Measuring Murine Natural Killer Cell Cytotoxicity using a Lactate Dehydrogenase Assay

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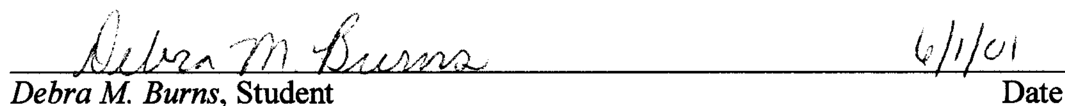

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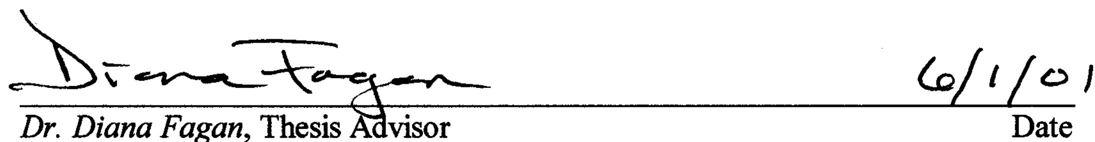

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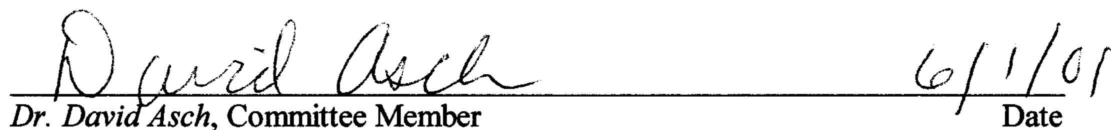

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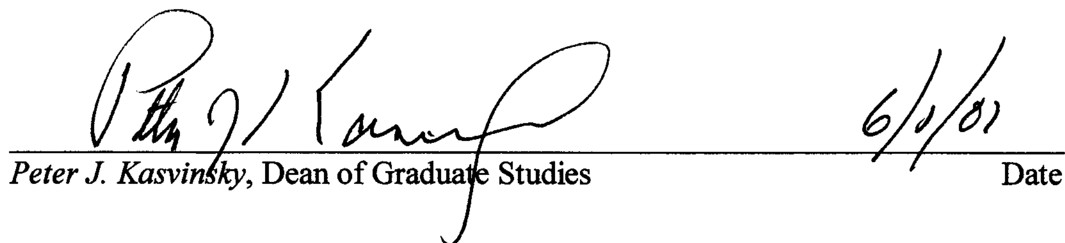

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ABSTRACT

Natural killer cells are among the first line of immune defense against virus-infected or tumor cells. Natural killer cells possess the ability to lyse target cells by releasing enzymes that perforate the target cell membrane. The lactate dehydrogenase assay measures the amount of lactate dehydrogenase, an intracellular enzyme, released by the target cell when it is lysed. When the LDH interacts with the substrate mix, the conversion of a tetrazolium salt (INT) into a formazan product produces a colorimetric reaction that can be measured using a standard microplate reader. The amount of lactate dehydrogenase released is an indication of how much the natural killer cells are killing the target cells. L1210, P815, Yac-1, and MPC 11 cells lines were tested for their usefulness as target cells when using this assay and murine spleen cells as effector cells. The results indicate that the MPC 11 (45% cytotoxicity) and Yac-1 (19% cytotoxicity) cell lines are targeted by murine NK cells and are useful positive controls for measuring murine NK cell cytotoxicity with the LDH assay. In our study, cytotoxicity was also observed with the L1210 cells, however, as these results are in contrast with previous reports, additional tests are required to confirm these results. The data also indicates that the P815 cell line is not targeted for lysis by murine NK cells. This cell line would be useful as a negative control in murine NK cell cytoxicity studies. While these studies have shown that the LDH assay can be used to measure murine NK cell function, the assay is not effective at all effector cell concentrations. The cytotoxicity at the higher E:T ratios was masked by the high amount of murine NK cell spontaneous LDH release. The high levels of spontaneous LDH release requires that careful titration of effector cells be performed when measuring murine NK cell cytotoxicity using the LDH assay.

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Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
CD	cluster of differentiation
CFDA	carboxy-fluorescein-diacetate assay
Cr	chromium
CTL	cytotoxic T lymphocyte
DMEM	Dulbeco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EuDTPA	europium chelated to diethylenetriaminopentiaacetate
FAD	flavin adenine dinucleotide
FCS	fetal calf serum
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITIM	immunoreceptor tyrosine-based inhibitory motif
LDH	lactate dehydrogenase
MHC	major histocompatability complex
MAFA	mouse mast cell function-associated antigen
NADH	reduced nicotinamide adenine dinucleotide
NK	natural killer cell

PBS	phosphate-buffered saline
T_c	T cytotoxic cell
TCR	T cell receptor
T_H	T helper cell
TNF	tumor necrosis factor

Introduction

The immune system is a defensive system utilized by the body to prevent infection and diseases (Kuby, 1997). There are two major components of the immune system: innate (nonspecific) immunity and acquired (specific) immunity. Innate and acquired immunity do not work independently of one another. In fact, they work together to eliminate foreign invaders.

Acquired Immunity

Cellular components of the acquired immune system specifically recognize foreign microorganisms and molecules based on the epitopes, the site on an antigen (any foreign molecule that binds to an antibody or T cell receptor) that is recognized and bound by antibodies or T cells. This branch of the immune system possesses four general characteristics: antigen specificity, diversity, immunologic memory, and self/nonself recognition. Antigen specificity allows the body to recognize and distinguish many differences among antigens. Billions of different antigen structures can be recognized and removed due to the amount of diversity displayed by the immune system. Immunologic memory (a result of memory cell production in response to an antigen) of acquired immunity allows the immune system to rapidly respond to a second encounter of the same antigen resulting in removal of the antigen before disease symptoms begin. Finally, the cellular components of the acquired immune system are able to distinguish between foreign cells and cells that are self, which prevents autoimmune diseases from occurring.

The cellular components of the acquired immune system are the B lymphocytes and T lymphocytes. Even though both B cells and T cells are cellular components of the acquired immune system, their effector cell responses are different. These responses can be grouped into two categories: humoral and cell-mediated responses. The humoral response involves B lymphocytes (B cells) that interact with a foreign invader, or antigen, and differentiate into plasma cells that release immunoglobulins (also called antibodies). The secreted antibody binds to the antigen and facilitates its removal from the body, thus acting as the effector of the humoral immune response. Cell-mediated responses are a result of activation of T lymphocytes (T cells), macrophages, and natural killer cells (NK). The cell-mediated component of acquired immunity results from T cell activation. T cell activation results in the proliferation and differentiation of T cells into T helper and T cytotoxic cells. Antigen complexed with major histocompatability molecules (MHC) I or II are presented on antigen presenting cells (B cells, macrophages or dendritic cells) to the T cytotoxic and T helper cells. T cytotoxic cells function to kill antigen infected cells. T helper cells, if activated, function to activate T cytotoxic cells, NK cells, and B cells. The activation of these cells will result in the removal of antigen.

MHC complexes are tightly linked clusters of genes. The products of these genes are associated with intercellular and self/nonself recognition (Kuby, 1997). MHC I genes encode a glycoprotein that is located on the surface of almost all nucleated cells and is responsible for presenting peptide antigens of altered self cells to the T cytotoxic cells. Antigens complexed with MHC class I molecules serve as the activating signal for T cytotoxic cells (CD8+). Cluster of differentiation molecules (CD) are located on the cell membrane of leukocytes and are used to differentiate leukocyte subpopulations. They are

identified by monoclonal antibodies. All monoclonal antibodies that interact with the same membrane molecule are grouped into a cluster of differentiation complex. MHC II genes encode glycoproteins that are expressed on antigen presenting cells. MHC class II combined with a foreign peptide activates T helper cells (CD4+). Along with the MHC molecules, the T cell receptor also complexes with CD3, another membrane molecule. This TCR-CD3 complex binds with a peptide-MHC complex on an antigen presenting cell, the T helper cell is activated via signal transduction between the TCR-CD3 and MHCII-Ag complex (Kuby, 1997). The TCR-CD3 and MHCII-Ag complex provides the first signal for activating T helper cells. An antigen-presenting cell provides the second signal, a co-stimulatory signal. The interaction between B7, a molecule on the membrane of the antigen presenting cell, and CD28, a molecule on the membrane of the T helper cell, commonly induces the co-stimulatory signal needed to activate a T helper cell to undergo clonal expansion, a rapid growth in cell number (Kuby, 1997).

The other T cells that elicit effector responses are T cytotoxic cells. T cytotoxic cells are activated by the interaction of the MHC I complex with antigen on the surface of altered self cells. The result of this interaction is the differentiation of the T cytotoxic cell into an effector cell called a cytotoxic T lymphocyte (CTL). Cytotoxic T lymphocytes are CD8+ and MHC class I restricted, which means that they only recognize antigen that is complexed with MHC I molecules. They perform cytotoxicity in two ways. The first is by binding to the target cell and releasing cytotoxic proteins such as perforin and granzymes that will perforate the target cell membrane. The second way CTLs kill target cells is via a membrane bound Fas ligand that is located on their surface. In order for Fas protein to be activated, it must first be bound by an antibody. If

activated, the Fas ligand will bind to the Fas receptor on the target cell surface. In both cytotoxic mechanisms, the primary means of killing the target cell is by inducing apoptosis (cell suicide).

