Effects of antibodies to membrane antigens on in vitro immune responses
by James Alan Harp

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
The mechanisms by which cells become activated and interact to produce immune responses have not been clearly defined. Cell surface molecules are thought to play a role in these events but the relevant molecules for the expression of many immune functions have not been identified. Thus, the major goal of this study was to apply a method of analysis, perturbation of membrane antigens by specific antibody, with the potential for identifying molecules relevant to function, to several major surface molecules of cells participating in in vitro models of immune function.

The results of these studies indicated that the presence of antibody to the Thy 1 molecule could inhibit generation of murine cytotoxic T lymphocytes in some cases but not others. It was also shown that antibody to another murine cell surface molecule, T200, could modulate and ultimately suppress the proliferative response to alloantigen as well as the generation of cytotoxic T cells. The suppressive effects were seen when antibody was added either at the initiation of culture or at various times after initiation.

These effects were shown to be unique to antibody interaction with the T200 molecule since antibodies to other cell surface molecules either had no effect or suppressed with different kinetics. The antibody alone was not mitogenic for murine splenocytes but could enhance the mitogenic effect of the plant lectin, concanavalin A. Antibodies to an allotypic determinant on the T200 molecule, Ly 5.1, also had suppressive effects on proliferation and generation of cytotoxicity.

In sum, these results contribute to the body of evidence suggesting that activation and interactive events leading to immune function may require the participation of several major cell surface molecules, possibly through formation of membrane complexes.

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A thesis submitted in partial fulfillment
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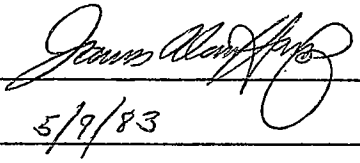
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ABSTRACT

The mechanisms by which cells become activated and interact to produce immune responses have not been clearly defined. Cell surface molecules are thought to play a role in these events but the relevant molecules for the expression of many immune functions have not been identified. Thus, the major goal of this study was to apply a method of analysis, perturbation of membrane antigens by specific antibody, with the potential for identifying molecules relevant to function, to several major surface molecules of cells participating in in vitro models of immune function.

The results of these studies indicated that the presence of antibody to the Thy 1 molecule could inhibit generation of murine cytotoxic T lymphocytes in some cases but not others. It was also shown that antibody to another murine cell surface molecule, T200, could modulate and ultimately suppress the proliferative response to alloantigen as well as the generation of cytotoxic T cells. The suppressive effects were seen when antibody was added either at the initiation of culture or at various times after initiation. These effects were shown to be unique to antibody interaction with the T200 molecule since antibodies to other cell surface molecules either had no effect or suppressed with different kinetics. The antibody alone was not mitogenic for murine splenocytes but could enhance the mitogenic effect of the plant lectin, concanavalin A. Antibodies to an allotypic determinant on the T200 molecule, Ly 5.1, also had suppressive effects on proliferation and generation of cytotoxicity.

In sum, these results contribute to the body of evidence suggesting that activation and interactive events leading to immune function may require the participation of several major cell surface molecules, possibly through formation of membrane complexes.

INTRODUCTION

The manner in which cells are activated to participate in immune responses has not been clearly defined. The current dogma is that cells receive signals through molecules carried on their surfaces, i.e., membrane antigens. It is therefore logical to investigate the surface molecules on cells participating in immune responses in order to define mechanisms of activation and interactive events leading to immune function. An understanding of the role played by the relevant surface molecules in immune interactions would be a key step in the unraveling of processes which control immune function and would likely extend to other biological processes involving cell recognition and interaction such a embryological development and neural function. Preliminary to the understanding of the interactive mechanisms of these relevant molecules is the identification of the membrane antigens which are in fact involved in the immune functions to be studied. Thus, the major goal of this study was to apply a method of analysis, perturbation of membrane antigens by antibody, with the potential for identification of molecules relevant to function, to several major cell surface molecules of cells participating in in vitro models of immune function.

The first problem to be faced lies in defining the nature of immune responses to be investigated. Some form of defense mechanism for protection of the body against extracorporeal invasion has been

shown to exist in all vertebrate species studied and probably exists in all invertebrates as well, at least those of multicellular organization (13). Indeed, plants have been shown to possess systems of protection against pathogenic organisms that probably qualify as an immune response (1). The most intensively studied and best characterized animal system of immunity is that of the mouse. This system probably also represents the greatest level of complexity of immune response that has evolved so far. There is no reason to suspect from the data currently available that any animal, including humans with their self-proclaimed superior level of intellect, possesses a more sophisticated system for responding to challenge by foreign antigen than the mouse. The value of the mouse as an experimental model for problems of immunity in man becomes more apparent as more data on the two systems emerge. The differences in the way the immune response functions in mouse and man appear so minor as to be meaningless.

The acquired immune response (that aspect of immunity which must be stimulated by exposure to a foreign antigen) of mouse and man is expressed in two major ways (25). The first is the production of antibody, immunoglobulin reactive with specific antigens, which appears as a result of interaction of the initiating antigen with pre-programmed B lymphocytes which are then stimulated to clonal expansion and production of antibody. This antibody functions in the control and elimination of foreign antigen by increasing the susceptibility of the foreign material to destruction by phagocytic cells, complement-mediated inactivation, or loss of function through

interference with key structures for pathogenesis such as adherence pili of bacteria.

The second major arm of the immune response is cell-mediated immunity. In this form of immunity, specifically sensitized cells interact directly with foreign antigen to bring about its destruction or elimination, without the intervention of antibody. Examples of this are recognition and killing of cells infected intracellularly with viruses, or cells expressing tumor antigens as a result of malignant transformation. Other forms of cell-mediated immunity include hypersensitivity reactions resulting from release of soluble mediators by lymphocytes interacting with the sensitizing antigen. Allograft rejection is another manifestation of cell-mediated immunity in which artificially introduced tissues from another animal are recognized and reacted against by specifically reactive lymphocytes.

While the distinction is not absolute, especially since antibody production and regulation are known to involve interaction of several cell types, humoral (antibody-mediated) immunity is mainly the province of B (processed by the bursal equivalent) lymphocytes and cell-mediated immunity is the province of T (processed by the thymus) lymphocytes (18). Further discussion will center on T lymphocytes and their role in cell-mediated immunity, since these were the experimental models used in this study.

While the ultimate goal of studies in immunology is to relate the findings to events in the intact animal, it is often difficult or impossible to study narrowly defined phenomena in the face of the multitude of factors influencing the outcome of any manipulations

in vivo. For this reason much of the work in cellular immunology has centered on in vitro correlates of immune function. Probably the two most widely used of such models are the mixed lymphocyte culture (MLC) and the attendant generation of cytotoxic T lymphocytes (CTL). These reactions have long been felt to be reasonably accurate reflections of in vivo cellular responses to foreign antigen (11, 68). Since these two reactions were the basis for most of my experimental conclusions, I will attempt to briefly describe the interactions which are thought to be relevant to their manifestation.

A murine mixed lymphocyte culture is initiated by combining lymphoid cells of one mouse strain, designated A, with lymphoid cells of a second strain, B. Usually cells of strain B have been rendered immunologically unresponsive by radiation or chemical means, so the response in culture will be unidirectional. During the period of culture, cells of the responding strain react to the foreign antigen presented by the cells of the stimulating strain by undergoing enlargement (blastogenesis) and division (proliferation). A process of differentiation also occurs during this time which results in the production of a subset of cells which are capable of recognizing and killing cells bearing the stimulating antigens. This is the generation of cytotoxic T lymphocytes, CTL.

The measurement of these two parameters, proliferation and generation of cytotoxic cells, is a straightforward process. At any desired point in the course of the MLC, the culture can be pulse-labelled with a radioactive DNA precursor, usually tritiated thymidine (^{3}H-thymidine). This material will be incorporated into the newly

synthesized DNA of proliferating cells, and after washing away the unincorporated radioactive material, the amount of proliferation in a culture can be expressed as a function of the radioactivity incorporated into the cells over a given period of time. The relative amounts of cytotoxicity generated in the cultures can be measured by harvesting the cells from culture and exposing them to measured numbers of radioactively labelled target cells bearing the same antigens as the original stimulator cells in MLC. These targets are labelled with a substance, usually radiochromium (^{51}Cr) which is released into the supernatant upon lysis of the cell. After allowing the population of effectors to interact with the targets for a fixed length of time, the cells are pelleted by centrifugation and the radioactivity released into the supernatant can be measured as a relative indication of the cytotoxic potential of the effector cells.

The mechanisms by which these processes of proliferation and generation of cytotoxicity occur have been the subject of intensive investigation for many years in many laboratories. The currently accepted scenario is as follows. The gene products of the major histocompatibility complex (MHC) are thought to play key roles in the recognition and activation events resulting in immune functions both in vivo and in vitro. There are two main types of MHC antigens, class I, the serologically defined antigens of which K and D are thought to be the most relevant in murine MLC, and class II, the lymphocyte activating determinants or Ia antigens (30). In murine MLC, one of the initial steps is thought to be the presentation of foreign Ia antigens by adherent cells to a subclass of T lymphocytes

in the responding population, the T helper cell. This presentation of antigen triggers the helper cell to express receptors for a soluble mediator, interleukin-1 (IL-1) which is being produced by the adherent cell. The combination of these two signals, Ia antigen and IL-1, render the helper cell competent to produce a second mediator, interleukin-2 (IL-2, formerly known as T cell growth factor) which is directed towards a second subset of T cells, the cytotoxic cell precursor. This cell type is induced to express receptors for IL-2 by interaction with the class I (K,D) antigens of the stimulating cell population. These two signals, K,D antigen and IL-2 drive the cytotoxic cell precursor to differentiation as a CTL. Concurrent with these interactions is the clonal expansion of the cells being stimulated by these foreign antigens and soluble factors (44). It is important to remember that lymphocytes which are potentially reactive to all manner of class I and II antigens are initially present in a normal lymphoid cell population. It is the preferential stimulation and subsequent proliferation of the cells which can recognize the stimulating antigens that results in the generation of a large population of end stage cells capable of specifically killing target cells bearing the initial stimulating determinants. Apparently the soluble mediators IL-1 and IL-2 are not antigen specific in that they will stimulate any cells bearing receptors for them. The specificity arises at the level of the stimulating antigens which induce receptors for these mediators only on the cells which are initially specific for antigen. Resting, or unstimulated cells in the population do not

express receptors for IL-1 and IL-2 and therefore do not respond to their presence (34).

It has been further suggested that these two main subsets of T cells (helpers and precursor/cytotoxic cells) interacting in these reactions can be characterized by the expression of differentiation antigens on their surfaces. These antigens belong to the Lyt series of antigens and the ones receiving most attention have been Lyt 1 and Lyt 2,3. Immature T cells express all three antigens but as differentiation proceeds they sort into two main groups, one carrying Lyt 1 and losing 2,3 and another carrying Lyt 2,3 and expressing little or no Lyt 1 (9). Selective depletion experiments have identified the helper cell in MLC as Lyt 1 bearing and the cytotoxic cell and its precursor as Lyt 2,3 bearing (10). Recent work has indicated that this distinction may not be absolute depending on the type of stimulating antigens (49, 63) but the model at least provides a framework for thinking about cellular interactions in in vitro immune responses. Also arising during these interactions is another subclass of T cells, the suppressor cell (also bearing Lyt 2,3) which may serve to down-regulate the response of the other cells in the population (2).

The major piece missing from the puzzle of immune cell action in MLC is the nature of the structure which recognizes the stimulating antigens. Its existence must be assumed due to the exquisite specificity of the effector cells for the antigenic determinants of the initial stimulator cells. Years of intensive investigation by the worldwide community of immunologists have resulted in agreement on

one point; the T cell receptor is not the immunoglobulin molecule used by B cells for antigen recognition (27). Beyond this is chaos and the world awaits the discovery (and the discoverer) of the elusive T cell receptor.

A third widely used in vitro method of studying cellular immune functions is the use of polyclonal activators, or mitogens. These are substances, mainly plant lectins or bacterial cell wall products which, when added to populations of lymphocytes, stimulate the proliferation of a large portion of them without regard to their antigenic specificity (43). Through use of these substances, large numbers of cells in an activated state can be obtained for study without the need to build up an initially small fraction of cells reactive with a given specific antigen. It is thought that the molecular mechanisms of activation by antigen and by polyclonal activators are similar, and so this method has been widely used in attempts to elucidate the mechanisms by which lymphocytes are induced to proliferation and differentiation.

It was discovered early on that certain of these polyclonal activators preferentially stimulated either T or B lymphocytes (3, 26). Two of the most widely used are concanavalin A (Con A), a plant lectin that is specific for T cells, and lipopolysaccharide (LPS), a component of gram negative bacterial cell walls which preferentially stimulates B cells. These are the mitogens I used in my studies.

Now that the types of in vitro immune responses to be investigated have been defined, the next question is the method which

will be used to investigate the role of surface molecules in their manifestation.

One method for investigating the possible function of a membrane antigen is to include antibody to that antigen in functional assays of in vitro immunity. If the antigen to which the antibody is directed plays a role in the function being assayed, then the combination of the antibody with the antigen should alter or occlude the further participation of that antigen in the response, resulting in a perturbation of the end result. Thus, modulation of an in vitro immune function by antibody to a membrane antigen suggests a relationship between the function and the antigen.

It was through studies in which antibody to the Lyt 2,3 lymphocyte membrane antigens was used to block cytotoxic effector cell function that this molecule (these two antigens have been shown to be carried on the same molecule, ref. 37) was implicated as playing a role in T cell mediated cytotoxicity (23, 45, 59). Similarly, the involvement of both Lyt 1 and Lyt 2,3 in the proliferative response to alloantigens was suggested by the effects of antibodies to these molecules on MLC and the generation of cytotoxic cells (24, 46).

It should be emphasized that the studies described above and the work to be described in this thesis involve addition of antibodies in the absence of complement. If complement is present, the formation of the antigen-antibody-complement complex on the surface of the antigen bearing cell could result in the lysis of that cell and its removal from further participation in the response being assayed.

In fact, the addition of antibody in the presence of complement to MLC was the method used by Cantor and Boyse (10) to define the interactions of the subsets of lymphocytes in MLC and generation of cytotoxic cells, i.e., they removed entire subsets (Lyt 1+ or Lyt 2,3+ cells) by complement mediated lysis and then looked at the effect on function. In studies with antibody addition in the absence of complement, no subsets of cells are removed entirely from the reaction. Rather, specific molecular interactions are selectively perturbed by the interaction of the antibody with its cognate antigen, and thus we can now look beyond the cellular requirements for immune interaction and begin to examine the molecular requirements.

At this point it is also pertinent to discuss briefly the differences between conventional and monoclonal antibodies. Conventional antibodies are produced by injecting a substance, the immunogen, into an animal which will see it as foreign and make an antibody response to it. Usually a series of injections are performed in order to raise the amounts of circulating antibody in the blood of the recipient to high levels. Serum can then be harvested from the animal, and if desired, enriched for immunoglobulin through various biochemical purification steps. By using purified antigen preparations for the injections, it is possible to elicit antibody which will be relatively specific for the immunogen. However, the antibody obtained in this manner will always be heterogeneous with respect to antibody subclass and relative binding affinity since it is the product of a number of B cell clones which were able to react with the stimulating antigen (22).

A monoclonal antibody avoids this problem since it is by definition the product of one clone of antibody-secreting cells and is therefore homogeneous. A recipient animal is primed with antigen as in the production of conventional antibody, but then lymphoid cells, usually splenocytes, are removed from the recipient and fused to an appropriate continuous cell line. A successful fusion results in a cell with the antibody-secreting characteristics of the lymphoid parent and the ability to grow in continuous culture inherited from the other parent cell line (31). These fused cells, or hybridomas, can be cloned by limiting dilution methods to assure origin from a single fusion, and tested for their ability to secrete antibody specific for the desired antigen. Once the hybridoma clones of interest are identified, they can be expanded to huge numbers in tissue culture or appropriate carrier animals and correspondingly large amounts of monoclonal antibody can be collected from culture supernatants or ascitic fluid. If so desired, these preparations can then be purified to give homogeneous monoclonal antibody to defined antigens (39).

I will now turn to a discussion of the membrane antigens I chose to examine by the method of antibody addition to functional assays (see Appendix B) and a summary of the results I obtained.

One of the markers that distinguishes murine T cells from other lymphocytes is the Thy 1 antigen (52). This antigen is expressed on virtually all cells undergoing processing in the thymus and continues to be expressed after these cells are exported to the peripheral tissues. It is not expressed on B cells. While the Thy 1

antigen defines a set of lymphocytes, it is not restricted to these cells. It is found on many other cell types including neuronal cells in the brain (53).

It is reasonable to assume that the Thy 1 antigen is present on cell surfaces for reasons other than as a convenient marker for immunologists. Its distribution on various cell types further suggests a role that is not restricted to immune function, for example, cell-cell recognition, or activation events common to many cell types. Previous studies have suggested that antibodies to murine Thy 1 or its homolog in human systems may perturb immune function (12, 48, 51). These findings have not been consistent, however, and the role of Thy 1 in the immune response is still not clearly defined.

A portion of my studies deals with the effect of additon of monoclonal anti-Thy 1 on the generation of cytotoxic cells. My results suggest that this antibody could reduce the in vitro generation of cytotoxic T lymphocytes against allogeneic targets in some cases but not in others. The manifestation of this blocking effect appeared to be related to the degree of genetic disparity between responding and stimulating cells in the MLC.

Another cell surface molecule which appears likely to play a role in immune functions is the T200 molecule. This molecule has been shown to carry non-polymorphic determinants (T200 antigens) as well as at least one set of allelic determinants (Ly 5.1 and Ly 5.2) (50, 60). The molecule has been shown to be present on a wide range of cells of hematopoietic lineage, including lymphocytes participating in immune responses (56, 64, 69). Previous studies have suggested that movement

of T200 in the cell membrane may influence movement of other molecules including those coded for by the MHC (5), and that binding of T200 by antibody can block the activation of lymphocytes by rabbit anti-mouse brain serum (38). Other work indicates involvement of T200 in the response to mitogens (15, 19).

