ANTIBODY SUPPRESSION OF LYMPHOMA GROWTH IN A SYNGENEIC TUMOR SYSTEM

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ABSTRACT

ANTIBODY SUPPRESSION OF LYMPHOMA GROWTH
IN A SYNGENEIC TUMOR SYSTEM

By

William W. Freimuth

Rabbit antiserum (anti-BAΘ) directed against the brain-associated theta (BAΘ) antigen was reactive with a surface component of S49A lymphoma cells. Coating the tumor cells with anti-BAΘ antibodies suppressed lymphoma growth in syngeneic mice. Preincubation of the lymphoma cells with anti-BAΘ before subcutaneous inoculation of mice prevented lymphoma growth in 85% of the hosts. Injection of anti-BAΘ directly into the tumor area 48 hours after a lethal tumor challenge prevented tumor growth. The lack of protection against tumor growth by normal rabbit serum (NRS) or chemically inactivated anti-BAΘ when preincubated with lymphoma cells or injected directly into the tumor area demonstrated that there were not non-specific factors in these sera which could suppress lymphoma growth. The ability of antibodies directed against a non-tumor specific antigen (BAΘ) to prevent or inhibit tumor growth *in vivo* in a syngeneic system represents a new and successful approach in antibody suppression of tumor growth.

Spleen cells from mice surviving previous tumor challenge were unable to provide passive protection against a lethal challenge

dose of tumor cells. Mice that survived a previous tumor inoculation 30 or 100 days earlier were rechallenged with unmodified or anti-BAΘ coated lymphoma cells. Immunological memory could not be established in these mice since the survivors did not demonstrate any suppression of tumor growth. However, factors which could agglutinate tumor cells were detected in the sera of mice inoculated with unmodified or antibody coated tumor cells. This result suggested that there was a humoral immune response to the lymphoma cells.

Sera from survivor and tumor bearing mice in which agglutinating factors were detected demonstrated no direct cytolytic ability when incubated with lymphoma cells in the presence of complement. Antibody coated tumor cells were not lysed by complement from normal mouse sera. Anti-BAΘ did not appear to be toxic to S49A lymphoma cells in the absence of complement since prolonged *in vitro* incubation of antibody coated cells did not reduce tumor cell viability.

To determine if syngeneic spleen cells could lyse unmodified or antibody coated lymphoma cells *in vitro*, a cytolytic assay using ^{51}Cr label was tested as a means of detecting membrane disruption. Spleen cells of survivor and tumor bearing mice did not demonstrate detectable cytolytic activity against unmodified lymphoma cells. In contrast when lymphoma cells were preincubated with anti-BAΘ before incubation with spleen cells from normal donors, a significant increase in percent specific ^{51}Cr release was observed. Spleen cells from survivor and tumor bearing mice had equal ability as compared with normal spleen cells to mediate antibody dependent cell mediated cytotoxicity (ADCMC). Lymphoma cells preincubated with NRS or sera of survivor mice were not killed by spleen cells of normal, survivor,

or tumor bearing mice. These results suggest that anti-BAΘ mediated cytolysis of tumor cells is specific, and survivor sera does not contain antibodies which could mediate ADCMC. Since only anti-BAΘ coated lymphoma cells were destroyed *in vitro* by spleen cells of normal syngeneic donors, antibody dependent cell mediated cytotoxicity is proposed as the mechanism of anti-BAΘ suppression of lymphoma growth *in vivo*.

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William W. Freimuth

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INTRODUCTION

Regulation of neoplastic growth by host defense mechanisms, first suggested by Ehrlich in 1909 (23), has been demonstrated in both man and animals. Modern tumor immunology has attempted to understand the immune defense mechanisms, and to learn how to repair or renew the protective capacity when neoplastic growth occurs. The importance of immunological prevention of tumor growth in the clinical setting was recently brought to the forefront of cancer research by Burnet's theory of "immunological surveillance" (5). Burnet proposes the formation of neoplastic cells as a common event during the entire life of vertebrates. He further postulates that malignant cells that do appear are normally eradicated by thymus-derived cells, after the immunological surveillance system recognizes the tumor specific antigens on the cell surface of neoplastic cells. However, impairment of the immune system by an inherited defect, immunosuppression, or reduced efficiency associated with increasing age may allow neoplastic growth to develop and, if unabated, the tumor may progress to fatal pathogenesis.

The importance of a functioning immune system was recently demonstrated in rodents that were thymectomized, treated with immunosuppressive drugs, or administered anti-lymphocyte serum. Good et al. (38) found that all of these animals had a significant increase in primary tumors after infection with certain oncogenic viruses or

following treatment with chemical carcinogens. Humans and mice that were immunosuppressed for transplantation of a foreign graft were ten times more likely to develop neoplasms of a leukocytic origin (38). Although the immune system is vital in the rejection of tumors, it has recently been demonstrated that there are several mechanisms of tumor "escape" from immunological destruction that involve immunological dysfunction. A subclass of thymus-derived cells can actually suppress the cellular immune response to tumor cells (62), and the antibody response to a variety of antigens (34). It has been suggested by Kaliss (57) that the humoral response directed against tumor allografts may be detrimental, since recognition of neoplastic cells may be prevented by masking tumor specific antigens. Recently, it was proposed by the Hellstroms (46) that immune complexes can block the efferent and afferent immune responses to tumor cells.

A large variety of immunological responses against neoplastic cells has been observed. Studies by Cerottini and Brunner (8,9), LeClerc et al. (67), and Plata et al. (87) have demonstrated that T-cells can effectively mediate destruction of allografts and neoplastic cells. Evans et al. (25,27) have shown that macrophages can be very potent killers of tumor cells. Recent evidence by Herberman et al. (51) and Kiessling et al. (59) have suggested the presence of "natural killer" cells which are responsive to certain viral induced antigens. Antibody directed against tumor cells, which has the potential to induce the cytolysis of tumor cells in the presence of complement, has been implicated by MacLennan (72) as the mediator of antibody dependent cell mediated cytotoxicity. The cytotoxic

mechanisms of each of these cellular and humoral responses against tumor cells, and their overall relationship to each other and the host's defense against tumor proliferation has been extensively reviewed (9,47,50,64).

The great potential and variety of immunological responses to tumor cells has often proven to be inadequate, even in immunologically competent hosts. Immunotherapy has been developed to help or replace deficiencies of the immune system. The immunologically competent host and the immunologically impaired host have been aided in their defense against tumor cells by passive transfer of immunologically competent cells or antibodies, and by active stimulation of the host's own immune system (73,99).

The following investigation is a study of an immunotherapeutic approach to stimulate the host to specifically destroy neoplastic cells in a syngeneic system. Effective suppression of tumor growth in syngeneic mice by administration of allogeneic or xenogeneic antiserum directed against tumor cells has been successfully demonstrated (14,39,42,93,130). However, antibody directed against antigens present on normal cells, which are also associated with the same neoplastically transformed cells, has not been studied directly. Therefore, rabbit antiserum specific for mouse brain associated theta (BA0) antigen, which is directed against theta antigen found on thymus-derived lymphocytes and lymphomas, could be utilized in this investigation to study its capacity to suppress lymphoma growth. The suppressive capacity of this antiserum was examined by preincubation of tumor cells with antiserum, or by its direct injection into the area of tumor inoculation. The *in vitro* finding of destruction

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of antibody coated tumor cells by syngeneic spleen cells from normal donors suggested that an antibody dependent cell mediated cytotoxic reaction occurred. This phenomenon is discussed as a possible mechanism for the observed antibody suppression of tumor growth *in vivo*.

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LITERATURE REVIEW

Tumor Specific Antigens

Surface changes in transformed cells create new tumor specific antigens (TSA) that can be recognized by the immune system of the host. Tumor specific antigens have been found on the cell surface of most tumors studied. However, Prehn (90) has observed that "spontaneous" tumors did not appear to have TSA which could induce tumor rejection in an immunologically competent host. Several correlations have been found between the TSA on the tumor cell and the inducing carcinogen. Chemical and radiation induced tumors have unique characteristic antigens that rarely cross-react with each other (64). Prehn (89) in 1957 and Klein (63) in 1960 first demonstrated that methyl-cholanthrene (MCA)-induced tumors in mice have different TSA from mouse to mouse, even in the same strain. They also showed that two MCA-induced tumors in a single mouse had different TSA. Chemical and radiation induced tumors often have histologically and pathologically identical neoplasms but different TSA (64). In contrast, viral induced tumors usually have at least one immunologically identical antigen. The TSA in tumors induced by viruses are occasionally parts of a budding viral envelope or are coded for by the virus (18). However, most alterations in normal surface components during the transformation process are coded for by the host. Dulbecco (18) has demonstrated that viral and chemical

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induced tumors can have different antigens internally, such as the intranuclear T-antigens of many DNA virus induced neoplasms. However, in every tumor system studied the TSA must be on the outer surface of the plasma membrane to cause an immunological rejection of that tumor.

A finding in many tumors was the presence of tumor associated embryonic antigens that have structures similar or identical to those found during embryogenesis or early development, but not present in adult life (10). The appearance of embryonic antigens has suggested that many malignant cells have dedifferentiated or uncovered masked neonatal antigens and given rise to rapidly dividing cells found during fetal life, which have lost the ability to respond to signals that inhibit growth.

In vitro and *in vivo* Methods

A variety of *in vitro* and *in vivo* assays have been created to measure the degree and type of immune responsiveness to neoplastic cells. Commonly used *in vivo* methods are adoptive transfer of sensitized lymphocytes into irradiated or normal animals; the Winn test (126), in which immune leukocytes and tumor cells are mixed together before injection; passive transfer of serum; and measurement of survival time or the tumor growth rate after a specific immunological treatment. The most popular *in vitro* assays include the colony inhibition assay, microcytotoxicity test, release of radioisotopes, and lymphocyte stimulation. The colony inhibition assay, designed by the Hellstroms (45), and its modification, the microcytotoxicity assay (113), measure the ability of leukocytes to inhibit the

formation of tumor colonies. Cytotoxicity measured by the release of radioisotopes has been employed in many tumor systems (4,7,50). Viable target cells are labeled with the radioisotope, and when the target cells die or their membrane is disrupted, the radioisotope is released into the media. Cellular or humoral immunity can be measured by radioisotope release after incubating immune lymphocytes or cytolytic antibodies and complement with labeled target cells. Lymphocyte stimulation measures the response of lymphocytes when admixed with target cells. The amount of incorporation of tritiated thymidine is related to the recognition and proliferative response by lymphocytes (15). An extensive review of these topics has been made by Herberman (50). All of the assays used to detect cytotoxicity mediated by the immune system have their limitations and measure various parameters of cytolysis, but they do provide a handle for an understanding of the complexities of the host response.

Immune Responses to Neoplastic Cells

The immune response to neoplastic cells can involve a broad spectrum of cellular and humoral responses. Cellular immune responses appear to be the more prevalent mechanism for inhibition of tumor growth and destruction of tumor cells. The various types of cellular responses which are measured by *in vitro* cytotoxicity assays are: 1) T-cell mediated cytotoxicity, 2) macrophage mediated cytotoxicity, 3) antibody dependent cell mediated cytotoxicity, and 4) natural killer cells. The role of cellular responses in the host's defense against neoplastic cells has been reviewed recently (9,47,50). Antibody directed against tumor antigens can also be beneficial to

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the host's response to tumor growth in the presence of complement, but some evidence suggests that antibody alone may inhibit the immune response. For any given tumor system any one or combination of the above mechanisms may be a potent immunological factor in abrogating tumor growth.

The concept of cell mediated cytotoxicity against neoplastic growth originated in 1955 when Mitchison (77) showed that immunity to tumor allografts containing foreign histocompatibility antigens could be adoptively transferred with lymphoid cells but not serum. The experiments of Klein et al. (63) in 1960 demonstrated that tumor specific immunity was mediated by cells in the lymph nodes or spleen from mice immunized to tumor specific antigens. In 1961 Winn (126) developed a neutralization test in which syngeneic tumor growth was inhibited when tumor cells were inoculated together with lymphocytes from allogeneic donors previously immunized against alloantigens of the tumors.

T-Cell Mediated Cytotoxicity

Allogeneic tumor systems. In many tumor systems, T-cell mediated cytotoxicity is thought to be the major immunological deterrent to neoplastic growth. The first direct evidence to support this concept for mediation of cytotoxicity by sensitized T-cells was demonstrated in an allogeneic mastocytoma tumor system by Cerottini et al. (8) in 1970. The following lines of evidence in allogeneic models provided the basis for this dogma. If B-cells were removed from an immune spleen cell population by immunoadsorbent columns, the B-cell depleted spleen cells were still cytotoxic to target cells *in vitro* (36,67),

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and could prevent tumor growth in lethally irradiated mice (9,110). If T-cells of an immune spleen cell population were destroyed by anti-Thy (θ) and complement, the cytotoxicity against the tumor cells by the remaining spleen cells was completely abrogated (36,66,67,86). In experiments by Cerottini et al. (8,9) and Freedman et al. (33), lethally irradiated mice were reconstituted with bone marrow or immune T-cells before injection of allogeneic tumor cells. The immune T-cells prevented tumor growth, while mice reconstituted with bone marrow and thymocytes died within two weeks.

The T-cell mediated cytolysis of neoplastic cells has been demonstrated to be a specific reaction. Partially purified immune T-cells could be adsorbed onto monolayers of sensitizing target cells, but not onto antigenically unrelated monolayers (9,110). Cytotoxic T-cells from mice sensitized to hapten coated tumor cells recognized haptens on the tumor cell surface and specifically destroyed the hapten coated neoplastic cells (17). Cerottini and Brunner (4) have shown during T-cell mediated destruction of target cells *in vitro* that antigenically unrelated bystander cells remained viable. Unlabeled target cells, added to a mixture of cytotoxic T-cells and ^{51}Cr-labeled tumor cells, competitively inhibited the T-cell mediated killing of labeled target cells (4,9).

Partial characterization of effector cell. Characterization of cytotoxic T-lymphocytes has been limited to antigenic markers and sedimentation velocity studies. Cantor et al. (7) have recently studied the Ly antigens on T-cells and correlated killer cells with the Ly-2,3 subclass which appears late in ontogeny. During allograft

rejection of various tumor cells the accumulated data suggest a differentiation pathway of T-cells into cytotoxic lymphocytes *in vivo*. Shortman et al. (107) found that the cytotoxic T-cell progenitor in the normal mouse spleen was a dense small lymphocyte. The early effector cells were light in density and corresponded to blast cells. As the response proceeds, the cytotoxic T-cells appear to pass through a series of maturation steps reflected by the increase in cell density and decrease in size (9,107). Recent evidence suggests that after these cells end their cytotoxic activity they can act in a memory response and differentiate into blast cells which produce accelerated cytotoxic T-cell formation upon secondary stimulation (9). The above results suggest that further differentiation or maturation of cytotoxic lymphocyte precursors occurs after they leave the thymus and populate the peripheral lymphoid tissues (9).

Syngeneic tumor systems. The initial concept of tumor specific immunity in syngeneic tumor systems was demonstrated by Gross (43) in 1943, Foley (32) in 1953, and Prehn and Main (89) in 1957. These researchers inoculated mice with syngeneic MCA-induced sarcoma cells, excised subsequent tumor nodules, and then rechallenged the mice with similar tumor cells, which the immunized mice rejected. Cell mediated cytotoxicity in syngeneic tumor systems was first shown by Klein et al. (63) in 1960. These investigators found that lymph node cells from mice immunized against syngeneic MCA induced sarcomas and mixed with tumor cells *in vitro* could neutralize tumor growth upon injection into sublethally irradiated mice.

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The study of T-cell mediated cytotoxicity in syngeneic tumor systems has been limited to the last few years. The most intensively examined syngeneic tumor system has been the Moloney sarcoma virus (MSV)-induced tumors, since spontaneous regression occurred about two weeks after viral immunization. Fefer et al. (29) in 1967 demonstrated that regression of MSV-induced sarcomas was mediated by immune lymph node cells. LeClerc et al. (67) in 1972 found cell mediated cytotoxicity to be predominantly T-cell dependent as measured by the ^{51}Cr release assay. Pretreatment of immune spleen cells with anti-Thy(0) and complement abrogated cytolytic activity, while nylon wool removal of B-cells and macrophages did not alter cytotoxicity (67,120). Lamon et al. (65,66) studied the same system with the microcytotoxicity assay and found cytotoxic T-cells in spleens and lymph nodes just prior to tumor development and just after tumor regression. However, these researchers found that a non-T-cell subpopulation was also cytotoxic *in vitro*.

Additional support for the formation of cytotoxic T-cells in syngeneic tumor systems was provided by studies in other spontaneous, chemical, and viral induced systems (12,36,65). Immune spleen cells from mice sensitized to a syngeneic plasmacytoma when transferred to sublethally irradiated syngeneic mice could prevent neoplastic growth (9). Allison (1) reported that transfer of syngeneic lymphoid cells from mice specifically immunized to a polyoma virus could prevent tumor growth. In both cases previous anti-Thy and complement treatment abrogated protection. In the first example rabbit anti-mouse light chain sera, which in the presence of complement will lyse B-cells, had no effect on the killing. Due to the

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limited number of syngeneic studies, the role of cytotoxic T-cells in syngeneic tumor systems remains to be elucidated.

Comparison of syngeneic and allogeneic tumor systems. In a few studies, the same tumor was examined in both syngeneic and allogeneic systems. Veit et al. (120) demonstrated that the MSV-induced sarcoma resulted in a qualitatively similar response by T-cells as the peak activity occurred on similar days, but quantitatively the allogeneic response was two- to fivefold greater than the syngeneic response. In a mastocytoma tumor system, Cerottini and Brunner (9) found that the number of ^{51}Cr-labeled immune T-cells injected into heavily irradiated hosts five days after subcutaneous (S.C.) inoculation of allogeneic and syngeneic tumor cells were four- to eightfold greater at the site of the allograft than at the site of syngeneic or antigenically unrelated allografts. One reason cell mediated cytotoxicity in allogeneic recipients was more vigorous than syngeneic recipients could have been that the allogeneic host's immune response was directed against histocompatibility (H-2) alloantigens, while the syngeneic host responded to the less immunogenic tumor specific or viral related antigens.

Mechanisms of T-cell mediated cytotoxicity. T-cell mediated lysis is thought to occur in three phases: (a) an initial stage in which the target cell is recognized by the appropriate receptor site on the T-cell membrane; (b) a protein synthetic phase; and (c) a final secretory phase which leads to eventual lysis of the target cell. This scheme which has been proposed by Henney (49) agrees

with the concept of cytolytic activity of effector T-cells being dependent on antigen induced mediator synthesis and its subsequent delivery.

The necessity of protein synthesis prior to the lytic process has been suggested by the effects of irreversible inhibitors of protein synthesis on cytotoxicity. Secretory activity was suggested by the modulation of cytolysis by cyclic-AMP levels (increased c-AMP concentration decreased cytolysis), and the inhibition of cytolysis by colchicine and EDTA (74). Inhibition of secretory processes by these agents has been consistently observed in a wide variety of cells (49).

