Humoral enhancement of metastasis : circulating IgG interactions with tumor-bearing lymphocytes
by Cheryl Juline Aslakson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
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Abstract:
Using an inbred BD-IV rat metastatic model, Starkey et al. (21) had previously shown that the metastasis enhancing moieties in serum from tumor-bearing rats reside with the IgG2b fraction. Since all our previous work was done using the lung colony assay, the current study verified that metastatic (but not local) tumor enhancement could also be demonstrated in a spontaneous metastasis system. However, the lung colony assay was clearly most advantageous for quantitation. Modulation of the immune system by TBS (serum from tumor-bearing rats) during the enhancement of metastasis was studied. It was found that the Helper:Suppressor T cell ratio (H: S ratio) for peripheral blood leukocytes (PBLs) from enhanced tumor-bearing rats was greatly reduced when compared to nonenhanced tumor-bearing rats. The kinetics of this decline were also investigated. The H:S ratio for enhanced tumor-bearer splenoc ytes and PBLs started to decline as early as day four and continued to do so until the end of the experiment on day twenty. On the other hand, the H : S ratio for nonenhanced tumor-bearing rats transiently peaked on day four and declined thereafter. Flow cytometric analyses of lymphocytes from non tumor-bearing rats and tumor-bearing rats revealed that IgG from TBS bound to a subset of T lymphocytes. Similar results were obtained for lymphocytes removed from enhanced tumor-bearing rats. IgG2b was the predominantly binding isotype for enhanced tumor-bearing PBLs and it appeared to be bound in vivo; binding by other isotypes was not significant. Preferential binding of one isotype by nonenhanced tumor-bearing and non tumor bearing PBLs was not demonstrated. Tumor bearer splenocytes, sorted on the basis of their ability to bind TBS IgG, were shown to enhance experimental metastasis in the lung colony assay. All of the sorted cells were T lymphocytes and a majority of the T cells expressed the suppressor T cell phenotype. These studies suggest that suppressor T cells are involved in humoral enhancement of metastasis. The presence of these suppressor cells may also explain problems associated with the adoptive immunotherapy of cancer.

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DEDICATION

I would like to dedicate this thesis in memory of my father, John Conrad Aslakson. It is his interest in education which has sustained and given me the courage to complete my master's degree.

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ABSTRACT

Using an inbred BD-IV rat metastatic model, Starkey et al. (21) had previously shown that the metastasis enhancing moieties in serum from tumor-bearing rats reside with the IgG_{2b} fraction. Since all our previous work was done using the lung colony assay, the current study verified that metastatic (but not local) tumor enhancement could also be demonstrated in a spontaneous metastasis system. However, the lung colony assay was clearly most advantageous for quantitation. Modulation of the immune system by TBS (serum from tumor-bearing rats) during the enhancement of metastasis was studied. It was found that the Helper:Suppressor T cell ratio (H:S ratio) for peripheral blood leukocytes (PBLs) from enhanced tumor-bearing rats was greatly reduced when compared to nonenhanced tumor-bearing rats. The kinetics of this decline were also investigated. The H:S ratio for enhanced tumor-bearer splenocytes and PBLs started to decline as early as day four and continued to do so until the end of the experiment on day twenty. On the other hand, the H:S ratio for nonenhanced tumor-bearing rats transiently peaked on day four and declined thereafter. Flow cytometric analyses of lymphocytes from nontumor-bearing rats and tumor-bearing rats revealed that IgG from TBS bound to a subset of T lymphocytes. Similar results were obtained for lymphocytes removed from enhanced tumor-bearing rats. IgG_{2b} was the predominantly binding isotype for enhanced tumor-bearing PBLs and it appeared to be bound *in vivo*; binding by other isotypes was not significant. Preferential binding of one isotype by nonenhanced tumor-bearing and nontumor bearing PBLs was not demonstrated. Tumor bearer splenocytes, sorted on the basis of their ability to bind TBS IgG, were shown to enhance experimental metastasis in the lung colony assay. All of the sorted cells were T lymphocytes and a majority of the T cells expressed the suppressor T cell phenotype. These studies suggest that suppressor T cells are involved in humoral enhancement of metastasis. The presence of these suppressor cells may also explain problems associated with the adoptive immunotherapy of cancer.

INTRODUCTION

The demonstration by Foley in 1953 that methylcholanthrene-induced sarcomas were immunogenic in mice, was the foundation of tumor immunology (for review see Brodt in reference 1, Hellstrom et al. in reference 2). Foley removed an established sarcoma and showed that the mouse was resistant to a second tumor challenge. Corroborative reports followed in 1957, by Prehn and Main (3), and in 1960, by Old et al. (4) and Klein et al. (5). These studies excluded the possibility that the antigenicity of methylcholanthrene-induced tumors was the result of genetic heterogeneity in the inbred mouse strains used (3,4). Klein et al.'s data revealed that the autochthonous host could also be immunized to some extent against challenge with cells of its own tumor (5).

Tumor immunogenicity is not a phenomenon restricted to chemically-induced tumors. Ultraviolet (UV) light-induced melanomas are also immunogenic (for review see Kripke in reference 6). Surprisingly, many human tumors and spontaneously arising tumors in experimental animals proved to be either weakly immunogenic or non-immunogenic (for review see Brodt in reference 1, Baldwin in reference 7).

The immunity that chemically-induced experimental tumors invoke is cell-mediated and has been transferred

by immune cells from tumor-bearing hosts (for review see Hellstrom et al. in reference 2, North in reference 8). Further *in vitro* studies revealed that the immune cells were thymus-derived lymphocytes (for review see North in reference 8). Passive immunization, the practice of injecting tumor-bearing animals with pooled syngeneic tumor bearer serum, did not confer immunity (for review see North in reference 8).

