CYTOFLUOROMETRIC EVALUATION OF THE ANTIGEN BINDING CELL RESPONSE TO TUMOR ASSOCIATED ANTIGENS IN THE MOUSE

by

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(Professor in Charge of Thesis)

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To my wife, Anamaria and

my children, Ian Joshua and

Jordan Paul

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STATEMENT OF THE PROBLEM

Specific recognition of antigen by cell surface receptors on immune cells triggers their differentiation into either effector or regulatory cells. The immune status of an individual may be assessed by measuring various aspects of cellular differentiation (eg. blast transformation, release of lymphokines). Each of these aspects however represents a secondary event subsequent to direct interaction of an immune cell with antigen. This primary interaction can be measured directly using the antigen binding cell (ABC) assay. Autoradiograpic and rosette forming cell techniques have been used to document the presence of ABC's in both human and animal systems. Although ABC's have been observed in normal animals, the level of antigen specific ABC's increases significantly after immunization. Depending upon the assay and conditions employed, T cells, B cells and macrophages can be shown to bind antigen. Because of limitations of previous technology, ABC's to tumor antigens have not been adequately documented. The functional involvement of ABC's in the immune response to tumor challenge remains to be established.

The purpose of this thesis is threefold: 1) using a newly described cytofluorometric technique we will document the presence of antigen binding cells to tumor-associated antigens in a murine melanoma tumor model; 2) we will define the cell types involved in the ABC response and the mechanism of antigen recognition; 3) we

will evaluate which component(s) (antigens) in the complex melanoma antigen preparation are recognized in the antigen binding cell assay.

The specific aims of this thesis are to:

1) Document the presence and specificity of ABC's in C57BL/6 mice injected with the syngeneic P51 melanoma tumor line.

2) Define the kinetics and magnitude of the ABC response.

3) Determine the cell type(s) and mechanism involved in binding melanoma associated antigens.

4) To investigate which component(s) in the melanoma antigen preparation are recognized by the antigen binding cell.

INTRODUCTION

The immune response to an antigen challenge represents an interaction of many cell types and factors. Both humoral and cellular host mechanisms may be activated following administration of an immunizing dose of antigen. Similarily, exposure of the immune system to tumor stimulates a variety of cells and factors which have cytotoxic or cytostatic tumor associated activity in vitro. In addition to these tumor reactive cells, a variety of blocking factors, including anti-tumor antibody, immune complexes, tumor antigen, T-suppressor cells and in some circumstances, macrophages may interfere with a protective immune response to tumor challenge. The following is a review of the immune response to tumor antigen and the involvement of ABC's in this response.

I. REVIEW OF CELLS AND NON-CELLULAR FACTORS IMPLICATED IN THE IMMUNE RESPONSE TO TUMOR

A. Antibody Independent Effector Cells

Passive transfer experiments have implicated the T cell as being crucial in providing protection against tumor challenge. The mechanism by which these cells provide protection may involve

cytotoxic reactions (16,18,20,50,66). Current work concerning the mechanism of cytotoxic T cell killing in vitro suggests two basic controlling elements. In order for an animal to exhibit a cytotoxic T cell response the immune system must recognize both histocompatibility and tumor associated determinants on the immunizing cells. The cytotoxic potential of the killer cells generated after this recognition cannot be expressed unless the immunizing and target cells share identical histocompatibility (K and/or D) loci of the mouse H-2 complex and tumor associated determinants (9,19,88). However, studies conducted using T cell deficient animals have not demonstrated consistent significant increases in the incidence of spontaneous tumors as compared to normals. Thus, the extent of T cell involvement in providing protection against tumor development in vivo remains unknown.

Natural Killer (NK) cells, demonstrated in rodents (24,40) and man (83) represent an effector cell population which is postulated to exist without prior immunization. These cells are thought to play a crucial role in providing the host with an immune surveillance system for protection against developing malignancies (32,39,57). In the murine system, NK cytotoxic activity has been demonstrated against a wide spectrum of tumors. Unlike the cytotoxic T cell, the NK cell is not H-2 restricted. In fact, NK activity may not be species specific, since mouse NK cells have documented cytotoxic activity against human leukemia cell lines (24). Natural killer cells have been characterized as being

distinct from either mature T cells (42) or B cells (33,41) since they lack theta or immunoglobulin cell surface determinants. The NK cell is non-adherent and non-phagocytic, thus eliminating it from the monocyte-macrophage series (41,84). Natural killer cells have been documented to possess a distict age and organ distribution, with maximum activity at 4-6 weeks of age in the mouse and complete cessation of activity by 12 weeks of age (41). The mechanism of cellular killing as mediated by NK cells is largely unknown. A model proposed by Roder (63) requires cellular contact and the recognition of an NK target antigen on the tumor cell surface via a hypothetical NK receptor. Whereas NK cells are believed by some to function independently of antibody, it has been demonstrated that many mouse strains produce natural antibodies against virally induced tumors (49,57). The possibility that these structures may serve as specific receptors, following passive absorption to the NK cell, must be considered.

B. Antibody Dependent Effector Mechanisms

The production of specific anti-tumor antibody has been demonstrated in a variety of model systems (6,36,58,67,69). Antibody may be demonstrated at early stages of tumor development, but tends to disappear or to become complexed with tumor antigen prior to metastasis (3,14,78). After tumor resection, serum antibody to the tumor may again be demonstrated (61).

The benefit of cytotoxic antibody in vivo is difficult to assess directly: however, the combination of tumor antibody and various effector cells is considered crucial in the host's immune response to tumor challenge. In vitro studies have demonstrated that macrophages (58,67,70), lymphocytes (68), and plateletes (47,48,68) possess specific anti-tumor reactivity in the presence of cytophilic tumor antibody.

The killer (K) cell can lyse tumor target cells in the presence of specific anti-tumor antibody (65). The K cell is non-phagocytic and lacks characteristic T or B cell determinants (65). Cellular lysis of target cells occurs following interaction of tumor antibody with the Fc receptor on the K cell surface. This mechanism of target cell destruction is termed antibody dependent cell mediated cytotoxicity (ADCC).

The cytotoxic and/or cytostatic effects of macrophages on tumor cells suggests their involvement in controlling tumor growth. Activation of macrophages against a tumor is the final step in a series of complex cellular interactions. Lymphocytes and macrophages accumulate non-specifically at a site of inflammation and interact with each other either directly (cellular contact) or indirectly (soluble mediators) resulting in the development of cytotoxic macrophages. Macrophages may be rendered cytotoxic for tumor cells via interaction with immune lymphocytes (15) or may be armed by a T cell factor, distinct from antibody, termed soluble

macrophage activating factor (SMAF) (82). The SMAF armed macrophage possesses cytotoxic activity against the immunizing tumor cell.

Macrophage killing of tumor cells may be either specific or nonspecific. Specific antibody mediated killing may occur through Fc receptors present on the macrophage cell surface. Evidence in vivo for the effectiveness of "arming" non-immune macrophages with tumor specific antibody has been demonstrated. Johnson and co-workers (36) suggest that as a tumor mass increases, the effectiveness of antibody mediated killing decreases, perhaps due to depletion of non-immune effector cells, specifically macrophages, rather than a limitation of antibody.

Wood and co-workers have documented macrophage infiltration into both human (86) and murine (85) tumors. In an attempt to assess the in vivo relevance of these tumor associated macrophages, Wood and Gillepsie (85) removed tumors from animals and depleted the tumor associated macrophage population. Reinjection of the tumor cells, depleted of the infiltrating macrophages, into naive recipients resulted in a significant reduction in survival rate as compared with animals who received tumor cells with associated macrophages. These results suggest that macrophages may have some role in vivo in regulating tumor growth.

Nonspecific tumoricidal activity of macrophages may be induced through the use of adjuvants or chronic infections (34).

Zarling and co-workers (87) have shown that spleen cells from mice injected with SV-40 transformed cells will provide protection against tumor challenge to naive animals. Indirect evidence suggests the macrophage as being the primary cell type involved in tumor protection. The importance of the macrophage as an effector cell with cytotoxic potential against the tumor or as a cooperative cell type involved in the generation of tumor effector cells of another lineage was left unresolved. Boraschi and Meltzer (5) have described a defect in macrophage activation in P/J mice using an *in vitro* tumor cytotoxicity assay. The *in vivo* manifestations of this macrophage defect in relation to infections and tumor development remain unresolved. Recent evidence suggests that the P/J mouse may have an increased incidence of chemically induced colorectal adenocarcinomas and may be less responsive in delayed type hypersensitivity reactions (8,71).

Macrophages have also been postulated to interfere with tumor development by inhibiting, rather than killing, tumor cells. Pasternack and co-workers (58) have documented *in vitro* the ability of murine macrophages, in association with antibody, to cause tumor cytostasis.

C. Interfering Factors

As described above, exposure of the host's immune system to an immunizing dose of tumor cells results in the activation of a

variety of cell types and mechanisms involved in the prevention of tumor development. However, to account for the observation that tumors frequently occur in immunologically competent hosts, a number of factors have been postulated to interfere with this protective immune response to tumor challenge. The Hellstroms (29) originally proposed that anti-tumor antibodies may be responsible for blocking cell surface tumor antigens, thus preventing these surface determinants from being recognized by cytotoxic effector cells. This concept of antibody blocking has been expanded to include blocking by immune complexes (76). Serum factors may also interact directly with cytotoxic effector cells, resulting in the inhibition of the host's immune response to tumor challenge. This direct interaction with cytotoxic effector cells may be due to either tumor antigen in soluble form or tumor antigen complexed with anti-tumor antibody (44).

Recent work has demonstrated that a variety of tumors induce the formation of suppressor T cells which directly interfere with the immune response to tumor challenge (21,75). A suppressor T cell factor has been identified which is responsible for inhibiting tumor effector cell reactivity. This antigen-specific suppressor cell factor(s) has a molecular weight < 65,000 daltons and lacks immunoglobulin constant region determinants. Recent investigation has demonstrated that this factor possesses I-J subregion determinants of the mouse H-2 histocompatibility complex (59). Perry and co-workers (59) have shown the suppressor factor,

administered to non-immune mice may elicit the development of tumor specific suppressor T cells. Greene and collegues (22) have demonstrated a reduction of syngeneic tumor growth in mice after in vivo administration of Anti-I-J antisera. It is postulated that this means of immunotherapy reduces T-suppressor cell activity which is inhibiting development of tumor destructive cytotoxic T cells.

Macrophages have also been demonstrated to be involved in the enhancement of tumor growth. A number of investigators have noted the appearence of macrophage-like suppressor cells after BCG treatment. Recently Klimpel, and co-workers (43) have demonstrated an in vitro inhibition of cytotoxic T cells by BCG-induced, macrophage-like suppressor cells. These cells are non-adherent and lack theta surface determinants. While the suppressor cells possess Fc receptors, these structures are not involved in suppression. Cellular contact was required for suppressor activity, and this action was independent of prostaglandin synthesis. Suppression of cytotoxic T cells resulted from the macrophage-like suppressor cell acting at the T-helper cell level which interfered with either the development and/or proliferation of cytotoxic T cells.

Eggers and Wunderlich (11) have documented an inhibition of in vitro cytotoxic activity in spleen cells from mice with progressively growing tumors as compared to normal spleen cells. This hyporesponsiveness was due to a nylon-adherent, theta negative cell, possibly a macrophage.

Recent evidence suggests that prostaglandins, produced by macrophages, have an inhibitory effect on lymphocyte function. Prostaglandin synthesis may be intiated by macrophages either through direct contact with various stimuli (Corynebacterium parvum, immune complexes or Fc fragments of antibody) or indirectly through lymphokines secrected from antigen activated lymphocytes. Increases in various cellular functions (antibody synthesis, delayed hypersensitivity and tumor resistance) have been observed in vivo after indomethacin treatment, which is an inhibitor of prostaglandin synthesis (73,81).

The immune response to tumor challenge involves numerous cell types and cytotoxic mechanisms which are postulated to be involved in vivo in tumor destruction. These effector cells may function either independently or in concert to reject a developing tumor. Whereas the cytotoxic T cell has been considered the piviotal cell in this response, a number of non-T cells have been documented to possess tumoricidal activity in vitro. The development or rejection of a tumor thus represents an intricate balance between effector cells and interfering factors of the immune system.

D. Assessment of the Immune Response to Tumor Antigen

Perhaps the most classical method of assessing humoral and

cellular immunity to conventional antigens is the skin test (31,53). Skin testing with tumor extracts has been carried out in tumor patients with mixed results. Patients may show reactions to tumor extracts especially in the early stages of tumor development, but individuals with advanced malignancies are often found to be hyporesponsive to testing with tumor antigens (30). These variable results may be due to interfering factors such as humoral antibody, circulating tumor antigen, immune complexes or various forms of therapy (56).

A number of *in vitro* assays such as blast transformation (17), macrophage or leucocyte migration inhibition (51), macrophage chemotactic inhibition (72) and leucocyte adherence inhibition (25,26) have been used with variable results to measure metabolic events which occur following recognition of tumor antigen. However, the relationship of these tests to protective tumor immunity is largely unknown. Cytotoxic assays provide a more direct method of evaluating an individual's ability to function against a tumor cell target. However, extention of these *in vitro* results to tumor control *in vivo* is lacking.

II. REVIEW OF ANTIGEN BINDING CELL ASSAYS TO DETECT BINDING OF CONVENTIONAL AND TUMOR ANTIGENS

A. ABC ASSAYS

A variety of techniques have been used to document the appearance of antigen binding cells (ABC's). Antigens may be radiolabelled (27,28,35,74) and then mixed with immune cells. Quantitation of the amount of labelled antigen bound to cells is detected using autoradiographic techniques. Fluorescent labelled antibodies (23) have also been used to bind antigen selectively. These complexes of antibody and antigen may then be detected using a fluorescent activated cell sorter (FACS) or fluorescent microscopy. Antigen coated immunoadsorbants (eg. collagen) have also been used effectively for either the enhancement or selective depletion of an ABC population (50,77). The rosette forming cell assay has been widely used for detection of antigen binding cells (80). Incubation of immune cells with antigen coated RBC's results in the formation of rosettes (immune cells bound to a number of antigen coated RBC's).

Use of any of the presently available methods for detection of ABC's requires that the method 1) detects all potential ABC types and 2) allows for an efficient and reliable method for quantitation of ABC's.

Autoradiography has been shown to demonstrate selectively high avidity ABC's, such as B cells. The demonstration of T cell binding by this technique is more difficult and was not initially recognized (4). An examination of some parameters of ABC development has shown

that B cells have significantly greater numbers of antigen receptors than T cells and thus bind antigen more effectively. In addition, incubation of immune cells with antigen at 37C instead of 4C had no influence on the frequency of B-ABC's, however these temperature differences increased T cell binding 2-3 fold. Fluorescent labelling of antigen, and then quantitation of the resulting ABC's has been used to detect B cells (23). The use of immunoadsorbants represents a good method for enriching or selectively depleting a population of ABC's. However, this method may bind some cells non-specificically.

The rosette forming cell assay has an advantage over the previously described techniques for detection of ABC's because this method has been used to document the involvement of antigen binding T cells, B cells and macrophages (4,55,80). The major limitation with this assay has been the lack of a reliable method, other than fluorescent microscopy, for quantitation of antigen specific ABC's. Quantitation of ABC's by fluorescent microscopy is time consuming and relies on a relatively small data base for the evaluation of statistical significance. Recently Tong, et. al. (80) described a cytofluorometric procedure for the detection and quantitation of antigen specific ABC's. This method utilizes laser optics and electronic detection and can quantitate ABC's based upon the parameters of particle size and nuclear fluorescence (see appendix #1). This method of quantitating ABC's is rapid and provides a reliable data base from which to evaluate the statistical significance of an ABC population.

B. DEMONSTRATION OF ANTIGEN BINDING CELLS TO CONVENTIONAL ANTIGENS

In normal animals a subpopulation of cells exist with receptors for antigen, apparently without prior exposure to the antigen. The presence of ABC's to a variety of antigens has been observed in nonimmune animals and after immunization, ABC's increase over normal levels (1,2,7,37,52,80). Merrill and Ashman (52) have documented changes in the ratio of T and B ABC's and cell surface isotypes of B ABC's specific for SRBC's, using an *in vitro* assay system. The maximum increase in ABC's occurred on day 4 of culture and represented a 10 fold increase in T-ABC's and a 4 fold increase in B-ABC's. Examination of isotype surface receptor changes after interaction with antigen suggests an increase in IgG bearing antigen specific ABC's.

As described earlier, ABC's have been documented to be B cells, T cells, and macrophages. The importance of these ABC's in the immune response has been well established. Antigen binding B cells have been shown to be antibody producing cells (23,38) while T ABC's have cytotoxic and suppressor functions (10,13). T-helper cells are apparently unable to bind free antigen directly (45,12) since this subpopulation of T cells consistently fails to bind to immunoadsorbant columns. T-helper cell binding probably requires interaction both with antigen and with Ia determinants of the H-2 histocompatibility complex. Antigen binding T cells have also been

shown to be effector cells involved in delayed type hypersensitivity responses (13).

The nature of the antigen receptor on the B cell has been established as surface immunoglobulin (28,64), while the nature of the T cell receptor remains less clearly defined. Current data indicates the involvement of variable region genes of the heavy chain in antigen recognition by T cells (46). Shared idiotypes between T and B cell receptors, specific for the synthetic polymer Tyr(TMA) were evaluated using an ABC assay by Prange and co-workers (60). They were able to demonstrate blocking of antigen binding by T cells using idiotypic specific antisera.

The mechanism of antigen specific recognition by macrophages appears to be cytophilic antibody arming (54,62,79,80). The importance of the "armed" macrophage in vivo is unknown. As described previously, however, antibody dependent cell mediated cytotoxicity has been shown in vitro to be an effective method of tumor cytotoxicity.

C. DEMONSTRATION OF ANTIGEN BINDING CELLS TO TUMOR ANTIGENS

The use of the ABC assay for evaluation of cellular recognition of tumor antigens has been limited relative to conventional non-replicating antigens. Mazo and Shellam (50) using tumor

membrane extracts coated on collagen gels documented an enrichment of *in vitro* tumor cytotoxic activity of the adherent cell population. The non-adherent cell population had significantly reduced tumor cytotoxic ability as compared to unfractionated spleen cells. In additional studies Mitchell and co-workers (54) described the formation of tumor specific immune rosettes between whole tumor cells and macrophages after administration of viable tumor cells. The mechanism of recognition of the tumor cell by the macrophage was through cytophilic tumor specific antibody which was linked to the macrophage through the cell's Fc receptor. Subsequent work (62) has shown that immune complexes of tumor cells and antibody can indirectly suppress Fc receptors on the macrophage. Treatment with both whole anti-tumor antibody and tumor cells, but not with either alone, elicited macrophages incapable of fixing cytophilic anti-tumor antibody and thus the animals failed to form tumor specific rosettes. A postulated mechanism described the interaction of immune complexes with naive T cells bearing Fc receptors, which led to the development of suppressor T cells which inhibited the expression of macrophage Fc receptors.

Recently Tong, and co-workers (79) have evaluated the immune response of a group of patients with squamous cell carcinoma of the head and neck using a panel of partially purifed KCl tumor cell preparations. These studies have established the presence of tumor specific ABC's in these patients and a lack of ABC reactivity to control tumor preparations. Macrophages were identified as the

predominant ABC type responding in the rosette forming cell assay. Arming of these macrophages with tumor specific antibody was the mechanism of tumor antigen recognition and binding. Further investigation has demonstrated that recurrent squamous cell carcinoma patients lack detectable tumor specific ABC's, as compared to patients with active disease or those in remission. Whether this suggests a loss or inactivation of tumor ABC's or a "masking" which prevents detection in this assay is unknown. However, these data do suggest the exciting possiblity that tumor specific ABC's may be important in preventing recurrent tumors.

III. Summary

The immune response to tumor challenge results in a complex series of cellular interactions which may be either specific or non-specific. While a plethora of information has been obtained describing the *in vitro* tumor cytotoxic potential of T cells, NK cells, K cells, macrophages and antibody, the *in vivo* relevance of any of these factors in protection against tumor development remains undetermined. The use of tests such as leucocyte migration inhibition, blast transformation and leukocyte adherence inhibition represent indirect evaluations of secondary parameters resulting from the interaction of an antigen with an immunocompetent cell. Attempts to correlate these parameters of cellular differentiation with immune status have frequently been unsuccessful. Direct methods for evaluation of immune reactivity include skin testing and

cytotoxic assays. Skin testing is often unacceptable because it also assesses *in vivo* blocking factors which may interfere with the interpetation of an individual's immune status. Cytotoxic assays evaluate the direct interaction of immune cells with antigens; however attempts to correlate *in vitro* cytotoxic reactivity to *in vivo* immune status have often been contradictory.

The antigen binding cell assay, which measures the primary interaction of antigen and immune cells, has been used to demonstrate the specific enhancement of either effector or regulatory cells after antigen challenge *in vivo*. This assay has the advantage over other techniques used to assess an individuals immune status since it documents the recognition and subsequent binding of antigen by immune cells directly, and does not require evaluation of a secondary parameter of this interaction. While a tremendous amount of information has been gained from ABC assays using conventional antigens, employment of this method for detection of cells responding to tumor challenge has been limited. The use of the ABC assay in a tumor model may provide important clues as to the nature of the host's immune response to tumor challenge.

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CYTOFLUOROMETRIC EVALUATION OF THE ANTIGEN BINDING CELL RESPONSE TO TUMOR ASSOCIATED ANTIGENS IN THE MOUSE:

I. KINETICS, SPECIFICITY AND CELL IDENTIFICATION

ABSTRACT

The antigen binding cell (ABC) assay has been used to evaluate the cellular recognition by mononuclear cells from C57BL/6 mice injected with keyhole limpet hemocyanin (KLH), viable melanoma cells or a KCl extract of the melanoma tumor cell line. Using a competitive inhibition assay with soluble antigen, we have demonstrated the specificity of ABC's which are found in immunized animals. The kinetic profiles of the ABC responses to KLH and replicating melanoma cells are very similar. Responses to both antigens appear by day 4 after antigen administration, reach maximum magnitude by days 5 to 7, and then decline significantly by day 10. The ABC response seen after injection of the tumor extract is quite distinct however; ABC's reach maximum levels by day 3 and remain elevated for an extended period of time (at least through day 19). There are also significant differences in the cell types which bind these antigens. Macrophages are the exclusive ABC type detected in response to injection of replicating melanoma cells; in contrast, T cells, B cells and macrophages are all seen as part of the antigen specific ABC's detected in spleen cells from animals injected with KLH. Animals injected with the soluble tumor extract have a non-staining cell population, in addition to T cells, B cells and macrophages, as part of their antigen specific cellular response.

INTRODUCTION

The encounter of antigen with a competent immune system results in a number of measurable events which reflect to varying degrees an individual's immune status. Correlates of immunity include cellular proliferation (2), production of factors (ie. MIF, LIF) (11,17), or membrane changes (9) to name a few. The earliest point of measurable immune recognition, however, is the specific binding of antigen to an immunocompetent cell. Evaluation of this interaction may be assessed directly using the antigen binding cell (ABC) assay. This assay has been used by others to demonstrate the binding of antigen by a variety of cell types, including T cells (6,7,13), B cells (4,7,13) and macrophages (14,15,16,19,20). Functional studies of these cell types have revealed that ABC's are directly involved as either effector or regulatory cells in the immune response to antigen.

The purpose of the present study is to define differences in the cellular mechanisms by which animals respond to either conventional or tumor antigens. We have used a rosette forming cell assay to evaluate the kinetics, specificity, magnitude and cell identification of ABC's from C57BL/6 spleens after immunization with either keyhole limpet hemocyanin (KLH), replicating melanoma cells or a KCl extract of the melanoma tumor. Our results indicate that KLH and melanoma specific ABC's may be detected after appropriate

immunization but that differences in the kinetics of the ABC responses occur. In addition, T cell, B cell and macrophage binding accounts for the majority of the specific ABC's seen after administration of either KLH or soluble melanoma tumor antigen, while the ABC response detected after injection of replicating tumor cells is exclusively mediated by macrophages. The mechanism by which these macrophage-ABC's gain specificity for tumor antigen will be described in an accompanying paper.

MATERIAL AND METHODS

1. Animals: C57BL/6 female mice, obtained from Simonson Labs were used throughout this study. Animals were used between the ages of 6-12 weeks.

2. Description of the Tumor: The P51 murine melanoma (18) was maintained by in vitro transfer every 3 to 4 days. The tumor was grown in RPMI-1640 media containing antibiotic-antimycotic and supplemented with 10% Fetal Calf Serum (FCS). The KCl extraction of the tumor was performed essentially as described by Meltzer (12). Briefly, approximately 10^8 cells were mixed with 15 mls of hypertonic 4M KCl, and left overnight at 4C. The mixture was then dialyzed extensively, first against water and then saline. After each dialysis the solution was centrifuged at 40,000 X g for 30 minutes at 4C and the cell pellets discarded. The protein concentration of the tumor extract was established using a Bio-Rad assay. This method is a dye-binding assay which is based on the differential color change of a dye in response to various concentrations of protein (Bio-Rad Laboratories).

3. Immunization Protocol: C56BL/6 female mice were injected intraperitoneally (IP) with either KLH, viable melanoma cells or a KCl extract of the melanoma. KLH was administered in incomplete

Freunds Adjuvant at a concentration of 100 µg KLH/mouse in 0.2 ml. Viable melanoma cells were administered IP in saline, with each animal receiving $2x10^6$ viable cells in 0.2 mls. Administration of the tumor extract was given IP in saline, with each animal receiving approximately 60 µg of protein in 0.2 ml volume.