Innate Immunity

The second major component of the immune system is innate immunity. The innate immune system is composed of anatomic, physiologic, endocytic, phagocytic, and inflammatory defensive barriers. Examples of anatomic barriers are the skin and mucous membranes. Both retard the entry of pathogens (disease-causing organisms) into the body or aid in clearing the body of any pathogens that may have been able to enter. Physiologic barriers include body temperature, pH levels, and chemical mediators. Body temperature and a fever response inhibit the growth of some pathogens. The acidic pH levels of the stomach kill most ingested pathogens. Chemical mediators such as lysozyme, interferon, and complement components facilitate the removal of pathogens via phagocytosis. Lysozymes are enzymes present in tears, saliva, and mucous secretions, and they digest peptidoglycan in the bacterial cell wall and function as a nonspecific antibacterial agent (Kuby, 1997). Interferons (IFN) are glycoproteins produced and secreted by certain cells that induce an antiviral state in other cells and also help to regulate the immune response. Complement components are serum proteins that participate in an enzymatic cascade, which generates a membrane attack complex that mediates cell lysis by creating a pore in the membrane of the target cell. Specialized cells such as macrophages, monocytes and neutrophils will then perform phagocytosis:

the engulfing of foreign particles. Finally, inflammatory barriers such as vasodilation and increased capillary permeability promote an influx of phagocytic cells into an infected tissue area, which leads to removal of the pathogen. Vasodilation is an increase in the diameter of blood vessels, which causes tissue redness and heat production in the inflamed tissue (Kuby, 1997). Increased capillary permeability allows for an influx or surge of fluid and cells from the dilated capillaries into the tissue. The fluid will pool in the tissue and cause swelling of the damaged or infected tissue, which will activate various immune response systems such as the kinin, clotting, and fibrinolytic systems. These systems promote the influx of white blood cells to the infected or damaged area. Macrophages are also activated to clear antigen from the affected area and to promote healing of the area.

Natural Killer Cells

Natural killer (NK) cells, the main cellular component of the innate immune system, represent a unique lymphocyte population of cells that are distinct from both T and B cells (Suzuki *et al.*, 1992). NK cells are derived from lymphoid stem cells in the bone marrow (Le Gros *et al.*, 1984)(Carson and Caligiuri, 1996). Lymphoid stem cells give rise to T cells, B cells, and NK cells (Kuby, 1997). Natural killer cells are CD3 negative large granular lymphocytes that express CD16 (Papa and Valentini, 1994). CD3 is a complex that was previously described as important in signal transduction of the T cell receptor. It is found on T cytotoxic and T helper cells. The lack of CD3 is one of the factors that differentiate NK cells from T cells. CD16 is a membrane receptor for the C-

terminus, or Fc region, of the immunoglobulins (antibody) IgG. IgG plays an important role in antibody dependent cellular cytoxicity (ADCC)(See cytotoxicity section).

While CD 16 and CD 3 are surface markers that distinguish NK cells from T cells, NK cells share a number of surface markers that are similar to T cell markers. These markers include Lyt-5, Thy-1, and Qa-1 (Le Gros *et al.*, 1984). Qa-1 is an MHC class I ligand on murine cells (Rohrer *et al.*, 2000). Natural killer cell recognition of Qa-1 results in the inhibition of target cell lysis (Vance *et al.*, 1998). Thy-1 is a membrane protein that is expressed upon entry into the thymus by progenitor T cells. Thy-1 is a marker of all thymus-derived lymphocytes (T cells) in the mouse (Kuby, 1997).

Natural Killer Cell Cytotoxicity

Natural killer cells possess cellular characteristics that allow them to kill certain tumor and infected cells, without prior sensitization and without restriction by major histocompatabiliy (MHC) antigens (Robertson and Ritz, 1990) (Yokoyama, 1995). This means that NK cells do not need prior exposure to antigen to be cytotoxic, and they do not have to recognize an antigen complexed with a specific MHC molecule as do T cells. Unlike T and B cells, natural killer cell cytotoxicity is not antigen specific because they do not have a specific antigen receptor. While natural killer cells do not have antigen specific receptors, they must bind the target cell in order for cytotoxicity to occur. Natural killer cells are capable of killing target cells by both non-MHC-restricted cytotoxicity (natural killer function) and by antibody-dependent cellular cytotoxicity (ADCC) (Robertson and Ritz, 1990). In ADCC, target cells are coated with an antibody

and are lysed by the natural killer cells that possess the Fc receptors for immunoglobulin. The Fc receptor is a cell-surface receptor specific for the non-antigen-binding fragment of an immunoglobulin molecule (Kuby, 1997). When natural killer cells recognize a target cell coated with antibody (IgG), the CD16 surface molecule on the NK cell will allow the NK cell to bind to the IgG molecule on the target cell, which will result in lysis of the target cell (Kuby, 1997).

While target recognition is the first step involved in NK cell cytotoxicity, two different receptor-mediated events determine whether the target cell is killed. They are activation and inactivation of the NK cell receptors (Helander and Timonen, 1998). The NK cell binds to the various target cells via receptor/ligand binding. Natural killer cells have four different types of target cell receptors. These receptors are adhesion molecules, triggering receptors, inhibitory receptors, or co-stimulatory receptors (Helander and Timonen, 1998). Natural killer cell receptors differ from T cell receptors in three major ways: 1). NK cells do not express rearranged receptors as the T cell does (Wang and Yokoyama, 1998); 2). Natural killer cells do not have antigen specific receptors, but bind to target cells through non-specific adhesion (Wang and Yokoyama, 1998); and 3). Selectivity for killing of cells is determined by cytotoxicity inhibitory signals. NK cell cytotoxicity is suppressed by signals delivered from the NK MHC receptor (Wang and Yokoyama, 1998). Thus, target cells that have lost MHC expression do not suppress NK cell killing.

The NK activation receptors include CD2, CD16, CD28, CD69, LY-6, Ly-49D, NKR-P1, 2B4, NKG2D, CD/NKG2A/C/E (Mason, 1996). The activation receptor NKR-P1 is a natural killer cell receptor that binds to oligosaccharides that are expressed on the

surface of tumor and virus-infected cells, which will often have abnormal patterns of glycosylation. The interaction between the NKR-P1 receptor with the oligosaccharide ligand signals the NK cell to kill the infected or tumor cell (Kuby, 1997).

Another murine NK cell receptor has been discovered which is homologous to the human NKp46 receptor, which is known to trigger natural cytotoxicity by NK cells (Biassoni *et al.,* 1999). The murine receptor, MAR-1 belongs to the immunoglobulin superfamily that is characterized by two C2-type Ig-like domains, a transmembrane portion containing a positively charged residue and a cytoplasmic tail lacking the immunoreceptor tyrosine-based activation motif (ITAM) (Biassoni *et al.,* 1999). The MAR-1 receptor is expressed on the surface of cell transfectants, and it is also selectively expressed on the surface of NK cells (Biassoni *et al.*, 1999). The exact role of MAR-1 in cytotoxicity is still unclear.

The main inhibitory receptors are Ly-49D/H, CD94, NKG2, KLRG1(previously called mouse mast cell function-associated antigen (mMAFA), and gp49A/B (Rohrer *et al.*, 2000). The killing of specific target cells by NK cells is mainly determined by the inhibitory receptors, rather than the stimulatory receptors (Rohrer *et al.*, 2000). Many of the inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMS), which give the receptor its inhibitory function (Rohrer *et al.*, 2000). ITIMS have been found in murine NKR-P1B receptors, suggesting that this receptor may have inhibitory functions as well as stimulatory functions. ITIMS have also been located in the NKG2A inhibitory receptor, KLRG1, and gp49B (Rohrer *et al.,* 2000).

The inhibitory receptors are categorized into two main groups: calcium dependent lectin-like receptors and the immunoglobulins-like receptors (Rohrer *et al.*, 2000). The

C-type lectin receptors are Ly-49, CD94, KLRG1, and NKG2. In order for NKG2 to be active, a heterodimer must form between the NKG2A and C isoforms or the NKG2A and E isoforms with the CD-94 C-type lectin receptor (Rohrer *et al.*, 2000). The heterodimer will then recognize Qa-1, and MHC I ligands (Rohrer *et al.*, 2000). The inhibitory receptor, KLRG1 (previously mMAFA) has a known ITIM located in its sequence. This indicated that this receptor has inhibitory effects on NK cell activation. This receptor is expressed on murine NK cells and virus activated CD 8+ T cells (Rohrer *et al.*, 2000). The exact role of this receptor is unknown.

The inhibitory receptor Ly-49 inhibits the activation receptor NKR-P1. It does this in the presence of an MHC I molecule. When Ly-49 binds to the MHC I molecule, it sends a negative signal to suppress the activation of the NKR-P1 receptor. Thus the NK cell is not activated to kill. In contrast, when a cell has reduced or no MHC class I expressed on the surface, the Ly-49 signal is also reduced, which allows the NKR-P1 signal to induce the killing of the target cell (Mason *et al.*, 1995).

Recently, an inhibitory form of 2B4, a murine natural killer cell receptor, has been characterized. The receptor 2B4 is found on the surface of all murine natural killer cells as well as T cells displaying non-MHC-restricted cytotoxicity. It has been shown that the 2B4 gene encodes two different products: 2B4L (150 amino acid cytoplasmic tail) and 2B4S (93 amino acid cytoplasmic tail) (Schatzle *et al.*, 1999). It has been discovered that these two forms of the receptor have different functions. Receptor 2B4L is an inhibitory receptor that inhibits killing of Yac-1 and P815 tumor target cells (Schatzle *et al.*, 1999).

After the natural killer cell binds to the target cell via a receptor/ligand interaction, degranulation occurs and the granules inside of the NK cells release cytotoxic compounds (Wright, 1983). The granules within the NK cells are continuously present and do not need to be activated. T cells must first encounter antigen and have time to mature before developing cytotoxic granules. The cytotoxic compounds released at the junction of the bound cell are perforin and granzymes. Perforin inserts into the cell membrane of the target cell and begins to polymerize in the presence of calcium, which causes the formation of a transmembrane pore. Granzymes A and B are able to flow through the transmembrane pore to the target cell. Granzyme A is the most abundant of the granzymes within the NK granules (Spitzer and Meadows, 1999). Granzyme B induces DNA fragmentation that will lead to apoptosis (cell suicide) of the target cell (Liu *et al.*, 1989).

Cytokine production and NK cell proliferation also occur as a result of the contact between natural killer cells and other cells, and the presence of soluble cytokines (Helander and Timonen, 1998). A cytokine is a protein that regulates the intensity of an immune response by acting on immune cells (Kuby, 1997). The various cytokines that are released by NK cells, include interleukin (IL)-3, IL-4, interferon (IFN)-gamma, and tumor necrosis factor (TNF) (Kuby, 1997). The cytokine IL-3 is released by NK cells to act on hematopoetic and mast cells to support the growth and differentiation of those cells. IL-4, another cytokine, acts as a costimulator for antigen-primed B cells, stimulates proliferation, differentiation, and class switch of activated B cells, up-regulates class II MHC expression of resting B cells and macrophages, induces proliferation of T cells, increases the phagocytic activity of macrophages, and stimulates the growth of mast cells

(Kuby, 1997). The release of IFN from NK cells acts to inhibit viral replication in uninfected cells, enhance macrophage activity, increase MHC class I and II expression on many different cell types, induce class switch to IgG in proliferating B cells, inhibit proliferation of TH2 cells, and mediate certain inflammatory cells (Kuby, 1997).