Six years after Foley's discovery, Burnett reformulated Paul Erlich's pre-immunology theory regarding the relative lack of tumors in animals and humans (for review see Brodt in reference 1, Schwartz in reference 9). This theory, referred to as the immune surveillance theory, assumed an active role for thymus-dependent cellular mechanisms in searching out and eliminating cancerous cells *in situ*. The appearance of any tumors indicated a failure on the part of the host's immune system to fulfill this function (for review see Brodt in reference 1). This theory was later expanded and revised to include the non-T cell dependent cytotoxic cells such as natural killer (NK) cells, macrophages and cells mediating antibody-dependent cellular cytotoxicity (ADCC) (for review see Baldwin in reference 10).

Many objections have been raised against immune surveillance as the means for controlling the appearance of

cancerous cells (9,11,12,13). First, immunosuppressed animals and human patients develop tumors mainly of the lymphoreticular system (11,12,13). According to the surveillance theory, tumors should arise in all tissue types. Second, nude mice which are athymic and patients with immune deficiency syndromes do not develop an excess of tumors (12,14). It has been argued that the lack of thymus-dependent cellular mechanisms is compensated for by natural surveillance mediated by NK cells, macrophages and cells mediating ADCC. Athymic nude mice have high NK cell levels and develop very few spontaneous tumors (12,13). NK cells may not be the only cells responsible for abrogation of in situ tumors. Beige mice which have low NK levels develop spontaneous tumors at the same rate as their normal littermates and nude mouse strains (12,13). However, the complexity of the cancer problem did not crystallize within the confines of the immune surveillance theory. At the time the theory was proposed, it afforded answers to perplexing questions and spurred much research, opening up new areas of study in tumor immunology.

The demonstration that tumors were immunogenic was followed by attempts to explain how these tumors escaped the surveillance mechanisms of their hosts. Even though active immunity to tumors was mediated by T lymphocytes, studies then focused on why injections of serum from tumor-

bearing animals into tumor bearer hosts resulted in prolonged tumor survival (15,16,17). Immunologic enhancement, the phrase adopted to describe this phenomenon, was defined as the successful establishment of a tumor and its progressive growth resulting in the death of the host (18). Tumor growth has been passively enhanced using tumor bearer serum (19,20), antibody fractions from tumor bearer serum (21,22), antilymphocyte serum (23), alloantibodies (17), monoclonal antibodies (24), anti-idiotypic antibodies (25,26,27), immune complexes (28,29,30,31), immune factors (32) and $F(ab')_2$ immunoglobulin fragments (22,33). Immunologic enhancement is not limited to primary tumor growth; metastatic disease has also been enhanced (21,24).

Almost all antibody classes have been implicated at one time or another as being able to enhance tumor growth. In the rat IAR6-1-RT7-4b hepatocarcinoma model, the IgG_{2b} fraction of tumor-bearing serum enhanced experimental metastasis (21). Allogeneic enhancement of the A/J-derived sarcoma Sa 1 in CBA mice was attributed to the antibody classes IgG_2 and IgG_1 by Duc et al. (15). The growth of the mineral oil plasmacytomas, MOPC-315 and MOPC-460, induced in BALB/c mice was enhanced by the IgG_1 fraction of alloantibodies prepared in CBA or CEH mice (17). Sacchi et al. (24) prepared monoclonal antibodies to a Lewis Lung

Carcinoma-associated antigen and found that the monoclonal antibody 135-13C affected the growth of the primary tumor and its metastases differently. The primary tumor showed a reduction of 20-25% in tumor weight compared to controls, while lung metastases were increased two-fold (24). Antibodies of the IgG subclass are not exclusive in their ability to enhance tumor growth. IgM antibodies have occasionally been implicated in prolonging the survival of tumor tissue (17).

Investigators have also reported that similar antibody subclasses are also effective immunotherapeutic agents against experimental cancers (34,35,36). Denkers et al. (34) have shown that significant inhibition of the AKR/J SL 2 lymphoma cells was mediated by the anti-Thy-1.1 IgG_{2a} antibody. IgG_1, IgG_{2b} and IgG_{2c} displayed less anti-tumor activity (34). Herlyn and Koprowski (35) were also able to inhibit the growth of a human tumor in nude mice using a monoclonal IgG_{2a} antibody. Suppression of the mouse plasmacytoma MOPC 315 by the IgG_2 fraction of syngeneic antitumor globulin also corroborates the fact that the same isotypes which enhance tumor growth in one system are the tumor rejecting isotypes in another system (36).

Differences seen in the ability of a given isotype to enhance or restrict tumor growth may be attributed to many factors. These include the route of serum administration,

the dilution factor of the serum and the methods used to prepare the isotypes or serum for injection.

Many mechanisms have been proposed to explain enhancement of tumor growth. Blocking factors in the form of anti-tumor antibody, circulating antigen-antibody complexes or free-circulating antigen found in the serum from tumor-bearing animals may be capable of binding to target cells and/or effector lymphocytes (30,37,38,39). These blocking factors could conceal antigens on tumors, protecting them from immune effector cells (37,39). Alternatively, these factors could render effector cells ineffective by binding to them (37,39). Oldstone et al. (30) were able to remove blocking factors from tumor-bearing serum by absorbing the serum with the appropriate tumor cells.

Specific classes of immunoglobulins in rodents and man are capable of activating complement. Immunoglobulins which activate the human complement system are IgM, IgG_1, IgG_2 and IgG_3 (40). Analogous complement activating classes in the mouse are IgM, IgG_{2a} and IgG_{2b} (41) and in the rat are IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c} (42). Complement activation and target cell lysis are important defense mechanisms against tumor cells (8,10).

Antibodies which do not activate complement may instead enhance tumor growth. Bodurtha et al. (43)

reported on the presence of complement-activating antibodies in ten patients with malignant melanoma in relation to tumor metastasis. They found that one melanoma patient with extensive metastatic disease lacked complement-dependent cytotoxic antibodies. The other nine patients, who had no evidence of visceral metastases, possessed complement-dependent cytotoxic antibodies in their sera (43). Demonstration of anti-complement (AC) activity which may be due to anti-complement antibodies has been documented in humans with malignant melanoma. AC activity sometimes coincident with tumor recurrence was reported by Gupta et al. (44). AC activity was demonstrated in 45% of the sera drawn from human patients with malignant melanoma compared to 10% of normal, healthy controls (44).