4. Antigen Binding Cell Assay

A. Preparation of RBC's: Red blood cells were collected in heparinized capillary tubes from C57BL/6 mice by rupturing the orbital sinus. The cells were washed twice in RPMI-1640 and resuspended at a 2.5% final (v/v) concentration in TRIS-buffered saline (TBS) (0.01M, pH 7.4). Antigen was then coupled to the red cell surface using chromium chloride (3). Efficient coupling of proteins to the surface of the red cell using chromium chloride requires that a number of procedures be carefully followed. The chromium chloride solution (0.1%, pH 5) should be "aged" for approximately 30 days prior to use. The solution should be added dropwise to the mixture of protein and red cells. It is crucial that the chromium chloride solution be added last, as it is rapidly inactivated. In addition, the reaction mixture should be free of phospate ions which interfere with the coupling reaction. Therefore, one ml of $CrCl_3$ (0.01%) in TBS was added dropwise to one ml of washed RBC's in TBS and one ml of antigen diluted in TBS to the appropriate final concentration (150 µg of KLH and 20 µg of

soluble melanoma extract). The mixture was left undisturbed for 4-5 minutes at room temperature after which time the cells were washed twice in RPMI-1640 and resuspended to a final concentration of 1% (2.5 ml) in RPMI-1640. KLH was coupled to the red cell surface to detect KLH ABC's, while the KCl extract of the melanoma tumor was conjugated to the red cell surface for identification of rosette forming cells in animals injected with either viable melanoma cells or the soluble tumor extract.

B. Preparation of Spleen Cells: Spleens were removed and minced into single cell suspensions. The cells were treated for 10 minutes at 4C with 0.83% NH_4Cl for lysis of RBC's. After being washed twice in RPMI-1640 the cells were adjusted to a final cell concentration of $1.25X10^7$ cells/ml in RPMI-1640.

C. Preparation of RFC's: Equal volumes (100 µl) of antigen conjugated RBC's and washed spleen cells were combined with 300 µl cold RPMI-1640. The rosetting mixture was centrifuged at 4C for 5 minutes at 200 x g, and then incubated at 4C for 60 minutes before being evaluated for rosette forming cells.

5. Quantitation of Rosette Forming Cells: Quantitation of RFC's was done using a cytofluorograph (Ortho Instruments model ÷4802A). The cytofluorograph has been used to quantitate rosette forming cells based upon the parameters of nuclear fluoresence and particle size (light scatter). Essentially, three populations of cells are

present in the rosetting mixture; non-immune white cells, antigen coated RBC's and rosetted cells. Addition of the nuclear stain acridine orange to the rosetting mixture allows for distinction of nucleated cell populations, (single cells and rosettes) from antigen coated RBC's in the rosetting mixture. RBC's which lack a nucleus are not stained. Use of the second parameter, particle size, allows discrimination of single cells from the larger rosetted cell population. The RFC response to a specific test antigen is then calculated as:

% SPECIFIC RFC RESPONSE = (% Response to test antigen) -
(% Response to control antigen)

Triplicate readings were obtained for each antigen using an average of 10^4 cells per reading

6. Indirect Fluorescent Staining: Identification of the cell types involved in the RFC response was assessed using indirect fluorescent staining. Briefly, $1.25X10^7$ cells in one ml of RPMI-1640 containing 0.3% bovine serum albumin (BSA) were incubated with a 1/20 dilution of rabbit anti-mouse theta or a 1/10 dilution of rabbit anti-mouse Ig reagent (Cedarlane Laboratories). The cells were incubated at 4C for 45 minutes, washed in RPMI-1640 and resuspended to the original one ml volume in RPMI-1640 plus 0.3% BSA. A fluorescein labelled goat anti-rabbit antisera (Cappel Laboratories) was added at a 1/50 dilution when used with the

anti-theta reagent and at a 1/10 dilution when used with the anti-Ig reagent and the mixture incubated for an additional 45 minutes at 4C. The cells were washed twice and resuspended in approximately 0.3 ml of RPMI-1640. Fluorescent cells were quantitated on a Leitz fluorescent microscope. These concentrations of antisera resulted in the staining of 60-70% of spleen cells and 6% of thymocytes as B-cells and 20-30% of spleen cells and 100% of thymocytes as T cells. No staining was observed with the goat anti-rabbit reagent alone. For detection of T-ABC's, mononuclear cells were treated with the appropriate reagents prior to being used in the ABC assay. Detection of B-ABC's however required forming ABC's initially, and then incubating the rosetting mixture with the appropriate antisera.

7. Adherent Cell Depletion:

A. G-10 Depletion: Adherent cells were depleted using the technique of Ly and Mitchell (10). Briefly, Sephadex G-10 beads (DIFCO) were incubated overnight in RPMI-1640 containing 5% FCS. A 35 cc. syringe was plugged with glass wool and the G-10 column poured to a final volume of approximately 15 cc. Spleen cell populations (4-5 mls at 10^7 cells/ml) free of RBC's were passed over the column. A total of 20 ml of column eluant was collected and the cell concentration was readjusted to $1.25X10^7$ cells/ml before use in the ABC assay. Generally, 60-70% of the cells were recovered following passage over the G10 column, with 90% macrophage depletion.

B. Carbonyl Iron Adherence: Adherent cell depleted populations were also obtained using carbonyl iron ingestion. Briefly, $5X10^6$ cells were incubated with 40 µg of washed carbonyl iron filings for 45 minutes at 37C with rotation. The iron adherent cell population was removed using a magnet. The remaining cells were washed twice in RPMI-1640 and readjusted to the appropriate cell concentration ($1.25X10^7$ cells/ml) for use in the Antigen Binding Cell Assay.

8. Macrophage Identification: Identification of macrophages was examined morphologically by staining with a differential nuclear and cytoplasmic stain, acridine orange. Briefly, 10 µl of acridine orange (0.1mg %) was added to the rosetting mixture. An aliquot of the stained cells were extracted with a pasteur pipette and examined under a fluorescent microscope (Leitz). Either 200-300 nucleated cells were examined per sample, using triplicate readings per antigen. Mononuclear cells with 3 or more attached RBC's were considered rosette forming cells. Macrophages were also identified as esterase positive cells.

9. Competitive Inhibition: Specificity of ABC's was established by incubating soluble antigen (either test or control antigen) at a 2.5 fold excess with mononuclear spleen cells ($1.25X10^7$) for 30 minutes at room temperature. An ABC assay, as described above, was then performed using the mixture of mononuclear spleen cells incubated with soluble antigen, and red cells coated with either test or control antigens.

10. Statistics: A Mann-Whitney U test was used to evaluate significant differences ($p < 0.05$) in binding of test versus control antigens.

RESULTS

Using a cytofluorometric procedure for quantitation of rosette forming cells from normal mouse spleens we established a consistent background measurement of approximately 5%. However this background value does not represent an actual 5% binding of normal mouse spleen cells to antigen coated RBC's, but is rather a composite of at least three components: WBC clumps, cellular coincidence and actual rosettes. Microscopic evaluation of normal mouse spleen cells which have been incubated with various Ag-RBC conjugates has established an actual ABC background level of 1% (Fig. 1). In addition, however, WBC clumps were observed in the rosetting mixture. While these clumps are not ABC's they would be detected as such by a cytofluorometric evaluation of these, and account for approximately 1.6% of the cytofluorometric measurement. In addition, when normal mouse spleen cells are incubated with unconjugated syngeneic RBC's a cytofluorometric background value of approximately 2.5% is observed. This is most likely due to cellular coincidence which results from the passing of spleen cells and RBC's in front of the laser of the cytoflorograph at precisely the same time. These are registered as rosettes, even though the spleen cells are not binding the antigen coated RBC. Therefore we have an expected cytofluorometric background value in normal mice of approximately 5% (WBC clumps, ABC's and machine background). This predicted background value corresponds well with the actual cytofluorometric response observed to KLH and melanoma extract coated syngeneic RBC's

incubated with normal mononuclear spleen cells (Fig. 1).

We have used acridine orange staining for identification of macrophages and indirect fluorescent staining for identification of T cells and B cells to determine the cellular identification of ABC's in normal or immune spleens. These data indicate that the T cell, B cell or macrophage proportion of background ABC's to a variety of antigens is the same in normal and immune animals (Table 1).

Having established the constant ABC background levels observed with cells collected from normal mice to various control antigen-RBC's, we asked if increases in antigen specific ABC's could be detected in appropriately immunized animals. ABC's were detected in mice injected with either KLH, replicating melanoma cells or the soluble melanoma extract, but no significant ABC responses were observed to these antigens using spleen cells collected from normal animals (Fig. 2). The appearance of antigen specific ABC's observed after antigen injection, and the lack of antigen specific binding by normal mouse spleen cells suggested the specificity of these ABC's. To evaluate further the specificity of these ABC's competitive inhibition assays were performed. Incubation of KLH-ABC's from KLH immunized animals with either media or soluble tumor extract had no significant effect on the magnitude of the KLH-ABC response (Fig. 3). However prior incubation with soluble KLH significantly inhibited KLH antigen binding. In a similar manner preincubation

with media or KLH had no effect on the magnitude of melanoma-ABC's; however, incubation with the soluble melanoma tumor extract significantly inhibited the melanoma-ABC response (Fig.3).

Since antigen specific ABC's to either KLH or tumor antigen were detected it was of interest to establish the kinetics of the ABC response to these antigens. A significant antigen binding cell response to KLH is present as early as 4 days following administration of antigen (Fig.4A). The response reaches a maximum at day 5-6 and then declines by day 10. In a similar manner the ABC response detected after injection of viable melanoma cells is maximum between days 3 to 6, slightly earlier than the KLH response, and again gradually declines until the response is completely lost by day 12 after antigen administration (Fig. 4B). Loss of ABC's after injection of replicating melanoma cells occurs prior to palpable tumor development (day 25). A significant ABC response to the KCl extract of the melanoma tumor is present by day 3 and the ABC response remains at a maximum level for a longer period (7 days) after antigen administration than the ABC response to either KLH or replicating melanoma cells (fig 4C). Moreover 19 days after injection of the soluble tumor extract a significant ABC response is still observed; however, this response is lost by day 25.

In order to determine cell types involved in the antigen specific ABC response seen after administration of either KLH, replicating melanoma cells or soluble tumor extract, morphologic

cell identification of macrophages and indirect fluorescent staining for T and B cells was performed. We established that the predominant ABC types seen after administration of KLH were T and B cells (Fig. 5). Approximately 74% of the KLH antigen specific ABC's were T cells and 22% were B cells. Macrophages were not significantly involved (4%) in this antigen specific response. The ABC response seen after injection of replicating melanoma cells is due exclusively to macrophages and no significant antigen binding T cells or B cells were detected (Fig. 5). Animals injected with the soluble melanoma tumor extract show an increase in antigen specific binding by macrophages, T cells, B cells and non-staining cells. These observed increases over background levels are approximately 20%, 29%, 30% and 21% respectively (Fig. 5).

The involvement of adherent cells in the antigen specific ABC response of spleen cells obtained from animals injected with either KLH, replicating melanoma cells or soluble tumor extract was evaluated using selective cellular depletion techniques. Removing G-10 and carbonyl iron adherent cells from KLH immune spleen cells, and then reconstituting this depleted cell population to the original concentration, enriched for KLH specific ABC's (table 2). This is consistent with cell morphology identification data which suggests macrophages (adherent cells) are not significantly involved in the KLH antigen specific ABC response. In contrast, these same techniques for depletion of adherent cells show a significant reduction in the ABC response seen after administration of either

viable melanoma cells or the soluble tumor extract. These data are again consistent with cell morphology results which suggest that macrophages represent the exclusive ABC type seen after injection with replicating melanoma cells. The ability of these depletion techniques to completely eliminate the antigen specific ABC response seen after injection with the soluble tumor extract does not agree completely with microscopic evaluation which suggests only 20% of these ABC's are macrophages (Fig. 5).

DISCUSSION

The ABC response of splenic lymphocytes observed after immunization of C57Bl/6 mice with either soluble KLH, replicating melanoma cells or a KCl extract of the melanoma tumor was investigated. We have established the kinetics, specificity and cell types involved in the ABC response to these antigens. Quantitation of ABC's was performed using a cytofluorometric procedure as established by Tong and co-workers (20). Determination of the percentage of ABC's found in immunized animals requires a method sensitive enough to detect small changes (approximately 1%) in antigen binding in immune versus non-immune mononuclear spleen cell populations. A variety of techniques, including direct microscopic observation and autoradiography, have been used by others for quantitation of ABC's (1,5,18). The cytofluorograph has the advantage over these techniques in that it can analyze a large number of cells in a short time period. Quantitation of ABC's found in a spleen cell population are routinely based on a sample size of at least 10^4 cells. Since ABC's constitute approximately 1% of our sample, it is important statistically to develop a large enough data base from which to judge the significance of these rosette values detected in immunized animals as compared to control ABC levels seen in normal mice. Comparison of the ability to detect ABC's cytofluorometrically or via direct microscopic evaluation has established a significant correlation ($r=0.92$) between these two techniques (Appendix I).

The present study has established the following points: 1) ABC's, detected after incubation of normal spleen cells with antigen coated RBC's have been identified and quantitated. Macrophages, T cells, B cells and a non-staining population of cells are all involved in this ABC response to control antigens. 2) The percentage of the ABC types detected after incubation of normal spleen cells with control antigen coated RBC's do not differ significantly from those observed after incubation of immune spleen cells incubated with control antigen coated RBC's. 3) The ABC responses seen after injection of either KLH, viable melanoma cells or a KCl extract of the melanoma tumor are antigen specific. Specificity of these ABC's is demonstrated by i) an increased level of the matched ABC's after injection with appropriate antigen and ii) the ability of appropriate free antigen to significantly inhibit the ABC response, while incubation with media or nonrelevant antigen had no effect on the magnitude of the response. 4) The kinetic profiles of the ABC response to KLH and replicating melanoma tumor cells are very similar. The ABC responses reach a maximum level (approximately 1%) between days 5-6 and then decline. Whether ABC's are lost or simply masked at later time points is currently unknown. In contrast, the ABC response seen after administration of soluble tumor extract is similar in magnitude to the response seen with spleen cells from animals injected with either KLH or replicating tumor, however, after injection of the soluble tumor extract the ABC response persists for approximately 7 days longer than do ABC's detected after administration of either KLH or tumor

cells. 5) Evaluation of the ABC type(s) involved in the response to these antigens indicate that a) after administration of KLH, T and B cells are the predominant antigen binding cell types, b) in marked contrast, macrophages represent the exclusive antigen specific ABC type seen after administration of replicating melanoma cells and c) after immunization with soluble tumor extract, T cells, B cells, macrophages and a non-staining cell accounted for the ABC population. The non-staining ABC population detected as part of the background ABC response seen with normal or immune spleen cells to soluble tumor extract apparently are not T cells, B cells or macrophages. These cells may represent natural killer (NK) cells which have been shown to bind and kill a variety of tumor cells without prior immunization.

Depletion of adherent cells by either G-10 or carbonyl iron ingestion, resulted in the loss of the ABC response of spleen cells from animals injected with the tumor extract. Morphologic identification of the cell types involved in this response showed that macrophages represented only 20% of the ABC's, with the remainder of the ABC response consisting of T cells, B cells and a non-staining cell population. The possibility that cells other than macrophages are also depleted by these methods for removal of adherent cells must be considered and may account for this observed discrepency. Alternatively, macrophages may be required for expression of ABC's, perhaps through release of a factor.

The results of this study demonstrate that different cell types are involved in the response to soluble versus replicating antigens. Both T and B-ABC's have appeared in response to particulate and soluble antigens. Merrill and Ashman (13) have demonstrated a shift in T/B cell binding after *in vitro* sensitization of mouse spleen cells with sheep red blood cells (SRBC). Higgins and Choi (6) have detected T-ABC's in response to soluble human gamma globulin (HGG) in bursectomized (B-cell deficient) chickens. The importance of the present study is that it establishes the cellular mechanisms by which animals respond, as evaluated using ABC's, to conventional versus tumor antigens. The fact that C57BL/6 mice respond to a challenge of replicating melamona cells with exclusively macrophage-ABC's, while the ABC response to KLH or soluble tumor extract involves mainly T and B cells, suggests different mechanisms whereby these animals may deal with a tumor challenge versus challenge with a conventional non-replicating antigen *in vivo*. The biological significance of macrophage ABC's in regulation of tumor development remains to be established, however.

Recently Tong and co-workers (19) have evaluated ABC responses in patients with various malignancies, using primarily a patient population with squamous cell carcinoma of the head and neck. The involvement of macrophage-ABC's, which are antigen specific due to adsorbed cytophilic antibody on their surface has been demonstrated. Further ABC analysis of individuals sensitized to

either KLH or PPD has also shown the involvement of macrophages armed with cytophilic antibody. The difference seen in the ABC type responding to conventional antigen, as described by Tong, and in the present study may be due in part to i) different cell sources (human peripheral blood versus mouse spleen) or ii) route of andministration of antigen (dermal versus IP in the mouse).

In the present study, the demonstration that macrophages are the exclusive ABC type observed in response to replicating tumor cells suggests the possible involvement of these cells in regulation of tumor growth. An inverse relationship exists between detection of tumor-ABC's and tumor development. Melanoma specific ABC's are observed prior to detection of palpable tumor developement, suggesting the possibility they may be important in controlling tumor growth when the malignancy is small but are lost when the tumor begins to escape immunological control. Alternatively, the presence of these cells may aid the escape of tumor cells from immune regulation by suppressing tumor immune cell activity. In the accompanying paper we will establish the involvement of macrophage "arming" as the mechanism of antigen specific recognition by melanoma macrophage-ABC's.

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FIGURE 1

Microscopic and cytofluorometric analysis of background values of ABC's to control antigens observed in normal animals.

Microscopic and Cytofluorometric Analysis of Antigen Binding by Normal Mouse Spleen Cells

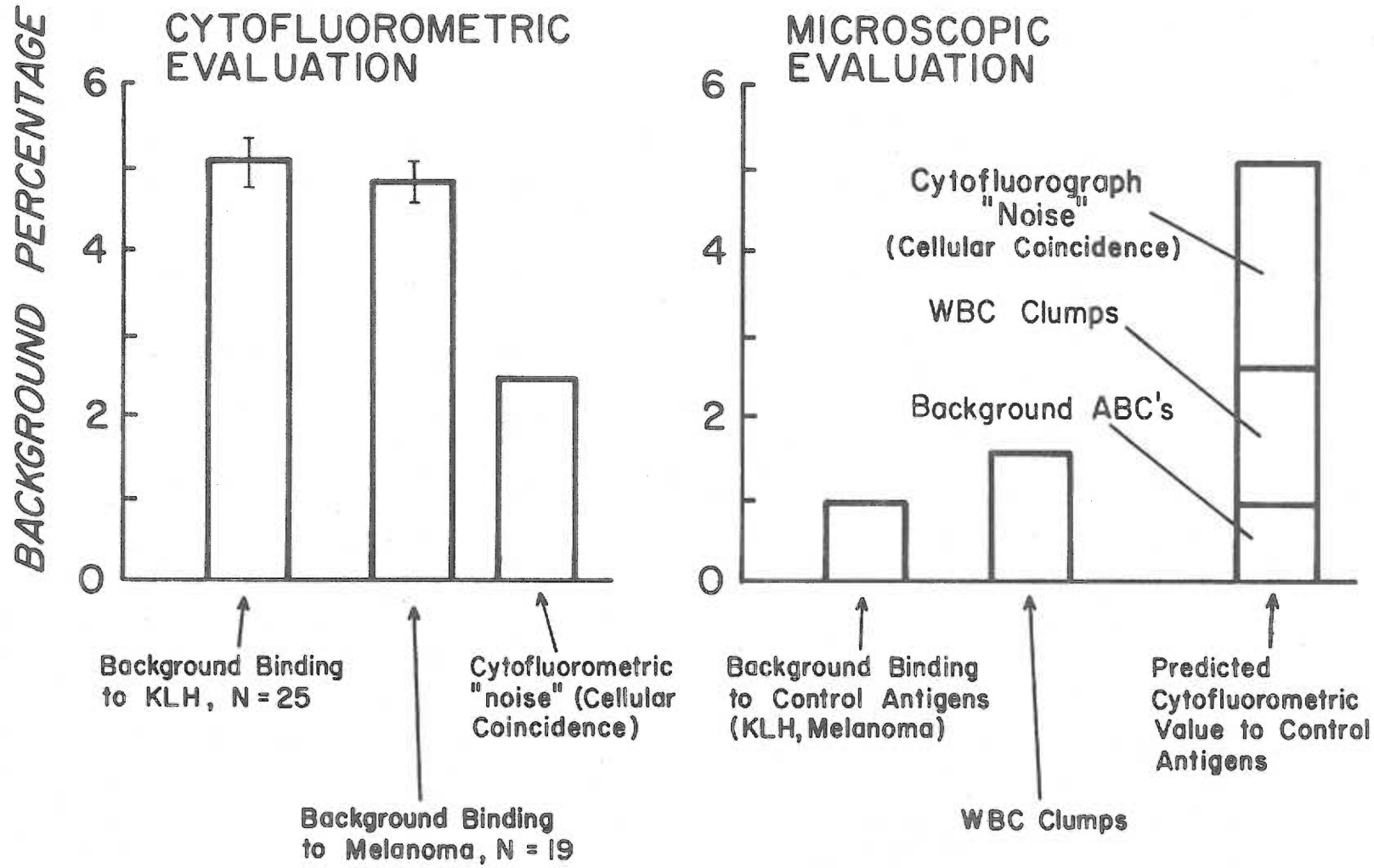


TABLE I

ANALYSIS OF BACKGROUND ABC's TO CONTROL ANTIGEN

CELLULAR COMPOSION OF ABC's

Immunizing ANTIGEN	MACROPHAGE[b]	T-ABC's[c]	B-ABC's[c]	NON-STAINING ABC's
-	2.5% ± 2.9 ($\bar{N}$=4)[a]	32% ± 2.2 ($\bar{N}$=4)	15.9% ± 2.3 ($\bar{N}$=6)	49.6%
+[d]	0.96% ± 2.5 ($\bar{N}$=7)	34% ± 4.6 ($\bar{N}$=4)	17.5% ± 3.5 ($\bar{N}$=5)	47.4%
Average Values	1.5% ± 2.6 ($\bar{N}$=11)	33.3% ± 3.5 ($\bar{N}$=8)	16.7% ± 2.6 ($\bar{N}$=11)	X=48.5%

[a]ABC determined if a nucleated cell binds 3 or more RBC's coated with control or immunizing antigen

[b]Determined by morphologic identification

[c]Determined by indirect fluorescence staining

[d]Antigens included KLH and melanoma tumor

[e]Number of experiments performed. In each experiment 10-50 ABC's were examined

FIGURE 2

Antigen Binding Cell Response of Immune and Non Immune animals.

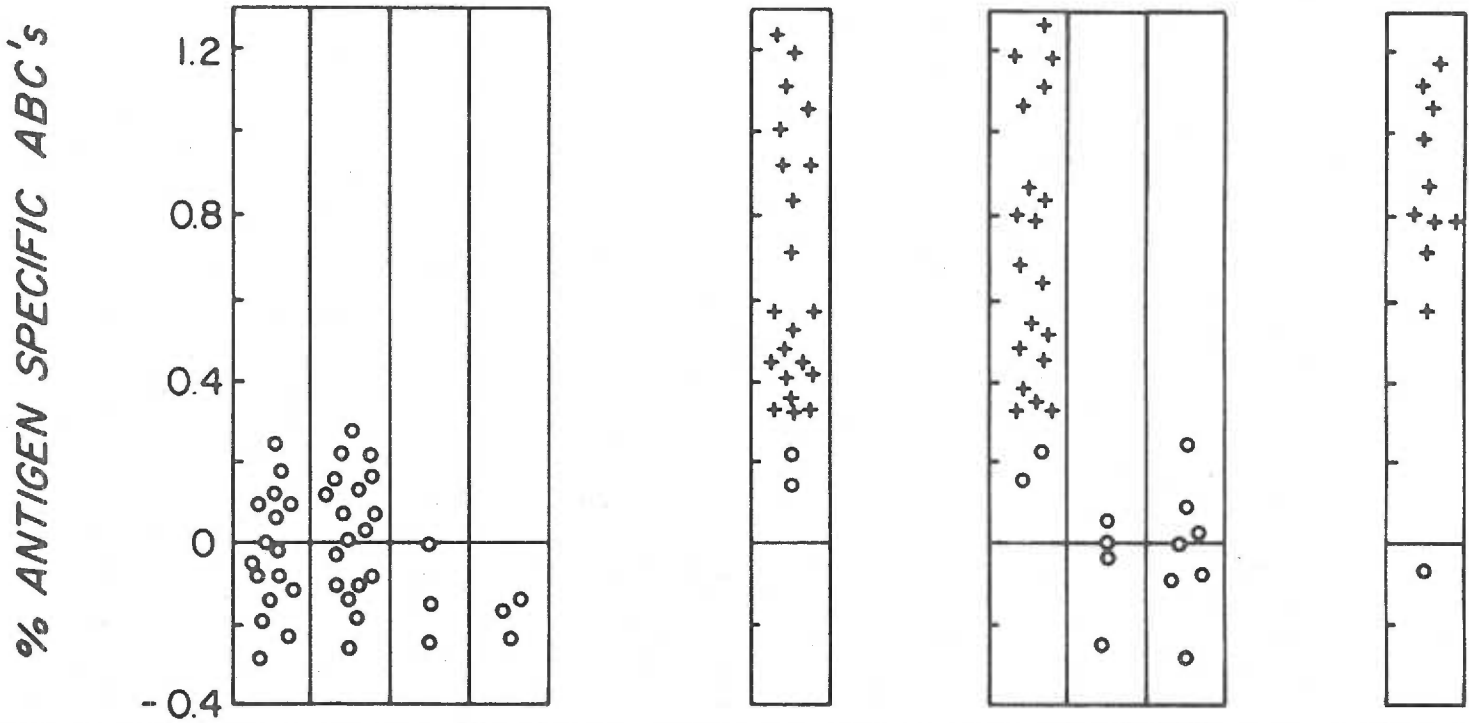
Significance is established at the $p < 0.05$ level, using a Mann-Whitney U statistical evaluation.

