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

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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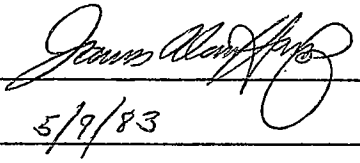
Signature 

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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

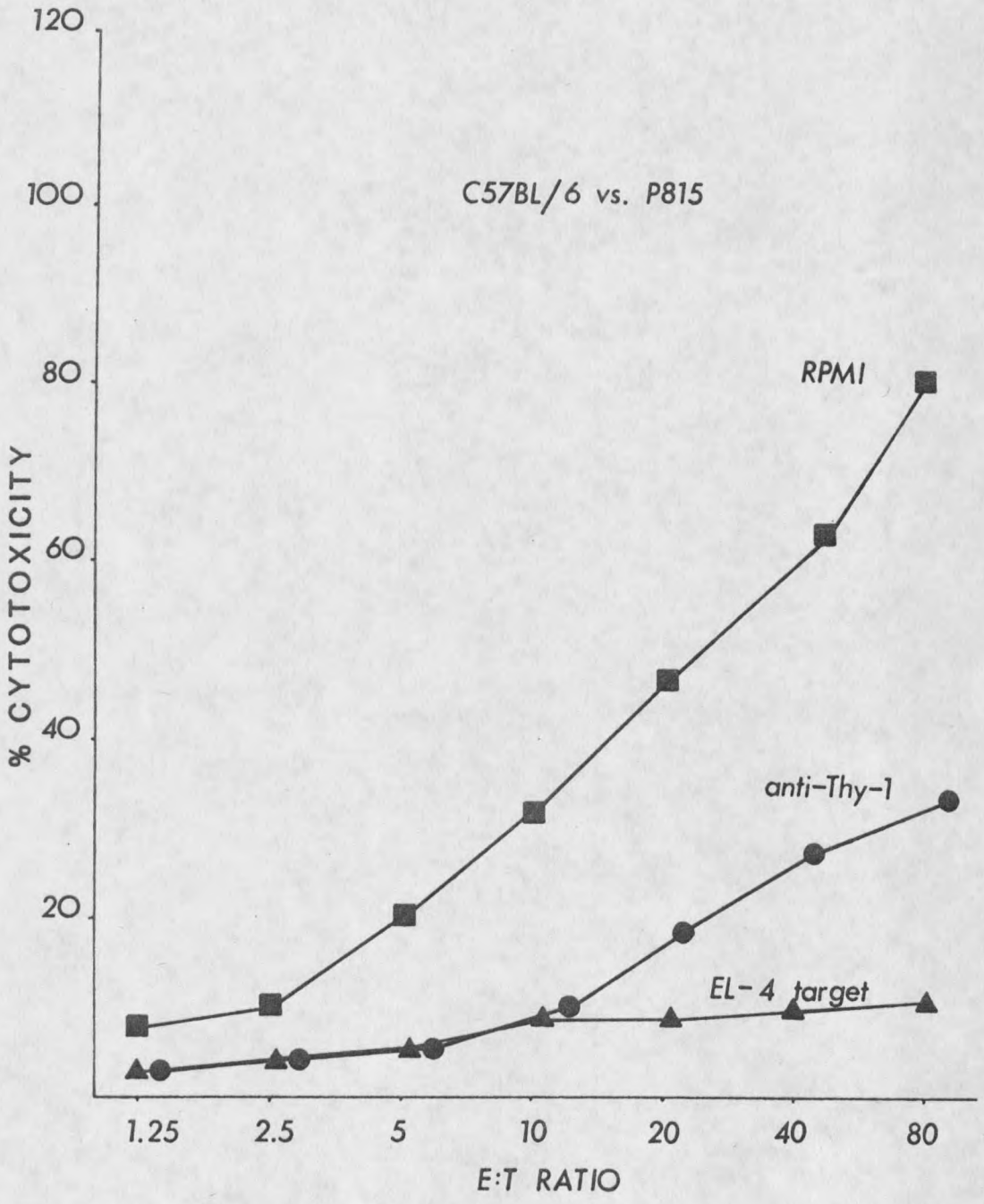

Figure 1. Modulation of the generation of cytotoxic effector cells by anti-Thy 1; 3 x 10^7 C57BL/6 splenocytes stimulated in MLC with 10^6 mitomycin C treated P815 cells (see text). Effectors were harvested at day 3 of MLC and assayed for cytotoxicity against P815 target cells. ■ represent cytotoxicity 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.