Previous investigations using the technique of antibody addition to functional assays have indicated a significant role for the T200 molecule. Antibody to an allelic determinant (anti-Ly 5.1) has been reported to block the effector phase of T cell-mediated cytotoxicity by some workers (45) but not by others (41). This alloantiserum has also been reported to reduce cytotoxicity mediated by natural killer cells (6, 29). Robin Small at Montana State University has shown that antiserum to the Ly 5 antigen can block antibody-dependent cellular cytotoxicity, and Sandra Ewald and Pamela Refling at MSU have demonstrated a reduction in oxidation-induced activation of lymphocytes in the presence of anti-Ly 5 (unpublished observations).

I have studied the effects of addition of antibodies to both allelic and nonpolymorphic determinants of the T200 molecule on in vitro immune responses. I have found that a monoclonal antibody to a nonpolymorphic determinant (anti-T200) modulated proliferation in MLC and the generation of allospecific cytotoxic cells. These modulations were seen as a late suppression of both responses, in some cases preceded by enhancement. The kinetics of this suppression differed from those seen with addition of anti-Lyt 2, and no suppressive effects were seen with monoclonal antibodies to other cell surface

molecules. In addition, I found that the mitogenic response to Con A, a T cell mitogen, appeared to be enhanced by the presence of monoclonal anti-T200, while the response to LPS, a B cell mitogen, was unaffected or even slightly suppressed.

The mechanism by which antibody to this cell surface molecule modulates immune responses remains unknown. In a preliminary investigation of the role played by soluble mediators, I did find that levels of IL-2 were reduced in the supernatants of MLC to which anti-T200 had been added.

I further demonstrated that conventionally produced antiserum to an allodeterminant of the T200 molecule, anti-Ly 5.1, suppressed the generation of cytotoxic cells in a restricted manner, i.e., these effects were seen only when the responding cell population carried the Ly 5.1 allele. Due to the problems inherent in the use of antiserum for these investigations, particularly the modulating effects of normal serum on MLC, I further characterized and extended these findings by use of a monoclonal antibody to Ly 5.1. In a series of preliminary experiments I found that the monoclonal anti-Ly 5.1 suppressed the proliferative response of responding cells in MLC. These assays were done with congenic mouse strains which differed only in their expression of the Ly 5 alloantigen. The anti-Ly 5.1 showed suppression of proliferation only to the responders carrying the Ly 5.1 allele. No effects were seen on the congenic responders carrying the Ly 5.2 allele, with which the antibody would not be expected to react. At the concentrations of monoclonal anti-Ly 5.1 I used in these experiments,

no effects were seen on the generation of cytotoxic effector cells by either responder population.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2^b), BALB/c (H-2^d), CBA/J (H-2^k), SJL/J (H-2^s), and A.SW (H-2^s) mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and bred in our laboratory. H-2 recombinant mice, B10.A (H-2^a), B10.A(2R) (H-2^{h2}), and B10.T(6R) H-$2^{\gamma 2}$) were kindly provided by Dr. Jack Stimpfling, McLaughlin Institute, Great Falls, Montana. C57BL/6-Ly 5.2 congenic mice were obtained from Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York. Athymic (nu/nu) mice on the BALB/c background were obtained from the colony maintained at Montana State University. Mice of both sexes were used at 6 to 30 weeks of age.

Cell Lines

Several cultured tumor cell lines were used as stimulators in MLC and as targets for ^{51}chromium release assays. These were the P815 mastocytoma of DBA/2 (H-2^d) origin, El-4 lymphoma of C57BL/6 (H-2^b) origin, and 2PK3, a B cell lymphoma of BALB/c (H-2^d) origin. The cell lines were maintained in vitro by passage in RPMI 1640 (Irvine Scientific, Santa Ana, California) supplemented with 10% heat inactivated fetal calf serum (Sterile Systems Inc., Ogden Utah) and 2 millimolar (mM) L-glutamine (Irvine) hereafter referred to as complete medium.

Antibodies

Hybridoma cell culture numbers 30-G12 (antiT200), 30-H12 (anti-Thy 1.2), and 30-C7 (anti-LGP100a) were obtained from Dr. Noel Warner, University of New Mexico, Albuquerque, New Mexico and were maintained in vitro in complete medium. These cell lines produce rat monoclonal antibodies of subclass IgG_{2a} (anti-T200 and anti-LGP100a) or IgG_{2b} (anti-Thy 1.2) and have been described in detail elsewhere (36). See also Appendix B. Supernatant fluid was collected from the hybridoma cultures, pooled, and precipitated with 50% ammonium sulfate. The precipitate was centrifuged, resuspended, and dialyzed against Tris (hydroxymethyl)-aminomethane hydrochloride (Tris HCl) buffer (Sigma Chemical Co., St. Louis, Missouri) at pH 8.0, then passed over a (diethylaminoethyl)-Sephacel (DEAE-Sephacel, Pharmacia, Piscataway, New Jersey) column as previously described (4). Fractions were eluted with a stepwise gradient of sodium chloride (NaCl) in Tris HCl buffer beginning with 0.05 molar (M) NaCl and proceeding in 0.05 M increments. The IgG antibody was recovered in the initial 0.05 M NaCl fraction, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, ref. 33) and indirect fluorescent antibody testing (IFA, ref. 28). The antibody-containing fraction was then concentrated and dialyzed against RPMI 1640 without fetal calf serum, sterilized by membrane filtration, and aliquots stored frozen until use in functional assays.

Monoclonal antibodies anti-$H2K^k$ and anti-Lyt 2 were purchased as purified antibody preparations from Becton-Dickinson, Sunnyvale,

California. Anti-H2K^k (clone 11-4.1) is a mouse IgG_{2a} antibody and anti-Lyt 2 (clone 53-617) is a rat IgG_{2a} antibody. These commercial antibody preparations were dialyzed against RPMI, filter sterilized and frozen in small aliquots for use in assays. All of the above monoclonal antibody preparations were adjusted with medium to give equivalent protein concentrations of approximately 250 micrograms per milliliter (µg/ml) and were added to assays at 2-4% of the total volume in the well unless noted otherwise.

Conventional anti-Ly 5.1 serum was produced by immunizing SJL/J mice (Ly 5.2) with spleen, thymus, and lymph node cells of A.SW mice (Ly 5.1) as described by Komuro et al. (32, 58). Serum was harvested by tail bleeding of hyperimmunized SJL/J mice and was filter sterilized and heat-inactivated at 56°C for 30 minutes before addition to assays. This antiserum had a titer of 1:160 by complement-mediated microcytotoxicity testing on BALB/c thymocytes and was negative against SJL/J thymocytes. Normal mouse serum (NMS) was collected from the appropriate unimmunized strains of mice, sterilized, and heat inactivated before use.

Monoclonal anti-Ly 5.1 was purchased from New England Nuclear, Boston, Massachusetts as a crude hybridoma preparation in ascites fluid (NEI-020). I purified this material by column chromatography with DEAE Affi-Gel Blue (Bio-Rad, Richmond, California) following previously published procedures (7). In brief, the crude preparation was diluted tenfold with 0.02 M Tris HCl buffer at pH 7.2 and then dialyzed overnight against the same buffer. The dialyzed material was loaded on to the column with several volumes of starting buffer.

Fractions were eluted with a stepwise gradient of NaCl in starting buffer at concentrations of 0.025 M, 0.05 M, and 0.1 M NaCl. Immunoglobulin was eluted at both 0.025 M and 0.05 M NaCl as determined by IFA and SDS-PAGE stained by the silver stain method (40). These fractions were dialyzed against RPMI without fetal calf serum, filter sterilized, and stored frozen in small aliquots for use in functional assays.

Mixed Lymphocyte Cultures

Mice were killed by cervical dislocation and the spleens removed aseptically into complete medium plus 10 mM N-2-hydroxyethyl-piperazine N'-2-ethanesulfonic acid (HEPES) (Sigma), 5×10^{-5} M 2-mercaptoethanol (Bio-Rad), 100 Units/ml penicillin, 75 micrograms (µg)/ml streptomycin and 2.5 µg/ml fungizone (Irvine). This was the medium used for all MLC, CML, mitogen stimulation, and soluble factor assays. Single cell suspensions of splenocytes were prepared by repeated passage through a 10 ml syringe and 22 gauge needle and erythrocytes were removed by hypotonic lysis with distilled water. Cells were then centrifuged and resuspended in medium. Stimulator splenocytes were adjusted to a concentration of 2×10^{7} cells/ml and incubated with 30 µg/ml of mitomycin C (Sigma) for 1 hour at 37°C in 5% carbon dioxide (CO_2) air mixture followed by three washes through fetal calf serum. After the final wash the cells were resuspended in medium. Responder splenocytes were prepared in a similar manner but the mitomycin C treatment was omitted.

Cultures were established in 96-well flat-bottom microplates (Linbro ISFB96TC) with either 2 x 10^5 responding cells and 6 x 10^5 stimulating cells (1:3 ratio) or 3 x 10^5 cells of each type (1:1 ratio) in a total volume of 200 microliters (µl)/well. Cultures were replicated a minimum of three times.

Proliferation was assessed at the times indicated by adding 1 microcurie (µCi)/well of tritium labelled thymidine (^{3}H-thymidine) (New England Nuclear) and incubating for an additional 4-5 hours. The wells were harvested by aspirating the contents onto glass fiber filter strips (Whatman GF/A) with a multiple automated sample harvester (Otto Hiller, Madison, Wisconsin, ref. 21) followed by extensive rinsing with distilled water to lyse the cells and remove unincorporated ^{3}H-thymidine. The glass fiber filter discs, containing the nuclear material from the cultured cells, were then dried, removed from the filter strips, and the incorporated radioactivity was measured by liquid scintillation counting in Aquasol (New England Nuclear) on a Beckman LS100C scintillation counter (Beckman Instruments, Palo Alto, California).

Cytotoxicity Assays

Effector cells were generated in primary MLC by combining splenocytes of responding mice at a concentration of 2 x 10^6 cells/ml with mitomycin C treated stimulator splenocytes (2-6 x 10^6 cells/ml) or mitomycin C treated tumor cell stimulators (6.7 x 10^4 cells/ml). Tests were performed in tissue culture flasks (Falcon 3013) at 20 ml total volume, 24-well tissue culture plates (Falcon 3047) at 2 ml

volume, or 96-well V-bottomed microtiter plates (Linbro 76-023-05) at 0.2 ml total volume/well. Cells were harvested at the times indicated by extensive scraping and resuspension with a disposable plastic pipette. These cell suspensions were washed and resuspended in medium. The cells were then assayed for cytolytic activity in a modified version of the ^{51}chromium release assay as described by Brunner et al. (8). A brief description follows. Serial dilutions of effector cells were made in 96-well V-bottomed microtiter plates. Effector cells were in a final volume of 100 µl/well. Target cells were incubated with 250 µCi ^{51}chromium (New England Nuclear) for 1 hour at 37°C in fetal calf serum, washed twice to remove residual unbound ^{51}chromium, and added to effector cell dilutions at a concentration of 10^4 cells in 100 µl of medium. Plates were centrifuged at 200 times gravity for 10 minutes and incubated 4 hours at 37°C in 5% CO_2/air. Plates were then recentrifuged at 250 times gravity for 10 minutes at 4°C. Without disturbing the cell pellets, 100 µl of supernatant was removed from each well for counting in a Beckman Biogamma counter (Beckman Instruments). Data are expressed as percent specific cytotoxicity calculated as follows:

$$\frac{\text{Experimental } ^{51}\text{Cr release} - ^{51}\text{Cr release in medium alone}}{\text{Total } ^{51}\text{Cr release in detergent} - ^{51}\text{Cr release in medium}} \times 100$$

The data are presented as the means of triplicate samples. Standard deviations were in all cases less than 5% of the mean.

Mitogen Stimulation Assays

Splenocytes were prepared similarly to the methods used for MLC. Responders were suspended in medium and added to 96-well flat bottom microplates at a concentration of 2-3 x 10^5 cells/well. Total volume of medium in the wells was 200 µl. Concanavalin A (Miles Yeda, Rehovot, Israel) was added to the wells at concentrations of 0.33 to 5 µg/ml. Bacterial lipopolysaccharide (LPS) was generously provided by Dr. J. A. Rudbach, University of Montana, Missoula, Montana. It was added to appropriate wells at concentrations of 2.5 to 10 µg/ml. Cultures were incubated for 48 hours and then pulse labelled with 1 µCi of ^{3}H-thymidine/well. After an additional 5 hours of incubation, wells were harvested and the incorporated radioactivity of the cells was measured by liquid scintillation counting. Data are expressed as the means of the incorporated radioactivity measured in triplicate samples.

Soluble Factor Assays

Culture supernatants from MLC were assayed for the presence of Interleukin-2 following the procedure of Gillis et al. (16). In brief, 100 µl of serially diluted supernatant from MLC was mixed with an equal amount of medium containing 4 x 10^3 CTLL-2 cells. These cells (obtained from Dr. Paul Baker, Veterinary Research Laboratory, Montana State University, Bozeman, Montana) are a cloned T cell line totally dependent upon the presence of IL-2 for their continued proliferation in culture. The mixtures of cells and supernatants were incubated in 96-well microplates for 24 hours and then given a 4 hour pulse of

^{3}H-thymidine (1 μCi/well). The wells were then harvested and the incorporated radioactivity of the CTLL-2 cells was measured by liquid scintillation counting. Amounts of IL-2 in the supernatant being assayed are expressed in arbitrary units defined by probit analysis of the ^{3}H-thymidine incorporation stimulated by the test supernatant relative to the incorporation induced by a standard preparation of IL-2 (16).

RESULTS

Effect of anti-Thy 1.2 on the Generation of Cytotoxic Cells

C57BL/6 spleen cells (H-2^b) when cultured as responding cells to P815 tumor cell stimulators (H-2^d) showed reduced generation of cytotoxic effector cells as measured against P815 targets when anti-Thy 1.2 (without complement) was added at the initiation of MLC (Fig. 1). In contrast, C57BL/6 responders to BALB/c spleen cell stimulators (H-2^d) generated an equal level of cytotoxicity to P815 targets in either the presence or absence of anti-Thy 1.2 in MLC (Fig. 2). In these and other experiments, it should be noted that no significant cytotoxicity was seen against EL-4 tumor cell targets (H-2^b), thus demonstrating the specificity of the effector cells for the priming alloantigens.

This first series of experiments compared the effects of the anti-Thy 1.2 on generation of cytotoxic cells against tumor cell stimulators or splenocyte stimulators. Since tumor cells differ from normal splenocytes in a multitude of ways, the differences I saw in the blocking effects of anti-Thy 1.2 between these two systems could therefore be due to a multitude of causes. In order to more critically examine the blocking phenomenon, I next compared the results of stimulation of responders by two different tumor cell lines. As shown in Fig. 3 and Fig. 4, anti-Thy 1.2 reduced the cytotoxicity against P815 targets when the initial stimulation came from P815 but

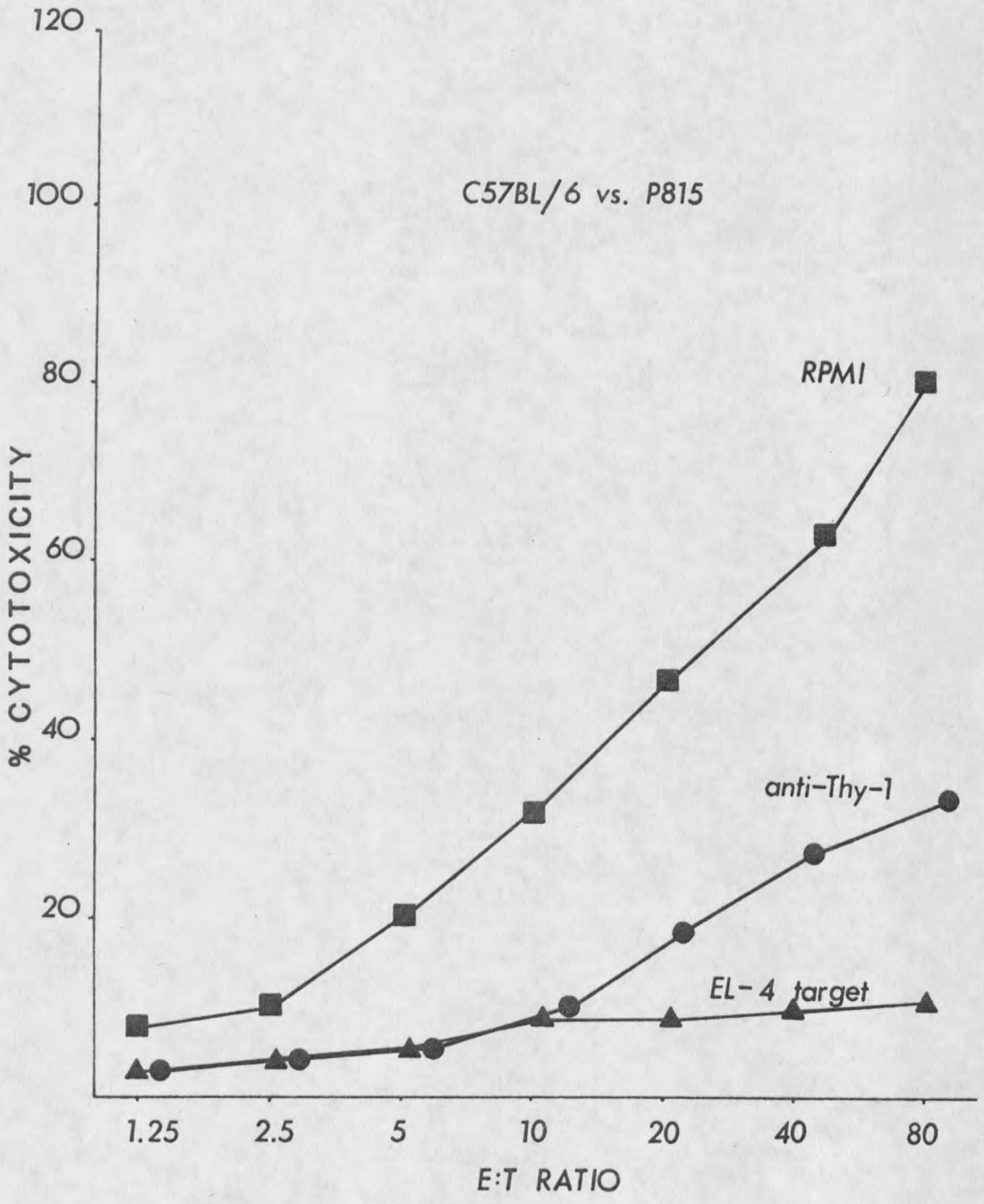


Figure 1. Modulation of the generation of cytotoxic effector cells by anti-Thy 1; 3 x 10^7 C57BL/6 splenocytes stimulated in MLC with 10^6 mitomycin C treated P815 cells (see text). Effectors were harvested at day 3 of MLC and assayed for cytotoxicity against P815 target cells. ■ represent cytotoxicity generated in cultures with no antibody added, ● cultures receiving 9% v/v anti-Thy 1 at initiation, ▲ cytotoxicity of cultures with no antibody added, against a non-specific target, EL-4.

