



Inhibition of alloantigen-specific T-cell mediated cytotoxicity by antisera specific for the mouse Ly-5 antigen
by Brian Scott Davis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:

Ly-5 is an antigenic determinant on the lymphocyte surface molecule T200. Two allelic forms exist: Ly-5.1 and Ly-5.2. Anti-Ly-5.1 serum was prepared by immunizing SJL (H-2S, Ly-5.2) mice with lymphoid tissue from A.SW (H-2s, Ly-5.1) mice. Inhibitory effects of anti-Ly-5.1 serum on alloantigen-specific T lymphocyte-mediated cytotoxicity were studied. Specificity for the Ly-5.1 antigen was demonstrated in two ways: 1) indirect immunofluorescence assays detected anti-Ly-5.1 antibodies on the surface of Ly-5.1+ thymocytes but not on the surface of congenic Ly-5.2+ thymocytes; 2) alloantigen-specific cytotoxicity was inhibited when Ly-5.1+ effector cells, but not congenic Ly-5.2+ effector cells, were treated with anti-Ly-5.1 serum.

Inhibition by anti-Ly-5.1 serum was shown to occur at the level of the effector cell when Ly-5.1+ cytotoxic T-lymphocytes (CTL) were pretreated with anti-Ly-5.1 serum and washed; subsequent cytotoxicity of untreated targets was inhibited. Cytotoxicity by congenic Ly-5.2+ CTL pretreated with anti-Ly-5.1 serum and washed was not inhibited.

Untreated CTL were not inhibited in their killing of Ly-5.1+ targets which were pretreated with anti-Ly-5.1 serum and washed. The use of Ly-5- Mil cells as targets did not alter the inhibition of alloantigen-specific cytotoxicity by anti-Ly-5.1 serum.

Inhibition by antisera was not an artifactual phenomenon of the experimental system. Antiserum specific for the H-2Kk antigen on CBA effector cells had no effect on cell-mediated killing whereas antiserum specific for Ly-5.1 inhibited.

The viability of CTL after a 2 hr incubation with Ly-5.1 antiserum, was determined by trypan blue exclusion and retention of ⁵¹Cr by radiolabeled CTL. Anti-Ly-5.1 treatment did not cause CTL death and thus, loss of viability is not the mechanism of inhibition by anti-Ly-5.1 serum.

Preliminary results showed that CTL could escape from inhibition by anti-Ly-5.1, presumably by shedding or modulating cell surface antigen-antibody complexes, and that after reexpression of Ly-5.1, these same CTL could be inhibited again by anti-Ly-5.1 serum.

Conjugate formation between in vivo-generated CTL and the alloantigen-specific target P815 cells, was not inhibited by anti-Ly-5.1 serum.

Time course studies showed that addition of anti-Ly-5.1 as late as 30 minutes after mixing of targets and effectors inhibited cytolysis. This approximately corresponds to the calcium-dependent step programming for lysis; however, it cannot be determined from these experiments whether Ly-5 is involved before or after this event.

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CYTOTOXICITY BY ANTISERA SPECIFIC FOR THE MOUSE LY-5 ANTIGEN

by

Brian Scott Davis

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in

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Ly-5 is an antigenic determinant on the lymphocyte surface molecule T200. Two allelic forms exist: Ly-5.1 and Ly-5.2. Anti-Ly-5.1 serum was prepared by immunizing SJL (H-2^S, Ly-5.2) mice with lymphoid tissue from A.SW (H-2^S, Ly-5.1) mice. Inhibitory effects of anti-Ly-5.1 serum on alloantigen-specific T lymphocyte-mediated cytotoxicity were studied. Specificity for the Ly-5.1 antigen was demonstrated in two ways: 1) indirect immunofluorescence assays detected anti-Ly-5.1 antibodies on the surface of Ly-5.1⁺ thymocytes but not on the surface of congenic Ly-5.2⁺ thymocytes; 2) alloantigen-specific cytotoxicity was inhibited when Ly-5.1⁺ effector cells, but not congenic Ly-5.2⁺ effector cells, were treated with anti-Ly-5.1 serum.

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Untreated CTL were not inhibited in their killing of Ly-5.1⁺ targets which were pretreated with anti-Ly-5.1 serum and washed. The use of Ly-5⁻ M11 cells as targets did not alter the inhibition of alloantigen-specific cytotoxicity by anti-Ly-5.1 serum.

Inhibition by antisera was not an artifactual phenomenon of the experimental system. Antiserum specific for the H-2K^k antigen on CBA effector cells had no effect on cell-mediated killing whereas antiserum specific for Ly-5.1 inhibited.

The viability of CTL after a 2 hr incubation with Ly-5.1 antiserum was determined by trypan blue exclusion and retention of ⁵¹Cr by radiolabeled CTL. Anti-Ly-5.1 treatment did not cause CTL death and thus, loss of viability is not the mechanism of inhibition by anti-Ly-5.1 serum.

Preliminary results showed that CTL could escape from inhibition by anti-Ly-5.1, presumably by shedding or modulating cell surface antigen-antibody complexes, and that after reexpression of Ly-5.1, these same CTL could be inhibited again by anti-Ly-5.1 serum.

Conjugate formation between in vivo-generated CTL and the alloantigen-specific target P815 cells, was not inhibited by anti-Ly-5.1 serum.

Time course studies showed that addition of anti-Ly-5.1 as late as 30 minutes after mixing of targets and effectors inhibited cytolysis. This approximately corresponds to the calcium-dependent step programming for lysis; however, it cannot be determined from these experiments whether Ly-5 is involved before or after this event.

INTRODUCTION

Various murine cell surface antigens have been described serologically, biochemically and functionally. Different compartments of lymphoid cells, as well as different levels of maturation within a compartment are defined by the cell surface antigens they express.

Cell Surface Antigens of the Murine Major Histocompatibility Complex. Cell-cell interactions involved in specific cellular immune responses depend upon distinction between self and nonself as determined by antigens of the Major Histocompatibility Complex (MHC), known as H-2 in the mouse. The MHC is a region of genes located on chromosome 17 which encodes three classes of molecules denoted I, II and III. Two categories of class I genes are 1) those located on the left side of H-2 which code for the cell-surface transplantation antigens K, D, and L, and 2) those located on the right side of H-2 denoted the Qa-2,3 and Tla regions. Whereas the class I MHC antigens of the K and D/L regions are transplantation antigens involved in graft rejection, those of the Qa-2,3 and Tla regions are provisionally denoted as hematopoietic differentiation antigens (Hood et al., 1983).

Class I antigens are integral membrane polypeptides with a molecular weight of 45,000, noncovalently associated with a 12,000 dalton polypeptide, β_2 -microglobulin which is encoded by a gene on chromosome 2 in the mouse.

Because scientists cannot tell which, if either of two models is correct, recognition of foreign or nonself antigen has somewhat perplexed them. Those two proposed models are, first, the dual receptor hypothesis which states that a cytotoxic T-lymphocyte (CTL) recognizes foreign antigen with one receptor while recognizing self-class I antigens with a separate receptor, and second, the modified self hypothesis which describes a single receptor which recognizes both foreign antigen and self-class I antigens concomitantly (Zinkernagel, 1978). Many experiments have been performed in attempts to identify which of these two models is correct; however, the results have failed to prove one over the other.

MHC-restricted T-cell responses involving class I antigens are typically cytolytic. In its simplest form, MHC-restricted cytolysis involves the recognition of self-H-2K or H-2D antigen in association with viral antigen leading to subsequent lysis of the virus-infected cell (Zinkernagel and Doherty, 1980). Other examples of H-2K/D-restricted cytolysis include cells chemically modified or conjugated with haptens, and cells allogeneic to effector

cells at minor histocompatibility loci. Whether these processes occur via the modified self or dual receptor model, they occur most efficiently when effector and target cells share H-2K or D alleles (Klein et al., 1977).

Cytotoxic T-cells which develop in response to class I allogeneic differences recognize only the H-2K/D antigens on target surfaces prior to cytolysis. It is hypothesized that alloantigenic recognition proceeds in the same manner as does recognition of viral antigens except that allo-H-2K/D antigens serve as a single signal. The particular sites on the class I molecule which are recognized are uncertain, might be limited to only a few, and might be related to desotope (determinant sequence) regions (Goodman and Sercarz, 1983).

The second class of MHC genes encode the class II antigens which are located between those for H-2K and H-2D/L antigens. This region, cumulatively termed the I-region, has been divided into five distinct subregions based on recombination analysis: I-A, I-B, I-J, I-E and I-C. I-A and I-E genes produce two class II heterodimeric molecules which range in molecular weight from 27-29,000 to 30-33,000. These I region-associated (Ia) antigens are found on the surfaces of macrophages, B-cells, dendritic and other accessory cells, and T-cells with helper or amplifier functions. Other class II antigens have been

characterized by functional and biochemical means (Krupen et al., 1982). Suppressor T-cells express Ia antigens which were originally mapped to the I-J region by classical genetic studies. By 1983, researchers found that only 2-3.4 kilobases of DNA existed in the I-J subregion (Hood et al., 1983); it was concluded that, at the molecular level, the I-J gene did not exist. More recently, however, it has been proposed that the expression of I-J antigens is controlled by a non-H-2 gene on chromosome 4 and perhaps involves the modified antigenic expression of I-E gene products (Hayes et al., 1984).

Altogether, class II molecules serve as restricting elements that permit regulatory T-cells, such as helper-, suppressor- and amplifier-T-cells, to view antigen in the context of self on the surfaces of other T-cells, macrophages or B-cells.

Non-MHC Cell Surface Antigens. Several non-MHC murine cell surface antigens have been described in the last twenty years. These include Thy-1, Lyt-1, Lyt-2, Lyt-3, Qa1,2,3,4,5, TL, and T200 (Ly-5).

The first antigen outside of the MHC to be described was Thy-1 (Reif and Allen, 1964) which is a 24,000 dalton glycoprotein (Letarte-Muirhead et al., 1975) having two allelic forms--Thy-1.1 and Thy-1.2 (Snell and Cherry, 1972). Since a similar antigen is found on thymus cells,

thymic epithelial cells, peripheral T-cells, brain tissue cells, epidermal cells and fibroblasts (Campbell and Williams, 1981), Thy-1 is not absolutely restricted to thymus-derived lymphocytes; however, T-cells are the only immune cells to express this antigen and thus Thy-1⁺ usually denotes thymus-derived lymphocytes. Thy-1 is the most abundant surface molecule on rodent thymocytes; nevertheless, its function is still unknown after nineteen years of study (Williams and Gagnon, 1982). The wide distribution of Thy-1, its sequence homology with immunoglobulin variable and constant domains, the significant similarity with an invertebrate homolog, and rat behavioral studies using anti-Thy-1 antibodies would imply a significant role for this glycoprotein, but many questions remain unanswered.

Other cell surface antigens which have received a great deal of study are the Lyt antigens. They are, for the most part, confined to T-cells. Lyt-1 is a 70,000 dalton glycoprotein encoded by a gene on chromosome 19. Lyt-2 and Lyt-3, which may be two antigenic determinants on the same molecule, are 35,000 dalton glycoproteins encoded by chromosome 6. Two allelic forms of each Lyt antigen exist, for example, Lyt-1.1 and Lyt-1.2. These antigens have become the most descriptive for T-cells in various stages of maturation or various compartments of lymphoid

tissue and distinguish T-cells with different functions (Shiku et al., 1975; Kisielow, et al., 1975; Cantor and Boyse, 1975; Stutman et al., 1977; Wettstein et al., 1979). The $\text{Lyt-1}^+2,3^+$ phenotype is characteristic of thymic cortical lymphocytes. $\text{Lyt-1}^+2,3^-$ peripheral lymphocytes, which are derived from $\text{Lyt-1}^+2,3^-$ cortical thymocytes, are characteristically T-helper cells, while $\text{Lyt-1}^-2,3^+$ cells are either T-suppressor cells or cytotoxic effector T-lymphocytes. Suppressor cells are described as also expressing H-2I-J gene products and Ia antigens whereas cytotoxic effector cells express the TL45 (145,000 dalton) antigen (Kimura and Wigzell, 1978).

The 44,000 dalton glycoprotein TL (thymus leukemia) antigen is controlled by the gene Tla. There are at least three TL alleles (TL1, TL2, TL3) and TL antigens are typically expressed on immature thymocytes in only some strains of mice (Boyse et al., 1965). All peripheral T-cells, as well as lymph node cells, are TL^- ; however, not all TL^- lymphocytes are fully matured cells. Approximately 5% of thymocytes are TL^- . These cells are hydrocortisone resistant and considered to be mature T-cells ready to exit the thymus.

In addition, Qa and TL antigens are a series of six antigens which can be used to define different subsets of T-cells. Qa1 and Qa2 are products of two distinct loci and

are expressed on thymocytes as well as peripheral T-cells (Flaherty, 1981). Qa3, Qa4, and Qa5 are expressed only on peripheral T-cells and it has not been determined whether or not they are encoded by separate genes. Along with other antigenic phenotypes, the Qa antigens describe more specific levels of T-cell function: $\text{Lyt-1}^+2,3^-$, Qa^- cells are helper cells for B-cell responses and $\text{Lyt-1}^+2,3^-$, Qa^+ are amplifier cells for suppressor cell functions (Flaherty et al., 1979).

Cellular Immune Responses. Cell surface antigens are used to differentiate cells involved in various cell-mediated immune responses. Specific cellular immune responses to antigenic stimulation involve thymus-derived lymphocytes (T-cells) and occur in two phases: the afferent and the efferent phases. The afferent arm of the immune response involves the generation or sensitization of immunoeffector cells from precursor forms. This generation involves three steps: 1) antigen recognition/presentation, 2) cellular proliferation, and 3) differentiation of precursor cells into effector cells.

Initial recognition of antigen usually occurs by an Ia^+ macrophage. Such macrophages process antigens and present them to $\text{Lyt-1}^+2,3^-$ T-helper cells and B-cells. During responses to alloantigen or virus-modified self antigens, macrophages release a soluble helper factor,

Interleukin-1 (IL1) in response to antigen. Helper T-lymphocytes recognize foreign Ia antigens. In response to a combination of Ia-antigen disparity and macrophage-derived IL1, T-helper lymphocytes produce and release other soluble factors, most notably Interleukin-2 (IL2) (Smith et al., 1979). IL2 subsequently promotes the proliferation of T-helper lymphocytes, as well as other immunoregulatory cells, and thereby produces a cascade or amplification system in cellular immunity.

Pre-cytotoxic T-lymphocytes (pCTL) require at least two signals for maturation and differentiation: 1) antigen recognition and 2) IL2. A third signal, T-cell Replacing Factor (TRF) or Cytotoxic Differentiation Factor (CDF) might also be required. Pre-cytotoxic T-cells recognize either viral antigens in the context of self-H-2 antigens, or they recognize an allogeneic difference in either H-2K or H-2D antigens. Subsequent interaction with IL2 causes antigen-stimulated cells to proliferate in a clonal fashion. Further response to soluble factors (TRF, CDF) results in the differentiation into cytotoxic effectors--Lyt-1⁻2,3⁺ CTL.