Cytotoxicity proceeds linearly as a function of time, and the extent of lysis is dependent on the concentration of lymphocytes (9,49). One lymphocyte can kill more than one target cell as it can have multiple contacts with neighboring target cells (9,49). Recognition is temperature dependent. At 4°C, cytotoxicity is completely inhibited if cell-cell contact is not made, while at 15°C the induction of lysis slows down to 1/71 the rate at 37°C (74). Cerottini and Brunner (9) have demonstrated that the cytotoxic reaction is specific since animals immunized against two different specificities of histocompatibility antigens will develop two populations of specific cytotoxic T-cells each carrying one type of receptor. Receptors could be removed by protease, and would be rapidly replaced once the protease was removed. Once a cytolytic T-cell comes in contact with a target cell, no other cell is required for cytotoxicity (49).

The requirement of direct contact of cell membranes of the effector T-cell with the target cell has been indicated by a variety of

experiments. Killing cannot occur when target and effector cells are separated by a viscous liquid (49), a semi-permeable membrane (49), or dextran particles (74). Weiss (123) performed electron microscopic and immunofluorescent studies of the interaction between lymphocytes and target cells. His work revealed sizable portions of lymphocytic membrane lying in close contact with the target cell membrane and the existence of intercytoplasmic connections between interacting cells.

Once cell-cell contact is made, the cytotoxic T-cell reorganizes the target cell membrane to effect lysis. Within a few minutes the lytic process will begin even if T-cell contact is broken off by EDTA or shearing forces (74). Cell membrane movement between the attached cells appears to be important for lysis, since cytochalasin B, which disrupts the contractile network of microfilaments associated with the membrane, inhibits T-cell mediated cytotoxicity (49). T-cell mediated cytotoxicity appears to be similar to antibody dependent complement mediated lysis in that the target cells in both cases have increased permeability, and increased swelling due to osmosis (9,49,74).

Antibody Dependent Cell Mediated Cytotoxicity

The role of cytotoxic antibody and complement in the host's defense against neoplastic growth has been questioned for many years. Kaliss in 1958 first suggested that antibody alone may actually enhance tumor growth (57). Möller's studies in 1962 demonstrated that most tumor cells were resistant to isogenic antibody and complement (78). Möller suggested that the low density of TSA may be

responsible for insufficient fixation of complement. More recently, Fish et al. (31) and Lesley et al. (68) suggested that the presence of only a few susceptible areas on the fluid tumor cell membrane which complement could effect would explain the inability of antibody to lyse the cells. However, a new role of anti-tumor antibody has been demonstrated in association with cell mediated cytotoxicity (CMC) (72,88,112).

The observation of Möller (79) in 1965 that normal mouse lymph node cells would kill allogeneic sarcoma cells in the presence of xenogeneic anti-mouse spleen cell antibody first suggested a cellular cytotoxic mechanism dependent on antibody. Perlmann and Holm (83) in 1968 were the first researchers to demonstrate that antibody directed against target cell antigens could render cells susceptible to lysis by non-sensitized lymphocytes. Recently, the general phenomenon of antibody dependent cell mediated cytotoxicity (ADCMC) has been described by Perlmann (84) and MacLennan (72) as the binding of antibody to the target cell antigen, and the binding of the Fc portion of the cell bound antibody to an unsensitized effector cell, eventually leading to target cell lysis. Most of the original work was done in mice using chicken red blood cells (CRBC), and allogeneic or xenogeneic antibodies directed against these cells (83,84,102). Rabbit anti-CRBC preincubated with CRBC coated these cells and made them susceptible to lysis by the appropriate effector cell. However, normal rabbit serum (NRS) preincubated with CRBC could not mediate lysis as measured by a ^{51}Cr release assay.

The extent of lysis appears to depend on the concentration of effector cells and antigen density on the target cells. Weiderman

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et al. (124) have suggested that a critical antigen density on the surface of target cells was necessary for the Fc portions of the cell bound antibody to be close enough to associate with effector cells. Perlmann (84) has demonstrated that approximately 5-7 effector cells were needed to kill one target cell. MacLennan (72) demonstrated that cytotoxicity varied with the logarithm of the number of effector cells incubated with antibody coated target cells.

Several studies have appeared only recently that demonstrate the importance of ADCMC in tumor systems of mice, rats, and humans. MacLennan (72) in 1969 was one of the first researchers to demonstrate that antibody coated Chang cells (neoplastic human liver cells) could be destroyed by normal mouse lymphoid cells. Scornik et al. (100) demonstrated that anti-EL-4 serum incubated with EL-4 lymphoma cells rendered the tumor cells susceptible to normal allogeneic spleen cells, while normal mouse serum (NMS) could not induce any cytotoxicity. Using the colony inhibition assay, Pollak et al. (88) demonstrated that the target cells of a viral induced adenocarcinoma, MSV-induced sarcoma, or a MCA-induced sarcoma could be lysed by normal allogeneic lymph node cells, if the tumor cells were preincubated with the appropriate antiserum from tumor immune mice. Antibody dependent cell mediated cytotoxicity appears to be tumor specific since non-sensitized lymphoid cells and sera from animals bearing a tumor of a different origin were not inhibitory to neoplastic growth (88). Normal lymphoid cells of syngeneic or allogeneic mice could lyse EL-4 lymphoma cells coated with mouse or rabbit anti-EL-4 antibodies, but not with those tumor cells incubated with NRS or NMS (129). Normal human peripheral blood lymphocytes

(PBL) have also been implicated in killing only those tumor cells preincubated with the specific antisera (117).

Specificity of ADCMC or suppression of ADCMC was further demonstrated by Stolfi et al. (112), who used two different syngeneic mammary tumor virus (MTV)-induced breast tumors designated A and B. The A tumor preincubated with isoimmune sera from mice with surgically removed tumors would induce ADCMC upon addition of normal lymph node cells, but not with A tumor cells preincubated with B isoimmune sera, nor with B tumor cells preincubated with A isoimmune sera. These results showed the specificity of the sera and ADCMC. Also the lymph node cells of mice bearing A tumors greater than one gram had decreased CMC and decreased ADCMC to A tumor cells preincubated with isoimmune A sera, but not to B tumor cells preincubated with isoimmune sera (112). The antigenic specificity of suppression suggested that only a small subgroup of the effector cells of ADCMC may actually participate against any one antigen.

Nature of antibody. The nature of the antibody required for mediating ADCMC in rats, mice, and humans appears to be of the IgG class of immunoglobulins (72,84). Studies with CRBC have shown that all subclasses of IgG can mediate ADCMC to a similar degree, while only IgG_1 and IgG_3 can fix complement or allow phagocytosis by macrophages (84,100). Aggregated myeloma proteins of IgG_{1-3} could inhibit ADCMC (72,84). IgM or IgA directed against TNP on CRBC could not mediate ADCMC, and aggregated IgM or IgA could not block ADCMC (84). Only the IgG fraction of anti-EL-4 sera in mice (129), and anti-rat lymphoma (16) in rats, could mediate ADCMC. In all

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systems tested the only antibody that could induce ADCMC was of the IgG class.

The essential portion of the immunoglobulin that is bound by the effector cell is associated with the Fc fragment. Binding Fab or $F(ab')_2$ fragments of myeloma IgG will not inhibit ADCMC (72). Binding of Fc fragments to the effector cell can inhibit ADCMC, suggesting that the effector cell has a receptor for the Fc portion of the cell bound antibody (72,84). MacLennan (72) has suggested that after plasmin digestion of rabbit anti-CRBC IgG the remaining 107 amino acids in the C terminal piece contain the determinant that binds to the effector cells.

Antibody can be present on target cells which does not fix complement, yet has the capacity to activate effector cells to perform ADCMC. MacLennan (72) and Perlmann (84) have diluted rabbit anti-CRBC sera to 10^{-6} of the original concentration and still were able to induce ADCMC, while complement mediated lysis of CRBC required antisera diluted no more than 10^{-2}. One explanation for this observation could be the distribution of cell surface antigens and their bound antibodies was not favorable for complement mediated lysis, since complement generally requires that several IgG molecules be in close proximity to be activated (68,124). This requirement could explain how small amounts of IgG not in close proximity on the cell surface can mediate ADCMC, but cannot induce complement mediated lysis.

It appears that the IgG molecule undergoes conformational change upon binding to a specific antigen that helps it bind to the effector cell. Perlmann (84), MacLennan (72), and Zighelboim et al.

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(129) have demonstrated that pretreatment of lymphocytes with antisera requires much greater amounts (10^4 times with CRBC) of antibody for killing than preincubation with target cells, probably because free antibody was loosely bound to the Fc receptor of the effector cells. Also 40 times the amount of anti-CRBC can be retained on effector cells if it was complexed to CRBC rather than being free (72,84). Immune complexes of antibody coated target cells can competitively inhibit ADCMC much easier than free or aggregated antibody (72,84). MacLennan has demonstrated that the most effective inhibitors of ADCMC were small soluble immune complexes with a large number of exposed Fc pieces of immunoglobulin that could firmly bind to Fc receptors on effector cells (72). These results suggest that there is some beneficial change in antibody structure that allows a more avid binding to the non-specific effector cell.

Characterization of the effector cell. Characterization of the effector cell for ADCMC has been a process of elimination of known cells. The effector cell, designated as the K-cell, has been found to be most abundant in the unsensitized spleen, peritoneal exudate, and peripheral blood lymphocytes, but no K-cell activity has been found in the thymus or thoracic duct (42,72,102). Thymectomy and low dose irradiation did not change the ADCMC against antibody coated Chang cells (72). The same level of ADCMC was acquired in systems using CRBC or tumor cells in athymic nude mice, or in mice treated with anti-Thy and complement (42). Since removal of adherent and phagocytic cells did not reduce the killing of antibody coated Chang cells, the macrophage did not appear to be important in mediating

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ADCMC in this system (72). However, macrophages had the ability to kill antibody coated CRBC by phagocytosis, but this was a different mechanism from ADCMC (72). Plasma cells and B-cells did not have the ability to mediate ADCMC, suggesting that none of the known immune cells were the effector cell of ADCMC (84).

The murine K-cell appears to be a new classification of cells and has some defined characteristics. Greenberg et al. (42) described the murine K-cell as a non-adherent, non-phagocytic lymphoid cell that lacks both surface immunoglobulin and theta antigen, and was found in low concentrations in the spleens of mice. Perlmann et al. (85) suggested that the effector cells have complement receptors, possibly for C3b and C3d components of complement. K-cells have an Fc receptor as shown by binding and competitive inhibition studies with immune complexes. Schirmacher et al. (98) demonstrated that the Fc receptor of K-cells was different from that of EA rosette forming cells and was not associated with Ia antigens. Recently, another type of effector cell has been described by Greenberg et al. (42), who designated it the myeloid K-cell. This is an adherent monocytic and granulocytic cell with an Fc receptor, and can be subdivided into phagocytic and non-phagocytic classes. Similarly, the human effector cell is a medium to large size mononuclear cell that is non-adherent, non-phagocytic, and has an Fc receptor (117).

Mechanism of ADCMC. The mechanism of ADCMC involves direct cell contact, and is energy dependent. The cytolytic reaction was inhibited when the temperature was lowered to 4°C, or by drugs which inhibit anaerobic or aerobic energy production (101,118). The

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importance of cell-cell contact in cytolysis was suggested by inhibition of ADCMC by cytochalasin B (101,118). Scornik et al. (100) demonstrated that there was not a soluble mediator present as non-sensitized bystander cells were not killed, while neighboring antibody coated cells were destroyed. Supernatants from these active reactions of ADCMC did not have any cytotoxic effect when added to a new culture.

The accumulated data at present suggest a general mechanism for cytolysis of antibody coated target cells by K-cells. The effector K-cells bind to antibody coated target cells through the Fc receptor present on the K-cell (72,84,101). Scornik et al. (101) proposes that after contact was made cell to cell interactions began and binding was irreversible. Target cells became damaged soon after contact with K-cells, and a certain degree of swelling was detectable (101). After a critical membrane lesion was established, the intracellular components began to leak out and critical intracellular concentrations of important ions such as Na^+ were not maintained (84,101). Evidence reported by Perlmann (84) suggests that during the lytic process the effector cell remains bound to the target cell until the damaged cell disintegrates. They also found that some of the antibody could be reutilized.

ADCMC and CMC Occurring Simultaneously

A few studies have concentrated on determining whether ADCMC and CMC occur simultaneously in one animal bearing a tumor, and what their interrelationships are. Xenogeneic and syngeneic studies indicated that CMC and ADCMC were independent of each other. In a

^{51}Cr release assay, DeLandazuri et al. (16) demonstrated that unlabeled antibody coated lymphoma cells would only inhibit ADCMC, while unlabeled and uncoated lymphoma cells would inhibit CMC only. If Fc receptor bearing cells were depleted by adsorption on antibody coated sheep red blood cells (SRBC), only ADCMC was abolished. Anti-Thy and complement treatment or adsorption of immune lymphocytes on syngeneic lymphoma cells abrogated CMC only (16).

Target cells from mice with surgically removed tumors pretreated with isoimmune serum before addition to syngeneic immune spleen cells significantly increased lysis of target cells as compared to target cells preincubated with NMS (16,112). The above results suggest that immune spleen cells in these tumor systems have at least two populations of cells: killer T-cells and K-cells. Stolfi et al. (112) and DeLandazuri et al. (16) demonstrated that immune spleen cells depleted of T-cells by anti-Thy and complement were 2-3 times more active in ADCMC than normal spleen cells. The increased ADCMC may be due to an increased number of the same type of effector cells, or a non-T-cell population such as macrophages (112). It appears that ADCMC has a very important role in eradicating neoplastic cells, and its true potential in the immune response is just becoming understood as is the role of the macrophage.

Macrophage Mediated Destruction of Neoplastic Cells

Macrophage killing of neoplastic cells was first demonstrated by Granger and Weiser (40) in 1964. They detected allogeneic tumor cell destruction by immune macrophages *in vitro*. Soon afterwards evidence was presented that immune macrophages could transfer to new

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hosts some ability to resist tumor allografts (41). At present, the role of the macrophage in the immune response to tumor cells has become more important with a better understanding of its specificity, interactions with other lymphoid cells, and the receptors on its cell surface that can bind to target cells. New evidence has been acquired that demonstrates macrophage destruction of tumor cells, macrophage infiltration of tumors, macrophage prevention of metastasis, and soluble factors produced by macrophages which inhibit tumor cell growth.

Armed and activated macrophages. A scheme proposed by Evans et al. (25,27) suggests that normal macrophages can progress into two active states. In the first state the normal macrophage becomes "armed" by appropriate hyperimmunization, *in vitro* incubation with spleen cells of hyperimmune mice, or exposure to cell free supernatant obtained from immune spleen cells that were cultured with the specific immunizing antigen (26). Armed macrophages were cytotoxic to specific target cells only. When an armed macrophage comes in contact with the specific immunizing antigen a second time, the armed macrophage transforms to become an activated macrophage (26,27). After direct contact between an activated macrophage and a target cell, the actual destruction of the target cell was non-specific (25,27).

Macrophages can be armed in the presence of immune T-lymphocytes, or their soluble products, after incubation with the immunizing antigen. Soluble products found in cell free supernatants of spleen cells from immunized mice incubated with tumor cells that could arm

macrophages were designated specific macrophage arming factor (SMAF) (26,86). Several investigators have suggested that SMAF could arm macrophages to kill neoplastic cells, but not normal cells (26,86, 119). Evans et al. (26) have characterized SMAF as a 50,000-60,000 molecular weight substance, while Peissens et al. (86) think SMAF may be macrophage inhibition factor. The production of SMAF appears to depend on immune T-cells, since anti-Thy and complement would prevent immune spleen cells from making SMAF when incubated with antigen (26,86). Macrophages could be armed *in vivo* by a single injection of hyperimmune spleen cells, but not with unsensitized spleen cells, suggesting the necessity of other immune cells to stimulate macrophages into an immunologically active role.

Mechanism of cytolysis. Immune macrophages recognize and bind to target cells after acquiring cytophilic factors. Granger and Weiser (41) in 1966 first suggested that these factors may be antibodies. More recently, Evans et al. (26) have suggested that these cytophilic factors may be SMAF, and appear to have specific affinity for a surface component of the sensitizing target cell. Macrophage killing of target cells appears to be due to some form of cell-cell contact or the production of a soluble toxin. Evans et al. (27) suggest that neoplastic cells are killed after membrane contact between the macrophage and the target cell. These investigators demonstrated that lymphoma cells in contact with immune macrophages for up to 24 hours could not replicate, and after 48 hours almost all lymphoma cells were dead. Phagocytosis occurred only after target cells were not viable and were disintegrating. In

contrast, Reed et al. (94) suggest that a non-specific soluble macrophage toxin was elaborated by viable macrophages after attachment to the tumor cell surface. The toxin production peaked in 8-16 hours at the same time tumor cell death peaked; then it disappeared over the next 24 hours. The toxin has been characterized by Reed et al. (94) as a 45,000 M.W. macrophage product, and may represent a lysozymal enzyme. Therefore, it appears immune macrophages bind specifically to target cells and disrupt the target cell membrane by a soluble toxin or some mechanism involving cell-cell contact.

Macrophage infiltration of tumors and control of metastasis. The macrophage has been shown to infiltrate growing *in vivo* tumors, and has been associated with the control of metastasis in some tumor systems. Evans et al. (28) found that macrophages, added to animals depleted of macrophages by anti-macrophage serum and complement treatment, infiltrated the progressively growing tumors. Several investigators have isolated macrophages from tumors *in vivo* (21,28, 44,119). These macrophages could proliferate *in vitro* but not in the presence of tumor cells. When the tumor cells were cultured without macrophages they proliferated ten times faster, suggesting a mutual suppressive activity by both macrophages and tumor cells (28,119). Haskill et al. (44) demonstrated that macrophages isolated from tumors were non-specific and more effective in killing tumor cells than peritoneal macrophages from tumor bearing mice, which were tumor specific in their killing. In a variety of experimental tumors, Evans et al. (28) found that 4-56% of the total cell population were macrophages of host origin derived from circulating blood

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monocytes, rather than self-replication of macrophages within the tumor.

In six different rat sarcomas in syngeneic recipients Eccles et al. (21) observed that the more macrophages found in the tumor the less metastasis occurred, and the larger the threshold dose required for fatal tumor growth. The tendency to metastasize was inversely related to immunogenicity of tumor cells. Even though an immune response was initiated to these proliferating tumors, it was not enough to induce macrophage infiltration by itself. These results suggested that an effective immune response at the site of tumor proliferation must occur to control neoplastic growth (21). The above experiments indicate that macrophages have an active role in killing tumor cells, preventing metastasis, and probably help in reducing the soluble tumor antigen load by phagocytizing disintegrating tumor cells.

Natural Killer Cells

A "natural" killer cell has been discovered in normal mice that was cytotoxic for some viral induced tumor cells. Kiessling et al. (59) demonstrated that spleens of normal young adult mice contained "naturally occurring" killer cells specifically directed against eight different Moloney leukemia lines. Studies by Kiessling et al. (59) and Herberman et al. (51) have demonstrated natural killer cells were present in one- to three-month-old mice, most frequently in the spleen, and to a lesser extent in lymph nodes, bone marrow, PBL, and peritoneal exudate, but not in the thymus. Killer cells were not removed by passage through nylon wool or

anti-immunoglobulin columns, and they were not sensitive to anti-Thy and complement (51,59). The natural killer cells did not perform ADCMC and were not inhibited by aggregated IgG (51,59). These investigators suggest that the killer cell was naturally occurring, yet they claim the effector cell response was Moloney leukemia virus specific, which suggests the killer cell was sensitized against virally-induced antigens rather than being a "naturally occurring" cell.

