Inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC) by LY-5 antisera
by Robin Marie Small

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

Abstract:
Antibodies to different cell surface antigens expressed on mouse spleen cells were tested for their ability to inhibit antibody-dependent cell-mediated cytotoxicity (ADCC) of antibody-coated sheep red blood cells (Ab-SRBC) in the absence of complement. Of the antibodies tested only those to Ly-5 or H-2 antigens significantly inhibited ADCC. Inhibition by Ly-5.1 antiserum was shown to be allele specific by experiments using c57BL/6-Ly-5.1 and C57BL/6-Ly-5.2 mice congenic for the Ly-5 locus. Inhibition by Ly-5 antiserum appeared not to be due to competition for the Fe receptor (FcR), since in mixing experiments third-party thymus cells treated with Ly-5 antiserum did not inhibit the cytotoxic activity of untreated cells. In comparing inhibition induced by antisera to H-2d, H-2k, and Ly-5 antigens, Ly-5.1 antiserum was more inhibitory at nearly every dilution tested. In addition, F(ab')2 and Fab fragments of Protein A-purified Ly-5.1 antibody were inhibitory to BALB/c spleen effector cells in ADCC of Ab-SRBC whereas fragments of H-2 antibodies had no effect. Since ADCC of tumor cells and erythrocytes may share a common lytic mechanism, several antisera to cell surface antigens found on spleen cells were tested for inhibition of ADCC to antibody-coated P815 tumor cells (Ab-P815). As seen in ADCC to Ab-SRBC, anti-Ly-5.1 was a more potent inhibitor than antibody against either H-2k or H-2d antigens. These results suggest that the Ly-5 molecule is important in cell-mediated killing processes.

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Robin Marie Small

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ABSTRACT

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INTRODUCTION

Antibody-dependent cell-mediated cytotoxicity (ADCC) is an integral part of cell-mediated immunity. It is a process used by the body by which leukocytes combine with a target in the presence of specific antibody to the target and cause destruction of that target. Foreign material such as parasites, bacteria, virus-infected cells and tumor cells are all suitable targets for ADCC. Antibody is formed by plasma cells against anything in the body which is not "recognized" as self. These antibodies, (termed immunoglobulin or Ig) once attached to the target antigens, will trigger various cells to combine with and destroy the target. Several different mechanisms for this are evident. ADCC is one such mechanism in which cells bearing receptors (FcR) for the Fc (tail) portion of antibodies will combine with the antibody-coated target and cause lysis of that target. More than one target cell can be lysed by a single effector cell and it does not require the activation of complement (C′). Direct contact between effector cells and target cells is required for lysis to occur. ADCC was originally shown in the laboratory (in vitro) by Perlmann et al. (1). Although ADCC has been best studied in vitro, and may quite possibly be merely an experimental

phenomenon, its importance in vivo has been implicated in a number of conditions. Some of these conditions include patients with leukemia (2), kidney transplants (3,4,5) and various immunodeficiencies (6). Koren (7) has published a standardized method for measuring levels of human ADCC activity.

There are many different cell types which have the capacity to perform ADCC. There is strong evidence that certain lymphocytes, other cell types including monocytic or macrophage-like cells, granulocytes and platelets all have ADCC activity to particular targets under appropriate conditions. Most investigators agree that the lymphocytes performing ADCC bear no distinguishing B or T-cell markers (8) and these cells have therefore been termed "null" cells. They are also referred to as K-(for killer) cells, not to be confused with NK or natural killer cells. Although ADCC was originally described with lymphocytes, other cell types including granulocytes, monocytes, macrophages, and even platelets appear to have ADCC activity. A common denominator for all of these different cells is the use of FcR to combine with antigen-antibody complexes. Cells from spleen, lymph node, peripheral blood and tonsils have the capacity to perform ADCC. A great deal of time and effort has been devoted to enrich for cells with ADCC activity. Aside from removing B and T-cells using C′ and antisera to B

and T-cell surface antigens, no single method has been described for the separation of K-cells from other lymphoid cells. Many methods have been tried to no avail, such as size fractionation, density centrifugation, and adherence to plastic or glass surfaces. Even though ADCC activity to one target cell may be enriched in one fraction, activity against the same or other target cells is almost always evident in another fraction (9,10).

It is important to distinguish between phagocytosis and ADCC. Phagocytosis involves engulfment of the antibody-coated target cell followed up to 20 hr later by intracellular lysis of the target cell. Ethylenediaminetetraacetic acid (EDTA) and aminophylline are potent inhibitors of phagocytosis. Monocytes and macrophages are cells primarily involved in this activity. ADCC on the other hand, does not involve engulfment and intracellular lysis of a target but describes a binding event between the effector cell and target cell resulting in rapid (1-4 hr) extracellular lysis of the target cell. Extracellular lysis is not inhibited by EDTA and aminophylline. Lymphocytes as well as monocytes and macrophages have been implicated in this type of cytotoxicity. It is difficult to discriminate between monocyte-mediated extracellular lysis and lymphocyte-mediated extracellular lysis in most cytotoxicity assays. Both activities are probably being measured in

standard ^{51}Cr release assays. Attempts to remove monocytes from spleen cell preparations usually result in some loss of total ADCC activity. Antibody-dependent macrophages have been shown to be fairly efficient at lysing togavirus-infected cells in the mouse (11). The same is true for the lysis of herpes-simplex virus-infected cells in humans (12). Although it is probable that some phagocytic cells have the capacity to lyse target cells extracellularly, there appear to be some very clear differences between phagocyte and lymphocyte-mediated lysis. One difference is that monocyte-mediated cytolysis is inhibited by both aggregated and monomeric IgG in solution and lymphocyte-mediated cytolysis is only inhibited by aggregated IgG (13). This may, however, only reflect a difference in FcR affinity for Ig or a qualitative difference between types of FcR on lymphocytes and monocytes (14).