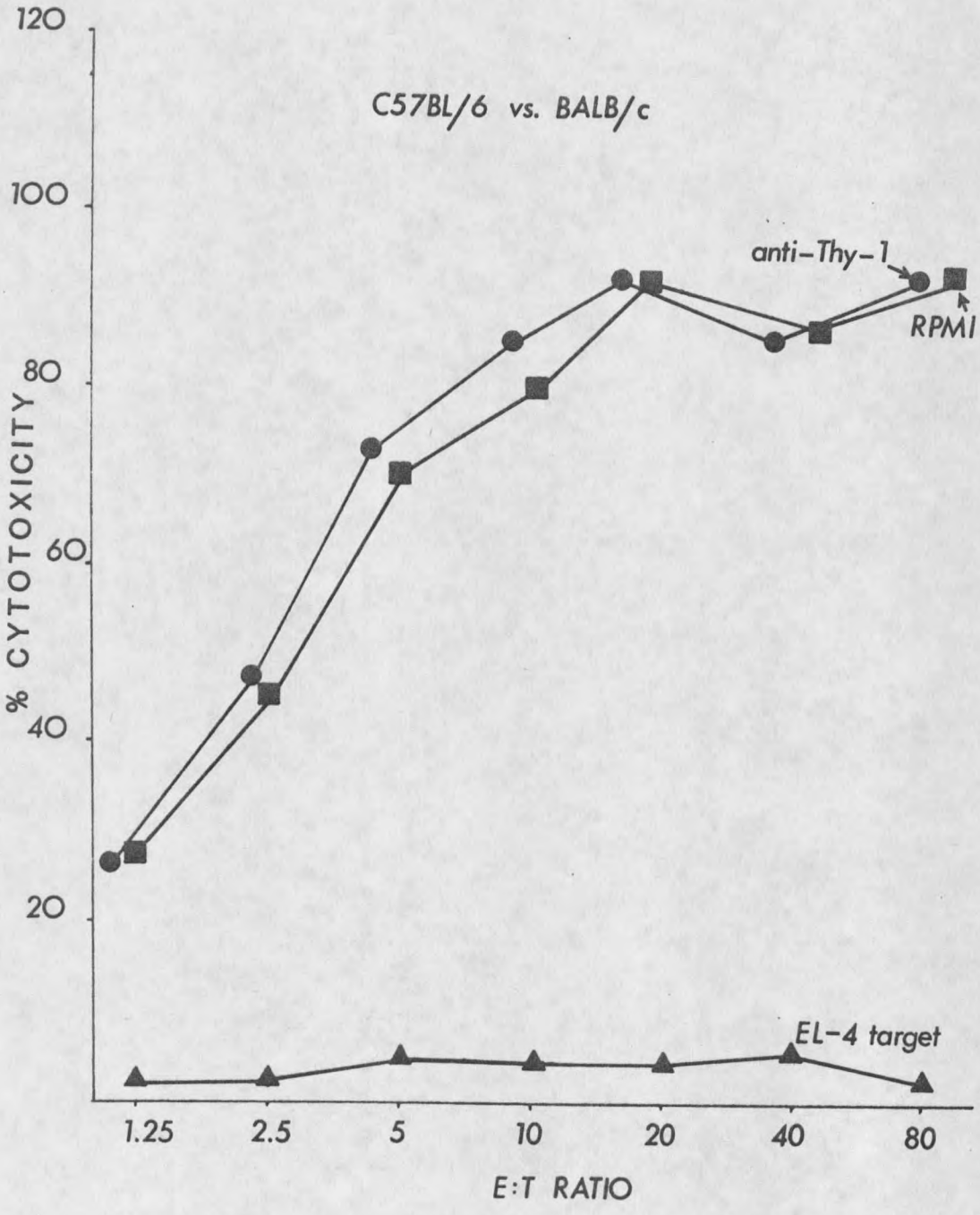


Figure 2. Legend similar to Figure 1 except C57BL/6 splenocytes were stimulated in MLC with 2.6 x 10^7 mitomycin C inactivated BALB/c splenocytes. 10% v/v antibody was added to the culture receiving anti-Thy 1.

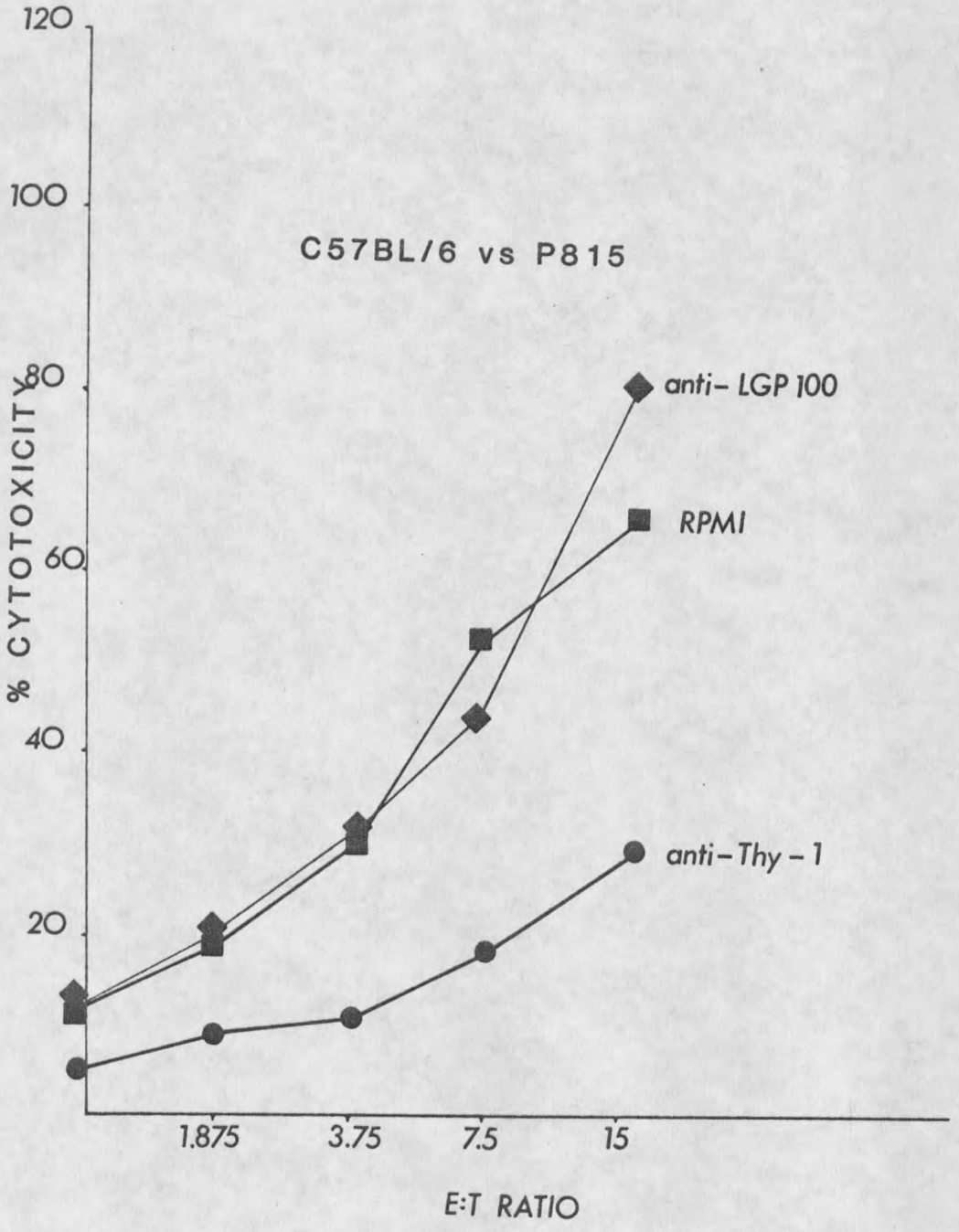


Figure 3. Modulation of the generation of cytotoxic effector cells by anti-Thy 1; 3 x 10^5 C57BL/6 stimulated with 10^4 P815 cells in 96-well microtiter plates as described in the text. Effectors were harvested at day 5 of MLC and assayed for cytotoxicity against P815 targets. ■ represent cytotoxicity generated in the absence of antibody, ● in the presence of 7.5% v/v anti-Thy 1, ◆ in the presence of 7.5% v/v anti-LGP100a.

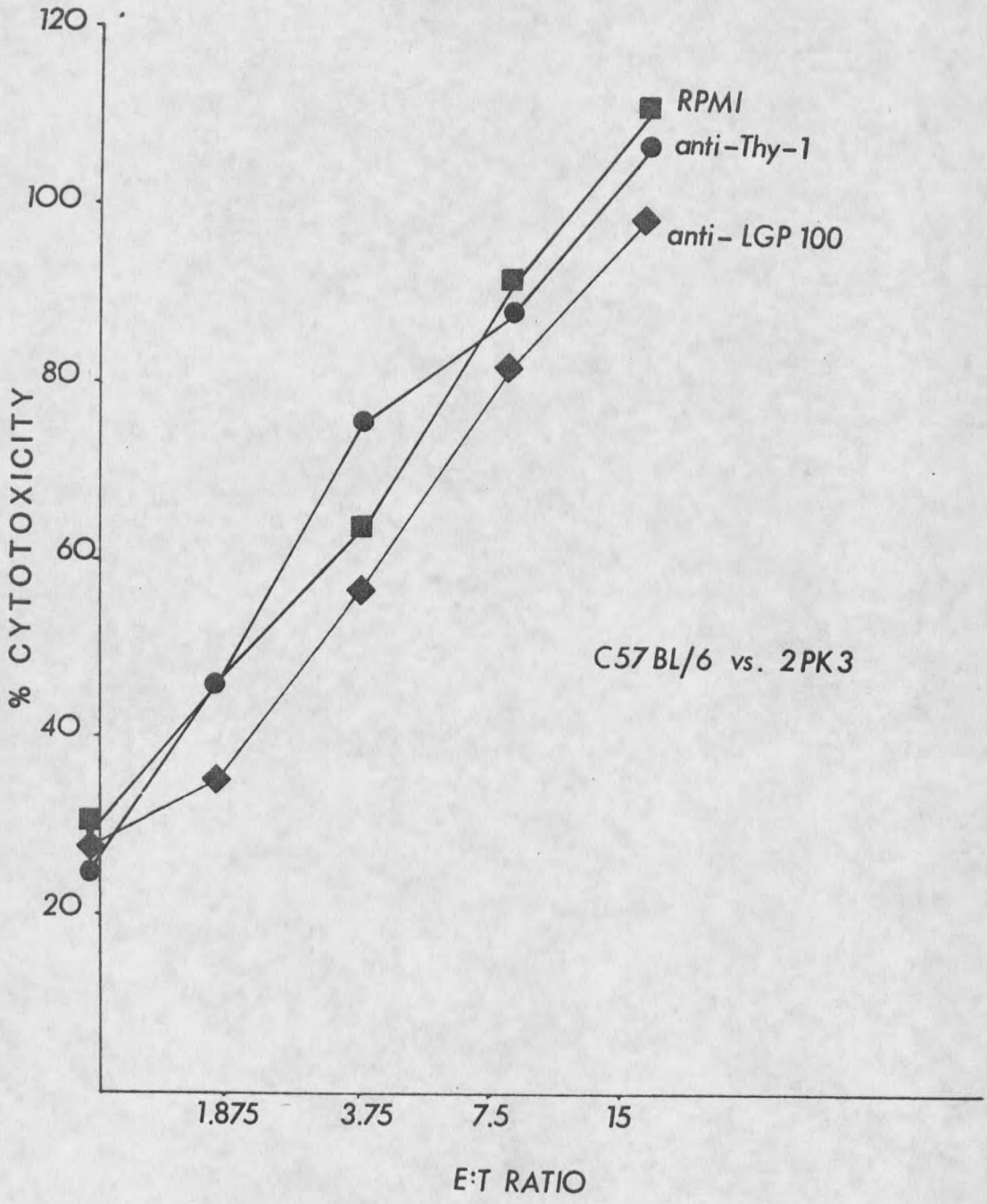


Figure 4. Legend similar to Figure 3 except C57BL/6 responders were stimulated with 10^4 2PK3 tumor cells.

not from 2PK3 tumor cells. It must be remembered, however, that while these two tumor cell lines share the same major histocompatibility determinants ($H\text{-}2^d$) there are still multiple differences between them, including the strains of origin (DBA/2 for P815 and BALB/c for 2PK3). Also seen in Figs. 3 and 4 is the lack of any significant effect on generation of cytotoxic cells when anti-LGP100a hybridoma antibody, carried through the same purification protocol as anti-Thy 1.2, is added to the MLC. This argues against the blocking effect of anti-Thy 1.2 being due to non-specific factors or the effects of column effluent from the purification procedure.

This second series of experiments still had the problem of comparing results of experiments in which tumor cell lines with multiple differences between them were used as stimulators for the generation of cytotoxic cells. In order to circumvent this problem, I next performed experiments using splenocyte stimulator cells from congenic mouse strains wherein the expression of cell surface antigens responsible for the stimulation of the responder cells could be more clearly defined. Since one of the major differences between the two tumor cell lines I used for stimulator cells in the earlier experiments involved the expression of Ia antigens (P815 is a low expressor and 2PK3 is a high expressor of Ia), I performed experiments using strains of congenic mice with recombinant H-2 haplotypes (Table 1) in which recognition of I region differences could be regulated by manipulation of responder and stimulator strain combinations. B10.A(2R) splenocytes recognize only a disparity at the D region of the MHC when stimulated by B10.A cells. With this combination of responder and stimulator

cells, anti-Thy 1.2 significantly reduced the generation of cytotoxic effectors as measured against P815 targets (Fig. 5). In contrast, B10.A(2R) responder cells stimulated by B10.T(6R) cells recognize a disparity across the entire MHC including the region coding for Ia. When this strain combination was used, no blocking was seen by anti-Thy 1.2 (Fig. 6).

Table 1. H-2 Genotypes of Mouse Strains Employed

	H-2 Region							
Strain	K	IA	IB	IJ	IE	IC	S	D
C57BL/6	B	B	B	B	B	B	B	B
BALB/c	D	D	D	D	D	D	D	D
DBA/2	D	D	D	D	D	D	D	D
B10.A(2R)	K	K	K	K	K	D	D	B
B10.A	K	K	K	K	K	D	D	D
B10.T(6R)	Q	Q	Q	Q	Q	Q	Q	D

Influence of anti-T200 on Proliferative Response in MLC

The addition of monoclonal anti-T200 without complement at the initiation of MLC resulted in a marked suppression of proliferation late in the response. In some experiments this effect was preceded by an early enhancing effect which was often quite striking (Fig. 7). This pattern of modulation of proliferation by anti-T200 was seen with several different strain combinations of responder and stimulator cells involving various degrees of genetic relatedness (Fig. 8). The manifestation of the late suppressive effect was not dependent upon the early enhancement, since some assays failed to show early increases of proliferation in response to the anti-T200 but still showed strong

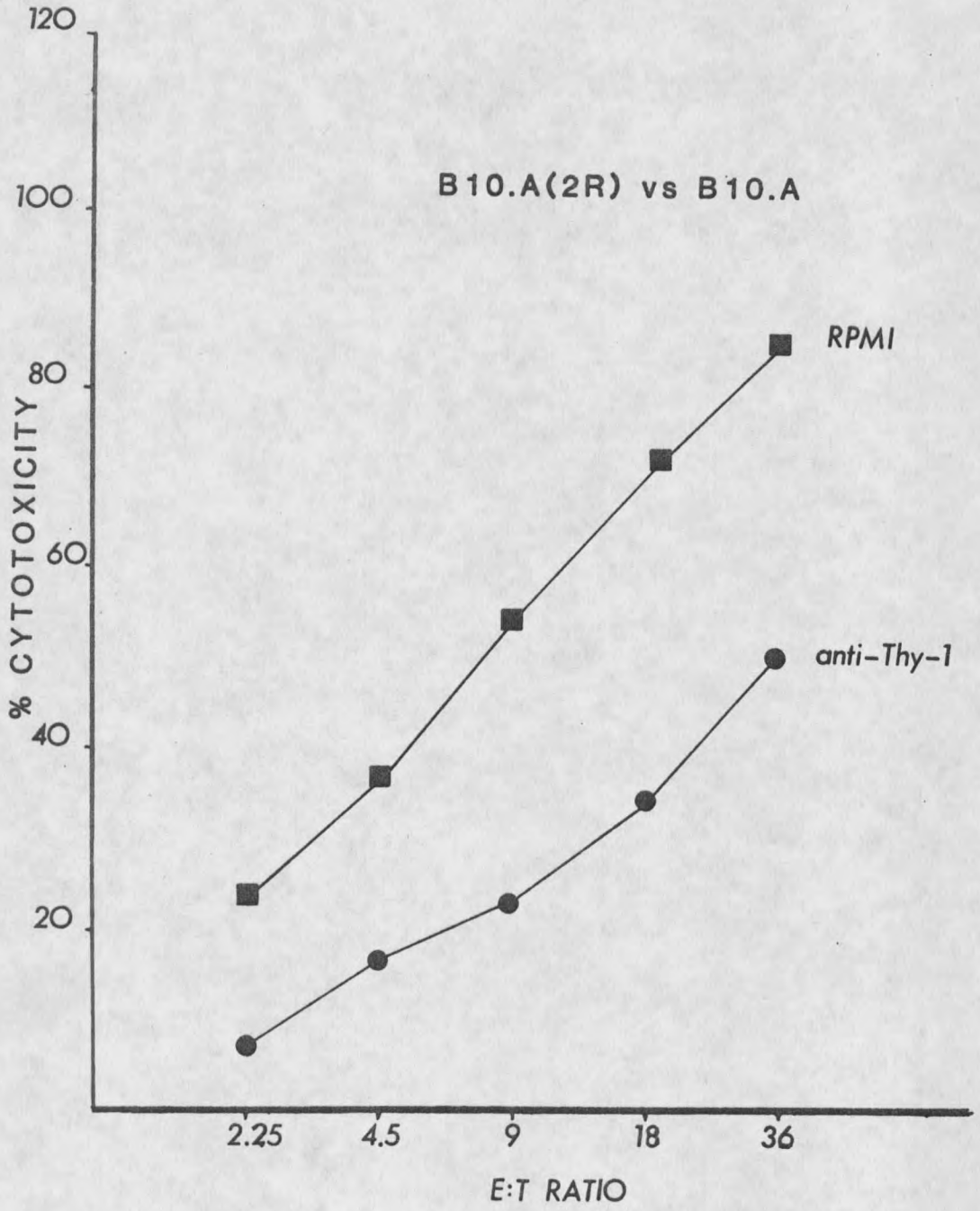


Figure 5. Modulation of the generation of cytotoxic effector cells by anti-Thy 1; B10.A(2R) splenocytes (H-2 haplotype k/d/b) responding to B10.A stimulators (H-2 haplotype k/d/d) at a 1:1 ratio in MLC. ■ represent cytotoxicity generated in cultures with no antibody added, ● cultures with 10% v/v anti-Thy 1 added at time 0, both measured atainst P815 targets ($H\text{-}2^d$) as described in the text.