The second phase of an immune response is the efferent arm of cellular immune responses. This phase is the actual effector phase and is exemplified, in the case of CTL, by the lysis of cells bearing the same (H-2 or H-2 plus virus)

antigens as those against which the CTL were generated. Cell-mediated cytotoxicity occurs through at least three steps: 1) antigen recognition/target cell binding, 2) "programming for lysis" (Wagner and Rollinghoff, 1974), also known as delivery of a "lethal hit" (Martz, 1975), and 3) killer cell-independent lysis (KCIL) of the target cell (Martz, 1977). After release of a programmed target cell, the CTL is available to recycle and kill other such targets for at least several cycles (Zagury et al., 1975; Martz and Benacerraf, 1976).

In the first step of CTL killing, target recognition and binding, the effector cell and target cell form a stable conjugate in which the membranes of both cells are in contact. This process is mediated by an antigen receptor on the CTL, the "T-cell receptor", in combination with other nonspecific adherence molecules. Adherence is relatively temperature insensitive and requires magnesium (Mg^{+2}) for stable binding between effector and target cells (Golstein and Smith, 1976; Plaut et al., 1976). Although Mg^{+2} is sufficient, calcium ions (Ca^{+2}) significantly synergize with Mg^{+2} during adhesion. Stable target binding in vitro occurs within 0.5 to 5 min at $37^{\circ}C$ after initiation of cell contact.

The second step of CTL killing, delivery of the "lethal hit", is mediated by an unknown mechanism, requires

anywhere from 5 to 30 min, occurs increasingly faster between 25°C to 40°C, and is dependent on the presence of Ca^{+2} (Martz et al., 1982b). This step is inhibited by chelators of divalent cations such as ethylenediamine tetraacetate (EDTA). Ethyleneglycol bis-(2-aminoethyl ether)-N,N'-tetraacetate (EGTA) is also frequently used since it binds Ca^{+2} 100-times more avidly than Mg^{+2} and thus allows target binding but inhibits programming for lysis. The observation that EGTA allows target binding but does not allow programming for lysis, while EDTA inhibits both, is used as further support for the hypothesis that magnesium ions are required for adhesion and calcium ions for delivery of the lethal hit.

The third step in CTL killing is the lysis of the target cell which occurs independently of the cytotoxic cell. Killer cell-independent lysis requires on the order of 90-100 minutes for completion, is the least temperature-sensitive of all three steps, and requires no divalent cations. Cell-mediated lysis is characterized by blebbing in the target cell membrane (zeiosis) and differs from complement-mediated lysis which involves swelling of the target cell (Sanderson, 1981). Zeiosis may be a rapid influx of water through Ca^{+2} -dependent potassium channels created during the lytic process (Berke, et al., 1984). Pores in target cell membranes, purportedly created by CTL,

have been described in electron microscopic studies (Henkart et al., 1982; Podack and Dennert, 1983); these may turn out to be the structures involved in programming for lysis. Furthermore, Russell and Dobos (1980) have reported that the target nucleus disintegrates moments after CTL contact but at a point much earlier than cytoplasmic membrane lysis. This further differentiates CTL killing from complement-mediated lysis which does not produce nuclear rupture. During KCIL, the effector cell is free to detach from the target and either to deliver lethal hits to other attached targets or to recycle to form new conjugates.

These three steps are a very simplified description of cell-mediated cytotoxicity based largely on temperature and cation requirements. Since the role of cations was studied in the mid-seventies, the picture of the cytolytic process has been modified little even though it has been studied much. Just as studies with divalent cations used inhibitory reagents, such as EDTA and EGTA, many other reagents have been used to elucidate the roles and mechanisms of cell surface molecules and metabolism in cell-mediated killing.

Inhibition Studies on T Cell-Mediated Cytotoxicity.
Investigations into the mechanism of cell-mediated killing usually involve attempts to inhibit the lytic process in order to delineate the necessary steps. Two approaches

include inhibition of cellular metabolism by treatment with drugs and inhibition of the functions of cell surface antigens by treatment with antibodies specific for these antigens.

Antibody blocking studies have been used to investigate the roles of various cell surface antigens on the effector cell: Lyt-1, Lyt-2 and Lyt-3, LFA-1, the newly described L3T4a, an unidentified antigen recognized by a rat anti-mouse activated T-cell serum (RAT*), Ly-5, β_2 -microglobulin, Thy-1, H-2 antigens, TL, LGP-100, Ly-6, immunoglobulins, various leukemia viral glycoproteins, and antigens defined by anti-idiotypic antisera, alloantisera and xenoantibodies (Martz et al., 1982a). Of all studied, only antibodies against Lyt-2, Lyt-3, LFA-1, L3T4a, RAT*, and Ly-5 have been shown to inhibit cytotoxicity in the mouse.

It is widely accepted that antibodies which specifically bind to a structure and interfere with a cellular function (in the absence of complement) must recognize a structure involved in that function. Recognition of target-cell H-2K or H-2D antigens is the basis of allo-antigen-sensitized CTL cytotoxicity; antibodies against target-H-2 antigens block target cell recognition and thus lysis. On the other hand, H-2K/D antigens on CTL are not

involved in such recognition and therefore antibodies against them have no effect on cytotoxicity.

Antibodies that inhibit a cellular function could do so in either of two ways: 1) "turn off" the effector cell globally and inhibit all related cell functions, or 2) interfere at a singular site which is directly or indirectly involved in that function, e.g. steric hindrance (Golstein et al., 1982). The first antibodies obtained which blocked effector cell killing were directed against target cell antigens such as H-2K and H-2D (Bonavida, 1974; Schrader and Edelman, 1976; Germain et al., 1975). Within the past five years, antibodies have been generated that block cell-mediated cytotoxicity, in the absence of complement, at the effector cell level.

The first antibodies to be studied in detail which block cell-mediated cytotoxicity (CMC) at the effector level, rather than the target level, were against Lyt-2,3 antigens (Nakayama et al., 1979; Shinohara and Sachs, 1979). Anti-Lyt-2,3 antibodies inhibit cytolysis of allogeneic as well as virus-infected syngeneic targets at the effector level (Nakayama, 1979; Shinohara and Sachs, 1979; Hollander et al., 1980). Experiments in which only effector cells (or target cells) were treated with and washed of excess antibody and combined with untreated target (or effector) cells confirm these findings (Pierres et al.,

1982; Hayot et al., 1982). Two types of experiments have shown that anti-Lyt-2 antibodies inhibit the target recognition step of CMC. First, inhibition of conjugate formation in the presence of Lyt-2 antibodies was reported (Hollander et al., 1981). Second, time course studies, comparing inhibition of CTL killing when addition of anti-Lyt-2 was varied relative to addition of Ca^{+2} in a Ca^{+2} -free medium, showed that anti-Lyt-2 only inhibited when added before or simultaneously with Ca^{+2} , but not after Ca^{+2} (Hollander, 1981; Shinohara, 1981).

An additional model of inhibition by anti-Lyt-2 has been proposed wherein Lyt-2 functions as a nonantigen specific receptor for class I MHC antigens (Swain, 1983).

The next antibody described which blocked cytotoxicity recognized a previously unknown cell surface antigen--LFA-1 (lymphocyte function-associated antigen-1) (Kurzinger et al., 1981; Davignon et al., 1981; Pierres et al. 1982; Dialynas et al., 1982). LFA-1 is a cell membrane molecule comprised of two noncovalently linked polypeptides of 180,000 and 95,000 daltons. It is expressed on both T- and B-cells as well as 80% of bone-marrow cells (Kurzinger et al., 1981). It seems unlikely that LFA-1 is involved in specific antigen recognition. Many similarities to the Mac-1 antigen, which has been described as a possible C3b receptor on macrophages and granulocytes, have been

reported (Springer et al., 1979). The LFA-1-associated structure might be a receptor for an as yet unidentified ligand (Martz et al., 1983).

Antibodies against LFA-1 inhibit cytotoxicity (Springer et al., 1982). Since such inhibition is additive with that of anti-Lyt-2, it is assumed that LFA-1 and Lyt-2 define two separate molecules. Furthermore, the two antibodies do not cross-inhibit one another, suggesting that they are topographically distinct (Sanchez-Madrid et al., 1982). In addition, LFA-1 expression does not correlate with MHC-recognition of class I or class II antigens (Dialynas et al., 1983b). As with Lyt-2, antibodies against LFA-1 inhibit only when added early relative to addition of Ca^{+2} . In fact, LFA-1 is believed to be involved in killing at an earlier event than is Lyt-2. Since anti-LFA-1 causes reversion of conjugate formation, LFA-1 as well as Lyt-2 may contribute to CTL-target avidity (Springer et al., 1982). Human homologs of both Lyt-2,3 and LFA-1 (Leu-2a and LFA-1, respectively) have also been identified (Evans et al., 1981; Springer et al., 1982).

In contrast to Lyt-2,3 antisera, anti-Lyt-1 does not inhibit CMC unless killing is directed against Ia antigenic differences (Swain et al., 1981). The newly described antigen, L3T4a, is also associated with class II reactivity and antibodies against L3T4a inhibit cytotoxicity.

Whereas anti-Lyt-2,3 and anti-LFA-1 antibodies inhibit only at very early stages in the lytic process, another antibody--RAT* (rat anti-mouse activated T-cell)--was shown to inhibit during programming for lysis as well as during the KCIL step (Hiserodt and Bonavida, 1981). The activity of RAT* can only be absorbed by "activated" T-cells (e.g. CTL, Concanavalin A (Con A)-induced blasts, etc.). This xenogeneic antiserum has some peculiar characteristics. For example, RAT* serum inhibits conjugate formation, but inhibits cytotoxicity of preformed conjugates without disrupting preformed conjugates. Nonetheless, the data indicate it is a very exciting reagent for studying effector cell structures.

Monoclonal antibodies against the newly discovered murine cell surface antigen L3T4a are, perhaps, the most recently described antibodies which inhibit antigen specific cytotoxicity (Dialynas et al., 1983b). The expression of L3T4a by T cell clones appears independent of the expression of Lyt-2 antigens and correlates with reactivity against class II MHC antigens; this reactivity includes antigen-specific cytotoxicity, antigen-specific proliferation, and release of lymphokines (Wilde et al., 1983). Inhibition of class-II specific cytotoxicity by anti-L3T4a antibodies was concluded not to inhibit the "lethal hit" but to interfere at a prior stage. L3T4a is probably

similar to the human Leu-3/T4 molecule which is related to the same function in class II antigen reactivity in humans (Dialynas et al., 1983a).

Lastly, the murine cell surface antigen Ly-5 was discovered by Komuro et al. (1975) after immunizing between mouse strains compatible for H-2 as well as known T-lymphocyte alloantigens (Lyt-1, Lyt-2, Lyt-3). SJL anti-A.SW sera were observed by complement cytotoxicity assays to recognize a single gene product on thymocytes and T-cells from several strains. Only two strains, SJL and STS, were found to express the antithetical antigen and were classified as Ly-5.2; most other known strains are classified as Ly-5.1.

The other identity of Ly-5 is T200. In 1979, Trowbridge described a murine cell surface antigen which was immunoprecipitated by a rat monoclonal antibody and had a molecular weight of 200,000. T200 was established as Ly-5 in 1980 by Omary et al. (1980b) and by Siadak and Nowinski (1980). Although rat anti-T200 antibodies could recognize murine Ly-5 molecules, they could not differentiate the two allelic forms, Ly-5.1 and Ly-5.2.

Ly-5 was originally believed to be limited to T lymphocytes. Because the BALB/c myeloma MOPC 70-A did not express Ly-5 as detected by complement-mediated cytotoxicity assays, it was surmised that B-lymphocytes did not

express Ly-5. It was later found that B-cells express Ly-5 antigens but they could not be detected by complement cytotoxicity assays. In fact, using other assays involving fluorescence or Protein A-sheep red blood cells, it was discovered that all normal hematopoietic cells, except erythrocytes and proerythroblasts, express Ly-5 antigens (Scheid and Triglia, 1979).

Nakayama et al. (1979), in a paper studying Lyt-2 and Lyt-3 antisera, first reported the inhibition of antigen-specific CTL cell-mediated cytotoxicity in the absence of added complement by Lyt-4.1 (Ly-5.1) antiserum. Inhibition was comparable to that of Lyt-2 and Lyt-3 antisera. This was reported again in 1982 (Nakayama, 1982) for Ly-5.1 antiserum with greater specificity than the original SJL anti-A.SW antiserum. In 1979, Cantor et al. (1979) reported inhibition of natural killer (NK)-mediated cytotoxicity in vitro by anti-Ly-5.1 serum in the absence of complement. Attempts to block xenogeneic antigen-specific CTL killing with Ly-5 antibodies in the absence of complement were unsuccessful (Davignon et al., 1981). Yakura, et al. (1983) reported inhibition of B-cell responses using a monoclonal antibody against the Ly-5 antigen, but saw no inhibition of cytotoxicity reactions and had less well-defined results on B-cell reactions using conventionally prepared anti-Ly-5 antisera (H. Yakura, personal communica-

tion). More recently, Newman et al. (1982, 1983) reported that a monoclonal antibody generated against the human homolog of T200 (Omary et al., 1980a) blocks human natural killer mediated cytotoxicity.

A great deal of evidence has accumulated that NK-cells and CTL have analogous mechanisms of killing (Hiserodt et al., 1982; Quan, et al., 1982). If there were an antibody that inhibited NK-killing but not CTL-killing, although the specific antigen was expressed on both types of cells, some difference might exist between NK- and CTL-killing mechanisms or in the role of that antigen in the lytic process (Seaman et al., 1981). However, an antibody which inhibited both types of killing would be further evidence for similarity of the two mechanisms.

At present many researchers have reported that anti-Ly-5 sera blocked NK cell-mediated cytotoxicity (Minato et al., 1980; Seaman et al., 1981; Brooks et al., 1983; Cantor et al., 1979; Pollack et al., 1979). Some of these researchers also studied CTL-mediated cytotoxicity and reported no inhibition by anti-Ly-5.

However, inhibition of T cell-mediated antigen-specific cytotoxicity by Ly-5 antisera has been studied by Harp, Davis, and Ewald (1984). In addition to inhibition of CTL killing, they have shown that anti-Ly-5.1/T200 antibodies inhibit alloantigen-stimulated mixed lymphocyte

reactions, in vitro generation of CTL, Con A blastogenesis and oxidative mitogenesis, and antibody-dependent cell-mediated cytotoxicity (ADCC) (Harp and Ewald, 1983; Harp, et al., 1984; Small et al., 1984). Their results agree with those of Nakayama with respect to inhibition of CTL killing and with Pollack et al. (1979) with respect to inhibition of ADCC. Furthermore, such inhibition of several immune responses parallels the effects of anti-L3T4a and anti-LFA-1 which inhibit the generation of CTL, cytotoxicity by CTL, and lymphokine release in response to antigen (Wilde et al., 1983; Dialynas et al., 1983a; Davignon et al., 1981). Likewise, antibodies against the human cell surface antigen, T3, which may be closely associated with the human "T-cell receptor", also inhibit generation of CTL, cytotoxicity by CTL, and antigen specific proliferation (Reinherz et al., 1980, 1982). An antibody with multiple inhibitory activities is not unique.