Complex interactions exist between a tumor and its host's immune response against it. In 1972, Niels Jerne (45) formulated and proposed the network hypothesis which described the immune system as a complex network of cells linked together via complementary interactions. These interactions are mediated by structures encoded in immunoglobulin V-region genes and have since been referred to as idiotypes. The immune system exists as a network where one idiotype is balanced by an anti-idiotype, which in turn is counter-balanced by an anti-anti-idiotype, and

so forth. Via this network, the immune system is maintained in a complex state of homeostasis until perturbed by the introduction of a foreign agent (for review see Monroe in reference 46). Alterations in the host's control over responsiveness toward a specific antigenic epitope affects the body's response to further antigenic stimuli. Eichman et al. demonstrated that anti-idiotypic antibodies can specifically suppress (47) or induce (48) immune responses to a particular antigen.

Idiotype networks have also been implicated in the immune control of tumor growth. In a study by Flood et al. (49), enhanced tumor growth was attributed to anti-idiotypic tumor antibodies. These investigators found that a UV-induced melanoma was able to grow progressively as a result of idiotypic-specific suppression of the tumor-specific immune response to the melanoma antigens (49). A corroborative study by Milburn and Lynch (25,26) reported on the anti-idiotypic antibody and anti-idiotypic cellular regulation of IgA expression in the MOPC-315 BALB/c plasmacytoma. The anti-idiotypic antibody regulated expression of the immunoglobulin while the anti-idiotypic T cells regulated secretion of immunoglobulin (25). On the other hand, inhibition of <u>in vivo</u> tumor growth has been induced by anti-idiotypic antibodies directed at distinct immunoglobulin idiotypes on the surface of the murine

plasmacytoma MOPC-460 (50) and against murine monoclonal antibody 8.2, an antibody specific for a human melanoma-associated cell surface marker P 97 (51).

It has been confirmed many times that serum from tumor-bearing animals can passively enhance tumor growth and metastatic disease. The mechanisms involved in this phenomenon have not yet been clearly defined. In addition, it is well documented that the adoptive transfer of tumor-associated lymphocytes (52,53,54,55) can also enhance tumor growth. Treves et al. (52) found that enhancing T lymphocytes from tumor-bearing C57BL/6 mice suppressed the host resistance to the syngeneic Lewis Lung-Carcinoma (3LL). T lymphocytes obtained from the enlarged spleen of the tumor-bearing mice were cytotoxic to 3LL target cells in vitro. However, these same spleen cells enhanced tumor growth in vivo when mice were injected i.v. with a mixture of spleen cells and tumor cells (52). Results reported by Uniel and Trainin (53) corroborate the data of Treves et al. Using the same syngeneic tumor line, 3LL, they found that thymocytes or thymus-derived lymphocytes could stimulate 3LL tumor growth in recipients. This enhancing effect was manifested by a higher number of tumor takes, acceleration of tumor growth and an increase in metastasis (53).

The progressive growth of tumors has been shown to be modulated by suppressor T cells (10,56). Results reported by Cheng et al. (57) found that the generation of immune effector T cells in mice with growing plasmacytomas was down-regulated by cyclophosphamide sensitive suppressor cells. Berendt and North (58) have provided evidence consistent with the hypothesis that concomitant immunity to the Meth A Fibrosarcoma decays as a result of the generation of a mechanism of T-cell-mediated immunosuppression. In addition, North and Dye (59) reported that the Ly $1^{+}2^{-}$ suppressor T cells down-regulated the generation of Ly $1^{-}2^{+}$ effector T cells during the progressive growth of a P815 mastocytoma. These and other findings suggest that the failure of the immune system to reject an immunogenic tumor is the result of the generation of suppressor T cells (54-59).

Suppressor T cells derived from tumor-bearing hosts have been shown to suppress immune functions in vitro (60,61). Clerici et al. (62) reported that suppressor cell activity, enriched by hydrocortisone treatment of 3LL tumor-bearing mice, down-regulated an in vitro lymphoproliferative response of tumor bearer mouse spleen cells to mitogens. The study undertaken by Bear (63) found that spleen cells from "late" tumor-bearing hosts (18-28 days post tumor cell inoculation) completely suppressed the

in vitro cytotoxic lymphocyte response to tumor immune cells. Mixed-lymphocyte culture responses of spleen cells from tumors induced by the murine Moloney Sarcoma Virus against allogeneic spleen cells were found to be markedly depressed as indicated by three parameters: lymphoblast counts, ^{3}H-thymidine incorporation and cell-mediated lysis (63).

Suppression of the immune system has been reported for many other diseases and has most often been reflected by changes in helper:suppressor T cells ratios. Included in this suppressed state are patients with Acquired Immune Deficiency Syndrome (64), Systemic Lupus Erythematosus (65) and leprosy (66,67). Changes in helper:suppressor T cell ratios have not been widely reported for cancer patients. Koyama et al. (68) did find changes in B and T lymphocyte counts in canine lymphosarcoma. Marked increases in B lymphocytes and decreases in T lymphocytes were observed in the peripheral blood, spleen and lymph nodes of dogs which had been diagnosed as having lymphosarcoma (68).

Immune regulation of tumor growth is complex; both the humoral and cellular arms of the immune system control tumor proliferation. The tumor may be destroyed or alternatively, allowed to grow unchecked by the immune system. I chose to investigate one of the manifestations

of unchecked tumor growth, the immunologic enhancement of tumor metastasis.

MATERIALS AND METHODS

Rats

BD-IV rats, originally obtained from Professor Rajewsky, Institute for Cell Biology, University of Essen, Germany and from Dr. Montesano, International Agency for Research on Cancer, Lyons, France, were bred by brother-sister matings and maintained under specific pathogen free (SPF) conditions at the Montana State University Animal Resources Center. Male rats, six to twelve weeks of age were used in these studies.