Cytotoxicity Assays

Natural killer cell function is demonstrated by the ability of isolated peripheral blood lymphocytes to lyse specific target cells without prior exposure to antigen (Papa and Valentini, 1994). The cytotoxicity of natural killer cells can be measured with several different single-cell and total cell assays.

The chromium release assay, a total cell assay, is most often used to measure NK cell cytotoxicity. It is an extremely sensitive assay and can be used with experimental protocols that allow for quantification of natural cytotoxicity reactions measured in lytic units (Korzeniewski and Callewaert, 1983). In brief, target cells are incubated with sodium chromate containing ^{51}Cr in order to radioactively label them. They are mixed with natural killer cells and NK ability to kill target cells is measured by amount of released chromium in the supernatant (Korzeniewski and Callewaert, 1983). This assay is accurate in measuring cytotoxicity of NK cells, but there are a number of drawbacks. First, there is abundant spontaneous release of chromium from target cells during long-term assays. The time required for labeling target cells and the loss of target cells during washes following labeling is also considerable. The chromium radioisotope, as well as the equipment needed for measuring the isotope, is extremely expensive. Finally,

radiation safety and disposal of the radioisotope poses a problem (Korzeniewski and Callewaert, 1983). Thus, there is an attempt to discover an assay that is both effective as well as safe and cost efficient.

Lanthanide europium chelated to diethylenetriaminopentiaacetate (EuDTPA) can also be used to label target cells such as tumor cells and lymphocytes. The target cells are labeled with 50 uM of europium in labeling buffer for twenty minutes in an ice bath. The labeling reaction is stopped by the addition of calcium chloride solution. The labeled target cells are mixed with various concentrations of effector cells and incubated for 2.5 hours at 37°C. Following incubation, the cells are centrifuged, and the supernatant is transferred to a flat-bottomed microtiter plate. Substrate mix is added, and the cells are allowed to sit for five minutes. If the effector cells bind to the labeled target cells, the target cell is lysed, and the europium is released into the supernatant. EuDTPA is fluorescent and can be measured using time-resolved flourometry (Volgmann *et al.*, 1989). The use of europium reduces background fluorescence when compared to other lanthanide ions or the chromium release assay and enhances the immunoflourescence assay's sensitivity because this compound's fluorescence decay time is 100-1000ns compared to 10-20ns, which is the decay time for fluorescent biological samples (Volgmann *et al.*, 1989). Europium does not decay as rapidly as other biological samples, thus it contributes less fluorescence to the background or media. This procedure does not involve radioactivity, however, it has a negative effect on the physiology of the target cells (Pacifici *et al.*, 1993). Both cell division and cell viability were shown to be impaired by higher levels of europium (Pacifici *et al.*, 1993). A second drawback is the

high price of the time resolved fluorometer (Volgmann *et al.*, 1989). Thus, this assay may not be as accurate or functional as other cytotoxicity assays.

Due to the cytotoxic effects europium has on the cellular physiology of the target cells, a non-cytotoxic europium method for labeling target cells has been proposed. In this modified procedure, only 1.0 uM of europium is added compared to the previously described amount of 50 uM (Pacifici *et al.*, 1993) (Volgmann *et al.*, 1989). The target cells were incubated for four days to ensure adequate growth and labeling. Following the incubation, effector cells are added to the target cells and incubated together for two or four hours at 37°C (Pacifici *et al.*, 1993). The fluorescence released following cell lysis by natural killer cells is measured using a time-resolved flourometer as previously described. Due to the lower concentration of europium used for labeling the target cells, this modified europium release assay procedure does not cause the amount of damage to the cellular physiology of the target cells as the original assay does (Pacifici *et al.*, 1993). However, the only target cell line tested using the modified procedure has been the K562 human erythroleukaemic cell line. It is unknown whether this procedure is useful with other human or mouse cell lines.

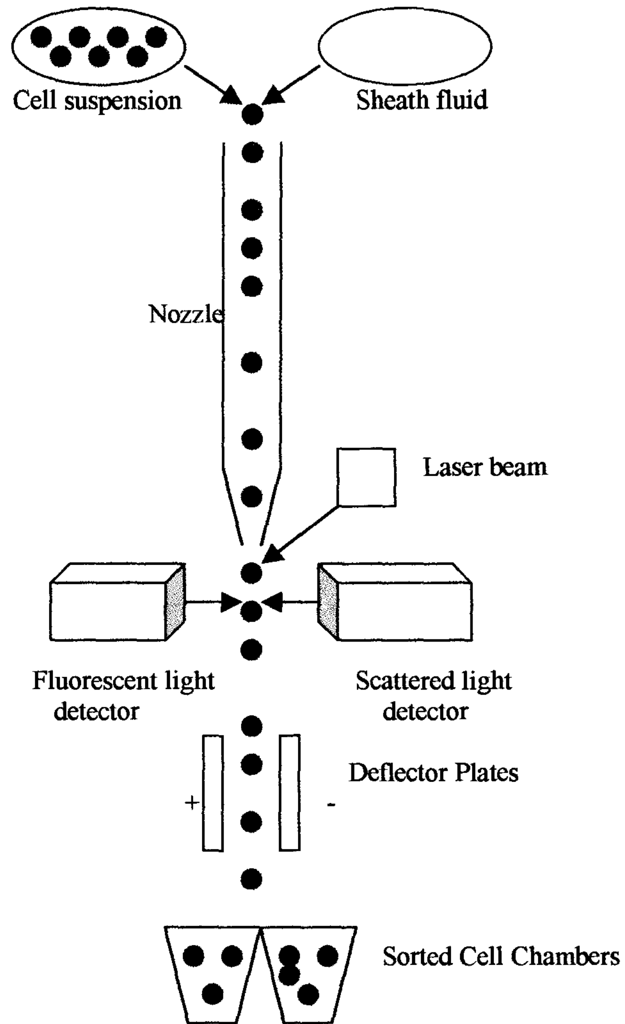
The carboxy-fluorescein-diacetate assay (CFDA) is another cytotoxicity assay being tested as an alternative to the chromium assay. In the CFDA assay, target cells are labeled with carboxy-fluorescein-diacetate, a fluorescent, non-radioactive compound by resuspending the cells in working solution and incubating them with the compound for thirty or sixty minutes. After labeling, the target cells are incubated for four hours with NK cells, and the supernatant containing the CFDA is read at 490nm in an automated microflourometer (Suzuki *et al.*, 1991). This test is more sensitive than the chromium

release assay. However, detection of target cell spontaneous release causes a decrease in the accuracy of detecting NK cell cytotoxicity (Hoshino *et al.*, 1991). Another drawback of the CFDA assay is the problem with identifying the dead and living target cells (Hoshino *et al.*, 1991). In addition to the described limitations, our laboratory is unable to use this procedure because we do not have access to an automated microplate flourometer.

Single-celled assays are also used in the detection of NK cell cytoxicity, and are more reliable than the total cell assays. Total cell assays involve numerous effector cells. It is difficult to determine which effector cells are responsible for the lysis of the target cells. Single-celled assays detect living and dead populations of target cells and effectors cells and distinguish these populations from one another (Papa and Valentini, 1994). Light microscopy or flow cytometry are typically used to determine the effector and target cell populations. To determine which cells are dead, the cells are stained with trypan blue or labeled with propidium iodide. When using trypan blue, living cells will appear clear under light microscopic conditions, while dead cells are unable to exclude the dye, which will permeate the plasma membrane resulting in the appearance of a blue cell. The use of propidium iodide, a red dye, will also stain dead cells red. Viable cells contain an intact plasma membrane and are capable of excluding the propidium iodide. When the cell dies, propidium iodide is able to permeate the membrane and bind the nuclear DNA by incorporating itself between the nucleotide sequences (Molecular Probes, 2001). Once the propidium iodide is incorporated into the DNA, the nucleus of the dead cells will fluoresce red. Propidium iodide is often used to label target cells when

using a flow cytometer. A drawback of using propidium iodide to measure cell death, is its tumorigenic properties (Papa and Valentini, 1994).

Most of the single-cell assays use flow cytometry to measure the number of live and dead cells in a population. Standard microscopy is tedious and subject to human bias. Normally, flow cytometry is used in conjunction with target cell labels such as dyes, which typically label dead cells. Light scatter patterns that occur with different cell types are measured using flow cytometry (Figure 1). When a sample of cells is passed through the flow cytometer, it is bombarded with a light source (laser). Once the light source hits the cells, it is scattered in different directions. A photodetector converts the collected light to electronic signals that are sent to a computer to be processed as digital signals (Rapley and Walker, 1998). Forward light scatter gives an indication of the cell's size and shape, whereas side scatter indicates granular contents within the cell. The fluorescence of any labeled cell type can also be determined using flow cytometry. After passing the cells through a flow cytometer, different cell types can be determined (effector and target) based on the amount of forward and light scatter they emit. Cell types can be distinguished from one another based on their distinct intracellular properties that can be measured by side scatter. The amount of intracellular components and granules can differentiate cell populations. Also, the use of forward light scatter can determine cellular size and shape. Dyes used for labeling cells are excited at various wavelengths. This excitation of the molecules in the dye causes the dye to fluoresce. If labeled with propidium iodide, dead cells will have a high amount of red fluorescence. The benefit of using flow cytometry is the capability to separate cell types as well as dead and living cells. Also, the killing activity and the binding capacity of the effector cells to


Cell suspension
Sheath fluid
Nozzle
Laser beam
Fluorescent light detector
Scattered light detector
Deflector Plates
+
-
Sorted Cell Chambers

the target cells can be measured (Papa and Valentini, 1994). Flow cytometric measurements have been shown to have similar results with the standard chromium release assay. However, when using propidium iodide to label target cells subjected to flow cytometry, there is a high level of leakage of the dye concurrent with the high spontaneous release that occurs from the target cell (Racz *et al.*, 1990). Dyes like propidium iodide also cause labeling of lymphocytes during the assay incubation period. This does not allow for accurate determination of target and effector cell populations (Johann *et al.*, 1995). Another drawback of using flow cytometry in conjunction with labeling dyes is the tedious data evaluation that limits the overall day-to-day use of this procedure (Racz *et al.*, 1990). Finally, the expense of the flow cytometry equipment can limit smaller labs from using this procedure. Our lab does not have access to a flow cytometer, which prevents us from using this technique to determine NK cell cytotoxicity.

