad[4]	35.9 (±4.7)	0/8	187.9 (±64.7)	8/8	8/8
gamma irrad[5]	34.8 (±4.5)	0/7	166.0 (±42.7)	7/7	7/7
gamma irrad[6]	32.0 (±1.6)	0/7	121.4 (±32.5)	7/7	7/7

[1] Silica injected ip, 1mg/mouse/week.

[2] Carrageenan injected ip, 1mg/mouse/week.

[3] Mice received irradiated B16F10 tumor cells (500 rads).

[4] 500 rads gamma irradiation on day of inoculation.

[5] 500 rads gamma irradiation on day 7 post-inoculation.

[6] 500 rads gamma irradiation on day 21 post-inoculation.

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