Obviously the host has a large number of cellular responses that can be focused on the destruction of neoplastic cells. However, even with such a vast and potent immune system the appearance of tumor growth is a common phenomenon. Studying the mechanisms by which malignant cells escape from immunological control should enhance the understanding of the actual level of immunological regulation of neoplastic growth *in vivo*.

Escape Mechanisms from Immune Surveillance

Despite the normally effective immune system with its great potential for a response to a specific antigen, there have been many experimentally supported mechanisms of escape from immunological control by neoplastic cells. The most prominent theories of escape mechanisms are: immunological enhancement, shedding of tumor specific antigens, blocking by immune complexes, antigenic modulation, and action of suppressor lymphoid cells. Any one of the above escape mechanisms could render a normally resistant host susceptible to tumor proliferation.

Immunological Enhancement

Immunological enhancement as originally defined by Kaliss (57) in 1958 was the successful establishment of a tumor homograft and its progressive growth as a consequence of the tumor's contact with specific antiserum prior to inoculation of an allogeneic tumor graft. Active enhancement was induced by injection of soluble tumor membrane before tumor challenge (57). Cruse et al. (11) demonstrated that antibodies directed against the whole antigenic profile of the tumor cell, especially the histocompatibility antigens in allografts, were much better at inducing enhancement than antibody directed against a few tumor isoantigens, which may leave some immunogenic membrane components exposed. Immunological enhancement was very specific since isoantibody directed against one allogeneic tumor could not enhance growth of a different H-2 tumor or a tumor with very immunogenic TSA (11). Mice were challenged with one tumor allograft to which it was sensitized, and then challenged with another tumor syngeneic to the first (128). Winn (128) found that the latter tumor survived if injected after the first, because antibodies directed against histocompatibility antigens were already present, while the original tumor was rejected.

Blocking antibodies have usually been found in the IgG class, with the exception of an observation by Winn (128), who found IgM enhanced tumor growth. Both complement fixing IgG_2 and non-fixing IgG_1 can enhance tumor growth; thus, cytolytic antibodies can also be enhancing antibodies. Investigators feel that the ability of antibody to fix complement is not important in tumor enhancement, although complement may have some other effect since complement

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depleted mice have greater enhancement of tumor growth (11,128). The $F(ab')_2$ and Fc fragments of enhancing IgG can enhance tumor growth, but whole IgG was about twice as effective as either fragment in enhancement (11). Cruse et al. (11) have suggested that the relative ability of IgG to enhance tumor growth was dependent on its affinity for antigen.

Several mechanisms have been proposed to explain the action of enhancing or blocking antibodies, including masking the antigen before its exposure to the host's immune cells (128), regulation of antibody production by feedback inhibition (11), and the Fc portion of the enhancing antibody binding to immunologically reactive cells and preventing recognition of tumor cells or reducing the effector cell's binding capacity to TSA (3,11). Preincubating allogeneic hyperimmune peritoneal macrophages with goat anti-mouse lymphoma antibody abrogated *in vivo* protection (19). Lesley et al. (68) and Cruse et al. (11) suggested that bivalent enhancing antibody may cause capping followed by endocytosis, change antigen density, and possibly remove antigen from the cell surface. An example of this mechanism is antigenic modulation of the thymus leukemia (TL) antigen in the presence of anti-TL antibodies (111). All of the above mechanisms are possible explanations for enhancement of allografts, but they do not entirely explain the survival of allogeneic or syngeneic tumors when enhancing antibody was absent from the sera and tumor cell surface, or in highly immunized animals with high levels of cytotoxic antibodies (2,3,46). Recent research has offered an alternative explanation that antibody binds to free

antigen, or that antibody bound to surface antigen is shed in the form of immune complexes.

Free Tumor Specific Antigens and Immune Complexes

The existence of blocking factors in serum of mice bearing progressively growing tumors was first described by the Hellstrom's in 1969 (46). Serum of human patients and animals with growing tumors abrogated *in vitro* destruction of neoplastic cells by immune spleen cells sensitized to the TSA of these tumors (2,3,46,47). The effect was transient as suppressed lymphocytes regained their activity when incubated *in vitro* without the blocking serum factors (47).

Antigen-antibody complexes were first suggested to be blocking factors by Sjorgen et al. (108) in 1971. The blocking serum which inhibited cytotoxic lymphocytes directed against MSV induced tumor cells was fractionated into substances greater than 100,000 M.W. and substances less than 10,000 M.W. The fraction containing substances greater than 100,000 M.W. was shown to contain antibodies which could be adsorbed by tumor cells. Individually these fractions lacked blocking activity, but when recombined the blocking activity was restored. Immune complexes have been found to be associated with the presence of various tumors in rodents (3,115). Baldwin demonstrated that the blocking activity of sera from hepatoma bearing rats could be eliminated when the sera were incubated with goat anti-mouse IgG, or adsorbed onto hepatoma cells. After Sephadex fractionation of the sera, blocking activity was found in the 7S

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fraction, suggesting that part of the blocking activity was associated with tumor specific IgG (3).

Some investigators have found free tumor specific antigen in the serum of tumor bearing mice at various stages of tumor growth (3,12,115). Sera of hepatoma bearing rats contained a large amount of free TSA during early tumor growth, and immune complexes plus free TSA during the late stages of tumor growth (3). Thompson (115) utilized a radioimmunoassay to detect soluble TSA of molecular weights less than 100,000 in the circulation of mice within 24 hours after a large inoculum of sarcoma cells. Thompson observed that the level of detectable soluble TSA was dependent on the challenge dose; the larger the initial dose of inoculum, the greater the level of soluble TSA that could be attained in the sera of challenged mice. One week after tumor inoculation the amount of TSA released by the tumor continued to increase at about the same rate as the increase in tumor cell population (115). However, in syngeneic mice successfully fighting the subsequent tumor growth of a large tumor challenge (due to Bacillus Calmette-Guerin [BCG] immunotherapy), the level of TSA fell continuously until none was detected (115).

Free TSA can specifically inhibit the killing of target cells by immune lymphocytes. Sjorgen et al. (108) demonstrated that soluble TSA could block immune lymphocyte cytotoxicity, but the TSA must remain bound to the immune lymphocyte. Solubilized TSA incubated for one hour with immune lymph node cells could inhibit the cytolytic response of these cells against syngeneic tumor cells, but not against other antigenically unrelated syngeneic neoplastic cells (3,108). Similar results were obtained with solubilized TSA of a

human colon carcinoma (47). The amount of inhibition in both cases increased with the amount of soluble TSA added to the reaction mixture.

The previous studies suggest that the soluble factors present in the sera of tumor bearing animals prevented immune lymphoid cells from killing neoplastic cells. These blocking factors were characterized as either immune complexes of anti-tumor antibody bound to soluble tumor antigens or soluble TSA alone. However, research performed to understand the mechanisms of suppression of the immune response to neoplastic cells by soluble factors led to the discovery that some cells of the immune system had the capacity to suppress the immune response against tumor cells.

Suppressor Cells

Along with soluble suppressor factors there appear to be cells in some tumor systems that contribute to the immunological suppression seen *in vivo*. Suppressor cells of thymus origin that are found in some tumor systems may be similar to those characterized in regulation of the antibody response (20,34,58). Experiments by Treves et al. (116) have shown that spleen cells from mice immune to a syngeneic lung carcinoma could kill tumor cells *in vitro* while they would enhance tumor growth *in vivo*. Anti-θ and complement treatment of splenocytes containing suppressor cells for a sarcoma (62), or a lung carcinoma (116) abrogated the suppressor activity. Further evidence of the T-cell nature of the suppressor cell was acquired by adult thymectomy, which reduced metastasis of the carcinoma (116). In mice with intact immune systems there was a marked suppression of

macrophage binding to leukemia cells shortly after an inoculation of leukemia cells. Adult mice thymectomized before initial tumor challenge allowed the macrophages to bind to leukemia cells *in vivo* in the presence of cytophilic antibody (62). The above experimental evidence indicated that suppressor T-cells could play an important role in enhancing tumor growth.

Suppressor T-cells appear to enhance tumor growth by suppressing anti-tumor mechanisms, rather than directly stimulating tumor cell proliferation. Treves et al. (116) observed that lethally irradiated recipients had similar tumor growth rates when tumor cells were mixed with spleen cells of either normal or tumor bearing mice. Spleen cells of tumor bearing mice injected separately or with tumor cells enhanced tumor growth in non-irradiated mice (116). A possible mechanism may be that adoptively transferred immune T-cells interact with normal T-cells in the recipient. Eardley (20) and Feldman (30) suggest that immune T-cells emit a signal recognized by the host's cells which generate immunosuppression by feedback inhibition, although both immune and normal spleen cells can function quite well on their own.

Adherent cells bearing surface immunoglobulin have also been implicated as suppressor cells. Spleen cells of mice bearing a MSV-induced sarcoma (61), a Gross virus induced lymphoma (35), or a MCA induced sarcoma (22) inhibited phytohemagglutinin (PHA) stimulated lymphocyte proliferation to syngeneic tumor cells, but did not inhibit cytotoxicity except in the latter case. Suppressor cells were characterized as macrophages, because of their adherence to glass, radioresistance, inactivation by carrageenan, and removal by

carbonyl iron and magnetism treatment. Eggers et al. (22) and Kirchner et al. (61) have suggested that inhibition of lymphocytes occurs at the sensitization stage before initiation of DNA synthesis, thus not affecting previously sensitized lymphocytes. In another study spleen cells from three different tumor bearing mice were isolated by velocity sedimentation techniques. Those cells which cosedimented with activated B-cells could suppress PHA stimulation of lymphocytes (61). It appears that in any tumor system there may be a variety of cells normally associated with the immune system that have the potential to enhance tumor growth.

Immunotherapy

A variety of immunotherapeutic approaches to control neoplastic growth by active or passive immunity and their experimental results have recently been reviewed (73,99). The following is a brief description of the types of immunotherapy that have been tested: 1) Prophylactic immunization against specific antigens of oncogenic viruses using viral capsid or TSA of infected cells. 2) Active non-specific immunization using adjuvants or agents such as BCG, *Corynebacterium parvum*, or dinitrochlorobenzene skin painting to induce delayed hypersensitivity. 3) Active specific immunotherapy by injecting x-irradiated or mitomycin treated autologous tumor cells, soluble tumor extracts, inert allogeneic or viable tumor cells that share common TSA with the autologous tumor, or by modifying the tumor cell membrane structure chemically, enzymatically, or by binding a strongly immunogenic substance to the cell surface before challenging the recipient. 4) Adoptive transfer of immunity

using immunologically competent and specifically sensitized lymphocytes of a donor with closely matched histocompatibility antigens. 5) Passive transfer of certain informational substances like "immune" RNA or transfer factor, which have the ability to transmit specific immunity to a non-immune population of immunocompetent cells. 6) *In vitro* immunization followed by adoptive transfer, by non-specifically stimulating autologous lymphocytes with mitogens, or specifically sensitizing lymphocytes by incubation with tumor cells treated with mitomycin C or irradiation. 7) Passive administration of antiserum cytotoxic to tumor cells, or unblocking antibodies. 8) Immunization against factors required for tumor growth. 9) Binding toxic materials such as ^{125}I, alkylating agents, diphtheria toxin, or toxic enzymes to tumor specific antibodies with the expectation that the toxic substance would be directly delivered to the tumor. Several of these methods have shown little or no protection against tumor growth. However, some immunotherapeutic approaches have met with varying degrees of success, depending upon the state of tumor progression, the type of tumor under study, and the immunological status of the host.

Antibody Suppression of Tumor Growth

One immunotherapeutic approach pertinent to the research to be presented is the use of cytotoxic antibody specifically directed against TSA, or the use of allogeneic or xenogeneic serum of animals immune to a specific tumor. The study of passive antibody suppression of tumor growth has been impaired recently by the concern that the administered antibody will enhance tumor growth, rather than abrogate

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it. In spite of the potential problems of immunological enhancement, several reports have suggested that appropriate antiserum can provide a successful immunotherapeutic approach to control neoplastic growth.

The first major successful study in antibody suppression of tumor growth was reported in 1956 by Gorer and Amos (39). They used alloantisera from CBA mice immunized by injection of EL-4 lymphoma cells. Unadsorbed antiserum injected I.P. into mice 24 hours before S.C. challenge of allogeneic tumor cells would prevent the appearance of tumors in almost 50% of the mice; normal CBA mouse serum had no effect. Mice injected up to seven days before tumor challenge with alloantisera were protected to the same degree, and mice injected up to two days later could prevent tumor appearance in 40% of the mice. Liver adsorbed anti-EL-4 injected I.P. into syngeneic C57Bl mice was found to delay tumor appearance and subsequent survival time by 10-14 days, indicating there was not any difference in the rate of tumor growth from control mice once the tumor appeared. Injection of alloantisera into syngeneic mice up to seven days before or one day after a tumor cell challenge provided some protection, although all mice finally succumbed to tumor growth.

Several other studies have demonstrated that suppression of tumor growth could occur to varying degrees when antisera directed against tumor cells was passively transferred into the challenged host (14,39,42,93). Davies et al. (14) demonstrated that tumor specific mouse alloantisera, adsorbed *in vivo*, and xenogeneic rabbit antisera to EL-4 and SB-1 lymphoma cells exhaustively adsorbed with spleen, were able to suppress syngeneic lymphoma growth *in vivo*.

Rao et al. (93) observed that liver and spleen adsorbed rabbit antiserum directed against Yoshida ascites sarcoma in rats could prevent tumor growth, if the antiserum was injected I.P. for seven consecutive days after the initial intraperitoneal tumor challenge. Both researchers found that injections of normal rabbit or normal mouse sera did not provide any protection against neoplastic growth.

Preincubation of tumor cells with tumor specific antisera could suppress neoplastic growth in syngeneic animals. In a series of studies, Shin et al. (104-106) have shown that if alloantisera directed against lymphoma cells were preincubated with tumor cells before injection, tumor appearance and subsequent death were delayed by seven days. Zighelboim et al. (130) demonstrated that preincubating EL-4 lymphoma cells with unadsorbed rabbit or allogeneic anti-EL-4 sera prevented palpable tumors from appearing when a lethal dose of these cells was inoculated I.P. in syngeneic mice. Preincubating lymphoma cells with normal mouse or rabbit sera did not provide any protection against neoplastic growth in either tumor system.

Mechanism of suppression. Only a few researchers have studied the mechanisms of antibody suppression of tumor growth and the cells involved (105,106,130). The role of complement in antibody suppression of lymphoma growth was studied in B10D2 mice deficient in the fifth component of complement (C5) (104). Heat inactivated alloantisera of AKR mice which lacked C5 did not contribute to the complement activity of the host. Mice deficient in C5 which received lymphoma cells preincubated with specific alloantiserum had a

prolonged survival of seven days as compared to mice which received lymphoma cells preincubated with normal mouse serum (104). Shin et al. (104) utilized C3H mice with an intact complement system and decomplemented them by injections of cobra venom factor which made C3 activity undetectable one day before antibody coated lymphoma cell challenge. Decomplementation had no effect on normal tumor growth or on the suppression of alloantibody coated lymphoma cells by the host (42,104). Addition of fresh normal mouse serum to rabbit antibody coated EL-4 cells did not induce their lysis *in vitro* or *in vivo* (130). These results suggest that C3 and C5 components of complement were not essential for antibody mediated destruction of lymphoma cells, and the suppressive effect was not mediated through tumor cell lysis activated by endogenous mouse complement.

A variety of cells which could be involved in *in vivo* antibody mediated suppression of tumor growth have been studied. Sublethal irradiation of mice one day before tumor challenge with antibody coated lymphoma cells completely abrogated the suppression of tumor growth (106). Shin et al. (106) also observed that peritoneal cells of normal mice, cell cultures containing 99% macrophages, or a large number of platelets could restore suppressive activity if admixed with tumor cells just before inoculation into the calf muscle of irradiated mice. Syngeneic peritoneal macrophages receiving thioglycallatecin (an inflammatory agent), followed five days later by 500R of x-irradiation, could restore suppressive activity. However, macrophages from mice that were only irradiated could not restore suppressive activity (106). Zighelboim et al. (130) found that I.P.

injection of thioglycollate three days before challenge of antibody coated EL-4 cells allowed greater protection against a higher dose of antibody coated tumor cells. T-cells were shown to be unimportant as anti-Thy and complement treatment of peritoneal cells or thymectomy of adult mice did not alter the *in vivo* suppression of lymphoma growth (130). The presence of antibody on lymphoma cell surface was required for suppression even with thioglycollate stimulated macrophages (106,130). It appears that macrophages play an important role in *in vivo* protection against antibody coated tumor cells, but thymus derived lymphocytes may also play a significant role.

Studies by Zighelboim et al. (129,130) and Hersey (42) have suggested that the mechanism of *in vivo* antibody mediated suppression of neoplastic growth may be ADCMC. *In vitro* experiments by Zighelboim et al. (129) showed that adherent cells and non-adherent cells of normal syngeneic spleens could kill antibody coated lymphoma cells. Adherent cells were 50-200% more effective at lysis of rabbit antibody coated EL-4 cells than non-adherent spleen cells. Macrophages from thioglycollate stimulated peritoneum were very effective in ADCMC. The killing was specific since lymphoma cells preincubated with NRS or uncoated bystander cells of unrelated syngeneic tumors were not killed (129). Hersey (42) demonstrated that rats receiving I.P. injections of tumor specific antiserum retained ADCMC activity against syngeneic lymphoma cells for 6-7 days after the first administration of antisera. The accumulated data suggest that passive antibody suppression of tumor growth *in vivo* is dependent on adherent macrophage-like cells and non-thymus derived lymphocytes

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that require the presence of antibody on the target cell surface, which may mediate ADCMC with the above effector cells.

In summary, the accumulated evidence from *in vitro* studies on the cell mediated immune response to neoplastic cells has demonstrated that a wide repetoire of cytotoxic cells have the capacity to destroy tumor cells. There are cells like the natural killer cells that may be available to destroy certain virally transformed cells, or those tumor cells that express their characteristic antigenic membrane changes. There are K-cells which can be converted to specific effectors of ADCMC upon contact with neoplastic cells coated with tumor specific IgG. Normal macrophages have been suggested to become specific cytotoxic macrophages after contact with soluble factors released by immune T-cells. Both the K-cell and the armed macrophage can effectively kill tumor cells, and may play a role in controlling metastasis. The T-cells, which have been designated the role of coordinator of the immune system, can induce B-cells to differentiate into antibody forming plasma cells, arm macrophages, or become specific cytotoxic killer cells. Any of these effector cells alone has been shown to estroy neoplastic cells *in vitro*. However, the *in vivo* situation would seem to involve all of these cells in relative proportions, and their success in inhibiting tumor growth would depend upon the immunological state of the host, the type of neoplasm, the initial dose, and the initial location of tumor growth.