Another single cell assay used to detect natural killer cell cytotoxicity is the CD-71 assay. CD-71 is a homodimeric glycoprotein, which is a transferrin receptor (Hoshino *et al.*, 1991). CD-71 is expressed on the commonly used target cell, K562, but it is not expressed on the surface of mononuclear cells (including NK cells). The transferrin receptor responds to a transferrin molecule coupled to iron. Iron is brought into the cell via transferrin receptor-mediated endocytosis. In the CD-71 assay, target cells are stained with FITC-conjugated anti-CD-71 monoclonal antibody. FITC is a green dye used to label cells in a similar manner as propidium iodide. The anti-CD-71 monoclonal antibody will bind to CD-71 located on the surface of the target cell. The target cells labeled with the anti-CD-71 monoclonal antibody are mixed with the NK cells in various

ratios and incubated for three hours. The cells are then measured by flow cytometry. Based on side scatter patterns, the CD-71 intensity of intact target cells will be higher than that of the dead or injured target cells (Hoshino *et al.*, 1991). There is significantly close correlation between the chromium release assay and the CD-71 assay cytotoxicity absorbance values. The CD-71 assay measured approximately 10% more cytoxicity than the chromium assay. A limitation of the CD-71 assay is that it cannot be used with effector cells that have high CD-71 levels of expression (Hoshino *et al.*, 1991). This assay also requires the use of a flourometer or flow cytometer, which smaller laboratories are unable to afford.

The previously described methods are not useful because our laboratory does not have access to the necessary equipment (i.e. Flow cytometer, flourometer). We also want to avoid the use of a radioactive isotope such as chromium. The method that will be tested in this study is the lactate dehydrogenase assay (LDH). The LDH assay will be used to measure the amount of murine natural killer cell killing of various murine target cell lines.

Lactate dehydrogenase (LDH) released from lysed tumor cells can be used as a simple and effective way of measuring the cytotoxicity of natural killer cells. In this total cell assay, peripheral blood lymphocytes or spleen cells are used as the effector cells at specific concentrations, and target cells are used in various concentrations. The target cells and the effector cells are incubated together and the amount of LDH released from the target cell is measured as a direct reflection of the effector cell cytotoxicity. LDH activity can be measured two different ways. The first is measuring the oxidation of NADH when pyruvate is converted to lactate. A microplate reader can measure the

decrease in NADH. The enzymatic reaction is started when NADH solution is added and allowed to run at room temperature (Decker and Lohmann-Matthes, 1988). The LDH inhibitor oxamate is added to terminate the reaction (Decker and Lohmann-Matthes, 1988).

LDH release can also be measured using an enzymatic test in which NAD+ is reduced through the conversion of lactate to pyruvate (Decker and Lohmann-Matthes, 1988). In this assay, NADH reduces FAD coupled to diaphorase (Decker and Lohmann-Matthes, 1988). This transfers $FADH_2$ electrons to the tetrazolium dye INT (Decker and Lohmann-Matthes, 1988). The enzymatic reaction is started by the addition of a solution containing NAD+ and diaphorase (Decker and Lohmann-Matthes, 1988). Termination of the reaction is achieved by the addition of LDH inhibitor oxamate. A microplate reader determines the amount of LDH release.

The LDH assay is inexpensive, rapid, and does not require the use of a radioactive isotope, thus making it more appealing to use than the standard chromium release assay. The LDH assay has many potential uses in research and will allow for more rapid measurements of natural killer cell cytotoxicity. Without the use of a radioactive isotope, the LDH assay is mush safer than the standard chromium release assay. Since there is no need for any radioactivity, this assay proves to be much cheaper, and laboratory money can be directed towards other areas of the research.

The LDH assay has previously been determined to be effective when used to measure natural killer cell cytotoxicity in conjunction with human target cell lines such as K562 (chronic myelogenous leukemia), HSB-2 (lymphoblast T-cell), CEM (lymphoblast T-cell), and HL-60 (promyelocytic) (Korzeniewski and Callewaert, 1983) (Kubo *et al.*,

1999). The LDH assay has been tested using YAC-1 murine tumor target cell line against the standard chromium release assay using the same cell line, and it proves to be just as effective (Decker and Lohmann-Matthes, 1988). The LDH assay has also been tested using human target cell lines (K562), Yac-1 and P815 murine cell lines (Decker and Lohmann-Matthes, 1988) (Promega Technical Bulletin, 1998). However, the use of the LDH assay in conjunction with murine target cells other than Yac-1 and P815 have not been adequately studied. Thus, there is a need to broaden the use of the LDH assay by utilizing numerous types of murine target cells in order to create an animal model that is adequate for studying murine natural killer cell cytoxicity.

To measure murine natural killer cell cytotoxicity, the types of target cells chosen and the optimal cell number must be taken into consideration when examining the effectiveness of the LDH assay. The cell lines that will be used as target cells for this study are L1210, P815, MPC11, and Yac-1 cells. L1210 murine lymphocytic leukemia cells arose from a tumor in the DBA murine strain. They are typically used for determining the cytotoxic activity of chemical agents and natural products (ATCC Bulletin, 1998). The P815 mastocytoma cell line was cultured from a DBA/2 mouse tumor. These cells are often used as target cells for determining T cell cytotoxicity but should not be killed by natural killer cells (ATCC Bulletin, 1998). MPC11 immunoglobulin secreting murine myeloma cells are cultured from a Merwin plasma cell tumor-11 that is carried in BALB/c mice. This cell line can produce gamma globulin molecules (IgG2b) as well as light chain dimers and free light chains (ATCC Bulletin 1998). Injecting A/Sn mice with Moloney Leukemia Virus (MLV) (ATCC Bulletin, 1998) induces the Yac-1 lymphoma cell line. Based on previous studies, P815 and

L1210 cell lines are thought to be insensitive to mouse NK cell cytotoxicity, whereas MPC11 and Yac-1 cells are thought to be sensitive to mouse NK cell targeting (Decker and Lohmann-Matthes, 1988)(Suzuki, *et al.*, 1991)(Lui, *et al.*, 1989).

Summary

The purpose of this study was to determine if the LDH assay is useful in measuring murine natural killer cell cytotoxicity towards murine effector cells. In this study, the Yac-1, P815, MPC 11, and L1210 cell lines will be tested for their effectiveness as NK cell targets using the LDH cytotoxicity assay. The Yac-1, P815, MPC 11, and L1210 cell lines have previously been test in this laboratory and have been determined to possess adequate levels of total LDH and to have low levels of spontaneous LDH release for use in these studies (Schmidt, 1999). To answer our question, the target cells were incubated with murine spleen cells, and the amount of cytotoxicity was calculated. The cytotoxicity at each E:T ratio for each cell line was analyzed and compared to previous studies to determine if the LDH assay is an adequate test for measuring murine NK cell cytotoxicity.

Materials

Dulbecco's Modified Eagle's Medium (DMEM) (containing high glucose, and pyridoxine hydrochloride), RPMI media 1640 powder without phenol red, with L-glutamine, fetal calf serum (FCS), L-glutamine, and MEM sodium pyruvate (100x) were purchased from Gibco BRL Life Technologies (Grand Island, New York). The cell lines L1210, P815, MPC11, and Yac-1 cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD). Swiss Webster mice were purchased from Charles River Company (Wilmington, MA). The Cytotox 96 non-radioactive cytotoxicity assay was purchased from Promega (Madison, WI). Included in the Cytotox 96 kit are lysis solution (9% Triton X-100), stop solution (1M acetic acid), and PBS substrate solution (0.2g/L KCl, 8.0g/L NaCl, 0.2g/L KH_2PO_4, 1.15g/L Na_2HPO_4, and 1% bovine serum albumin).

Methods

Cell Culture

P815, L1210, and MPC11 cells were grown in DMEM with high glucose (4.5%), with L-glutamine (29.2%), sodium pyruvate (0.37%) and FCS (10%). Yac-1 cells were grown in RPMI media FCS (10%), L-glutamine (29.2 mg%) and sodium pyruvate (0.37%). All cell types were incubated at 37°C and 5% CO_2. Cells were fed every 48-72 hours under sterile conditions.

Target cell stocks were frozen in media containing 10% dimethyl sulfoxide (DMSO) at a concentration of 1×10^6 cells/mL. The target cell aliquots were placed into cryopreservation vials and stored in a sealed styrofoam container at -70°C for 24 hours. The cells were transferred to liquid nitrogen for permanent storage.

Cell Counting

Cell counting with a hemacytometer was done to determine the number of cells/ml of media. Cells and trypan blue dye were mixed 1:1 and added to a hemacytometer chamber. The cells were counted using a hemacytometer grid. Dead cells (purple) were excluded. The number of cells/ml was established using the following calculation:

$$\text{Cells/ml} = (\text{average \# per large square}) \times 10^4/\text{ml} \times \frac{1}{\text{Dilution}}.$$

Cytotoxicity assay

A female Swiss Webster mouse was selected and placed into a carbon dioxide gas chamber to be sacrificed. The spleen was removed, placed in a 50ml conical tube and placed on ice. One milliliter of incomplete RPMI media was placed in a well of a 24 well culture plate. The spleen was placed in the well containing the RPMI media, and any fat on the spleen was removed under sterile conditions. Using a plunger from a sterile 10ml syringe, the spleen was mashed through a tissue sieve into a petri dish until there were no large pieces intact. The petri dish containing the spleen cells was washed several times with sterile media, and the spleen cells were placed in a 50ml conical tube. The final volume in the 50ml conical tube was brought up to 45ml. The spleen cells were placed on ice for five minutes. All but the last 5ml which contained blood cells and fat clumps were transferred to a new 50ml conical tube, centrifuged, and resuspended in RPMI media without phenol red or FCS at a concentration of 3×10^5 cells/ml. Serial dilutions were performed to achieve the effector cell concentrations of 20,000 cells/ml, 10,000 cells/ml, 5,000 cells/ml, 2,200 cells/ml, 1,250 cells /ml, and 0 cells/ml.