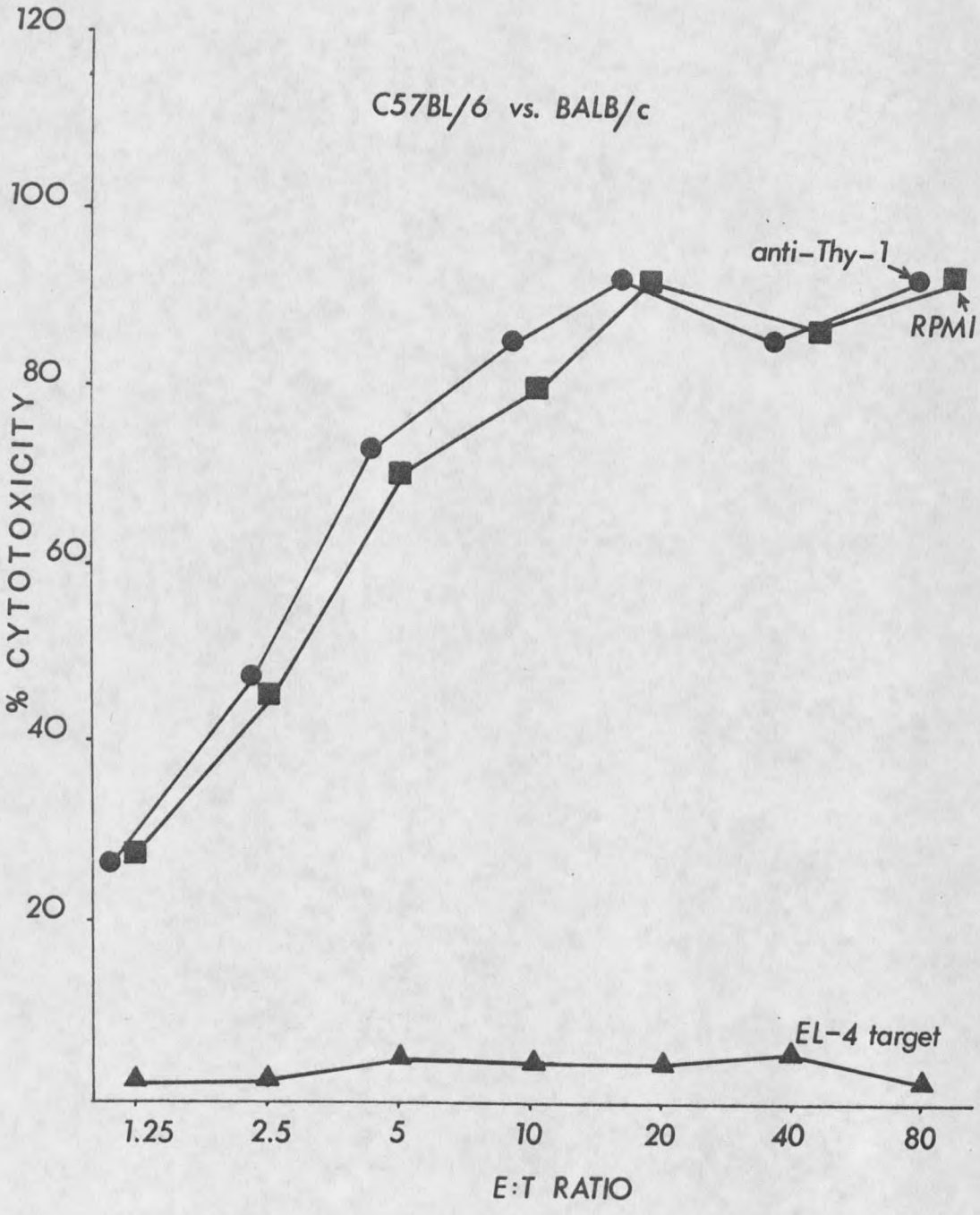

Figure 2. Legend similar to Figure 1 except C57BL/6 splenocytes were stimulated in MLC with 2.6 x 10^7 mitomycin C inactivated BALB/c splenocytes. 10% v/v antibody was added to the culture receiving anti-Thy 1.