Some investigators feel that ADCC to erythroid target cells is mediated only by adherent phagocytic cells whereas ADCC to tumor cells is mediated by non-adherent, lymphocytic cells (15,16). Greenberg et al. (17) show that both phagocytic and non-phagocytic cells can lyse antibody-coated chicken red blood cells (Ab-CRBC) whereas only non-phagocytic cells are active against alloantibody-coated SL-2 lymphoma cells. Non-phagocytic cells have also been shown to lyse certain virus-infected mouse cells (11). In

human peripheral blood there are two mononuclear cell populations responsible for ADCC to virus-infected target cells. One is an adherent cell, primarily phagocytic, and looks like a monocyte or a macrophage. These cells cause lysis of virus-infected cells only after 8 hr of incubation. The other cell type is nonadherent, looks like a small to medium-sized lymphocyte and produces target cell damage within 2 hr of incubation (12). Purified preparations of human polymorphonuclear leukocytes (PMN) have been shown to lyse tumor cells in an antibody-dependent, non-phagocytic manner (18). Aside from lymphocytes and phagocytic cells, human and rat platelets also have the ability to lyse antibody-coated erythrocytes and have been implicated in ADCC of Schistosoma mansoni larvae. This activity requires FcR on the platelet surfaces and IgE antibody (19,20).

At least two other cell-mediated killing processes operate by extracellular lysis; cytotoxic T-lymphocyte (CTL) killing and NK cell lysis. Cell-mediated cytolysis (CMC) of allogeneic or virally-infected syngeneic target cells by sensitized thymus-derived cytotoxic T-lymphocytes (CTL) has been well studied (see review, ref. 21). This type of cytolysis is restricted by molecules encoded in the major histocompatibility complex (H-2) on the effector cells and requires that the target cell bear foreign (non-self) H-2

antigens or viral antigens which are seen in the context of self H-2. Antibody and C′ are not required. Three steps for CMC by CTL have been characterized: (i) recognition of target and Mg^{++}-dependent conjugate formation with the target cell, (ii) Ca^{++}-dependent "programming" for lysis, and (iii) Ca^{++}-independent killer cell-independent lysis (22). Although the cells that mediate this function are T-cells and therefore clearly separable from K-cells, the mechanism of lysis may be similar.

NK cells or natural killer cells are spontaneous killer cells which are not H-2 restricted, can kill a variety of tumor and virally-infected targets, and require no presensitization, antibody, or C′ to perform cellular lysis. They are thought to be involved in immune surveillance and tumor destruction in vivo (23). These cells resemble K-cells in that they are non-adherent, non-phagocytic, bearing no common T or B-cell markers (with the possible exception of Thy-1) and requiring direct contact with the target for extracellular lysis to occur. Their tissue distribution, size, and morphological characteristics are also the same as K-cells (see review, ref. 24). Peak levels of cytolytic activity are evident during the same 4 to 9 week old age span in mice. NK and K cells have comparable sensitivities to X-irradiation, metabolic inhibitors, prostaglandins, interferons, and

temperature variations. Evidence is strong that these two activities may in fact be mediated by an overlapping population of effector cells (24,25). At the single-cell level, a human lymphocyte is capable of binding to and lysing both NK and ADCC targets (26). Ojo and Wigzell (27) provide evidence: a) that NK cells are equivalent to K-cells and are the only mouse cells that can kill antibody-coated tumor target cells, and b) that these cells are distinct from those cells mediating ADCC to antibody-coated erythroid target cells. Morphologically both NK and K cells have characteristics of large granular lymphocytes. This is evident in the rat, mouse, and human systems (28,29). It has not yet been possible to enrich for one activity without enriching for the other.

The issue over the origin of NK/K cells has been further confused by evidence suggesting certain similarities between CTL and NK cells. The mechanism of natural cell-mediated cytolysis (NCMC) performed by NK cells can be broken down into three discrete steps similar to those in CMC by CTL (30). Recently, several investigators observed that allospecific T-cell clones acquired the ability to lyse NK-sensitive target cells after prolonged culture conditions in the presence of T-cell growth factors (31,32). Also, many cloned NK cell lines and fresh NK cells express mRNA which encodes one of the chains of the T-cell antigen

receptor (33).

NCMC has been studied at the genetic level as well as at the cellular level. Inbred strains of mice differ in the levels of NCMC to various target cells (34). Differences in activity can be seen within a single strain of mouse against sublines of the same tumor cell. This diversity in activity probably reflects differences in genes that regulate the recognition of distinct target cell antigens. ADCC has not yet been studied in depth at the genetic level. A few studies do however show that levels of ADCC and NCMC are similar in the more common strains of laboratory mice (see review, ref. 35).

The cationic requirements for various types of cell-mediated killing processes appear to be very different in the human and mouse systems (36). CTL and NK cells require both Ca^{++} and Mg^{++} for lysis of allogeneic target cells. Lysis of antibody-coated sheep red blood cells (Ab-SRBC) by unsensitized human blood cells or mouse cells does not require Mg^{++} or Ca^{++} but is enhanced by Mg^{++}. Lysis of antibody-coated Chang human liver tumor cells by unsensitized human blood cells is Ca^{++}-dependent and Mg^{++}-independent, yet spontaneous lysis of uncoated Chang tumour cells is largely dependent on both cations. Human NK, ADCC, and CTL effector cells all appear to assemble pores on target cell membranes (37,38). These pores

increase membrane permeability and promote target cell lysis. These studies suggest that a final lytic step may be shared by NK, ADCC, and CTL lytic pathways.