Tissue Culture Media

The RT7-4bs tumor cell line was cultured *in vitro* in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS)(HYCLONE LABORATORIES, Logan, UT), 5ug/ml insulin (CALBIOCHEM, La Jolla, CA), 100 U/ml penicillin (GIBCO, Grand Island, NY) and 100ug/ml streptomycin (GIBCO). This formulation constitutes complete RPMI medium. Tumor cells used for subcutaneous and intravenous injections were washed and resuspended in Ca^{+2} and Mg^{+2} -free Tyrode's balanced saline (CMF). The medium used for fluorescence-activated cell sorter (FACS) analysis of lymphocyte preparations and for FACS sorting of splenocytes was McCoy's 5A medium (GIBCO) supplemented with

5% heat-inactivated FBS (CM). Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin, fraction V (BOEHRINGER MANNHEIN BIOCHEMICALS, Indianapolis, IN)(PBS-BSA) and PBS-BSA with 0.01M NaN_3 (SIGMA CHEMICAL COMPANY, St. Louis, MO) were used for the determination of T and B cell surface antigens.

Tumor Cell Lines

The tumor cell lines, RT7-4bs (4bs) and RT7-4b-Ls (4b-Ls), were derived from IAR6-1-RT7, a dimethylnitrosamine-transformed culture of BD-IV rat parenchymal cells (69,70). Characterization of these cells lines is described in detail elsewhere (71,72). These tumor lines were maintained in BD-IV rats by subcutaneous transplant of each tumor every three weeks. RT7-4bs cells were obtained as primary cultures from minced subcutaneous tumor tissue passed through a 200 mesh stainless steel screen into complete RPMI medium. Cell suspensions were pelletized, washed twice in complete RPMI medium and resuspended in complete RPMI medium. Cells were plated at 1 x 10^4/sq cm in 25 cm^2 tissue culture flasks (NUNC, Denmark) and incubated at 37°C in a humidified atmosphere of 7% CO_2 in air. Once a week, cell lines were harvested for passage by gentle trypsinization [0.05% trypsin (CALBIOCHEM) and 0.02% ethylenediaminetetraacetic, disodium salt (EDTA) (SIGMA CHEMICAL COMPANY) in CMF] for five minutes at room

temperature, followed by trypsin inactivation with complete RPMI medium. The cells were then replated at a 1:4 split ratio.

Sera

Blood was obtained from nontumor-bearing male rats under ether anesthesia via cardiac puncture. In an analogous fashion, blood was also obtained from tumor-bearing rats which two weeks earlier had been transplanted with small pieces of the 4bs and 4b-Ls tumors subcutaneously. The blood was allowed to clot, and then centrifuged at 4°C, 750xg for twenty minutes. Serum fractions from nontumor-bearing rats (NRS) were collected and pooled. Likewise, the serum fractions from the 4bs and 4b-Ls tumor bearing rats (TBS) were collected and pooled. Pooled NRS and TBS were divided into aliquots and stored frozen at -20°C until use. Freeze-thaw cycles were avoided.

Spontaneous Metastasis Assay

4bs tumor cells were removed from the tissue culture vessels by gentle trypsinization as described earlier. The monodispersed tumor cells were pelletized, washed twice with CMF and resuspended at a concentration of 5×10^6 cells/ml in CMF. Tumor cell viability was assessed by trypan blue dye exclusion and cell suspensions which were

greater than 95% viable were used for inoculation. Five hundred thousand tumor cells were injected subcutaneously into the right flank of each rat. Two hours post tumor cell injection, each rat in the control group was injected intraperitoneally (i.p.) with 0.2ml NRS and each rat in the experimental group was injected i.p. with 0.2ml TBS. Sera injections were continued every second day thereafter. Subcutaneous tumor growth was monitored every second day with vernier calipers and tumor volume was calculated using the formula $4/3\,\pi\,r^3$. At the time of necropsy, total body weight was recorded and the rats were examined for evidence of metastatic disease. Lungs, liver, spleen and the subcutaneous tumor were removed, weighed and fixed in Bouin's fixative (73). The number of spontaneous lung metastases was enumerated using a dissecting microscope.

Lung Colony Assay

RT7-4bs tumor cells were harvested from the tissue culture vessels as described earlier and resuspended at 5×10^4 cells/ml in CMF. Tumor cell viability was assessed as previously described. Ten thousand cells in 0.2ml total inoculum were injected into the lateral tail vein of each rat. Two hours after intravenous (i.v.) injection of 4bs tumor cells and every two days thereafter, rats in the control group and the experimental group were injected with serum as described for the spontaneous metastasis assay.

Post-mortem investigation included examination for extra-pulmonary metastases and recording of total body, lung, liver and spleen weights. The lungs, liver and spleen were fixed in Bouin's fixative, and the number of tumor lung colonies enumerated using a dissecting microscope.

Determination of Leukocyte Surface Phenotypes

Lymphocyte Preparation. Spleens were removed and dispersed to single cell suspensions by passing through a 200 mesh wire screen. Peripheral blood leukocytes (PBLs) were isolated from freshly drawn blood obtained via cardiac puncture. The blood was mixed with heparin (SIGMA CHEMICAL COMPANY) (50mg/ml; 0.1ml/3ml of whole blood), centrifuged at 4°C, 750xg for twenty minutes and the buffy coat removed. Erythrocytes were removed from cell preparations by hypotonic lysis using a six second exposure to sterile distilled water (74). Cells were washed twice and resuspended in PBS-BSA at a concentration of 2×10^7 cells/ml. Two million cells per well were dispensed into a flat-bottomed, 96-well, microtiter plate (NUNC), centrifuged at 4°C, 188xg for five minutes and the excess supernatant was removed.

T Cell Determinants. The following mouse monoclonal antibodies (mab), which were purchased from BIOPRODUCTS FOR SCIENCE, Inc., Indianapolis, IN, were used to label cell surface determinants on rat lymphocytes:

1.) W3/25. This mab labels the rat helper T cell subset functional in mixed lymphocyte reactions, graft versus host reactions and antibody responses (75,76),

2.) MRC OX-8. This mab labels a T cell subset which mediates suppression of antibody formation (77). It also recognizes a determinant expressed on precursor cytotoxic T cells (78), and

3.) MRC OX-19. This mab recognizes a determinant expressed on all thymocytes and peripheral T cells but does not bind to B cells, macrophages, natural killer cells, mast cells or other cell types (78).