Only the studies by Nakayama and associates and those of Ewald and associates have reported inhibition of CTL-killing by Ly-5 antiserum. The reasons why Nakayama succeeded in inhibiting CTL-killing with Ly-5 antisera where others failed are not known. Since some (Davignon et al., 1981; Newman, 1982; Seaman et al., 1981; Yakura, personal communication) who reported no inhibition by Ly-5 antibodies used monoclonal antibodies, the value of monoclonal anti-

bodies in the study of such inhibition is suspect. In fact, Harp and Ewald (unpublished results) have attempted to inhibit CTL-killing with monoclonal anti-Ly-5.1 and have not.

Brooks et al. (1982) attempted to inhibit CTL killing with polyconal Ly-5.1 antiserum. Whereas they inhibited cytotoxicity with anti-Lyt-2 serum, they reported no inhibition by anti-Ly-5.1 serum. Their data do show, however, a 25% reduction in cytotoxicity at serum dilutions of 1:40 or less as compared with dilutions of 1:160. They report this difference as not significant and it is certainly meager relative to inhibition by anti-Lyt-2 serum. It will be difficult to determine the reason for the differing results of these researchers until more is known about the nature of anti-Ly-5.1-mediated inhibition as obtained by those who observe it.

As more work is done on cell-mediated immune responses, undoubtedly more antibodies will be described which inhibit them. It is not sufficient to report that such antibodies have been found. More detailed analyses of their action should reveal the nature of their activity and thus elucidate some of the mechanisms of immune responses.

My work describes inhibition of alloantigen-specific T-cell mediated cytotoxicity by anti-Ly-5.1 serum, establishes the specificity of the alloantiserum, and attempts

to elucidate the mechanism of inhibition by determining which event of the lytic process anti-Ly-5.1 serum inhibits.

The crux of these studies on the specificity of Ly-5.1 antisera involved using congenic pairs of mice, one Ly-5.1⁺, the other Ly-5.2⁺, as sources of effector cells. Studies on the mechanism of inhibition involved a) determining whether inhibition occurs at the level of the effector or the target cell, and b) eliminating antibody-mediated killing of effector cells as the simple means by which cytotoxicity is inhibited. Further studies of the inhibitory mechanism were done to delineate the events which anti-Ly-5.1 serum inhibited (target recognition/binding, "programming for lysis", etc.).

MATERIALS AND METHODS

Media

Tumor cells were cultured in vitro in RPMI 1640 (Irvine Sci., Santa Ana, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sterile Systems, Ogden, UT) through a 0.45 micrometer (um) filter (Millipore Corp., Bedford, MA).

Mixed lymphocyte cultures and cultures testing for cell-mediated cytotoxicity were incubated in RPMI medium further supplemented with 10 millimolar (mM) N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma Chemical, St. Louis, MO), 5×10^{-5} M 2-mercaptoethanol (2ME) (Bio-Rad Laboratories, Richmond, CA), 100 Units/milliliter (ml) penicillin, 75 micrograms (ug)/ml streptomycin, 2.5 ug/ml fungizone (Irvine) and 2 mM L-glutamine (Irvine), hereafter referred to as complete RPMI medium.

Hanks Balanced Salt Solution (HBSS) (Irvine) was prepared from powder as directed on the bottle, filtered and stored at 4°C.

Mice

C57Bl/6 (H-2^b), SJL/J (H-2^S), A.SW (H-2^S), and CBA (H-2^k) mice were obtained from Jackson Laboratories, Bar

Harbor, ME. BALB/cByJ (H-2^d) were donated by Dr. Jim Cutler, Montana State University. C57Bl/6-Ly-5.2 (H-2^b) mice were obtained from Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York, NY. Breeding colonies of all strains listed were established in our animal facilities. Mice of both sexes were used at 8 to 50 weeks of age.

Cell Lines

Tumor cell lines carried in vitro included the following: 1) P815, a dimethyl benzanthrene-induced mastocytoma of DBA/2 (H-2^d) origin, 2) EL4, a spontaneous lymphoma of C57Bl/6 (H-2^b) origin, 3) M11, a plasmacytoma of BALB/c (H-2^d) origin which is Ly-5⁻ (Lanier et al., 1981), and 4) BW5147 (H-2^k) a spontaneous, Thy-1.1⁺, cortisone and PHA sensitive thymoma from AKR mice.

Antisera

Conventionally generated antisera against the Ly-5.1 antigen were prepared by the method described by Komuro et al. (1975). SJL/J mice (Ly-5.2) were immunized intraperitoneally with 5×10^7 pooled cells from the spleens, thymuses and lymph nodes of A.SW mice (Ly-5.1) approximately every two weeks. Starting with the fifth immunization, blood was collected by supraorbital bleeding 7 and 10 days after injection. Sera were collected from clotted

blood after centrifugation and were heat-inactivated at 56°C for 15 min. Sera were tested by immunofluorescence for presence of anti-Ly-5.1 activity and pooled. Aliquots were frozen and stored at -20°C until future use. Normal mouse sera (NMS) from unimmunized SJL/J mice were collected and treated in a similar manner.

Conventional antiserum specific for the H-2K^k molecule was produced at and received from the National Institutes of Health (catalog no. Y1-9-03-15-03). This antiserum was produced by immunizing (A.TL x 129)F₁ mice (H-2K^{s/b}, I^{k/b}, S^{k/b}, D^{d/b}) with tissue from A.AL mice (H-2K^k, I^k, D^d). This serum contains activity only for the H-2K^k antigen.

Indirect Immunofluorescence Assay

Sera from individual donor mice were assayed for antibody activity by the indirect immunofluorescence assay (IFA). Sera assaying positively on Ly-5.1⁺ cells (thymocytes from most strains of mice) and negatively on Ly-5.1⁻ cells (B6-Ly-5.2 or SJL/J thymocytes) were considered to be anti-Ly-5.1 sera. Antisera were diluted, typically 1:25, 1:50, 1:100, 1:200 and 1:400, in HBSS with 0.02% NaN₃. Briefly, 5 x 10⁵ cells in HBSS were placed in each well of a 96-well flat-bottom microtiter plate (Falcon catalog no. 3072). The plate was centrifuged at 200 x G for 5 min and excess medium was removed. Fifty microliters of each anti-

serum at each dilution were added to wells. The plate was incubated at 4°C for 30 min followed by centrifugation at 200 x G for 5 min. Excess antiserum was removed carefully by three washes with 200 ul HBSS per well. Next, 50 ul of FITC-labelled rabbit anti-mouse immunoglobulin secondary antiserum, diluted 1:50 in HBSS, was added to each well. The contents of the wells were mixed and the plate incubated at 4°C for 30 min. The plate was centrifuged and cells in each well washed three times as before. Finally, 50 ul of 1:1 glycerol-HBSS were added per well and each well scored for fluorescence using an Olympus IM inverted microscope mounted with a model BH-RFL fluorescence vertical illuminator. The fluorescence illuminator was equipped with B(DM-500 + O-515) dichromic mirrors, no barrier filter, and a 500 FITC JCB 5-0 filter. The power source was a 100-watt mercury power supply (Chi U Tech Corp., Bayside, NY).

Mixed Lymphocyte Cultures

Mice were killed by cervical dislocation and the spleens removed aseptically into complete RPMI medium. Single cell suspensions of splenocytes were prepared by forcing spleens through an ethanol/flame-sterilized wire screen, and then repeatedly through a 22 gauge needle on a 10 ml syringe. Erythrocytes were removed by hypotonic

lysis. Splenocytes used as responder cells were centrifuged and resuspended in complete RPMI medium. Splenocytes used as stimulator cells were treated with 30 ug/ml mitomycin-c (Sigma Chem.) for 1 hr at 37°C in a humidified atmosphere of 5% carbon dioxide (CO₂) in air, at a concentration of approximately 2-3 x 10⁷ per ml. Stimulator cells were then centrifuged and resuspended in 1 ml complete RPMI medium, layered over 10 ml FCS and centrifuged. The cells were washed through FCS once more. Finally, both responder and stimulator cell populations were resuspended in RPMI medium and counted in a hemocytometer.

Equal numbers, 2 x 10⁷ each, of responder and stimulator cells were added to 25 cm² tissue culture flasks (Falcon catalog no. 3013) in a total volume of 12 ml complete RPMI medium. The flasks were incubated upright for 4 days at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cytotoxicity Assays

Mixed lymphocyte culture-generated cytotoxic T-lymphocytes were harvested by centrifugation after 4 days of incubation. Cells were washed at least once, resuspended in complete RPMI medium and counted in a hemocytometer. CTL-mediated cytotoxicity was assayed in a 2 hr ⁵¹Chromium (⁵¹Cr) assay against either of two target cell types: radiolabelled tumor cells (e.g. P815) or radio-

labelled 3-day Lipopolysaccharide (LPS)-induced blasts (25 ug/ml LPS (Difco Lab, Detroit, MI)). These assays were modified from that described by Brunner et al. (1968). Briefly, target cells were radiolabelled by incubating approximately 3×10^7 washed tumor cells or 1×10^7 Ficoll-Hypaque-enriched LPS-blasts with 200 microcuries (uCi) Sodium ^{51}Cr chromate ($\text{Na}^{51}\text{CrO}_4$) (New England Nuclear, Boston, MA) for 1 hr at 37°C in 200 ul FCS. Next, radiolabelled target cells were washed twice through 5 ml FCS as previously described and resuspended in complete RPMI medium. Cytotoxic T-cells were diluted several times (e.g. 1:2, 1:4, 1:8, 1:16) in 100 ul triplicates and delivered into wells of a 96-well V-bottom microtiter plate (Linbro cat. no. 76-023-05). After an appropriate dilution, $1-3 \times 10^4$ target cells in 100 ul were added 1) to each well containing diluted CTL, 2) to triplicate wells containing only medium, and 3) to triplicate wells containing 100 ul detergent which lyses all cells (ZAP). Plates were centrifuged at $200 \times \text{G}$ for 5 min to initiate contact between CTL and targets. After incubation for 2 hr at 37°C in humidified air with 5% CO_2 , plates were recentrifuged at $350 \times \text{G}$ for 10 min at 4°C . Carefully, 100 ul of supernatant were removed from each well and counted for gamma-radioactivity in a Beckman Biogamma counter (Beckman Instruments). Alternatively, 100 ul of assay supernatant were dissolved

in 2 ml Multisol (Isolabs, Akron, OH) and counted in a Packard Tri-Carb 460CD liquid scintillation counter using window settings identical to those for counting tritium (^3H). Data were obtained as counts per minute (cpm) and percent cytotoxicity was calculated by the formula:

$$\% \text{ Cytotox.} = \frac{\text{cpm with CTL} - \text{cpm in medium alone}}{\text{cpm in detergent} - \text{cpm in medium alone}} \times 100$$

Time course assays were performed with the following differences: 1) 96-well flat-bottomed (Costar, Cambridge, MA) plates were used instead of V-bottom plates; 2) plates were centrifuged only briefly to initiate contact between effector and target cells, which requires approximately 15 min, and the contents of all wells were resuspended by gentle pipeting; 3) reagent additions were made at various times before ($t=-15$ min) or after ($t=0, 15, 30, 45, 60, 90, 120$ min) addition of targets, and each well was mixed by gentle pipetting. All reagent-treated wells were incubated a total of 120 min from the time of target addition before harvesting supernatants. Controls included targets without effectors in the presence of each reagent (e.g. anti-Ly-5.1, EGTA).

Serum Treatment For Cytotoxicity Assays

Unless otherwise noted, all antiserum treatments involved preincubating effector cells with 10 μl of anti-

Ly-5.1 or NMS per well for 15 min at 4°C in the absence of target cells. After antibody had been allowed to bind to effector cells, target cells were added and the plate centrifuged.

Other treatment protocols, such as effector or target cell pretreatment and wash, involved addition of 50 ul serum to a pool of 10^6 - 10^7 effectors or 10^5 targets, in a 1.5 ml Eppendorf tube (Van Waters & Rogers Sci., Seattle, WA) followed by a 30 min incubation at 4°C, and two washes by centrifugation in complete RPMI medium. This treatment approximately corresponds to or exceeds the same amount of antiserum per cell as was used in six assay wells--triplicates at two effector-to-target ratios--in experiments in which antiserum was present throughout the assay.

In Vivo Generation of Cytotoxic T-Lymphocytes

Peritoneal exudate lymphocytes were generated by injecting C57Bl/6 and congenic C57Bl/6-Ly-5.2 mice intraperitoneally with 3×10^7 P815 cells in HBSS. Ten days later, mice were killed by cervical dislocation and 3-5 ml of HBSS were injected into the peritoneum. The abdomen was massaged and fluid was removed with a pasteur pipet through a hole in the peritoneum. In order to maximize cell recovery, the peritoneal cavity was washed a second time with

HBSS and fluid again removed. These peritoneal exudate cells were centrifuged, resuspended in 10 ml of fresh HBSS and incubated for 1 hr in a plastic petri plate at 37°C to allow macrophages and other adherent cells to attach. The plate was then carefully rinsed twice, avoiding any contact which would detach adherent cells. Nonadherent cells were centrifuged and washed once in complete RPMI medium. These cells were considered as peritoneal exudate lymphocytes (PEL). Alloantigen specific cytotoxicity against P815 targets was consistently at least 8-10 times greater than that generated by in vitro MLC as judged by the effector-to-target ratio required to achieve 40-50% cytotoxicity.

Determination of Conjugate Frequency

One hundred microliters containing 10^5 PEL and 100 ul containing 10^5 target cells were mixed in a 10 x 75 mm test tube, centrifuged and incubated for 15 min at 37°C. The pellet was gently resuspended once with a variable micropipetor set at 100 ul. An aliquot was then transferred to a hemocytometer and lymphocyte-target conjugates were enumerated microscopically. Tumor-target cells were easily distinguished by size, color and refractivity.

From 150 to 200 PEL were counted and scored for conjugate association. A conjugate was scored if one target and one lymphocyte were juxtaposed with visible contact between

their membranes. Adjustment through the plane of focus was frequently necessary to confirm contact. Cells present in clumps, or lymphocytes clumped around one or more target cells were not scored or counted. A systematic progression through all grids and regions of one hemocytometer counting chamber was performed. Running counts of target cells, lymphocytes and conjugates were made. Target cell concentration, lymphocyte concentration, and percent conjugate frequency were calculated.

Replicate counts were performed; however, emphasis was given to examining all effector-target combinations as quickly as possible in order to equalize treatments. Serum treatments were scored and calculated as blind tests in order to eliminate prejudice.

Determination of CTL Viability

CTL viability after incubation with sera was determined by trypan blue exclusion or by percent release of ^{51}Cr from radiolabelled effector cells. One hundred microliters of cell suspension were added to 100 μl 0.4% trypan blue (Sigma Chem.) in pH 7.4-phosphate buffered saline and mixed. A drop was placed onto a hemocytometer and five red cell grids were counted for viable cells (clear) and for dead cells (blue) after 3 min elapsed.