Molecules on the surface of effector cells are the units by which effector cells recognize and combine with target cells. These molecules may also be involved in delivery of the lethal hit and subsequent lysis of the target cell. By studying these cell surface molecules it is possible to gain an insight into their function and evolutionary significance to the immune system. Cell surface antigens coded for by the major histocompatibility complex (MHC) in the mouse, known as H-2 antigens, are extensively involved in cell-cell interactions (for review, see ref. 39). The H-2K, H-2D, and H-2L regions of the MHC code for class I antigens which are found on nearly every cell in the body and are important in transplantation immunity. Differences in these genes dictate whether one mouse strain will accept a tissue graft from another strain of mouse. The Qa-2,3 and Tla regions of the MHC code for class I antigens involved in hematopoietic cell differentiation. H-2K and H-2D are serologically detectable antigens and have a molecular weight of 45,000. These molecules are directly involved in CTL restriction. The I region of the mouse MHC codes for class II Ia (immune-associated) antigens which are found on some

T-cells, macrophages, and B-cells and are involved in the regulation of T-cells. The Ia antigens are more restricted as far as their tissue distribution and consist of dimers having sizes of 28,000 and 30,000 daltons. Similar major cell surface antigens are coded for by the HLA complex in the human major histocompatibility system (MHS). Many other cell surface antigens coded for by genes located outside of the mouse MHC have been described. The Thy-1 antigen is a glycoprotein originally found on mouse thymocytes (40). Its distribution includes cells from brain tissue, epidermal cells, and fibroblasts (41). Although the Thy-1 molecule is expressed as one of two allelic forms (Thy-1.1, Thy-1.2) on T-cells from every strain of mouse tested, no significant role has yet been ascribed to it. The Lyt antigens are also found on mouse T lymphocytes and are thought to be involved in T-cell differentiation and function. Lyt-1, 2, and 3 antigens as well as antigens coded for in the Tl and Qa regions of the mouse MHC have a very specific distribution on various subsets of T-cells performing distinct functions in the immune system (42,43). Lyt-$1^{+}2,3^{-}$ lymphocytes are characterized as T-helper cells and are involved in T-cell regulation and B-cell help. Lyt-$1^{-},23^{+}$ lymphocytes are either T-suppressor cells or cytotoxic effector T-lymphocytes (CTL).

All mouse cells (except erythrocytes) derived from

hemopoietic stem cells express the T200 molecule (44). The T-cell form has been characterized as a molecule with a molecular weight (Mr) = 170-200,000 and is referred to as T200. The B-cell form is slightly larger, Mr = 210-220,000, and is referred to as B220 (45,46,47). A human homologue of the mouse T200 glycoprotein has been chemically characterized by peptide mapping (48) and a similar glycoprotein with a size of 150,000 daltons has been described for rat thymocytes (49). Ly-5 is an alloantigen that is found on the T200 molecule and has two allelic forms, Ly-5.1 and Ly-5.2. It was originally described by Komuro et al. (50) in 1975 as a T-cell differentiation antigen but has since been found on other cells as well. During biochemical synthesis of the Ly-5 molecule different molecular weight species are expressed by B-cells and T-cells (51). There have been several monoclonal antibodies reactive with determinants on T200 that react with either T-cells or B-cells, but not both, implicating a difference in structure of T200 molecules on B-cells and T-cells (45,52,53). The B-cell form of T200 (B220) appears to function in the regulation of antigen-driven B-cell differentiation (54).

Biochemically, T200, as studied in a murine lymphoma cell line, appears to be a phosphorylated transmembrane glycoprotein (55). There is a protease-resistant domain

that is exposed on the exterior cell surface and contains most of the mannose oligosaccharide units. This fragment has a size of 100,000 daltons by sodium-dodecyl-sulfate-polacrylamide gel electrophoresis (SDS-PAGE). The other domain is located on the cytoplasmic side of the cell membrane and is sensitive to digestion with trypsin. H-2 and Ia antigens have also been shown to be transmembrane proteins. Biochemical analysis of mouse high molecular weight surface glycoproteins by Ewald and Refling (56) has demonstrated certain proteolytic activities associated with the Ly-5 molecule.

Fc receptors are cell surface entities that serve many important functions in the immune system. The term FcR was coined more to describe a binding event than an actual molecule on the surface of cells. It has been postulated that the FcR may be comprised of many different cell components rather than a single molecule (57). The fact that artificial phospholipid membranes (liposomes) can bind hydrophobically to the Fc region of immunoglobulin (58) demonstrates that nearly any cell may have the capacity to non-specifically bind Fc. Different cells bind Ig Fc with varying degrees of avidity and whether or not it is possible to detect this binding event in an assay system determines if a cell can be classified as having an FcR. Two FcR for IgG on mouse peritoneal macrophages have been characterized.

FcRI binds IgG_{2a} and FcRII binds IgG_1 and IgG_{2b}. Several FcR have also been characterized on human blood monocytes (for review, see ref. 59). FcR are found on a wide variety of cells including monocytes and macrophages (60), B-cells, K-cells, and even activated T-cells. The affinity of various FcR for different classes and subclasses of Ig varies from cell to cell. FcR bind different sub-classes of IgG with varying degrees of avidity (61). Although IgM antibodies can induce ADCC in the absence of IgG antibodies, their efficiency on a molar basis is much less (62). FcR specific for IgG on phagocytic cells are involved in the stimulation of phagocytes and the ingestion of microorganisms. The interaction of IgG complexes with FcR on platelets leads to aggregation and vasoactive amine release. FcR for IgE, expressed on basophils and mast cells, are thought to be involved in degranulation during allergic reaction to allergins (63,64,65). Although mainly IgG- and IgM-specific FcR are involved in ADCC, IgE induces platelets to perform ADCC. FcR, like other membrane molecules, are subject to capping by polyvalent antibodies under permissive conditions (37°C, no azide). It is possible then that during a cytolytic event such as ADCC, FcR may function passively to concentrate the antibody-coated target cells to a region on the effector cell where lysis can occur. In contrast to a passive function, FcR may

actively trigger a cytolytic event upon binding to immune complexes.