Much evidence has been accumulated to demonstrate an immune response can actually stimulate tumor growth. Suppressor T-cells and macrophage-like cells can enhance tumor growth possibly by

suppressing the immune response directed against the tumor cells. The specific antibody response to tumor cells has been implicated in providing a means in which neoplastic cells can mask their immune recognition sites. Soluble tumor specific antigens have been suggested to bind to tumor specific antibodies or remain free in the serum. It has been suggested that these soluble factors have the capacity to specifically inhibit cell mediated responses against tumor cells. The present understanding of tumor immunology suggests that the immune response to neoplastic cells is two sided, and any manipulation of the immune response for immunotherapeutic purposes must look at the potential dangers as well as the potential benefits.

MATERIALS AND METHODS

Mice

Two- to four-month-old male and female BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME) and Flow Laboratories (Dublin, VA). All mice in each experiment were age and sex matched. Rechallenged mice were older, but they had appropriate age and sex matched controls.

Tumor and Its Growth Characteristics

The S49A lymphocytic lymphoma is a solid tumor that was induced by mineral oil in female BALB/c mice in 1966 at the Salk Institute (San Diego, CA) (54). The S49A lymphoma was a gift from the Salk Institute. The S49A lymphoma was passed in a cell suspension in Eagle's Minimal Essential Medium (MEM) and injected subcutaneously (S.C.) in the abdomen of syngeneic mice.

In the S49A lymphoma, there is a definite relationship between time of tumor appearance and survival and the dose of tumor challenge. Results of a dose-response experiment for challenges of 10^4, 10^5, 10^6, and 10^7 viable cells demonstrated that the first day of tumor appearance as measured by palpation was 18, 15, 10, and 4 days, respectively (results not presented here). Once the tumor appeared, the time until death in all four groups was about 20 days. Several other experiments have confirmed this observation that the dose of

tumor challenge is only important in determining the first appearance of the tumor, and once the tumor is palpable the growth rate is the same regardless of the initial tumor concentration. In 18 months of passing the S49A lymphoma, the virulence increased, although fluctuation was observed. The fluctuations of tumor growth were probably due to the difference in the size of the tumor that was used for passage, vascularization of tumor, tumor immunogenicity, the general state of health of the host, and any immune response to the tumor.

The lowest tumor dose tested for tumor induction was 10^3 viable lymphoma cells. Eighty percent of the mice challenged with 10^3 tumor cells acquired palpable nodules, while 10^4 or more viable lymphoma cells caused tumors in 100% of the mice injected.

The lymphoma grew in all directions from the point of subcutaneous injection and formed a solid tumor. There was marked splenomegaly associated with tumor growth which was probably due in part to the migration of a considerable number of lymphoma cells to the spleen. A S.C. injection of 10^6 viable spleen cells from a large tumor-bearing mouse caused tumor formation in a secondary host. The lymphoma eventually invaded the peritoneal cavity and death usually occurred soon thereafter, although the pathology leading to death was not defined.

Tumor Cell Line

The *in vitro* cell line S.49.1 was derived from the *in vivo* S49A tumor and was provided by Dr. R. Patterson. The S.49.1 cell line was maintained in Dulbecco's modified Eagle's medium that was

supplemented with penicillin, streptomycin, and mycostatin (each agent 100 units/ml medium) and 10% heat inactivated (56°C, 30 min) fetal calf serum (Grand Island Biological Company, Grand Island, NY). The S.49.1 cells were cultured at 37°C in a humidified atmosphere of 5-10% CO_2. The S.49.1 cell line has been characterized with the following antigenic markers and properties: thymus leukemia (TL) antigen, theta (Thy-1.2) and histocompatibility ($H\text{-}2^d$) antigenic markers (55). The above properties suggest the S.49.1 cell originated from a thymocyte or a T-lymphocyte. Average diameter of S.49.1 cells was 15 microns (54). Other properties of the S.49.1 cells were their sensitivity to lysis by 10^{-5} M thymidine, 10^{-4} M dibutryl c-AMP, 10^{-6} M hydrocortisone, 10^{-5} M thioguanine, 5 µg/ml phytohemagglutinin, and 10 µg/ml concanavalin A (91).

Antisera

Antiserum directed to mouse brain associated theta antigen (BAΘ) was produced in rabbits by the method of Golub (37). CBA/J brains were removed and teased with forceps and a 21 gauge needle in phosphate buffered saline (PBS), pH 7.4; 0.5 ml per brain. The resultant cell suspension was combined with an equal volume of complete Freund's adjuvant and emulsified. Dutch Belt rabbits (Center for Laboratory Animal Resources, Michigan State University) were injected intramuscularly (I.M.) in the leg with 0.5 ml of the emulsion, on a weekly basis for three weeks. One week after the last injection, blood was obtained from the marginal ear vein; serum was obtained after allowing the blood to clot. The antiserum was adsorbed extensively for one hour with BALB/c liver cells, BALB/c

RBC, and agarose. BALB/c liver cells were teased into a cell suspension with forceps and washed three times before adsorption of serum. BALB/c RBC were collected in Alsever's solution. The adsorbed serum was then partially purified by three 50% ammonium sulfate precipitations (48) and dialyzed against three changes of PBS in 48 hours. Normal rabbit serum (NRS) was obtained from the same Dutch Belt rabbit before sensitization. During the course of the experiments antisera from three rabbits were used.

Cytotoxic Titer

The cytotoxic titer of each antiserum was obtained in the following manner: One million target cells, either S49A cells, S.49.1 cells, or thymocytes in 0.1 ml of MEM were combined with 0.05 ml of appropriate dilutions of antiserum and 0.1 ml of 1:4 dilution of guinea pig serum as the source of complement. As controls the cells were combined with only complement or only antiserum. The cells were incubated for one hour at 37°C in a humidified CO_2 incubator. Trypan blue dye was then added to the cells, and exclusion of the dye represented cell viability. The titer was the reciprocal of the last dilution of antiserum giving less than 50% viability.

Binding Trinitrophenol TNP to Anti-BAΘ

Eisen's method (24) of binding TNP to protein was followed in coupling TNP to anti-BAΘ. Recrystallized trinitrobenzene sulfonic acid (TNBS) was added to anti-BAΘ serum in a ratio of 0.1-8.0 mg TNBS per mg protein in anti-BAΘ serum. An equal amount of sodium carbonate was added, so that the solution was about pH 9.2. The

solution was wrapped in aluminum foil and allowed to react overnight at room temperature. The unbound TNP was removed by dialysis against three changes of distilled water in 48 hours. The amount of TNP bound to anti-BAθ was measured with a spectrophotometer at 348 mμ. At this wavelength the entire absorbance is due to TNP-aminoacid conjugates which are mostly lysine, with an extinction coefficient of $E^{1\%}_{348}$ = 15,400. For the purpose of calculating the number of TNP molecules bound per molecule of IgG, the molecular weight of IgG was assumed to be equal to 160,000. Anti-BAθ to which TNP was bound was designated anti-BAθ-TNP.

Immunofluorescence

Fluorescein was bound to anti-BAθ-TNP by the following method: One milliliter of carbonate-bicarbonate buffer (9 parts 0.5 M $NaHCO_3$ to 1 part 0.5 M Na_2CO_3) was added to 5.0 ml of 5 mg/ml anti-BAθ-TNP to bring the pH to 9.0-9.5. One hundred fifty micrograms of dry fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratories, Baltimore, MD) per mg of protein in the antiserum were added to the solution and the mixture reacted for one hour on a shaker at room temperature. Excess FITC was removed by passing the FITC-anti-BAθ-TNP solution over a Sephadex G-25 column. Five hundredths milliliter of fluorescein-conjugated anti-BAθ-TNP were allowed to react with 2×10^5 S.49.1 cells in 0.25 ml MEM, or with 2×10^5 SRBC in 0.25 ml of MEM, for one hour at room temperature. Both cell suspensions were washed three times with MEM and placed on a microscope slide for observation under a fluorescence microscope.

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Protein Assay

The concentration of protein in the antisera was obtained by the method of Lowry (71), or by the measurement on a spectrophotometer of absorbance at 280 mμ minus the absorbance at 320 mμ, with an extinction coefficient for immunoglobulin $E^{1\%}_{280}$ = 13.6. The measurement at 320 mμ corrected for the non-specific absorbance due to debris in the antiserum.

Host Responses to Lymphoma Growth

Assay for Antibody Suppression of Tumor Growth

A BALB/c mouse bearing a S49A tumor was sacrificed by decapitation and a portion of the tumor was removed aseptically. The excised portion of the tumor was teased into a cell suspension with broad forceps and passage through 21 and 25 gauge needles, respectively. The S49A cells were washed once in MEM by centrifugation for 8 minutes at 380 x g and the pellet resuspended in fresh MEM. Viability was determined by trypan blue exclusion and found to be between 50-70%. One tenth milliliter of MEM containing 10^6 S49A lymphoma cells was incubated with 0.1 ml of undiluted anti-BAΘ, anti-BAΘ-TNP, NRS, or MEM for one hour at 37°C in a humidified CO_2 incubator. The tumor cells which were bound with anti-BAΘ or anti-BAΘ-TNP were designated as S49A-BAΘ and S49A-BAΘ-TNP, respectively, and were diluted to a concentration of 10^5 viable cells/ml (60-90% viable). One tenth milliliter of the cell suspension was injected S.C. into the abdomen of each mouse. Each group of mice injected with S49A lymphoma cells preincubated with anti-BAΘ,

anti-BAΘ-TNP, NRS, or MEM contained between 3-10 mice. The first day of tumor appearance, tumor size, and survival time were recorded. The first day of tumor appearance was determined by palpation. Tumor size was determined by the mean diameter of the tumor (mm) by a ruler every other day. In addition, on days 2, 5 and 8 after the primary injection of 10^4 viable S49A cells, 0.1 ml of either a 1:2 dilution of anti-BAΘ, anti-BAΘ-TNP, or NRS was injected into the same area as the original injection in groups of mice containing between 4-9 mice.

Winn Test

The Winn test is an experimental assay that permits comparison of the *in vivo* effectiveness of spleen cells of treated mice versus spleen cells of normal untreated mice to inhibit tumor growth (126). The original experimental design by Winn was to demonstrate cell mediated immunity to allogeneic tumor cells in hyperimmune spleen cells when transferred together to a new syngeneic host. The Winn test has been modified for a completely syngeneic system to study the effects that spleen cells of survivor and tumor bearing mice have on the growth of S49A lymphoma cells *in vivo*.

One hundred thousand or a million viable spleen cells from normal, survivor, or tumor bearing mice were added to 10^3 or 10^4 viable S49A or S49A-BAΘ cells so that a 100:1 ratio of spleen cells to tumor cells was achieved. One tenth milliliter of the mixture was injected S.C. into the abdomen of each normal mouse in groups of 3-7 mice. The first day of tumor appearance, tumor size and survival time were recorded to determine if spleen cells of

sensitized or tumor bearing mice admixed with uncoated or antibody coated tumor cells could transfer protection to normal syngeneic mice.

Concurrently with the Winn test, 10^7 viable spleen cells from normal, survivor, or tumor bearing mice were injected intravenously (I.V.) in the tail vein of five normal syngeneic mice. One week later 10^4 viable S49A cells were injected S.C. into the previously injected mice and into a control group of six normal mice. The appearance of tumor growth and survival time were recorded to determine if protection could be transferred by sensitized spleen cells or if tumor cells were present in tumor bearing mice.

Plaque Assay

The modified method of Jerne (56) was used to measure antibody producing cells. Sheep red blood cells (SRBC) used in this assay were coated with TNP by the method of Rittenberg and Platt (95).

Agglutination Tests

Sera from tumor-bearing survivors and normal mice were tested for agglutinating antibody. Rabbit anti-BA0 and NRS were used as controls for agglutination by antibody and non-specific agglutination, respectively. In addition, rabbit anti-mouse immunoglobulin was used to determine if freshly excised tumor cells had mouse immunoglobulin on their cell surface. A serial dilution of 0.05 ml of mouse or rabbit sera from 1:2 to 1:64 was made in each tube. To each tube 0.45 ml containing 2×10^6 S49A cells in MEM was added and mixed. The tubes were incubated for one hour at 37°C in a CO_2 incubator. The tubes were then centrifuged for five minutes at 300 x g. The cells were slowly resuspended by a manually created vortex. The

agglutination titer was taken as the reciprocal of the last dilution showing visible clumping. In addition, a kinetic study of tumor growth in anti-BAΘ treated and untreated mice was performed to determine if there was any relationship between tumor growth and appearance of agglutinating factors in the sera of these mice. Mice were bled from the tail vein 8, 16 and 23 days after tumor inoculation and sera were collected and usually pooled from 2-3 mice.

The results from mouse serum are expressed as:

$$\text{Agglutination Factor} = \frac{\text{Experimental Titer}}{\text{Normal Mouse Serum (NMS) Titer}}$$

where experimental titer = NMS titer, agglutination factor = 0.

Antibody Dependent Cell Mediated Cytotoxicity

The ^{51}Cr release cytotoxicity assay was modified from that of Brunner and Cerottini (4). In the ^{51}Cr release assay, specific cytotoxicity varies linearly with the logarithm of the number of immune lymphoid cells over a range of cell concentrations causing between 15 and 75% lysis (7,9). (Cytotoxicity is measured by the release of ^{51}Cr from dead cells into the medium.) Specificity has been demonstrated by the competitive inhibition of lysis of labeled target cells by immune lymphoid cells in the presence of other antigenically identical target cells, but not in the presence of unrelated target cells (9). A minimum of 5-10 x 10^3 ^{51}Cr labeled target cells are required for a measurable reaction. A large number of lymphocytes are needed, which may be a limiting factor. Radioactive chromates with hexavalent chromium easily penetrate biological membranes. Once inside, the chromate, a strong oxidizing reagent,

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will readily react with protein to form a strongly bound complex containing reduced trivalent chromium (76). Due to the large size of the protein-chromium complex, it is unlikely to escape from a viable cell. An advantage of this assay was that it could be run in 4-6 hours, which prevented lymphoid cell sensitization.

A possible pitfall in the interpretation of ^{51}Cr release has been the relatively high spontaneous release of ^{51}Cr from target cells. Chromium is a strong oxidizing agent and a known toxin (76) which may cause greater cell death, thus a corresponding greater spontaneous release. Rajam et al. (91) demonstrated that 2 µg/ml of ^{51}Cr can be toxic to cells. However, the ^{51}Cr release assay has been the method of choice of the majority of researchers who have measured ADCMC *in vitro*. Therefore, this technique was used to determine if the ADCMC phenomenon was present in the S49A lymphoma system.

A suspension of S49A cells was prepared aseptically and adjusted to 2×10^7 viable cells/ml in Dulbecco's medium with 10% FCS; the cells were 90% viable. To 1.0 ml of this cell suspension was added 200 µCi of $Na_2{}^{51}CrO_4$ in saline (New England Nuclear, Boston, MA; specific activity equal to 470 mCi/mg). The mixture was placed in a humidified CO_2 incubator at 37°C for one hour with occasional shaking. After centrifugation for five minutes at 380 x g, the ^{51}Cr labeled S49A cells were adjusted to 2×10^6 cells in 0.2 ml. Two tenths milliliter of the appropriate mouse or rabbit sera to be tested was added and mixed well. The tubes were placed in a CO_2 incubator for one hour at 37°C and then washed once in Dulbecco's modified medium. Spleens were aseptically removed from 2-4 mice

and teased into a single cell suspension which was adjusted to 4×10^7 cells/ml in Dulbecco's supplemented medium.

For the cytotoxicity assay 0.25 ml of the spleen cell suspension was added to 1×10^5 S49A ^{51}Cr labeled cells in 0.25 ml of medium in a 13 x 100 mm plastic capped tube (Falcon, Oxnard, CA). Tests were done in triplicate when possible. All tubes were incubated for 5-7 hours at 37°C in a humidified CO_2 incubator. One milliliter of cold Dulbecco's supplemented medium was added to each tube and the contents were manually mixed until the cells were resuspended. The cells were centrifuged at 380 x g for 8 minutes. One milliliter of the supernatant was removed and added to vials for counting in a gamma counter. Maximum ^{51}Cr release was determined by three cycles of freeze-thawing 10^5 ^{51}Cr labeled S49A cells. Spontaneous release was measured from 10^5 ^{51}Cr-labeled S49A cells from all experimental groups incubated for 5-7 hours in medium only (i.e., without spleen cells). The effects of complement were measured by adding 0.25 ml of a 1:10 dilution of guinea pig serum in Dulbecco's supplemented medium to treated and untreated tumor cells. The results are expressed as:

$$\text{Percent Specific } ^{51}Cr \text{ Release} = \frac{\text{Experimental Release (CPM)} - \text{Spontaneous Release (CPM)}}{\text{Maximum Release (CPM)} - \text{Spontaneous Release (CPM)}} \times 100$$

Statistical Analyses

One-tailed Student's t-test was used to measure the difference between the means of data of various experimental groups. A chi-square test was used to measure the significance of the difference between percentages of the number of mice which died from lymphoma growth.

RESULTS

Binding TNP to Anti-BAΘ and Its Effect on Cytotoxic Titer

The original objective of the following experiments was to enhance the immunogenicity of murine lymphoma cells by directly binding antibody coupled with hapten (anti-BAΘ-TNP) to the neoplastic cell membrane. Challenge by the modified tumor cell may then induce an immune response that would lead to the retardation or inhibition of tumor growth. The effects of coupling TNP to anti-BAΘ were studied to determine if certain quantities of TNP bound to an antibody molecule could inhibit the cytotoxicity of anti-BAΘ serum or prevent anti-BAΘ immunoglobulin from binding to target cells.

A limited range of TNP molecules per immunoglobulin molecule did not destroy cytotoxic activity of anti-brain associated theta antibody. Anti-BAΘ-TNP in groups 4-7 of Table 1 show a gradation in the activity of the antiserum. Smaller amounts of TNP bound per molecule of immunoglobulin yielded higher cytotoxic titers. In groups 5 and 6 the level of percent dead reached only 26 or 46% of undiluted anti-BAΘ-TNP and maintained this percentage to a 1:16 dilution in both cases. The percent dead cells changed very little over five twofold dilutions. These results suggest there may be a threshold level of TNP molecules that can be bound before the immunoglobulin was inactivated.

Table 1. Relationship of the number of TNP molecules bound to anti-BAθ immunoglobulin on the cytotoxic titer of the antiserum

Antiserum	Target Cell	TNP Added (gm) / Protein (gm)	# TNP Molecules / Immunoglobulin	Mean Cytotoxic Titer[b]
1. Anti-BAθ	S49A	---	0	16
2. Anti-BAθ-TNP	S49A	0.174	29	32
3. Anti-BAθ	S.49.1	---	0	32
4. Anti-BAθ-TNP	"	1.041	N.D.[e]	0
5. "	"	0.647	71	0[c]
6. "	"	0.395	60	0[d]
7. "	"	0.176	26	32
8. "[a]	"	N.D.	68	0
9. "[a]	"	N.D.	38	32
10. Anti-BAθ[a]	"	---	0	32
11. Anti-BAθ[a]	thymocytes	---	0	128

a = Antisera used in antibody suppression of S49A lymphoma growth.
b = Represents average of 2-4 experiments.
c = Observed 26% dead from undiluted to 1:16 dilution of antisera.
d = Observed 46% dead from undiluted to 1:16 dilution of antisera.
e = Not done.