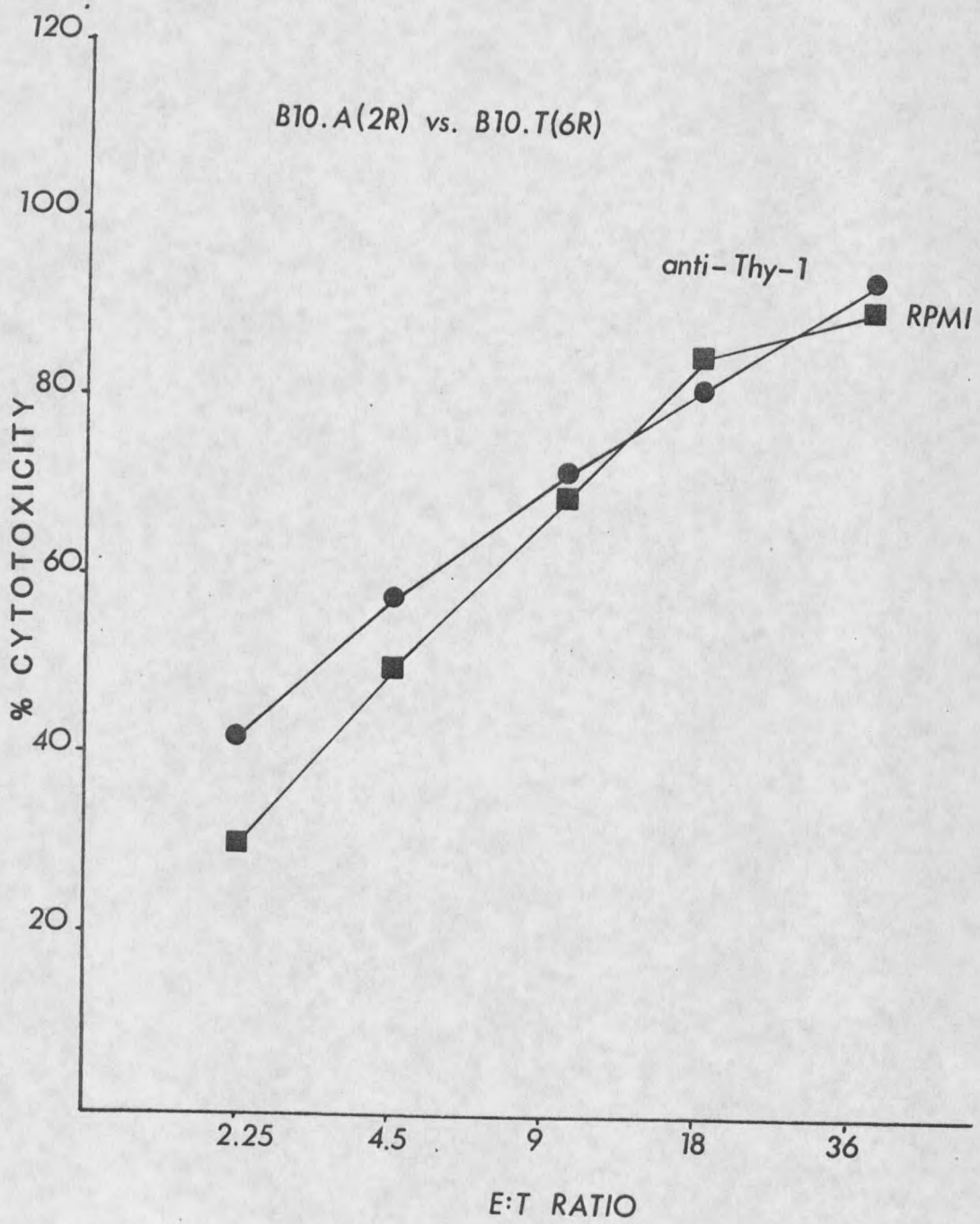


Figure 6. Legend similar to Figure 5 except B10.A(2R) splenocytes (k/d/b) were stimulated with B10.T(6R) splenocytes (q/q/d).

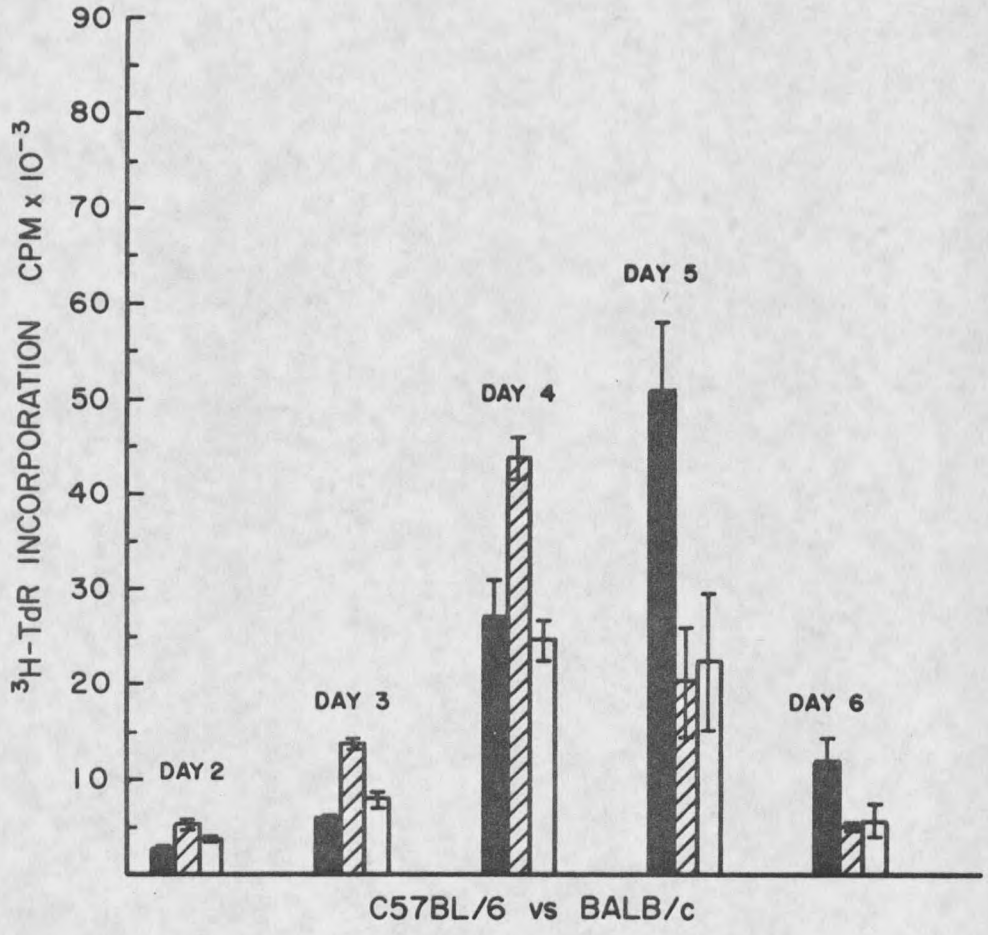


Figure 7. Proliferative response in MLC of C57BL/6 splenocytes stimulated with mitomycin C inactivated BALB/c splenocytes (at a 1:3 ratio) in the absence of antibody (solid bars), with anti-T200 added at the initiation of cultures (hatched bars), or with anti-T200 added 24 hours after initiation of cultures (open bars). See text for details.

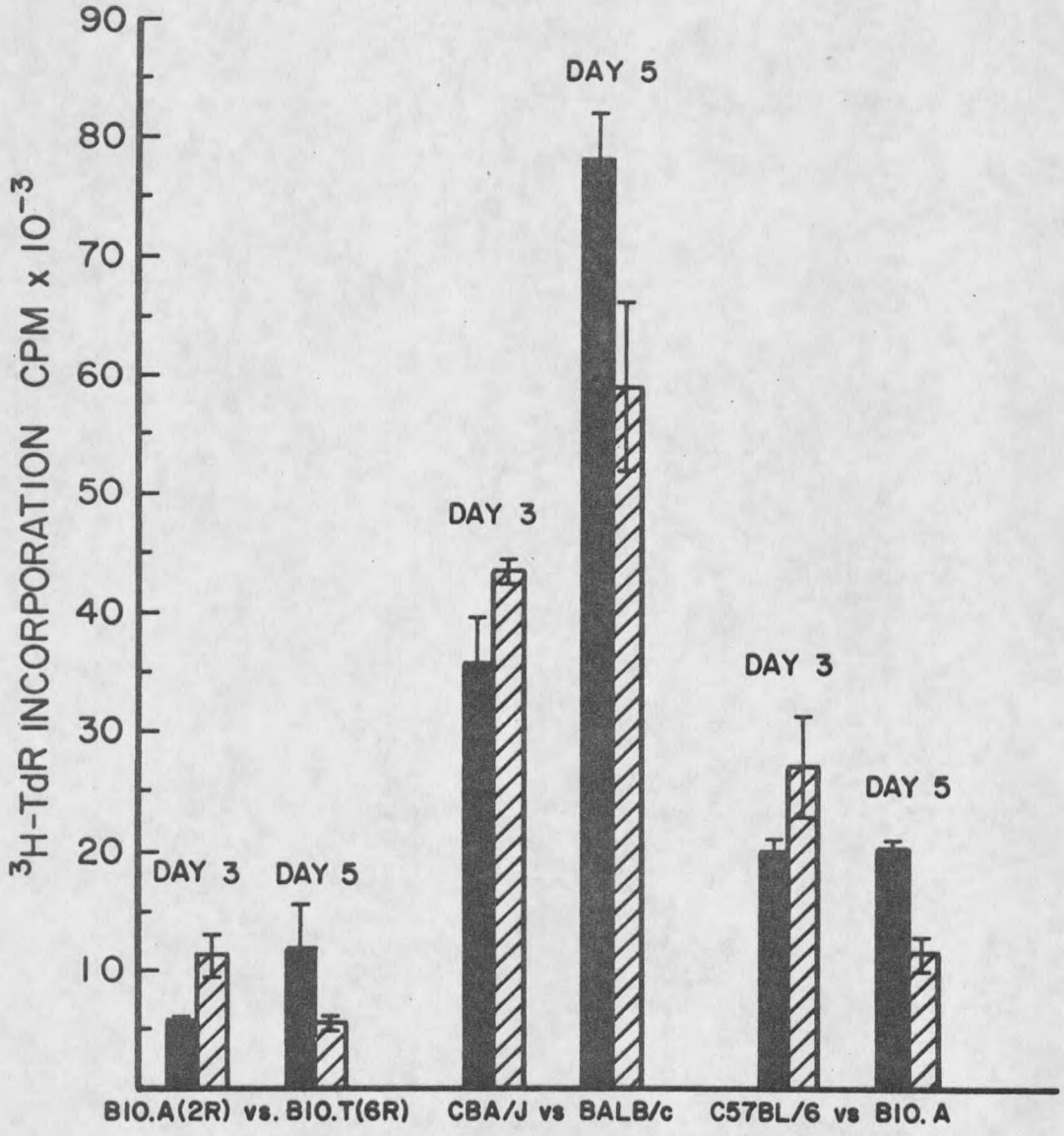


Figure 8. Effect of anti-T200 antibody on MLC cultures initiated with various combinations of responding and stimulating cells. Cultures were set up with a 1:3 ratio of responders to stimulators. Solid bars represent proliferation in cultures without antibody; hatched bars cultures with antibody added at time 0 of MLC.

suppression by day 4 or 5 of MLC (data not shown). Further evidence for the separation of the two effects was given by experiments in which antibody was added 24 hours after the start of the culture. This protocol eliminated the early enhancement but the late suppressive effect remained intact (Fig. 7).

Since the addition of anti-T200 to MLC was seen in many cases to cause an early increase in proliferation, I wished to exclude the possibility that the antibody was acting as a mitogen for the responding cells rather than augmenting proliferation in response to alloantigen. I set up parallel cultures in which the anti-T200 was added to responder cells in the absence of allogeneic stimulators. As seen in Table 2, anti-T200 had no enhancing effect on the proliferation of responders cultured in the absence of alloantigen on any of the days tested.

Effect of anti-T200 on the Generation of Cytotoxic Cells

Monoclonal anti-T200 without complement was added at the initiation of MLC and effector cells were harvested at the times indicated for testing in the ^{51}Cr release assay. As shown in Fig. 9, the effect of anti-T200 on generation of cytotoxicity against allogeneic targets was similar to the effect on proliferation seen in the previous series of experiments, that is, early enhancement followed by suppression. Again, as with modulation of proliferation in MLC, the late suppression of the response was the more consistent effect. The degree of this suppression appeared to be related to the genetic origin of the responder and stimulator populations which were

Table 2. Effects of anti-T200 on Responder Cells in the Absence of Alloantigen[a]

Responder	Stimulator	anti-T200	CPM[b] day 3	CPM[b] day 4	CPM[b] day 5
C57BL/6	-	-	961±64	1,096±143	705±141
C57BL/6	-	+	773±187	704±84	805±121
C57BL/6	BALB/c	-	7,168±751	21,999±1687	31,330±3427
B10.A(2R)	-	-	618±47	1,371±394	1,447±191
B10.A(2R)	-	+	613±114	1,036±160	1,133±124
B10.A(2R)	B10.T(6R)	-	5,631±436	15,044±2,027	11,845±3,643
CBA/J	-	-	7,954±498	10,708±908	11,997±2,159
CBA/J	-	+	5,318±672	7,657±1208	7,350±999
CBA/J	BALB/c	-	35,911±3,656	61,132±4,766	78,121±4,216

[a]Cultures set up in 96 well microplates (see text) with 2 x 10^5 responders and 6 x 10^5 stimulators (where present).

[b]Counts per minute ^{3}H-thymidine incorporation, Mean±SD

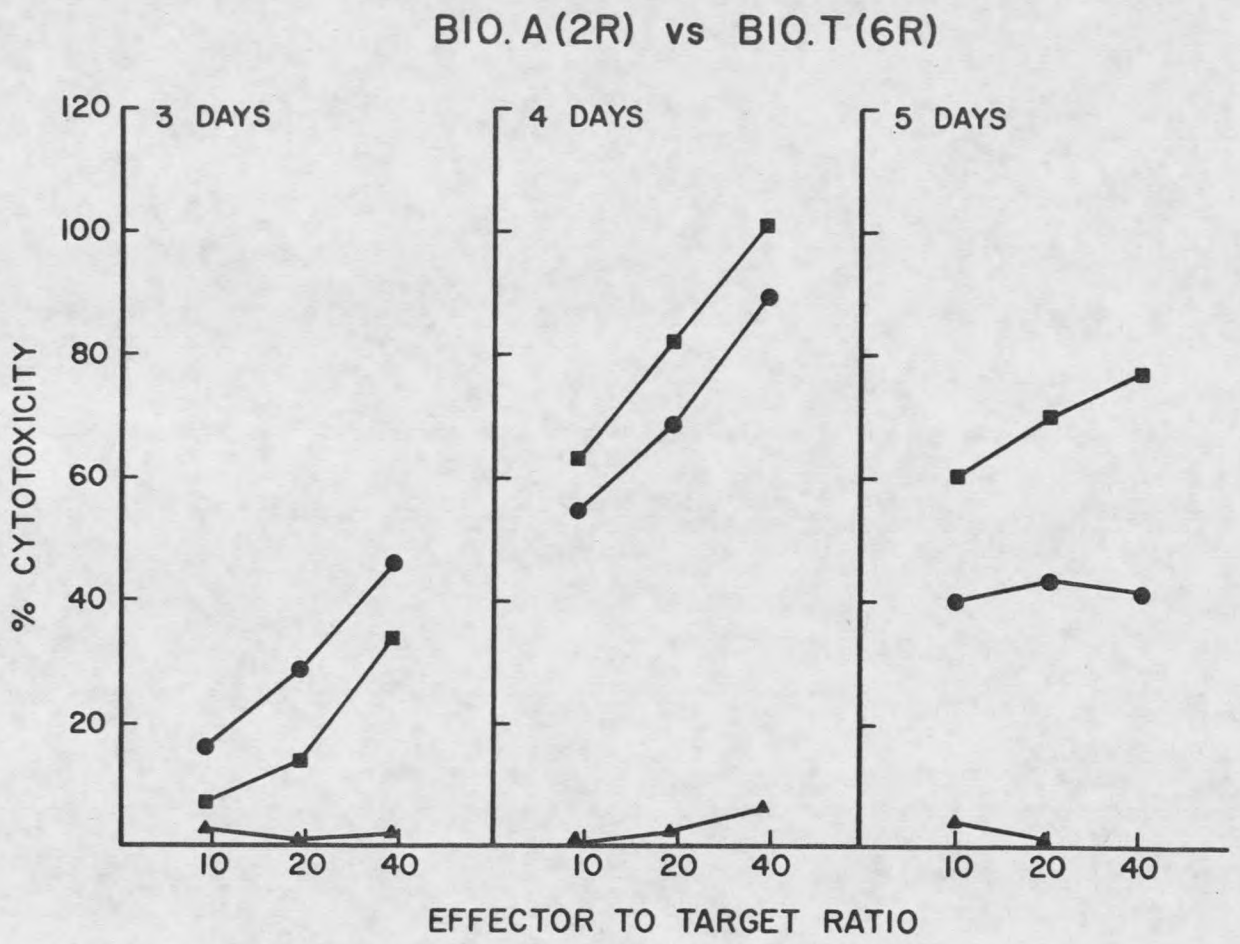


Figure 9. Modulation of the generation of cytotoxic effector cells by anti-T200; B10.A(2R) responding to B10.T(6R) at a 1:1 ratio in MLC. ■ represent cytotoxicity generated in cultures with no antibody added, ● cultures with 15% v/v anti-T200 added at time 0, both measured against P815 target cells. ▲ represent cytotoxicity of cultures with no antibody added, against a non-specific target, EL-4.

used to generate the cytotoxic cells as well as the relative numbers of each cell type used to initiate the MLC (Table 3). For example, B10.A(2R) cells as responders were more easily suppressed than SJL/J responders. Also, as the responder to stimulator ratio dropped, suppression was much more evident.