Percent viability was calculated by the formula:

$$\% \text{ Viability} = \frac{\text{number of viable (clear) cells}}{\text{total number of cells counted}} \times 100$$

An alternative method of determining cell viability, used with antiserum treated CTL, involved ^{51}Cr -labelling CTL and observing percent ^{51}Cr release after incubation with serum. Radiolabelling of CTL was performed the same as for Ficoll-Hypaque-enriched LPS blasts (see "Cytotoxicity Assays" above). Percent viability was calculated by the following formula:

$$\% \text{ Viability} = \left(1 - \frac{\text{CPM released spontaneously from CTL}}{\text{CPM after detergent treatment}}\right) \times 100$$

RESULTS

Specificity of Anti-Ly-5.1 Antiserum for the Ly-5.1 Antigen

Functional specificity of anti-Ly-5.1 serum can be demonstrated in two ways: 1) indirect immunofluorescence assays (IFA), using congenic B6-Ly-5.1 cells or B6-Ly-5.2 cells as substrate, detect any antibodies whether directed toward Ly-5 or toward other antigens on the B6 background; and 2) cell-mediated cytotoxicity assays test inhibition of killing in the absence of complement in assays using two cell types, one Ly-5.1 and one Ly-5.2, as either effectors or targets, or using an Ly-5⁻ cell line as a target.

Specificity for Ly-5.1 was first shown by immunofluorescence assays using thymocytes from both C57Bl/6 and C57Bl/6-Ly-5.2 mice (Table 1).

Table 1. Indirect Immunofluorescence Assay of Anti-Ly-5.1 Serum.

Anti-Serum Dilution	C57Bl/6 thymocytes		C57Bl/6-Ly-5.2 thymocytes	
	anti-Ly-5.1	NMS	anti-Ly-5.1	NMS
1:25	+++	-	+	+
1:50	+++	-	-	-
1:100	+	-	-	-
1:200	+	-	-	-
1:400	+	-	-	-

Next, specificity for Ly-5.1 was demonstrated in CMC assays which determined whether anti-Ly-5.1 serum inhibited at the level of the effector cell and whether it was specific for effectors expressing the Ly-5.1 antigen. Addition of anti-Ly-5.1, heat treated to inactivate complement, to a CMC assay containing BALB/c (H-2^d, Ly-5.1) effectors and SJL LPS-blasts (H-2^s, Ly-5.2) targets inhibited cytotoxicity relative to medium alone and to addition of NMS (Figure 1). Conversely, the addition of Ly-5.1 antiserum to assays containing SJL effectors and radiolabelled BALB/c LPS-blast targets, where the antibody could react with target but not effectors, did not inhibit cell-mediated killing.

In order to investigate whether antibodies with specificities for antigens other than Ly-5 might be the inhibitory antibodies in the antiserum, effectors were generated from C57Bl/6 or C57Bl/6-Ly-5.2 congenic mice, both by in vitro mixed lymphocyte culture and by in vivo immunization with P815 tumor cells. Subsequent cytotoxicity was assayed for inhibition by anti-Ly-5.1 serum. As shown in Figures 2 and 3, anti-Ly-5.1 serum inhibited cytotoxicity only when the effector cells possessed the Ly-5.1 allele. This was shown both for effector cells generated in vitro from normal splenocytes (Figure 2) as well as those generated in vivo as PEL from immunized mice (Figure 3).

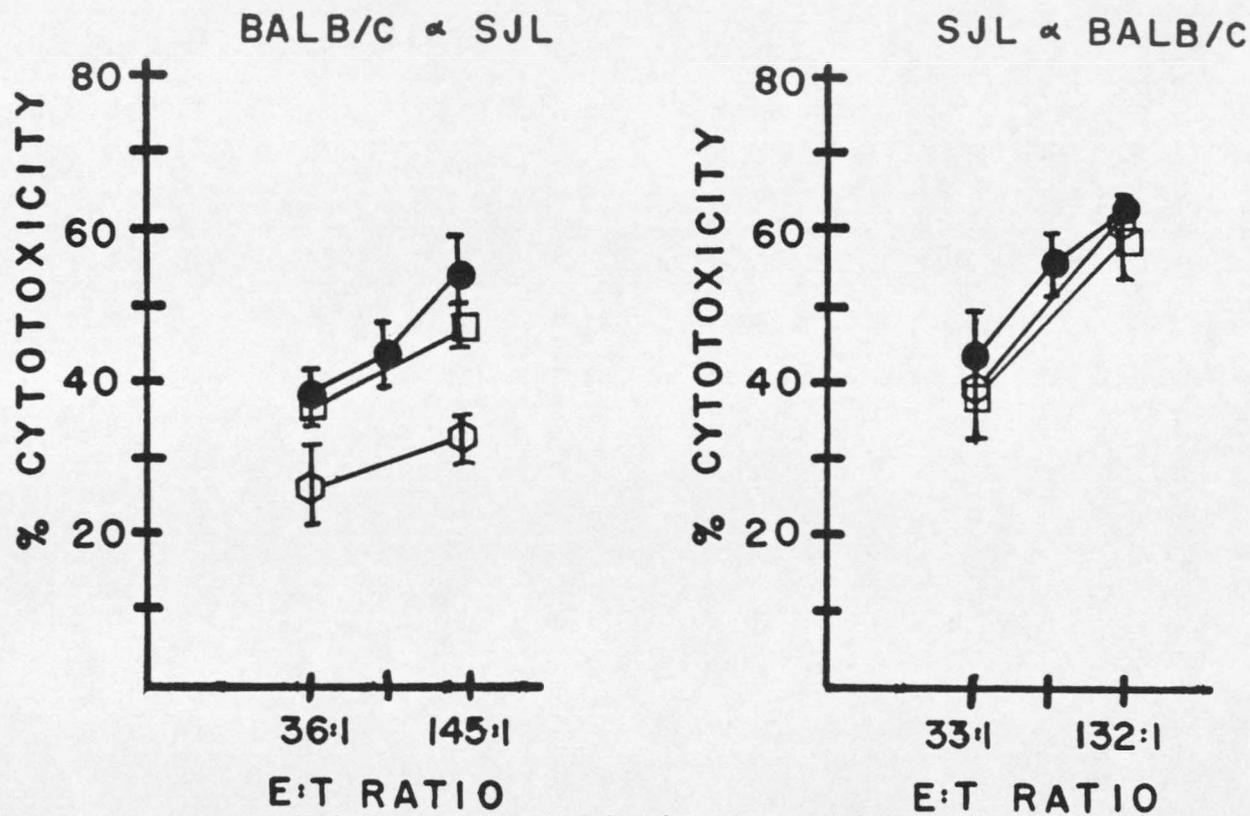


Figure 1. Inhibition of CTL killing by anti-Ly-5.1. BALB/c splenocytes were cultured in vitro with mitomycin-c treated SJL splenocytes. Conversely, SJL splenocytes were cultured in vitro with mitomycin-c treated BALB/c splenocytes. On day 4 of the MLC, cytotoxicity was assayed against SJL-LPS blasts and BALB/c-LPS blasts respectively. ● represents RPMI medium only; □ represents addition of 10 ul NMS per well; ◻ represents addition of 10 ul anti-Ly-5.1 per well.

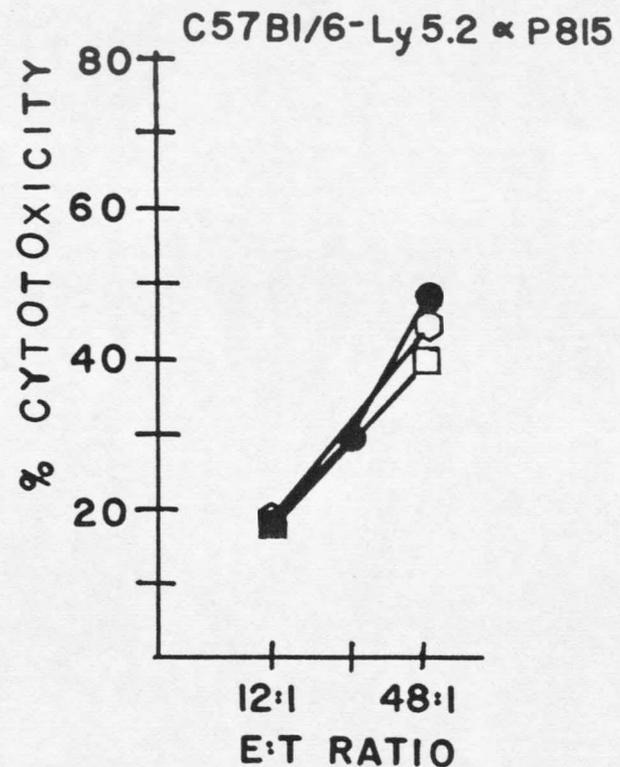
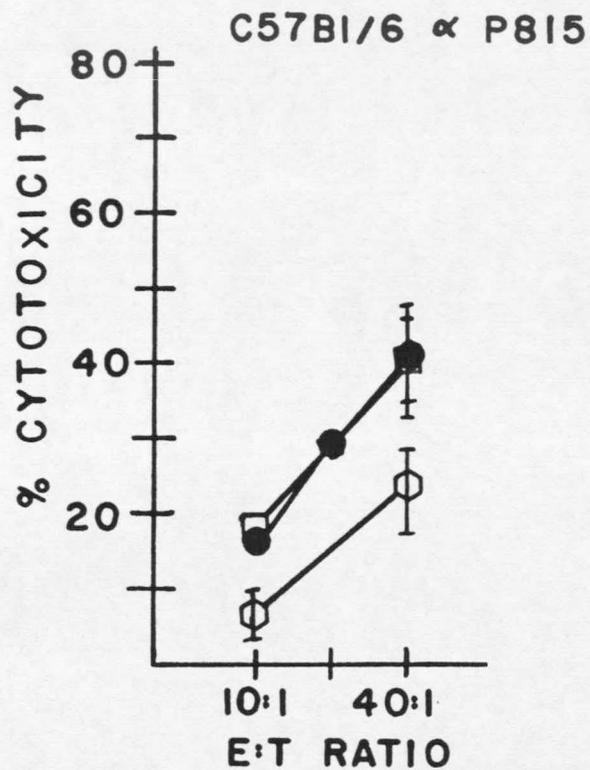


Figure 2. Specificity of anti-Ly-5.1-mediated inhibition of CTL killing. C57Bl/6 splenocytes or congenic C57Bl/6-Ly-5.2 splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. On day 4 of culture, CMC was assayed against P815 targets.

● represents RPMI medium only; □ represents addition of 10 ul NMS per well; ○ represents addition of 10 ul anti-Ly-5.1 per well.

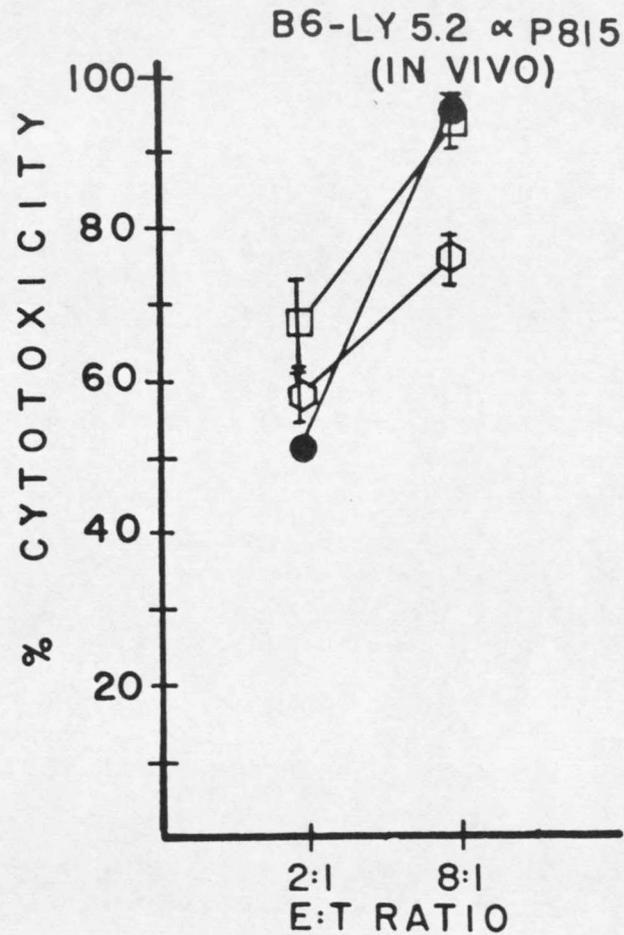
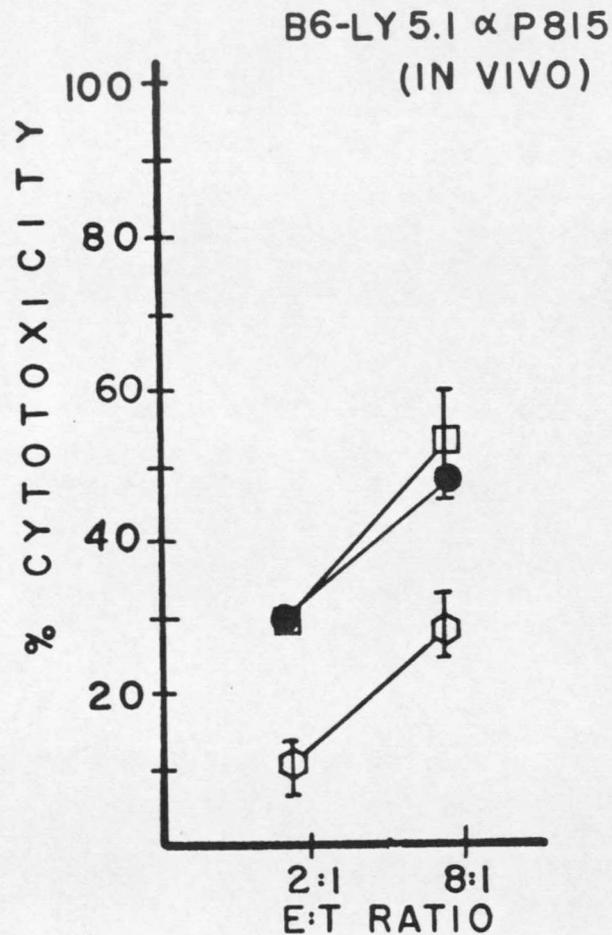


Figure 3. Inhibition of Peritoneal Exudate Lymphocytes (PEL)-mediated cytotoxicity. Congenic C57Bl/6 and C57Bl/6-Ly-5.2 mice were immunized intraperitoneally with P815 cells. Ten days later, PEL were removed and CMC was assayed against P815 targets. ● represents RPMI medium only; □ represents addition of 10 ul NMS per well; ⬡ represents addition of 10 ul anti-Ly-5.1 per well.

Inhibition by Anti-Ly-5.1 at the Level of Effector Cells

The presence of target Ly-5 antigens might affect inhibition of CTL-mediated killing by anti-Ly-5.1 serum in either of two ways: 1) synergistically by antibodies bound to target antigens sterically hindering recognition, binding or killing, or 2) antagonistically by removing inhibitory antibodies through absorption or by facilitating conjugate formation. The experiment illustrated in Figure 1 examined this issue somewhat, but further experiments were done.

The experiment shown in Figure 4 compared inhibition of cytotoxicity by anti-Ly-5.1 serum with congenic B6 anti-BALB/c (H-2^d) effectors assayed against either P815 cells (Ly-5⁺, H-2^d) or M11 (H-2^d), an Ly-5⁻ cell line (Lanier et al., 1981), as targets. In all cases, inhibition was specific for Ly-5.1⁺ effectors and did not vary with different target cells.