One of the best ways to study the function of molecules on the surface of cells is to treat those cells with antibody specific for a molecule of interest and study what effect it has on normal cellular functions. If the antibody (in the absence of C′) blocks a specific function of a cell then that molecule is probably involved in that function. Several molecules on the surface of CTL have been studied this way. Antisera to the Lyt-2, Lyt-3, LFA-1 (lymphocyte function-associated antigen-1) (66), and Ly-5 (67,68) antigens block CMC by CTL bearing those antigens. These molecules have therefore been postulated to be involved in cytolysis by CTL. An antiserum, called RAT*, produced in rats against alloimmune mouse CTL has also been shown to block CTL functions (69). RH1, an antiserum raised in rats immunized with human activated alloimmune lymphocytes, analogous to RAT*, was found to block not only CTL but also NK and K-cell cytolysis in the absence of C′ (70). Harp and Ewald (71) demonstrated in 1983 that monoclonal antibody to the T200 molecule could modulate the generation of CTL in culture in the absence of C′. Nakayama et al. (68) and Harp et al. (67) demonstrated that the killing phase of CTL was suppressed by treatment of effector cells with Ly-5 alloantisera. Harp et al. (67) also showed

that treatment with Ly-5 alloantisera suppressed the generation of CTL and the activation of splenocytes by mitogens. They showed that this effect was allospecific in that only mice whose splenocytes were positive for the Ly-5.1 antigen were inhibited by anti-Ly-5.1. Furthermore generation and effector phases of CTL killing by spleen cells from F_1 heterozygotes bearing both the Ly-5.1 and Ly-5.2 alleles were inhibited only by a combination of Ly-5.1 and Ly-5.2 antisera (67). Recently, Le Francois and Bevan (72) have produced a monoclonal antibody to a determinant shown to be on the T200 molecule and this monoclonal antibody selectively reacts with CTL and inhibits their killing activity.

Antisera against T200, Ly-5, and NK-1.1 (an antigen reported only on NK cells), and a monoclonal antibody directed against FcR (73,74) have been shown to block NK cell lysis of various target cells. Seaman et al. (73) have shown that a monoclonal anti-T200 antibody inhibits murine NK cell activity. The observation that anti-Ly-5 blocks NK cell cytolysis was first reported by Kasai et al. (75). Pollack et al. (76) in 1980 found that ADCC to antibody-coated SL-2 T-lymphoma target cells was inhibited by Ly-5 antisera in the absence of C′. Antisera to NK-1 also reduced ADCC activity. Antisera directed against other cell surface antigens such as Lyt 1, 2, 3 and Thy-1 had no effect

on ADCC in the absence or presence of C′. NK cytolysis of YAC-1 lymphoma target cells was similarly inhibited by NK-1 and Ly-5 antisera. Unfortunately, $F(ab')_2$ fragments of the inhibitory antibodies were not made and tested for inhibition of ADCC. The FcR on K-cells are uniquely sensitive to blockade by Fc regions of any antibody either complexed with antigen, aggregated or in monomeric form, and in particular when antibody is bound to the effector cell (77). Without using fragments of antibody devoid of Fc it is impossible to know if the inhibitory effects were due to a specific blocking of an effector molecule on the killer cell by the Fab portion of the antibody or simply due to non-specific blocking of FcR.

Antisera to other antigens on K-cells have been studied only minimally for blocking of ADCC. Since Ia antigens have been shown to be non-covalently associated with FcR on B-cells (78), Schirrmacher et al. (79) tested to see if FcR on K-cells were also associated with Ia antigens. They found that K-cell FcR were not blocked by $F(ab')_2$ fragments of anti-Ia sera. Halloran et al. (80) conducted inhibition studies on FcR positive cells that rosette with erythrocyte-antibody (EA) complexes. These cells include other cell types in addition to K-cells. They found that whereas the formation of EA rosettes by FcR bearing cells was blocked by both undigested and $F(ab')_2$ fragments of

anti-H-2K and anti-H-2D antibodies, ADCC of Ab-SRBC was inhibited only by undigested anti-H-2 antibodies.

My studies support Halloran's findings in that $F(ab')_2$ fragments of antibodies to MHC antigens had no effect on ADCC to antibody-coated sheep red blood cells (Ab-SRBC). I report that intact antibody and $F(ab')_2$ and Fab fragments of antibody to the Ly-5 antigen did inhibit ADCC to Ab-SRBC in a specific manner. Anti-Ly-5.1 also appeared to block ADCC of tumor targets. My data suggest that the Ly-5 molecule on spleen effector cells may be involved in the lysis of antibody-coated target cells.

MATERIALS AND METHODS

Mice

BALB/c, SJL, A.SW, CBA and C57BL/6(B6) mice were bred in this laboratory from mice originally obtained from Jackson Laboratories (Bar Harbor, ME). B6-Ly-5.2 mice congenic with the B6(Ly-5.1) strain were obtained from Dr. Edward A. Boyse (Memorial Sloan-Kettering Cancer Center, N.Y., N.Y.). Donors of spleen cells for ADCC tests were 4 to 9 weeks old. Donors of tissues for immunization were between 1 and 3 months of age and recipients were at least 8 weeks of age.

Media

Tumor cells lines were cultured in vitro in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sterile Systems Inc. Logan, UT), 2mM glutamine (Irvine Scientific), and 5×10^{-5}M 2-mercaptoethanol (BIO-RAD Laboratories, Richmond, CA); this medium is abbreviated RPMI-FCS. ADCC assays were conducted in RPMI-FCS. Cells used for immunizations and the indirect immunofluorescence assay were prepared in Hanks' Balanced-Salt Solution (HBSS) (Irvine Scientific) or in phosphate-buffered saline (PBS) pH 7.2.

Antisera

All antisera used in these experiments were heat-inactivated at 56°C for 30 min to remove C′ activity and centrifuged at 10,000 x g to remove aggregates before use in ADCC assays.

(a) Anti-sheep red blood cells (Anti-SRBC) antiserum: BALB/c mice were immunized with three weekly intravenous (i.v.) injections of 0.1 ml of 10% (v/v) SRBC (Colorado Serum Co., Denver, CO) in PBS. Thereafter, the mice were injected every 2 weeks with the same dose of antigen. They were bled from the retroorbital sinus 7 and 10 days after each immunization, starting after the third injection. Sera from individual bleedings had hemagglutination titers between 1280 and 2560 against SRBC.

(b) Anti-P815 antiserum: CBA mice ($H\text{-}2^k$) were given weekly intraperitoneal (i.p.) injections of 1×10^6 P815 cells [DBA/2 mouse ($H\text{-}2^d$) mastocytoma] in PBS for 8 to 10 weeks. Mice were then boosted with $5 - 10 \times 10^6$ P815 cells every 2 weeks and bled 7 and 10 days after each immunization. Serum was pooled and tested regularly for activity by indirect immunofluorescence (IIF) on P815 cells.

c) Lyt-1.1 and Lyt-1.2 antisera: These sera were generously provided by Dr. Ian F. C. McKenzie, Victoria, Australia. They were produced by the method of Shen et al. (81).