Specificity of the Modulations by anti-T200

It was possible that some or all of the effects I observed using anti-T200 were not due to the reaction of the antibody with its cognate antigen, but rather to other effects such as Fc receptor intervention. To test this, I added to assays a monoclonal antibody, anti-LGP100a, of the same isotype as anti-T200 (IgG_{2a}). The anti-LGP100a was incapable of reacting with the responders, and in some of the experiments both the responders and stimulators, since the C57BL/6 and B10 congenic mouse strains used in these studies express the alternative allele of this antigen, LGP100b. The antibody was harvested and purified using the same procedures as for the preparation of antiT200 and was added to the assays at equivalent concentration. As is shown in Figs. 10, 11, and 12, anti-LGP100a did not affect either MLC or CML with the exception of an occasional late enhancing effect on MLC (Fig. 11) which was opposite to the effect produced by anti-T200. In addition, Figs. 11 and 12 show that addition of monoclonal antibody to another determinant which was present on the responding cells, $H2K^k$, had no effect on either proliferation in MCL or the generation of CTL, in contrast to the effect seen with addition of anti-T200.

Table 3. Degree of Suppression of Generation of Cytolytic Activity by anti-T200 with Various Responder and Stimulator Combinations[a]

Responder	Cells/well	Stimulator	Cells/well	R:S ratio	% suppression[b] 20:1	% suppression[b] 10:1[c]
B10.A(2R)	4×10^6	B10.T(6R)	5×10^5	8:1	19	21
B10.A(2R)	3×10^6	B10.T(6R)	3×10^6	1:1	25	23
B10.A(2R)	2×10^6	B10.T(6R)	6×10^6	1:3	42	43
B10.A(2R)	2×10^6	B10.A	6×10^6	1:3	ND[d]	22
SJL/J	2×10^6	B10.T(6R)	6×10^6	1:3	14	17

[a] Measured against P815 target cells at day 5; for protocol used in generation, see text.

[b] Calculated as follows: $\frac{\% \text{ cytotoxicity without antibody} - \% \text{ cytotoxicity with antibody}}{\% \text{ cytotoxicity without antibody}}$

[c] Effector to target cell ratio in cytotoxicity assay.

[d] ND, not done.

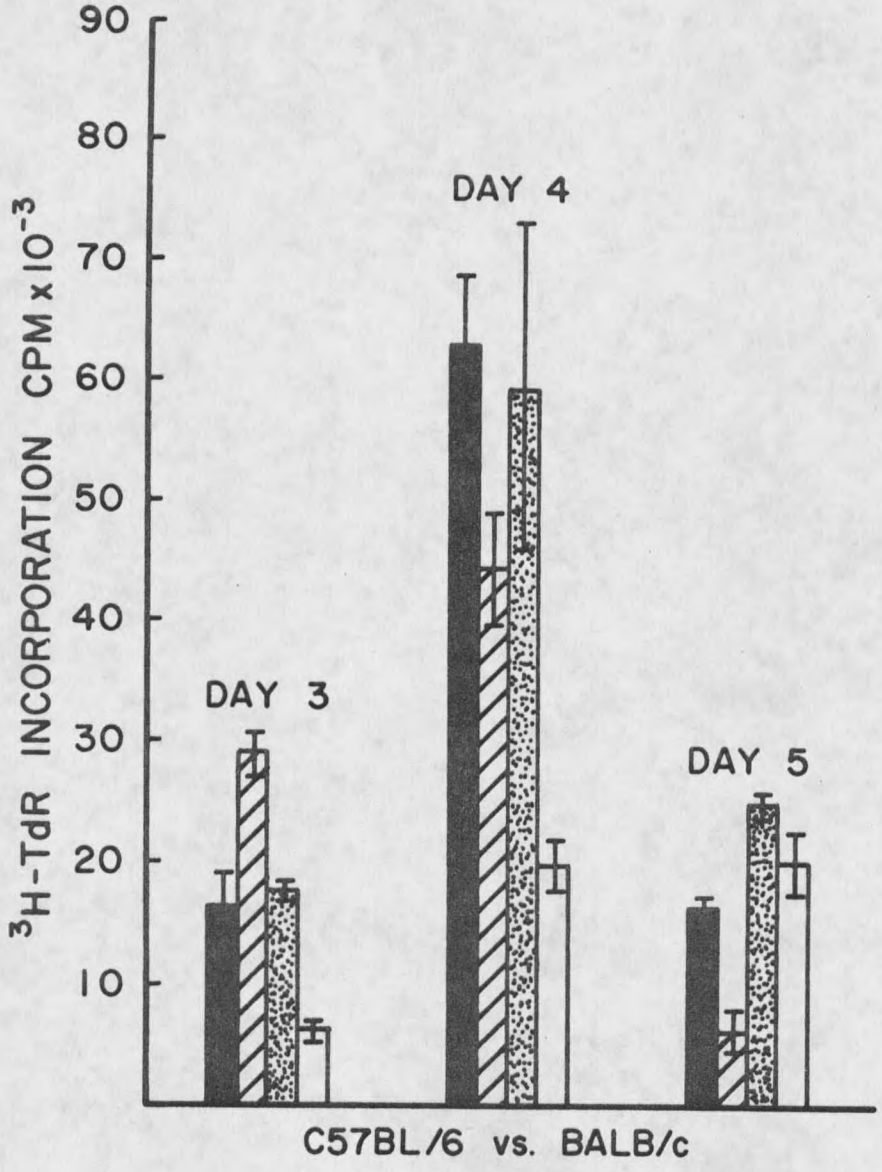


Figure 10. Proliferative response in MLC of C57BL/6 splenocytes stimulated with BALB/c splenocytes (at a 1:1 ratio) in the absence of antibody (solid bars), in the presence of anti-T200 (hatched bars, anti-LGP100a (stippled bars), or anti-Lyt 2 (open bars).

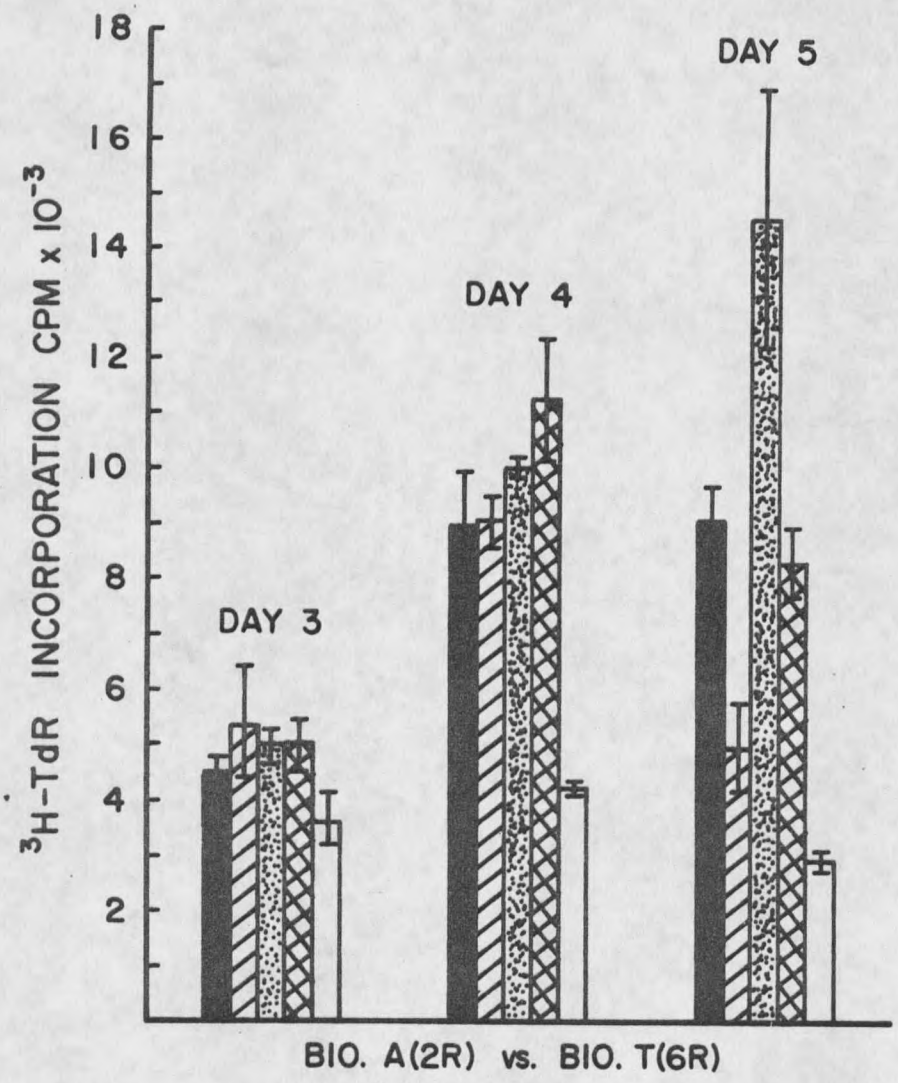


Figure 11. Proliferative response in MLC of B10A(2R) splenocytes stimulated with B10.T(6R) splenocytes at a 1:1 ratio. Legend is similar to Figure 10; cross-hatched bars represent culture in the presence of anti-$H2K^k$.

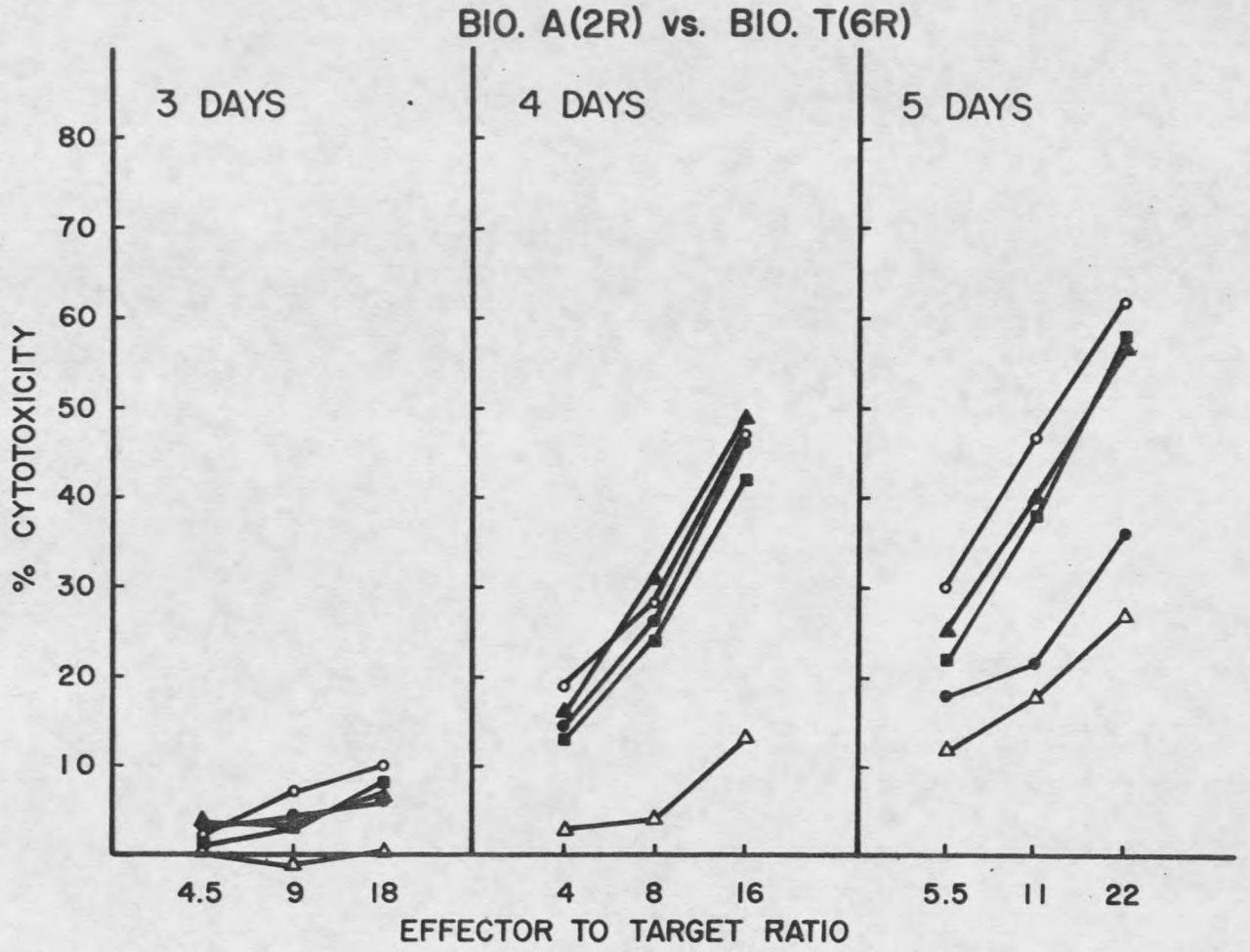


Figure 12. Specificity of the modulation of the generation of cytotoxic effector cells by anti-T200. MLC was initiated with a 1:3 responder to stimulator ratio. ■ represent cytotoxicity generated in cultures with no monoclonal antibody added, ● cultures initiated in the presence of anti-T200, ▲ anti-LGP100a, O anti-$H2K^k$, and Δ anti-Lyt 2.

Monoclonal antibody to the Lyt 2 molecule has been shown to suppress in vitro immune responses (23, 24, 46). I included this antibody in some of the assays and in agreement with previously published results found that anti-Lyt 2 suppressed both proliferation in MLC and the generation of cytotoxic T cells (Figs. 10, 11, 12). It is interesting to note that the kinetics of this suppression by anti-Lyt 2 differed markedly from those seen with anti-T200. Suppression by anti-Lyt 2 was seen early in both MLC and generation of CTL, and I observed a tendency for MLC to escape from suppression by anti-Lyt 2 late in the response (Fig. 10) in contrast to the suppressive effects of anti-T200 which were seen late in both MLC and the generation of cytotoxic cells.

Kinetics of Modulation by anti-T200

I had previously demonstrated that the addition of anti-T200 at 24 hours after the initiation of MLC abolished any enhancing effects while leaving suppression intact. Fig. 13 shows the results of an experiment in which anti-T200 was added to MLC at various times after initiation. A delay of as little as 8 hours after initiation in adding the antibody abolished the early enhancing effect while evidence of suppression could be seen even when addition was delayed up to 96 hours after initiation of MLC. These suppressive effects were more apparent in the experiment shown in Fig. 14 in which the initial enhancement was minimal. Fig. 15 illustrates the effects of addition of antibody at various times on the generation of cytotoxic cells. At day 4 of the assay, antibody added 8 hours or later after initiation

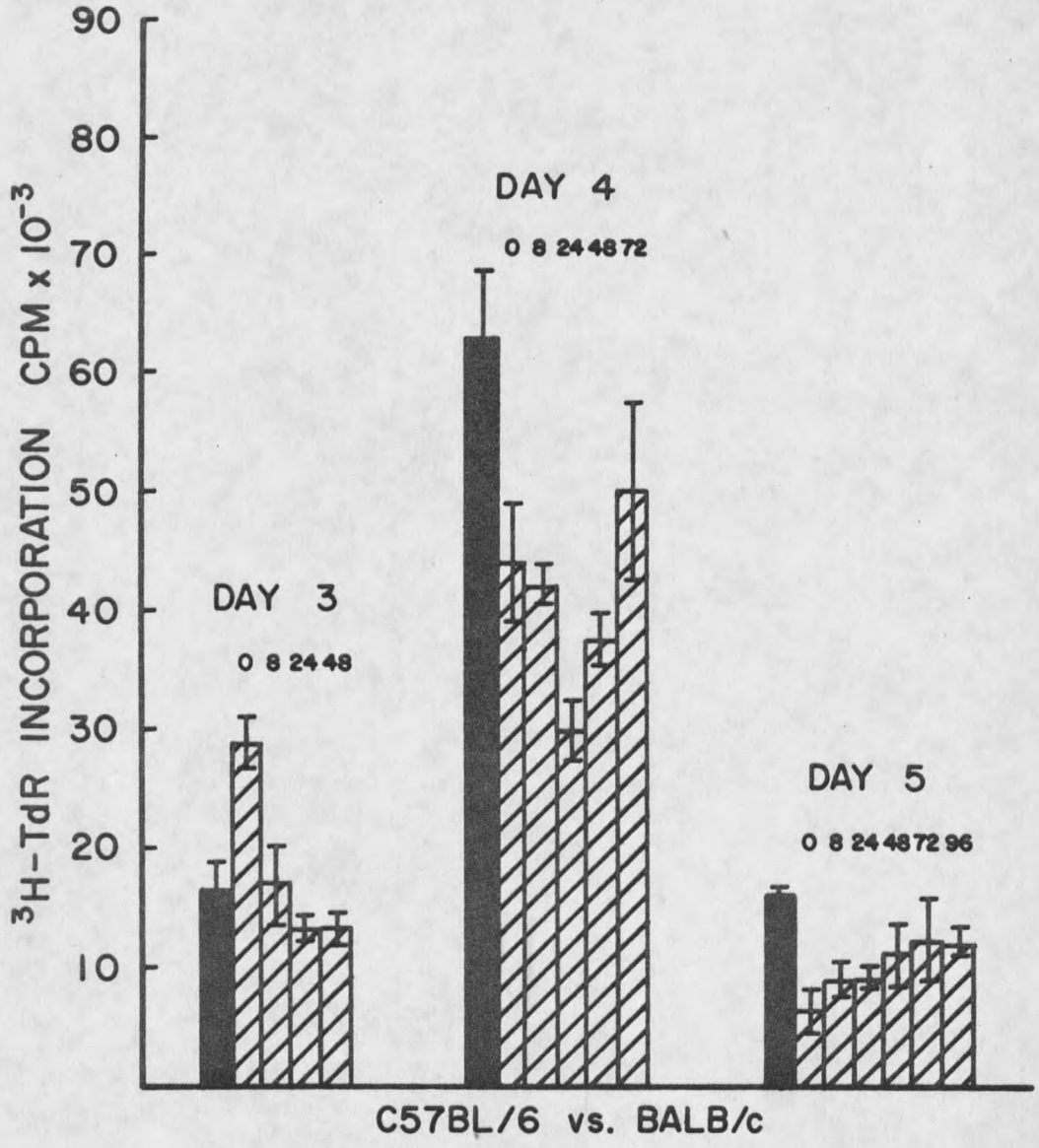


Figure 13. Effect of addition of monoclonal anti-T200 at various times after initiation of MLC (1:1 responder to stimulator ratio). Solid bars represent culture in the absence of antibody, hatched bars culture in the presence of anti-T200. Numbers above the bars represent hours after initiation of MLC at which antibody was added.