The target cells were washed three times with 5ml of RPMI media without phenol red or fetal calf serum. The cells were centrifuged between each wash at 4°C and 250 x *g* for 8 minutes. The target cells were counted with a hemacytometer using trypan blue to determine the number of living cells and diluted to a concentration of 3×10^5 cells/ml in RPMI media without phenol red or fetal calf serum. The target cells were added to all the experimental wells according to their previously determined optimal concentration (Schmidt, 1999). L1210 cells were added at a concentration of 10,000 cells/100µl; P815

at 5,000 cells/ 100μl; Yac-1 at 5,000 cells/100μl; MPC11 at 10,000 cells/100μl. The target cells were incubated alone to determine target cell spontaneous lactate dehydrogenase (LDH) release (Control #1, Figure 2). Target cells were incubated with 1x lysis buffer to determine target cell maximum LDH release (Control #2). Spleen cells were incubated alone at each effector cell concentration (0:1, 1.25:1, 2.5:1, 5:1, 10:1, and 20:1) to determine effector cell spontaneous LDH release (Control #3). Culture medium alone was added to 3 wells to determine background absorbance (Control #5). Lysis solution (10μl) was added to target cell maximum LDH release wells (Control #2), and medium containing lysis solution (1x) was included for volume correction control (Control #4). The plate was centrifuged at 250 x *g* for 4 minutes, and then incubated at 37°C with 5% CO_2 for 4 hours.

Following incubation, the plate was centrifuged at 250 x *g* for 4 minutes, and 50μl of the supernatant was transferred to a 96 well flat bottom non-sterile plate. The assay buffer was thawed, warmed to room temperature, and 12ml was removed and added to a bottle of cytotoxicity substrate mix. Resuspended cytotoxicity substrate mix (50 μl) was added to each well of the plate. The plate was covered with foil and incubated for 30 minutes. Stop solution (50μl) was added to each well to stop the enzymatic reaction. The absorbance was read at 490nm, and the percent of LDH released from the target cells was determined using the following calculation:

$$\frac{LDH_{experimental} - LDH_{effector\ cells} - LDH_{spontaneous}}{LDH_{maximal} - LDH_{spontaneous}}$$

$LDH_{experimental}$ is the LDH released from the coculture of the effector cells (E) and the target cells (T) at a specific E:T ratio (the number of effector cells to target cells). $LDH_{effector\ cells}$ is the amount of LDH released from separately cultured effector cells at specific

Target Spontaneous Release (control #1)	Culture Medium Background (control #5)										
			Effector Cell Spontaneous Release (control #3)								
Target Maximum Release (control #2)	Volume Correction Control (control #4)										
			Experimental wells								
		20:1	10:1	5:1	2.5:1	1.25:1	0:1				

concentrations. $LDH_{spontaneous}$ is the LDH released from separate cultures of target cells, and $LDH_{maximal}$ is the amount of LDH released from the target cells after detergent lysis.

The Cytotox 96™ Assay measures the amount of LDH released in culture supernatants. This is measured with a 30-minute coupled enzymatic assay, which results in the conversion of INT (a tetrazolium salt), into a red formazan product (Promega, 1992). The color that is formed due to this conversion is proportional to the number of lysed target cells (Promega, 1992). The visible wavelength absorbance data are collected from the 96-well plate reader.

Results

In order to determine if the LDH assay is suitable for measuring murine natural killer cell cytotoxicity, murine effector cells were incubated with murine target cells as described in the methods. The percent of cytotoxicity was calculated using the equation described in Methods. The cell lines tested were Yac-1, MPC11, L1210, and P815. Three experimental trials were completed for each cell line. Triplicate wells containing E:T ratios of 20:1, 10:1, 5:1, and 0:1 were used in all L1210 assays, P815 assays 1 and 2, and MPC 11 assay 1. Triplicate wells containing E:T ratios of 20:1, 10:1, 5:1, 2.5:1, 1.25:1, and 0:1 were used in P815 assay 3, MPC 11 assays 2 and 3, and all Yac-1 assays. As previously determined, the optimal number of target cells for each cell line was used in the experimental trials (Schmidt 1999). The mean percent cytotoxicity was calculated for the P815, Yac-1, and MPC 11 cell lines. The mean cytoxicity was not calculated for the L1210 cell line, as will be discussed. A One Way Analysis of Variance was used to determine significant differences between E:T ratios.

Yac-1

The data obtained from the three experimental trials are shown in Tables 1-3. In assay 1, the percent cytotoxicity at 20:1 was –10.2%; 10:1 was –11.6%; 5:1 was 3.7%; 2.5:1 was 3.7%; 1.25:1 was 3.0%; and 0:1 was –0.6% (Figure 3). The maximum response was seen at 5:1 and 2.5:1 E:T. In assay 2, the percent cytoxicity at 20:1 was –3.5%; 10:1 was –5.6%; 5:1 was –1.4%; 2.5:1 was 3.5%; 1.25:1 was 0.9%; and 0:1 was 0% (Figure 4).

Yac-1 Assay #1

TS	*20:1*	*10:1*	*5:1*	*2.5:1*	*1.25:1*	*0:1*
0.032	***ES***	***ES***	***ES***	***ES***	***ES***	***ES***
0.028	0.026	0.011	0.007	0.003	0.003	0.01
0.035	0.034	0.012	0.002	-0.001	0.001	0.005
	0.029	0.009	0.008	0.00	0.003	0.004
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.032	0.03	0.0107	0.006	0.0007	0.002	0.006
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.004	±0.004	±0.002	±0.003	±0.002	±0.001	±0.003
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.182	0.065	0.047	0.049	0.037	0.041	0.037
0.212	0.056	0.05	0.042	0.049	0.037	0.036
0.194	0.014	0.039	0.04	0.033	0.038	0.039
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.196	0.045	0.045	0.0437	0.039	0.038	0.037
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.015	±0.027	±0.006	±0.005	±0.008	±0.002	±0.002

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

Table 1

Yac-1 Assay #2

TS	*20:1*	*10:1*	*5:1*	*2.5:1*	*1.25:1*	*0:1*
0.022	***ES***	***ES***	***ES***	***ES***	***ES***	***ES***
0.031	0.095	0.053	0.025	0.005	0.006	0.00
0.034	0.011	0.054	0.028	0.01	0.003	0.002
	0.108	0.054	0.025	0.008	0.005	0.006
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.029	0.104	0.054	0.026	0.0075	0.005	0.0027
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.006	±0.008	±0.0006	±0.002	±0.0035	±0.0015	±0.0031
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.18	0.133	0.075	0.053	0.044	0.034	0.028
0.159	0.125	0.073	0.053	0.039	0.037	0.034
0.175	0.125	0.076	0.054	0.043	0.035	0.033
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.171	0.128	0.075	0.053	0.042	0.0353	0.032
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.006	±0.005	±0.002	±0.0006	±0.003	±0.002	±0.003