(d) Thy-1.2 antibody: Medium was harvested from

hybridoma cells, 30-H12 (Salk Distribution Center, San Diego, CA) grown in RPMI-FCS. These cells produce a rat monoclonal antibody to the Thy-1.2 antigen (82).

(e) H-2 alloantisera: Anti-H-$2K^k$, E-b.d., and E-b.k were obtained from the National Institutes of Health. E-b.d was raised in (B10 x 129J)F_1 mice immunized with B10.D2 tissues. This antiserum had a hemagglutination titer of 2560 and a cytotoxic titer of 1280 on B10.D2 cells as reported by literature accompanying NIH contract sera. It recognized numerous specificities in the K, I, D(L), and TL regions of the B10.D2 (H-2^d) major histocompatibility complex (MHC). E-b.k was raised in (B10 x 129/j)F_1 mice immunized with B10.K tissues. This antisera had a hemagglutination titer of 1280 and a cytotoxic titer of 5120 on B10.K cells. E-b.k recognized numerous specificities in the K, I, D(L), and TL regions of the B10.K (H-2^k) MHC. Anti-H-$2K^k$ antiserum was raised by immunizing (A.TL X 129)F_1 mice with tissues from A.AL mice [genotype: ($K^sI^kD^d$ x $K^bI^bD^b$) anti-$K^kI^kD^d$]. This antiserum had a titer of 256 on B10.K cells as determined by complement-mediated cytotoxicity and recognized the H-2.11, 23,25 specificities in the K region of the B10.K MHC.

(f) Monoclonal antibodies: Monoclonal anti-H-$2K^k$ was obtained from Becton Dickinson (Sunnyvale, CA). Monoclonal anti-Ly-5.1 was obtained from New England Nuclear (Boston,

MA). Both monoclonal antibodies were of the mouse IgG_{2a} subclass.

(g) Ly-5 antisera: Ly-5 antisera were raised in two strain combinations. Ly-5.1 antiserum, raised in SJL (Ly-5.2) mice immunized with cells from A.SW (Ly-5.1) mice as described by Komuro et al. (50), was used in all of the experiments except the specificity studies (Table 4 and Figure 7). At least 1×10^7 cells were pooled from the spleen, thymus, and lymph node of A.SW mice and injected i.p. into SJL mice every 2 weeks for at least 8 weeks. Serum from individual mice was collected from blood obtained from the retroorbital sinus 7 and 10 days after immunization and tested for activity on A.SW thymus cells by IIF. Serum having high levels of activity on A.SW thymocytes and no activity on SJL thymocytes was pooled and stored at $-80^{\circ}C$ for use in inhibition assays.

Ly-5.2 antiserum (used in Figure 7) raised by Rolf Taffs at Montana State University, was made by immunizing (B10 x A.SW)F_1 mice with lymphoid cells from SJL mice in the same manner described above.

The Ly-5.1 antiserum used in the specificity experiments was raised in a strain combination using mice congenic with B6 for the Ly-5 locus and is described in the results.

In each case, normal mouse sera from nonimmunized age-matched mice from the same strain were collected and

pooled for use as a control for the antibodies and any other factors normally found in mouse sera.

Indirect Immunofluorescence Assay

The binding efficiency of antibodies in our antisera was tested on cells bearing the appropriate antigens using an indirect immunofluorescence assay (IIF) modified for our purposes by Dr. S. Ewald at Montana State University from a procedure described by Mishell and Shiigi (83). Briefly, 5×10^5 cells in HBSS with 0.02% NaN_3 were placed in each well of a 96-well flat-bottom microtiter plate (Falcon). The plate was centrifuged at 200 x g for 5 min and excess medium was aspirated and discarded. The cells were then resuspended in 50 ul of dilutions of antiserum made in HBSS + 0.02% NaN_3 and incubated for 30 min at 4^oC. The plate was centrifuged at 200 x g for 5 min and excess antiserum was aspirated and discarded. Each well containing cells was washed carefully three times with 200 ul of HBSS + 0.02% NaN_3 without centrifuging between washes. Cells were then resuspended in 50 ul of fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulin (Miles Scientific, Naperville, IL) diluted 1:50 in HBSS + 0.02% NaN_3 and incubated for 30 min at 4^oC. The plate was then centrifuged and washed three times as before. Cells in each well were finally resuspended in 100 ul of 1:1 glycerol-HBSS

and the cells were scored on a scale of 0-4 for positive fluorescence. An Olympus IMT inverted microscope equipped with BH-RFL reflected high fluorescence attachments, dichroic mirrors, and a FITC filter, was used to score for fluorescence. Control wells containing cells incubated with FITC-labeled rabbit anti-mouse immunoglobulin alone were also included.

To compare binding activities of various antisera on cells to be used in ADCC, spleen cells from appropriate strains of mice were used for IIF.

Titers for antisera reported in Results were calculated as the reciprocal of the dilution of antiserum giving approximately one-half of the maximum amount of fluorescence seen with the lowest dilution of antiserum.

Hemagglutination Assay

To determine the titer of the anti-SRBC antiserum used in ADCC assays, a hemagglutination assay as described by Herbert (84) was used. One-hundred microliters containing doubling dilutions of anti-SRBC serum in PBS were added to duplicate wells of a 96-well round-bottom microtiter plate (Falcon). Dilutions ranged from 1:10 to 1:20,480. Control wells containing PBS alone were also included. One-hundred microliters of a 1.0% packed cell suspension of washed SRBC were added to each well and the plate incubated for 2 hr at room temperature. The titer of the antiserum was taken to

be the reciprocal of the highest serum dilution giving an unequivocally positive reaction.