+ represents significant values; O represents non-significant values.

Antigen specific ABC's are determined by comparing control Ag-RBC's with Test Ag-RBC's. ABC percent represents the increase in binding of the test Ag-RBC over control Ag-RBC.

$$\% \text{ ABC's} = \frac{(\text{ABC's to test Ag-RBC's}) - (\text{ABC's to control Ag-RBC's})}{\text{Total mononuclear cells}} \times 100$$

Antigen Binding Cell Response of Immune and Non-immune Animals



IMMUNIZATION	none	none	none	none		KLH		Mel	Mel	Mel		T.E.
TEST Ag-RBC	KLH	Mel	EL-4	NMSp		KLH		Mel	EL-4	NMSp		Mel
# POS./# TESTED	0/16	0/19	0/3	0/3		22/24		19/21	0/4	0/7		10/11

o non-significant

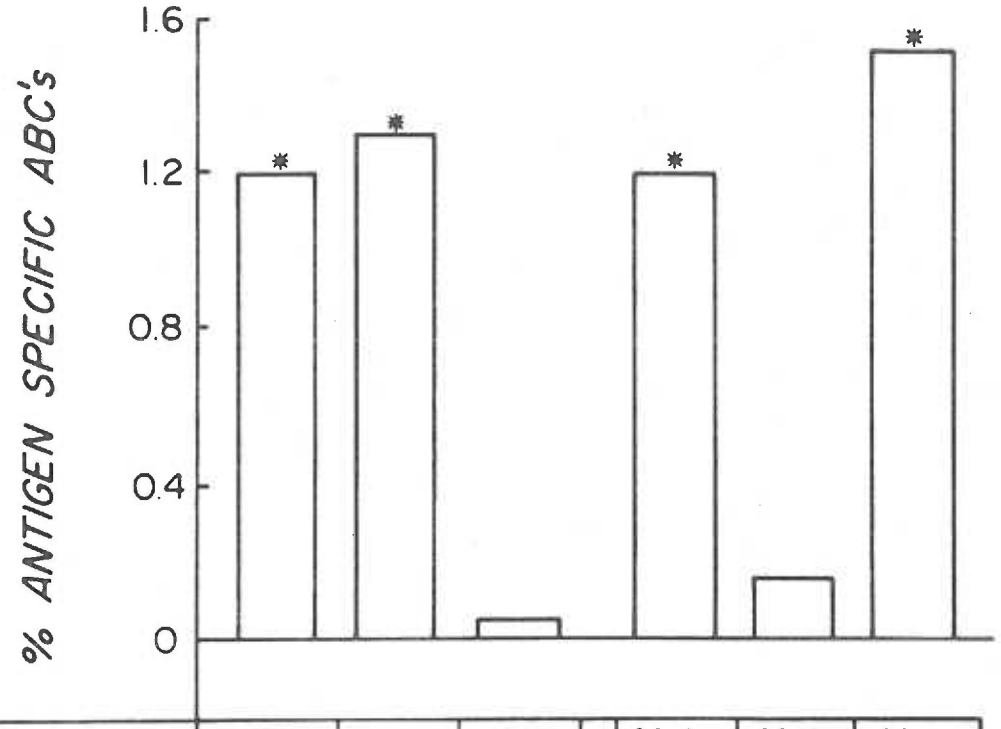
+ significant increase over control ($p < 0.05$)

FIGURE 3

A competitive inhibition assay, using a 2.5 fold excess of soluble antigen, was done to establish the specificity of the antigen binding cell response seen after injection of C57BL/6 mice with either KLH or replicating melanoma cells.

Significance is determined at the $p < 0.05$ level using a Mann-Whitney statistical analysis. This is representative of three experiments.

Competitive Inhibition Assay of ABC's Seen in Immune Animals



IMMUNIZED TO	KLH	KLH	KLH		Mel. Cells	Mel. Cells	Mel. Cells
TEST Ag-RBC	KLH-RBC	KLH-RBC	KLH-RBC		Mel.-RBC	Mel.-RBC	Mel.-RBC
COMPETITIVE INHIBITOR	Media	MSE[a]	KLH		Media	T.E.[b]	KLH

a KCl Extract of Normal Mouse Spleen
b Melanoma Tumor Extract
* Significant Over Control ($p<0.05$)

FIGURE 4

Kinetics of the specific Antigen Binding Cell response after administration of either A) KLH, B) replicating melanoma cells or C) tumor extract

Significance is established at the $p < 0.05$ level using a Mann-Whitney statistical analysis. Data presented is representative of 2 to 5 experiments per time point.

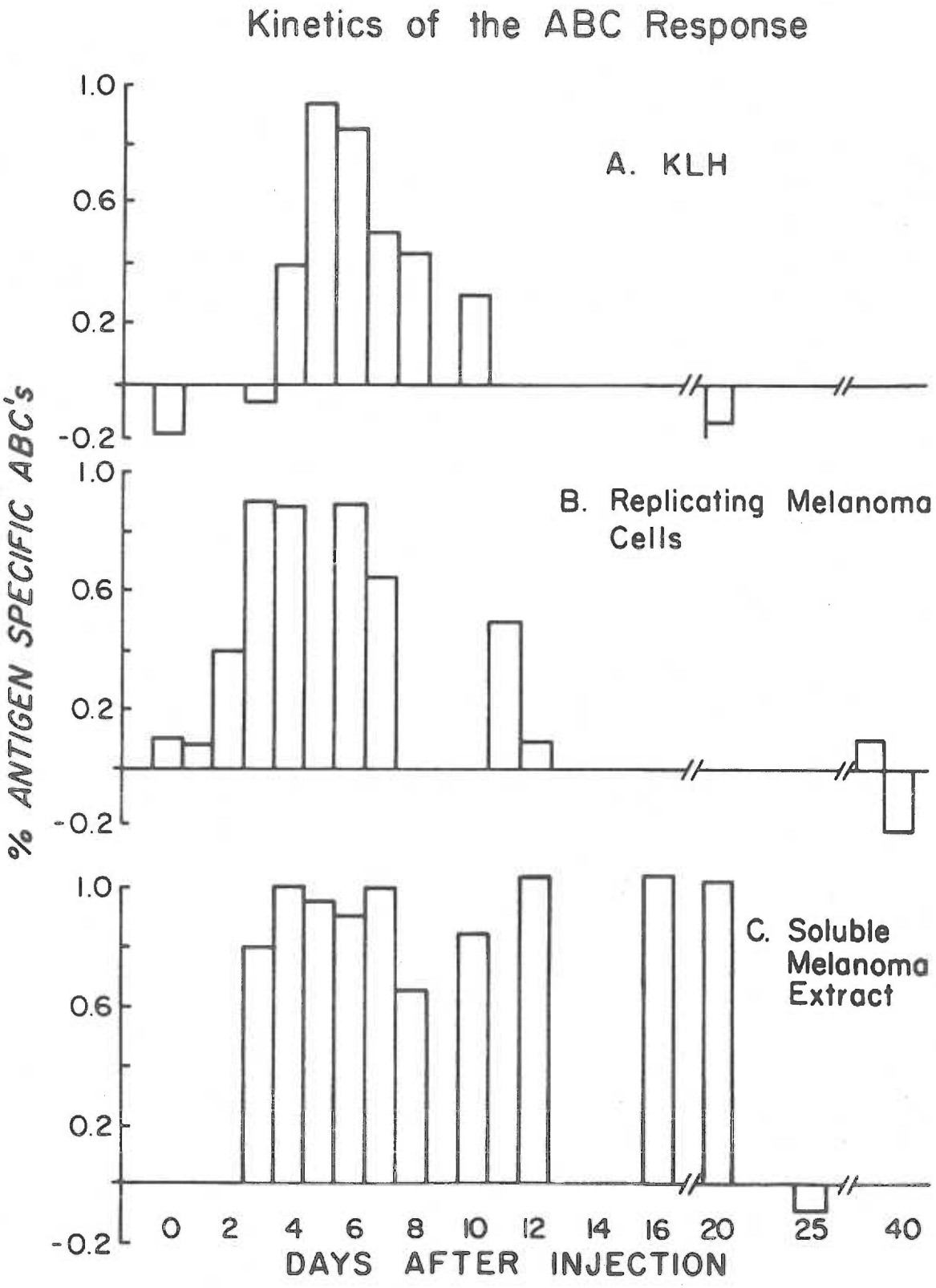

Kinetics of the ABC Response
% ANTIGEN SPECIFIC ABC's
1.0
0.6
0.2
-0.2
A. KLH
B. Replicating Melanoma Cells
C. Soluble Melanoma Extract
0 2 4 6 8 10 12 14 16 20 25 40
DAYS AFTER INJECTION

FIGURE 5

Comparison of T cell, B cell, macrophage and non-staining ABC involvement in the antigen specific binding response of lymphocytes from C57BL/6 mice injected with either KLH, replicating melanoma cells or soluble tumor extract.

Composition of ABC Response

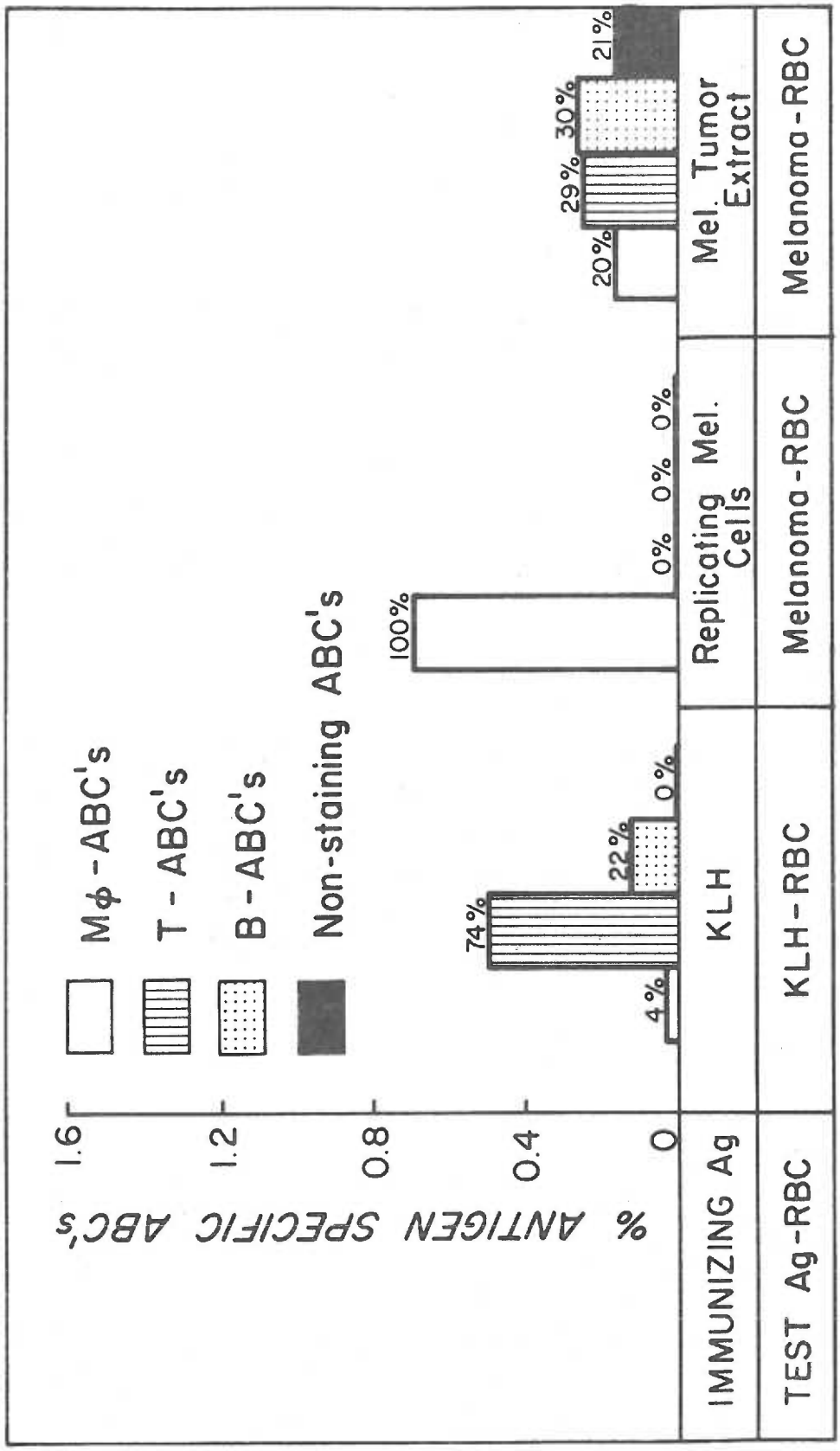


TABLE 2

Effect of Removal of Adherent Cells on ABC's

Antigen Injected	ABC Before Adh.[a] Cell Removal	ABC After Adh. Cell Removal	Method
KLH	0.72%*	1.38%*	G-10
KLH	0.30%*	0.58%*	Carb. Fe
Melanoma Cells	0.65%*	0.09%	G-10
Melanoma Cells	1.08%*	0.10%	Carb. Fe
Melanoma Extract	0.81%*	-0.38%	G-10
Melanoma Extract	0.73%*	-0.08%	Carb. Fe

[a]Adherent cells

*Significant value ($p < 0.05$)

CYTOFLUOROMETRIC EVALUATION OF THE ANTIGEN BINDING CELL RESPONSE TO TUMOR ASSOCIATED ANTIGENS IN THE MOUSE:

II. MECHANISM OF TUMOR ANTIGEN RECOGNITION

ABSTRACT

We have used the antigen binding cell (ABC) assay to evaluate the cellular recognition of tumor antigen by mononuclear cells after injection of C57BL/6 mice with replicating melanoma cells or a soluble KCl extract of the melanoma tumor. We have previously established that the ABC type involved in response to injection of replicating tumor antigen is a macrophage. In addition, macrophages represented 20% of the antigen specific ABC response seen after injection of soluble tumor antigen. In this paper we will show that plasma collected from animals injected with either replicating melanoma cells or the tumor extract is capable of specifically "arming" normal sleen cells to become melanoma-ABC's. Antibody, purified by affinity column chromatography, is responsible for providing antigen specificity to the macrophage. The cell which is being "armed" in the normal spleen cell population is an adherent cell (macrophage). Incubation with an anti-mouse immunoglobulin reagent significantly inhibits the ABC responses seen in animals injected with either replicating melanoma cells or soluble tumor extract, suggesting that recognition of antigen by tumor specific ABC's is mediated by cytophilic antibody.

INTRODUCTION

The immunologically competent host possesses a variety of cells which are potentially capable of tumor destruction. In vitro studies have established the tumoricidal capabilities of T cells (2,3,13,21), natural killer (NK) cells (6,9,15,20), K cells (20) and macrophages (1,7,23). The importance of the macrophage in immune function has been well established: antigen presentation, suppressor cell activity, and tumor killing represent a few of the immunologic capabilities of these cells. The cytotoxic and/or cytostatic effects of macrophages may exert specific or non-specific control over neoplastic cell growth. Specific killing may occur through Fc receptor bound antibody present on the surface of the macrophage or through interaction with immune lymphocytes (1). By the latter mechanism macrophages may become specifically cytotoxic after "arming" by a T cell factor, distict from antibody, termed soluble macrophage activating factor (SMAF) (23). Non-specific tumoricidal activity of macrophages may be induced through the use of adjuvants or after chronic infection.

The ease with which tumors may be induced in vivo suggests the importance of regulatory mechanisms which inhibit the function of cells known to have cytotoxic activity against tumor cells in vitro. Shed tumor antigen (11), blocking antibodies (5) or masking of tumor antigens have all been proposed as potential inhibitors of

in vivo tumor destruction. More recently the involvement of T-suppressor (Ts) cells which are specific for chemically induced tumor antigens have been identified (4,16). Subsequent work has established that a product of these cells, termed T-suppressor cell factor (Ts-F), carries the I-J+ marker and possesses tumor specificity (16). The postulated in vivo function of Ts cells and Ts-F is to interfere with cytotoxic T cell activity.

Klimple and co-workers (10) have demonstrated that BCG-induced macrophage-like suppressor cells are also capable of inhibiting cytotoxic T cell activity. These cells act upon T helper cells which are required for either the development and/or proliferation of cytotoxic T cells.

To evaluate the cellular recognition of antigen by splenic lymphocytes from animals injected with either conventional or tumor antigens we have used the antigen binding cell (ABC) assay. We have previously demonstrated that the predominate ABC types seen in response to administration of keyhole limpet hemocyanin (KLH) or soluble melanoma tumor extract were T and B cells (see accompanying paper) In contrast, macrophages represented the exclusive ABC type seen after injection of replicating melanoma cells. An examination of the ABC response after administration of KLH or melanoma cells demonstrated similar kinetic profiles. Maximum levels of ABC's were detected within 4 to 6 days after antigen administration and then declined. In contrast, the ABC kinetics after administration of the

KCl tumor extract remained at a maximum level for an extended period following antigen administration (through day 19).

In this paper we will examine the mechanism by which macrophage-ABC's found in the spleen of C57BL/6 mice injected with either replicating melanoma cells or soluble tumor extract gain specificity for antigen. Our studies show that antibody from immune plasma, collected from animals injected with either of these antigens, is able to "arm" normal spleen cells to form specific ABC's. Additionally we have established that the predominant cell which is being "armed" in the normal spleen is an adherent cell. The potential importance of these melanoma-ABC's in tumor protection will be discussed.

MATERIAL AND METHODS

Animals: C57BL/6 female mice, obtained from Simonson Labs were used throughout this study. Animals were used between the ages of 6-12 weeks.

2. Description of the Tumor: The P51 melanoma (8) was maintained by *in vitro* transfer every 3 to 4 days. The tumor was grown in RPMI-1640 media containing antibiotic-antimycotic and supplemented with 10% Fetal Calf Serum (FCS). The KCl extraction of the tumor was performed essentially as described by Meltzer (14) and has been described in detail in the accompanying manuscript. The protein concentration of the tumor extract was established using a Bio-Rad assay.

3. Immunization Protocol: C56BL/6 female mice were injected intraperitoneally (IP) with either KLH, viable melanoma cells or a KCl extract of the melanoma. KLH was administered in Incomplete Freunds Adjuvant at a concentration of 100 µg KLH/mouse in 0.2 ml. Viable melanoma cells were administed IP in saline, with each animal receiving $2x10^6$ viable cells in 0.2 mls. Administration of the tumor extract was given IP in saline, with each animal receiving approximately 60 µg of protein in 0.2 mls.

4. Antigen Binding Cell Assay

A. Preparation of RBC's: Red blood cells were collected in heparinized capillary tubes from C57BL/6 mice by rupturing the orbital sinus. The cells were washed twice in RPMI-1640 and resuspended at a 2.5% final (v/v) concentration in TRIS-buffered saline (TBS). Antigen was then coupled to the red cell surface using chromium chloride. Important parameters involved in this procedure have been described in the accompanying manuscript. One ml of $CrCl_3$ (0.01%) was added dropwise to one ml of washed RBC's and one ml of antigen diluted in TBS to the appropriate final concentration (150 µg KLH, 20 µg of melanoma extract). The mixture was left undisturbed for 4-5 minutes at room temperature at which time the cells were washed twice in RPMI-1640 and resuspended to a final concentration of 1% (2.5 ml) in RPMI-1640. KLH was coupled to the red cell surface to detect KLH ABC's, while the KCl extract of the melanoma tumor was conjugated to the red cell surface for identification of rosette forming cells in animals injected with either viable melanoma cells or the soluble tumor extract.

B. Preparation of Spleen Cells: Spleens were removed and minced into single cell suspensions. The cells were treated for 10 minutes at 4C with 0.83% NH_4Cl for lysis of RBC's. After being washed twice in RPMI-1640 the cells were adjusted to a final cell concentration of $1.25X10^7$ cells/ml in RPMI-1640.

C. Preparation of RFC's: Equal volumes (100 µl) of antigen conjugated RBC's and washed spleen cells were combined with 300 µl

cold RPMI-1640. The rosetting mixture was centrifuged at 4C for 5 minutes at 200 x g, and then incubated at 4C for 60 minutes before being evaluated for rosette forming cells.

5. Quantitation of Rosette Forming Cells: Quantitation of RFC's was done using a cytofluorograph (Ortho Instruments model #4208A). The cytofluorograph has been used to quantitate rosette forming cells based upon the parameters of nuclear fluoresence and particle size (light scatter). Essentially, three populations of cells are present in the rosetting mixture; non-immune white cells, antigen coated RBC's and rosetted cells. Addition of the nuclear stain acridine orange to the rosetting mixture allows for distinction of nucleated cell populations, (single cells and rosettes) from antigen coated RBC's in the rosetting mixture. RBC's which lack a nucleus are not stained. Use of the second parameter, particle size, allows discrimination of single cells from the larger rosetted cell population. The RFC response to a specific test antigen is then calculated as:

% SPECIFIC RFC Response = (% Response to test antigen) -
(% Response to control antigen)

Triplicate readings were obtained for each antigen using an average of 10^4 cells per reading

6. Blocking of the ABC Response with Anti-Ig Antisera: Blocking of

the RFC response was assessed using rabbit anti-mouse Ig antisera (Cedarlane Laboritories). Briefly, $1.25X10^7$ cells in a total volume of one ml of RPMI-1640 plus 0.3% Bovine serum albumin were incubated with a 1/10 dilution of mouse anti-Ig. The cells were washed twice and used at the appropriate cell concentrations, as described above, in the ABC assay. This concentration of rabbit anti-mouse Ig, when combined with a 1/10 dilution of fluorescein labelled goat anti-rabbit antisera (Cappel Laboritories) results in the staining of 60-70% of spleen cells and 6% of thymocytes. No staining was noted with the fluorescein labelled reagent alone.

7. Adherent Cell Depletion:

A. G-10 Depletion: Macrophages were depleted using the technique of Ly and Mitchell (12). Briefly, Sepadex G-10 beads (DIFCO) were swollen overnight in RPMI-1640 containing 5% FCS. A 35 cc. syringe was plugged with glass wool and the G-10 column poured to a final volume of approximately 15 ml. Spleen cell populations (4-5 mls at 10^7 cells/ml) free of RBC's were passed over the column. A total of 20 ml. of column eluant was collected and the cell concentration was readjusted to $1.25X10^7$ cells/ml before use in the ABC assay. Generally, 60-70% of the cells were recovered following passage over the G10 column, with 90% macrophage depletion.

B.Carbonyl Iron Adherence: Macrophage depleted cell populations were also obtained using carbonyl iron ingestion. Briefly, $5X10^6$ cells

were incubated with 40 µg of washed carbonyl iron filings for 45 minutes at 37C with rotation. The iron adherent cell population was removed using a magnet. The remaining cells were washed twice in RPMI-1640 and readjusted to the appropriate cell concentration ($1.25X10^7$ cells/ml) for use in the Antigen Binding Cell Assay.

8. Affinity Column Chromatography: Rabbit anti-mouse Ig (Cedarlane Laboratories) was conjugated to cyanogen bromide activated Separose 4B beads (Pharmacia) and plasma collected from either normal or immune animals was passed over the affinity column. Bound material was eluted using 3M sodium thiocyanate. The eluant was dialyzed against saline. The eluted material was antibody as demonstrated by its specific reaction with the Rabbit anti-mouse Ig (Cedarlane Laboratories) reagent in gel diffusion.

9. Statistics: A Mann-Whitney U test was used to evaluate significant differences ($p < 0.05$) in binding of test versus control antigens.

RESULTS

We have established previously by morphologic criteria and adherence properties that macrophages are the exclusive ABC type involved in response to replicating melanoma tumor cells. Macrophages also constitute approximately 20% of the ABC response seen after injection of soluble tumor extract. The specificity of these ABC's has been established by inhibiting the ABC response after incubation with soluble tumor extract but not with media or KLH.

If macrophage-ABC's are recognizing and binding antigen by cytophilic antibody it should then be possible to "arm" normal spleen cells with plasma collected from animals with macrophage ABC's. Indeed, incubation of normal spleen cells with plasma collected on different days after injection of either replicating melanoma cells or the soluble tumor extract was able to "arm" normal spleen cells to become melanoma specific-ABC's (Fig. 1). It is important to note that the immunized plasma donor need not have a significant cellular ABC response in order to have plasma which is capable of arming normal cells. In 5 separate studies, 1 of which is shown in Fig. 1, a positive ABC response was obtained after "arming" of normal spleen cells with plasma obtained from donors which lacked a significant cellular ABC response. Incubation with normal plasma did not result in the formation of antigen specific

rosettes. The "arming" ability of immune plasma was tumor specific in the sense that plasma collected from animals injected with the non-cross reactive thymoma EL-4, was unable to "arm" normal spleen cells to produce a significant melanoma specific ABC response.