The indirect immunofluorescence (IIF) microtitration technique was used to determine T lymphocyte subsets. Fifty ul of a 1:50 dilution of mabs W3/25, MRC OX-8 and MRC OX-19 was added to the centrifuged cells and incubated at 4°C for one hour. After the cells were washed twice with PBS-BSA containing azide, 50ul of a 1:32 dilution of the second antibody, a fluorescein isothiocynate (FITC) conjugated goat anti-mouse IgG antibody (CALBIOCHEM) was added and the incubation continued for another hour at 4°C. The cells were washed twice in PBS-BSA containing azide and resuspended in PBS-BSA containing azide (79). One hundred cells from each labeled cell preparation were

counted and evaluated for fluorescence using a Leitz Ortholux II microscope (E. LEITZ, Inc., Rockleigh, NJ).

The helper:suppressor T cell ratio (H:S ratio) was expressed as follows:

$$\frac{\text{\# positive fluorescent cells labeled by W3/25}}{\text{\# positive fluorescent cells labeled by MRC OX-8}}.$$

The percentage of T cells was expressed as the:

$$\frac{\text{\# positive fluorescent cells labeled by MRC OX-19}}{\text{\# total cells counted}} \times 100.$$

B Cell Determinants. The IIF antibody labeling procedure, a modification of the method described by Moller (80), was used to label B lymphocytes. One-tenth milliliter of a 1:64 dilution of a rabbit anti-rat IgM antibody (MILES LABORATORIES, Elkhart, IN) was added to the centrifuged cells and incubated at 4°C for one hour. After the cells were washed twice with PBS-BSA containing azide, 0.1ml of 1:100 dilution of the second antibody, a FITC-conjugated goat anti-rabbit antibody (COOPER BIOMEDICAL, Inc., Malvern, PA) was added and the incubation continued for an additional hour at 4°C. The cells were washed and resuspended in PBS-BSA containing azide. One hundred cells were counted using a fluorescence microscope and evaluated

for fluorescence. The percentage of B cells was expressed as:

$$\frac{\#\text{ positive fluorescent cells labeled by anti-IgM}}{\#\text{ total cells counted}} \times 100.$$

Fluorescence Activated Cell Sorting Analyses

Animal and Cell Preparation. Male rats were prepared for FACS analyses in an analogous fashion for those used in the experimental lung colony assay. Ten thousand 4bs tumor cells were injected into the lateral tail vein of each rat. Two hours after the tumor cells were injected, each rat in the control group was injected with 0.2ml NRS i.p. and each rat in the experimental group with 0.2ml TBS i.p. Sera injections were continued every second day until day fourteen. On day fourteen, the animals from the control and experimental groups and two age/sex matched nontumor-bearing rats were bled under ether anesthesia via cardiac puncture. Blood was mixed with heparin as previously described, and centrifuged at 4°C, 750xg for twenty minutes. Serum was discarded and the buffy coat saved. Spleens and thymuses were removed and were dispersed to single cell suspensions by passing through a 200 or 70 wire mesh screen, respectively. Erythrocytes were removed from cell preparations using hypotonic lysis with sterile distilled water as described earlier. The nylon wool

nonadherent splenocyte fractions were collected after incubation of splenocytes on nylon wool columns for forty-five minutes at 37°C (81). All cell suspensions were washed twice with CM and resuspended in CM at 2 x 10^7 cells/ml.

Binding Parameters of IgG from NRS and TBS. Preliminary experiments revealed that the optimal working dilutions for NRS, TBS and the FITC-conjugated rabbit anti-rat IgG antibody were 1:1, 1:12.5 and 1:50, respectively. One-tenth milliliter of each cell suspension was incubated with CM, NRS or TBS for thirty minutes at 37°C. After washing twice with CM, the cell suspensions were incubated with 0.1ml FITC-conjugated rabbit anti-rat IgG antibody (MILES YEDA, Ltd, Rehovot, Israel, distributed by MILES LABORATORIES) for thirty minutes at 37°C. The cells were washed twice with CM and resuspended in a total volume of 1.0ml CM.

The intensity of fluorescence of the TBS IgG-labeled cells was determined using a fluorescence-activated cell sorter, FACS 440 (BECTON-DICKINSON, MOUNTAIN VIEW, CA). The FACS 440 was interfaced with the Consort 40 equipped with the LACELL programs, ACQ4 and DISP 4 (BECTON-DICKINSON). The 488 nm line of a 164-02 Argon-ion laser (SPECTRA PHYSICS, Mountain View, CA) operated at 100 mW, was used to excite the fluorochrome. Forward scatter, 90 degree

scatter and fluorescence intensity were acquired on samples of 10,000 events using the ACQ4 program. The DISP 4 program was used to calculate the intensity of fluorescence, reflecting the amount of rat IgG bound after incubation with NRS and TBS. The data gathered from samples incubated with CM was used to discriminate between nonspecific fluorescence and fluorescence emitted from TBS IgG-labeled cells. Contaminating traces of red cells, nonviable cells and debris were excluded by appropriately setting the forward scatter threshold (82).

Binding Parameters of IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c} from NRS and TBS. Commercial antibodies used for these studies included the following:

1.) The monoclonal antibody MARG 2b-8 (BIOPRODUCTS FOR SCIENCE, Inc.). This antibody is a mouse monoclonal IgG_1 antibody prepared against the $gamma_{2b}$ heavy chain of the rat IgG_{2b} immunoglobulin (83), and

2.) The polyclonal antibodies:

a.) Goat anti-rat IgM (COOPER BIOMEDICAL, Inc.),

b.) Sheep anti-rat IgG_1 (MILES LABORATORIES),

c.) Goat anti-rat IgG_{2a} (PEL-FREEZ BIOLOGICALS, Rogers, AR), and

d.) Goat anti-rat IgG_{2c} (MILES LABORATORIES).