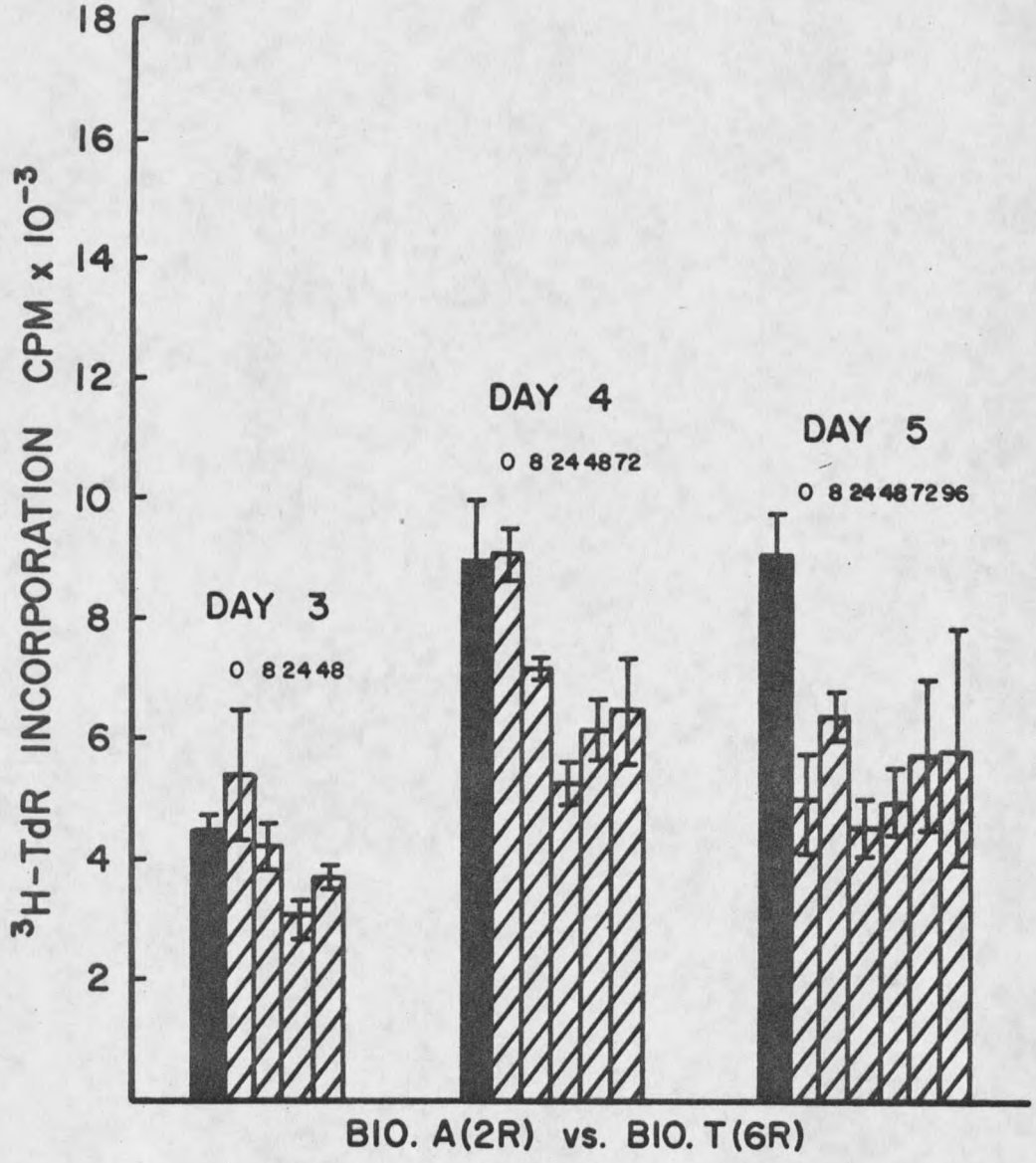


Figure 14. Effect of addition of monoclonal anti-T200 at various times on MLC in an experiment in which initial enhancement was minimal. Legend as described for Figure 13.

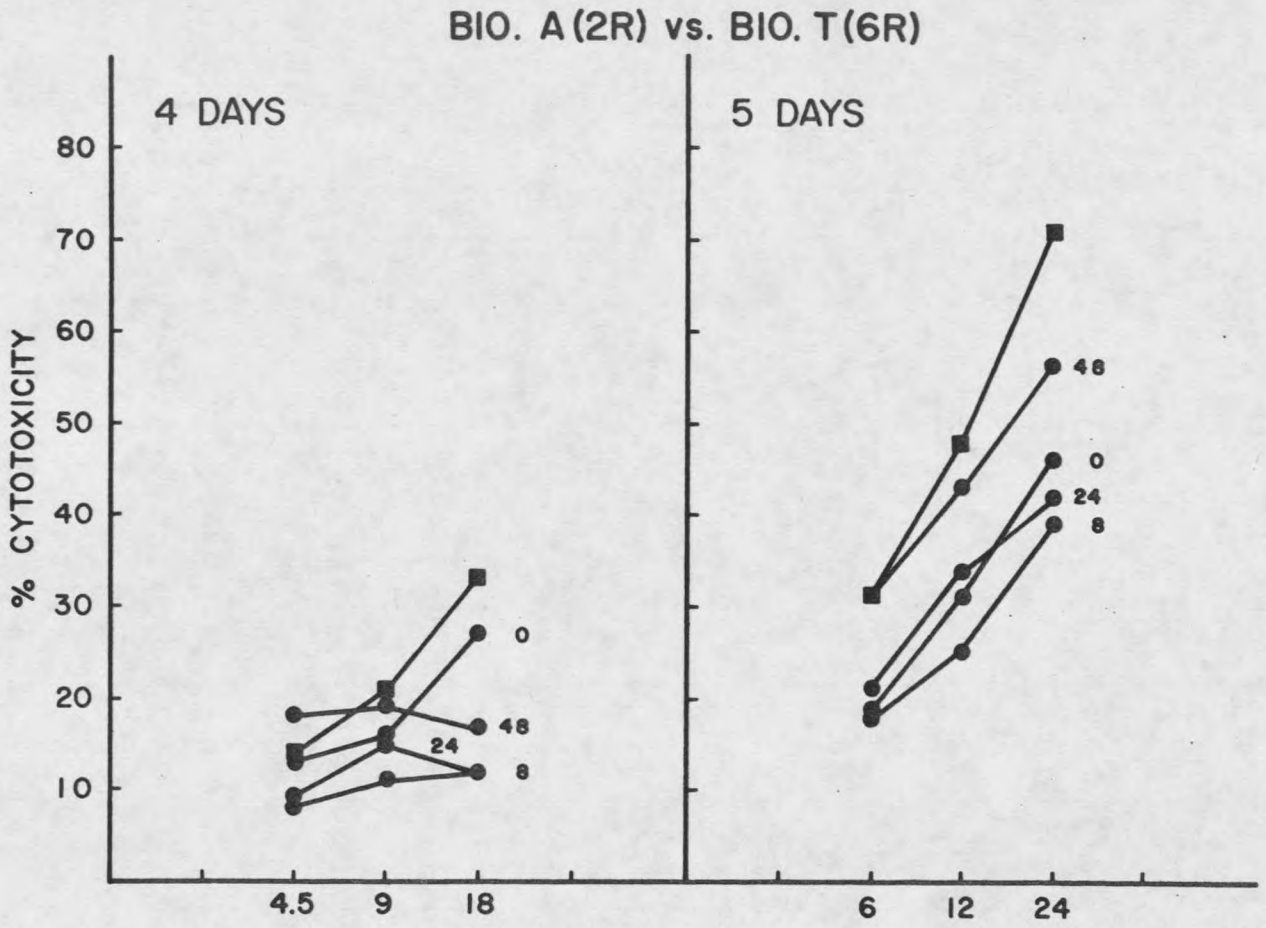


Figure 15. Effect of timed addition of anti-T200 on the generation of cytotoxic effector cells. MLC was initiated with a 1:3 responder to stimulator ratio. ■ represent cytotoxicity generated in the absence of added antibody, ● represent cytotoxicity generated in the presence of anti-T200 added at various times after initiation of culture. Numbers accompanying the lines represent hours of addition.

of MLC gave more evidence of suppression of the generation of cytotoxicity than antibody added at the beginning of culture. By day 5 of the assay, the cytotoxicity generated in the cultures which received antibody at 0, 8, or 24 hours after initiation all showed a similar degree of suppression while cultures receiving antibody at 48 hours were less suppressed. Addition at 72 and 96 hours after initiation gave results similar to addition at 48 hours (data not shown).

These results are in contrast to the kinetics of suppression by anti-Lyt 2 added at different times after initiation of culture. As seen in Table 4, when anti-Lyt 2 was added at 24 or 48 hours after initiation of MLC, no suppression of proliferation was seen. The effect on generation of cytotoxicity also differed from that seen with addition of anti-T200. Table 4 shows that on day 4 of assay, anti-Lyt 2 added 24 or 48 hours after initiation had no effect on the generation of cytotoxicity. By day 5, only cultures receiving antibody at 48 hours after initiation showed any suppressive effect at all, whereas on day 5 addition of anti-T200 at 48 hours or later after initiation resulted in a greatly diminished suppressive effect (Fig. 15).

Effect of anti-T200 on Mitogenic Responses

As was shown in previous experiments, anti-T200 by itself was not mitogenic for responding cells in the absence of alloantigen. To determine whether the antibody would affect the response to a mitogenic stimulus from another source, the following experiments were performed.

Table 4. Effects of addition of anti-Lyt 2 at various times on in vitro responses[a]

Addition of antibody	Day of assay	Proliferation[b]	% cytotoxicity[c]
none	3	16,522±2767	ND[d]
0 hr	"	6,194±886	"
24 hr	"	15,649±974	"
48 hr	"	15,678±6390	"
none	4	62,860±5767	80%
0 hr	"	19,863±2394	66%
24 hr	"	46,861±2921	76%
48 hr	"	68,367±10,731	78%
none	5	16,111±708	69%
0 hr	"	20,246±2580	77%
24 hr	"	23,841±451	66%
48 hr	"	22,854±5437	60%

[a]C57BL/6 responders cultured with BALB/c stimulators.

[b]Counts per minute of ^{3}H-thymidine incorporation±Standard deviation.

[c]Measured against P815 targets at a 5:1 effector to target ratio.

[d]Not done.

Splenocytes from a C57BL/6 mouse were cultured with several different concentrations of mitogens in either the presence or absence of anti-T200. Table 5 shows that the addition of anti-T200 greatly enhanced the response to Con A at two of three concentrations tested. In contrast, the response to LPS was relatively unaffected, or even slightly suppressed by the addition of anti-T200 to the assays. This would suggest that the anti-T200 is preferentially interacting with T rather than B cells since Con A is a T cell mitogen and LPS is a B cell mitogen.

Effect of anti-T200 on Responses of Splenocytes from Athymic Mice

A further suggestion that the anti-T200 was exerting its effects primarily through interaction with T cells was given by some preliminary experiments with athymic (nu/nu) mice. These mice are almost totally lacking in T cell function (55) and therefore if the anti-T200 is exerting its effects through interaction with T cells, little or no effect should be seen on the responses of in vitro assays with splenocytes from nu/nu mice. Table 6 shows this to be the case. The inclusion of anti-T200 in Con A stimulation assays of BALB/c splenocytes (the background strain of the nu/nu mice) had the expected effect of enhancing the proliferative response. In contrast, anti-T200 had no effect on proliferation in response to Con A by nu/nu cells. Also seen in Table 6 is the difference between effects of anti-T200 on the response to alloantigen by either BALB/c or nu/nu splenocytes. As expected from the results of previous experiments, the presence of the anti-T200 suppressed the proliferative response of BALB/c

Table 5. Effect of anti-T200 on Mitogenic Responses[a]

Mitogen	anti-T200	CPM[b]
Con A 1.25 µg/ml	-	49,830±3258
Con A 1.25 µg/ml	+	85,883±1494
Con A 2.5 µg/ml	-	53,531±1288
Con A 2.5 µg/ml	+	78,294±3007
Con A 5 µg/ml	-	30,860±1357
Con A 5 µg/ml	+	30,320±7413
LPS 2.5 µg/ml	-	16,656±1476
LPS 2.5 µg/ml	+	12,269±770
LPS 5.0 µg/ml	-	23,634±3075
LPS 5.0 µg/ml	+	21,693±1053
LPS 10 µg/ml	-	25,810±2606
LPS 10 µg/ml	+	19,205±4079

[a]Cultures set up in 96 well microplates with 2 x 10^5 C57BL/6 splenocytes/well.

[b]Counts per minute ^{3}H-thymidine incorporation, Mean±SD.

Table 6. Effect of anti-T200 on Responses of BALB/c or nu/nu Splenocytes

Responder	Stimulation	anti-T200	CPM[a]
BALB/c	none	-	2,302±553
"	"	+	1,547±124
"	Con A[b]	-	38,926±4005
"	"	+	54,725±3442
nu/nu	none	-	2,790±408
"	"	+	2,000±208
"	Con A	-	8,751±260
"	"	+	7,252±735
BALB/c	alloantigen[c]	-	10,041±797
"	"	+	5,522±734
nu/nu	"	-	1,315±300
"	"	+	1,064±84

[a]Counts per minute of ^{3}H-thymidine incorporation standard deviation.

[b]Concanavalin A added to assays at a concentration of 0.33 µg/ml. Results shown are for a 48 hour incubation period.

[c]Responders stimulated with C57BL/6 splenocytes in MLC as previously described. Results are for day 5 of MLC.

splenocytes measured on day 5 of MLC. Anti-T200 added to MLC in which nu/nu splenocytes were used as responders had no effect.

Effect of anti-T200 on Amounts of IL-2 Present in Supernatants from MLC

A preliminary investigation into the role of soluble mediators in the modulation of immune responses by anti-T200 was conducted by assaying the relative amounts of IL-2 present in supernatants from MLC initiated in either the presence or absence of the antibody. Table 7 shows the results of experiments in which supernatant was harvested from MLC on successive days and quantitated for the presence of IL-2 as described by Gillis et al. (16). The amounts of IL-2 in supernatants from cultures receiving anti-T200 at initiation were markedly reduced at day 3 of the assay compared to supernatants from cultures receiving no antibody. This effect is not due to any residual anti-T200 in the supernatant interfering with the soluble factor assay. Control assays in which anti-T200 was added directly to an IL-2 standard showed that the antibody had no effect on the ability of preformed IL-2 to stimulate the proliferation of the CTLL-2 cells used in the test (data not shown).

Effect of Conventional anti-Ly 5.1 Serum on Generation of Cytotoxic Cells

Conventional antiserum to an allodeterminant of the T200 molecule, Ly 5.1, was added at the initiation of MLC and its effect on the generation of cytotoxic cells was examined at days 4 and 5.

Table 7. Effect of anti-T200 on Amounts of IL-2 in Supernatants from MLC

Responder	Stimulator	Day of MLC	anti-T200	IL-2
C57BL/6	BALB/c	2	-	0.36U
"	"	2	+	0.36U
"	"	3	-	0.42U
"		3	+	<0.07U
"	"	4	-	<0.07U
"	"	4	+	<0.07U
B10.A(2R)	B10.T(6R)	2	-	0.42U
"	"	2	+	0.32U
"	"	3	-	0.35U
"	"	3	+	<0.07U
"	"	4	-	<0.07U
"	"	4	+	<0.07U

A profound suppressive effect was seen at day 4 when anti-Ly 5.1 was added to cultures involving several different strains of mice (Table 8).

In order to examine the specificity of this effect, cultures were set up in which the responder splenocytes were genetically either positive or negative for the Ly 5,1 alloantigen. As seen in Fig. 16, anti-Ly 5.1 blocked generation of cytotoxic cells by A.SW responders ($H\text{-}2^s$, Ly 5.1+) but not by SJL/J responders ($H\text{-}2^s$, Ly 5.2+). This result indicates that it is necessary for the cognate antigen to be present on the responding cells in MLC for the anti-Ly 5.1 to affect the generation of cytotoxic cells. It is also of interest to note that the suppression seen on day 4 with the A.SW responders was largely overcome by day 5, thus arguing against any non-specific toxic effect of the antiserum for the cytotoxic cells or their precursors.