The involvement of antibody in providing immune plasma with antigen specificity was evaluated by passing normal and immune plasma over a rabbit anti-mouse Ig affinity column. The bound immunoglobulin was evaluated for its "arming" ability using normal spleen cells. Results from two experiments (Table 1) show that this purified antibody was able to "arm" normal spleen cells for the development of melanoma specific ABC's. The eluant from column passed normal plasma was unable to arm normal spleen cells.

Since plasma collected from immune animals is able to specifically "arm" normal spleen cells to become melanoma specific ABC's, it should then be possible to inhibit this "arming" response by prior incubation of normal spleen cells with normal plasma containing antibodies which are not melanoma specific. Indeed, prior incubation with normal plasma significantly inhibited the "arming" ability of plasma collected from animals injected with either replicating melanoma cells or the tumor extract (Fig. 2).

To establish which cell was being "armed" in the normal mouse spleen population various cellular depletion techniques were employed. Depletion of adherent cells by G-10 passage or carbonyl

iron ingestion prior to "arming" with plasma collected from tumor immunized animals significantly inhibited the ABC responses when compared to non-depleted cell populations (Table 2). These data suggest that an adherent cell, probably a macrophage, is the cell type in the normal spleen which is being armed in vitro with immune plasma.

We have established previously that the ABC response detected after injection of replicating melanoma cells reaches a maximum level between days 5 to 7 and then declines to background level by day 12. In order to establish if the loss of ABC's seen at these late times after injection of replicating melanoma cells was due to a lack of splenic ABC's (macrophages) or was due to in vivo blocking factors, plasma was collected from animals injected with melanoma cells at various times and attempts made to "re-arm" autologous spleen cells (Table 3). Plasma collected from animals injected with replicating melanoma cells is able to "arm" autologous spleen cells to produce a significant ABC response. This "re-arming" ability was not dependent upon the donor animal having a significant cellular ABC response prior to "arming" since animals injected 10 or 13 days prior with replicating melanoma cells lacked significant cellular ABC's but were still able to "arm" autologous spleen cells.

Animals injected with viable tumor cells begin to develop palpable tumors approximately 25 days after injection of cells. During this period of tumor development, there is an increasing

amount of tumor antigen presented to the hosts immune system (17). We wished to to examine plasma collected from animals before and after palpable tumor development for it's ability to "arm" normal spleen cells to become melanoma specific ABC's. Plasma collected from animals as late as 13 days after tumor cell injection was capable of arming normal spleen cells, however plasma collected 25 days after tumor cell injection was unable to provide a significant arming effect (Fig. 3).

Since macrophage-ABC's, detected after injection of either replicating melanoma cells or soluble tumor extract, apparently are binding antigen by cytophilic antibody attached to their surface, it should then be possible to inhibit ABC formation by pre-incubating donor spleen cells with an anti-mouse Ig reagent. Our results indicate that anti-mouse Ig significantly inhibits the ABC response in animals injected with either replicating melanoma cells or the tumor extract as compared to untreated control cells (Fig. 4).

DISCUSSION

In the present study we have examined the mechanism of antigen specific recognition by melanoma-ABC's. Several points have been established: 1) Plasma collected from animals injected with either replicating melanoma cells or soluble tumor extract is able to "arm" normal spleen cells to become melanoma specific ABC's. This "arming" response is melanoma specific since incubation with either normal plasma or plasma collected from animals injected with an antigenically distinct tumor, EL-4, does not result in a significant ABC response. 2) "Arming" of normal spleen cells with plasma collected from animals injected with replicating melanoma cells is not restricted to donors who have a significant splenic ABC response, but can also be seen in animals which lack a significant cellular ABC response. 3) The plasma "arming factor" has been identified as antibody. 4) The cellular population in the normal spleen which is being "armed" after incubation with immune plasma is an adherent cell (macrophage). 5) Prior incubation of normal spleen cells with normal plasma inhibited the subsequent "arming" ability of immune plasma. This result suggests that the factor (antibody) in normal plasma is occupying sites on the adherent cell surface (Fc receptors?) which then inhibits "arming" by immune plasma. 6) Treatment of splenic ABC's from animals injected with either replicating melanoma cells or soluble tumor extract, with an anti-mouse Ig reagent, significantly inhibited the ABC response, suggesting the ABC receptor is surface immunoglobulin. 7) Our

previous data has indicated that the ABC response after injection of replicating melanoma cells reaches a maximum between days 4 to 6 and is then lost after day 12. The question was asked if animals which had lost their cellular ABC response still had "armable" macrophages in their spleens. "Re-arming" experiments, incubating spleen cells with autologous plasma from these animals, demonstrated a significant ABC response. Therefore, even though tumor injected animals lack detectable ABC's in their spleen, their plasma is able to "arm" autologous macrophages. 8) Plasma collected from animals 25 days after injection of viable tumor cells is unable to "arm" normal spleen cells for the generation of an antigen specific ABC response. The possible involvement of soluble or complexed tumor antigen, which is known to be increased in animals with palpable tumors, may be a mechanism to explain the lack of "arming" ability of this plasma.

We have evaluated the cellular recognition of melanoma antigen by splenic lymphocytes from C57BL/6 animals injected with replicating melanoma cells in the hope of better understanding the biological significance, if any, of these melanoma specific ABC's. Tong and co-workers (22) have recently shown the involvement of antibody armed macrophage ABC's in a patient population of individuals with squamous cell carcinoma of the head and neck. However the biological significance of these ABC's obviously could not be established.

Rao and Bonavida (17), have demonstrated in rats that soluble tumor antigen is shed into the serum after injection of a Gross virus associated lymphoma. Maximum levels of the tumor antigen in the serum corresponded with greatest tumor growth. One model which could explain the loss of the ABC's 12 days after melanoma cell injection (Fig. 5) involves the inhibitory effects of soluble tumor antigen. In our model, melanoma specific ABC's, perhaps with tumoricidal capabilities, would be lost due to binding of free tumor antigen by cytophilic antibody on the melanoma-ABC surface or through a T-dependent suppression of the macrophage receptor due to the presence of tumor antigen-antibody immune complexes (18,19). Formation of immune complexes may also reduce the "arming" ability of plasma collected from animals with a developing tumor.

If melanoma-ABC's are indeed involved in tumor protection, it should be possible to demonstrate an effect on tumor development by selectively enhancing for melanoma-ABC's in the tumor challenged animal. Since we know that the melanoma-ABC's are macrophages, it will be possible to "arm" an adherent cell enriched normal spleen cell population <u>in vitro</u> and then establish its effectiveness <u>in vivo</u> in altering tumor development.

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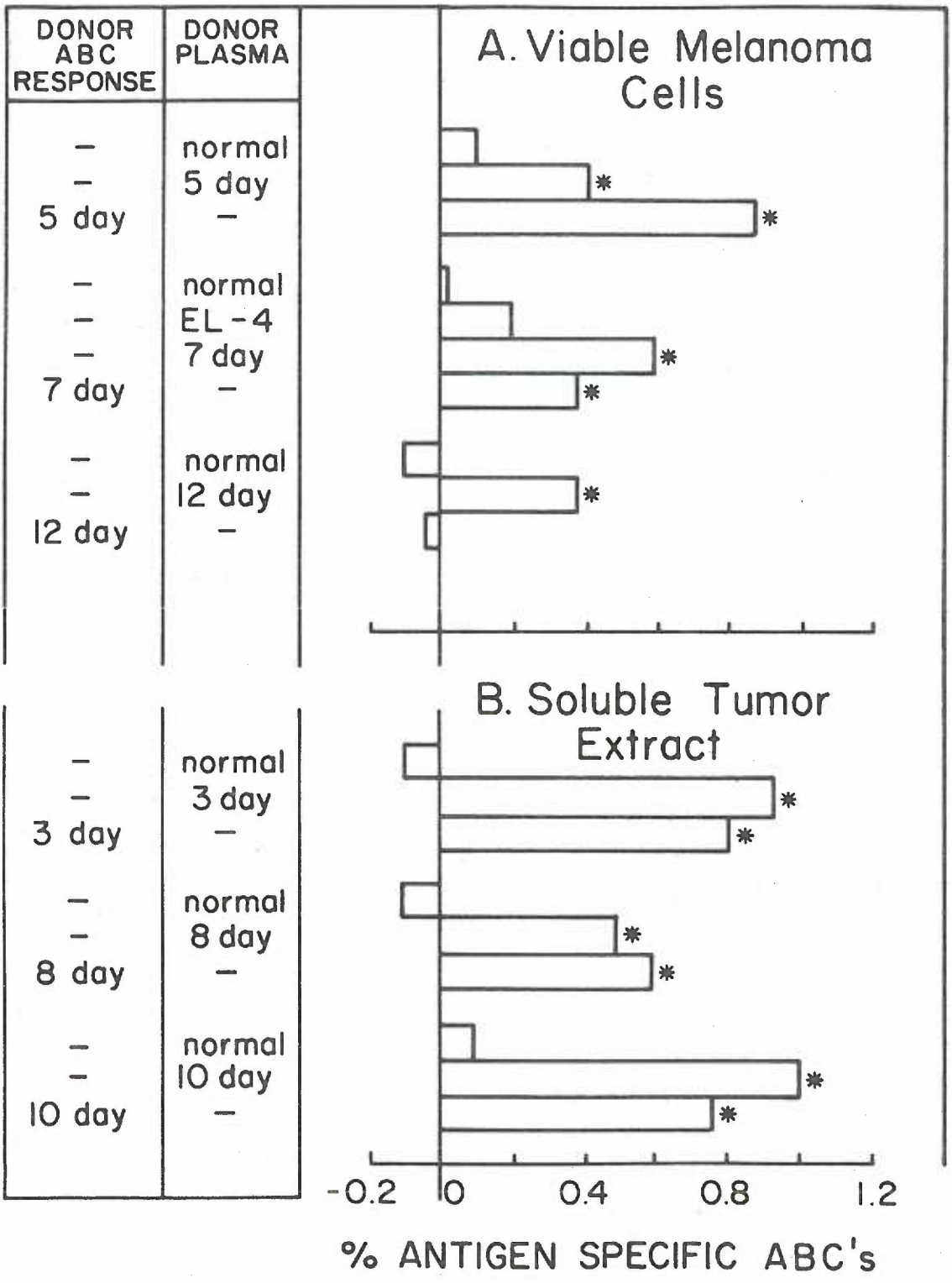
FIGURE 1

Ability of plasma collected from animals injected with either A) replicating melanoma cells or B) tumor extract to "arm" normal spleen cells to become melanoma specific ABC's.

* Melanoma ABC's are significantly elevated ($p < 0.05$) relative to control ABC's.

Each data point is representative of from 2 to 4 separate experiments.

Antigen specific ABC's are determined by comparing the ABC response to the immunizing antigen minus the ABC response to a control antigen.



* Significant Over Control ($p<0.05$)

TABLE I

ARMING OF NORMAL SPLEEN CELLS FROM MELANOMA IMMUNIZED MICE

Source of Antibody	% Antigen Specific ABC	
	EXPERIMENT	
	I	II
Normal Mice	0%	0.25%
P51-Immune Mice	3.0%*	2.0%*

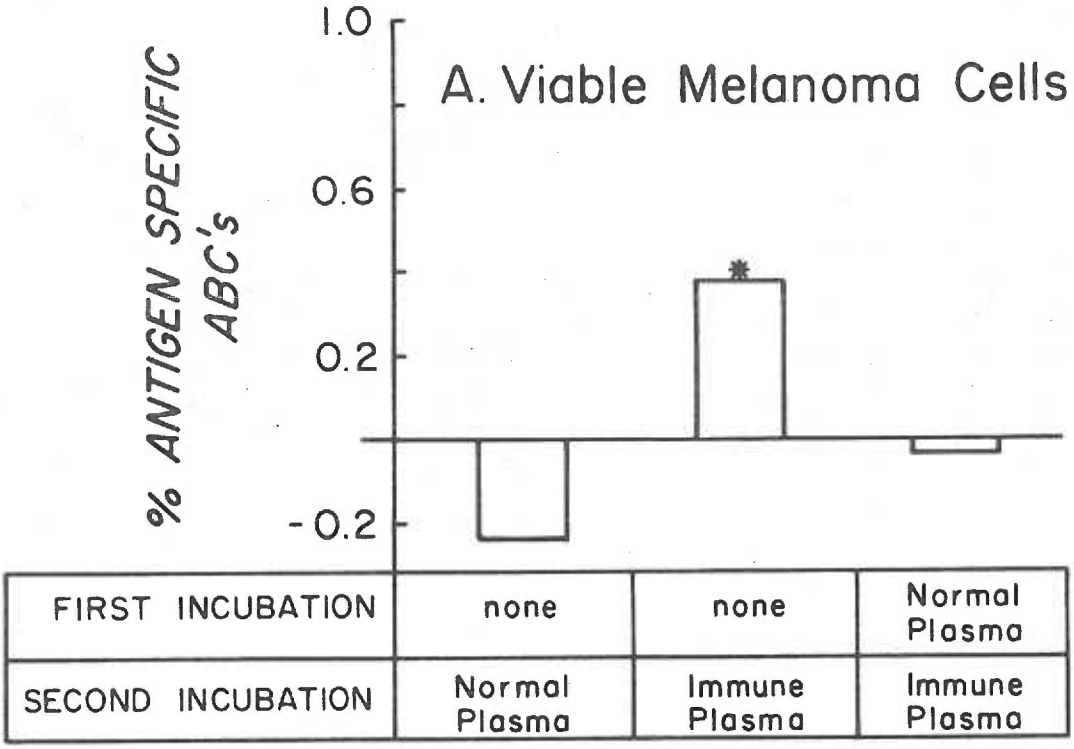
* Indicates significant ($p < 0.05$) ABC response

FIGURE 2

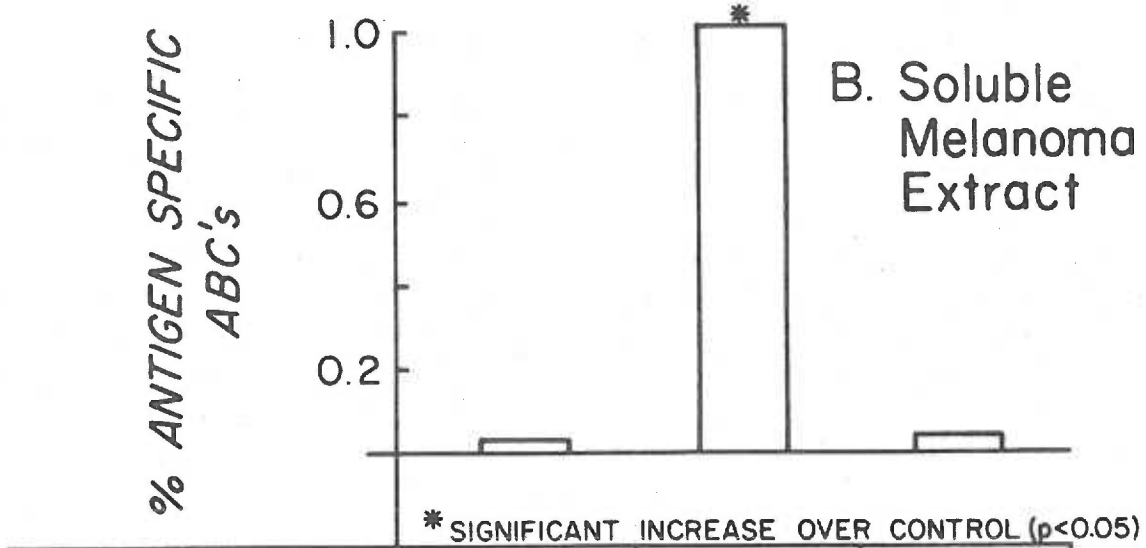
Pre-incubation with normal plasma blocks the "arming" of normal spleen cells with plasma collected from animals injected with either A) replicating melanoma cells or B) tumor extract.

This experiment is representative of 2 separate experiments which gave similar results.

Pre-incubation with Normal Plasma Blocks the "Arming" of Normal Spleen Cells by Immune Plasma.



FIRST INCUBATION	none	none	Normal Plasma
SECOND INCUBATION	Normal Plasma	Immune Plasma	Immune Plasma



* SIGNIFICANT INCREASE OVER CONTROL ($p<0.05$)

FIRST INCUBATION	none	none	Normal Plasma
SECOND INCUBATION	Normal Plasma	Immune Plasma	Immune Plasma

TABLE 2

REMOVAL OF ADHERENT CELLS FROM NORMAL MOUSE SPLEEN CELLS: EFFECT ON "ARMING" WITH IMMUNE PLASMA.

Plasma Collected from donors injected with:	Immune plasma arming (% ABC) of normal mouse spleen cells.		Adherent Cell Depletion Technique
	Untreated	Adh. Cell depleted	
Melanoma Cells	0.34%*	-0.11%	Carbonyl Iron
Melanoma Extract	0.68%*	0.05%	Sephadex G-10
Melanoma Extract	0.96%*	-0.05%	Carbonyl Iron

* Indicates significance at the $p < 0.05$ level.

TABLE 3

ARMING OF SPLEEN CELLS WITH EITHER NORMAL OR AUTOLOGOUS PLASMA

DAYS AFTER INJECTION WITH MEL. CELLS	ARMING (% ABC) NORMAL PLASMA	AUTOLOGOUS PLASMA
7	0.38%*	1.23%*
10	-0.10%	0.71%*
13	-0.12%	0.98%*
38	0.08%	0.23%*[a]

*Significant value above control ($p < 0.05$)
[a]Used 5 day immune plasma rather than autologous plasma.

Figure. 3

ARMING ABILITY OF PLASMA COLLECTED FROM ANIMALS INJECTED WITH REPLICATING MELANOMA TUMOR CELLS

Arming of Normal Spleen Cells with Plasma Collected from Animals Injected with Replicating Melanoma Cells

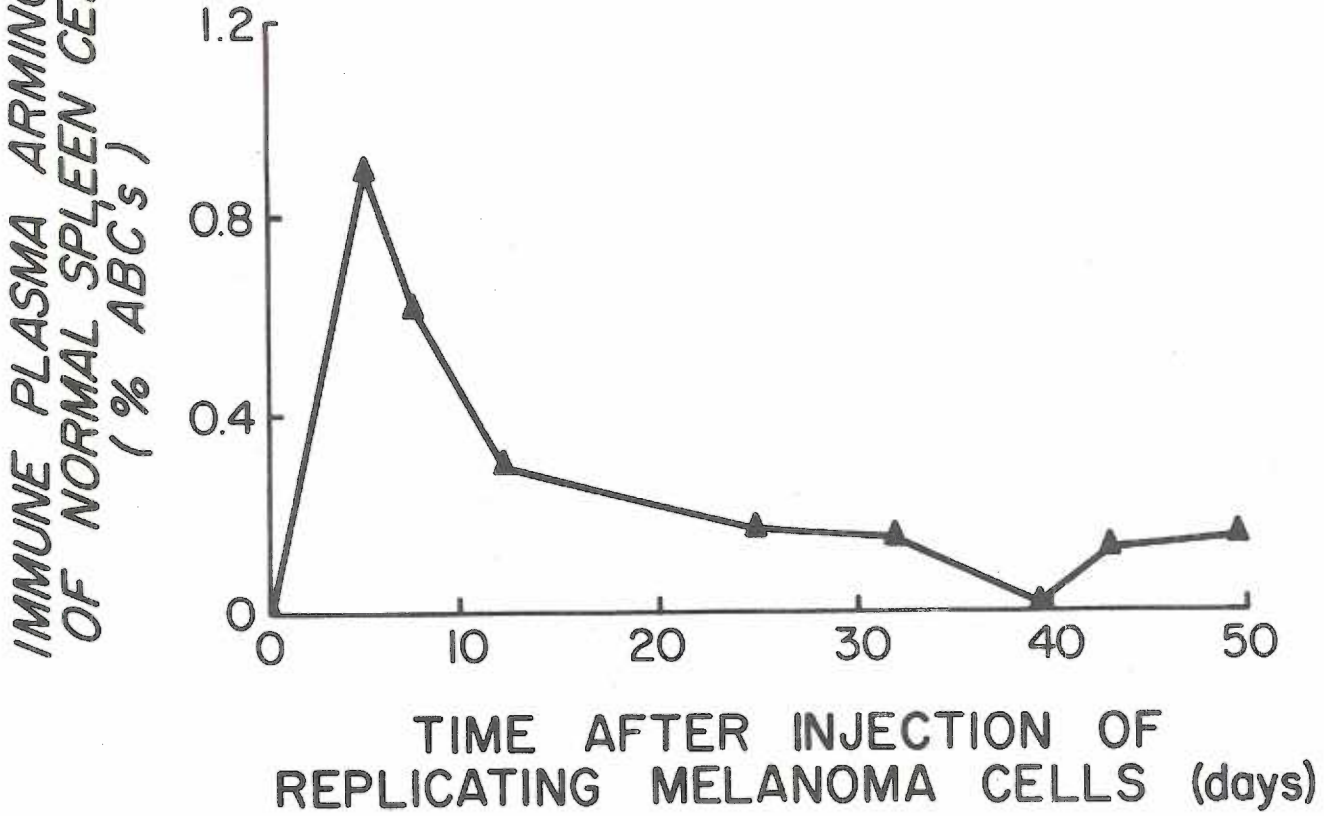
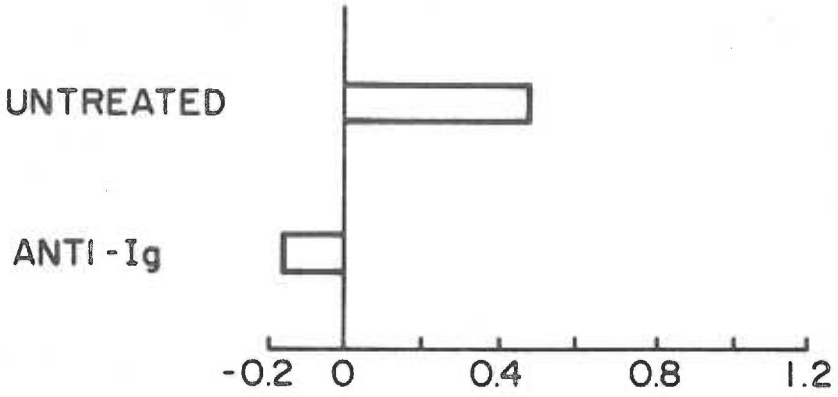


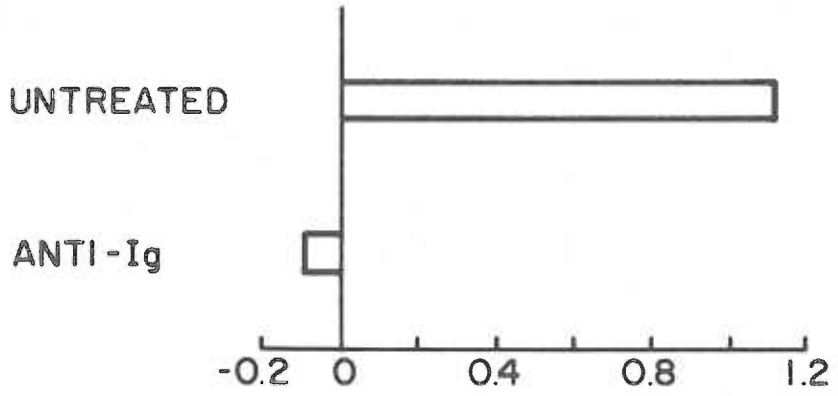
FIGURE 4

Effect of treatment of spleen cells with anti-Ig antisera on the ABC response seen in mice injected with either A) replicating melanoma cells or B) tumor extract.