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

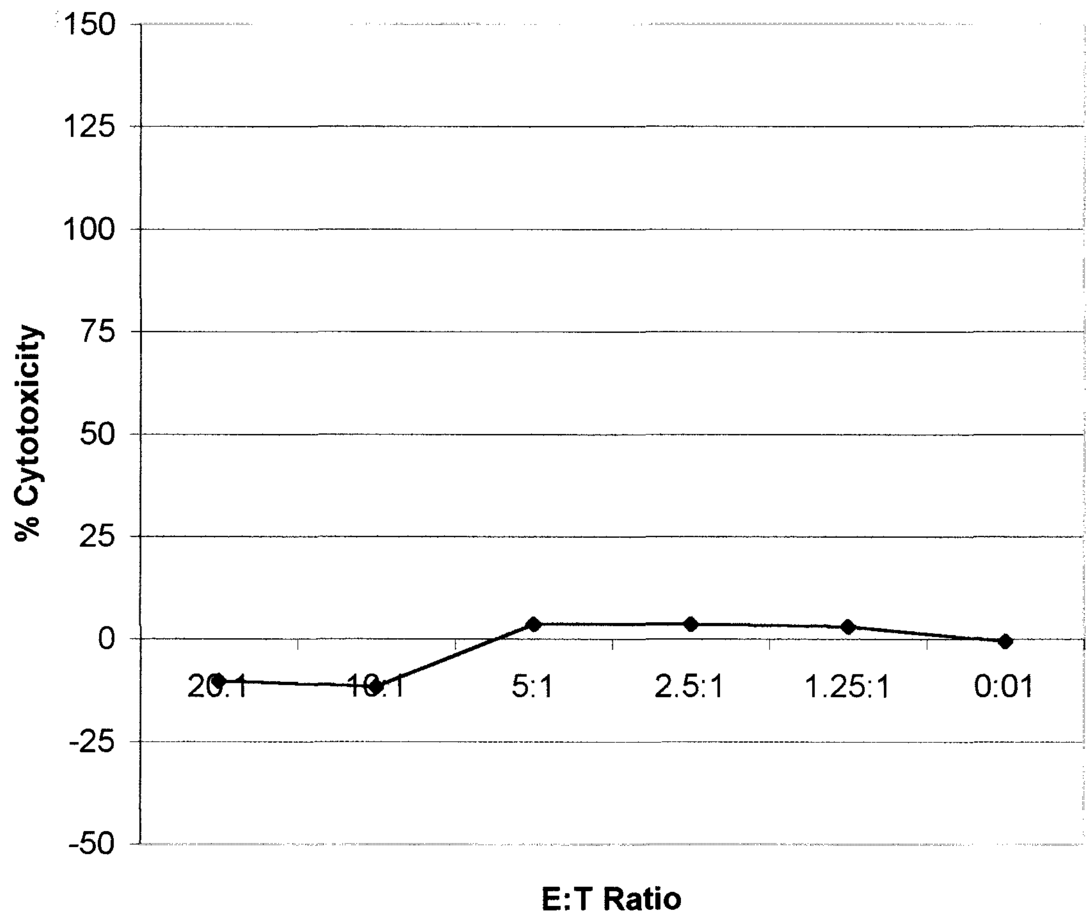
Table 2

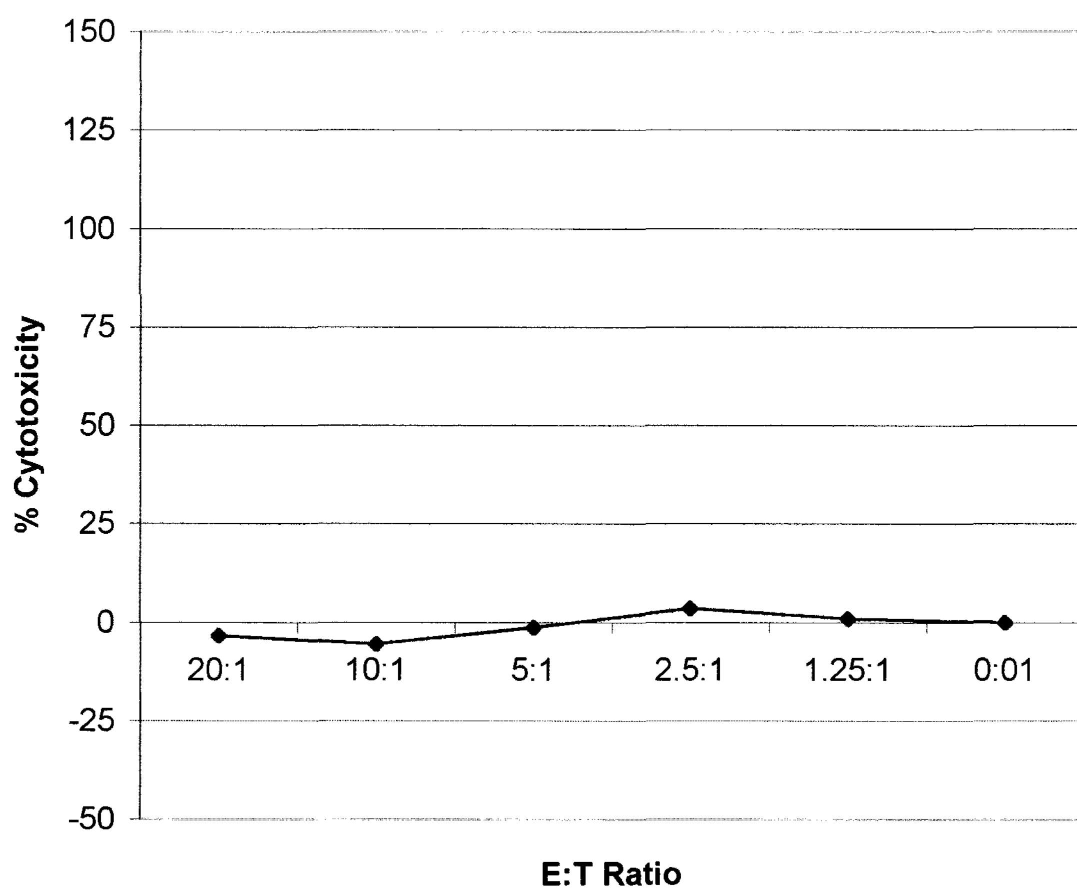
Yac-1 Assay #3

TS	*20:1*	*10:1*	*5:1*	*2.5:1*	*1.25:1*	*0:1*
-0.002	***ES***	***ES***	***ES***	***ES***	***ES***	***ES***
0.001	0.161	0.152	0.102	0.064	0.026	0.032
0.001	0.175	0.155	0.111	0.064	0.028	0.03
	0.172	0.153	0.104	0.063	0.027	0.033
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.00	0.17	0.15	0.106	0.064	0.027	0.032
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.002	±0.007	±0.002	±0.005	±0.0006	±0.001	±0.002
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.013	0.171	0.155	0.112	0.065	0.031	0.035
0.012	0.172	0.149	0.109	0.075	0.033	0.033
0.016	0.170	0.152	0.113	0.073	0.034	0.035
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.014	0.171	0.152	0.111	0.071	0.033	0.034
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.002	±0.001	±0.003	±0.002	±0.005	±0.002	±0.001

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD- Standard deviation

Table 3

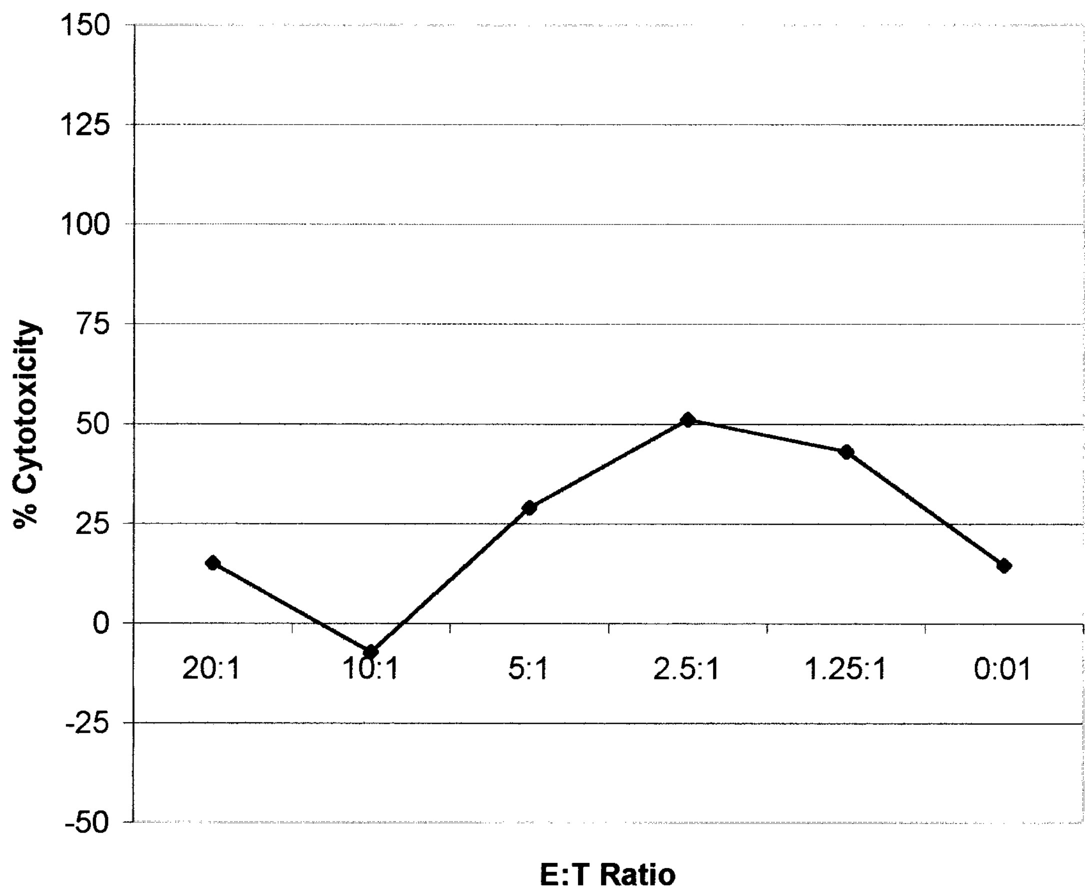

Yac-1 Assay 1
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio

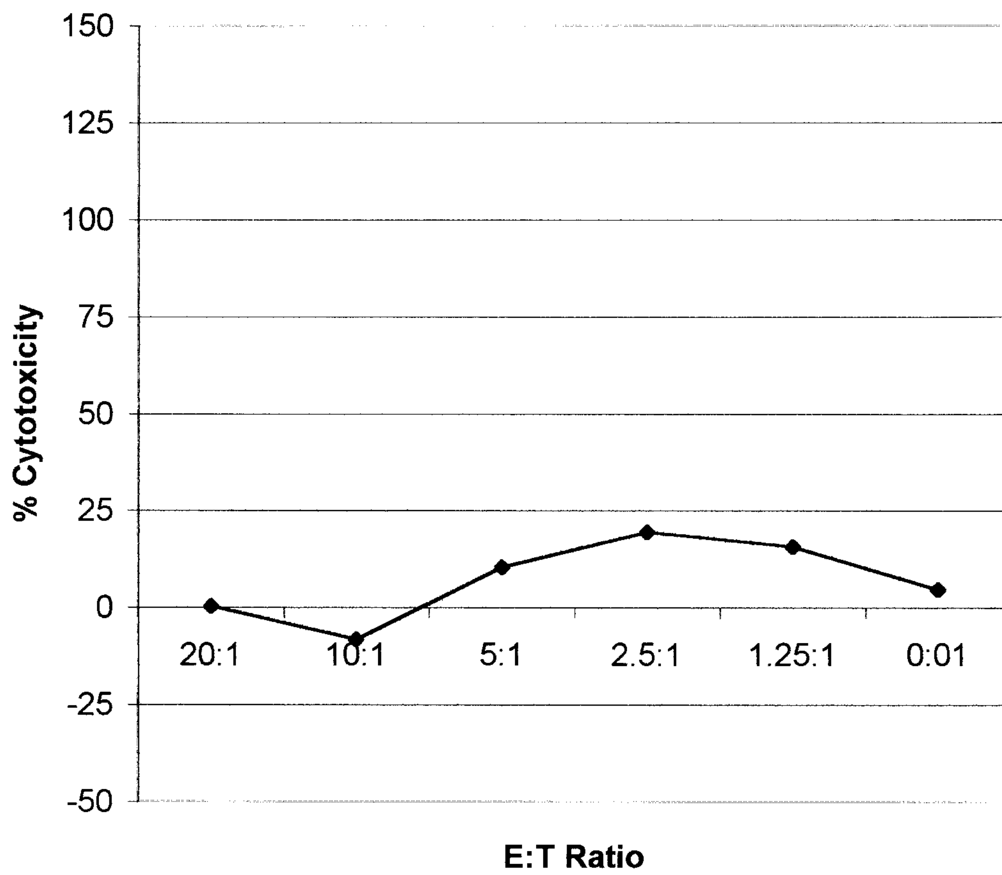

Yac-1 Assay 2
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio

The maximum response was at 2.5:1 E:T ratio. In assay 3, the percent cytotoxicity at 20:1 was 15%; 10:1 was –7.3%; 5:1 was 29%; 2.5:1 was 51%; 1.25:1 was 43%; and 0:1 was 14.6% (Figure 5). The maximum response was again at 2.5:1 E:T ratio. The mean percent cytotoxicity at each E:T ratio was calculated (Figure 6). The mean percent cytotoxicity at 20:1 was 0.4% (± 12.8); 10:1 was –8.2% (± 3.1) ; 5:1 was 10.4% (± 16.3); 2.5:1 was 19.4% (± 27.4); 1.25:1 was 15.6% (± 23.7); and 0:1 was 4.7% (± 8.6). The maximum response was seen at the 2.5:1 E:T ratio. A One Way Analysis of Variance showed that there are no significant differences between the mean values at each E:T ratio ($p = 0.45$).