These polyclonal antibodies were conjugated to FITC (COOPER

BIOMEDICAL, Inc.) using the method developed by Hapner and Hapner (84).

PBLs were incubated with CM, NRS, and TBS (using the antibody dilutions given on page 21) for thirty minutes at 37°C. Cell preparations were incubated further with MARG 2b-8 for thirty minutes at 37°C followed by incubation with a FITC-conjugated goat anti-mouse IgG antibody for thirty more minutes at 37°C. Similarly, following incubation with CM, NRS, and TBS, the same cell preparations were incubated with the conventional FITC-conjugated antibodies for thirty minutes at 37°C to determine the binding parameters of these antibody isotypes from NRS and TBS to PBLs.

The labeled cells were analyzed on the FACS 440 using the same laser setting, and data acquisition and analysis programs as previously described. The percentage of positive fluorescent cells was calculated as previously described under Binding Parameters of IgG from NRS and TBS.

Fab Antibody Fragment Preparation. The IgG fraction of TBS was obtained by precipitation with saturated ammonium sulfate (SIGMA CHEMICAL COMPANY) and then enzymatically digested with mercuripapain (COOPER BIOMEDICAL, Inc.) using the method described in Selected Methods in Cellular Immunology (85). Fab fragments were purified from the digested material by passage over a Protein A-Sepharose CL-4B column (PHARMACIA FINE CHEMICALS,

Sweden) and diluted to a final volume equal to the 1:12.5 TBS dilution used for other FACS analyses. The quality of the papain digestion was assessed using a 7.5% Sodium Dodecyl Sulfate-PAGE gel under nonreducing conditions (86). PBLs were incubated with CM, NRS and TBS (using the antibody dilutions given on page 21) and the antibody fragments as previously described for the Binding Parameters of IgG from NRS and TBS. The labeled cells were analyzed on the FACS 440 using the same laser setting, and data acquisition and analysis programs as previously described. Also, the percentage of positive fluorescent cells was calculated as previously described under Binding Parameters of IgG from NRS and TBS.

Adoptive Transfer of Splenocytes Previously FACS-Sorted on the Basis of Binding TBS IgG. Ten thousand 4bs tumor cells, prepared as previously described for the lung colony assay, were injected into the lateral tail vein of a male BD-IV rat. Two weeks after tumor cell injection, the spleen was removed and dispersed to a single cell suspension by passing through a 200 mesh wire screen. Erythrocytes were removed using hypotonic lysis with sterile distilled water as previously described. The cells were washed twice with CM supplemented with 5ug/ml insulin, 100ug/ml penicillin and 100U/ml streptomycin and resuspended at 2 x 10^7 cells/ml in supplemented CM. Twenty

million cells were incubated with 1.0ml TBS (using the antibody dilution given on page 21) for thirty minutes at 37°C. The cells were washed with supplemented CM and then incubated with 1.0ml FITC-conjugated rabbit anti-rat IgG antibody for thirty minutes at 37°C. The cells were sorted, using the 488 nm line of the 164-02 Argon-ion laser operated at 100 mW in log mode on the Fluorescence One Channel, on the basis of binding TBS IgG. Operation of the laser and settings for the forward scatter threshold were analogous to those used for ascertaining the binding patterns of antibodies from NRS and TBS. The cells were sorted over two hours, collected into complete RPMI medium and held on ice until the sort was completed. Five hundred thousand IgG positive-sorted cells were injected i.p. into each of five experimental rats, which two hours earlier had been injected with 1×10^4 4bs tumor cells as previously described for the lung colony assay. Control rats, which had also been injected two hours earlier with 4bs tumor cells i.v., were injected i.p. with 0.2ml 0.85% NaCl (SIGMA CHEMICAL COMPANY). Three weeks after tumor cell injection, the animals were killed and necropsied as described for the lung colony assay. Tumor lung colonies were counted under a dissecting microscope.

IgG-positive, sorted splenocytes and unsorted splenocytes were typed by IIF for helper T cells,

suppressor T cells and total T cells. Twenty million splenocytes were prepared for sorting by incubating with 1.0ml TBS (using the same antibody dilutions given on page 21) for thirty minutes at 37°C. After washing twice with CM, the cells were incubated with 0.1ml of a 1:1 dilution a rabbit anti-rat IgG antibody (COOPER BIOMEDICAL, Inc.) conjugated to rhodamine tetramethyl isothiocyanate (COOPER BIOMEDICAL, Inc.) as previously described for FITC. The fluorescently-labeled splenocytes were sorted on the basis of IgG bound from TBS. The 514 nm line of the Argon-ion laser was operated at 100 mW with the DF 575/25 band pass filter in place, and the sorting was monitored in the log mode on the Fluorescence Two channel. The forward scatter threshold was set as previously described for the cells which had been sorted and injected for adoptive transfer. The cells were collected into complete RPMI medium and held on ice as previously described. After incubation for two hours in complete RPMI medium, the sorted splenocytes as well as unsorted splenocytes were labeled with mabs W3/25, MRC OX-8 and MRC OX-19. Labeled cells were counted and the data calculated as previously described under Determination of Leukocyte Surface Phenotypes.

When it was discovered that TBS complexes on FACS sorted splenocytes, labeled with either rhodamine or fluorescein, would not cap off even after overnight

incubation at 37°C, similar cell preparations were subjected to light trypsin treatment in attempts to remove the complexes. After the splenocytes were labeled with TBS and FITC-conjugated anti-rat IgG antibody as previously described, 0.1ml trypsin was added to the splenocytes. Following trypsin addition, at thirty-second intervals up to two minutes and then at two minute intervals up to ten minutes, the cells were recovered from trypsin with complete RPMI. Following trypsinization, the cells were assessed for complex removal using fluorescence microscopy.

Histological Examination

Lungs and subcutaneous tumors from animals in the various experimental and control groups were excised, fixed in Bouin's solution, and processed routinely for paraffin embedding. Sections were stained with hematoxylin and eosin (H & E) or Periodic-Acid Schiff and hematoxylin (PAS-H).