EFFECT OF TREATMENT WITH ANTI-Ig ON ABC's



EFFECT OF ANTI-Ig TREATMENT ON ABC RESPONSE OF ANIMALS INJECTED WITH REPLICATING MELANOMA CELLS

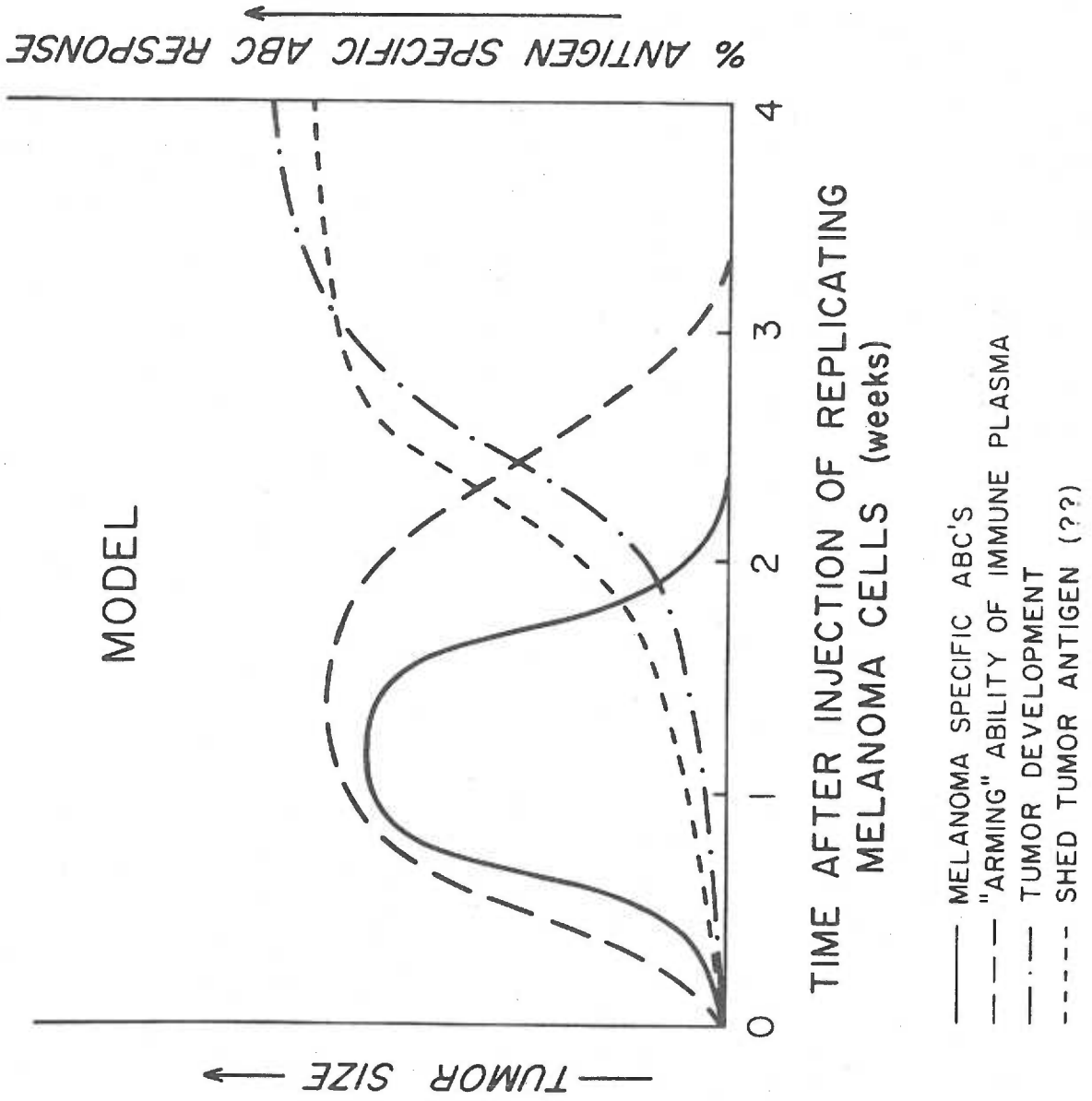


EFFECT OF ANTI-Ig TREATMENT ON ABC RESPONSE OF ANIMALS INJECTED WITH MELANOMA TUMOR EXTRACT

% Ag-SPECIFIC ROSETTES

FIGURE 5

Postulated effect of developing tumor and shed tumor antigen on the ABC response and "arming" ability of plasma collected from animals injected with replicating melanoma cells.


% ANTIGEN SPECIFIC ABC RESPONSE
TUMOR SIZE
MODEL
0
1
2
3
4
TIME AFTER INJECTION OF REPLICATING MELANOMA CELLS (weeks)
MELANOMA SPECIFIC ABC'S
"ARMING" ABILITY OF IMMUNE PLASMA
TUMOR DEVELOPMENT
SHED TUMOR ANTIGEN (??)

ANTIGEN BINDING CELL-RADIOIMMUNOPRECIPITATION (ABC-RIP) ASSAY IN ANALYSIS OF POSSIBLE TUMOR ASSOCIATED ANTIGENS IN MOUSE MELANOMA

Abstract

A novel method has been employed to analyse antigens, including tumor antigens, that are bound by mononuclear cells from immunized mice. Extracts of mouse tumor cell lines (P51 melanoma and EL-4 lymphoma), mouse spleen, mouse plasma and keyhole limpet hemocyanin (KLH), were radiolabelled with ^{125}I and mixed with mononuclear cells from C57BL/6 mice, either non-immune or immune to P51 melanoma or to KLH. After incubation, the cells were washed, the bound antigens extracted from the cell pellet with NP-40 and subjected to polyacrylamide gel electrophoresis under dissociating conditions. Autoradiograms of gels revealed the molecular heterogeneity of the iodinated products bound by the cells. As expected, the results appear to indicate that mice immunized with KLH bind KLH proteins that are not bound by cells from non-immune mice. Mononuclear spleen cells from C57Bl/6 mice immunized with the syngeneic P51 melanoma line bind three distinct proteins in the P51 extract not found in the EL-4 extract. However, non-immune spleen cells also bind these same proteins, although to a much lesser extent. The results suggest that normal C57Bl/6 mice have a pre-existing low level of P51 binding cells which can be increased upon exposure to replicating tumor cells.

INTRODUCTION

We have recently developed a cytofluorometric antigen binding cell (ABC) assay to quantitate cells capable of recognizing and binding to conventional and tumor antigens. Incubation of cells with antigen coated syngeneic RBC's results in the formation of rosettes, immune cells surrounded by a number of antigen coated RBC's (11,12). Specific increases in cells binding tumor antigens have been observed in human tumor patients (11) as well as in mice bearing the syngeneic P51 melanoma (manuscript #1). This method however does not provide information concerning the nature of the antigen(s) being recognized by the responding cells. We have developed further this approach by using radiolabelled tumor cell extracts to ask which of the molecule(s) in the extract are bound by host cells. The data presented here suggest that cells from both normal and immune mice selectively recognize three proteins from the syngeneic P51 melanoma cell line. Moreover, the quantity of "tumor associated antigen" bound to the spleen cells is increased by immunization. This same approach using radiolabelled KLH has demonstrated binding of a specific component by mononuclear spleen cells obtained from KLH immune mice.

MATERIAL AND METHODS:

1. Animals: C57BL/6 mice, aged 6-12 weeks, were obtained from Simonson Laboratories.

2. Tumors: The P51 melanoma and the EL-4 lymphoma are both of C57BL/6 origin. Both cell lines were maintained *in vitro* in RPMI-1640 with 10% fetal calf serum (FCS) and transferred every 3-4 days. The melanoma cells are treated with 0.25 % trypsin in PBS for 10 minutes at 37C to release the cells from the culture flasks prior to use.

3. Tumor cell and control tissue extraction: Cell extractions were performed using a modification of the method as described by Metzler et al. (7). Briefly, approximately 10^8 cells were mixed with 15 ml of hypertonic 4M KCl overnight at 4C and then dialysed extensively first against water and then against saline. After each dialysis the solution was centrifuged at 40,000 x g for 30 minutes at 4C, and the cellular pellets discarded.

4. Keyhole limpet haemocyanin preparation: KLH was prepared as described previously by Vandenbark, and co-workers (13).

5. Immunization: C57BL/6 mice recieved a single intraperitoneal(IP) injection of either $2X10^6$ viable melanoma cells in saline or of KLH (100 µg) in 0.2mls in Incomplete Freunds adjuvant (IFA) 4-8 days

prior to use.

6. Iodine labelling: ^{125}I-Iodine (Amersham, with a specific activity of 13 - 17 mCi/µg) was used for labelling of the tissue extracts and mouse serum. Briefly, 50 µg of protein was mixed with with 400 µl of TRIS-buffered saline (TBS), 10 µl of Chloromine-T (1mg/ml) and 1 mCi ^{125}I-Iodine, and the mixture was incubated on ice for 20-30 minutes. The addition of Chloromine-T allows tyrosine molecules to be labelled with ^{125}I-Iodine by acting as an electron acceptor. The reaction was stopped by the addition of 10 µL of sodium metabisulfate (1mg/ml in distilled water), which acts as an electron donor. Free and protein bound ^{125}I-Iodine were separated using a 5 ml Sepadex G-10 column. One ml fractions were collected from the column using RPMI-1640 with 1% BSA as eluant. The fractions were counted and radiolabelled protein fractions were combined. In general, 2-3 fractions were combined for each labelled protein extract and stored at -70C.

7. Antigen binding cell-radio immunoprecipitation (ABC-RIP) assay: Four to eight days after injection with either P51 melanoma cells or KLH, animals were sacrificed by cervical displacement and spleens removed. Aged matched normal animals were used as controls and treated in an identical fashion. Single cell suspension of the spleens were prepared, washed once for 10 minutes at 200 x g with RPMI-1640 and red blood cells were lysed with NH_4Cl. The cells were washed three times with RPMI-1640 following NH_4Cl treatment,

and cell pellets containing either 10^7 mononuclear spleen cells (antigen binding) or 10^8 mononuclear spleen cells (absorption) were prepared. The cell pellets were immediately suspended in 100 to 200 µl of ^{125}I-Iodine labelled antigens, containing from 25 X10^6 to 50X10^6 cpm, and placed on ice. After various times of incubation, the cell-antigen mixtures were centrifuged for 5 minutes and the supernatants were collected. The cells were then washed three times with RPMI-1640 with 1% BSA and finally with RPMI-1640 alone. To avoid interference of proteins which were nonspecifically bound to the plastic tubes used, cells were transferred to fresh tubes after each centrifugation. The pellets were counted for bound ^{125}I-iodine after the final wash. Membrane bound proteins were extracted from the cell pellets by mixing them thoroughly with 100 to 200 µl of extraction buffer (0.5 % NP-40). These mixtures were kept on ice for 20 minutes, after which time the cell debris was pelleted and the supernatants were counted and saved for further analysis on polyacrylamide gels.

8. SDS-polyacrylamide gel electrophoresis: 40 µl of extract from each of the cell pellets were mixed with 20 µl of sample buffer (3.75% SDS, 3.75% 2-Mercaptoethanol, 35% glycerol and 0.005% Bromophenol blue in 0.15 M TRIS-HCl, pH 6.8) and the mixtures were left at 90C for 10 minutes. The samples were then electrophoresed on a Model 220 vertical slab gel electrophoresis cell (Biorad Laboratories) using a 13% separating gel and 3% stacking gel. The samples were run at constant voltage at 40 V for 16-18 hours and the

gels were dried on a gel dryer (Biorad Laboratories). Autoradiography was performed using high resolution intensifying screens (Kronex) and Kodax X-OMAT XR-5 films. Exposure time was adjusted according to the amount of radioactive labelled protein which had been applied to the gels. Exposure times ranged from 12 hours with 10^5 cpm/sample, to 5 days with 2000-5000 cpm/sample. Molecular weight markers, obtained from Pharmacia Fine Chemicals (Piscataway,New Jersey) were labelled with ^{125}I-Iodine as described above and included in each analysis.

RESULTS

Previous work has shown that the rosette forming cell assay is capable of detecting antigen specific ABC's in animals immunized with either P51 melanoma tumor cells or KLH (manuscript #1). The increase in rosette forming cells documented in mononuclear spleen cells from immunized animals was observed between 4 to 8 days after antigen injection and was antigen specific (Fig. 1). These results indicate that spleen cells from immune mice bind distinct molecules in the KLH and tumor cell preparations and that this reaction might be immunologically specific.

In order to analyse further the specificity of the antigen binding response, mononuclear spleen cells from control and P51 immunized mice were mixed with radiolabelled extracts from either P51 melanoma cells, EL-4 tumor cells, normal spleen cells or normal mouse plasma. After 15, 60 or 120 minutes incubation on ice, the cpm bound to the cell pellets were determined. Results depicted in Fig. 2 show that a substantial amount of radiolabelled antigens (0.2 % of spleen cell extract, 0.7 % of P-51 melanoma extract, 2.0 % of EL-4 thymoma extract and 3.5 % of normal mouse plasma) were bound to the cells after 15 minutes incubation. Moreover the binding increased during the two hour incubation period. It is important to note that spleen cells derived from the P51 immune mice bound almost twice as many counts as did spleen cells from normal mice after 1 hour incubation. In 3 of 6 subsequent experiments, an increase in

binding of the P51 extract by P51 immune cells as compared to normal cells was observed. It seemed possible that a high background binding of the P51 extract to normal cells might preclude routine discrimination of specific binding. Adsorption experiments were designed to circumvent this difficulty. The P51 extract was absorbed with (10^8) normal or immune spleen cells prior to use. A representative experiment (Table 1) demonstrated that about half of the counts bound to 10^7 cells could be absorbed with 10^8 cells regardless of whether the cells used for absorption were derived from immune or control mice. The slight increase (10 - 15 %) in counts selectively bound by immune cells both with non-absorbed extract as well as with extract absorbed with normal, but not with immune cells, suggests preferential binding of some of the components in the extract to immune cells.

Treatment of the cell pellets for 20 minutes on ice with the extraction buffer released about 50% of of the radiolabelled antigens (Table 1). These extracted antigens were then applied to SDS-polyacrylamide gels, electrophoresed and autoradiograms produced. Three distinct bands, representing molecules with apparent molecular weights of 15,000, 17,000 and 33,000 daltons were seen in the extraction of cell pellets treated with non-absorbed tumor-cell extract (Fig. 3). These bands are clearly weaker in extractions from cell pellets treated with absorbed tumor cell extract. No clear difference was seen in the intensity of the bands bound by normal or immune cells and immune cells do not bind

molecules not bound by control cells.

As may be seen in Fig. 3, the original, untreated tumor extract contains a large number of bands of various molecular sizes in addition to the three which seem to be selectively bound to spleen cells. Longer exposure of the gel in Fig. 3 demonstrated, however, that all these bands could be found in the treated preparations, although their concentration was far lower than that of the three selectively bound molecules. Further studies showed that the presence of all the components in the melanoma extract seen after incubation with spleen cells resulted from non-specific binding to the polystyrene tubes used in these experiments. In subsequent experiments, cells were always immediately transferred into fresh tubes after each cell wash. This reduced the amount of counts bound to approximately half, and increased the relative amount of counts bound to immune cells versus control cells (Table 2).

In an attempt to clarify further the question of specificity, experiments were carried out with mice immunized either with replicating P51 melanoma cells or with KLH in IFA. The conventional cytofluorometric ABC assay demonstrated a specific increase in both KLH and P51 ABC's (Table 3). Using the ABC-RIP assay (Table 4), similar increases in counts bound by immune versus control cells were observed. SDS-PAGE autoradiograms (Fig. 4) of binding to the melanoma tumor extract again showed the presence of the three molecular species of approximately 15,000, 17,000 and 33,000

daltons, although in these experiments the largest of the three molecular bands (33,000 dalton), was indistinct and dispersed. The intensity of the bands corresponded well with the counts bound and extracted from the pellets, the strongest binding occurring with P51 immune cells and the weakest, in fact, with KLH immune cells. Cellular binding to the KLH preparation (Fig. 5) demonstrated several bands, and the KLH immune cell extraction appeared to have at least one band with an apparent molecular weight of approximately 100,000 daltons, which is not found in control or P51 immune cell extraction.

The SDS-PAGE results indicate that in the P51 system, spleen cells from immunized mice bind selective components in the P51 tumor extract, this binding being present, although weaker in normal cells. In the KLH system however, the immune cells bind component(s) in KLH which appear not to be recognized by normal cells.

DISCUSSION

In the present study we have examined the physiochemical nature of the molecules in tumor cell preparations recognized by normal and immune host cells. Extensive work in many laboratories has been carried out in order to define structures specific for tumor cells both in human and animal tumor models (1,3,5). Most of these studies have started from the assumption that malignant cells express structures not present on normal cells. Consequently one of the approaches used for detection of these tumor associated antigens (TAA) has used either xenogeneic antisera (rendered tumor specific by absorptions) or monospecific hybridoma antibodies which react only with the appropriate tumor cell (10).

The approach taken in the present study was based on our previous findings that an immune response in hosts towards a tumor could be monitored using the antigen binding cell assay. In this technique either mouse spleen cells or human peripheral blood mononuclear cells are mixed with autologous erythrocytes chemically coated with tumor antigen or nonrelevant control antigens. These antigen specific ABC's are detected and quantitated using a cytofluorometric procedure as described by Tong, and co-workers (12). These same authors have shown that patients with primary squamous cell carcinoma of the head and neck have an increase in antigen specific rosettes whereas patients with recurrent disease do not (11).

In several recent studies, radioimmunoprecipitation assays(RIP) have been employed in the study of tumor associated antigens. In these RIP assays, tumor cell membrane proteins are labelled with radioactive iodine ^{125}I, and the TAA is precipitated with a monospecific antibody, usually one generated using hybridoma technology (6,10). The complex formed by antibody and tumor antigen is selectively separated from the reaction mixture either with a secondary antibody or with Staphylococcus aureus, protein A, which will bind to the Fc portion of antibody. In the present study we have used a similar approach to separate specific molecules within the tumor cell extract. We have used immune spleen cells to bind the putative TAA from the KCl tumor extract. There is a fundamental difference between the RIP assay and the presently used method, since in radio-immunoprecipitation, the specific antibodies will separate molecules which are unique for the tumor but not necessarily connected with the defence mechanism of the host. In the ABC-RIP assay, the separated molecules are recognized and bound by host cells but are not necessarily unique for the tumor cells.

The results obtained in the present study show that antigen binding cells can be used effectively to characterize and possibly purify molecules present in the heterogenous tumor cell extracts towards which the host cells are reacting. A striking feature in the present study was the selectivity with which the host cells reacted with only a few molecules in the tumor extract. Only about 0.5-1.0 % of the radiolabelled antigens mixed with host spleen cells

were bound to 10^7 cells. On the other hand, absorption studies indicated that almost all of the the reacting antigens were removed when the amount of cells was increased. Therefore antigen binding cells do bind preferentially with the relevant molecules. Absorption studies using red blood cells have demonstrated no binding of components from either the KLH or melanoma tumor preparations. The binding of the 3 components from the tumor extract by either normal or melanoma immune spleen cells is not due to properties of these components which would cause them to stick to any cell type. The nonspecific binding in the system may be due to antigen binding by other cells in the suspension and/or binding to the plastic tubes. It is noteworthy, however, that binding of protein to the tube was totally nonselective, the molecules in the tumor cell extract binding relatively equally.

Although there seems to be a strong selectivity in the reaction of antigen binding cells with the tumor antigen mixture, differences in immunological specificity were observed between binding of a syngeneic tumor cell extract and KLH. KLH immune spleen cells, but not normal cells, reacted with at least one molecule from the antigen preparation. This was expected, as it seemed likely that non-immune animals would not have detectable levels of cells capable of recognizing KLH. It is notewortly however, that with KLH, in addition to binding of a specific band by immune cells, both immune and non-immune cells bound several other molecules in the KLH mixture. The reaction towards the P51 extract was similar in that

spleen cells from both immune and non-immune animals were capable of binding three proteins selectively. With KLH, as well as P51, the binding was stronger with immune spleen cells than with non-immune cells. Therefore the difference in the immune and nonimmune cell binding to some KLH components as well as to P51 may only be quantitative. In the P51 system, antigen reacting only with immune cells may exist, although below detectable levels.

The relationship of the molecules in P51 which can be characterized with the ABC-RIP assay to molecules which might be unique to the tumor cells is unknown. It is of interest that the three protein bands could not be demonstrated in a normal mouse spleen cell extract nor in the syngeneic control tumor line EL-4. It is of interest however that the estimated molecular weights are close, but not identical, with proteins associated to the mouse major histocompatibility complex (MHC). Hypothetically, therefore, it would be possible that the molecules characterized here would represent altered MHC gene products (1,5,8).

The cell type(s) operating in the ABC-RIP assay is unknown. In the ABC assay, depletion of adherent cells will abolish ABC's against P51 but not against KLH. Also, reaction to the P51 extract can be transferred from immune to non-immune mice with immune antibody. It has been proposed that the major cell type reacting with P51 in the ABC assay is a macrophage armed with cytophilic tumor associated antibody. In the ABC-RIP assay, the finding that

both immune and non-immune cells could recognize three proteins in the P51 extract would be consistent with the presence of natural killer (NK) cells since this cell demonstrates tumor reactivity without prior immunization (2,4,9). The results from criss-cross experiments, with P51 and KLH immune mice, however, are more compatible with the findings from the ABC assay. Thus present results suggest that low levels of antibody capable of recognizing some molecules on syngeneic tumor cell membrane preexists in non-immune mice. Injection of mice with tumor cells results in an increase in the reacting cell population.

In conclusion, several questions, including the reacting cell type and the mechanism of binding remain to be answered with the ABC-RIP assay. It is important to notice, however, that the method outlined here presents ample possibilities to analyse and characterize molecules in malignant cells towards which the host cell is reacting. It is possible that the antigens in the tumor cell extracts that are characterized by their reaction with host cells may be more important than the antigens characterized with heteroantisera.

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FIGURE 1

Antigen Binding Cell Response of Animals Immune or Nonimmune to KLH or Melanoma Tumor

Antigen Binding Cell Response of Immune and Non-immune Animals

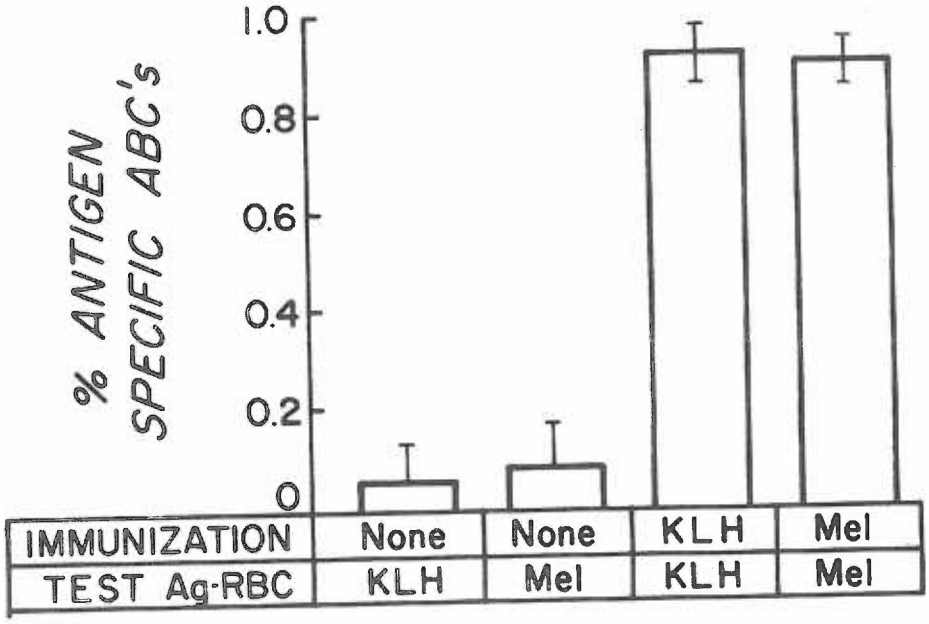


FIGURE 2

Radiolabelled Antigen Bound by Immune and Nonimmune Cells

×—× Nonimmune spleen cells

Δ—Δ Melanoma immune spleen cells

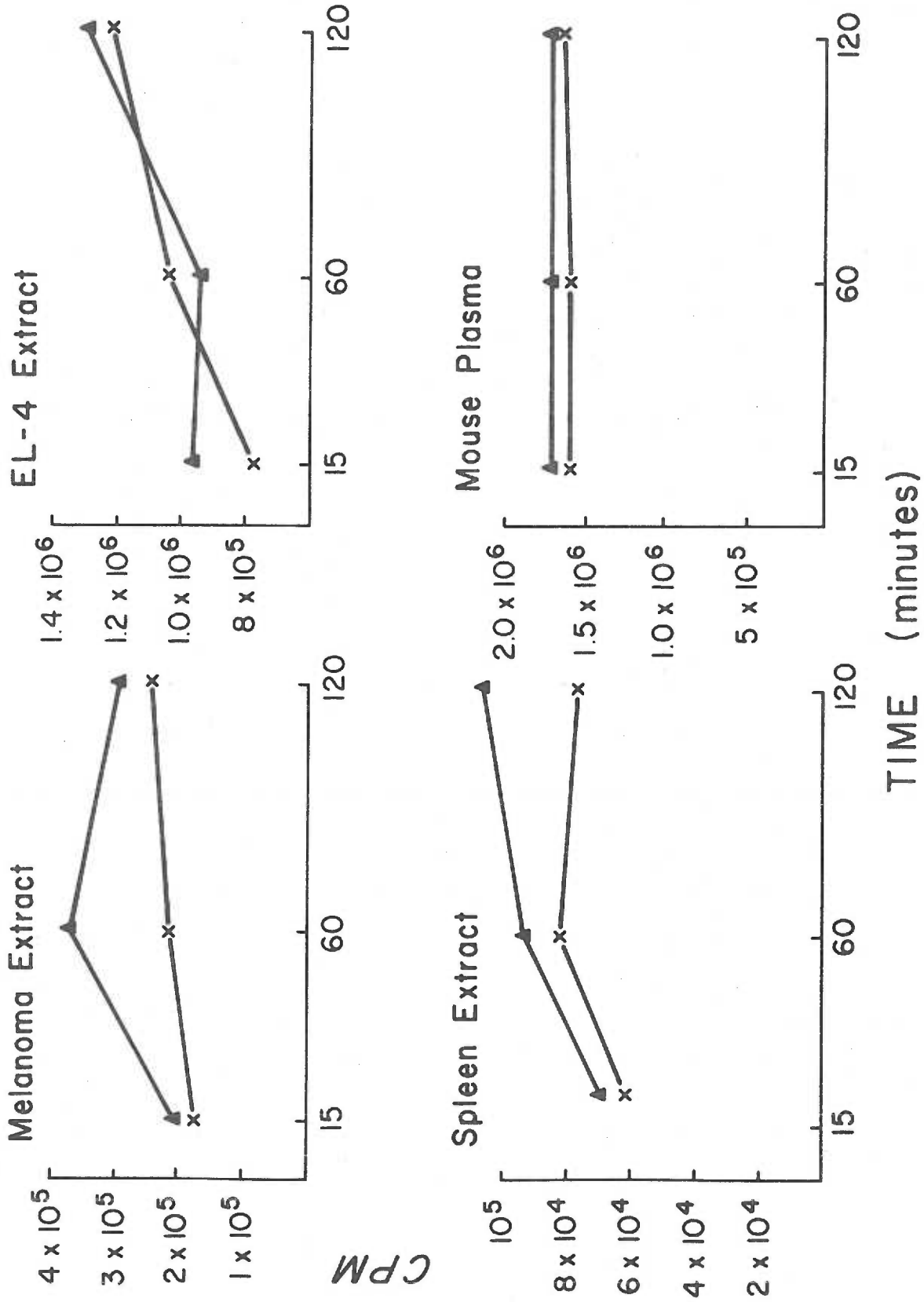

Melanoma Extract
EL-4 Extract
Spleen Extract
Mouse Plasma
CPM
TIME (minutes)
4 x 10^5
3 x 10^5
2 x 10^5
1 x 10^5
1.4 x 10^6
1.2 x 10^6
1.0 x 10^6
8 x 10^5
2.0 x 10^6
1.5 x 10^6
1.0 x 10^6
5 x 10^5
10^5
8 x 10^4
6 x 10^4
4 x 10^4
2 x 10^4
15
60
120