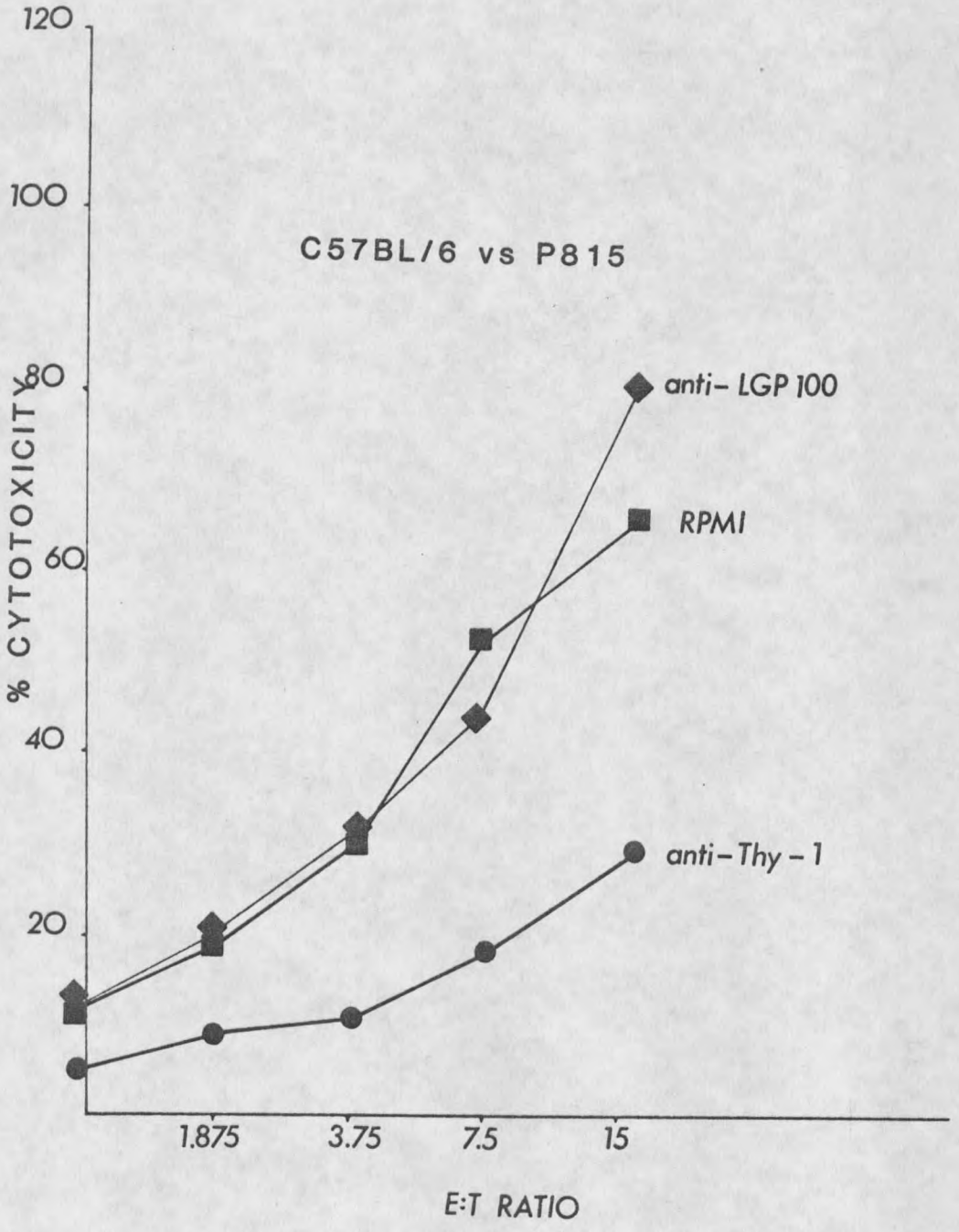

Figure 3. Modulation of the generation of cytotoxic effector cells by anti-Thy 1; 3 x 10^5 C57BL/6 stimulated with 10^4 P815 cells in 96-well microtiter plates as described in the text. Effectors were harvested at day 5 of MLC and assayed for cytotoxicity against P815 targets. ■ represent cytotoxicity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