Statistical Analyses

Differences in the number of lung metastases between experimental groups were analyzed using the non-parametric Mann-Whitney U Two-Tailed Test and were considered significant at $p < 0.02$.

RESULTS

Enhancement of Metastasis using the Lung Colony Assay and the Spontaneous Metastasis Assay

Chronic injection of TBS enhanced lung colonization by the 4bs tumor using the experimental lung colony assay. As Table 1 shows, chronic injections of TBS enhanced lung colonization 2.7 times over control animals injected with NRS. The average number of pulmonary tumor nodules was 173 for enhanced, experimental rats and 65 for control rats (Table 1.)

The spontaneous metastasis assay was used to investigate whether subcutaneous tumor growth and spontaneous lung metastasis could be modulated in an analogous fashion. As shown in Table 1, spontaneous metastasis was enhanced in animals chronically injected with TBS. Values for spontaneous metastases from "serum-enhanced", experimental rats correspond to 58 metastatic tumor lung nodules and from control rats, 17 metastatic tumor lung nodules (Table 1).

Chronic injection of TBS, however, did not significantly affect the growth rate of the primary, subcutaneous tumor (Figure 1.) As shown in Figure 1, the latent period for the appearance of the primary tumors and the growth rates were similar for both groups.

TABLE 1. THE EFFECTS OF TUMOR BEARER SERUM ON METASTASIS USING THE SPONTANEOUS METASTASIS ASSAY AND THE LUNG COLONY ASSAY

REGIMEN	RAT GROUP	MEAN NUMBER OF PULMONARY TUMOR COLONIES (RANGE) P[a]
LUNG COLONY ASSAY		
BD-IV rats were injected with $1x10^4$ 4bs cells i.v. Two hours after 4bs cell injection, control rats received 0.2ml NRS[b] i.p. and experimental rats 0.2 ml TBS[b] i.p. Sera injections continued every second day until necropsy at Day 20.	Controls (n = 5)	65 (35 - 120)
	Experimentals (n = 5)	173 (94 - 457) P < 0.0087
SPONTANEOUS METASTASIS ASSAY		
BD-IV rats were injected with 5×10^5 4bs cells subcutaneously. Sera injections were given in a fashion analogous to the lung colony assay until necropsy at Day 27.	Controls (n = 8)	17 (1 - 63)
	Experimentals (n = 7)	58 (0 - 263) P = 0.3

[a]P-values were calculated using Mann-Whitney U Two-Tailed Test.

[b]NRS = serum from normal rats; TBS = serum from tumor bearing rats.

Histologic examination of lung sets from control rats and experimental rats showed that the nodules counted were indeed tumor colonies. There was no discernible difference in lung morphology between the two groups.

In summary, metastatic tumor lung colonization was enhanced using the experimental lung colony assay and the spontaneous metastasis assay. Humoral enhancement was essentially restricted to metastasis. Since metastasis was enhanced using either assay, we chose to use the lung colony assay, which is inherently a better assay for quantitation, for the remainder of these studies.

Changes in T Cell and B Cell Phenotypes for Leukocytes from Enhanced Tumor Bearing Rats and Nonenhanced Tumor-Bearing Rats

TBS modulation of the immune system during the enhancement of metastasis was studied. First, changes in the H:S ratios were investigated for enhanced tumor-bearing rats. As shown in Table 2, fourteen days after i.v. inoculation of 4bs tumor cells, the H:S ratio for PBLs from enhanced tumor-bearing rats was greatly reduced when compared to nonenhanced tumor-bearing rats. The H:S ratio for PBLs from enhanced tumor-bearing rats was 0.57 $\pm$ 0.03 and from nonenhanced tumor-bearing rats was 1.49 $\pm$ 0.21 (Table 2). This reduction was restricted to circulating PBLs and was not seen for splenocyte preparations. The H:S