MPC 11

The data obtained from the three experimental trials is in Tables 4-6. In all three experimental assays, cytotoxicity was observed (Figures 7-9). In assay 1, the cytotoxicity at 20:1 was 0.6%; 10:1 was 13%; 5:1 was 28%; and 0:1 was –21% (Figure 7). The maximum response was seen at 5:1. In assay 2, the percent cytotoxicity at 20:1 was 12%; 10:1 was 5%; 5:1 was 51%; 2.5:1 was 2.6%; 1.25:1 was 3%; and 0:1 was 18% (Figure 8). The maximum response was at the 5:1 E:T ratio. In assay 3, the percent cytoxicity at 20:1 was 14.6%; 10:1 was 8.5%; 5:1 was 56%; 2.5:1 was 2.8%; 1.25:1 was 4%; and 0:1 was 14% (Figure 9). Again, the maximum response was seen at the 5:1 ratio. The mean percent cytotoxicity at each E:T ratio was calculated (Figure 10). The mean percent cytotoxicity at 20:1 was 6.25% (± 7.4); 10:1 was 8.8% (± 4.0); 5:1 was 45% (±14.9);


Yac-1 Assay 3
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio


Yac-1 % Mean Cytotoxicity vs E:T Ratio
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio

MPC11 Assay #1

TS	***20:1***	***10:1***	***5:1***	***0:1***
0.033	***ES***	***ES***	***ES***	***ES***
0.033	0.028	0.005	-0.002	0.042
0.050	0.042	0.007	-0.002	0.042
	0.024	0.011	0.002	0.043
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.039	0.031	0.008	-0.0007	0.042
SD	***SD***	***SD***	***SD***	***SD***
±0.01	±0.009	±0.003	±0.002	±0.0006
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.153	0.069	0.056	0.049	0.045
0.205	0.072	0.053	0.055	0.044
0.284	0.072	0.100	0.163	0.044
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.21	0.07	0.07	0.089	0.039
SD	***SD***	***SD***	***SD***	***SD***
±0.07	±0.002	±0.03	±0.064	±0.009

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

Table 4

MPC11 Assay #2

TS	*20:1*	*10:1*	*5:1*	*2.5:1*	*1.25:1*	*0:1*
0.02	***ES***	***ES***	***ES***	***ES***	***ES***	***ES***
0.02	0.191	0.126	0.062	-0.001	0.011	0.001
0.017	0.224	0.126	0.058	0.023	0.017	0.01
	0.241	0.124	0.067	0.013	0.015	0.009
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.019	0.22	0.125	0.062	0.012	0.0143	0.007
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
± 0.0017	± 0.254	± 0.0012	± 0.0045	± 0.007	± 0.003	± 0.005
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.265	0.264	0.154	0.067	0.036	0.024	0.035
0.242	0.266	0.155	0.277	0.037	0.036	0.019
0.254	0.267	0.157	0.256	0.039	0.06	0.149
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.254	0.266	0.155	0.2	0.037	0.04	0.068
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
± 0.012	± 0.002	± 0.002	± 0.12	± 0.002	± 0.02	± 0.071

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release

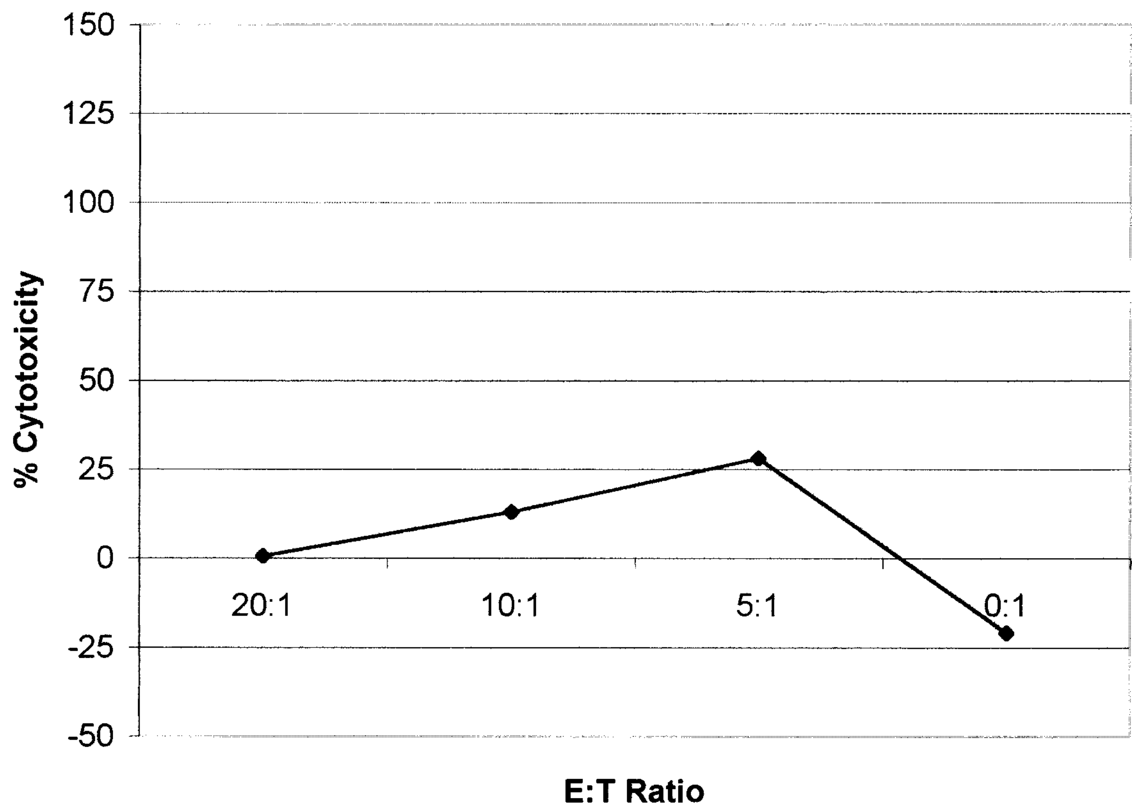
Table 5

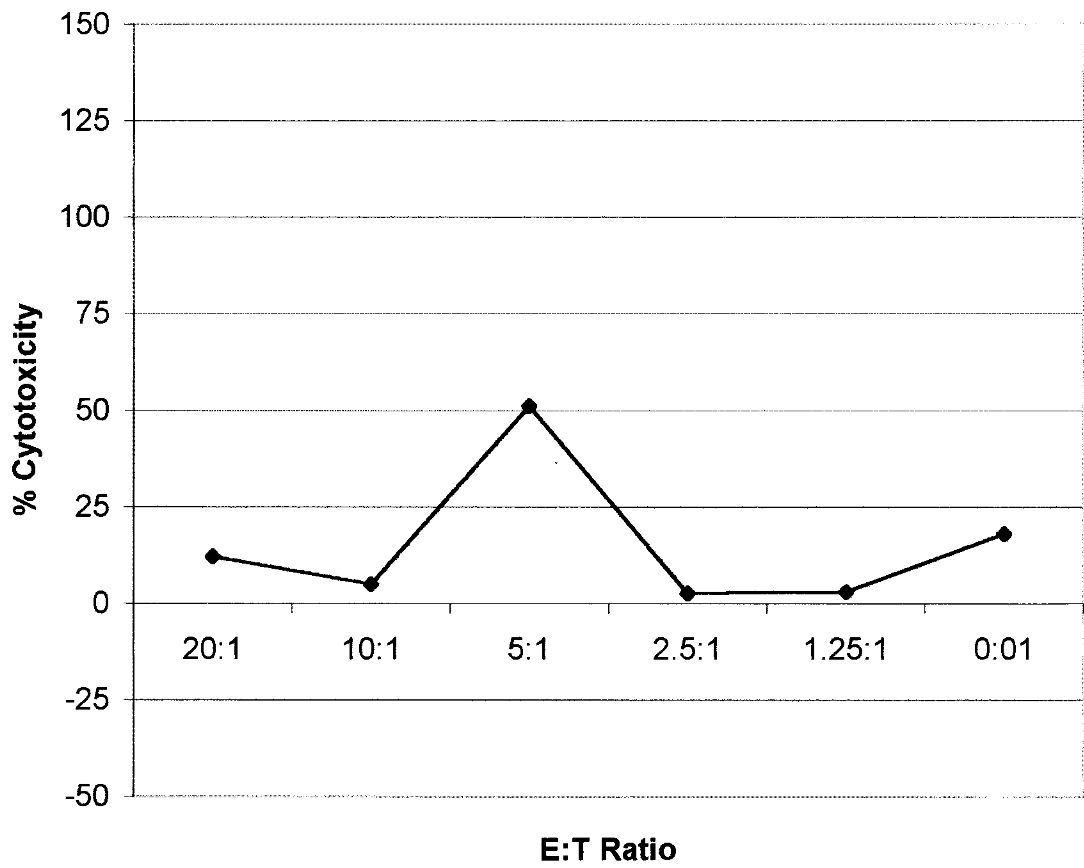
MPC11 Assay #3

TS	*20:1*	*10:1*	*5:1*	*2.5:1*	*1.25:1*	*0:1*
0.018	***ES***	***ES***	***ES***	***ES***	***ES***	***ES***
0.016	0.224	0.147	0.076	0.007	0.022	0.006
0.014	0.257	0.148	0.073	0.03	0.019	0.011
	0.279	0.152	0.076	0.026	0.015	0.009
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.016	0.253	0.15	0.075	0.021	0.019	0.0087
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.002	±0.028	±0.003	±0.002	±0.012	±0.004	±0.0025
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.27	0.304	0.181	0.083	0.044	0.03	0.034
0.254	0.304	0.188	0.328	0.041	0.047	0.03
0.264	0.306	0.188	0.305	0.047	0.059	0.144
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.262	0.305	0.186	0.24	0.044	0.045	0.069
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.008	±0.001	±0.004	±0.14	±0.003	±0.015	±0.065