These data indicated that anti-Ly-5.1 serum inhibited at the level of the effector cell with specificity for the Ly-5.1 antigen. To further support this hypothesis, anti-serum treat-and-wash studies were performed. In these experiments, anti-Ly-5.1 treated effector cells were washed of excess antibodies, combined with untreated targets and assayed for cell-mediated cytotoxicity. Likewise anti-Ly-5.1 treated target cells were washed, combined with

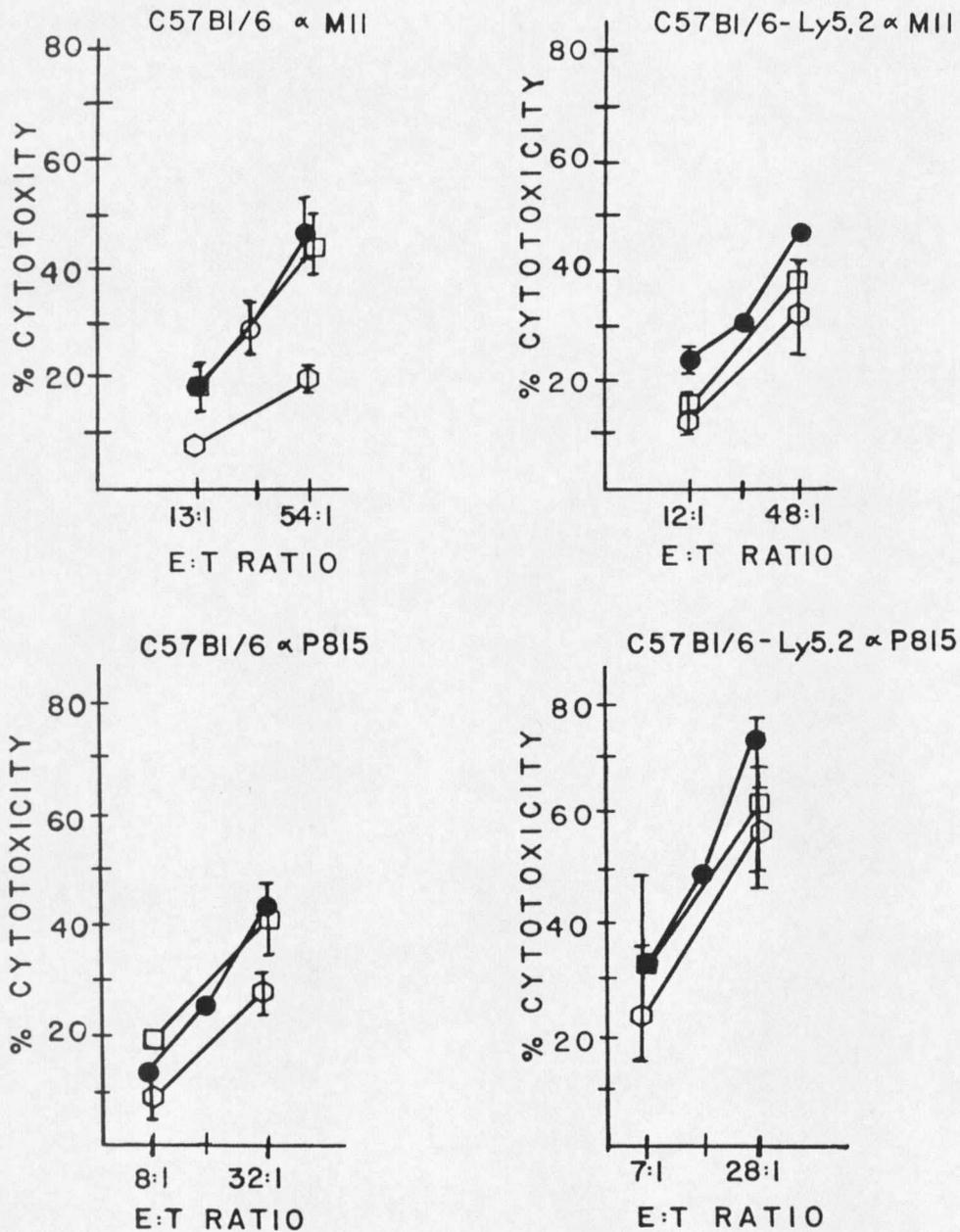


Figure 4. Inhibition of CTL killing against an Ly-5⁺ target (P815) and an Ly-5⁻ target (M11). C57Bl/6 splenocytes or congenic C57Bl/6-Ly-5.2 splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. Four days later, CMC was assayed against both targets. ● represents RPMI medium only; □ represents addition of 10 ul NMS per well; ○ represents addition of 10 ul anti-Ly-5.1 per well.

untreated effector cells, and assayed for cytotoxicity. Antibody binding to the treated cells would be the only source of inhibitory activity. Pretreatment of B6 effectors with anti-Ly-5.1 serum followed by washing resulted in inhibition of killing (Figure 5) whereas pretreatment and washing of B6-Ly-5.2 effectors did not (Figure 6). Experiments involving addition of antiserum without washing showed specificity for the Ly-5.1 allele as previously demonstrated. Conversely, when target cells were pretreated with anti-Ly-5.1 serum and washed of excess antibody, inhibition was not observed.

A trivial explanation for these results may be that antibodies bound to effector cells might eventually dissociate, bind to and inhibit at the target level. In order to rule out this possibility, M11 targets were used in a similar experiment (Figure 7). Because M11 cells do not express Ly-5 antigens, antibodies dissociating from pretreated CTL were unable to bind to target cells. Therefore, only Ly-5.1 antibodies bound to effector cells which persisted through washing inhibited cytotoxicity.

Dose-dependent inhibition of cytotoxicity by anti-Ly-5.1 serum is shown in Figure 8. Addition of increasing amounts (5, 10, 20 ul) of Ly-5.1 antiserum resulted in increasing inhibition of cytotoxicity by B6 effectors relative to that involving the addition of equal amounts of

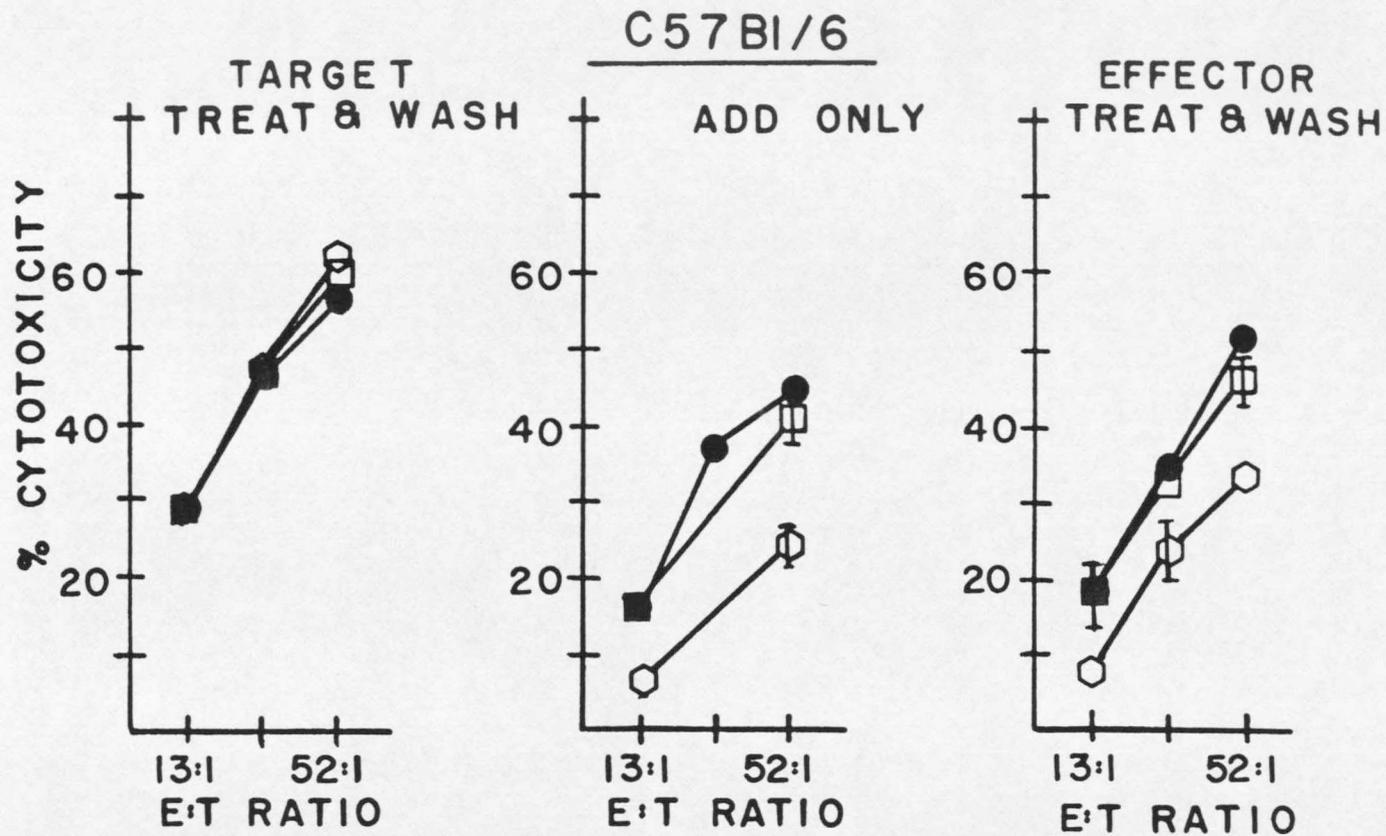


Figure 5. Inhibition of CTL killing after pretreatment with anti-Ly-5.1. C57Bl/6 (Ly-5.1) splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. On day 4, CMC was assayed in three different ways: 1) P815 targets were pretreated with serum, washed and to untreated CTL; 2) CTL were pretreated with serum for 15 min then P815 targets were added; 3) CTL were pretreated with serum, washed and added with P815 targets. Serum treatments were comparable to previous experiments. ● represents RPMI medium only; □ represents pretreatment with NMS; ○ represents pretreatment with anti-Ly-5.1.

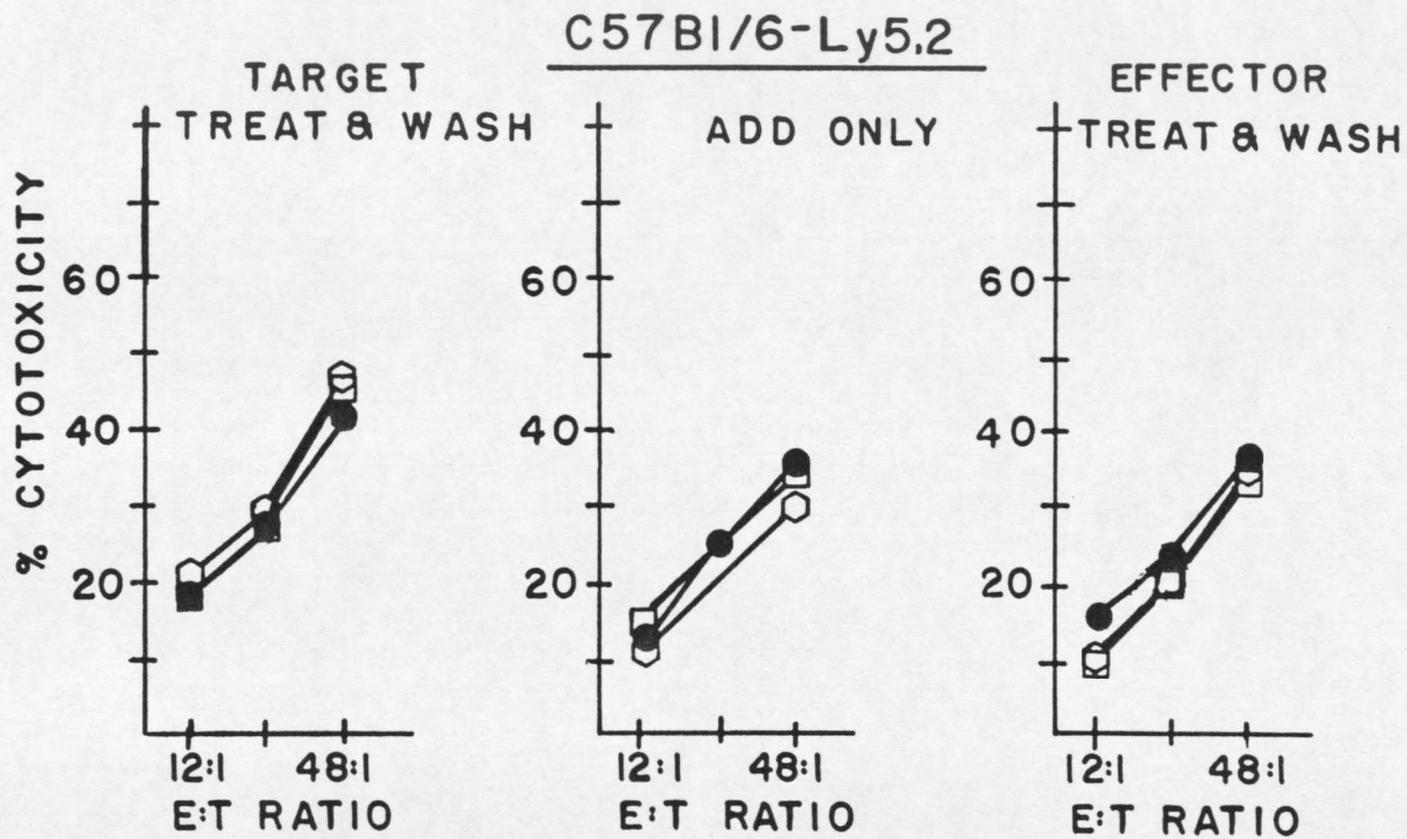


Figure 6. Specificity of anti-Ly-5.1 mediated inhibition of CTL killing after pretreatment with anti-Ly-5.1 and wash. C57B1/6-Ly-5.2 splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. On day 4, CMC was assayed in three different ways: 1) P815 targets were pretreated with serum, washed and added to untreated CTL; 2) CTL were pretreated with serum then P815 targets were added; 3) CTL were pretreated with serum, washed and added to P815 targets. ● represents RPMI medium only; □ represents treatment with NMS; ⬡ represents treatment with anti-Ly-5.1.