When the antiserum was tested against various target cells different cytotoxic titers often resulted. For example, anti-BAΘ or anti-BAΘ-TNP tested against S49A cells generally resulted in a twofold decrease or similar titers as compared when S.49.1 cells were the target cells. The cytotoxic titer of anti-BAΘ against thymocytes was always four- to eightfold greater than against S.49.1 cells. Similar results with thymocytes and syngeneic lymphoma cells were observed by Golub and Herzenberg (personal communication).

Although not shown, an immunofluorescent technique was used to observe anti-BAΘ-TNP (29 TNP/immunoglobulin) binding to S.49.1 cells. Fluorescein conjugated anti-BAΘ-TNP was incubated with S49.1 cells, or SRBC as a control, and washed three times with MEM before observation with a fluorescence microscope. There was a well defined fluorescence of replicating and non-replicating S.49.1 cells. The SRBC did not show fluorescence. This result suggested that anti-BAΘ-TNP binds specifically to lymphoma cells.

Antibody Suppression of Lymphoma Growth

The following series of experiments were designed to determine the ability of anti-BAΘ and anti-BAΘ-TNP to suppress lymphoma growth *in vivo*. Different approaches to antibody treatment of lymphoma cells were tested in an attempt to find a protocol that could prevent tumor growth. The possibility of a host response to anti-BAΘ-TNP or the TNP moiety of the antibody-hapten complex bound to tumor cells was examined for its role, if any, in tumor suppression.

The first reasonably successful attempt to demonstrate retardation of tumor growth occurred when S49A cells were incubated at 4°C

for 30 minutes with either anti-BAθ or anti-BAθ-TNP before tumor inoculation (Table 2). These preliminary data showed a significant delay in tumor appearance, and thus increased survival time. The time between the first day of tumor appearance and death was about 20 days for all groups tested, suggesting similar growth rates. Measuring the diameters of all the tumors on a daily basis displayed parallel growth rates. These data suggest that the antibody or antibody-hapten complex inhibited the growth of the original tumor, possibly by mediating the death of many of the inoculated tumor cells. Anti-BAθ was more successful at retarding tumor growth than anti-BAθ-TNP, suggesting that TNP molecules bound to anti-BAθ-TNP may impair binding of antibody to tumor cells. This result correlates well with the lower cytotoxic titers of anti-BAθ-TNP *in vitro*.

Mice were sensitized to anti-BAθ-TNP in an attempt to form memory cells responsive to this antibody-hapten complex. Upon challenge by S49A-BAθ-TNP cells a secondary response against the "foreign" antibody-hapten complex could then destroy the coated tumor cells. Mice were sensitized to anti-BAθ-TNP by two S.C. injections of 50 μg of anti-BAθ-TNP in complete Freund's adjuvant, or by injecting S.C. 10^7 anti-BAθ-TNP coated thymocytes into the area of eventual tumor challenge 10 days prior to challenge. These sensitized mice did not show any protective effect as compared with non-treated mice (Experiment II, Table 2). These results suggested that the host response to antibody coated tumor cells was not due to memory cells directed against the cell bound antibody-hapten complex.

Table 2. Anti-BAθ and anti-BAθ-TNP suppression of S49A lymphoma growth[a]

Exp.	Group	First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Tumor Mice / Total Mice
I	10^4 S49A cells preincubated with:			
	Medium	11.0 ± 1.4	27.6 ± 3.2	5/5
	Anti-BAθ	21.6 ± 7.9*	40.6 ± 8.5*	5/5
	Anti-BAθ-TNP	15.4 ± 3.6*	38.6 ± 3.8**	5/5
II	$2x10^5$ S49A cells preincubated with:			
	Medium	9.2 ± 1.1	30.0 ± 0.0	5/5
	Anti-BAθ	16.4 ± 2.7**	36.8 ± 3.5**	5/5
	Anti-BAθ-TNP	11.7 ± 1.6*	31.8 ± 0.4*	5/5
	Anti-BAθ-TNP sensitized[b]	10.7 ± 1.1	30.0 ± 0.0	3/3
	Thymocyte-BAθ-TNP[c]	11.0 ± 1.7	30.0 ± 0.0	3/3

a = antiserum incubated with cells for 30 minutes on ice

b = mice were injected twice S.C. with 50 μg of anti-BAθ-TNP in complete Freund's adjuvant 10 days before tumor challenge of $2x10^5$ S49A-BAθ-TNP cells

c = mice were sensitized by injecting 10^7 thymocytes bound with anti-BAθ-TNP 10 days before tumor challenge of $2x10^5$ S49A-BAθ-TNP cells

Student's t test *p<0.05 **p<0.005

The antibody response to the TNP moiety of anti-BAΘ-TNP bound to S49A cells in mice injected S.C. with S49A-BAΘ-TNP was measured by the Jerne plaque assay with TNP coated SRBC (94). The results shown in Table 3 indicate that mice primed twice with 50 μg of anti-BAΘ-TNP in complete Freund's adjuvant had a threefold increase in plaque forming cells over normal mice. Injection of 10^5 S49A-BAΘ-TNP cells into normal or survivor mice did not result in a significant increase in plaque forming cells to TNP. This result suggests that the measurable level of antibody response to TNP was not a crucial factor in the suppression of tumor growth by anti-BAΘ-TNP.

As a further attempt to demonstrate antibody mediated suppression of coated tumor cells, S49A lymphoma cells were preincubated with antiserum or NRS for one hour at 37°C before animal inoculation to increase the number of antibodies that were bound to tumor cells. A small dose of 10^3 S49A cells was used to reduce the initial tumor mass which would otherwise allow the rapidly dividing tumor cells to "outpace" any immune response. The results of this preliminary experiment (Experiment I, Table 4) showed that anti-BAΘ and anti-BAΘ-TNP coated S49A cells did not cause a palpable tumor over 100 days after the initial challenge. Two of the three control mice challenged with 10^3 S49A cells incubated in NRS acquired tumors as quickly as mice challenged with untreated tumor cells.

The effectiveness of injections of anti-BAΘ or anti-BAΘ-TNP during the initial stages of tumor growth was examined. Mice from each of the above groups were selected for S.C. injection with 0.1 ml of either anti-BAΘ or anti-BAΘ-TNP directly into the area of

Table 3. Antibody response to TNP in spleens of mice challenged with anti-BAθ-TNP coated S49A cells

Experimental Group	No. of Mice	Number of Direct Plaques[c] Avg. ± SE x 10^{-3}
Control	2	46 ± 13
Anti-BAθ-TNP[a]	2	153 ± 74
S49A-BAθ-TNP[b]	2	60 ± 7
100 Day Survivors[b]	2	50 ± 9

a = Mice were primed by two S.C. injections of 50 μg of anti-BAθ-TNP in complete Freund's adjuvant, the last injection being one week before assay.

b = All mice were injected 10 days prior to assay with 10^5 S49A-BAθ-TNP cells.

c = Representative results of one of two experiments with the same number of mice. Average is derived from duplicates from each mouse, and there were not any detectable indirect plaques.

Table 4. Suppression of S49A lymphoma growth by antibody

Exp.	Experimental Group	First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Mice with Tumors / Total Mice
I	10^3 S49A cells preincubated with:			
	Medium	15.6 ± 0.6	42.3 ± 6.1	3/4
	NRS	17.0 ± 1.0	52.5 ± 3.5	2/3[@]
	Anti-BAΘ	no tumors	no tumors	0/4[@]
	Anti-BAΘ-TNP	no tumors	no tumors	0/4[@]
II	Mice injected with 0.1 ml anti-BAΘ or anti-BAΘ-TNP on Days 2, 5, and 8 after challenge with S49A cells[a]			
	Medium	17	35	1/4[b]
	Anti-BAΘ	no tumors	no tumors	0/4[@]
	Anti-BAΘ-TNP	no tumors	no tumors	0/4[@]

a = These mice were from the groups in Experiment I and were given injections of anti-BAΘ (0.1 ml) or anti-BAΘ-TNP (0.1 ml) into the same area of the initial tumor challenge on the days indicated.

b = The one tumor bearing mouse had an intraperitoneal tumor.

c = No palpable tumors were observed over the 100 days after tumor inoculation.

Chi-square value: [@]<0.05.

tumor challenge on the second, fifth, and eighth days after primary tumor challenge. The results of Experiment II, Table 4, suggested that mice challenged with S49A-BAθ or S49A-BAθ-TNP did not have any visible tumor growth. The presence of an intraperitoneal tumor in one mouse was explained by the fact that if the mouse was injected I.P. with tumor cells, then S.C. injections of antiserum probably would not have reached the tumor cells at all or in insufficient quantities to lead to eradication of all tumor cells.

Another set of experiments was performed to support the previous results which suggested that anti-BAθ and anti-BAθ-TNP could suppress or prevent tumor growth. These experiments were also designed to demonstrate that the antibodies specifically directed against the BAθ antigen on S49A cells inhibited tumor growth and not some non-specific factor in NRS or in hyperimmune rabbit serum. The data presented in Experiment I of Table 5 indicated that 100% of the mice injected with S49A cells preincubated with media or NRS acquired fatal tumor growth. Anti-BAθ was the only rabbit serum that could prevent tumor growth *in vivo* in a significant number (5 of 7) of mice when preincubated with S49A cells before inoculation. The fact that all seven mice injected with S49A cells preincubated with anti-BAθ-TNP acquired tumors suggested that the 38 TNP molecules coupled to each immunoglobulin reduced the ability of the antibody-hapten complex to bind to S49A cells *in vitro* and subsequently reduced suppression of tumor growth *in vivo*. The decreased suppression of tumor growth observed when TNP was coupled to anti-BAθ as compared to unmodified anti-BAθ caused the elimination of anti-BAθ-TNP from further experimentation.

Table 5. Antibody suppression of S49A lymphoma growth

Exp.	Experimental Group	First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Mice with Tumors / Total Mice
I	10^4 S49A cells preincubated with:			
	Medium	12.8 ± 1.0[a]	37.2 ± 7.4	10/10
	NRS	15.7 ± 2.1*	46.8 ± 5.4*	7/7
	Anti-BAθ-TNP (inactive)[b]	17.8 ± 3.4**	42.2 ± 4.0	6/7@
	Anti-BAθ	18.0 ± 1.0**	42.0 ± 1.4	2/7@
	Anti-BAθ-TNP[c]	18.4 ± 1.5**	44.0 ± 5.6	7/7
II	Mice injected on Days 2, 5, and 8 with 0.1 ml of:[d]			
	NRS	16.7 ± 2.6**	43.0 ± 8.7	7/7
	Anti-BAθ-TNP (inactive)[b]	16.1 ± 3.0**	45.7 ± 7.9	7/7@@
	Anti-BAθ	no tumors	no tumors	0/7@@
	Anti-BAθ-TNP	no tumors	no tumors	0/7@@

a = All values are relative to media control, but if values are compared to NRS, only anti-BAθ-TNP is significantly different.
b = Inactive due to an excess of TNP bound per immunoglobulin (68 TNP/Ig) and did not have a demonstrable cytotoxic titer.
c = Anti-BAθ-TNP cytotoxic titer = 32, and 38 TNP/Ig.
d = Mice were injected with 10^4 S49A cells preincubated in medium, prior to injection of NRS or anti-BAθ into the same area of tumor challenge, on the days indicated after the lymphoma cell inoculation.
Student's t test: *$p<0.05$ **$p<0.005$ Chi-squared value: @<0.05 @@<0.005.

Preincubation of S49A cells with normal rabbit serum delayed tumor appearance and subsequent death by 3-9 days compared to mice that were injected with S49A cells preincubated with medium (Experiment I, Table 5). However, two other experiments (Experiments II and III, Table 6) did not demonstrate a significant delay in tumor appearance or subsequent death in any of the 17 mice tested. These results suggest that the protective capacity of NRS when preincubated with tumor cells was minimal.

Anti-BAΘ was inactivated by binding excess TNP molecules per immunoglobulin (68 TNP/Ig). This inactivated anti-BAΘ-TNP did not have any cytotoxic activity as measured in the cytotoxicity assay (Table 1). Inactivated anti-BAΘ-TNP preincubated with S49A cells did not prevent tumor growth and it impaired tumor progression by only five days (Experiment I, Table 5). When inactive anti-BAΘ-TNP or NRS was injected three times after initial tumor challenge, tumor cell proliferation occurred in all mice that were tested (Experiment II, Table 5). In contrast, multiple injections of anti-BAΘ or active anti-BAΘ-TNP suppressed 100% of tumor growth. These results suggested that a functionally active immunoglobulin specifically directed against the BAΘ antigen was required to prevent growth of the S49A lymphoma, rather than a non-specific factor in hyperimmune rabbit serum or NRS.

Anti-BAΘ Suppression of S49A Lymphoma Growth in Normal and Survivor BALB/c Mice

This set of experiments was designed to determine if mice that had survived a previous experience with anti-BAΘ or anti-BAΘ-TNP coated tumor cells would show any immunity to a rechallenge of 10^4

Table 6. Anti-BAθ suppression of S49A lymphoma growth in survivor mice

Exp.	Experimental Group	First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Mice with Tumors / Total Mice
I	10^4 S49A cells preincubated with:			
	Medium	12.8 ± 1.0	37.2 ± 7.4	10/10
	Rechallenge of 100-125 day survivors,[a] 10^4 S49A cells preincubated with:			
	Medium	13.3 ± 1.2	34.0 ± 4.4	15/17
II	10^4 S49A cells preincubated with:			
	Medium	11.6 ± 1.4	28.9 ± 5.1	8/8
	NRS	11.7 ± 2.1	29.3 ± 2.2	9/9[@]
	Anti-BAθ	20.5 ± 4.9**	37.0 ± 4.2*	2/7
	Rechallenge of 100 day survivors,[b] 10^4 S49A cells preincubated with:			
	Medium	11.4 ± 1.2	27.2 ± 3.6	8/9
	Anti-BAθ	17.3 ± 2.9**	36.2 ± 4.5*	6/9

Table 6 (continued)

Exp.	Experimental Group	First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Mice with Tumors / Total Mice
III	10^4 S49A cells preincubated with:			
	Medium	15.3 ± 1.3	36.3 ± 4.6	8/8
	NRS	16.3 ± 2.5	36.6 ± 6.9	8/8@@
	Anti-BAΘ	no tumors	no tumors	0/8
	Rechallenge of 30 day survivors,[b] 10^4 S49A cells preincubated with:			
	Medium	14.7 ± 1.7	34.7 ± 2.6	7/8@
	Anti-BAΘ	15.0 ± 0.0	41.0 ± 7.0	2/8

a = Mice used were survivors of a tumor challenge 100-125 days earlier with 10^3 or 10^4 S49A cells treated with anti-BAΘ.

b = Mice used were survivors of a previous challenge with 10^4 S49A cells and treated with anti-BAΘ on days 2, 5, and 8 after tumor challenge.

Student's t test: *$p<0.05$ **$p<0.005$ Chi-square values: @<0.05 @@<0.005.

unmodified S49A cells or 10^4 S49A-BAΘ cells into the same area as the first injection. The first experiment in Table 6 provided evidence that mice surviving a previous tumor challenge (10^3 or 10^4 S49A-BAΘ or S49A-BAΘ-TNP cells, or 10^3 or 10^4 S49A cells followed by multiple anti-BAΘ injections) 100-125 days earlier could not mount any significant defense against tumor growth.

The data in Experiments II and III of Table 6 demonstrated that survivors of an earlier challenge of 10^4 S49A-BAΘ cells or 10^4 S49A cells followed by injections of anti-BAΘ 2, 5 and 8 days later were not protected against subsequent tumor challenge. The rate of tumor appearance and survival times were not different from control mice or NRS treated mice as all but two of the 17 rechallenged mice succumbed to fatal tumor growth. The amount of time between initial injection and rechallenge (either 30 or 100 days) had no effect on the survivor's response. Survivors rechallenged with S49A-BAΘ cells showed little protection, or possibly an increased susceptibility to fatal tumor growth rather than an increased resistance against target cells previously encountered. These results suggest that survivor mice have no memory response of any significance to rechallenge by 10^4 S49A cells and may even be more susceptible to a secondary tumor challenge of anti-BAΘ coated cells than normal mice were to primary challenge of S49A-BAΘ cells.

Suppression of Lymphoma Growth by Injections of Anti-BAΘ into Tumor Area

This set of experiments was designed to determine the degree of protection against tumor cell proliferation which could be obtained by injecting anti-BAΘ into the area of inoculation of unmodified

lymphoma cells. The data in Experiment I of Table 7 showed that none of the mice that received S.C. injections of anti-BAθ into the tumor area 2, 5 and 8 days after the tumor challenge acquired a palpable tumor nodule. Multiple injections of NRS did not prevent fatal tumor growth in any of the seven mice treated. Similar results were obtained in another experiment (Experiment II, Table 7). Eight of nine mice that received multiple injections of NRS died from tumor proliferation and their survival times were similar to those of control mice injected with unmodified lymphoma cells. Tumor growth was prevented in 18 of the 20 mice that received multiple injections of anti-BAθ. The fact that two mice acquired intraperitoneal tumors after multiple injections of anti-BAθ suggested that an error was made during injection as mentioned earlier, and further exemplified the importance of the anti-BAθ being able to reach its target cell so it can invoke its protective mechanism.

Further experiments were performed to determine the frequency of anti-BAθ injections required to inhibit tumor growth and if varied amounts of NRS could possibly suppress tumor growth. The results signify that one injection of anti-BAθ two days after initial tumor challenge was sufficient to prevent a palpable tumor (Table 8). The inability of a single injection or multiple injections of NRS to prevent tumor growth in any of the 26 treated mice supports the suggestion that there was not a non-specific factor present in rabbit serum which prevented tumor proliferation.

In an attempt to determine how long anti-BAθ treatment could suppress tumor growth, six mice bearing tumors with diameters ranging from 7-15 mm were examined. After multiple injections of anti-BAθ

Table 7. Suppression of lymphoma growth by injection of anti-BAΘ into tumor area

Exp.	Experimental Group	First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Mice with Tumors / Total Mice
I	Mice injected on days 2, 5, and 8 with 0.1 ml of:[a]			
	NRS	16.7 ± 2.6	43.0 ± 8.7	7/7 @@
	Anti-BAΘ	no tumors	no tumors	0/7 @@
	Control[b]	12.8 ± 1.0	32.7 ± 7.4	10/10
II	Mice injected on days 2, 5, and 8 with 0.1 ml of:[a]			
	NRS	13.9 ± 2.9	30.9 ± 7.5	8/9
	Anti-BAΘ	12.5 ± 0.7	26.0 ± 2.8	2/20 c@@
	Control[b]	11.6 ± 1.4	28.9 ± 5.1	8/8

a = Mice were injected with 10^4 S49A cells preincubated in medium prior to injection of NRS or anti-BAΘ into the same area of tumor challenge on the days indicated after the initial lymphoma cell challenge.

b = Mice injected with 10^4 S49A cells preincubated in medium and not injected with serum.

c = Two tumor bearing mice acquired intraperitoneal tumors.