I wished to determine the optimal range of concentration of antiserum that would result in the reduction of generation of cytotoxic cells. Table 9 shows the results of an experiment in which increasing amounts of anti-Ly 5.1 or SJL/J normal serum were added to MLC and the generation of cytotoxic cells assayed at day 4. With A.SW responder cells, the anti-Ly 5.1 showed significant blocking effects relative to SJL/J normal serum (the recipient strain for production of the antibody when from 20 to 160 µl of either antibody or normal serum was added. At 320 µl the normal serum became suppressive for the generation of cytotoxicity. As expected, there was no difference between the effects of anti-Ly 5.1 and normal serum

Table 8. Degree of Suppression of Cytolytic Activity by anti-Ly 5.1[a]

Responder[b]	Stimulator	% suppression[c]
B10.A(2R)	B10.A	79
B10.A(2R)	B10.T(6R)	69
B10.A(2R)	BALB/c	69
CBA/J	BALB/c	69
CBA/J	B10.T(6R)	90
C57BL/6	BALB/c	44

[a]Measured against P815 target cells at day 4.

[b]2×10^6 responders cultured with 6×10^6 stimulators in MLC.

[c]Calculated as in Table 3, for a 10:1 effector to target cell ratio.

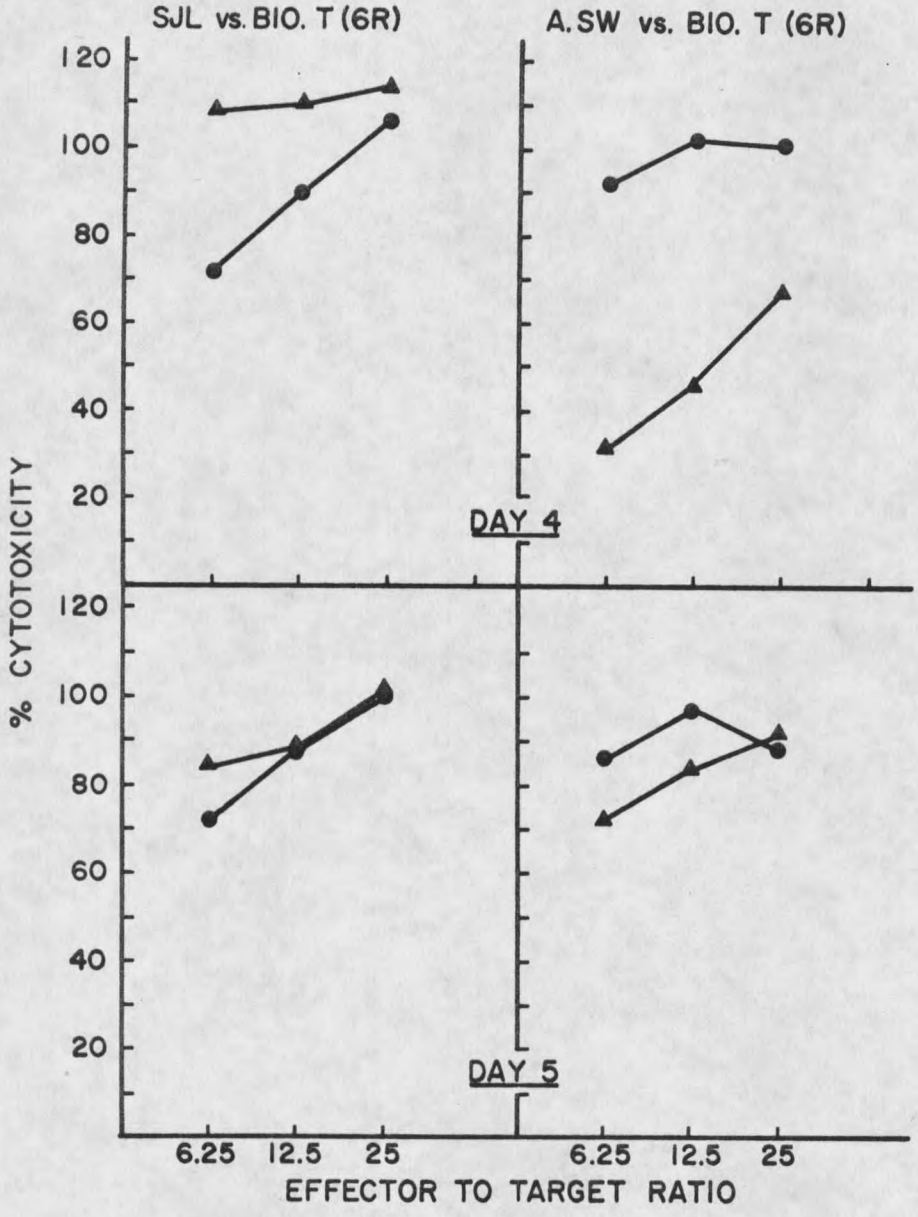


Figure 16. Effects of addition of Ly 5.1 antiserum on the generation of cytotoxic effectors by SJL/J or A.SW splenocytes. MLC was initiated at a 1:3 ratio of responders to B10.T(6R) stimulators. ▲ represent cytotoxicity generated in cultures receiving anti-Ly 5.1, ● represent cytotoxicity generated in cultures receiving SJL/J normal mouse serum. Cytotoxicity was measured against P815 targets.

Table 9. Effect of anti-Ly 5.1 Serum on Generation of CTL

Responders[b]	Amount of antiserum or NMS	% cytotoxicity[a]	
		anti-Ly 5.1	NMS
A.SW	10 µl	60	83
A.SW	20 µl	53	88
A.SW	40 µl	11	87
A.SW	80 µl	4	88
A.SW	160 µl	6	77
A.SW	320 µl	2	2
SJL/J	10 µl	21	25
SJL/J	20 µl	82	80
SJL/J	40 µl	86	56
SJL/J	80 µl	73	79
SJL/J	160 µl	60	18
SJL/J	320 µl	24	0

[a]Measured on day 4 against P815 targets at a 12.5:1 effector to target ratio.

[b]2×10^6 responders cultured with 4×10^6 B10.T(6R) stimulators in MLC.

when tested against the Ly5.2+ responder strain, SJL/J. These observations further support the conclusion that the suppressive effects seen with anti-Ly 5.1 are due to the reaction of the antibody with its specific antigen, and are not due to non-specific factors such as the effects of normal mouse serum.

Modulation of in Vitro Responses by Monoclonal anti-Ly 5.1

The results obtained using antiserum to Ly 5.1 were not completely definitive due to the multispecific nature of the conventionally produced antibody. Therefore, I used a monoclonal antibody to the Ly 5.1 determinant in order to further examine the specificity of the effects previously seen using conventional antiserum. Table 10 shows the results of an experiment in which monoclonal anti-Ly 5.1 was added at the initiation of MLC with either C57BL/6 or C57BL6-Ly 5.2 splenocytes as responders. The antibody caused a reduction in the proliferative response of C57BL/6 cells at days 4 and 5 of the assay, but no effect was seen on the proliferation of cells from the congenic partner, C57BL/6-Ly 5.2, which does not carry the cognate antigen. While these results should be considered preliminary, they strongly suggest that the reduction of prolieferation is due to the reaction of the antibody with the appropriate antigen since the antibody is monospecific and the only difference between the two responder strains is the expression of Ly 5.1.

I also attempted to determine the effect of this monoclonal antibody on the generation of cytotoxic effector cells by C57BL/6 and

Table 10. Effects of Monoclonal anti-Ly 5.1 on Proliferation in MLC[a]

Responders	Anti-Ly 5.1	Day of Assay	CPM[b]
C57BL/6	-	3	13,593±892
"	+	"	13,631±1330
"	-	4	31,815±4996
"	+	"	23,000±4368
"	-	5	12,044±2557
"	+	"	6,072±622
C57BL/6-Ly 5.2	-	3	26,045±2905
"	+	"	25,272±1399
"	-	4	33,820±3556
"	+	"	32,256±7005
"	-	5	4,465±618
"	+	"	6,064±1320

[a]Cultures were initiated with 4×10^5 responders and 2×10^5 BALB/c stimulators in 96-well microplates.

[b]Counts per minute ^{3}H-thymidine incorporation, Mean±SD.

C57BL/6-Ly 5.2 responders. In this experiment, no modulation of the response was seen using cells of either strain as responders at any of the antibody concentrations tested (data not shown).

Effect of Absorption on Suppression by Anti-T200

Previous experiments using other antibody preparations as controls indicated that the modulation of in vitro responses by anti-T200 was due to a specific antigen-antibody interaction. In order to further substantiate that the suppressive effect of the anti-T200 preparation on MLC was due to the antibody component, an absorption procedure was performed. A 1-40 dilution of monoclonal anti-T200 in RPMI (200 µl total volume) was absorbed by two sequential 30-minute incubations on ice with 7.5×10^8 pooled spleen, thymus, and lymph node cells from C57BL/6 mice. The absorbed preparation was filter sterilized, and 12 µl/well of this material was added to MLC. Another series of wells received 10 µl of a 1-40 dilution of unabsorbed anti-T200. Results are shown in Table 11.

Table 11. Effect of Absorption on MLC Suppression by anti-T200[a]

Addition to assay	CPM[b]
none	3393±1028
anti-T200	1428±171
absorbed anti-T200	5851±1417

[a]MLC initiated with C57BL/6 responders and BALB/c stimulators in a 1:1 ratio

[b]Counts per minute of ^{3}H-thymidine minus incorporation by responder cells alone, Mean±SD, measured on day 5 of assay.

This experiment indicates that absorption of the antibody preparation by lymphoid cells bearing the cognate antigen, a procedure which should remove specific antibody activity, results in a loss of the ability of the preparation to mediate suppression in MLC. This result argues that the suppressive effect is due to action of the antibody itself and not to other factors present in the preparation. In fact, the absorbed material caused an apparent enhancement of proliferation, suggesting a non-specific effect which was unmasked by removal of the antibody-mediated suppression.

Differential Effects of Harvesting Method on Apparent Suppression of MLC by anti-T200

Cells pulse-labelled with ^{3}H-thymidine were harvested from MLC on days 3, 4, and 5 of the assay by the usual method, i.e., deposition on glass fiber filters and rinsing with distilled water (see Materials and Methods) or by sequential rinsing with 0.85% saline, 5% trichloracetic acid (TCA), and methanol (21). Both sets of filters were then dried, and the discs placed in scintillation vials with Aquasol for measurement of radioactivity. Results are presented in Table 12.

Table 12. Effect of Harvest Method on MLC Suppression by anti-T200[a]

Method	anti-T200	CPM[b] day 3	CPM[b] day 4	CPM[b] day 5
Dist. H_2O	-	19,235±2127	26,323±2666	7,114±1028
"	+	37,770±4604	23,677±6267	5,149±171
TCA	-	18,422±2432	36,669±4022	10,212±256
"	+	8,296±1137	7,026±309	2,071±163

[a]MLC initiated with C57BL/6 responders and BALB/c stimulators in a 1:1 ratio.

[b]Counts per minute ^{3}H-thymidine incorporation, Mean±SD.

As seen from this result, harvesting the cells after pulse-labelling with ^{3}H-thymidine by the TCA method resulted in a greater reduction of measured radioactivity in cultures receiving anti-T200 at initiation. The suppressive effect was also apparent on all three days of assay, compared to the early enhancement followed by suppression seen when cultures were harvested by the distilled water method. One explanation for this observation is that the distilled water harvesting procedure does not break open the nucleii of the cells, and therefore both ^{3}H-thymidine incorporated into DNA as well as that maintained in the intranuclear pool is represented in the total count of radio-activity. In contrast, the TCA harvest method effectively disrupts the nuclear membrane and the remaining radioactivity represents only that incorporated into DNA.

The implications of this observation for the mechanisms of modulation by anti-T200 are provocative, but much more investigation needs to be done before any conclusions can be drawn.

DISCUSSION

The molecular mechanisms involved in the initiation and functional phases of immune responses remain largely unknown despite vigorous investigation in many laboratories. The examination of the role played by surface molecules of cells participating in immune responses may prove to be one of the most fruitful means for elucidating these mechanisms. I have examined the effects of antibody to some of the major surface molecules on immune cells in order to define their relevance to certain in vitro functions. It is hoped that these studies may contribute a piece to the eventual solution of the puzzle of cell interactions.

By definition, a T cell expresses the Thy 1 antigen on its surface. Since T cells play a central role in immune responses, both directly and as accessory cells, it seems reasonable that this antigen might have some relevance to the special functions performed by these cells. Indeed, there is some evidence that this might be the case. Antibody to a non-polymorphic determinant of Thy 1 has been reported by Norcross et al. (48) to act as a mitogen for murine peripheral T cells. Hollander and co-workers reported that anti-Thy 1 monoclonal antibody can block MLC induced proliferation (24). In addition, several groups have reported that antibodies to OKT3, a possible human homolog of Thy 1, can inhibit proliferation of human T cells induced by both mitogens and alloantigens (51, 54). These antibodies have

also been reported to cause a reduction of both the generation and the effector phases of CTL killing (12, 65).

I examined the effect of monoclonal antibody to Thy 1, in the absence of complement, on the generation of cytotoxic T cells. I was able to demonstrate a fairly consistent suppressive effect on the generation of C57BL/6 ($H\text{-}2^b$) CTL against P815, a tumor cell line of the $H\text{-}2^d$ haplotype, but not against BALB/c splenocytes, also $H\text{-}2^d$ (see Figs. 1 and 2 in Results section). Initially, it seemed likely that this suppression could have been due to some peculiarity inherent in the use of tumor cell stimulators for the generation of cytotoxic cells. However, experiments using another tumor cell line, 2PK3 ($H\text{-}2^d$) as stimulators resulted in no suppressive effect by anti-Thy 1 in contrast to the effect seen when P815 was the stimulator (Figs. 3 and 4). One of the major differences between these two tumor cell lines is in the expression of class II major histocompatibility antigens (Ia). P815 is a low or non-expresser while 2PK3 expresses very large amounts of Ia. It has been reported that the generation of cytotoxic cells in the presence or absence of Ia differences between responders and stimulators may occur via different mechanisms, possibly by the activation of different subsets of helper cells (63). One possible explanation for the difference in susceptibility to blocking of CTL generation by anti-Thy 1 in the different systems I examined could have been the differential susceptibility of the mechanisms generating CTL in the presence or absence of Ia stimulation to the effects of the antibody. Since there are numerous differences in addition to Ia expression between BALB/c splenocytes, P815, and

2PK3 tumor cells which could explain this differential susceptibility to blocking effects by anti-Thy 1, further experiments were in order.

Congenic mice, which differ only at known genetic loci, are a powerful tool for examining roles of a given antigen, since differences at defined loci can be examined in the context of identical background genes. I was able to obtain congenic mice with recombinant H-2 haplotypes such that responder-stimulator combinations in MLC could be set up which allowed for genetic recognition of class I (K or D) differences with or without recognition of Ia antigen (see Table 1).

Initially, it appeared that these experiments supported the hypothesis that Ia recognition regulated the ability of anti-Thy 1 to suppress generation of CTL. In the presence of an Ia disparity between congenic responders and stimulators, anti-Thy 1 had no suppressive effect (Fig. 6). On the other hand, in combinations where responders and stimulators differed only at class I loci and no Ia disparity was present, anti-Thy 1 reduced the generation of CTL (Fig. 5). These results have been somewhat clouded by further experiments that suggested that manipulation of experimental parameters may also influence the ability of anti-Thy 1 to block CTL generation. In some experiments with congenic mice, blocking was seen by anti-Thy 1 even in the presence of Ia differences (data not shown). Due to the direction of research dictated by funding in the laboratory, it has not been possible to pursue these investigations. It is therefore not possible to conclude from the results at hand whether anti-Thy 1 can differentially block generation of CTL depending on the degree of genetic disparity between responders and stimulators or if other

mechanisms can account for the results seen. However, these observations of suppressive effect on an in vitro immune function by anti-Thy 1 do fit into a pattern of effects seen by others, a fact which tends to minimize the possibility that the effects I saw were artifacts of the experimental system.

Another cell surface molecule which may play a role in immune functions is the T200 molecule. At present, the manner in which this molecule may participate in these responses is unclear. However, a number of investigators have presented evidence that it plays a major role in cellular events. Bouguignon et al. (5) showed that capping of T200 by specific antibody resulted in the co-capping of H-2, TL, and Thy 1 antigens on mouse thymoma cells. Maino and co-workers (38) suggested the participation of T200 in a membrane activation complex involving an Fc receptor interaction between accessory and responding cells to a rabbit anti-mouse brain antibody. Gordon, Favero, and co-workers (15, 19) have shown that the T200 glycoprotein may be one of the major binding sites for Con A, and that it may also be involved in oxidation-induced activition of lymphocytes. In sum, these results suggest a significant role for the T200 molecule in cellular activation.

A further indication for an important functional role for the T200 antigen is the ability of antibody to determinants on the T200 molecule to block various in vitro activities of lymphocytes. Nakayama and his colleagues (45) were able to block the effector stage of cytotoxic T cell-mediated killing with antiserum to Ly 5.1. This blocking was at a level comparable to that seen with antiserum to

Lyt 2,3 determinants. Other workers have shown blocking of natural killer cell-mediated cytotoxicity by anti-Ly 5.1 serum (6, 29, 41) and also by antibody to non-polymorphic determinants on the T200 molecule (57). Work at Montana State University in the laboratory of Dr. Sandra Ewald has indicated the anti-Ly 5 serum can block antibody-dependent cell-mediated cytotoxicity, oxidation-induced activation of lymphocytes, and the effector phase of CTL killing.

I have shown that antibody to a non-polymorphic determinant on the T200 molecule can alter the course of proliferation to alloantigens by murine splenocytes (Figs. 7 and 8). This modulation was seen as a late suppressive effect, often preceded by early enhancement. The enhancing effect was not seen in all cases, and was only seen when the anti-T200 was added to the assay at time 0 of MLC. These observations are compatible with a model in which the antibody is able to activate a subset of cells within the splenocyte population to increased proliferation within a very limited time frame, i.e., the first 8 hours of culture as shown in Figs. 13 and 14, once this early stage was passed, addition of antibody resulted in suppression only. It seems possible that the antibody then acted on a second subset of cells, which may not have been susceptible to its action in the very early stages of culture. In any case, some type of suppressive mechanism was available for activation by antibody additon essentially throughout the entire course of the MLC, since addition of antibody even at hour 96 to a 120 hour culture resulted in some suppression of the response (Figs. 13 and 14).