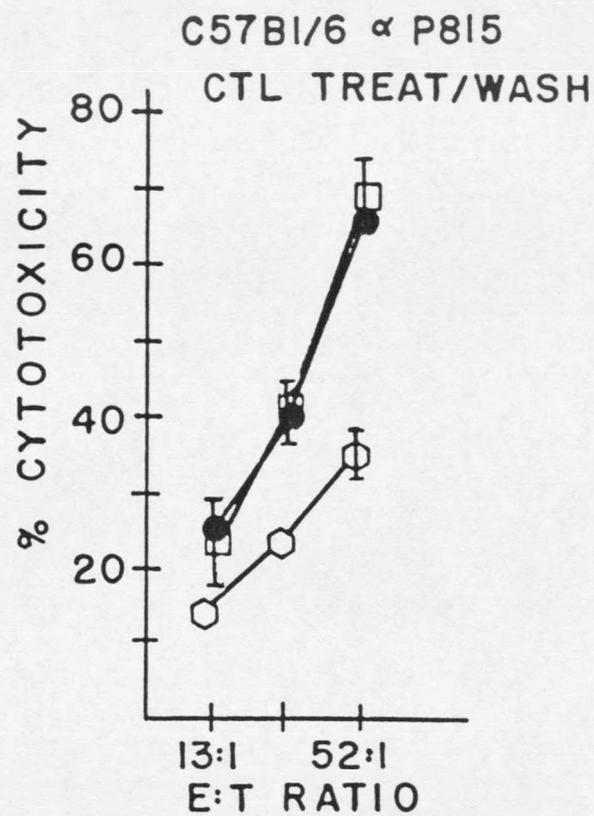
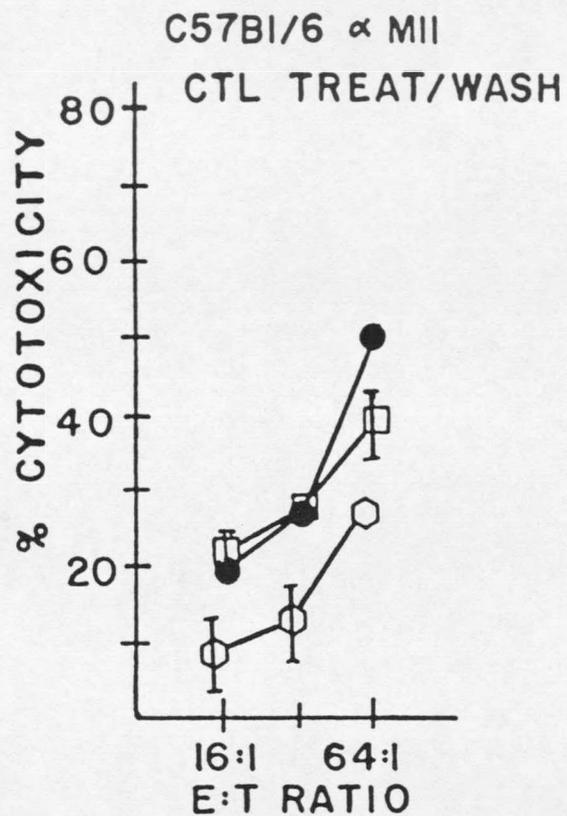


Figure 7. Inhibition of anti-Ly-5.1 treated and washed CTL when assayed against an Ly-5⁺ target (P815) and an Ly-5⁻ target (M11). C57Bl/6 splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. On day 4, CMC was assayed after CTL were pretreated with serum, washed and added to targets. ● represents untreated effectors; □ represents pretreatment with NMS; ○ represents pretreatment with anti-Ly-5.1.

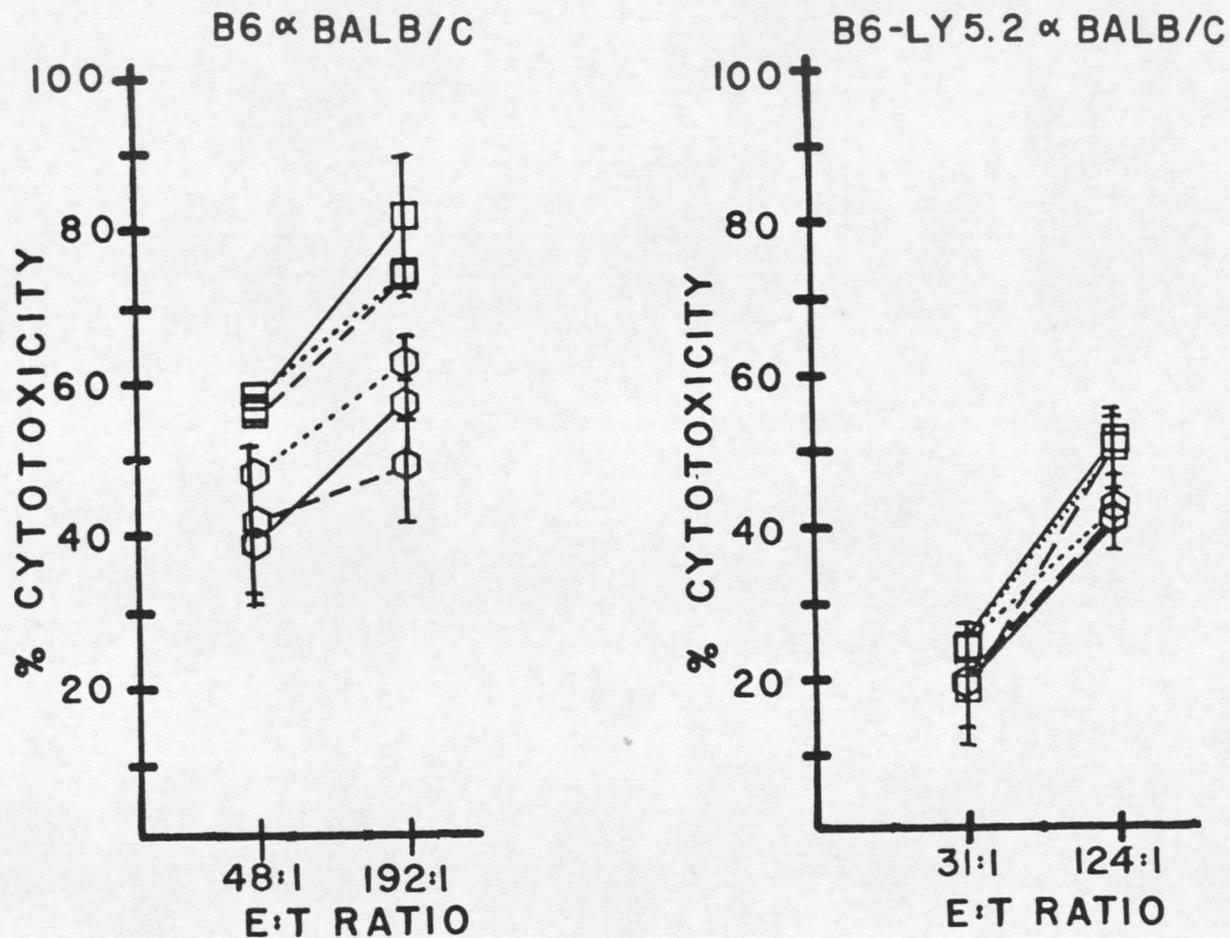


Figure 8. Dose-dependent inhibition of CTL killing by anti-Ly-5.1. C57B1/6 splenocytes or C57B1/6-Ly-5.2 splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. On day 4, CMC was assayed against P815 targets. represents addition of 5 ul serum per well; — represents addition of 10 ul serum per well; --- represents addition of 20 ul serum per well. □ represents addition of NMS; ○ represents addition of anti-Ly-5.1.

NMS. Addition of equal volumes of Ly-5.1 antisera to wells containing B6-Ly-5.2 effectors resulted in no inhibition of killing.

Activity of Anti-Ly-5.1 Compared to Anti-H-2K^k

Inhibition of cell-mediated cytotoxicity by antiserum can occur in any of three ways: 1) by binding to molecules which are directly involved in the cytolytic process and interfering with that process; 2) by binding near such molecules and sterically interfering with their function; or 3) by binding at sites unrelated to the cytolytic mechanism and causing some unknown perturbation of cell actions. In order to determine whether the inhibitory activity of anti-Ly-5.1 serum was specific for molecules involved in the cytolytic process, another conventional antiserum specific for the H-2K^k molecule was tested for inhibition of killing by CBA (H-2K^k, Ly-5.1) effectors.

As shown in Figure 9, anti-H-2K^k antibodies bound to CBA effector cells had no effect on cell-mediated killing, whereas anti-Ly-5.1 antibodies bound to the same effector cell did inhibit.

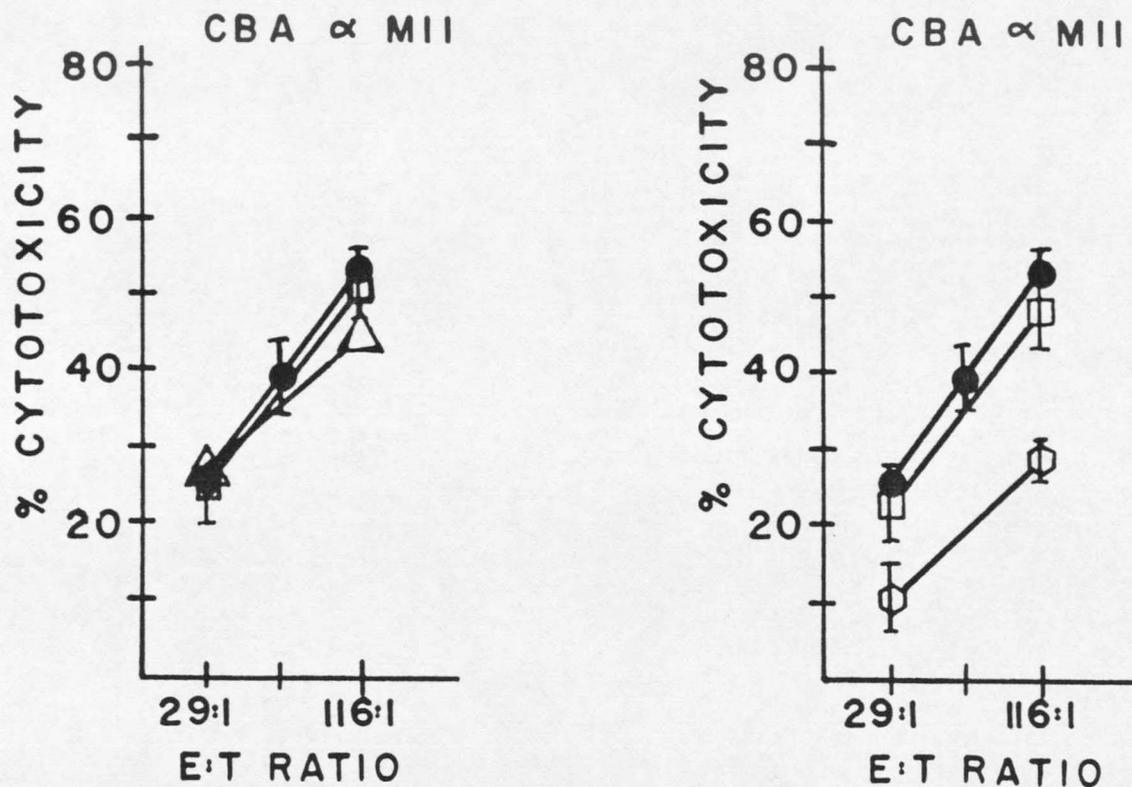


Figure 9. Inhibition of CTL killing by anti-Ly-5.1 but not by anti-H-2K^k. CBA (H-2^k) splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. On day 4, CMC was assayed against Ly-5⁻ targets (M11). ● represents RPMI medium only; □ represents addition of 10 ul anti-Ly-5.1 per well; △ represents addition of 10 ul anti-H-2K^k per well.

Mechanism of Inhibition by Anti-Ly-5.1 Antiserum

Ly-5 antisera may inhibit CTL by any of a number of different mechanisms. In order to ascertain possible mechanisms for the inhibitory activity of anti-Ly-5.1 sera, experiments were done to observe whether anti-Ly-5.1 serum induced effector cell death.

Table 2 shows data from trypan blue exclusion assays for effector cell viability in CMC assay wells after 2 hr incubation with serum at 37°C.

Table 2. Percent Viability (Via) by Trypan Blue Exclusion and Percent Cytotoxic Activity (Ctx) of Effector Cells^a in Medium, Normal Mouse Serum, or Anti-Ly-5.1 Serum.

Expt.	Medium		NMS		Anti-Ly-5.1	
	Via	Ctx ^b	Via	Ctx ^b	Via	Ctx ^b
1	---	89.7 ^c	41.9	88.0 ^c	41.5	61.5 ^c
2	---	43.2 ^d	47.1	52.4 ^d	47.2	16.0 ^d

^a C57Bl/6 anti-BALB/c CTL

^b Alloantigen-specific cytotoxicity against P815 targets

^c 52:1 Effector-to-Target ratio

^d 52:1 Effector-to-Target ratio

In two separate experiments, the presence of anti-Ly-5.1 serum inhibited CMC 31% and 69% relative to the presence of NMS. However, no significant differences in effector cell viability were observed in samples treated with Ly-5 antiserum or NMS.

As shown in Tables 3 and 4, percent release of ^{51}Cr from radiolabelled effector cells was not significantly different in the presence of NMS or anti-Ly-5.1 serum. In contrast, the percent release of ^{51}Cr from radiolabelled target cells by CTL killing was inhibited when anti-Ly-5.1 was added to these same effectors.

Table 3. Effects of Anti-Ly-5.1 Serum on ^{51}Cr Release from Radiolabelled Effector Cells as Compared to the Effects of Anti-Ly-5.1 Serum on Cell-Mediated Killing of Labelled Targets by the Same Unlabelled Effectors.

Treatment	Viability of CTL ^a (Radiolabeled CTL)	Specific Killing of Targets by CTL ^a (Radiolabeled P815)
Medium	95.6% ^b	89.7% ^c
NMS	95.4%	88.0%
Anti-Ly-5.1	95.7%	61.5%

^a C57Bl/6 anti-BALB/c CTL

^b Viability calculated as described in Materials and Methods

^c 52:1 Effector-to-Target ratio

In another experiment, shown in Table 4, both ^{51}Cr radiolabelled B6 and B6-Ly-5.2 effectors were incubated in the presence of anti-Ly-5.1 serum. These effector populations were tested for CMC at the same time and showed typical killing and inhibition by anti-Ly-5.1 serum (data not shown).

Table 4. Anti-Ly-5.1 Treatment of ^{51}Cr -labelled C57Bl/6 or C57Bl/6-Ly-5.2 Effectors.

Anti-Ly-5.1 added	Percent $^{51}\text{Chromium}^a$ from:	
	C57Bl/6 CTL	C57Bl/6-Ly-5.2 CTL
10 ul	1.26%	-10.5%
5 ul	1.57%	-4.8%

^a Relative to medium only

Negative percent release of radiolabel relative to medium indicates that the presence of mouse serum may stabilize targets against spontaneous lysis or release of radiolabel. This is further indicated where twice as much antiserum (10 ul) resulted in less percent release of radiolabel than did the lesser amount of antiserum (5 ul).

Because this antiserum has been shown to be specific in its inhibition of CMC, any antibody-mediated effector lysis should be seen only with B6 CTL. Since no difference in viability was seen between treatment with anti-Ly-5.1 of either B6 or B6-Ly-5.2 effectors, I conclude that antibody-mediated inhibition of CMC is not due to antibody-mediated lysis of effector cells.