Purification of IgG and Production of $F(ab')_2$

One milliliter of conventional antiserum or normal SJL mouse serum was applied to stoppered plastic Quik-Sep minicolumns (Isolabs Inc., Akron, OH) containing a one milliliter bed volume of Protein A-Sepharose (Pharmacia, Piscataway, NJ) equilibrated in 100 mM PBS pH 8.0. After 60 min incubation at room temperature, the minicolumns were unstoppered and placed in 15 ml conical centrifuge tubes and the entire column assembly was centrifuged for 2 min at 50 x g as described by Parkinson et al. (85). Columns were washed with PBS to remove unbound serum proteins. Antibody was eluted with sequential applications of 40 mM glycyl-tyrosine in 100 mM PBS followed by PBS alone (86). Eluates were pooled and then concentrated and desalted in 50k ultrafiltration membrane cones (Amicon, Danvers, MA). The concentrated IgG was finally exchanged into 0.025 M acetate, 0.14M NaCl buffer, and the pH adjusted to 4.5 with dilute acetic acid; a portion (2 mg) was incubated with 10 ul of 1 mg/ml pepsin (Worthington Diagnostic, Freehold, NJ) in PBS for 30 hr 37°C in a CO_2 incubator (87). A separate portion of each immunoglobulin preparation was incubated in the same buffer without pepsin under identical conditions as a control for the digestion procedure.

After incubation, both digested and undigested samples were adjusted to a pH of 8.0 with 3N NaOH. The digested samples in a 1 ml volume were cleared of remaining intact immunoglobulin and pFc´ fragments by application to Protein A-Sepharose minicolumns; the unbound fraction was then incubated for 30 min with 5 mg of Protein A-Sepharose beads in a 1.5 ml conical tube. Samples were centrifuged at 10,000 x g to remove Protein A-Sepharose, and the supernatant fraction was recovered. Digested and undigested samples were dialyzed extensively against PBS (10 mM pH 7.2) and ultracentrifuged at 100,000 x g for 1 hr to remove aggregates before their use in ADCC. Molecular weights of the fragments were determined by comparison to a known mouse $F(ab')_2$ standard (Cappel Labs, West Chester, PA) and other molecular weight standards including mouse IgG, bovine serum albumin, and phosphorylase B (Sigma Chemical Co., St. Louis, MO) on 8.5% SDS-polyacrylamide gels (88) stained with silver (BIO-RAD, Richmond, CA). Protein concentrations were assessed using a Gilford multimedia densitometer attached to a Gilford system 2600 spectrophotometer. The $F(ab')_2$ fragments and intact IgG controls of each antiserum and NMS were tested for inhibition of ADCC at equivalent protein concentrations.

ADCC to Antibody-Coated Sheep Red Blood Cells (Ab-SRBC)

Effector cells were adjusted to a concentration of 1-8 x 10^7 cells/ml in RPMI-FCS. Doubling dilutions of effectors were made in tubes and then aliquoted, 100 ul/test well, into Linbro 96-well conical bottom plates.

Sheep red blood cells (1 x 10^8) were washed three times in RPMI-FCS and resuspended in 0.5 ml FCS. Two hundred fifty microcuries $Na_2{}^{51}CrO_4$ (200-500 Ci/g, New England Nuclear, Boston, MA) were added and the cell suspension incubated at 37°C for 1 hr. The red cells were washed twice by centrifugation over a cushion of FCS and finally resuspended to a concentration of 1-5 x 10^6 cells/ml in RPMI-FCS. Anti-SRBC antiserum or BALB/c normal mouse serum (as a negative control) was added to the labeled SRBC suspension to a concentration of 1:100. One hundred microliter aliquots of ^{51}Cr-labeled SRBC at a concentration of 1-5 x 10^6 cells/ml and containing 1:100 anti-SRBC or BALB/c NMS were added to each well containing effectors, so that the total volume of the incubation mixture was 200 ul and the final concentration of anti-SRBC (or NMS) was 1:200. In addition, control wells containing only 100 ul ^{51}Cr-labeled SRBC plus serum plus 100 ul medium were set up to test for spontaneous ^{51}Cr-release in the absence of effectors. Wells with 100 ul ^{51}Cr-labeled SRBC plus 100 ul medium received detergent (Zap-O-Globin, Coulter

Diagnostics, Hialeah, FL) to determine maximum release. All tests were performed in duplicate or triplicate.

The plates were centrifuged at 150 x g before incubating at 37°C for 1 hr. At the end of the incubation period, the plates were again centrifuged at 150 x g at 4°C for 10 min. One hundred microliters of medium were removed from each well and placed in scintillation vials for counting in a biogamma counter (Beckman Instruments, Palo Alto, CA) for detection of ^{51}Cr release. Values for duplicate and triplicate samples were averaged and the percent specific ^{51}Cr release was calculated as:

$$\frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100\%$$

ADCC to Antibody-Coated P815 Tumor Cells (Ab-P815)

CBA or BALB/c effector cells were adjusted to a concentration of 1 x 10^7 cells/ml in RPMI-FCS. Doubling dilutions of effectors were made in tubes and then aliquoted, 100 ul/test well, into Linbro 96-well conical bottom plates.

P815 mastocytoma cells of DBA/2 (H-2^d) origin were labeled with 100 ul $Na_2{}^{51}CrO_4$ for 1.5 hrs at 37°C. Labeled P815 cells were washed twice by centrifugation over a cushion of FCS and resuspended to a concentration of 1 x 10^5 cells/ml in RPMI-FCS. Anti-P815 antiserum (anti-H-2^d) or

CBA normal mouse serum (absorbed with P815 cells) was mixed with the cell suspension at a 1:10 dilution and the suspension was incubated for 15 min at 4°C. To remove the excess anti-H-2^d, Ab-P815 were centrifuged to pellet and resuspended in fresh RPMI-FCS to the same volume. One hundred microliter aliquots of labeled Ab-P815 or NMS-treated P815 cells (at a concentration of 1 x 10^5 cells/ml) were added to each well containing effectors. In addition, values for spontaneous release were determined from wells containing heat-inactivated (45°C, 10 min) effector cells added to targets. To determine maximum ^{51}Cr-release, detergent was added to wells containing target cells. All tests were performed in triplicate.