TABLE 1

^{125}I-labelled P-51 melanoma extract bound and extracted from normal or P-51 immune[a] mouse spleen cells

Number of cells	Status of cell donor	Time of incubation	Absorption	Amount bound to cells cpm	%	Amount extracted increase cpm	increase
10^8	normal	60	no	2,977,700		1,095,000	
10^8	P-51 immune	60	no	2,436,350	- 18 %	773,520	-29%
10^7	normal	60	no	442,200		151,190	
10^7	P-51 immune	60	no	484,600	+ 10 %	164,590	+9%
10^7	normal	60	with P-51 immune cells	217,730		78,400	
10^7	P-51 immune	60	with P-51 immune cells	203,730	- 6 %	66,060	-15%
10^7	normal	60	with normal cells	212,090		65,630	
10^7	P-51 immune	60	with normal cells	243,880	+ 15 %	72,810	+11%

[a]Spleen cells harvested 4-6 days after immunization with replicating melanoma cells

FIGURE 3

SDS-Polyacrylamide gel of ^{125}I-Labelled Melanoma Tumor Extract Bound by Normal and Tumor Immune Spleen Cells

Channel 1: Molecular markers (MW X 10^{-3})

2: Tumor immune spleen cells (10^7) binding to melanoma tumor antigen

3: Normal spleen cells (10^7) binding to melanoma tumor antigen

4: Tumor immune spleen cells binding to melanoma tumor antigen, absorbed with tumor immune spleen cells

5: Normal spleen cell binding to melanoma tumor antigen absorbed with tumor immune spleen cells

6: Tumor immune spleen cell binding to melanoma tumor antigen absorbed with normal spleen cells

7: Normal spleen cell binding to melanoma tumor antigen absorbed with normal spleen cells

8: Tumor immune spleen cell(10^8) binding to melanoma tumor antigen

9: Normal spleen cell(10^8) binding to the melanoma tumor antigen

10: Melanoma tumor antigen extract

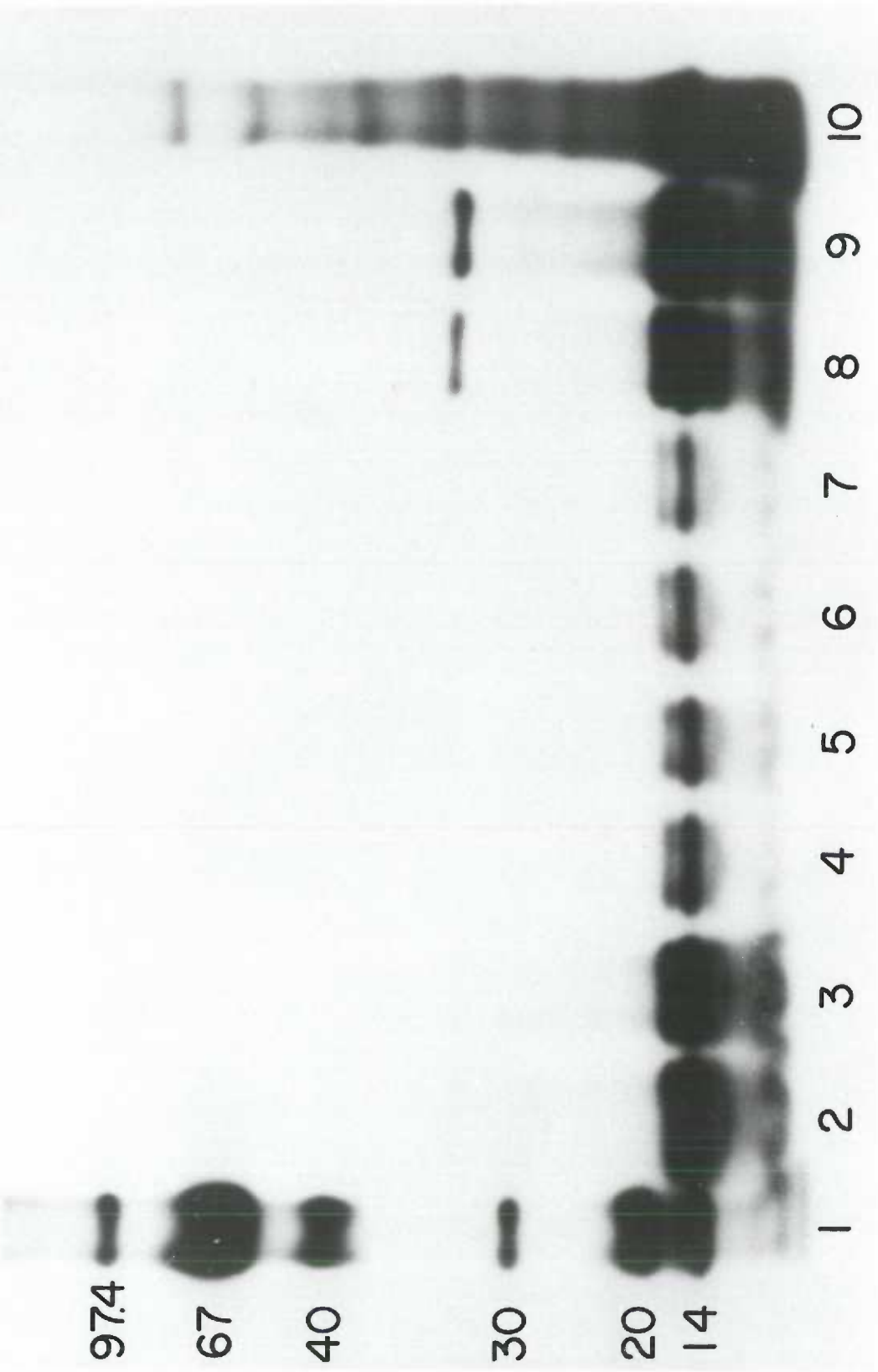

1
2
3
4
5
6
7
8
9
10
97.4
67
40
30
20
14

TABLE 2

^{125}I-labelled P-51 melanoma extract bound to and extracted from normal and P-51 immune mouse spleen cells

Cell No.	Donor Status	Time	Absorption	CPM Bound	Increase	Extracted	Increase
10^7	normal	15	no	220,500		71,640	
10^7	P-51 immune	15	no	291,670	+ 32 %	97,730	+ 36 %
10^7	normal	60	no	368,240		121,720	
10^7	P-51	60	no	511,470	+ 46 %	167,840	+ 38 %
10^7	normal	60	no	384,610		109,020	
10^7	P-51 immune	60	no	535,130	+ 39 %	160,260	+ 47 %

TABLE 3

CYTOFLUOROMETRIC EVALUATION OF ABC's

EXPERIMENT	IMMUNIZATION	% ANTIGEN SPECIFIC ABC's
#1	None	-0.18%
	KLH	1.04%*
	P51	0.43%*
#2	None	-0.05%
	KLH	0.86%*
	P51	0.87%*

* Significant $p < 0.05$ level

TABLE 4

^{125}I-Labelled Tumor Antigen or KLH Bound and Extracted From Normal and Immune Spleen Cells

Experiment 1:

Donor Status	Antigen	CPM Bound	Increase	CPM Extracted	Increase
Normal	P51	368,240		121,720	
P51 Immun.	P51	511,470	38%	167,840	38%
KLH Immun.	P51	421, 510	14%	155,580	28%
Normal	KLH	84,290		31,110	
P51 Immun.	KLH	94,360	10%	36,810	18%
KLH Immun.	KLH	108,430	27%	49,580	59%

Experiment 2:

Normal	P51	556,010		150,611	
P51 Immun.	P51	808,900	46%	204,775	36%
KLH Immun.	P51	498,525	-12%	184,620	18%
Normal	KLH	73,455		27,165	
P51 Immun.	KLH	98,070	33%	25,085	-8%
KLH Immun.	KLH	105,440	44%	49,755	83%

FIGURE 4

SDS-Polyacrylamide Gel of ^{125}I-Labelled Melanoma Tumor Extract Bound by Normal and Tumor Immune Spleen Cells

Channel 1 and 4: Binding of the melanoma tumor extract by normal spleen cells

2 and 5: Binding of the melanoma tumor extract by tumor immune cells

3 and 6: Binding of the melanoma tumor extract by KLH immune spleen cells

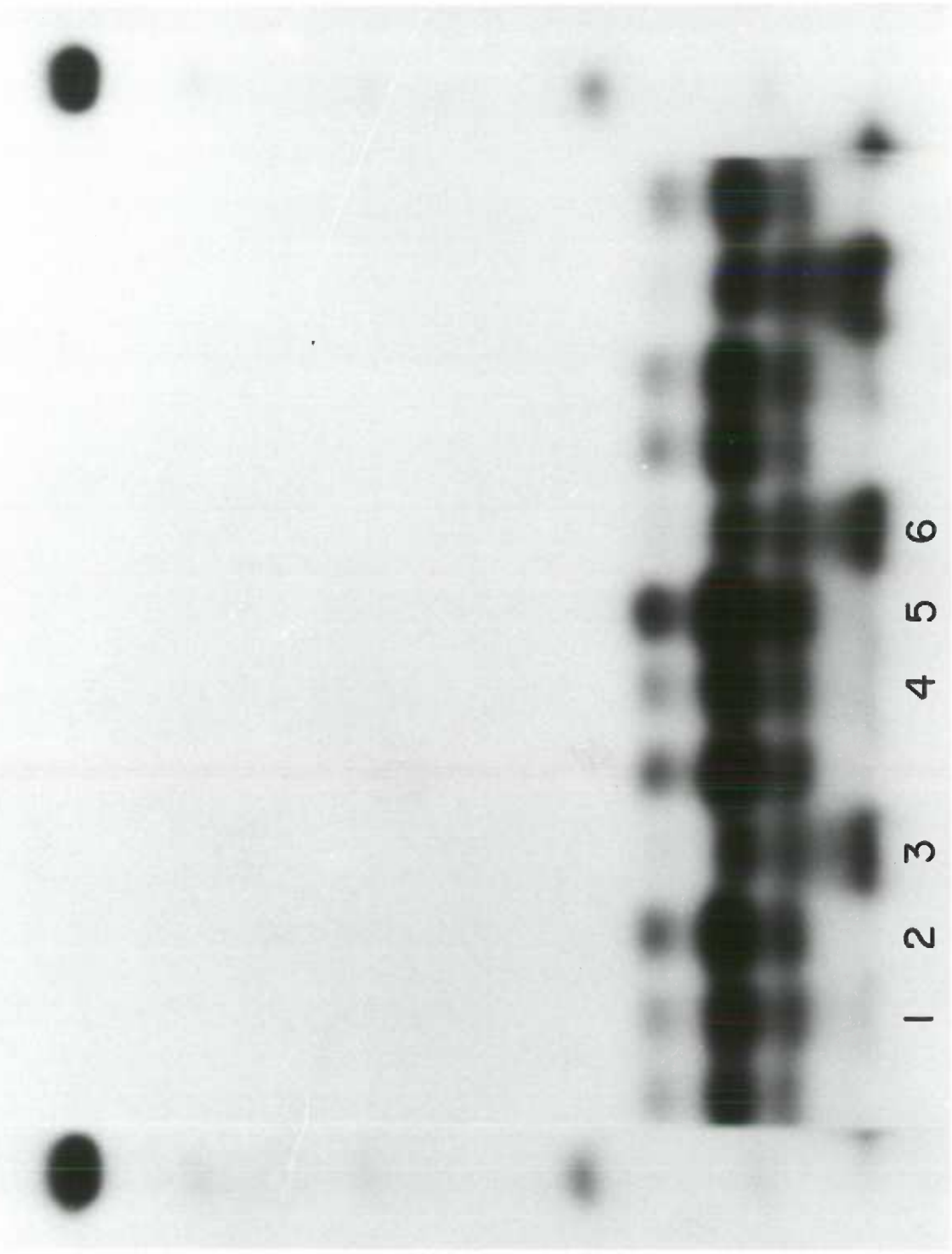

1
2
3
4
5
6

FIGURE 5

SDS-Polyacrylamide Gel of ^{125}I-Labelled KLH Bound by Normal and KLH Immune Spleen Cells

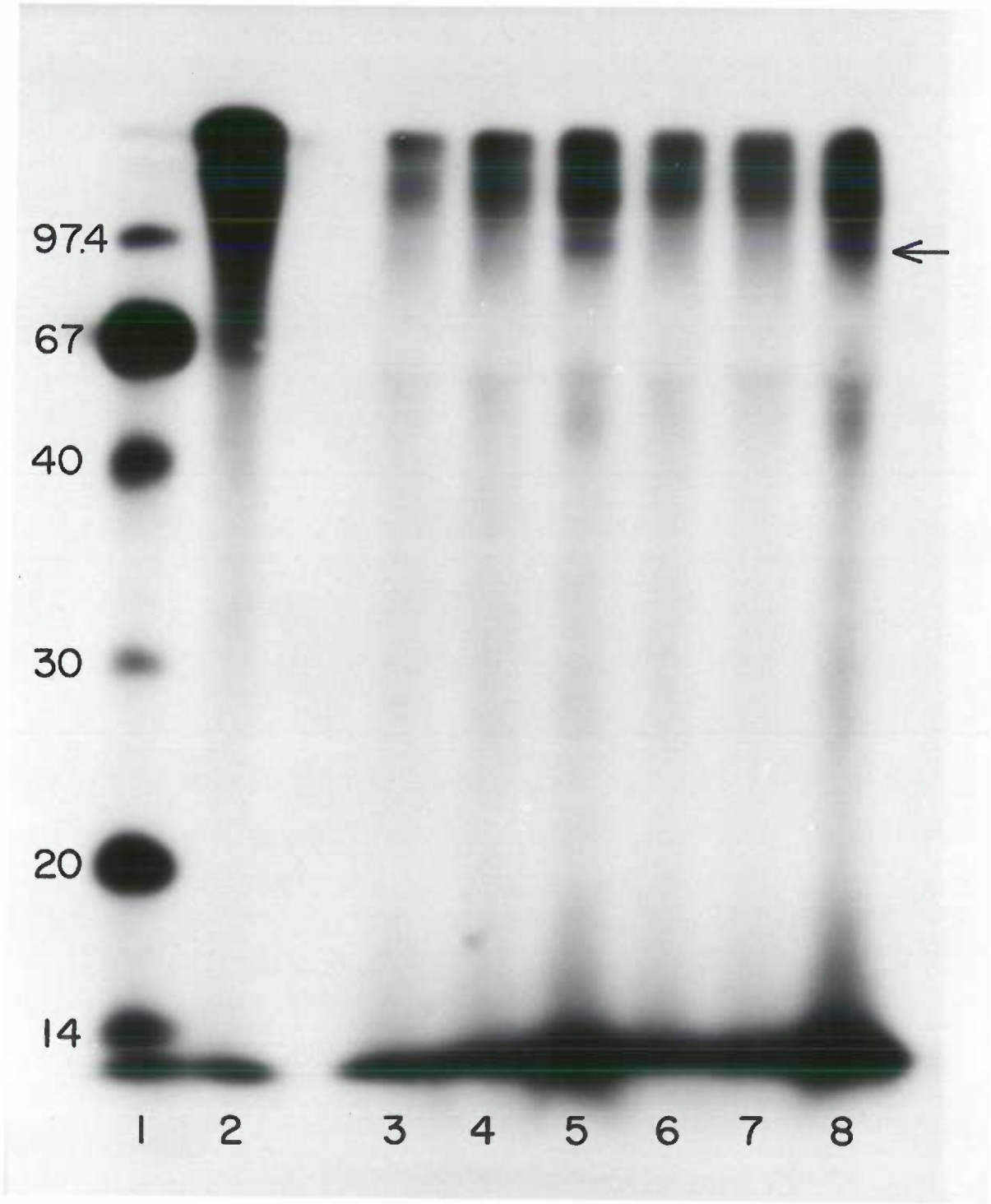
Channel 1: Molecular weight markers

2: KLH preparation

3 and 6: Normal spleen cells binding to KLH

4 and 7: Tumor immune spleen cells binding to KLH

5 and 8: KLH immune spleen cells binding to KLH


97.4
67
40
30
20
14
1
2
3
4
5
6
7
8

DISCUSSION AND SUMMARY

We have evaluated the cellular recognition of antigen by immune cells using the antigen binding cell (ABC) assay. This method measures the primary interaction of antigen and cells, mediated through cell surface receptors, rather than examining an indirect event which is the result of this interaction (eg. blast transformation, MIF release, etc.). The importance of ABC's as effector and regulatory components of the immune response to antigen has been extensively reviewed. In this thesis it was our intention to delineate the ABC response in mice to conventional and tumor antigens. It was felt that an understanding of the ABC response to conventional antigen, in terms of the kinetics and responding cell types, would provide a basis from which to contrast the immune response to tumors. The results from this thesis work have established the following points:

1) Antigen binding cells are found in spleen cells collected from animals injected with either KLH, replicating melanoma cells or a KCl extract of the melanoma tumor.

2) These ABC's are antigen specific and are found only in animals injected with the appropriate antigen.

3) The kinetic profiles of the ABC response observed after injection of KLH or replicating melanoma cells are very similar.

ABC's to these antigens are present at day 4 after antigen administration and reach a maximal response by day 6, eventually declining to background levels. The profile of the ABC response seen in animals injected with soluble tumor extract is similar in magnitude to the ABC response observed after injection of either KLH or replicating melanoma cells; however, the ABC response after administration of soluble tumor extract is of longer duration (at least through day 19).

4) The cell types involved in the ABC response to either KLH, replicating melanoma tumor cells or soluble melanoma extract are quite distinct. T and B cells represent the predominate antigen binding cells observed after administration of KLH. In contrast, animals injected with replicating melanoma tumor cells generate an antigen specific ABC response which is mediated exclusively by macrophages. Injection of soluble tumor extract results in antigen binding by T cells, B cells, macrophages and a non-staining cell population (possibly NK cells).

5) The mechanism of tumor antigen recognition by melanoma specific ABC's is mediated by cytophilic antibody. Antibody from animals injected with replicating melanoma cells is able to specifically "arm" normal spleen cells for the generation of tumor specific ABC's.

The immune response which an animal generates to an antigen represents a complex series of direct (cellular contact) and indirect (soluble mediators) interactions for generation of effector or regulatory cells. The objective of this thesis was to evaluate the immune response in mice to conventional and tumor antigens. The results from this work suggest distinct mechanisms by which animals respond to replicating and non-replicating antigens. The importance of this work is that it provides information concerning the immune response to these antigens using an in vitro assay which has been shown to correlate with in vivo immune status. The in vivo biological significance of tumor macrophage ABC's is unknown. Preliminary evidence obtained in this thesis suggests the involvement of these tumor specific ABC's in regulating tumor growth (Appendix II).

The ability of an animal to generate effector cells which possess specific in vitro cytotoxic activity against tumor cell targets, suggests that tumor associated antigens (TAA) are being recognized by the hosts immune system. The nature of these antigens remain to be described. An important conclusion from the work presented in this thesis is that the ABC assay detects tumor specificity. In an attempt to examine the components responsible for this tumor specificity we have employed a novel approach which combines the ABC assay and the principles of radioimmunoprecipitation. This method demonstrated the heterogeneity of the melanoma tumor extract which has been used in

the ABC assay and in addition established the following points:

1) Normal and tumor immune spleen cells bind components in the KCl tumor extract.

2) There is selective binding of three components in the tumor extract by both normal and immune cells. There is however no specific binding of a component by tumor immune cells which is not bound by normal spleen cells.

3) Tumor immune cells bind quantitatively more of the tumor extract than do normal or KLH immune cells. This increased binding by tumor immune spleen cells is due to increased binding of two components in the KCl tumor extract.

While conclusive statements concerning the isolation and identification of these putative tumor associated antigens cannot be made, the development of this methodology for investigation of tumor antigens has been worthwhile. It is possible that this methodology may eventually lead to a better understanding of the molecules capable of inducing a tumor specific immune response.

Appendix I.

CYTOFLUOROMETRIC EVALUATION OF ANTIGEN BINDING CELLS

The interaction of antigen with an immunocompetent cell results in a number of biochemical changes (increased lipid turnover, DNA and protein synthesis, ect.) which correlate, to varying degrees, with the immune status of an individual to a particular antigen. This interaction of antigen and immune cells may be assessed directly however using the antigen binding cell (ABC) assay. Antigen binding cells may be formed by incubating red blood cells, which have had antigen chemically coupled to their surface, with immune or normal syngeneic spleen cells (8). Antigen binding cells are seen as rosettes, or immune cells surrounded by a number of antigen coated RBC's. T cells (2,6), B cells (4,6) and macrophages (8) have all been demonstrated using ABC assays.

While formation of ABC's is not difficult, accurate quantitation of these cells remains a limiting factor in routine ABC evaluations. The proportion of ABC's seen to any antigen generally represents only about 1% of the total spleen cell population (7,8). Therefore detection and quantitation of ABC's must be sensitive enough to establish the statistical significance of this population of cells. Methods used for detection and quantitation of ABC's include autoradiography, microscopic evaluation or the fluoroescent activated cell sorter (FACS) (2,4).

Throughout this study we have used a cytofluorometric evaluation as first described by Tong, et. al. (8), for the detection and quantitation of ABC's (Fig. 1). We feel this method offers the

statistical reliability and speed which is required for analysis of ABC's. The cytofluorograph is able to distinguish populations of cells based upon various parameters. We routinely analyze an ABC population using the properties of nuclear fluorescence and particle size. Within a rosetting mixture there are essentially three populations of cells; non-immune cells, antigen coated RBC's and immune rosettes (Fig. 2). Addition to the rosetting mixture of acridine orange, a nuclear stain, results in staining of only mononuclear cells (non-immune cells and rosettes) while anucleated RBC's remain unstained. Rosetted cells can be discriminated from non-rosetted cells on the basis of the increased size of rosetted cells, which results in increased light scatter. The rosetted cells are therefore represented on the cytofluorograph oscilloscope as a distinct subpopulation, slightly displaced to the right of the major population of non-immune spleen cells (Fig. 2). Our studies have determined that a significant (R=0.92, Fig. 3) correlation exists between cytoflorometric and fluorescent microscopic techniques for the detection of antigen specific ABC's.

Within the normal spleen cell population there exists a proportion of cells capable of binding antigen without prior exposure to the antigen (1,3,5). The detection of antigen specific ABC's thus must represent the increase in rosette forming cells detected to the immunizing antigen above the proportion of ABC's seen to control antigens. As an example, Fig. 4 represents the background values obtained when using the cytoflorograph for

determination of ABC's found in normal and immune spleen cell populations. Spleen cells obtained from normal animals lack significant levels of antigen specific ABC's, while spleen cells obtained from immune animals demonstrate a significantly increased level of antigen specific ABC's to the appropriate immunizing antigen.

Cytofluorometric evaluation of normal spleen cell ABC's to a panel of antigens results in a consistent "background" level of approximately 5%. This value, observed when using the cytoflorograph, is a composite of at least three independent factors: 1) ABC's seen in normal spleen cells to any control antigen, 2) large cells and cell clumps and 3) cellular coincidence. Microscopic evaluation of rosetting mixtures formed after incubation of normal spleen cells and antigen coated RBC's has demonstrated an ABC background found to all antigens tested of approximately 1%. These levels remain unchanged for control antigen-RBC conjugates tested with spleen cells from immune animals immunized with irrelevant antigens. However, they increase significantly when tested with the immunizing antigen. In addition to the presence of ABC's seen after incubation of normal spleen cells and antigen-RBC conjugates, a consistent level of white cell clumping has been observed microscopically. This cell clumping would most likely be counted as rosettes on the cytofluorograph. Cellular clumping has been estimated to account for 1.0% to 1.5% of the background level observed when using the cytofluorograph.

Cellular coincidence, or the passage of white cells and RBC's in front of the cytofluorograph laser at the same time, even though they are unattached, will also contribute to part of the observed background. Passage of normal spleen cells and uncoated RBC's through the cytofluorograph results in an inherent background of approximately 2.5%. Therefore the background level observed with the cytoflorograph to a panel of antigens incubated with normal spleen cells is a combination of actual background rosettes, white cell clumping and cellular coincidence. This background level remains unchanged when tested with control antigen-RBC conjugates and either normal or immune spleen cells. When immune spleen cells are tested with the immunizing antigen-RBC conjugate, a significant increase in antigen specific ABC's is observed. This increase in detected antigen specific ABC's is due to an increase in the percentage of cells with receptors for the immunizing antigen (actual ABC's) and not due to an increase in one of the non-specific components which contribute to the background.