Another possible mechanism of action for inhibition by anti-Ly-5.1 serum would be inhibition or disruption of CTL-target cell conjugates. Antisera inhibiting or disrupting specific conjugate formation would be expected to lower the

frequency of observable conjugates. Studies on conjugate formation used cytotoxic cells generated in vivo, which are present in peritoneal exudate washings in a higher frequency than are those generated in vitro. This is shown by the difference in effector-to-target cell ratios needed to achieve comparable cytotoxicity in CMC assays involving either effector type (Glasebrook, 1978). Experiments to observe the effect of anti-Ly-5.1 serum on specific conjugate formation were performed using such in vivo generated effectors. Data from these studies of conjugate formation, along with respective data for inhibition of CMC, are shown in Table 5.

Table 5. Effect of Anti-Ly-5.1 Serum on Conjugate Formation by C57Bl/6 PEL Sensitized Against P815 (H-2^d) Cells.

Expt.	Target	H-2	Treatment					
			RPMI		NMS		Anti-Ly-5.1	
			Conj ^a	Cyto ^b	Conj ^a	Cyto ^b	Conj ^a	Cyto ^b
1	P815	d	15.4	73.5 ^c	14.7	81.0 ^c	16.3	57.0 ^c
	BW5147	k	3.6	-----	-----	-----	-----	-----
2	P815	d	13.2	-----	9.2	78.0 ^d	9.1	40.0 ^d
	M11	d	9.6	-----	-----	-----	-----	-----
	EL4	b	3.8	-----	-----	-----	-----	-----
	BW5147	k	3.1	-----	-----	-----	-----	-----

- ^a Conj = percent conjugates observed
^b Cyto = percent cytotoxicity assayed
^c 19:1 Effector-to-Target ratio
^d 10:1 Effector-to-Target ratio

Escape From Inhibition by Anti-Ly-5.1

It is known that when antibodies bind to cell surface antigens, those antigens are "modulated" and cleared from the cell surface. New antigen, unbound to antibody, is then expressed (Ritz, et al., 1980; Reinherz, et al., 1982). Subsequent exposure to antibody repeats this cycle. In an attempt to determine whether Ly-5 was modulated by exposure to anti-Ly-5 serum, CTL were pretreated with anti-Ly-5.1 serum and washed of excess antibodies. Cytotoxicity was then assayed at various times. These times were intended to allow for removal of Ly-5:anti-Ly-5 complexes from the CTL surface and expression of new Ly-5 antigens. Two such experiments gave differing results.

The data in Table 6 are from such an experiment in which antibody-treated and washed CTL were plated in 96-well microtiter plates in 100 ul of medium. After incubation times as indicated, radiolabelled target cells were added and the plate was centrifuged. After 2 hr further incubation, the plate was centrifuged again and supernatants were counted for ^{51}Cr as in previous assays. CTL not receiving targets at a given time were resuspended to optimize their continued viability for future assays.

Table 6. Escape from Anti-Ly-5.1-Mediated Inhibition by Pretreated and Washed CTL^a.

Time of Target Addition	Cytotoxicity by CTL ^a Pretreated with:	
	NMS ^b	Anti-Ly-5.1 ^b
0 hr	59.49%	43.10%
4	50.34%	34.00%
8	41.98%	31.22%
12	35.94%	27.94%
20	26.23%	15.23%
27	16.47%	12.69%
39	7.78%	11.62%

^a C57Bl/6 anti-BALB/c CTL against P815 targets

^b 75:1 Effector-to-Target ratio

Anti-Ly-5.1-treated CTL, after 27 hr incubation in the absence of antiserum, were still inhibited in their antigen-specific killing of P815 targets. It was concluded that CTL did not readily escape from inhibition by anti-Ly-5.1. It was also noted that the cytolytic activity of both populations declined with time. This indicated that culture conditions were not satisfactory to maintain CTL-activity over prolonged periods.

The data in Table 7 are from a repeat experiment with the difference that anti-Ly-5.1-treated congenic CTL were washed and incubated in a 1.5 ml Eppendorf tube in medium

containing 50% MLC supernatant. At the indicated times, CTL were mixed and 100 ul were added to microtiter wells along with radiolabelled target cells, centrifuged, incubated for 2 hr, and supernatant fluids harvested and counted for ^{51}Cr .

These results indicated that incubation of antiserum-treated effector cells in medium containing MLC-generated lymphokines allowed Ly-5 antigens to be reexpressed in 4 hr or less.

Table 7. Escape from Anti-Ly-5.1-Mediated Inhibition by Pretreated and Washed Congenic CTL Incubated in the Presence of 50% MLC-Generated Supernatants.

Time of Target Addition	Antigen-Specific Cytotoxicity by:			
	C57Bl/6 CTL ^a		C57Bl/6-Ly-5.2 CTL ^a	
	NMS	Anti-Ly-5.1	NMS	Anti-Ly-5.1
0 hr	44.39% ^b	31.90% ^b	53.78% ^c	55.42% ^c
4	31.87%	30.98%	41.22%	42.09%
12	34.68%	40.16%	42.35%	50.02%

^a CTL generated against BALB/c, assayed against P815

^b 84:1 Effector-to-Target ratio

^c 54:1 Effector-to-Target ratio

After it was demonstrated that CTL had escaped from inhibition by anti-Ly-5.1 serum, they could be assayed to determine if their Ly-5.1 antigens were reexpressed on their surface, i.e. could they be inhibited a second time? Table

8 contains data from an experiment using the same population of CTL involved in the same experiment summarized in Table 7. CTL, previously treated with anti-Ly-5.1 serum, washed, incubated for 18 hr in 50% MLC supernatant, and which were shown to have recovered from inhibition by anti-Ly-5.1, were again assayed for cytotoxicity against P815 targets. During this assay, some CTL received no further serum treatment, some were treated with NMS (no wash), and some were treated with anti-Ly-5.1 (no wash).

Table 8. Secondary Serum Treatment of CTL^a Treated 18 Hr Previously with Normal Mouse Serum or with Anti-Ly-5.1.

CTL	Serum Treatment ^a		Cytotoxicity ^b after 2nd Treatment
	1st	2nd	
B6	NMS	---	44.43%
	Ly-5.1	---	45.83%
B6-Ly-5.2	NMS	---	52.27%
	Ly-5.1	---	54.44%
B6	NMS	NMS	44.63%
	NMS	Ly-5.1	27.56%
B6-Ly-5.2	NMS	NMS	48.73%
	NMS	Ly-5.1	45.35%
B6	Ly-5.1	NMS	48.79%
	Ly-5.1	Ly-5.1	32.03%
B6-Ly-5.2	Ly-5.1	NMS	51.93%
	Ly-5.1	Ly-5.1	48.39%

^a Serum treated-CTL were the same as those used in Table 7

^b P815 target cells

As can be seen, no further treatment resulted in comparable killing by both pairs of congenic CTL. Secondary treatment with NMS of B6 CTL previously treated with anti-Ly-5.1 did not alter observed cytotoxicity. Conversely, treatment with anti-Ly-5.1 serum of NMS-treated B6 CTL did cause inhibition of cytotoxicity by 43%. A second treatment of NMS-treated B6 CTL with NMS did not affect killing whereas treatment of anti-Ly-5.1-treated B6 CTL with anti-Ly-5.1 resulted in 28% inhibition of killing. No treatment or combination of treatments of B6-Ly-5.2 CTL caused inhibition of cytotoxicity.

These data suggest that CTL might have modulated their cell surface Ly-5 antigens upon exposure to anti-Ly-5 antibodies, reexpressed Ly-5 molecules, and were again inhibited in their killing of alloantigen-specific targets by a second treatment with Ly-5.1 antiserum. Whether the presence of MLC-generated lymphokines induced or accelerated this modulation, or whether the incubation of CTL in a larger volume rather than in small microtiter wells maintained metabolic stability is not known; however, the decline in cytolytic activity with incubation time was not apparent in the latter escape-experiment as it was in the first.

Time Course Studies

By defining the time interval during which inhibition by anti-Ly-5.1 serum can occur, it might be possible to define which stage of CTL-killing is inhibited. The last experiments performed attempted to delineate this time interval. These time course studies involved adding various inhibitory reagents to CMC assays at different times during incubation and observing the degree of cytotoxicity at the end of the assay. If a reagent inhibits a process which occurs within a limited time interval, addition of that reagent after that time point would be expected to have no effect on cytotoxicity.

Inhibition by anti-Ly-5.1 serum was studied relative to inhibition by five millimolar (5 mM) ethylene glycol-bis(2-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) which chelates Ca^{+2} , inhibiting the Ca^{+2} -dependent delivery of the lethal hit (Martz, 1977). Delivery of the lethal hit occurs approximately 30 min after initiation of effector-target contact. If Ca^{+2} is chelated from the system by adding EGTA before that time, no killing is observed. Addition of EGTA at time points afterwards results in decreasing degrees of inhibition as irreversible lethal hits are sequentially delivered. A representative time course experiment is shown in Figure 10. When EGTA was added at 15 min prior to the addition of targets ($t=-15$),

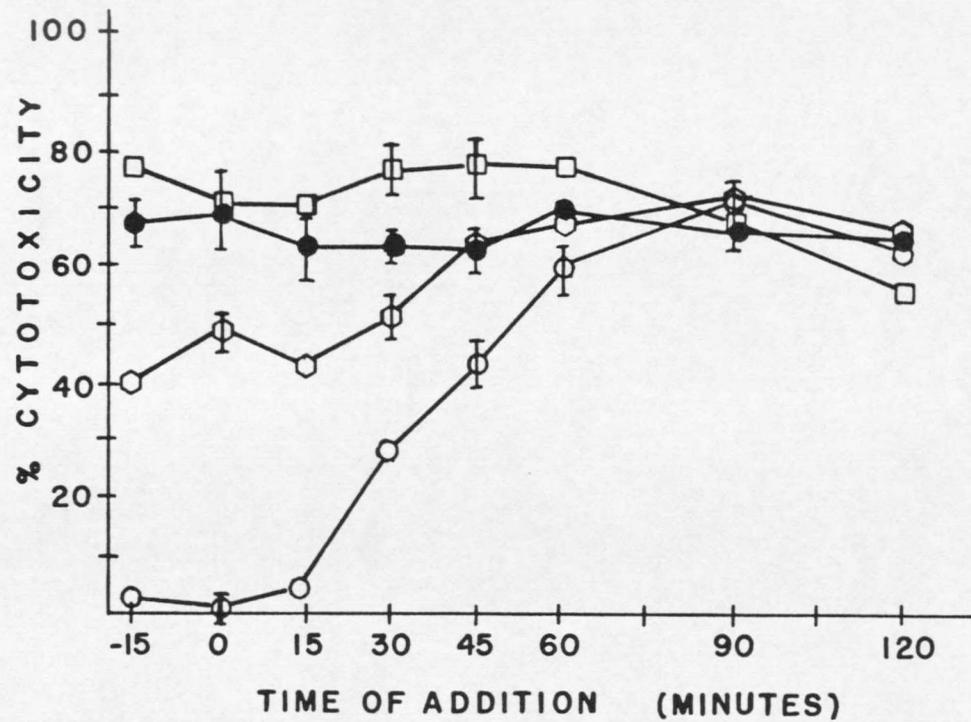


Figure 10. Time course of inhibition of CTL killing by anti-Ly-5.1 compared with that of EGTA. C57Bl/6 splenocytes were cultured with mitomycin-c treated BALB/c splenocytes. CMC was assayed on day 4 against P815 targets. Addition of RPMI medium, 5 mM EGTA, NMS or anti-Ly-5.1 occurred at time points indicated on the x-axis. Addition of targets was at t=0 min. All assays were incubated 120 minutes before harvesting. ● represents RPMI only; ○ represents addition of 10 ul 105 mM EGTA; □ represents addition of 10 ul NMS; ◇ represents addition of 10 ul anti-Ly-5.1 per well.

at the time of target addition ($t=0$), or 15 min after targets were added, cytotoxicity was inhibited 100%. When EGTA was added at time points 30, 45 and 60 min after targets, inhibition gradually diminished until observable killing was equivalent to that seen when RPMI medium was added.

Although the degree of inhibition observed was less than that with EGTA, an analogous pattern was seen when anti-Ly-5.1 was added to the CMC cultures. Addition of anti-Ly-5.1 at time points $t=-15$, $t=0$, and $t=15$ min consistently inhibited killing approximately 30-45% relative to the addition of NMS. Inhibition of cytotoxicity gradually diminished when antiserum was added at time points 30 min or more after addition of targets until by $t=60-90$ min no appreciable difference was seen as compared to NMS. These results cannot precisely differentiate whether anti-Ly-5.1 serum inhibits cell-mediated cytotoxicity at a stage occurring just before, just after, or coincidental with the Ca^{+2} -dependent step; however it appears that the event inhibited by EGTA and the event inhibited by anti-Ly-5.1 occur within a similar narrow time frame.

These time course studies would further support data indicating that anti-Ly-5.1 sera do not inhibit conjugate formation which occurs within the first few minutes of effector-target cell interaction.

DISCUSSION

I have studied the effects of anti-Ly-5.1 sera on alloantigen-specific murine cell-mediated cytotoxicity in vitro. My results address two aspects of inhibition by anti-Ly-5.1: specificity and mechanism.

Specificity of Anti-Ly-5.1 Sera. Antiserum specificity refers to the reactivities of inhibitory antisera. Reactivities other than those predicted by known alloantigenic differences between donor and recipient mice can arise because of unknown antigenic differences.

The production of anti-Ly-5.1 antiserum involved a producer strain of mice which was homozygous for the Ly-5.2 allele. These mice were immunized with tissue from mice of a strain carrying the Ly-5.1 allele. SJL mice were the first to be described as expressing Ly-5.2 as the alternate allelic form of Ly-5.1, which is found on cells of most other strains. If other antigenic differences existed between SJL and A.SW strains, e.g. the major or minor histocompatibility complexes, antibody activities other than anti-Ly-5.1 could have arisen.

A.SW mice have the same MHC haplotype as do SJL mice--both are H-2^S--but differ at the Ly-5 locus and at many other background genetic loci. In contrast, congenic pairs

of mice are genetically identical at all loci except for a selected locus and possibly other closely linked loci. Examples of congenic mice are the C57Bl/6 and C57Bl/6-Ly-5.2 strains; the latter mice were produced by breeding C57Bl/6 (H-2^b, Ly-5.1) mice to SJL mice, then backcross breeding the offspring to the C57Bl/6 strain while selecting for progeny carrying the Ly-5.2 allele on a C57Bl/6 background. After 22 backcrosses, these mice were considered congenic and have been made available from their breeders (E. A. Boyse, shipment literature).

Antisera produced by the immunization of C57Bl/6-Ly-5.2 mice with lymphoid tissue from C57Bl/6 mice would be highly specific for the Ly-5.1 and associated alleles. However, because of the limited availability of B6-Ly-5.2 mice, it has been easier to produce anti-Ly-5.1 serum immunizing SJL mice with the lymphoid tissue from A.SW mice. This antiserum is not necessarily specific only for the Ly-5.1 antigen because the strains are not congenic; therefore, Ly-5.1 specificity must be tested, preferably on cells from congenic mice.