The plates were centrifuged and counted and percent specific cytotoxicity was calculated as in ADCC to Ab-SRBC. Natural cytotoxicity was also measured by incubating effector cells with ^{51}Cr-labeled P815 cells in the absence of P815 antiserum or NMS. Unless otherwise stated, values for natural cytotoxicity did not exceed 5% of the level of killing seen in ADCC to Ab-P815.

In both ADCC to Ab-SRBC and ADCC to Ab-P815 assays, significant levels of specific cytotoxicity were seen within short incubation times, 1 hr and 4 hr respectively, whereas control wells containing NMS treated target cells usually showed less than 5% ^{51}Cr-release (data not shown). These

results indicate that killing requires the presence of specific anti-target antibody. Specific cytotoxicity was evident at several effector to target cell (E:T) ratios.

Inhibition of ADCC

Effector cells were either treated with antisera and washed before testing against antibody-coated target cells or the antisera were added to wells containing effector cells for the duration of the assay.

Percent inhibition was calculated as:

$$\left(1 - \frac{\text{specific cytotoxicity with test sera}}{\text{specific cytotoxicity in presence of normal mouse serum or medium alone}}\right) \times 100\%$$

C′-mediated Microcytotoxicity Assay

In order to compare cytotoxic titers of antisera on cells in the presence of complement, a two-step cytotoxicity assay described by North (89) was used. Antisera were diluted two-fold into RPMI-FCS and 4 ul of each dilution were placed into duplicate wells of a Cooke Microtiter V-bottom plate. Appropriate target cells were washed and suspended at a concentration of 5×10^5 cells/ml in RPMI-FCS before adding 4 ul of cell suspension to each well containing antiserum. Antisera were incubated with the cells for 10 min at room temperature. Rabbit C′ (Pel Freeze Biological) was diluted 1:10 in RPMI-FCS and 4 ul added to each well. Control wells containing cells alone, cells plus

C′ alone, and cells plus antiserum alone were also included. Plates were incubated for 45 min at 37°C in the presence of 5% CO_2. Fluid was removed from wells by blotting the edge of each well carefully with a cotton applicator stick. Wells were stained with 0.1% nigrosin for 10 min at room temperature. Dead cells and total cells in each well were counted using an inverted microscope. Counts from duplicate wells were averaged, and percent specific cytotoxicity was calculated as:

$$\frac{(\%\text{ dead cells experimental} - \%\text{ dead cells control})}{(100\% - \%\text{ dead cells control})} \times 100\%$$

Titer was reported as the reciprocal of the dilution of antisera giving 50% of the maximum cytotoxicity seen in the presence of antibody plus C′.

RESULTS

The Effect of Ly-5 Antiserum on ADCC to Ab-SRBC

Antisera to cell surface antigens expressed on mouse spleen cells were tested for their ability to inhibit ADCC to Ab-SRBC in the absence of complement. Previous experiments in our laboratory[1] had shown that Ly-5.1 antiserum inhibited ADCC to Ab-SRBC by spleen cells bearing the Ly-5.1 antigen. Figure 1 illustrates the inhibitory effect of anti-Ly-5.1 on ADCC by BALB/c ($Ly\text{-}5.1^+$) spleen cells when the Ly-5.1 antiserum was included in the assay at a final concentration of 1:20. Normal mouse serum from SJL mice (SJL NMS), used as a control for the antibodies normally found in SJL serum, had no effect at the same concentration. Antiserum to the alternate allodeterminant, Ly-5.2, which is not expressed by BALB/c spleen cells, also had no effect on ADCC activity. When SJL ($Ly\text{-}5.2^+$) spleen effector cells were tested for ADCC to Ab-SRBC in the presence of 1:20 anti-Ly-5.1, anti-Ly-5.2, or SJL NMS, only anti-Ly-5.2 had a significant inhibitory effect (Figure 2). Spleen cells from other strains of mice bearing the Ly-5.1 antigen such as CBA and C57BL/6 (B6) were also inhibited by

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[1]Previous experiments supervised by Dr. Sandra Ewald were done as undergraduate research projects by Robin M. Small and Shannon Walden.