Similar effects were seen on the generation of cytotoxic effector cells (Figs. 9 and 15). Assays in which anti-T200 was added 8 hours or later after initiation of the MLC showed reduced generation of cytotoxicity by day 4 of the assay, whereas cultures which received the antibody at the initiation of culture did not show suppression until day 5. This could have been due to the suppressive mechanism having to "catch up" and overcome the early enhancement which was often seen when the antibody was added at time 0. When antibody was added at 48 hours or more after initiation of the MLC, the ultimate suppressive effect was much less than that seen with earlier antibody addition (Fig. 15). The development of cytotoxic effector cells is a more complex process than the induction of proliferation in MLC, and perhaps these later additions of antibody did not allow sufficient time before termination of the cultures for the suppressive effects to become apparent.

I did not see suppressive effects on these in vitro immune responses by addition of monoclonal antibodies to other cell surface molecules, including one of identical isotype to the anti-T200 (anti-LGP100a), with the exception of anti-Lyt 2 (Figs. 10, 11, and 12). This monoclonal antibody to Lyt 2 (clone 53-6.7) had previously been shown by others to suppress both MLC and generation of cytotoxicity (23, 24). However, the kinetics of the suppression by anti-T200 appeared quite different from those seen with anti-Lyt 2. The effect of anti-Lyt 2 was most apparent in the early stages of MLC and in fact seemed to wane by day 5 of the response in some experiments (Fig. 10).

In contrast, anti-T200 had its greatest suppressive effects on day 5 of both MLC and generation of cytotoxicity.

I also repeated the time course experiments of Hollander et al. (24) and confirmed that suppressive effects of anti-Lyt 2 were lost if the antibody was added either 24 or 48 hours after initiation of the MLC (Table 4). In contrast, addition of anti-T200 at later times resulted in suppression equal to or sometimes greater than that seen when the antibody was added at time 0 (Figs. 13, 14, and 15). These results strongly suggest that the mechanisms of suppression by anti-Lyt 2 and anti-T200 are different.

The mitogenic response of lymphocytes is thought to be a polyclonal activation phenomenon that bypasses the need for engagement of specific antigen receptors on the responding cells (61, 67) although some evidence has been presented that perturbation of these receptors may be required for optimal mitogenesis (35). Early work indicated that purified populations of responding cells could be activated by mitogens (42, 67) but more recent evidence has suggested that accessory cells play a role in the activation and regulation of mitogenic responses (20, 47). In view of the results I obtained which showed that anti-T200 suppressed responses of lymphocytes to allogeneic stimulation but was not by itself mitogenic (Table 2), I was interested in examining the effect of the anti-T200 on the response of cells to known mitogens.

The addition of anti-T200 to the assay resulted in a marked augmentation of the proliferative response of C57BL/6 splenocytes to Con A (Table 5). It should be re-emphasized that the addition of

anti-T200 to responder cells in the absence of alloantigen or mitogen had no effect. The enhancing effect of anti-T200 on the Con A response was lost as the concentration of Con A added to the cells was increased. This could have been due to competition between the antibody and the mitogen for binding sites on the cells, resulting in a sub-optimal response. This sort of competition at high levels of mitogen has been reported by others (14). It is also interesting to note that at high levels of addition, Con A is less efficient at inducing a proliferative response. This could be due to an induction of suppressor cell function, a process which could be augmented by the addition of anti-T200.

The enhancing effect of anti-T200 on the response to Con A seemed paradoxical at first, considering that the previous modulating effects shown by the antibody were all suppressive. It should be remembered, however, that assays for proliferation to mitogens are conducted at 48 hours, since these responses tend to peak out rather quickly. The proliferation to alloantigen in MLC was often enhanced at day 2 or 3 of the assay (Figs. 7 and 8). With this in mind, the enhancement of mitogenesis by anti-T200 is not such a surprising observation. It should be mentioned at this point that these experiments with modulation of mitogenic responses were preliminary in nature; no other monoclonal antibodies were added to the assays as controls for the anti-T200. For this reason, any conclusions drawn from these results must be considered to be tentative.

Taken together with the time course experiments on the effects of anti-T200 on MLC and generation of cytotoxic cells, these

observations on the effects of the antibody on response to Con A would fit a model in which anti-T200 preferentially stimulates the early proliferation of a subset of suppressor cells, which then later serve to dampen the responses being tested. The results seen with the addition of anti-T200 to LPS stimulation of splenocytes (Table 5) are also compatible with this model in that the antibody could be activating regulatory cells to reduce the response of the B cells to LPS, thus giving an overall reduction of proliferation.

The preliminary experiments with athymic (nu/nu) mice are quite difficult to evaluate for at least two reasons. The first is that I was attempting to look at the effects of anti-T200 on what I feel to be basically T cell responses using splenocytes from animals with few or no T cells. The second reason, which is related to the first, is that as expected, these responses were quite low, and this makes it difficult to determine any modulating effects due to addition of the anti-T200. The one interesting observation to be made from these results is that no indication was given that the presence of the anti-T200 was able to interact with proliferative signals (either mitogen or alloantigen) and bestow competence on a population of cells, the nu/nu splenocytes, that would not normally respond to those signals (Table 6).

Interleukin-2, formerly known as T cell growth factor (TCGF), has been proposed as a key mediator in responses of lymphocytes, especially in proliferation and production of cytotoxic cells (17, 66). Thus, it seemed logical to examine the effect of anti-T200 on the amounts of IL-2 which were assayable in supernatants of antibody

suppressed MLC. These experiments showed a clear reduction in the IL-2 measureable in supernatants at day 3 of MLC (Table 7). The time lag which was seen between the reduction of IL-2 and the suppression of proliferation 24 to 48 hours) would suggest, but certainly not prove, a cause and effect relationship between the two events. Further studies on the kinetics of this reduction, and the effects of exogenous IL-2 on suppressive effects of anti-T200 need to be done before firm conclusions can be drawn. Relating these results to the model I have suggested for the mechanism of modulation of in vitro responses by anti-T200 is tenuous at best, but it is of interest to note that a recent report has shown that a suppressor cell arises after 3 days of MLC which is capable of absorbing IL-2 from the surrounding medium, thus diminishing the generation of CTL in the cultures (62). It is tempting to speculate that the addition of anti-T200 to my assays may have preferentially stimulated this type of suppressor cell, which then diminished the amount of IL-2 available in the cultures for further proliferation and differentiation of other responding cell types.

If the T200 molecule does play a role in the genesis of immune responses, as suggested by my results with antibody to a non-polymorphic determinant of the molecule, an important question to address is whether or not antibodies to other determinants on the molecule have similar effects. In order to investigate this, I produced a conventional antiserum to another determinant on the molecule, anti-Ly 5.1. Since this antiserum reacts only with cells of mouse strains which carry the appropriate allelic determinant, and

the anti-T200 recognizes the molecule on cells of all strains of mice, it follows that they must be recognizing different determinants. The fact that the anti-Ly 5.1 will react with cells of some strains of mice but not others also allows for the performance of experiments to examine the specificity of modulations caused by the antibody. This can be done by setting up assays in which the responding cells are either positive or negative for the determinant recognized by the antibody.

The initial series of experiments with this antiserum established that it was capable of profoundly suppressing the generation of cytotoxic cells (Table 8). This effect was seen with a number of different responder-stimulator combinations, and in some experiments addition of the antibody at the initiation of MLC virtually abrogated the generation of cytotoxic cells. It was interesting to note that the suppression was most apparent when assays for CTL were performed on day 4 of the MLC. This is in contrast to the suppressive effects of anti-T200 on CTL generation, which were always maximal at day 5 of MLC. It is important to note that the suppressive effects seen with anti-Ly 5.1 often waned by day 5 and a recovery of the cytotoxic activity in the cultures occurred (Fig. 16). This observation argues strongly against the suppressive effect having a trivial explanation such as a non-specific cytotoxic effect of the antibody for the effector cells or their precursors. Rather, it suggests that the cytotoxic cells are present in the culture but are being held in check, or perhaps delayed in maturation, by an active mechanism induced by the presence of the anti-Ly 5.1.

All of the responder cells in the initial series of experiments carried the Ly 5.1 allele and therefore would be expected to be susceptible to effects of the antiserum. The stimulator cells in these assays were likewise Ly 5.1+ and so it could not be determined if the effects seen were due to interaction of the antibody with antigen on the responding cells, stimulating cells, or both. I therefore performed a series of experiments comparing the effects of the antibody when SJL/J ($H\text{-}2^s$, Ly 5.2) or A.SW ($H\text{-}2^s$, Ly 5.1) splenocytes were used as responders to generate cytotoxic cells in MLC. It should be remembered that, although these two strains share the $H\text{-}2^s$ haplotype, they are not congenic, and therefore multiple differences may exist in their expression of surface antigens. Since these were also the donor and recipient strains used for production of the anti-Ly 5.1 serum, SJL/J cells would not be expected to react (since SJL/J is the strain in which the serum was produced), but it is possible for the serum to react with any antigens on A.SW cells which are not common to the two strains and not just Ly 5.1. Immunoprecipitation analysis of the antiserum did show, however, that antibody to Ly 5.1 was the major immunoreactive component.

The results of these experiments were quite clear in that they showed a total lack of suppressive effect on CTL generation when SJL/J cells were used as responders in MLC (Fig. 16). These experiments were done in parallel with MLC in which A.SW cells were the responders, using the same stimulators and the same targets for measurement of cytotoxic effector function. The results indicated that the antiserum must react with an antigen on the responding cell

population in order for effects to be seen. The cells used as stimulators were Ly 5.1+, but in the absence of the antigen on the responders, this was not sufficient to allow manifestation of suppression by the antiserum.

I noted that the SJL/J normal mouse serum used as a control in these assays had a sometimes striking enhancing effect on the generation of cytotoxicity compared to cultures incubated in medium alone. Since the anti-Ly 5.1 serum is essentially SJL/J normal mouse serum with the addition of antibodies stimulated by injection of A.SW cells into the recipient mice, I wished to examine the relationship between these effects more closely. I performed experiments in which varying amounts of either SJL/J normal mouse serum or anti-Ly 5.1 serum were added to MLC cultures with either SJL/J or A.SW cells as the responders. I found that even at very low concentrations of serum, anti-Ly 5.1 inhibited the generation of cytotoxicity by A.SW responders relative to normal mouse serum (Table 9). This effect was seen through a wide range of concentrations until, at very high levels of added serum, the normal mouse serum became toxic for the cells and abrogated the generation of cytotoxicity. In contrast, the effects of anti-Ly 5.1 serum and normal mouse serum were parallel throughout the range of concentrations tested when SJL/J cells were used as responders in MLC. The antibody did not show suppressive effects against SJL/J responders until levels of addition were reached at which the normal mouse serum also depressed CTL generation (Table 9). These results support the conclusion that there is a substance in the anti-Ly 5.1 serum, presumably antibody, that is not present in normal

mouse serum, that reacts with an antigen present on A.SW but not SJL/J responder cells, to cause suppression of the generation of cytotoxicity.

I hoped to overcome some of the difficulties inherent in using conventional antiserum in these studies, particularly the wild modulations of proliferative response in MLC caused by the presence of normal mouse serum, by the use of a monoclonal antibody to the Ly 5.1 determinant. In addition, I wished to determine the specificity of the effects in a more definitive manner through use of congenic mice which differed only in their expression of the Ly 5.1 antigen rather than at multiple loci as was the case with the SJL/J-A.SW responders. I was able to obtain C57BL/6-Ly 5.2 mice, congenic to the C57BL/6 line, and I column-purified a commercial preparation of monoclonal anti-Ly 5.1.

Table 10 shows the results of an experiment in which the effects of addition of monoclonal anti-Ly 5.1 on proliferation in MLC by either C57BL/6 or C57BL/6-Ly 5.2 splenocytes were compared. As shown, the antibody, added at the initiation of MLC, suppressed the proliferative response by C57BL/6 responders on days 4 and 5 of the assay, but had no effect on the proliferative response of the congenic partner, C57BL/6-Ly 5.2. This was the expected result and it supports an interpretation that the modulating effects of anti-Ly 5.1 on MLC were due to the reaction of the antibody with its cognate antigen and could not be attributed to trivial or non-specific processes.

I wished to demonstrate the specificity of the suppression of generation of CTL by anti-Ly 5.1 in a similar manner, and I set up the

appropriate experiments using monoclonal antibody and congenic mice as responders. Unfortunately, no suppressive effects were seen on either congenic partner at any of the antibody concentrations on either of the days tested. In this same assay, conventionally produced anti-Ly 5.1 serum did show evidence of suppression of CTL generation by the appropriate responder, so the lack of effect by the monoclonal antibody cannot be attributed to failure of the assay. There are several explanations for the lack of suppression by the monoclonal anti-Ly 5.1. First, one of the properties of monoclonal antibodies which make them so desirable is their homogeneous nature. In an assay system such as I used, this can result in their being less active than a conventionally produced antiserum with its heterogeneous mixture of antibodies with varying avidities and possibly multispecific reactivities to determinants on the target molecule. The suppressive effects of the conventional antiserum in this experiment were not particularly strong, and it is possible that the amounts of monoclonal antibody I added to the assay were not sufficient to produce measurable effects due to lower avidity.

Another possible explanation for the lack of effect on generation of cytotoxic cells by the monoclonal anti-Ly 5.1 is that it may be reacting with a determinant on the molecule that is not critical to function. There is no evidence concerning the number of allotypic determinants on the T200 antigen, and it is conceivable that there are more than one. If this is the case, conventionally produced antiserum to the molecule would likely contain antibody to all of the allodeterminants, including those whose perturbation by antibody would

result in an alteration of function. Since by definition, the monoclonal antibody reacts with only one determinant, there is no guarantee that a given monoclonal antibody to the molecule will react with a determinant the perturbation of which will result in a measurable effect on an in vitro assay. The fact that the antibody did affect proliferation in MLC does not negate this explanation for its lack of effect on generation of CTL. Generation of cytotoxic effector cells is a far more complex process than the induction of a proliferative response to alloantigen, and while perturbation of a given allodeterminant by the monoclonal antibody might be sufficient to produce a suppression of proliferation, it might not be sufficient, unless the antibody is present in overwhelming excess, to measurably affect the generation of cytotoxic effector cells. Further work, possibly with a series of monoclonal antibodies raised against determinants on the T200 molecule, needs to be done to clarify this.

In spite of the difficulties with the monoclonal anti-Ly 5.1, the sum of the results of experiments using antibodies to T200 contributes to the existing body of information suggesting an important functional role for this molecule in the activation and expression of in vitro immune responses. I demonstrated that antibodies to at least two different determinants on the molecule could suppress either proliferation to alloantigen, generation of cytotoxicity, or both. I also showed that antibody to a non-polymorphic determinant on the T200 molecule could enhance the response of murine splenocytes to a T cell mitogen.

The extent to which the T200 molecule may be involved in specific recognition or activation events in cellular responses remains open to question. It is still not possible to rule out the explanation that the observed effects are due to steric hindrance of another molecule physically adjacent to T200 on the membrane. This becomes less likely as evidence accumulates for the alteration of more and more functions by antibody to T200, especially if the antibodies can be shown to be reacting to different determinants. Nonetheless, I am cautious in interpreting these results as indicating a direct role for the molecule in cellular mechanisms of specific subsets of lymphocytes. Put another way, T200 is most likely not the elusive T cell receptor. The wide distribution of the molecule on hematopoietic cells would argue against this; on the other hand, this wide distribution does suggest a role in activation or interactive events common to many types of cells. It seems reasonable to envision the involvement of T200 in molecular complexes on cell surfaces which may form in response to external activation signals. Intuitively, and from evidence gathered by others (5, 38), it seems likely that such complexes might contain several major cell surface molecules, as well as antigen specific recognition molecules, which must aggregate and migrate in an ordered manner for the proper signal to be transmitted to the interior of the cell and result in ultimate expression of immune function, if such function is the destiny of that cell. It is highly provocative that one of the reports suggesting the formation of molecular complexes on cell surfaces identified two of the molecules

in the complex as Thy 1 and T200 (5). One would expect that a perturbation by antibody of any of the molecules in such a complex might alter the expression of any functions served by that complex. My work indicates that antibodies to both Thy 1 and T200 can indeed alter the expression of in vitro immune function.

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APPENDICES

Appendix A

ABBREVIATIONS USED IN THIS THESIS

Con A	Concanavalin A
CO_2	Carbon dioxide
^{51}Cr	Radioactive chromium
CTL	Cytotoxic T lymphocyte
DEAE	(diethyaminoethyl)
DNA	Deoxyribonucleic acid
E:T	Effector to target
^{3}H-TdR	Tritiated thymidine
HEPES	N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid
IgG	Immunoglobulin G
IFA	Indirect fluorescent antibody
IL-1	Interleukin-1
IL-2	Interleukin-2
LPS	Lipopolysaccharide
M	Molar
MHC	Major histocompatibility complex
ml	milliliter
MLC	Mixed lymphocyte culture
mM	millimolar
µCi	microcurie

µg	microgram
µl	microliter
NaCl	Sodium chloride
NMS	Normal mouse serum
nu/nu	nude/nude (homozygous genotype)
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
Tris HCl	Tris-(hydroxymethyl)-aminomethane hydrochloride
v/v	volume/volume

Appendix B

Table of antigens and antibodies discussed in this thesis.

Antigen	M.W.[a]	Distribution	Antibody	
			Species raised in	IgG subclass
$H2K^k$	45K, 12K[b]	all cells and tissues	mouse	$mIgG_{2a}$[c]
LGP100	100K	T and B lymphocytes	rat	$mIgG_{2a}$
Lyt 2,3	35K, 30K[b]	T lymphocytes	rat	$mIgG_{2a}$
Ly 5	200K	all hematopoietic cells except mature red blood cells	mouse mouse	conventional $mIgG_{2a}$
T200	200K	same as Ly 5	rat	$mIgG_{2a}$
Thy 1	23K	T lymphocytes, neuronal and epidermal cells	rat	$mIgG_{2b}$

[a] Molecular weight in kilodaltons (K).

[b] Molecule consists of two polypeptide chains.

[c] "m" indicates a monoclonal antibody.

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