The current trend in antibody studies is to produce antibodies of a single specificity from B-cell/plasmacytoma hybrids. Using monoclonal antibody from such hybridomas constitutes a major impetus in immunology today. Monoclonal antibodies have not yet been produced in our

laboratory for several reasons: 1) time and resources have not allowed for the intensive work involved; 2) experiments absolutely requiring monoclonal specificity have not yet become paramount; and 3) our experiences with two different monoclonal antibodies, one specific for Ly-5.1, the other for T200, as well as the results of other researchers, have led us to believe that the few monoclonal antibodies currently available somehow lack the activity that we have detected, perhaps because of epitope specificity or avidity.

Because they lack the monoclonal nature of hybridoma antibodies, SJL anti-Ly-5.1 sera must be assayed for Ly-5.1 specificity. Specificity was first demonstrated by immunofluorescence on thymocytes from congenic B6 mice (Table 1). Using congenic mice allowed the most accurate demonstration of Ly-5 specificity--genetic differences between the two strains are limited and the only known antigenic differences are alleles of Ly-5. Thymocytes from these mice were used for two reasons. Activity detected against thymocytes should be most similar to activity observed against thymus-derived cytotoxic lymphocytes. Secondly, since the IFA utilizes a secondary antibody, FITC-conjugated rabbit anti-mouse Ig, background fluorescence from surface Ig-expressing B-cells was minimized by using thymocytes.

The specificity of anti-Ly-5.1 sera was further demonstrated in cytotoxicity assays. Inhibition occurred only when the effector population expressed Ly-5.1; Ly-5.2 effector cells were not inhibited (Figures 1, 2). In order to ensure that each lot of Ly-5.1 antiserum would show this same specificity, all antisera were assayed for specificity using congenic pairs of effector cells.

In addition to the inhibition of cytotoxicity by in vitro-generated CTL, it was shown that anti-Ly-5.1 serum inhibits killing by in vivo-generated PEL (Figure 3). This is of interest because investigators have been unable to inhibit PEL using anti-Lyt-2 antibodies which do inhibit CTL generated in vitro in mixed lymphocytes cultures (MacDonald, Thiernesse, and Cerottini, 1981). Such inhibition of PEL may be more difficult because the affinity of PEL for targets is greater, they form conjugates more quickly and recycle more rapidly (Glasebrook, 1978). Since anti-Ly-5.1 serum was able to inhibit PEL-mediated killing, it is significant to conclude that such inhibition is not a trivial interference with the lytic process or that it is, in some other way, an artifact of in vitro-generated CTL-mediated killing.

The preceding experiments did not adequately address the question of whether anti-Ly-5.1 serum inhibits at Ly-5 molecules expressed on effector cells, target cells, or a

combination of both. The following experiments showed that inhibition occurred only when antibodies reacted with Ly-5.1 antigens on effector cells. When Ly-5.1⁺ effector cells were pretreated with anti-Ly-5.1 serum and washed of excess antibodies, inhibition of killing was observed (Figure 4, 5, 6). When M11 tumor cells, which do not express Ly-5 antigens (Lanier et al., 1981) were used as targets, inhibition occurred only when CTL expressed Ly-5.1 (Figure 7). Pretreatment and washing of targets, P815 or M11, resulted in no inhibition of their subsequent killing by untreated effectors.

I assumed that antibody-antigen dissociation was negligible and that the antibodies acted only at the level of the cells originally treated. My results demonstrate that inhibition using Ly5.1⁻ M11 targets was comparable to inhibition using Ly-5.1⁺ P815 targets. I therefore concluded that anti-Ly-5.1 serum inhibited only at the level of Ly-5 molecules on Ly-5.1-expressing effector cells.

Finally, the dose-dependent response to anti-Ly-5.1 serum showed that inhibition of cytotoxicity is a direct result of the presence of antiserum instead of an artifact of experimental manipulation (Figure 8).

Studies on the Mechanism of Inhibition. Studies on the mechanism of antibody-mediated inhibition of a cellular

function attempted to differentiate three possibilities: 1) the inhibitory antibody recognized structures involved in that cellular function; 2) the inhibitory antibody recognized cell surface structures somehow physically associated with those structures directly involved in the cellular function; or 3) the observed inhibition is somehow a result of antibody binding to the cell surface at sites unrelated to the cellular function.

An experiment using anti-H-2K^k serum studied whether anti-Ly-5.1 serum inhibited by binding to structures unrelated to the CTL-lytic process or to structures directly or indirectly related. H-2K and H-2D antigens are antigens recognized on target cells by cytotoxic cells which, in the case of alloantigen-specific killing, determine those targets susceptible to killing. H-2K and H-2D antigens on the effector cells have no role in this function (Zinkernagel and Doherty, 1979). I used a conventional anti-H-2K^k serum, prepared and donated by NIH, to determine whether antibodies recognizing structures not associated with killing would inhibit such killing by simply binding to effector cells. CBA CTL (H-2^k, Ly-5.1) were unaffected by the presence of anti-H-2K^k serum while anti-Ly-5.1 serum inhibited cytotoxicity as usual (Figure 9). This experiment showed that simple binding of antibody to the cell surface did not inhibit CTL killing and suggested that Ly-5 mole-

cules on CTL are involved in CTL-killing, unlike H-2K molecules.

In order to differentiate structures which are involved in cell-mediated cytotoxicity and those which are closely enough associated that antibodies against them also interfere, the problem becomes more complex. Indeed, Newman et al. (1983) have generated several monoclonal antibodies that immunoprecipitate human homologs of T200/Ly-5, two of which inhibit NK-mediated killing, and two of which do not. They concluded that the inhibitory antibodies recognize one site, site A, on T200 molecules and subsequently interfere with killing. Conversely, the noninhibitory antibodies recognize another site on the same molecule, site B, which does not interfere with killing. Once again, the question arises as to whether the A-site is at a position such that the antibodies interfere with the lytic function of the T200 molecule or such that they sterically interfere with the lytic function of another nearby molecule. It is a difficult question to answer.

Whether or not anti-Ly-5.1 antibodies produce global inactivation of effector cell was studied by Harp et al. (Harp, Davis, Ewald, 1984). Using C57Bl/6, congenic C57Bl/6-Ly-5.2, and (C57Bl/6 x C57Bl/6-Ly-5.2) F_1 effector cells, S. J. Ewald showed that anti-Ly-5.1 serum inhibited only homozygous Ly-5.1 CTL. Conversely, anti-Ly-5.2 serum

inhibited only homozygous Ly-5.2 CTL. The addition of both anti-Ly-5.1 and anti-Ly-5.2 antibodies was required to inhibit heterozygous Ly-5.1/Ly-5.2 CTL. It was concluded that antibodies which inhibited cytotoxicity specifically recognized structures directly involved in the lytic process or closely associated with structures directly involved and inhibited their normal operation.

Another way to approach this question would be to determine whether or anti-Ly-5.1 serum reduces the viability of CTL. CTL viabilities were determined during cell-mediated cytotoxicity assays. Two methods were used to detect antiserum-mediated lysis of CTL: 1) dead effector cells were detected microscopically as those unable to exclude trypan blue stain; and 2) lysis of radiolabelled effector cells was detected by measuring the amount of radiolabel, ^{51}Cr , released into culture supernatants. From the data it was concluded that no significant death of CTL is caused by anti-Ly-5.1 serum and thus cell death is not the mechanism whereby cell-mediated cytotoxicity was inhibited (Tables 2, 3, 4). Ly-5 antisera inhibit at singular sites of killing on effector cells and do not inactivate CTL globally either by causing cell death or another unknown state of nonresponsiveness.

If anti-Ly-5.1 serum does not inhibit cytotoxicity by inactivating cytotoxic lymphocytes, it might act by inhi-

biting one of the three stages of cell-mediated cytotoxicity: 1) target recognition and binding, 2) "programming for lysis" or delivery of the lethal hit, and 3) target lysis in the absence of effector cells. The first of these stages was assayed microscopically by observing formation of conjugates between effector cells and targets. The frequency of conjugate formation between sensitized effectors and target cells of the sensitizing haplotype should be significantly higher than that between effectors and target cells which are allogeneic to the sensitizing haplotype (Hiserodt and Bonavida, 1981).

Conjugate formation was determined for C57Bl/6 PEL sensitized against P815 (H-2^d) tumor cells. B6 anti-P815 PEL formed conjugates with H-2^d targets (P815 or M11) with significantly greater frequency than with targets of other H-2 haplotypes. Addition of anti-Ly-5.1 serum did not reduce conjugate formation whereas it did inhibit killing by the same PEL (Table 5). This demonstrated that anti-Ly-5.1 serum does not inhibit killing by interfering with the target recognition or the formation of stable conjugates.

The final experiments attempted to determine whether Ly-5.1 antiserum inhibited cytotoxicity at either the Ca⁺²-dependent delivery of the lethal hit or during CTL-independent target cell lysis. Addition of EGTA inhibits the Ca⁺²-dependent step of CTL-killing by chelating Ca⁺².

Assays involving the addition of EGTA at various time points during a 2 hr incubation delineate the time course of inhibition at this step (Figure 10). Although anti-Ly-5.1 serum did not inhibit as strongly as did EGTA, inhibition by both reagents was possible after addition at comparable time points--approximately 15 min. Addition of either anti-Ly-5.1 or EGTA 15 min or more after addition of targets resulted in diminished inhibition of cytotoxicity and both curves showed comparable diminution over the next 30-45 min until no differences were noted between all four reagents. From these data it was concluded that inhibition of CTL-killing by anti-Ly-5.1 serum occurred at a time point very near the delivery of the lethal hit, and certainly after conjugate formation.

Controversy over Inhibition by Anti-Ly-5.1. These results differ with those of other researchers who have reported no inhibition of CTL-mediated killing by anti-Ly-5 antibodies but have reported inhibition of NK cell-mediated killing (Minato et al., 1980; Davignon et al., 1981; Brooks et al., 1982). It should be noted that several differences exist between the methods presented here and those of others. These differences should be described.

Minato et al. (1980) assayed CTL killing with C57Bl/6 anti-CBA effectors against ConA-induced CBA blast cells during a 6 hr incubation period. Their Ly-5 antiserum was

the product of F_1 hybrid mice, (STS x E) F_1 , immunized with B6 leukemia tumor cells, anti-ERLD serum. Antiserum was preincubated with effector cells for 30 min at room temperature. Pretreated CTL were then washed of excess serum. It is impossible to correlate the titer of their anti-ERLD serum with that of our anti-A.SW serum. Furthermore, it cannot be ruled out that a 30 min preincubation at room temperature allowed bound antibodies to become capped, shed or internalized. Antibodies that did not bind to effector cells were removed by washing and unavailable to inhibit subsequent killing. It was also possible that escape from inhibition by anti-Ly-5 serum could have occurred during a 6 hr incubation. Finally, these authors observed a pattern of killing in the presence of NMS which indicates that nonimmune serum inhibited killing; no medium only control was shown for this experiment. It appears that their anti-Ly-5.1 serum inhibited cytotoxicity but not relative to NMS.

Davignon et al. (1981) studied inhibition of CTL-mediated killing differently. Whereas their effector cells were of C57Bl/6 origin, their stimulators/targets were xenogeneic lymphoma cells (BNL ϕ) from Brown Norway rats. Furthermore, their B6 anti-BNL ϕ CTL were stimulated twice, once in vivo, once in vitro. Their anti-Ly-5 antibody was a monoclonal antibody from culture supernatants of a rat anti-(B6 anti-BNL ϕ CTL) hybridoma. The specificity of

this monoclonal antibody did not differentiate between the Ly-5.1/Ly-5.2 allodeterminants. Because it apparently recognizes a species-specific epitope it is better described as an anti-T200 rather than an anti-Ly-5 antibody (Omary et al., 1980b; Siadak, et al., 1980). Using a similar rat anti-mouse T200 monoclonal antibody, I have failed to inhibit CTL killing (data not shown). Whether this was due to a property of xenogeneic specificity or of monoclonal antibodies is unknown; however, it may indicate that a certain epitope-specificity or antibody-antigen avidity is required for inhibitory activity.

In addition, Davignon et al. treated their effector cells, as did Minato et al., at room temperature for 30 min. Since these cells were not washed, and excess antibody was present, it is still possible that antibodies could bind to the cell surface and block CTL killing.

The methods of Brooks et al. (1982) are most similar to those presented here. CBA anti-C57Bl/6 effector cells were pretreated with SJL anti-A.SW (anti-Ly-5.1) serum for 20 min at 4°C and assayed against EL4 mouse tumor targets for 4 hr. They, however, reported that no significant blocking ($p > 0.05$) of CTL killing was observed with anti-Ly-5.1 serum. Their data do show an approximate 25% reduction of cytotoxicity at antiserum dilutions of 1:10 to 1:40 as compared with higher dilutions; no control experiments with

normal mouse sera were shown. This difference seems small in comparison with the 100% inhibition of CTL killing that they observed with anti-Lyt-2 serum. This could be due to the relative immunogenicity of Ly-5 allodeterminants as compared with that of Lyt-2 allodeterminants and the titers of the two antisera could have had quite different inhibitory activities.

With the further elucidation of the mechanism of inhibition by anti-Ly-5.1 serum, it may become possible to ascertain the reasons for these disparate findings.

In summary, the data presented show that SJL anti-A.SW serum inhibited CTL killing with specificity for the Ly-5.1 antigen on effector cells. This inhibition was dose-dependent and could be reversed with time, presumably by allowing antibodies bound to surface Ly-5 molecules to become capped and internalized or shed. New Ly-5 antigens were subsequently reexpressed on the cell surface. Furthermore, Ly-5.1 antiserum-mediated inhibition did not cause death of effector cells or interfere with the formation of conjugates between CTL and targets. Data suggesting that the time point of inhibition occurred closer to the calcium-dependent "programming for lysis" event also supports the findings that anti-Ly-5.1 serum did not inhibit conjugate formation.

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APPENDIX

COMMONLY USED ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
B6	C57Bl/6
B6-Ly5.2	C57Bl/6-Ly-5.2, congenic with C57Bl/6
CDF	Cytotoxic differentiation factor
CMC	Cell-mediated cytotoxicity
ConA	Concanavalin A
CTL	Cytotoxic T-lymphocytes
51-Cr	Chromium, atomic weight 51, gamma emitter
EDTA	Ethylene diamine tetraacetate
EGTA	Ethyleneglycol bis(2-aminoethyl ether)-N,N'-tetraacetate
FCS	Fetal calf serum, fetal bovine serum
FITC	Fluorescein isothiocyanate
HBSS	Hank's buffered saline solution
HEPES	N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid
Ia	I-region associated
IL1	Interleukin-1 (macrophage derived)
IL2	Interleukin-2 (lymphocyte derived)
KCIL	Killer cell-independent lysis
LFA-1	Lymphocyte function-associated-1 antigen
LPS	Bacterial lipopolysaccharide
MLC	Mixed lymphocyte culture, one-way
mM	Millimolar
NIH	National Institutes of Health, Bethesda, MD
NK	Natural killer cell
NMS	Normal mouse serum
pCTL	Cytotoxic T-lymphocyte precursor
PEC	Peritoneal exudate cells, all
PEL	Peritoneal exudate lymphocytes, nonadherent fraction
RAT*	Rat anti-mouse activated T-cell
RPMI	RPMI 1640, 10% FCS, HEPES, 2-ME, antimicrobics
T-cells	Thymus-derived lymphocytes
uCi	Microcuries
TRF	T-cell replacing factor
ul	Microliters