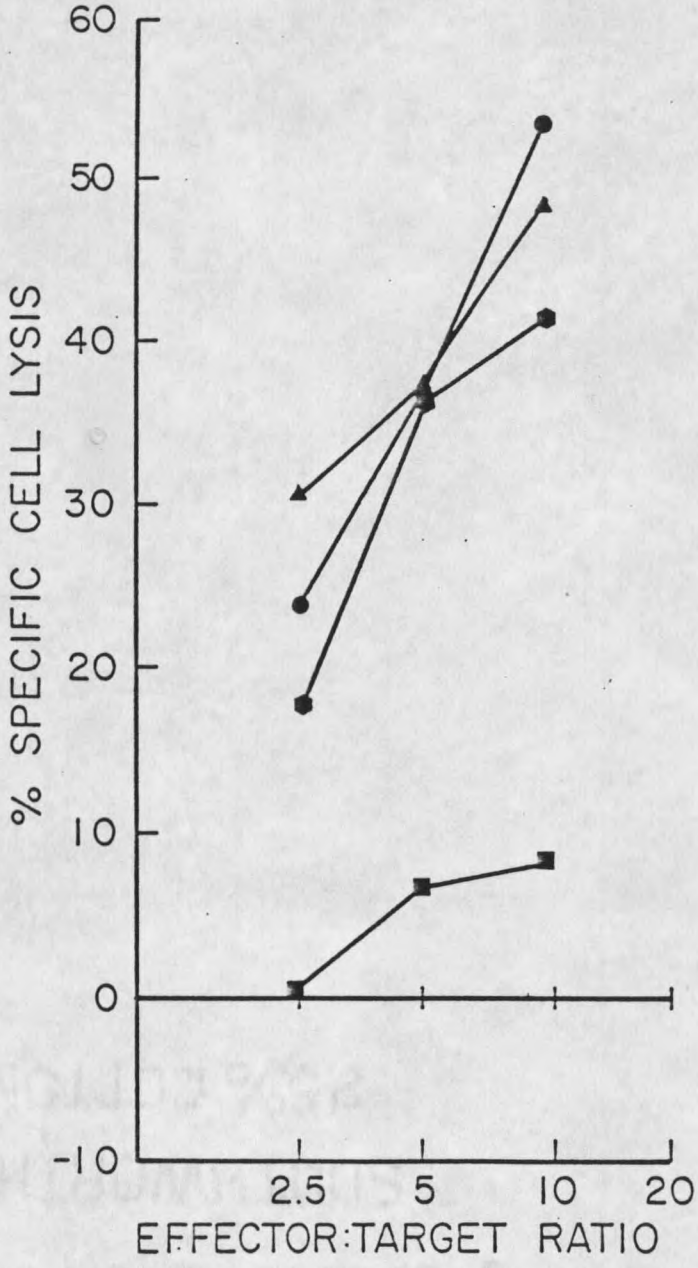

Figure 1. Inhibition of BALB/c spleen cells ADCC by anti-Ly-5.1. BALB/c spleen cells were incubated for 1 hr with ^{51}Cr-labeled Ab-SRBC at various E:T ratios in the absence of antibody (●—●), in the presence of 1:20 SJL NMS (▲—▲), 1:20 anti-Ly-5.1 (■—■), or 1:20 anti-Ly-5.2 (●—●).

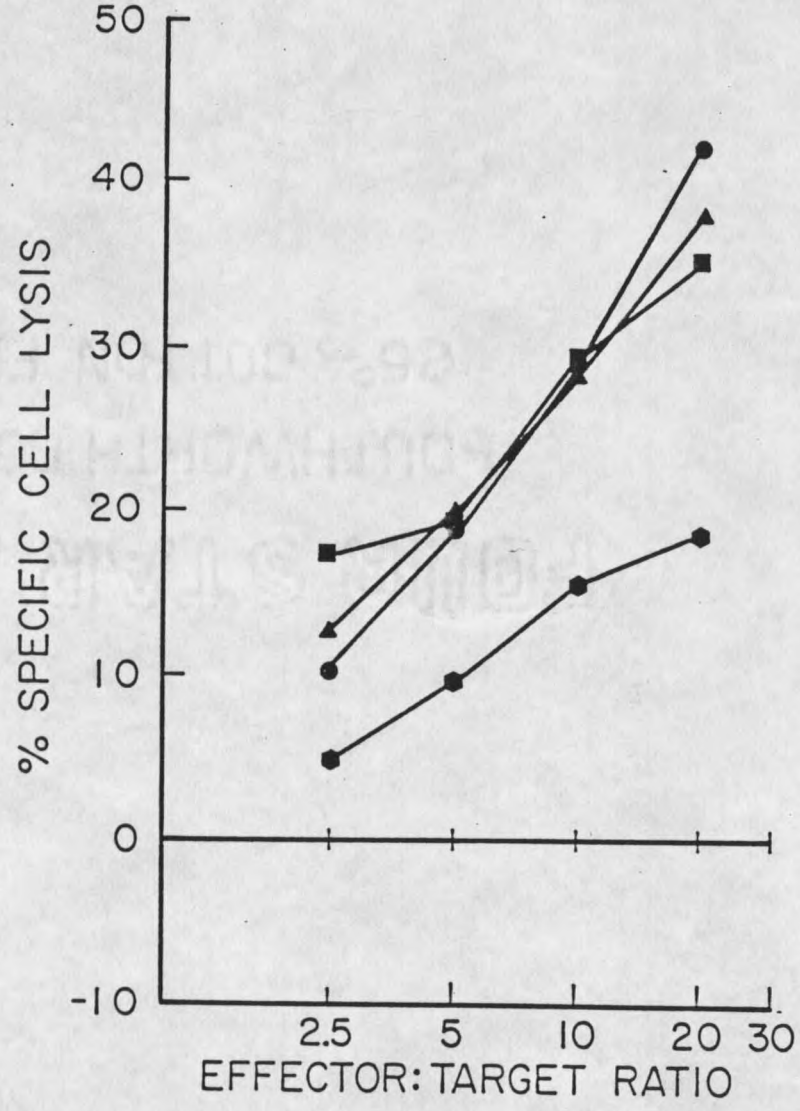

Figure 2. Inhibition of SJL spleen cell ADCC by anti-Ly-5.2. SJL spleen cells were incubated for 1 hr with ^{51}Cr-labeled Ab-SRBC at various E:T ratios in the absence of antibody (●—●), in the presence of 1:20 SJL NMS (▲—▲), 1:20 anti-Ly-5.1 (■—■), or 1:20 anti-Ly-5.2 (⬢—⬢).

Ly-5.1 antiserum. Results from these experiments are included in later sections. To test for dose-dependent inhibition of ADCC to Ab-SRBC, various dilutions of Ly-5.1 antiserum were mixed with BALB/c spleen effector cells before incubation with Ab-SRBC for 1 hr. The data in Figure 3 show that the inhibitory effect of Ly-5.1 antiserum decreased upon increasing dilution. SJL NMS had no effect at any of the same dilutions tested (data not shown). Inhibition by anti-Ly-5.1 was visible even at very low effector:target cell (E:T) ratios. When BALB/c spleen cells were treated for 10 min at 4°C with dilutions of anti-Ly-5.1, washed once, and tested for ADCC activity (data not shown), dose dependent inhibition was similar to that of Figure 3. These results indicate that Ly-5.1 antiserum does not have to be present in excess throughout the assay period in order to cause inhibition.

In order to rule out the possibility of anti-Ly-5.1 being toxic to the spleen effector cells, BALB/c spleen cells labeled with ^{51}Cr were incubated with unlabeled Ab-SRBC in the presence or absence of various concentrations of anti-Ly-5.1. Spleen cells that were not labeled with ^{51}Cr were also tested for inhibition of ADCC by anti-Ly-5.1. Table 1 shows that although spleen cells were inhibited in ADCC to Ab-SRBC by all dilutions of anti-Ly-5.1 tested, these same concentrations of antiserum did not cause significant ^{51}Cr-release from labeled spleen cells.