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

by Brian Scott Davis

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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 cytotoxici-city 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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Untreated CTL were not inhibited in their killing of $Ly\text{-}5.1^+$ targets which were pretreated with anti-Ly-5.1 serum and washed. The use of $Ly\text{-}5^-$ 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 $H\text{-}2K^k$ 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 ^{51}Cr 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.

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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 histocompatability 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 allo-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 Lyt-$1^+2,3^+$ phenotype is characteristic of thymic cortical lymphocytes. Lyt-$1^+2,3^-$ peripheral lymphocytes, which are derived from Lyt-$1^+2,3^-$ cortical thymocytes, are characteristically T-helper cells, while 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 T145 (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: Lyt-$1^+2,3^-$, Qa^- cells are helper cells for B-cell responses and 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 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°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, Conconavalin 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 cytolysis (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 cytotoxicty 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 $4^{o}C$.

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 ($H\text{-}2^d$) were donated by Dr. Jim Cutler, Montana State University. C57Bl/6-Ly-5.2 ($H\text{-}2^b$) 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 ($H\text{-}2^d$) origin, 2) EL4, a spontaneous lymphoma of C57Bl/6 ($H\text{-}2^b$) origin, 3) M11, a plasmacytoma of BALB/c ($H\text{-}2^d$) origin which is $Ly\text{-}5^-$ (Lanier et al., 1981), and 4) BW5147 ($H\text{-}2^k$) a spontaneous, $Thy\text{-}1.1^+$, cortisol 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)F_1 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% NaN_3. Briefly, 5 x 10^5 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 (CO_2) in air, at a concentration of approximately 2-3 x 10^7 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 10^7 each, of responder and stimulator cells were added to 25 cm^2 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% CO_2 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 ^{51}Chromium (^{51}Cr) assay against either of two target cell types: radiolabelled tumor cells (e.g. P815) or radio-

labelled 3-day Lipoploysaccharide (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}chromate ($Na^{51}CrO_4$) (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, $1\text{-}3 \times 10^4$ 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 x 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 x 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