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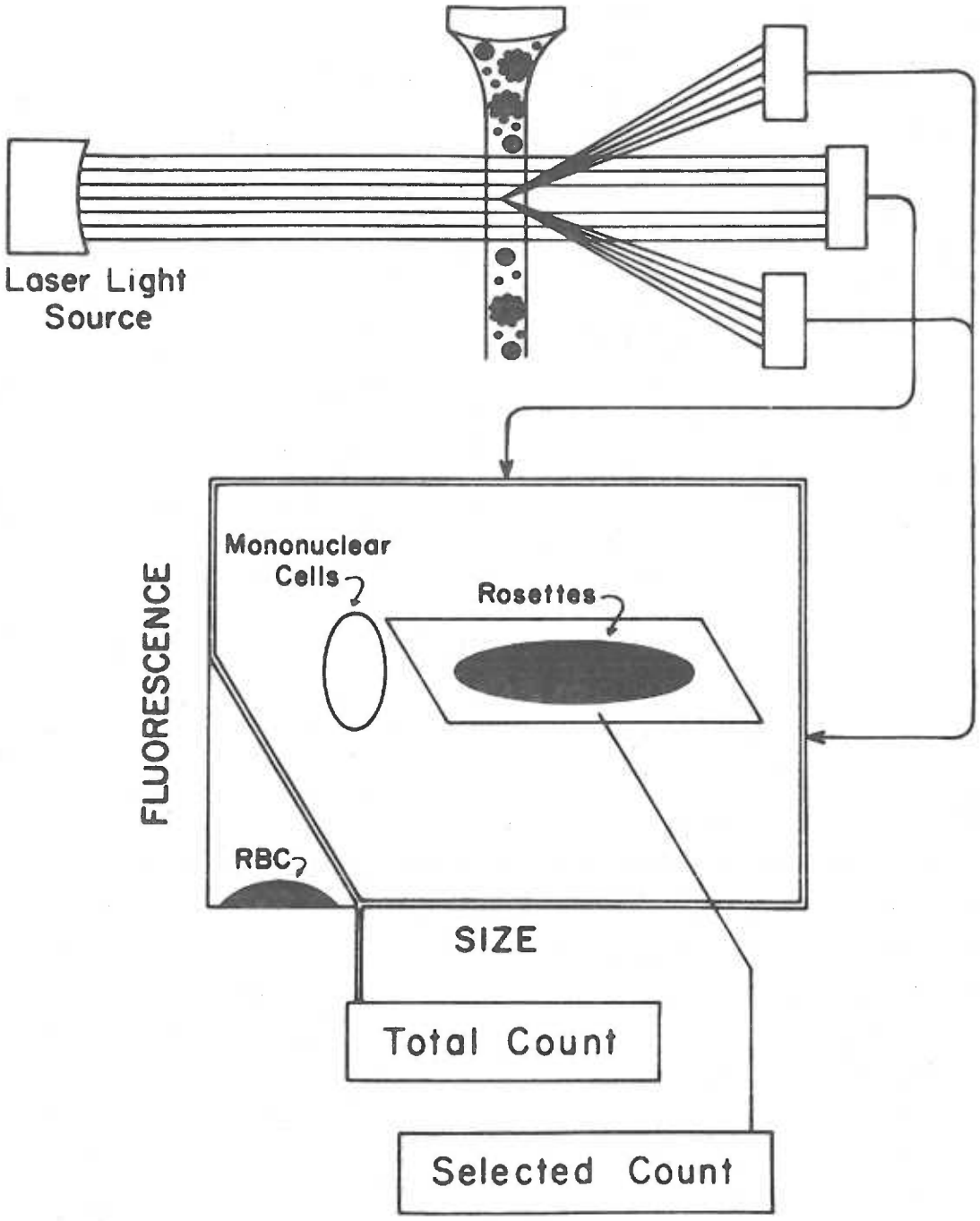
8. Tong, A. W., Vandenbark, A. A., Burger, D. R. and Vetto, R. M. Cytofluorometric evaluation of antigen specific rosette forming cells in humans. I. Correlation of immunity to conventional antigens with antibody mediated monocyte recognition. J. Immunol.(Submitted)

FIGURE 1

Graphic Representation of Cytofluorometric Detection of Antigen Binding Cells



GRAPHIC REPRESENTATION OF CYTOFLUOROMETRIC DETECTION OF ABC's

FIGURE 2

Antigen Binding Cell Assay

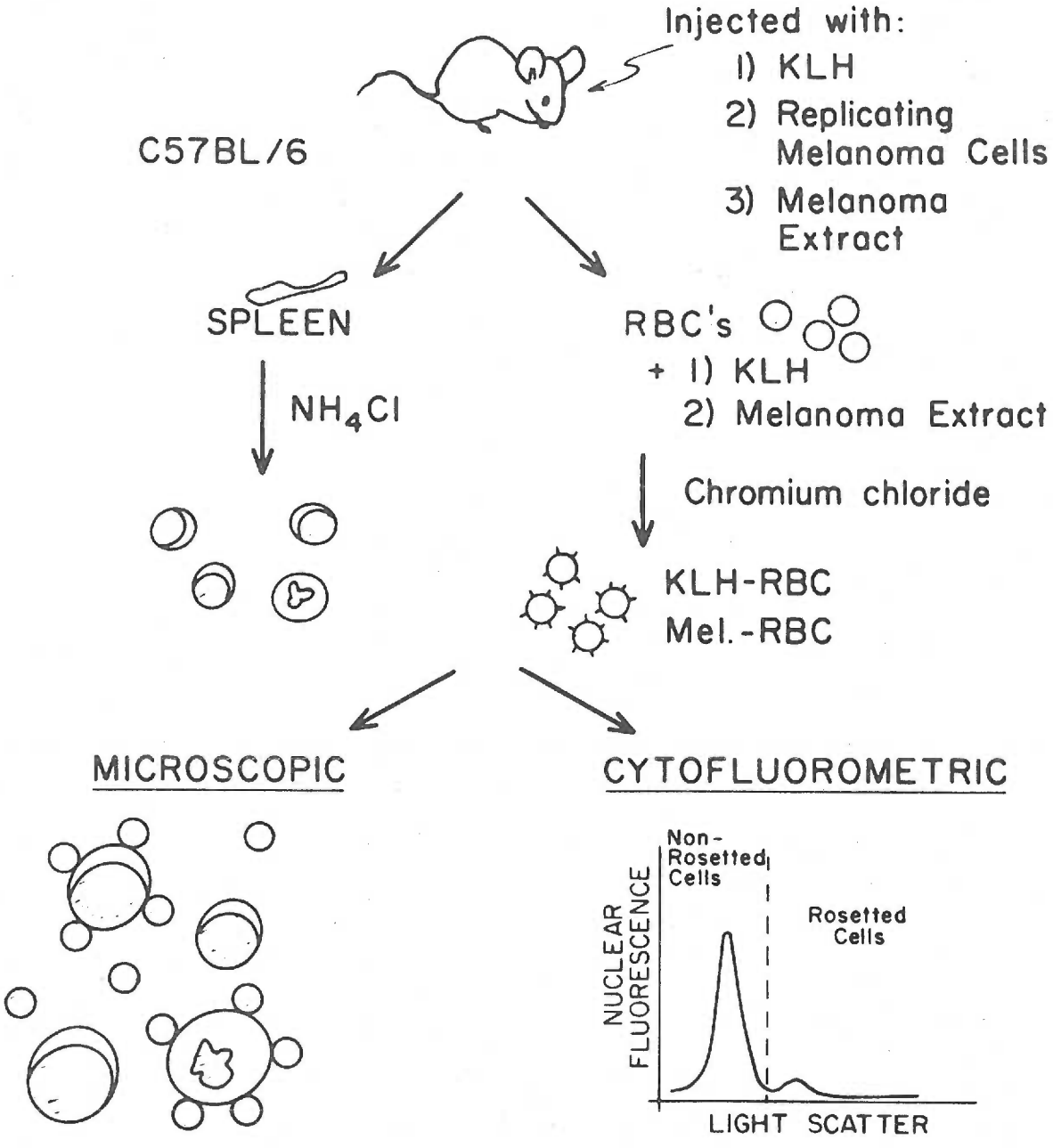

Injected with:
1) KLH
2) Replicating Melanoma Cells
3) Melanoma Extract
C57BL/6
SPLEEN
NH_4Cl
RBC's
+ 1) KLH
2) Melanoma Extract
Chromium chloride
KLH-RBC
Mel.-RBC
MICROSCOPIC
CYTOFLUOROMETRIC
Non-Rosetted Cells
Rosetted Cells
NUCLEAR FLUORESCENCE
LIGHT SCATTER

FIGURE 3

Correlation of Microscopic and Cytofluorometric Evaluation of Antigen Binding Cells

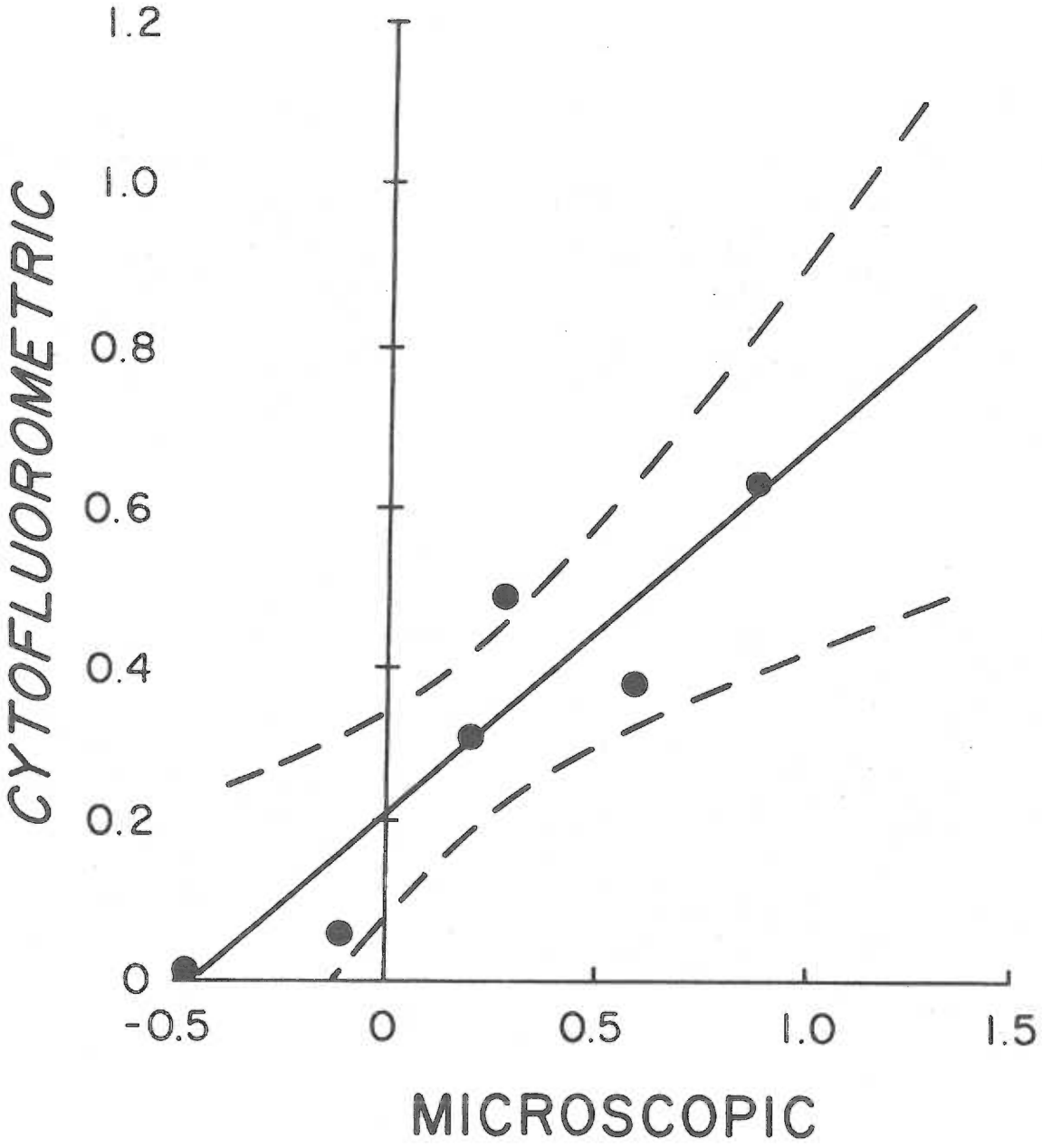

CYTOFLUOROMETRIC
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.5
0
0.5
1.0
1.5
MICROSCOPIC

TABLE 1

EXAMPLE OF SPECIFIC ABC RESPONSE[a]

AG-RBC	IMMUNIZING ANTIGEN	CYTOFLUOROMETRIC VALUES			X +/- SD	% ABC's
KLH-RBC	None	4.96	5.22	4.93	5.04 ± .16	N.S.[b]
EL4-RBC	None	4.98	5.16	5.20	5.11 ± .12	N.S.
MeL-RBC	None	5.10	5.08	5.00	5.06 ± .05	N.S.
KLH-RBC	KLH	5.88	5.92	6.02	5.94 ± .07	0.74%*
MeL-RBC	KLH	5.05	5.32	5.24	5.20 ± .14	
KLH-RBC	Melanoma	5.10	5.18	5.30	5.19 ± .10	
MeL-RBC	Melanoma	5.83	5.70	5.86	5.80 ± .09	0.61%

[a]% AG SPECIFIC ABC's = (% ABC response to the immunizing AG-RBC) - (% ABC response to a control AG-RBC).

[b] Not significant

** Significant increase (p 0.05).

Appendix II.

Effect of Whole Spleen Cell Populations which Contain Melanoma Specific Antigen Binding Cells in the *In Vivo* Regulation of Melanoma Tumor Development

Injection of viable tumor cells frequently activates both humoral and cellular responses. Tumor specific antibody and tumor specific cytotoxic cells have been documented in a variety of systems including the B16 murine melanoma model (3,4,6,7,12,13,14,15). The production of B16 specific antibodies has been observed after injection of either whole tumor cells (4) or after injection of a partially purified preparation of the B16 melanoma tumor (3). Since the B16 tumor is immunogenic, a number of studies have attempted to evaluate the in vivo significance of this immune response in protection against tumor development (1,2). Bystryn (3) noted that repeated injections of either irradiated or sublethal doses of viable melanoma cells induced resistence to a subsequent lethal dose of viable B16 cells. In a later study Bystryn noted that partially purified B16 tumor antigen, collected from in vitro culture supernatants of viable melanoma cells, provided specific protection against B16 tumor development in C57BL/6 mice. Brinckerhoff and Lubin (1) have documented a significant decrease in the incidence of B16 induced tumors when animals received two IP injections of muconomycin A treated B16 tumor cells prior to administration of viable B16 cells. Muconomycin A treatment inhibits protein and glycoprotein synthesis, and appears in the B16 melanoma system to enhance tumor immunogenicity. Wang and co-workers (18) have described a protective effect of immune RNA extracted from the spleen and lymph nodes of Hartley guinea pigs injected 14 days prior with B16 melanoma tumor. These workers documented a significant increase in

long term survival rate when mice were first injected with B16-immune RNA treated sygeneic lymphoid cells. This protective effect was tumor specific as Lewis lung carcinoma-immune RNA had no protective effect against B16 tumor induced metastasis. Burger and co-workers (2) have demonstrated the involvement of both humoral and cellular immune responses to injection of viable P51 melanoma tumor cells, (a sub-line of the B16 murine melanoma (11)). These investigators noted a lack of significant tumor protection in mice receiving a single injection of killed P51 melanoma cells in complete Freunds adjuvant inoculated 30 days prior to injection of viable tumor cells. From these studies however a correlation between increased tumor size and increased levels of P51 melanoma specific antibody was observed (2). This suggested that anti-tumor antibody may have a tumor enhancing function in vivo as has been postulated by others (10). Passive transfer experiments were performed in which recipient C57BL/6 animals received cell transfers of either peritoneal exudate, lymph node or spleen cells from donor animals immunized with the P51 melanoma tumor. The recipients who were then challenged with P51 tumor cells two days after cell transfer showed a reduction in the rate of tumor development and a significant reduction in tumor weights as compared to control animals who received only tumor cells. If recipient animals were challenged 16 days after passive cell transfer only those animals receiving peritoneal exudate cells showed a significant reduction in tumor development.

Melanoma specific antigen binding cells (ABC) may be generated in the spleens of normal mice after a single injection of $2X10^6$ viable melanoma cells (see manuscript #1). The maximal response of melanoma specific ABC's occurs between days 5 to 7, and ranges from 0.50 to 1.00%. The predominate ABC type in response to the melanoma tumor antigen is a macrophage. The importance of the macrophage *in vitro* in mediating tumor cytotoxicity has been well established (5,17). Suggestive evidence also supports an *in vivo* role for the macrophage in protection against tumor development (12,19). Since melanoma specific ABC's can be generated in the spleens of C57BL/6 mice, an attempt was made to evaluate *in vivo* what effect, if any, spleen cells which contain melanoma specific ABC's have on tumor development. C57BL/6 mice (female) were injected either intraparentally or subcutaneously with a mixture of 10^6 viable P51 melanoma tumor cells and variable numbers of syngeneic spleen cells. The variables in these experiments were 1) the number of spleen cells the animals received and 2) prior treatment of the animals from which the spleen cells were obtained. Spleen cells were obtained from three groups of animals: 1) normal animals. 2) animals who had received a single IP injection of $2X10^6$ melanoma tumor cells 6-8 days previously, or 3) animals who had received a single injection of a KCl melanoma tumor extract, which has also been demonstrated to induce melanoma specific ABC's (see manuscript #1). These treated and control spleen cell populations were mixed with $1X10^6$ viable P51 melanoma tumor cells at ratios of either 25:1 or 50:1 spleen cells:tumor cells and injected into untreated

recipients. To evaluate the effect of donor treatment on tumor development the following criteria were documented: 1) day of first palpable tumor, 2) day of death, 3) weight variations and 4) tumor size in recipients.

Experiment #1: All animals received an IP injection of the spleen cell:tumor cell mixture at a 25:1 ratio. There were 10 animals/group. A control group which received only melanoma tumor cells ($1X10^6$) was also included. Spleen cells were obtained from normal animals and from mice who had received a single IP injection of $2X10^6$ viable melanoma cells 7 days prior and had 0.55% melanoma specific ABC's in their spleen cell population. Spleens were also collected from animals who had received a single IP injection of the melanoma extract 8 days prior and had 0.63% melanoma specific ABC's in their spleen cell poulation.

Treatment	Mean Day to Tumor ± S.D.	Mean Day to Death ± S.D.
Tumor only	32.64 ± 8.66	37.27 ± 8.28
Normal cells:Tumor	34.90 ± 5.36	43.20 ± 6.30
P51 ABC's :Tumor	34.50 ± 4.72	38.80 ± 6.25
KCl P51 ABC's:Tumor	34.50 ± 6.95	37.00 ± 5.96

No significant differences exist between groups in either mean time to palpable tumor development or mean time to death. Analysis of weight development also revealed no significant group differences (Fig. 1).

Experiment #2: The effect of an IP injection at a 50:1 ratio of spleen cells:melanoma tumor was evaluated for an effect on tumor development. The concentration of the melanoma tumor cells remained constant at $1X10^6$ cells. There were 10 animals/group. Spleen cells were obtained from either 1) normal animals or 2) animals who had received a single IP injection of $2X10^6$ viable melanoma cells 8 days prior and had 0.50% melanoma specific ABC's in their spleen cell population.

Treatment	Mean Day to Tumor ± S.D.	Mean Day to Death ± S.D.
Normal cells:Tumor	50.13 ± 4.64	53.10 ± 8.90
P51 ABC's:Tumor	43.30 ± 6.68	47.00 ± 9.30

The mean time to first palpable tumor or time to death do not differ significantly between treatment groups. Analysis of the effect of treatments on weight (Fig. 2) showed a significant difference between groups. Animals receiving normal spleen cells plus tumor had increased weights as compared to animals receiving spleen cells containing melanoma specific ABC's plus tumor.

Experiment #3: All animals received a subcutaneous injection of the spleen cell:tumor cell mixture at a 25:1 ratio. Spleen cells were obtained from normal animals or from mice who had received a single IP injection of $2X10^6$ viable melanoma cells 6 days prior and had 0.57% melanoma specific ABC's in their spleen cell population. There were 10 animals/treatment group and 5 animals in a control group which received only melanoma tumor cells. Again the concentration of tumor cells remained constant at $1X10^6$ cells.

Treatment	Mean day to Tumor ± S.D.	Mean Day to Death ± S.D.
Tumor cells only	20.40 ± 3.29	40.80* ± 4.15
Normal spleen:Tumor	16.80 ± 1.75	34.50 ± 2.27
P51 ABC's:Tumor	18.70 ± 3.27	35.70 ± 2.16

The mean day to palpable tumor did not differ between treatment groups. There is however a significant difference among groups, $F_{(2,22)}=9.586$; $p < 0.05$ with respect to the mean day to death. Injection of spleen cells from either normal or treated animals plus tumor cells resulted in significant differences in day to death as compared with injection of tumor cells only. No significant difference exist however between the two treatment goups. The treatment time profile showing the effect on weight (Fig. 3) shows no significant difference. Analysis of the effect of treatment on tumor size shows a significant ($p < 0.05$) difference between each

treatment group. Animals receiving spleen cells containing melanoma specific ABC's had significantly smaller tumors than did animals receiving equal numbers of normal spleen cells. Animals receiving only tumor cells however had significantly reduced tumor sizes as compared to the two treatment groups.

Experiment #4: All animals received a subcutaneous injection of the spleen cell:tumor cell mixture at a ratio of 50:1. A control group receiving a subcutaneous injection of tumor cells only was also included (10^6). Spleen cells were obtained from normal animals or from mice who had received a single IP injection of $2X10^6$ viable melanoma cells 6 days prior and had 0.57% melanoma specific ABC's in their spleen cell population. There were 10 animals/treatment group and 5 animals in the control group which received only viable melanoma tumor cells .

Treatment	Mean day to Tumor ± S.D.	Mean to Death ± S.D.
Tumor only	15.00 ± 5.61	34.40 ± 5.13
Normal cells:Tumor	10.00 ± 1.94	32.10 ± 4.84
P51 ABC's:Tumor	12.00 ± 4.00	34.40 ± 4.90

Analysis revealed no significant group differences in either the time to first palpable tumor or the time to death. Analysis of the time profile of the effect of treatment on weight (Fig. 5) and the

effect of treatment on tumor size (Fig. 6) revealed that treatments caused significant differences between groups. Animals which had received spleen cells containing melanoma specific ABC's had increased weights and reduced tumor sizes as compared to animals receiving normal spleen cells plus tumor. Animals receiving only tumor cells again had the lowest weights and smallest tumors.

These experiments were designed to evaluate what in vivo effects spleen cells containing melanoma specific ABC's may have on the regulation of tumor growth. The following points may be concluded from these experiments: 1) Spleen cells which contain a subpopulation of melanoma specific ABC's do not provide absolute protection against tumor development. 2) Different treatments caused no significant differences in the mean time to first palpable tumor development. 3) The mean time to day of death differed significantly in only 1 out of 4 experiments. In this one study, animals receiving spleen cells from either normal or treated animals had significantly reduced survival rates as compared to animals who received only tumor cells; however no significant differences existed with respect to day of death when comparing the two treatment groups. 4) Some effect of treatment on weights was observed. The effect was not consistent however between experiments except to note that animals receiving only tumor cells consistenly had reduced weights as compared to animals receiving either normal spleen cells or spleen cells containing melanoma specific ABC's. 5) Analysis has revealed a significant effect of treatment on tumor

size. Animals receiving spleen cells containing melanoma specific ABC's demonstrated significantly reduced tumor size as compared to animals receiving normal spleen cells.

These data provide suggestive evidence (experiments 3 and 4) that spleen cell populations which contain melanoma specific ABC's may retard tumor development relative to normal spleen cells. A consistent finding in these experiments has been the lack of a significant difference detected to either the time required for development of palpable tumor or survival rates when comparing animals injected with either spleen cells containing melanoma specific ABC's or normal spleen cells.

Analysis of the effect of treatment on tumor size (experiments 3 and 4) demonstrated an enhancement of tumor growth in animals receiving either normal or P51-ABC containing mononuclear spleen cells as compared to animals receiving only tumor cells. This enhancement of tumor growth may be due to the transfer of T-suppressor cells (8,9,16) from both normal and treated spleen cells which interfere with tumor destruction. Bystryn (3) has noted that injection of normal cells prior to administration of viable tumor cells also resulted in a significant enhancement of tumor growth as compared with animals receiving only PBS plus tumor.

The in vivo effectiveness of a specific cell type in protection against tumor development is extremely difficult to assess with

accuracy. While a number of cell types have been shown to possess tumoricidal activity in vitro, the importance of these cells in vivo is largely unknown. The results from the present study suggest that melanoma specific ABC's may be involved in reducing tumor size when compared to administration of equal numbers of normal spleen cells. The biological significance of these results must be questioned, however, since spleen cell populations which contained melanoma specific ABC's had no effect on either the rate of tumor development or survival as compared to normal spleen cells.