Chi-square values: @ <0.05 @@ <0.005.

Table 8. Antibody suppression of lymphoma growth and frequency of antibody treatments[a]

Experimental Group		First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Mice with Tumors / Total Mice
Mice Injected With:	Day of Injection:			
NRS	2	14.2 ± 2.3	35.9 ± 5.7	9/9
NRS	2,5	14.8 ± 2.0	36.4 ± 6.9	9/9
NRS	2,5,8	13.3 ± 1.8	38.6 ± 2.6	8/8
Anti-BAΘ	2	no tumors	no tumors	0/9[@@]
Anti-BAΘ	2,5	47	72	1/9[@@]
Anti-BAΘ	2,5,8	no tumors	no tumors	0/9[@@]
---	(control)	14.4 ± 3.2	37.1 ± 7.3	9/9

a = Mice were injected S.C. in the same area of tumor challenge with 0.1 ml of either NRS or anti-BAΘ on the days indicated.

Chi-square values: [@@]<0.005.

little or no suppression of neoplastic growth was observed (data not presented here) in these mice, suggesting that anti-BAθ injections may prevent growth of tumors in early stages of development only.

Winn Test and Adoptive Transfer of Syngeneic Spleen Cells

The Winn test was utilized in this series of experiments to demonstrate if spleen cells of survivor mice exhibited cell mediated immunity when transferred with tumor cells into new syngeneic hosts. The effects of spleen cells from tumor bearing mice on the growth of S49A lymphoma cells *in vivo* were examined also. Preliminary experiments (data not shown) with 3-5 mice in each experimental group suggested that spleen cells from mice bearing a small tumor (<20 x 20 mm tumor) admixed with tumor cells before injection did not alter tumor growth. Mice injected with spleen cells of survivor mice admixed with tumor cells acquired tumors on the same day as mice injected with tumor cells alone. This result suggested that survivor spleen cells had no detectable effect on tumor proliferation. However, spleen cells from mice bearing a large tumor (>20 x 20 mm tumor) enhanced the appearance of a palpable tumor. The tumors appeared in 12 days rather than 17 days following injection of 10^3 S49A cells. The results of these preliminary experiments suggested three possibilities to explain the enhancement of tumor growth by spleen cells of mice bearing a large tumor or lack of protection by survivor spleen cells; there were not any "immune" cells present in the spleens of these animals; there were "suppressor" cells present which inhibited immune cells, or there were tumor cells

in the spleens of these mice. Since S49A lymphoma cells were derived initially from the thymus, it was possible that these cells had the ability to migrate to the spleen.

The following experiments (Table 9) were designed to determine whether spleen cells of tumor bearing mice contained tumor cells that enhanced tumor growth, and if survivor spleen cells were immune to anti-BAθ coated S49A cells. The results indicated the occurrence of a marked increase in the time of appearance of a palpable tumor when 10^6 spleen cells of tumor bearing mice were mixed with 10^4 S49A cells as compared to normal and survivor spleen cells (Experiment I, Table 9). Injecting anti-BAθ coated S49A cells admixed with normal spleen cells gave the expected result of delayed appearance of the tumor and increased survival time compared to uncoated tumor cells. However, it appears that 10^6 normal spleen cells may promote tumor growth, since 86% of these treated mice acquired tumors instead of the usual 15%. One million spleen cells from mice bearing a large tumor could induce a tumor by themselves, indicating a migration of lymphoma cells to the spleen sometime during tumor growth.

The second experiment in Table 9, performed simultaneously with the first experiment, was designed to study the effects of populating a normal syngeneic mouse with 10^7 spleen cells from normal, survivor, or tumor bearing mice when injected I.V. one week before tumor challenge. Normal and survivor spleen cell injections did not alter tumor growth as compared to 10^4 S49A tumor cell control. This result and that of the Winn test suggested that survivor spleen cells did not have the capacity to transfer cell mediated

Table 9. Enhancement of tumor growth by tumor bearing spleen cells: Winn test and adoptive transfer

Exp.	Experimental Group	First Day of Tumor Appearance Avg. ± SE	Survival Time (Days) Avg. ± SE	Mice with Tumors / Total Mice
I	10^4 S49A cells injected with 10^6 spleen cells from these mice:			
	Normal[a]	14.1 ± 2.1	30.8 ± 1.5	7/7
	30 Day Survivor	13.3 ± 1.1	30.0 ± 1.6	7/7
	Large Tumor Bearer	8.0 ± 1.0**	26.0 ± 3.7*	7/7
	10^4 S49A-BA0 cells injected with 10^6 spleen cells from these mice:			
	Normal	18.7 ± 7.4	37.0 ± 6.6*	6/7
	30 Day Survivor	16.0 ± 1.5	35.5 ± 5.1	6/7
	Large Tumor Bearer	7.4 ± 0.9**	24.3 ± 1.2**	7/7
	10^6 spleen cells of large tumor bearing mice[b]	7.6 ± 0.9**	25.2 ± 2.4**	5/5
II	Control 10^4 S49A cells	11.7 ± 2.0	28.5 ± 3.1	6/6
	10^7 spleen cells transferred I.V. (one week before 10^4 S49A cells) from:			
	Normal[c]	10.8 ± 1.8	28.2 ± 4.5	5/5
	30 Day Survivor	13.0 ± 0.8	24.4 ± 4.9	5/5
	Large Tumor Bearer	---[d]	9.4 ± 0.9**	5/5

a = Winn test where all results of Experiment I are compared with this group.
b = 10^6 spleen cells from the same source of large tumor bearing mouse used in the other groups and no S49A cells were injected.
c = All spleen cells transferred were from the same source used in Experiment I.
d = All mice died before any palpable tumor growth could be detected.
Student's t test: *$p<0.05$ **$p<0.005$.

immunity against lymphoma cells to other syngeneic mice. The dramatic result of injecting 10^7 spleen cells from mice bearing a large tumor was a swift death in only nine or ten days without any palpable tumor. The results from the two experiments strongly suggest that the increased rate of appearance of tumors and early death were due at least in part to the presence of tumor cells in the spleens of tumor bearing mice, thus increasing the initial tumor load.

Immune Response to S49A Cells and Antibody Coated Tumor Cells

Once the phenomenon of antibody suppression of S49A lymphoma growth was verified, a few experimental approaches were taken in an attempt to understand the mechanism of antibody suppression and to characterize inherent immunity against unmodified S49A cells. The experimental approaches taken were to examine the inherent antibody response by an agglutination assay and to examine the *in vitro* cytolytic responses displayed by spleen cells of normal, survivor, and tumor bearing mice against unmodified and antibody coated lymphoma cells in a ^{51}Cr release assay.

Agglutination Assay

The purpose of these experiments was to examine the possibility that mice challenged with untreated or antibody coated lymphoma cells produced an antibody response to the tumor cells. Although the nature of the agglutinating factors was not defined, it was presumed these factors were mainly antibodies directed against lymphoma cells. Agglutination of S49A cells was used to measure the humoral response to S49A cells since agglutination would measure an antibody response

even if the antibodies were ineffective in killing tumor cells. The data presented in Table 10 suggested that survivors of a S49A-BAθ or S49A-BAθ-TNP challenge acquired an agglutination factor of 8 (titer = 16) for about 35 days, which then gradually declined until there was not any detectable agglutination greater than that found in normal mouse serum (NMS) on the ninetieth day after the initial tumor challenge. Non-specific agglutination was found in NMS pooled from 5-10 mice at 1:2 or 1:4 dilutions of sera. Agglutination of S49A cells by a 1:32 dilution of anti-BAθ (NRS titer = 0) demonstrated that indeed antibody directed against a component on the tumor cell surface could agglutinate these cells. The data also indicate that mice with tumors smaller than 20 x 20 mm had a noticeable agglutinating antibody response at a 1:16 dilution of serum. However, as the tumor became larger, factors causing agglutination were not detectable in the serum. Not only did the agglutinating antibodies disappear from the serum of mice bearing a large tumor (titer = 0), but the factor in NMS that caused non-specific agglutination disappeared also. The progressively growing tumor may have adsorbed these factor(s) from the blood when the tumor load reached a certain state or size.

The above evidence suggested a correlation between the size of tumor growth and agglutination titer. Therefore, a study was performed on several groups of mice injected with unmodified or antibody coated tumor cells over a 23-day period of tumor growth. The results of Table 11 indicate that there was not a detectable antibody response eight days after tumor challenge. On the sixteenth day practically all mice had substantial agglutination factors of

Table 10. Agglutination tests of various mouse sera incubated with S49A cells

Experimental Group	Number of Mice[e]	Agglutination Factor[a]
Sera from mice bearing:		
Small tumor[b]	3	4
Large tumor[c]	6	0.5
Survivor mouse serum:		
Day 10[d]	2	8
Day 32	2	8
Day 35	1	8
Day 40	1	4
Day 40	1	2
Day 90	4	0

a = Agglutination Factor = $\frac{\text{Experimental Titer}}{\text{NMS Titer}}$, when experimental titer = NMS titer, agglutination factor = 0.
b = small tumor diameter (<20 x 20 mm).
c = large tumor diameter (>20 x 20 mm).
d = represents the number of days after initial tumor challenge in mice that remained tumor free
e = each mouse serum tested individually and all mice shown on a certain day had same agglutination factor

Table 11. Agglutination factor in mice challenged with uncoated and antibody coated S49A cells

Experimental Group[b]	No. of Mice	Agglutination Factor[a] Day 8	Day 16	Day 23
10^4 S49A cells preincubated with:				
Medium	3	0	0*(3)	0.5**(3)
NRS	3	0	2-4*(3)	2*(3)
Anti-BAθ	2	0	4	4*(1)
Rechallenge of 100 day Survivors				
Medium	2	0	4*(2)	2*(2)
Anti-BAθ	2	0	4*(1)	2*(1)
Mice injected on days 2, 5 and 8 with 0.1 ml of:				
NRS	2	0	4*(2)	0*(2)
Anti-BAθ	2	0	4	4-8

a = Calculated same as Table 10, but is measured on indicated day after the initial tumor challenge. The hyphenated numbers indicate that each mouse was tested separately; all others were pooled serum

b = Represents the same group of mice used in Experiment I, Table 5, and Experiment II, Table 7.

* = Number of mice with a small tumor (<20 x 20 mm).

** = Number of mice with a large tumor (≥20 x 20 mm).

2 or 4 whether they had a small tumor or not. On the twenty-third day most mice that had progressively growing tumors had reduced agglutination titers or none at all, as was the case with mice bearing a large tumor, while those mice without tumors retained the same titer. These results suggested that as the tumor was being rejected or was growing an antibody response against the lymphoma cells occurred, which would disappear as the tumor became quite large or was eradicated.

Rabbit anti-mouse immunoglobulin serum was used to examine if antibody was present on the surface of freshly excised S49A lymphoma cells. The addition of a twofold dilution of rabbit anti-mouse immunoglobulin serum to S49A cells from mice bearing a large tumor caused marked agglutination. This result suggested that the S49A cells have antibody bound to their membrane surface, which would agree with the theory that the tumor adsorbs specific anti-tumor antibodies.

Antibody Dependent Cell Mediated Cytotoxicity (ADCMC)

Although cell mediated immunity against S49A lymphoma cells was not demonstrated by adoptive transfer of survivor spleen cells or in the Winn test, other tests were available to study this phenomenon. The ^{51}Cr release assay has been a method of choice in the determination of cell mediated immunity and antibody dependent cell mediated cytotoxicity *in vitro*. Therefore, this method was used to study these phenomena in the S49A lymphoma system.

The following experiments were performed to determine if there was any cell mediated immunity by survivor spleen cells against S49A

cells *in vitro*. Also, sera from normal, survivor, and tumor bearing mice, or rabbit anti-BAθ or NRS were examined for their ability to induce ADCMC with normal spleen cells. Spleen cells from normal, survivor, and tumor bearing mice were tested to determine if any difference existed in their abilities to perform CMC or ADCMC. The cytotoxicity of antibody or any factors present in mouse or rabbit sera against lymphoma cells was tested by incubation of the sera with S49A cells. Complement was added to some of these samples to measure complement mediated lysis.

Spontaneous release of ^{51}Cr from the freshly extirpated lymphoma cells that were incubated with various mouse and rabbit sera was studied to determine if there were any cytotoxic factors present. Results, not presented here, from several experiments demonstrated that the various rabbit and mouse sera did not have a significant effect on ^{51}Cr release compared to S49A cells incubated only in Dulbecco's supplemented medium. The spontaneous release from all groups tested throughout all the experiments ranged from 27-36% of the maximum ^{51}Cr release during a 5-7 hour incubation. This range of spontaneous release from S49A cells was not uncommon for freshly excised tumor cells, which usually ranges between 10-35% for four hour incubations (4,7,109,112). Neoplastic cells from various types of tumors have their own characteristic range of spontaneous release, probably related to such factors as metabolic rate, rate of cell death, and permeability of the tumor cell membrane.

The following experiment was designed to determine if antibodies from various mouse or rabbit sera could bind to the tumor cells and fix complement that would cause complement mediated lysis. Complement

was added to tubes containing S49A cells and one of the following sera: NMS, survivor mouse sera, sera from tumor bearing mice, NRS, or anti-BAΘ. The data in Table 12 indicated that only anti-BAΘ caused significant and complete lysis (100-104% specific ^{51}Cr release) of all antibody coated tumor cells. This result suggests that 100% of the S49A cells preincubated with anti-BAΘ were coated with anti-BAΘ, while the other sera tested did not have a detectable ability to fix complement and induce cytolysis. Sera from mice bearing a large tumor demonstrated a suppression of specific ^{51}Cr release (0.4% as compared to 5.6% specific ^{51}Cr release by NMS when these sera were incubated with labeled S49A cells and complement [Experiment 1, Table 12]). This result may reflect the lack of some factor which was present in NMS which caused some non-specific lysis of labeled tumor cells, but was absent from the sera of mice bearing a large tumor.

The ^{51}Cr release cytotoxicity assay provided a means of determining whether cell mediated immunity or ADCMC played a role in the mechanism of anti-BAΘ suppression of lymphoma growth. Experiment I in Table 13 was designed to determine the effects of incubation of S49A cells with various mouse and rabbit sera on their reaction with normal spleen cells. Preliminary experiments, not presented here, with survivor spleen cells 14-21 days after initial tumor challenge and ^{51}Cr labeled S.49.1 and S49A cells showed no significant killing of the labeled target cells. The results of Experiment I, Table 13, indicated that none of the survivor or tumor bearing mouse sera had a significant effect on the killing of S49A cells by normal spleen cells as compared to NMS. The only serum preincubated with S49A cells demonstrating a marked increase in killing was anti-BAΘ with a +19.2%

Table 12. Complement mediated cytotoxicity of anti-BA0 coated S49A lymphoma cells

Experimental Group[b]	Experiment I % Specific ^{51}Cr Release[a] Avg. ± SE	Experiment II % Specific ^{51}Cr Release Avg. ± SE	Experiment III % Specific ^{51}Cr Release Avg. ± SE
S49A cells preincubated with:			
Medium	+0.7 ± 0.8	+3.1 ± 1.6	+7.9 ± 1.9
NMS	+5.6 ± 0.3	+5.5 ± 1.1	---
Survivor Mouse Sera	+3.2 ± 0.8	+4.7 ± 1.3	---
Survivor Mouse Sera	+4.0 ± 1.0	---	---
Sera from Mice Bearing a Large Tumor	+0.4 ± 0.6*[c]	---	---
NRS	+1.9 ± 0.3	+3.0 ± 0.9	+6.6 ± 4.6
Anti-BA0	+99.9 ± 1.7**	+100.0 ± 1.8**	+104.3 ± 2.9**

a = % Specific ^{51}Cr Release = $\frac{\text{Experimental (CPM) - Spontaneous (CPM)}}{\text{Maximum Release (CPM) - Spontaneous (CPM)}}$ x 100.

b = Guinea pig serum was the source of complement and was added to each tube after the S49A cells were preincubated with the indicated solution.

c = As compared to NMS.

Student's t test: *$p<0.05$ **$p<0.005$.

Table 13. Antibody dependent cell mediated cytotoxicity of anti-BAθ coated S49A cells by syngeneic spleen cells

Spleen Cells	S49A Cells Preincubated with:	Experiment I % Specific Release[a] Avg. ± SE	Δ%[b]	Experiment II % Specific Release Avg. ± SE	Δ%	Experiment III % Specific Release Avg. ± SE	Δ%
NORMAL	Medium	-4.7 ± 0.8		-6.9 ± 1.1		-3.9 ± 1.1	
	NMS	-1.1 ± 0.4		-6.3 ± 2.9		---	
	Survivor Mouse Sera	+1.4 ± 0.7		-4.4 ± 0.6		---	
	Survivor Mouse Sera	+1.0 ± 1.3		---		---	
	Sera (Large Tumor Bearer)	-5.0 ± 1.2		---		---	
	NRS	-2.3 ± 0.4		-6.8 ± 0.2		-5.6 ± 1.5	
	Anti-BAθ	+19.2 ± 0.2**	(21.5)	+0.9 ± 0.6*	(7.7)	+5.7 ± 0.7*	(11.3)
SUR-VIVOR	Medium	---		-5.9 ± 1.1		-2.7 ± 1.6	
	NMS	---		-3.8 ± 2.1		---	
	Survivor Mouse Sera	---		-4.6 ± 1.1		---	
	NRS	---		-9.6 ± 0.6		-4.5 ± 0.6	
	Anti-BAθ	---		-1.1 ± 1.3*	(8.5)	+3.4 ± 1.4	(7.9)

Table 13 (continued)

Spleen Cells	S49A Cells Preincubated with:	Experiment I % Specific Release[a] Avg. ± SE	Δ%[b]	Experiment II % Specific Release Avg. ± SE	Δ%	Experiment III % Specific Release Avg. ± SE	Δ%
SMALL TUMOR BEARER	Medium	---		-10.8 ± 0.7		---	
	NMS	---		-8.6 ± 0.1		---	
	Survivor Mouse Sera	---		-5.1 ± 1.4		---	
	NRS	---		-11.8 ± 0.7		---	
	Anti-BAθ	---		+0.9 ± 0.3*	(12.7)	---	

a = % Specific ^{51}Cr Release = $\frac{\text{Experimental (CPM) - Spontaneous (CPM)}}{\text{Maximum Release (CPM) - Spontaneous (CPM)}}$ x 100.

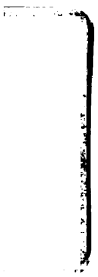
b = Δ% = % Specific ^{51}Cr Release (Anti-BAθ-NRS), which represents the only groups to demonstrate significant differences between values.

Student's t test: *$p<0.05$ **$p<0.005$.

specific ^{51}Cr release, while the NRS control (-2.3% specific ^{51}Cr release) had no effect. These results suggested that ADCMC may be an important mechanisms of killing S49A cells *in vitro*.