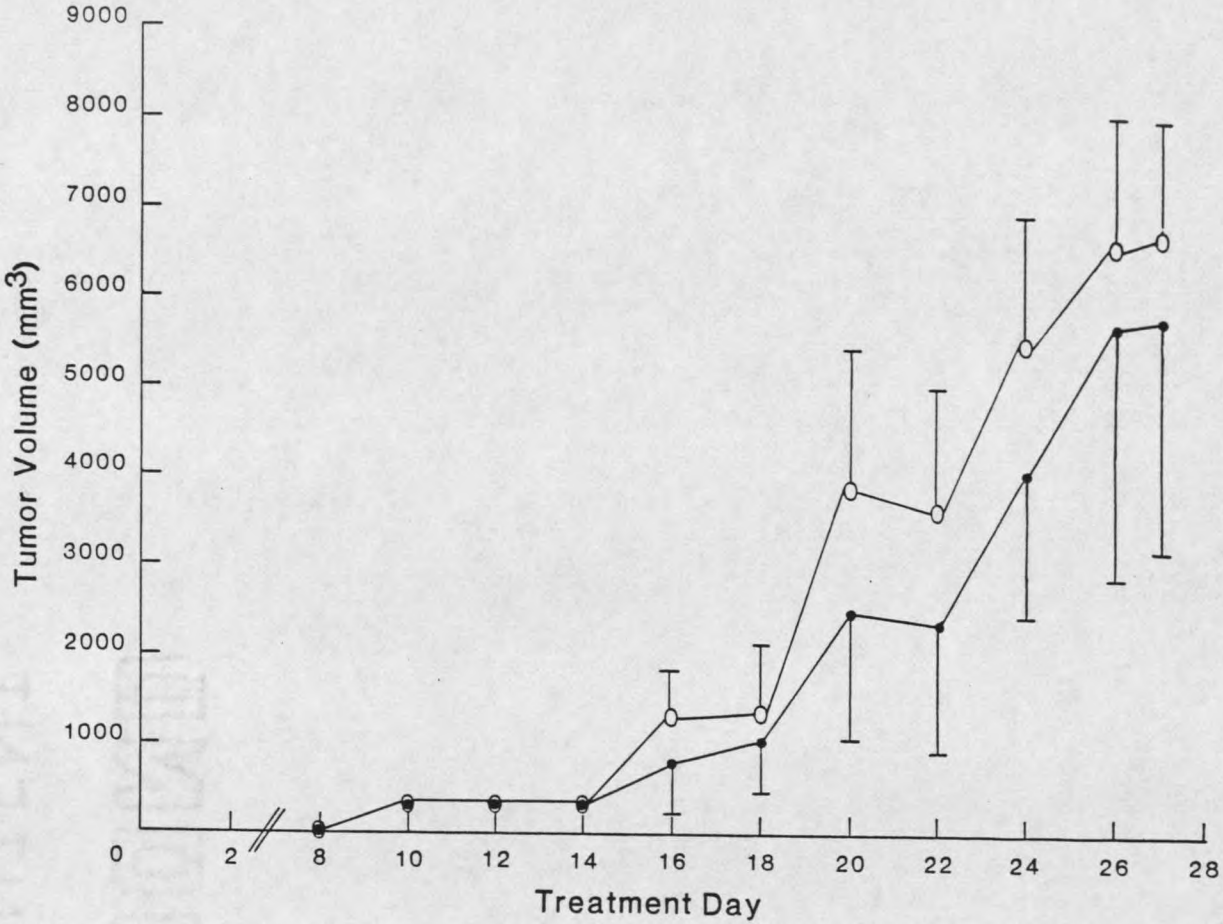

FIGURE 1. THE EFFECTS OF CHRONIC INJECTION OF TUMOR BEARER SERUM ON SUBCUTANEOUS TUMOR GROWTH. BD-IV rats were injected with 5×10^5 4bs cells subcutaneously. Two hours after tumor cell injection, control rats (●——●——●) received 0.2 ml NRS[a] and experimental rats (O——O——O) 0.2 ml TBS[a]. Sera injections were continued every second day until the experiment ended at day 27.

[a]NRS = serum from normal rats; TBS = serum from tumor-bearing rats.

ratios for splenocyte preparations from enhanced and nonenhanced tumor-bearing rats were similar, 0.84 ± 0.16 and 0.89 ± 0.21, respectively (Table 2). The H:S ratios established for nontumor bearer control rats were 1.63 ± 0.33 for splenocytes and 1.46 ± 0.10 for PBLs (Table 2).

Since a major reduction in the PBLs H:S ratio for "serum-enhanced" tumor-bearing rats was detected fourteen days after tumor inoculation, the kinetics of the helper:suppressor modulation was monitored every four days from the time of tumor cell injection until day twenty. Animals injected with 4bs tumor cells i.v. usually become moribund twenty-one days after injection of tumor cells. As shown in Figure 2, the immune systems of enhanced tumor-bearing rats started to change shortly after tumor injection. The H:S ratios for splenocyte and PBL preparations from "serum-enhanced" tumor-bearing animals started to decline as early as day four, although this was not as marked for splenocytes (Figure 2, Panels A and B). The H:S ratios for PBLs continued to decline until day sixteen when they stabilized. The H:S ratios for splenocytes continued to drop until the experiment was terminated. The lowest ratio, which was seen on day twenty, was 0.53 ± 0.05 for splenocytes (Figure 2, Panel A) and 0.52 ± 0.06 for PBLs (Figure 2, Panel B).

TABLE 2. HELPER : SUPPRESSOR T CELL RATIOS FOR "SERUM-ENHANCED" TUMOR - BEARING RATS, NONENHANCED TUMOR-BEARING RATS AND NONTUMOR-BEARING RATS

	SPLENOCYTES (HELPER:SUPPRESSOR ±S.E.)	PERIPHERAL BLOOD LEUKOCYTES (HELPER:SUPPRESSOR ± S.E.)
NONTUMOR-BEARING RATS (n = 4)	1.63 ± 0.33	1.46 ± 0.10
NONENHANCED TUMOR-BEARING RATS (n = 3)	0.89 ± 0.21	1.49 ± 0.21
ENHANCED TUMOR BEARING-RATS (n = 4)	0.84 ± 0.16	0.57 ± 0.03

Ten thousand 4bs tumor cells were injected i.v. into the lateral tail veins of four male BD-IV rats. Two hours later, two rats were injected i.p. with 0.2ml NRS[a] (nonenhanced tumor-bearing rats) and the remaining two rats were injected with 0.2ml TBS[a] (enhanced tumor-bearing rats). Sera injections continued every second day until day fourteen, at which time the helper:suppressor T cell ratios were evaluated by indirect immunofluorescence.

[a]NRS = serum from normal rats; TBS = serum from tumor-bearing rats.

On the other hand, the nonenhanced tumor bearers exhibited a slightly increased H:S ratio on day four, possibly reflecting a generalized early stimulation of immune activity as has been reported in several experimental systems (87) (Figure 2). H:S ratios for splenocytes and PBLs taken from nonenhanced tumor-bearing rats at day four were 1.54 $\pm$ 0.02 and 1.53 $\pm$ 0.07, respectively. After day four, the H:S ratios for splenocytes and PBLs started to decline and continued to do so until the experiment ended (Figure 2, Panels A and B). At the end of the experiment, the H:S ratio was 0.71 $\pm$ 0.02 for splenocyte and PBL preparations (Figure 2, Panels A and B). Control values for age/sex matched nontumor-bearing rats were 1.45 $\pm$ 0.07 for splenocytes and 1.45 $\pm$ 0.08 for PBLs.

H:S ratios changed for enhanced and nonenhanced tumor-bearing rats but this change was not accompanied by concomitant changes in overall B and T cell numbers. As shown in Table 3, B and T cell numbers did not change significantly when metastasis was enhanced. The percentages of T cells for splenocyte preparations from "serum-enhanced" tumor bearers were 50 $\pm$ 1% and PBLs 49 $\pm$ 1%. "Serum-enhanced" splenocytes comprised 27.5 $\pm$ 0.5% B cells and PBLs 29.5 $\pm$ 3.5% B cells (Table 3). B and T cell parameters were similar for nonenhanced tumor-bearers and