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FIGURE 1

Effect of Treatment on Weight in C57BL/6 Mice

Δ—Δ :Tumor cells only

o—o :25:1 ratio of normal spleen cells:tumor cells

×—× :25:1 ratio of spleen cells containing melanoma ABC's:tumor cells (tumor ABC's were generated by injecting donors with replicating melanoma cells)

×---× :25:1 ratio of spleen cells containing melanoma ABC's:tumor cells (tumor ABC's were generated by injecting donors with the soluble tumor extract)

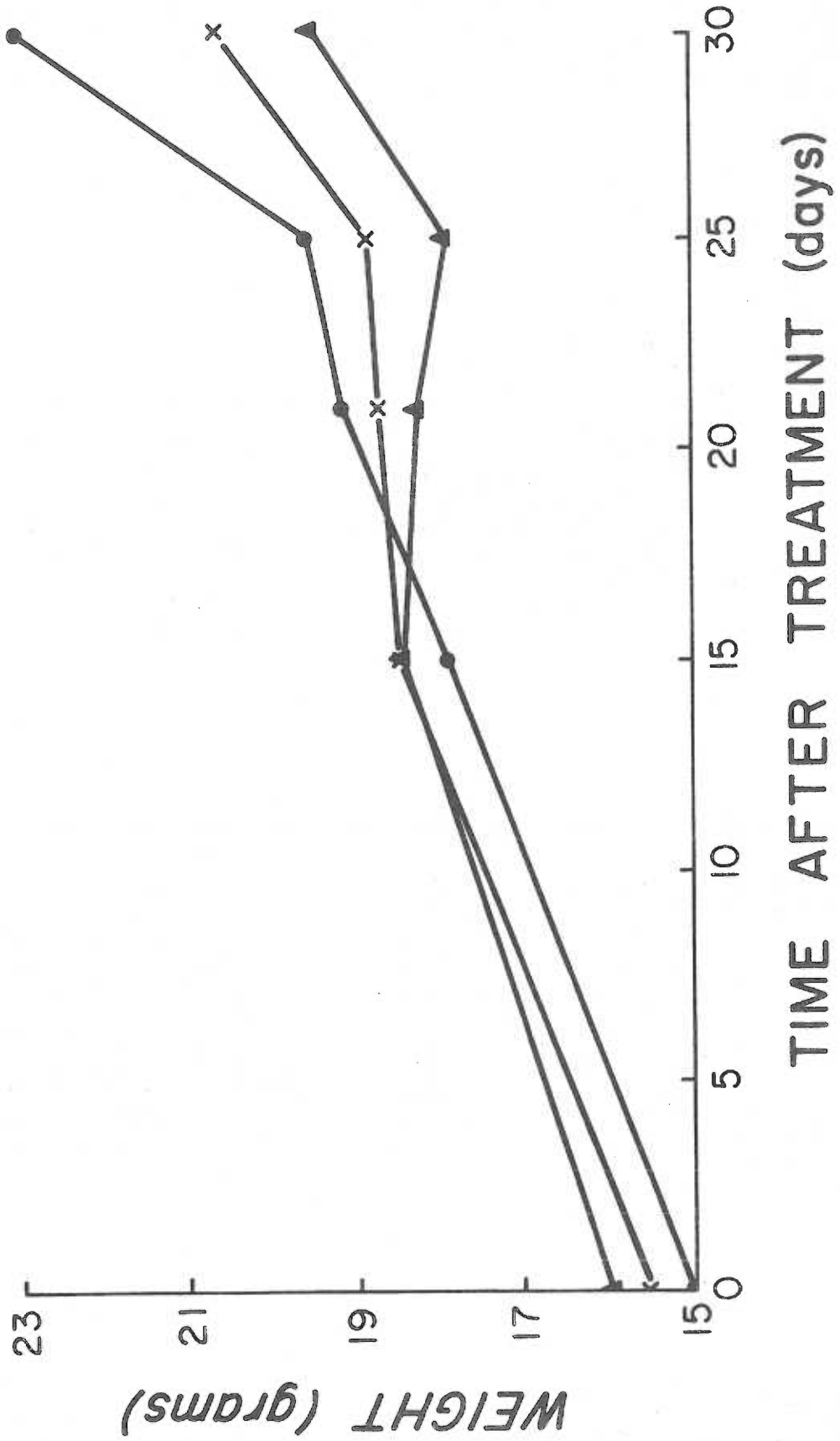

WEIGHT (grams)
23
21
19
17
15
0
5
10
15
20
25
30
TIME AFTER TREATMENT (days)

FIGURE 2

Effect of Treatment on Weight in C57BL/6 Mice

o—o :50:1 ratio of normal spleen cells:tumor cells

×—× :50:1 ratio of spleen cells containing melanoma ABC's:tumor cells(tumor ABC's were generated by injecting donors with replicating melanoma tumor cells)

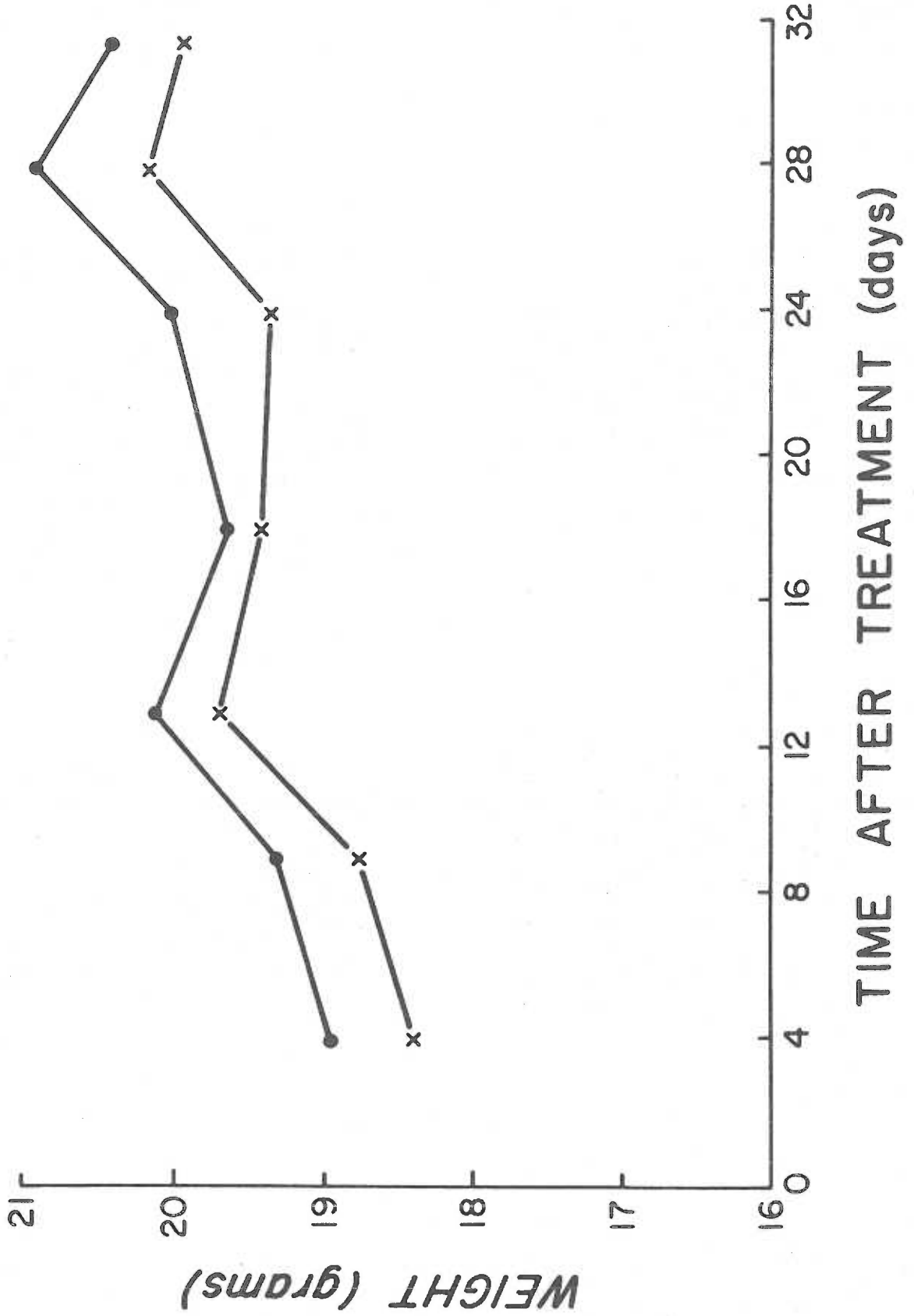

WEIGHT (grams)
21
20
19
18
17
16
0
4
8
12
16
20
24
28
32
TIME AFTER TREATMENT (days)

FIGURE 3

Effect of Treatment on Weight in C57BL/6 Mice

Δ—Δ : Tumor cells only

o—o : 25:1 ratio of normal spleen cells:tumor cells

×—× : 25:1 ratio of spleen cells containing melanoma ABC's:Tumor cells (tumor ABC's were generated by injecting donors with replicating melanoma cells)

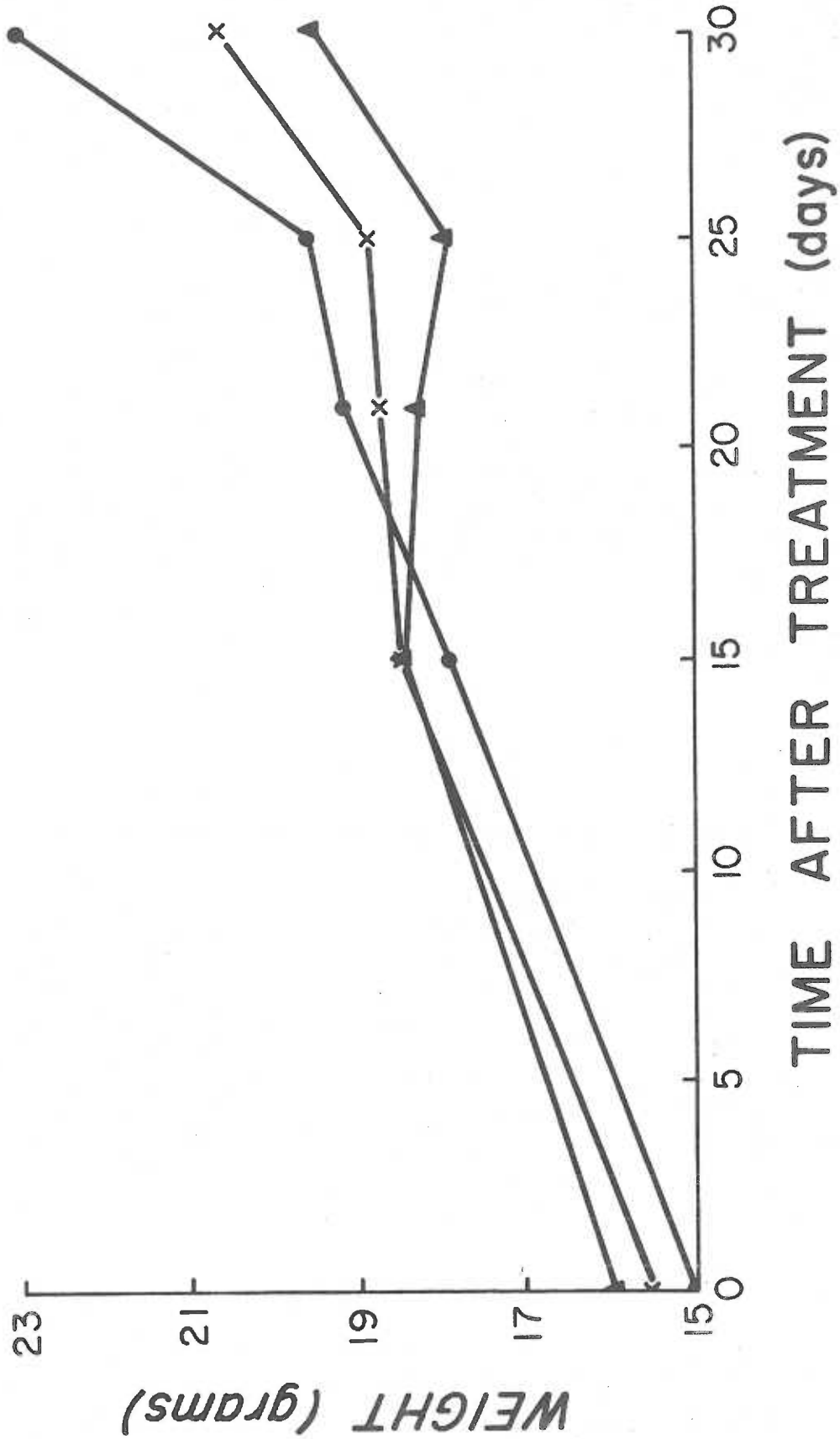

WEIGHT (grams)
23
21
19
17
15
0
5
10
15
20
25
30
TIME AFTER TREATMENT (days)

FIGURE 4

Effect of Treatment on Tumor Development in C57BL/6 Mice

△—△ : Tumor cells only

○—○ : 25:1 ratio of normal spleen cells:tumor cells

×—× : 25:1 ratio of spleen cells containg tumor ABC's:tumor cells(tumor ABC's were generated by injecting donors with replicating melanoma cells)

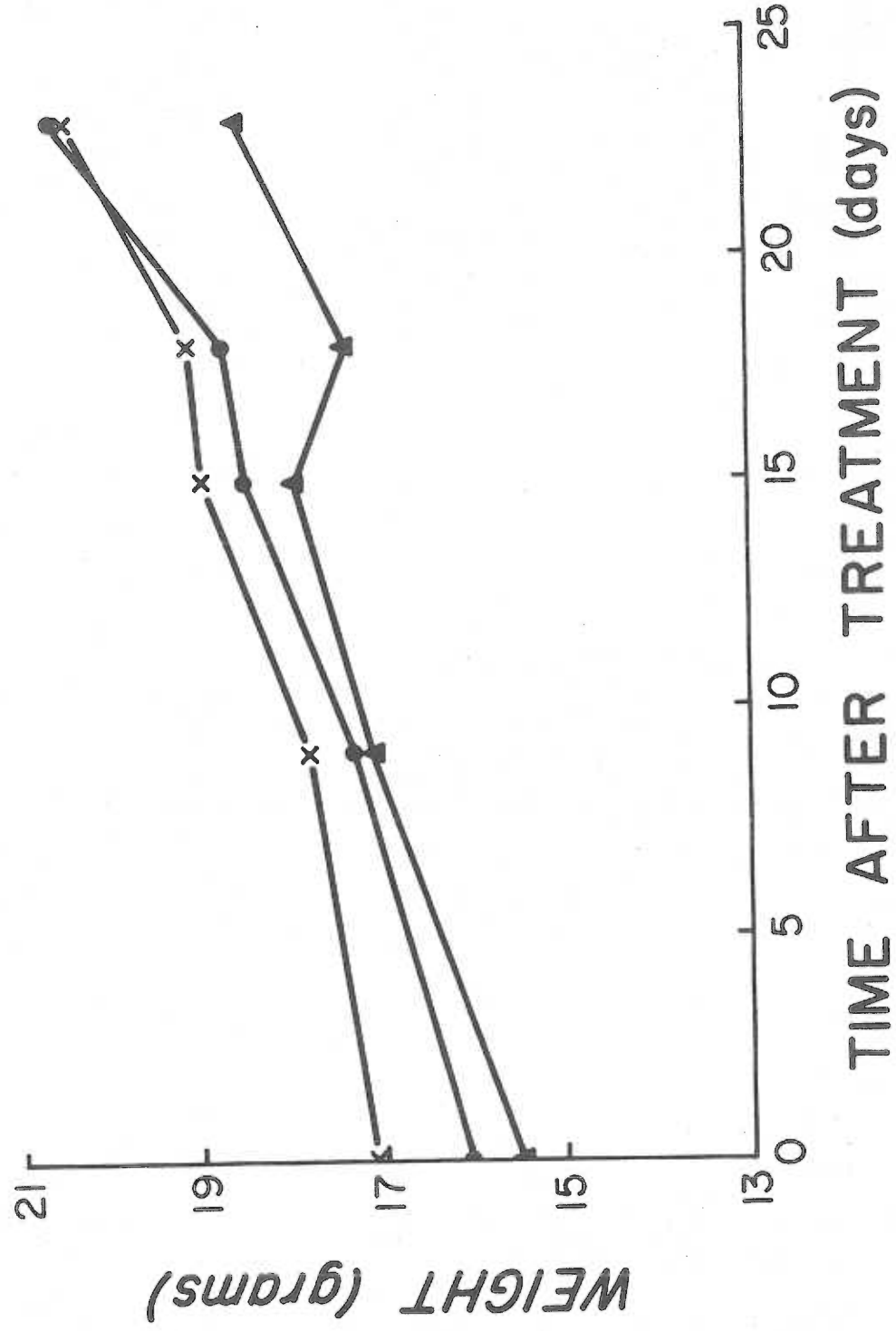

WEIGHT (grams)
21
19
17
15
13
0
5
10
15
20
25
TIME AFTER TREATMENT (days)

FIGURE 5

Effect of Treatment on Weight in C57BL/6 Mice

△——△ : Tumor cells only

○——○ : 50:1 ratio of normal spleen cells:tumor cells

×——× : 50:1 ratio of spleen cells containing melanoma ABC's:tumor cells(tumor ABC's were generated by injecting recipients with replicating melanoma cells)

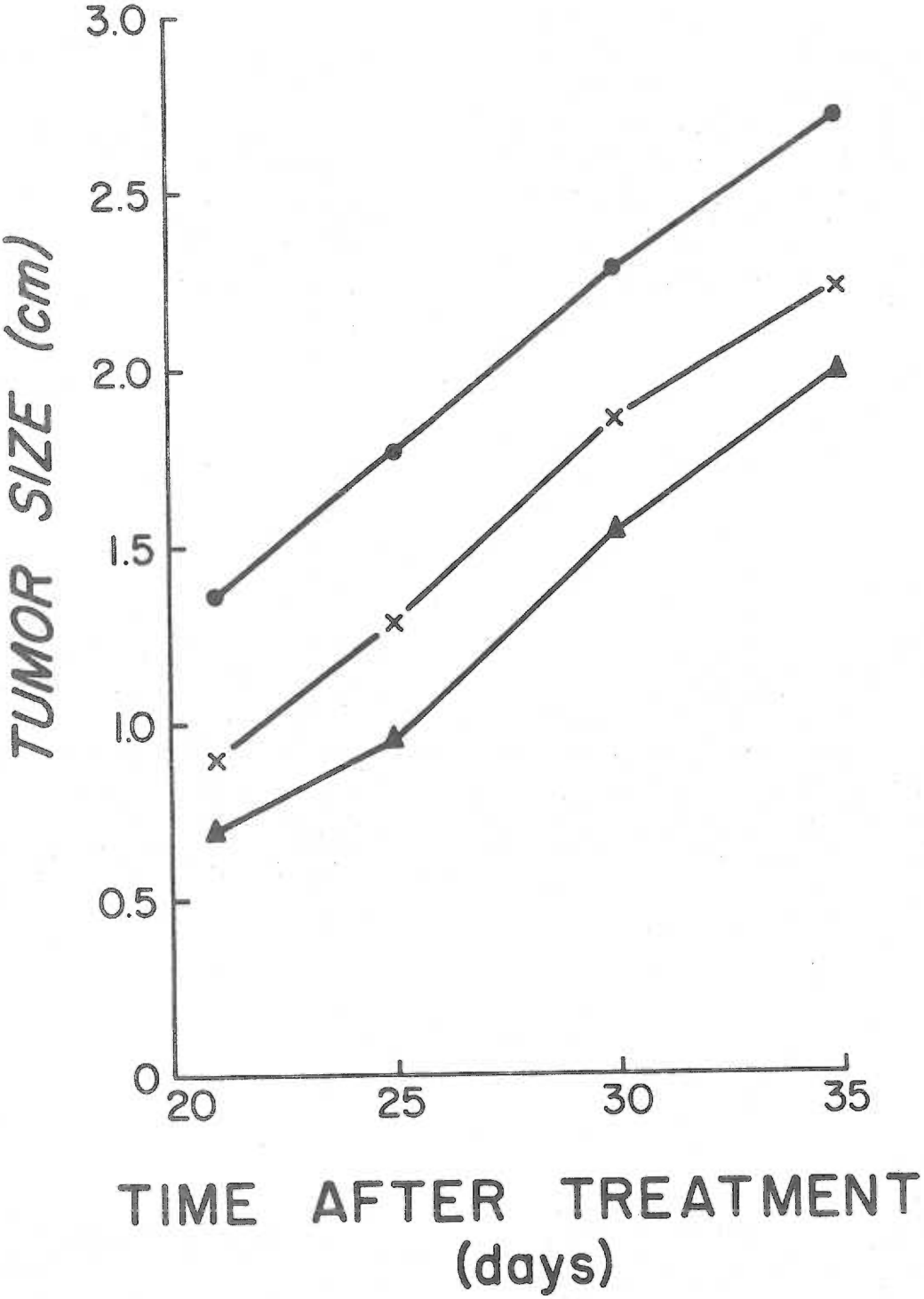

TUMOR SIZE (cm)
3.0
2.5
2.0
1.5
1.0
0.5
0
20
25
30
35
TIME AFTER TREATMENT
(days)

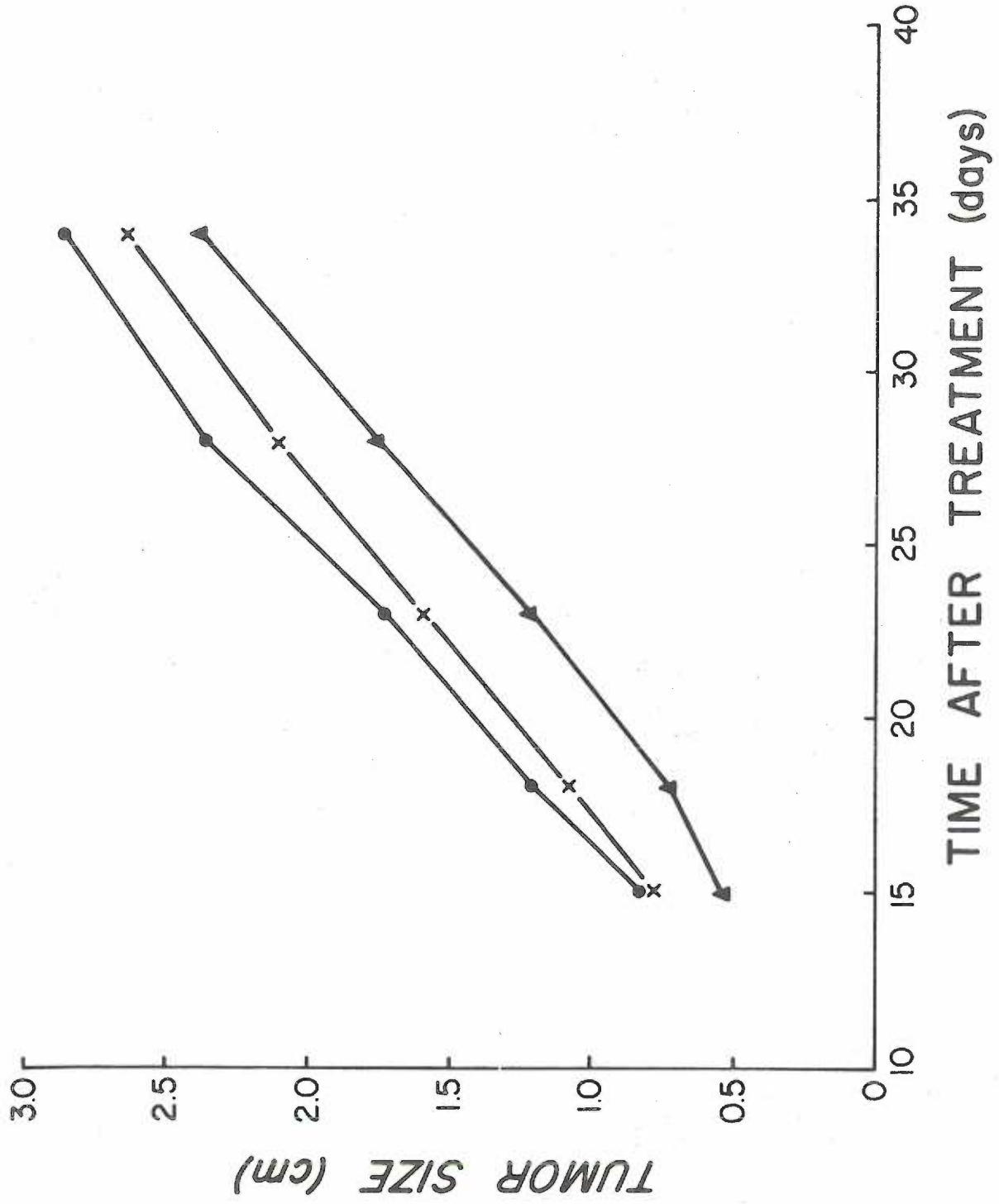
FIGURE 6

Effect of Treatment on Tumor Development in C57BL/6 Mice

Δ—Δ :Tumor cells only

○—○ :50:1 ratio of normal spleen cells:tumor cells

×—× :50:1 ratio of spleen cells containing melanoma ABC's:tumor cells(tumor ABC's were generated by injecting donors with replicating melanoma cells)


TUMOR SIZE (cm)
3.0
2.5
2.0
1.5
1.0
0.5
0
10
15
20
25
30
35
40
TIME AFTER TREATMENT (days)

ABBREVIATIONS

ABC	antigen binding cell
Ag	antigen
BCG	Bacillus Calmette Guerin
Ig	immunoglobulin
KLH	keyhole limpet hemocyanin
LAI	leukocyte adherence inhibition
MIF	migration inhibition factor
R	correlation coefficient
RBC	red blood cell
RFC	rosette forming cell
TBS	tris buffered saline
TAA	tumor associated antigen
WBC	white blood cell

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