Cell mediated cytotoxicity was not displayed in the first experiment. Therefore, a positive control experiment was performed (not shown here) to determine that CMC could occur when ^{51}Cr-labeled S49A cells and sensitized spleen cells were incubated together as measured by the ^{51}Cr release assay. Allogeneic BCF_1 mice were sensitized to S49A cells by two S.C. injections of 10^6 viable S49A cells. The sensitized BCF_1 mice were sacrificed one week following the second injection of S49A cells and their spleen cells were used as a control for CMC in the ^{51}Cr release assay. The BCF_1 spleen cells caused a significant (8%) increase in ^{51}Cr release from labeled S49A cells. This result was similar to results found with C57Bl spleen cells and ^{51}Cr-labeled S.49.1 cells (68). The low ^{51}Cr release may have been due to the low H-2^d antigen density found on the target cells (68). When S49A-BA0 cells were incubated with sensitized BCF_1 spleen cells, a 32% specific ^{51}Cr release was recorded, suggesting that anti-BA0 was effective in inducing ADCMC in an allogeneic system as well as a syngeneic system.

The ability of spleen cells from normal and survivor mice and mice bearing a small tumor to mediate ADCMC with anti-BA0 coated tumor cells was studied. The data presented in Experiment II, Table 13, again suggest that only anti-BA0 can induce a significant increase in the lysis of S49A cells with the three types of spleen cells tested. Spleen cells of normal and survivor mice and mice bearing a small tumor had similar abilities in performing ADCMC with



anti-BAΘ coated S49A cells recording Δ% of 7.7, 8.5, and 12.7, respectively. Survivor spleen cells, from mice challenged 45 days earlier, lacked the ability to kill unmodified S49A cells *in vitro*. This result suggested that these cells did not have a measurable CMC against syngeneic S49A cells *in vitro*. Spleen cells from mice bearing a small tumor did not have the ability to lyse untreated S49A cells *in vitro* as demonstrated by their -10.8% specific ^{51}Cr release.

A third experiment (Table 13) was utilized to confirm the earlier results of anti-BAΘ inducing ADCMC equally in normal and survivor spleen cells. Spleen cells from mice surviving two challenges of 10^4 S49A-BAΘ cells, the last challenge being 30 days earlier, demonstrated cytotoxicity against S49A cells preincubated with anti-BAΘ +3.4% specific ^{51}Cr release as compared to -4.3% specific ^{51}Cr release for S49A cells preincubated with NRS. Normal spleen cells had a similar ability to mediate ADCMC with anti-BAΘ coated S49A cells as indicated by +5.7% specific ^{51}Cr release. Also, as in the previous experiment, survivor spleen cells had no ability to kill uncoated S49A cells as indicated by -2.7% specific ^{51}Cr release.

The data from the three ^{51}Cr release experiments in Table 13 demonstrated that normal mouse sera, survivor mouse sera, sera from tumor bearing mice, or normal rabbit sera preincubated with S49A cells did not have the ability to mediate ADCMC. However, S49A cells preincubated with anti-BAΘ were lysed by spleen cells from normal, survivor, or tumor bearing mice. There was no significant difference in the killing of anti-BAΘ coated cells by spleen cells of survivor or tumor bearing mice as compared to spleen cells

from normal mice as exemplified by the Δ% in Experiments II and III in Table 13. This result and the inability of spleen cells from survivor and tumor bearing mice to lyse uncoated S49A cells suggested that these cells did not have the capacity to mediate CMC *in vitro*. Although the lysis of anti-BAθ coated target cells by syngeneic spleen cells varied from experiment to experiment, antibody dependent cell mediated cytotoxicity appeared to be the prevalent cytotoxic mechanism that killed anti-BAθ coated lymphoma cells *in vitro*.

DISCUSSION

The initial objective of these experiments was to bind to a tumor cell a specific foreign marker that would be strongly immunogenic *in vivo*, and to induce an immune response against the modified tumor cell that inhibited tumor growth. The antiserum anti-BAΘ was selected because of the strong immunogenicity of allogeneic or xenogeneic immunoglobulins in mice (13) and the presence of brain associated theta antigen on the surface of S49A lymphoma cells. To further increase the immunogenicity trinitrophenol (TNP) molecules were bound to immunoglobulins of anti-BAΘ, forming anti-BAΘ-TNP. The binding of TNP to anti-BAΘ did not enhance the effectiveness of the antibodies bound to lymphoma cells before injection but, instead, it partially abrogated the effectiveness of protection of anti-BAΘ. Anti-BAΘ was able to suppress lymphoma growth in a syngeneic system when tumor cells were coated with it before injection, or when it was injected directly into the area of tumor challenge two days after injection of tumor cells. Attempts to correlate the antibody protection with a cellular immune response were unproductive, suggesting that another mechanism was acting. After a variety of approaches to demonstrate cell mediated cytotoxicity (CMC) by sensitized spleen cells were attempted, it was discovered that normal spleen cells could lyse antibody coated tumor cells *in vitro*. The experimental

results suggested that ADCMC may be the mechanism of action of antibody suppression of lymphoma growth.

Trinitrophenol bound to anti-BAθ immunoglobulins inhibited the cytotoxic ability of anti-BAθ. The limited inactivation of anti-BAθ cytotoxicity seen with 71 and 60 TNP per immunoglobulin could be due to a certain percentage of the population of immunoglobulins having more TNP molecules bound than the threshold number (≈ 38 TNP/Ig). The threshold number was described as the maximum number of TNP molecules bound per immunoglobulin of anti-BAθ so that the cytotoxic activity of anti-BAθ was retained. Nord et al. (80) demonstrated that antitumor antibodies could be iodinated and have five-sixths (at 2 moles I/mole IgG) to one-third (at 21 moles I/mole IgG) of their antigen binding activity retained. Conformational changes may have prevented anti-BAθ from binding to BAθ antigen on S.49.1 cells or may have altered the immunoglobulin's receptor for complement so that the complement cascade could not be activated. Nord et al. (80) found that specific inactivation of antibody by iodination involved protein denaturation with increasing levels of iodination. TNP molecules bound in the antigen binding site of the immunoglobulin may have blocked antibody binding to antigen. To retain the activity of anti-DNP antibodies conjugated with the alkylating agent, Trenimon, Linford et al. (70) found it necessary to block the antigen receptor sites on the immunoglobulins with DNP before conjugation. In the present experiments, further evidence of loss of activity was indicated by the twofold reduction in cytotoxic titer of anti-BAθ-TNP as compared to anti-BAθ.

The results of antibody suppression of lymphoma growth indicated that tumor cells coated with anti-BAΘ had a much lower chance of forming tumors than uncoated cells. Even after two days of undisturbed tumor growth, anti-BAΘ injections prevented tumor growth in 100% of the mice. Anti-BAΘ-TNP did not appear to be as potent as anti-BAΘ in preventing tumor growth when S49A cells were coated with these preparations before injection. The decreased effectiveness of anti-BAΘ-TNP in the prevention of tumor growth when S49A cells were coated *in vitro* before injection could have been due to the TNP causing slight conformational changes in the immunoglobulin, reducing avidity and affinity for S49A cells. Nord et al. (80) demonstrated that extensive iodination of anti-MSV sera increased non-specific binding and decreased avidity for MSV induced lymphoma cells. Although preincubation of tumor cells with anti-BAΘ-TNP only delayed appearance of the tumor, this antiserum could effectively prevent any palpable tumor from forming when injected on days 2, 5 and 8 after tumor inoculation. One reason for the increased effectiveness of the multiple injections following tumor challenge in preventing tumor growth as compared to *in vitro* preincubation of antiserum with tumor cells could have been the presence of antibody for a longer period of time. Another factor could have been the abundance of antibody in tissues surrounding the tumor area *in vivo*.

Normal rabbit serum (NRS) did not have the ability to completely suppress tumor growth, but on occasion it slowed the initial advance of tumor appearance. NRS may have impaired tumor growth by non-specific adsorption of some immunoglobulins onto the tumor cell

membrane, which could have induced a cytolytic immune response. NRS could also have acted by non-specifically activating lymphoid and phagocytic cells in the local area of injection.

The inability of the inactivated anti-BAΘ or NRS to prevent tumor growth when tumor cells were pretreated with these sera before injection or when these sera were injected into the site of tumor challenge suggests that the suppression of S49A lymphoma cells by anti-BAΘ was a specific reaction. The ability of lymphoma cells to grow intraperitoneally, in spite of three S.C. injections of anti-BAΘ into the abdomen suggests that anti-BAΘ must bind to the tumor cell surface in sufficient quantity to afford protection.

Anti-BAΘ is an antiserum directed specifically against a group of determinants representing a common antigen (BAΘ) found on thymocytes, mature T-cells, and cells of the brain (37,122). The antiserum, produced in rabbits, was made highly specific for BAΘ antigen by adsorption with mouse liver and red blood cells of the BALB/c strain. Anti-BAΘ can suppress the *in vivo* growth of a lymphocytic lymphoma, that also bears BAΘ antigen, in syngeneic hosts. This result appears to be, at present, the first time that antibody made specifically against a non-tumor specific antigen could suppress tumor growth in a syngeneic system. All other successful attempts at antibody suppression of tumor growth utilized a relatively tumor specific antiserum. The tumor specific antisera were produced by inoculating tumor cells into rats, rabbits, or mice of a different strain. The antisera were either extensively adsorbed *in vitro* or *in vivo* to become tumor specific (14,52,104), spleen or liver

adsorbed (39,93,106), or not adsorbed at all yielding anti-mouse sera (130).

The extent of protection and frequency of injection required to achieve protection was also different from past studies. A single injection or multiple injections of anti-BA0 in the same area of tumor inoculation prevented tumor growth in nearly 100% of the animals given a lethal dose of tumor cells 48 hours previously. When lymphoma cells were preincubated with anti-BA0, 85% of the mice did not develop tumors at all, and the majority of mice that did succumb to tumor growth had prolonged survival. The findings of Gorer (39) demonstrated that alloantisera to EL-4 lymphoma cells, injected I.P. immediately after S.C. tumor challenge in syngeneic mice, could prolong survival 10-14 days. Gorer also found some protection was demonstrated if antisera were injected up to seven days before or one day after tumor challenge. Hersey showed that rabbit anti-rat lymphoma serum given I.P. twice a day before and two days after intracardial tumor challenge could prolong the survival of rats by eight days (52). In contrast to Gorer (39) and Hersey (52), Rao et al. (93) observed that tumor specific rabbit antiserum directed against a rat sarcoma could suppress intraperitoneal tumor growth in all syngeneic rats receiving antiserum I.P. on seven consecutive days after tumor challenge. Zighelboim et al. (130) found that EL-4 lymphoma cells preincubated in unadsorbed rabbit or allogeneic anti-EL-4 could not induce tumor growth in any syngeneic mice inoculated. This success may have been attributed to the wide spectrum of normal and tumor specific antigen to which the antiserum was directed. However, tumor cells and antiserum inoculated one after

another at the same intraperitoneal site could only prolong survival for nine days. Davies et al. (14) demonstrated that exhaustively adsorbed tumor specific rabbit antisera injected I.P. into syngeneic mice, 4-18 hours after a lethal dose of EL-4 lymphoma cells was injected I.P. and at 24-hour intervals for 3-4 days, would prolong survival for 2-8 days. Shin et al. (104,106) demonstrated that preincubation of 6C3HED lymphoma cells with spleen adsorbed allogeneic anti-6C3HED delayed tumor appearance and subsequent death by seven days. In comparison to previous studies, it appears anti-BAΘ used in equal or substantially lower quantities than that of tumor specific antisera provides a powerful tumor suppressive agent for the syngeneic S49A lymphoma system.

The greater protection produced by anti-BAΘ against tumor growth in comparison to the protection produced by tumor specific antisera in some of the previously mentioned syngeneic tumor systems may be explained by several factors. The inability of Gorer (39) and Hersey (52) to prevent tumor growth may have been due to the antisera being injected at a site different from the tumor inoculation, therefore substantially diluting out the amount of antibody that could reach and bind to tumor cells. Also, injections of tumor specific antisera into the peritoneal cavity could dilute the amount of antibody reaching intraperitoneal tumor cells as compared to the limited area of antibody diffusion following subcutaneous injection of anti-BAΘ into the tumor area. The use of alloantisera by Gorer (52), Shin (104), and by Davies (14) in some experiments may have been less efficient in mediating tumor destruction and had a lower potency as compared to xenogeneic rabbit antisera. Indeed, Davies

et al. (14) and Zighelboim et al. (130), who used alloantisera and rabbit antisera in their studies with syngeneic lymphoma cells, concluded that rabbit antisera were much more effective at inhibition of tumor growth than mouse alloantisera. The spleen adsorption of anti-lymphoma serum by Shin et al. (106) and the exhaustive adsorption (20-50 spleens/ml antisera) by Davies et al. (14) may have left only a few antibodies directed against tumor specific antigen that could suppress tumor growth. Although not directly examined, the class of antibodies found in rabbit anti-BAΘ sera and the avidity of antibody to S49A cells may have been such that ADCMC, one proposed mechanism of antibody suppression of tumor growth, was more potent with anti-BAΘ than with tumor specific antisera.

Some observations suggested that the immunogenicity of S49A cells in syngeneic BALB/c mice was low. A low dose of S49A lymphoma cells was required for fatal tumor growth in normal and survivor mice. Spleen cells of tumor bearing mice or survivors displayed no cytotoxicity to lymphoma cells *in vitro*. Agglutinating factor(s) were found in the serum of tumor bearing mice. This evidence suggested that unmodified S49A lymphoma cells were weakly immunogenic. Further studies, such as injection of irradiated lymphoma cells into syngeneic mice, would be required to determine the immunogenicity of S49A cells.

The previous observations suggesting that S49A cells were weakly immunogenic led to further agglutination experiments to determine to what extent there was a humoral response to these tumor cells *in vivo*. The results from the agglutination tests suggest that about ten days after tumor challenge measurable agglutinating factors were formed and increased in activity over time. Although these "factors" were

presumed to be antibodies directed against S49A lymphoma cells, they were designated "agglutinating factors" since their antibody properties were not defined. The level of agglutinating factors remained constant for about 25 days subsequently and gradually disappeared by the ninetieth day if the tumor was eradicated. In progressively growing tumors the level of agglutinating factors increased to a certain point, about 16 days after tumor challenge, and then declined. The decrease in agglutinating factors may have been caused by the adsorption of the factors onto tumor cells as the tumor load increased. Finally, when the tumor was quite large, all detectable agglutinating factors were absent. This absence could likewise be explained by the complete removal of the factors by the large tumor load.

The mechanism of anti-BAΘ suppression of lymphoma growth could have been due to a number of possibilities. Endogenous complement could have mediated the cytolysis of antibody coated cells. Anti-BAΘ or its binding to the tumor cell surface could have been toxic to tumor growth. The host's own cytolytic antibody with endogenous complement could have caused lysis of the lymphoma cells. Non-specific factor(s), in rabbit anti-BAΘ sera or in the sera of sensitized mice, could have killed tumor cells. Cell mediated cytotoxicity could have developed. Or, finally, antibody dependent cell mediated cytotoxicity could have destroyed antibody-coated tumor cells. The host could potentially respond by one or any combination of the preceding mechanisms.

The role of endogenous complement in mediating antibody suppression of tumor growth was investigated. Lymphoma cells coated with

anti-BAθ were incubated with undiluted fresh normal syngeneic mouse serum for two hours. The NMS did not facilitate any cytotoxicity with antibody coated cells in two experiments. Simultaneous controls of NMS with unmodified tumor cells did not demonstrate any cytolytic effects, while a known source of active complement (guinea pig serum) diluted 1:10 could induce 100% lysis of the antibody coated cells. In similar experiments Zighelboim et al. (130) also found that when rabbit antibody coated lymphoma cells were incubated with their respective syngeneic normal mouse serum for several hours, no lysis occurred. Hersey demonstrated that normal rat serum could not mediate the lysis of antibody coated rat tumor cells (52). These investigations suggest that either the antibodies could not fix complement or the normal sera could not induce cytolysis. Another possible explanation of these investigations was that the tumor cells were resistant to complement mediated lysis, which will be discussed in a later section. Winn has proposed that the likelihood of endogenous complement reaching the subcutaneous tumor cells while the antibody was still present on the tumor cell surface in sufficient quantities to mediate lysis was remote (127).

Mice naturally have low levels of complement in their blood, which in part may be due to a deficiency in one or more complement components (78). Terry et al. (114) suggested that the poor cytolytic ability of the sera of many different mouse strains including BALB/c may be due to the low levels of C3 activity found in these mice. The only evidence for normal BALB/c serum containing cytolytic complement was demonstrated by Rosenberg et al. (97), who found that the sera of male BALB/c mice mediated cytotoxicity when incubated with mouse

antibody coated guinea pig RBC. Female BALB/c mice had much lower cytolytic complement activity. In the present studies, no cytolytic activity attributed to complement could be identified with normal BALB/c serum and anti-BAθ coated tumor cells when the ^{51}Cr release assay was performed.

Some researchers have demonstrated that complement is not essential for antibody suppression of lymphoma growth. Shin et al. (104) demonstrated that lymphoma growth could be suppressed in mice deficient in C5 if tumor cells were preincubated in tumor alloantisera. Mice with intact complement systems were depleted of complement activity by cobra venom factor which made C3 activity undetectable (104). Although mice that were decomplemented one day before tumor challenge were susceptible to normal tumor growth, these mice could still suppress the growth of alloantibody coated lymphoma cells (104). Similar results were obtained by Hersey (52), who demonstrated that cobra venom factor-treated mice were depleted of complement to less than 1% of normal levels, yet these mice were still protected against tumor challenge by administration of tumor specific antisera. These results suggest the presence of complement is not necessary for antibody suppression of tumor growth.

The antibody coated tumor cells may have been killed *in vivo* by host cytolytic antibody and endogenous complement. Although an agglutination factor was observed in tumor bearing mice and survivors, it was not necessarily cytolytic antibody. The increase in agglutination titers did not seem to be important in the host's defense against the tumor since a noticeable titer was not always correlated with decreased tumor growth. However, the observable and reproducible

levels of agglutination factors indicated that there was some sort of a humoral response even if it was non-protective by itself. The presence of antibody on the tumor cell surface *in vivo* was suggested by the agglutination of freshly excised tumor cells by goat anti-mouse immunoglobulin. A similar finding of antibody on the surface of progressively growing tumor cells was shown by Fish et al. (31). The addition of fresh NMS or guinea pig serum as a source of complement to S49A cells did not reduce their viability, which suggested that host antibody present on *in vivo* tumor cells either did not fix complement or the tumor cells were resistant to lysis. Sera from survivor mice that showed agglutination were incubated with S.49.1 or S49A cells in the presence of guinea pig serum but no cytotoxicity was observed. The function of the agglutinating factors is undefined, but they may have enhanced tumor growth by acting as a blocking antibody or as part of an immune complex.

A humoral response to antibody coated cells may have been directed against the antibody or hapten moiety of the anti-BAΘ-TNP complex bound to S49A cells. Spleen cells of mice sensitized twice with anti-BAΘ-TNP coated thymocytes or lymphoma cells coated with anti-BAΘ-TNP were unable to mount a detectable antibody response to TNP. This result suggested that another mechanism, other than a cytolytic antibody response, was operational in antibody suppression of S49A lymphoma growth.

Several studies have indicated that most neoplastic cells are resistant to lysis by antibody and complement. Möller, in 1962, suggested that sensitivity to cytotoxic antibody by allografts is dependent upon spatial arrangement of suitable H-2 receptor sites

on cell surfaces (78). If antigenic sites are few or widely separated, the tumor cell-antibody complexes may be unable to bind sufficient complement at any single point on the cell surface to cause injury. Möller's results suggested that cytotoxicity could be mediated through localized concentration of complement on surfaces of cells rich in antigen receptors. A study of myeloma and lymphoma cells showed a threshold effect with a given amount of surface antigen, below which complement mediated killing was poor and above which complement killing was complete (68).

It is possible that S49A cells may have a "low" density of BAθ antigen on their surface. In an experiment utilizing iodinated anti-BAθ, not presented here, the number of iodinated anti-BAθ antibodies bound to BAθ determinants present on the tumor cell surface were approximately equal to those found on the much smaller thymocyte as reported by Vitetta et al. (121). Since the average diameter of a small lymphocyte such as a thymocyte is 7.5 microns, and the average diameter of S.49.1 and S49A cells is 15 microns (54), the surface area of the lymphoma would be about four times greater than that of a thymocyte. Considering the number of BAθ antigenic sites on lymphoma cells was similar to that found on thymocytes, the density of BAθ antigen on the surface of S49A cells would be four-fold less than that found on the surface of thymocytes. This observation may partially explain the four- to eightfold lower cytotoxic titer of anti-BAθ with S49A cells as compared to thymocytes. The previous results suggest that the "low" density of BAθ antigen on the lymphoma cell surface may have reduced the susceptibility of anti-BAθ coated cells to complement mediated lysis.

Besides antigen distribution, Ohanian et al. (81) found that the class of antibody bound to a cellular antigen and its location near a site susceptible to complement lysis was important in determining if cytolysis would occur. A similar mechanism could be operating in S49A tumor bearing mice if the host's antibody response, directed against S49A cells, was comprised of antibodies incapable of fixing any or enough complement to induce lysis.

Lymphoma growth may have been inhibited *in vivo* by the toxicity of anti-BAθ or its binding to the tumor cell surface. Chromium-labeled anti-BAθ coated cells incubated in culture conditions for seven hours released similar amounts of ^{51}Cr as cells incubated with media, NRS, or NMS, indicating an absence of toxicity. Binding anti-BAθ to S.49.1 cells did not alter the growth rate of these cells in culture even when present on the cell surface for 30 hours or more. In fact, studies by Shearer et al. (103) indicated that binding of antibody to L cells in the absence of complement could stimulate the growth rate by an aggregation mechanism similar to lectin stimulation. Shearer demonstrated that the interaction of rabbit antibody to L cell lines increased radioactive nuelcoside and nutrient uptake, correlated with an increased growth rate, allowing the cell line to grow beyond normally inhibitory cell concentration levels. Incubation of anti-BAθ, without exogenous complement in the presence of BAθ-bearing cells, is not toxic, while NMS alone will cause more damage to BAθ bearing cells than anti-BAθ (Golub, personal communication). These results suggest that anti-BAθ or its binding to lymphoma cells did not have any noticeable adverse effects upon tumor growth.

A non-specific factor in normal or hyperimmune rabbit sera may have been toxic to tumor cells. As mentioned earlier, the inability of NRS or inactivated anti-BAΘ to prevent fatal tumor growth indicated that there were not any non-specific factors in anti-BAΘ that could suppress tumor growth.

Evidence of a cell mediated response to antibody coated or uncoated tumor cells could be represented by the following: a secondary tumor challenge could be rejected or its growth rate inhibited. The transfer of sensitized spleen cells into a normal host before tumor challenge could delay tumor appearance. Survivor spleen cells admixed with uncoated or antibody coated tumor cells before tumor challenge could prolong survival or eradicate tumor growth. An increase in the percent specific release of ^{51}Cr from labeled tumor cells could occur when these cells were incubated with sensitized spleen cells. If any of these reactions were to occur, it would suggest that a cellular response could play an important role in the immune response against lymphoma cells.

A secondary challenge of mice surviving a primary challenge of lymphoma cells 30 or 100 days earlier was performed in an attempt to establish whether a memory response to lymphoma cells existed. The surviving mice did not show any protection against unmodified or anti-BAΘ coated lymphoma cells. These results suggested a lack of a detectable memory response to either tumor cell surface antigens or cell surface bound anti-BAΘ.

In three other studies in which antibody suppressed lymphoma growth, survivors were rechallenged 30-100 days after the initial tumor challenge. The three groups of investigators found that the

rechallenged mice demonstrated no protection against tumor growth (14,39,130). In one case antibody suppression of tumor growth in rats afforded protection to a tumor rechallenge six months later (93). This protection may have been due to the high dose (2×10^7 cells) of tumor cells injected, which could have stimulated the immune response. Another factor may have been that seven injections of rabbit anti-tumor sera could have non-specifically induced an immune response (93).

The lack of a detectable secondary response to S49A cells could have been due to short term cellular (T-cell) memory, as seen in second set rejection of transplantations (125) and in secondary humoral responses (58). Since 30 days past tumor cell inoculation was the earliest attempt at secondary challenge *in vivo*, the cellular memory that remained may have been insufficient to respond. Holden et al. (53) demonstrated that, three months after the initial injection of a spontaneously regressing MSV induced tumor, only 5% of the sensitized mice acquired tumors upon challenge of cross-reacting syngeneic leukemia cells. It was found that mice could be rechallenged up to six months after the primary inoculation of syngeneic plasmacytoma cells and survive (9). The tumor cells were rejected by a T-cell mediated cytotoxic response as demonstrated by abrogation of protection with anti-θ and complement. The duration of a T-cell memory response in syngeneic tumor systems can be long, but its nature and manner of inducement remain undefined.

The secondary immune response to a syngeneic tumor was tested *in vitro* by the ^{51}Cr release assay. Holden et al. (53) demonstrated that an *in vitro* secondary immune response could be measured for

only 14 days after a secondary challenge of syngeneic cross-reacting leukemia cells. Rollinghoff found detectable cell mediated cytotoxicity *in vitro* in immunized mice up to 35 days after the injection of spontaneously regressing MSV induced tumor cells (96). Both investigators demonstrated that the secondary immune response was mediated by T-cells. In the present study spleen cells of mice that had received their third inoculation of lymphoma cells 14-21 days earlier did not demonstrate detectable cytotoxicity in the ^{51}Cr release assay. The lack of detectable cytotoxicity by sensitized spleen cells suggests an absence of a memory response. Stolfi et al. (112) and De Landazuri et al. (16) also found that mice that survived a previous tumor challenge, due to antibody suppression of syngeneic breast tumor growth or syngeneic lymphoma growth, respectively, did not have a detectable T-cell mediated response.

A cell mediated memory response to tumor antigens has successfully been transferred into syngeneic hosts by the adoptive transfer technique or by the method of Winn (126). Adoptive transfer of lymphoid cells sensitized to a polyoma-induced tumor (1) or a plasmacytoma (9) could prevent tumor growth in new syngeneic irradiated hosts. The protection in both cases could be abrogated by anti-θ and complement, indicating T-cell dependency. In contrast, similar attempts in the S49A tumor system to transfer immunity against the antibody coated lymphoma cells in a 100:1 ratio with unmodified or antibody coated tumor cells before injection did not yield any protective capacity. Transferring 10^7 survivor spleen cells a week before tumor challenge did not alter tumor growth. The inability to transfer immunity into

syngeneic mice suggests that mice which survived a lymphoma cell challenge did not have a significant memory response.

Suppressor T-cells may have been present in mice challenged with S49A lymphoma cells and could have inhibited an immune response to the syngeneic tumor cells. This cell type could be a possible explanation for the slight enhancement of tumor growth in survivor mice that were rechallenged or when spleen cells or survivor mice were used in a Winn test or an adoptive transfer. Several researchers have demonstrated that suppressor T-cells are present in tumor-bearing mice and can enhance tumor growth (35,60,62). It is also possible that the lymphoma cells themselves could be suppressing an immune response. In a study utilizing two lymphoma cell lines, Feldman et al. (30) demonstrated that these two theta positive cell lines can produce a factor that can suppress humoral immunity.

The tumor challenge dose of 10^4 S49A lymphoma cells may be too low to induce an active memory response so that "sneaking through" may occur (75). Often a very low dose of weakly immunogenic cells, that slowly proliferate, can escape detection or attack from an immunologically competent host (64). It has been suggested that the mechanism of action is an active suppression of the immune response by suppressor T-cells, rather than an escape from immune recognition (75). To determine if the lack of a detectable cell mediated cytotoxicity to S49A lymphoma cells is due to a sub-immunogenic dose, a higher dose of antibody coated lymphoma cells such as 10^6-10^7 could be injected, and then test the hosts' spleen cells for CMC, or rechallenge any survivors.

Another type of cell mediated response that could have been involved in the killing of uncoated or antibody coated tumor cells was the macrophage. Specific cytotoxic tumor cell killing by macrophages has been demonstrated by several investigators (27,28,44,119). The proportion of macrophages found within proliferating tumors has been correlated with the state of the immune response to the tumor (21) and with control of metastasis (21,44). Evans et al. (25,26) have suggested that normal macrophages can be specifically armed by a soluble product from sensitized lymphocytes, or activated by a variety of non-specific stimulants. The armed macrophages can kill specifically, while activated macrophages were indiscriminate in their inhibition of tumor growth. Evans et al. (26) indicated that arming or activation of macrophages required more than one encounter with antigen. Although the role of macrophages in the suppression of anti-BAθ coated lymphoma cells was not explored, it is possible that macrophages participated in the destruction of these tumor cells. Normal macrophages or macrophage-like cells have been implicated in cellular destruction of antibody coated cells (105,129), and their role in the S49A tumor system will be discussed in a later section.

In the present experiments the coating of tumor cells with anti-BAθ or one injection of anti-BAθ at the tumor site suppressed lymphoma growth in mice that did not have a prior encounter with lymphoma cells. This result suggests that normal mice would have to quickly generate cytotoxic cells specifically directed against cell bound anti-BAθ or tumor specific antigens to provide protection against tumor growth. However, this possibility appears unlikely

considering lymphoma cells seem to be weakly immunogenic, and the lack of a detectable cell mediated response against unmodified or antibody coated lymphoma cells. Also, 10^4 uncoated tumor cells were nearly 100% lethal upon challenge of normal or survivor mice. Mice sensitized to anti-BAΘ before tumor challenge did not exhibit increased protection against antibody coated lymphoma cells. These observations suggest that destruction of syngeneic lymphoma cells coated with anti-BAΘ requires a mechanism dependent on cells already present in normal mice before tumor challenge.

Antibody dependent cell mediated cytotoxicity has recently been shown to be a very effective means of destroying cells coated with the proper class of antibody *in vitro* (52,88,109,112,129). The ADCMC reaction is an *in vitro* response to antibody coated target cells mediated by spleen, lymph node, or peritoneal cells of normal mice or mice inoculated with tumor cells (42,72). The present system of antibody suppression of lymphoma growth in normal syngeneic mice seems to fit the requirements of ADCMC. K-cells are present in the spleens of normal mice. These cells appear to be able to bind to the Fc portion of the antibody bound to tumor cells, which leads to the destruction of the tumor cells (72). The ADCMC phenomenon was demonstrated in the S49A tumor system by the ^{51}Cr release assay. Lymphoma cells were preincubated with NRS, media, or anti-BAΘ and then incubated with normal spleen cells of syngeneic BALB/c mice. In these experiments only those cells coated with anti-BAΘ demonstrated a significant release of ^{51}Cr above the controls, expressing specific killing of anti-BAΘ coated lymphoma cells. When sensitized syngeneic spleen cells of survivor or tumor bearing mice were added to antibody

coated cells, there was not any increase in cytotoxicity over that which was found with normal spleen cells. This result suggests that these spleen cells have an equal ability to mediate ADCMC.

Spleen cells of survivor and small tumor bearing mice were examined in the ^{51}Cr release assay to determine if a detectable cell mediated cytotoxicity to uncoated S49A lymphoma cells was present *in vitro*. These spleen cells did not demonstrate the significant increase of percent specific ^{51}Cr release over controls associated with cell mediated cytotoxicity. Zighelboim et al. (130) tested for non-antibody dependent cell mediated cytotoxicity in spleen cells of mice that survived a previous challenge of antibody coated lymphoma cells. These investigators did not find any cytolytic activity with uncoated EL-4 lymphoma cells in the presence of survivor spleen cells. These results suggested an absence of cell mediated cytotoxicity to uncoated S49A lymphoma cells by spleen cells of survivor and small tumor bearing mice.

Host antibody in mice challenged with S49A cells may have been able to mediate ADCMC. The presence of host antibody in mice after tumor challenge, capable of inducing ADCMC, was tested by preincubating fresh serum from tumor bearing or survivor mice with lymphoma cells before the lymphoma cells were incubated with spleen cells of normal and survivor mice. The level of ^{51}Cr release in these samples was similar to the release observed with lymphoma cells preincubated with medium alone. These results suggested that mice challenged with uncoated or antibody coated lymphoma cells did not have endogenous antibodies capable of inducing ADCMC.

Two reports have recently suggested that antibody suppression of tumor growth *in vivo* was due to ADCMC (52,130). Hersey (52) found ADCMC activity, as measured by the ^{51}Cr release assay, in the sera of rats up to seven days after the injection of tumor cells and rabbit anti-tumor sera. Hersey found that only the antibody directed against the tumor cell would show significant cytotoxicity, while the same antisera would not cause lysis with unrelated cells. Zighelboim et al. (130) performed the only previous study to utilize rabbit antibody coated lymphoma cells with normal syngeneic spleen cells. These investigators demonstrated that the antibody coated lymphoma cells showed a significant increase in ^{51}Cr release over spontaneous release in NRS controls (129). Specificity was demonstrated by the lack of ^{51}Cr release from bystander cells during ADCMC of unlabeled antibody coated tumor cells (129). Zighelboim et al. (129) also found that the IgG fraction of rabbit anti-EL-4 could mediate ADCMC. The IgG class of antibodies has been shown to be the nature of the antibody that mediates ADCMC in many tumor systems (72,112,130). The IgG fraction alone as well as unfractionated sera would suppress lymphoma growth *in vivo* if lymphoma cells were pre-incubated with it before injection. Gorer and Amos (39) and Davies et al. (14) demonstrated that the IgG fraction mediated antibody suppression of *in vivo* tumor growth, which suggests that ADCMC may have been the effector mechanism of antibody inhibition of tumor growth in these studies.

The suppression of lymphoma growth by one injection of anti-BA0 two days after the initial tumor injection may have been due to ADCMC. The anti-BA0 injected into the same area of the tumor

inoculation could reach all growing tumor cells. It is postulated that the continued presence of antisera, unlike tumor cells pre-incubated with anti-BAΘ, would allow enough time for the effector cells to reach the inoculation site. The better protection found in mice receiving *in vivo* injections of anti-BAΘ as compared to *in vitro* preincubation could be due to the longer presence of the antibody that mediates tumor cell lysis by effector cells *in vitro*.

The actual cell that mediates ADCMC *in vitro* and possibly *in vivo* has been studied by several researchers. The effector cell of ADCMC designated as the K-cell has been found to be most abundant in the spleen, peritoneal exudate, and peripheral blood lymphocytes in unsensitized mice (72,84,102). The murine K-cell has been described as a non-adherent, non-phagocytic lymphoid cell that lacks both surface immunoglobulin and theta antigen and has weak surface adherence properties (42,102). MacLennan has implicated a cell with the above properties as the effector cell that mediated ADCMC of antibody coated Chang cells (72). Studies by Zighelboim et al. (129) and Shin et al. (105,106) suggested that a macrophage-like cell mediated the antibody suppression of tumor growth *in vivo*. Recently, evidence reported by Greenberg et al. (42) has suggested that another type of effector cell, besides the lymphocytic K-cell, can mediate ADCMC. These investigators have characterized the macrophage-like cell as an adherent monocytic cell containing granules and designated it as a myeloid K-cell. Although the characteristics of the effector cell(s) that mediates ADCMC of anti-BAΘ coated lymphoma cells *in vitro* or the cell(s) that may mediate antibody suppression of tumor growth *in vivo* were not directly studied,

it is likely, from the above observations, that lymphoid or myeloid K-cells were responsible for the antibody mediated suppression of S49A lymphoma growth demonstrated *in vitro* and *in vivo*.

Model

A hypothetical model for antibody suppression of lymphoma growth is presented below. Lymphoma cells coated with anti-BAΘ *in vitro* are injected subcutaneously into the abdomen of normal mice. The lymphoma cells remain in the growth phase of the cell cycle until the cells adapt to the new environment, at which time the cells begin to replicate. The trauma of injection may initiate a minor inflammatory response that brings leukocytes into the tumor area. The myeloid or lymphoid K-cells in the regional lymph nodes or circulating in the blood that enter the inoculation area can recognize the Fc portion of the tumor cell bound anti-BAΘ. The Fc receptors on the effector K-cell surface firmly bind to the activated Fc portion of the cell bound antibody. Shortly thereafter, the K-cell has programmed the antibody coated tumor cell for destruction. The membrane interactions between the K-cell and the tumor cell lead to a deregulation of membrane transport of electrolytes, which allows osmotic swelling and subsequent lysis. Lymphoma cells not yet recognized by the K-cells will continue to actively synthesize and shed theta antigen along with its bound antibody. If a K-cell has not bound to the lymphoma cell before all of its anti-BAΘ is shed, the tumor cell will have escaped immediate recognition by the K-cell and can proliferate into a fatal tumor growth. Likewise, if the anti-BAΘ bound to tumor cells is shed before a sufficient immune

attack can be coordinated, another means of escape from immunological destruction is provided.

SUMMARY

Lymphoma growth in syngeneic mice was suppressed or completely eradicated by coating lymphoma cells with rabbit anti-BAΘ. Preincubation of lymphoma cells with anti-BAΘ or injection of the antisera into the same area of primary tumor inoculation 48 hours later inhibited tumor proliferation. The use of a highly specific antiserum directed against the common theta antigen, found on normal and neoplastic cells derived from the thymus, was a new and successful approach in antibody suppression of tumor growth. The inability of normal rabbit serum or chemically inactivated anti-BAΘ to suppress lymphoma growth demonstrated that non-specific factors which could suppress tumor growth were absent from these sera. The lack of a detectable cell mediated response *in vitro* by spleen cells of survivor mice, and the lack of protection against tumor challenge in sensitized mice, suggested that an immune response did not develop. Immunity to unmodified lymphoma cells could not be adoptively transferred to normal mice by spleen cells of sensitized mice. The complement of normal mouse sera was unable to mediate lysis of antibody coated lymphoma cells. The only detectable immune response to lymphoma growth was a factor in the sera of challenged mice which could agglutinate tumor cells but could not induce ADCMC or complement mediated lysis.

The possibility of suppression of lymphoma growth by antibody dependent cell mediated cytotoxicity was examined. This mechanism

was suggested by the significant increase of specific ^{51}Cr release from anti-BA0 coated lymphoma cells in the presence of normal syngeneic spleen cells. A model was discussed that proposes the destruction of antibody coated tumor cells was mediated by K-cells recognizing and binding to the Fc portion of the cell bound anti-BA0 before the antibody was shed from the tumor cell surface.

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