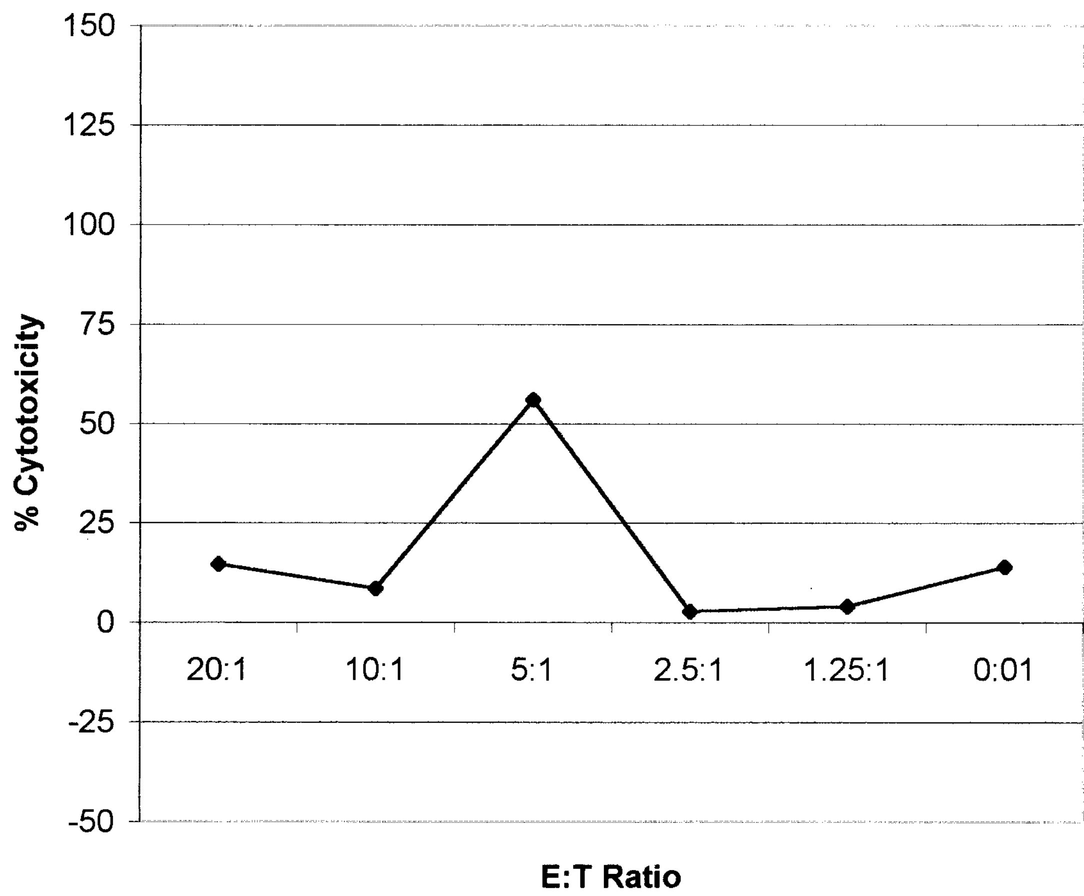
TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

Table 6

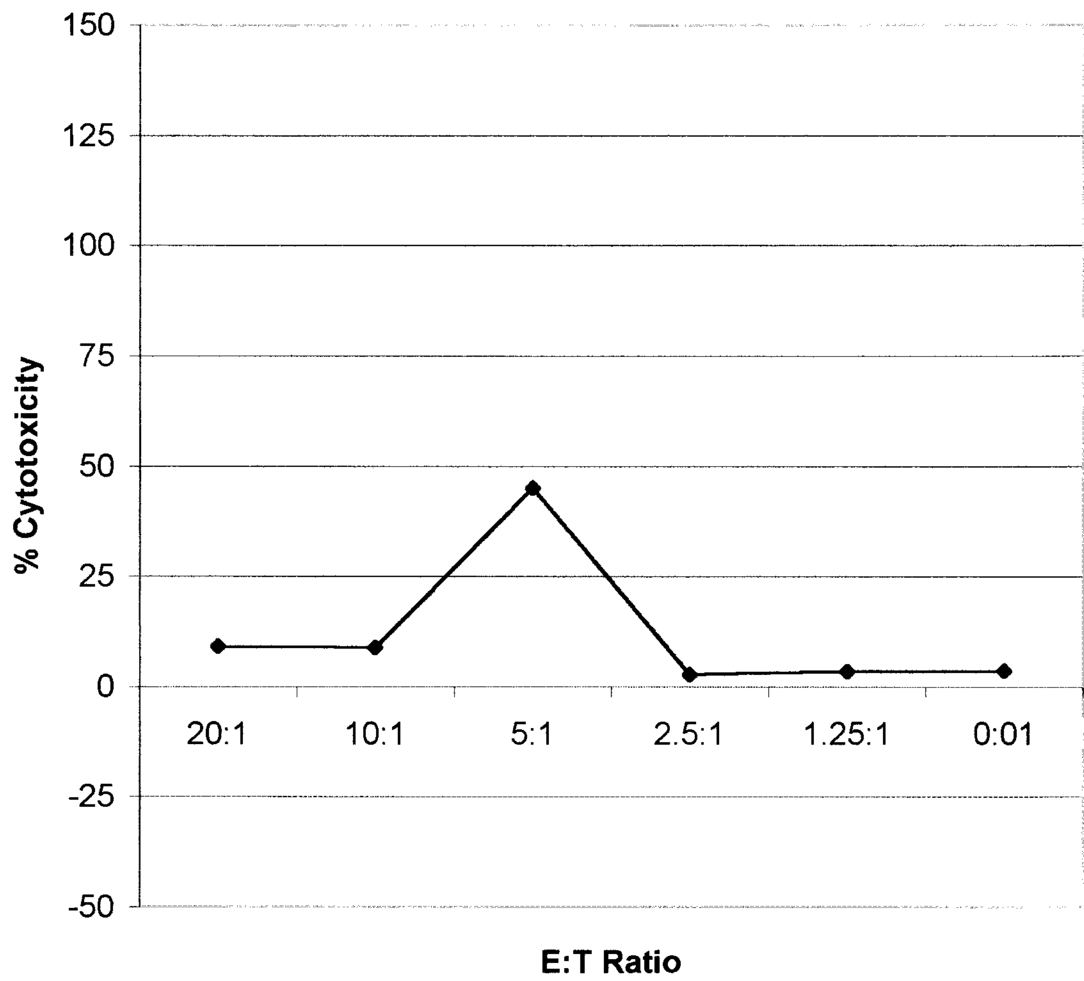

MPC 11 Assay 1
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
0:1
E:T Ratio



MPC11 Assay 2
% Cytotoxicity
150
125
100
75
50
25
0
-25
-50
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio



MPC11 Assay 3
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio


MPC11 Mean % Cytotoxicity vs E:T Ratio
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio

2.5:1 was 2.7% (± 0.14); 1.25:1 was 3.5% (± 0.7); and 0:1 was 3.6% (± 21.4). The mean maximum response was seen at the 5:1 E:T ratio (Figure 10). A One Way Analysis of Variance showed that there the 5:1 E:T ratio was statistically different from all other E:T ratios examined ($p = 0.014$).

L1210

The data obtained from the three experimental trials is shown in Tables 7-9. In assay 1, cytotoxicity was not observed (Figure 11). The percent cytotoxicity at 20:1 was –294%; 10:1 was –209%; 5:1 was –275%; and 0:1 was –11.4% (Figure 11). The negative values are due to the low amount of maximum target cell LDH release. This may have occurred because the target cell number was not optimized or the lysis buffer did not lyse the cells. In order to account for the problematic data, the percent cytotoxicity was calculated using the average target cell LDH release from assays 2 and 3. After recalculation of the data, the percent cytoxicity for 20:1 was 79.6%; 10:1 was 56.6%; 5:1 was 74.4%; and 0:1 was 3.1%. The adjusted data does not seem to follow a similar pattern of cytotoxicity as seen in assay 2. However, at the 5:1 ratio, the same percent cytotoxicity is seen. In assay 2, cytotoxicity was observed (Figures 12). In assay 2, the percent cytotoxicity at 20:1 was –24.79%; 10:1 was 14.28%; 5:1 was 74%; and 0:1 was –27.7% (Figure 12). The maximum response was seen with the 5:1 E:T ratio. In assay 3, the percent cytotoxicity at 20:1 was 113.5%; 10:1 was 109.5%; 5:1 was 130.5%; and 0:1 was –60% (Figure 13). In assay 3, the effector cell spontaneous LDH release was lower than other data indicates it should be (Tables 7-9), causing the percent cytotoxicity to appear unusually high.

L1210 Assay #1

TS	*20:1*	*10:1*	*5:1*	*0:1*
-0.014	*ES*	*ES*	*ES*	*ES*
-0.012	0.215	0.211	0.005	0.041
-0.001	0.195	0.137	0.057	0.042
	0.251	0.089	0.065	0.045
Mean	*Mean*	*Mean*	*Mean*	*Mean*
-0.009	0.22	0.15	0.042	0.04
SD	*SD*	*SD*	*SD*	*SD*
±0.007	±0.03	±0.06	±0.032	±0.002
TM	*Exp.*	*Exp.*	*Exp.*	*Exp.*
-0.021	0.519	0.213	0.283	0.042
-0.019	0.435	0.349	0.322	0.045
-0.25	0.456	0.402	0.22	0.041
Mean	*Mean*	*Mean*	*Mean*	*Mean*
-0.097	0.47	0.32	0.28	0.043
SD	*SD*	*SD*	*SD*	*SD*
±0.133	±0.04	±0.1	±0.05	±0.002

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD- Standard deviation

Table 7

L1210 Assay #2

TS	*20:1*	*10:1*	*5:1*	*0:1*
0.046	***ES***	***ES***	***ES***	***ES***
0.086	0.424	0.349	0.240	0.081
0.084	0.484	0.384	0.213	0.084
	0.466	0.389	0.210	0.082
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.072	0.46	0.37	0.22	0.08
SD	***SD***	***SD***	***SD***	***SD***
±0.023	±0.031	±0.22	±0.02	±0.002
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.343	0.440	0.446	0.458	0.089
0.330	0.475	0.473	0.431	0.084
0.253	0.499	0.513	0.515	0.091
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.31	0.47	0.48	0.47	0.09
SD	***SD***	***SD***	***SD***	***SD***
±0.05	±0.03	±0.034	±0.043	±0.004

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

Table 8

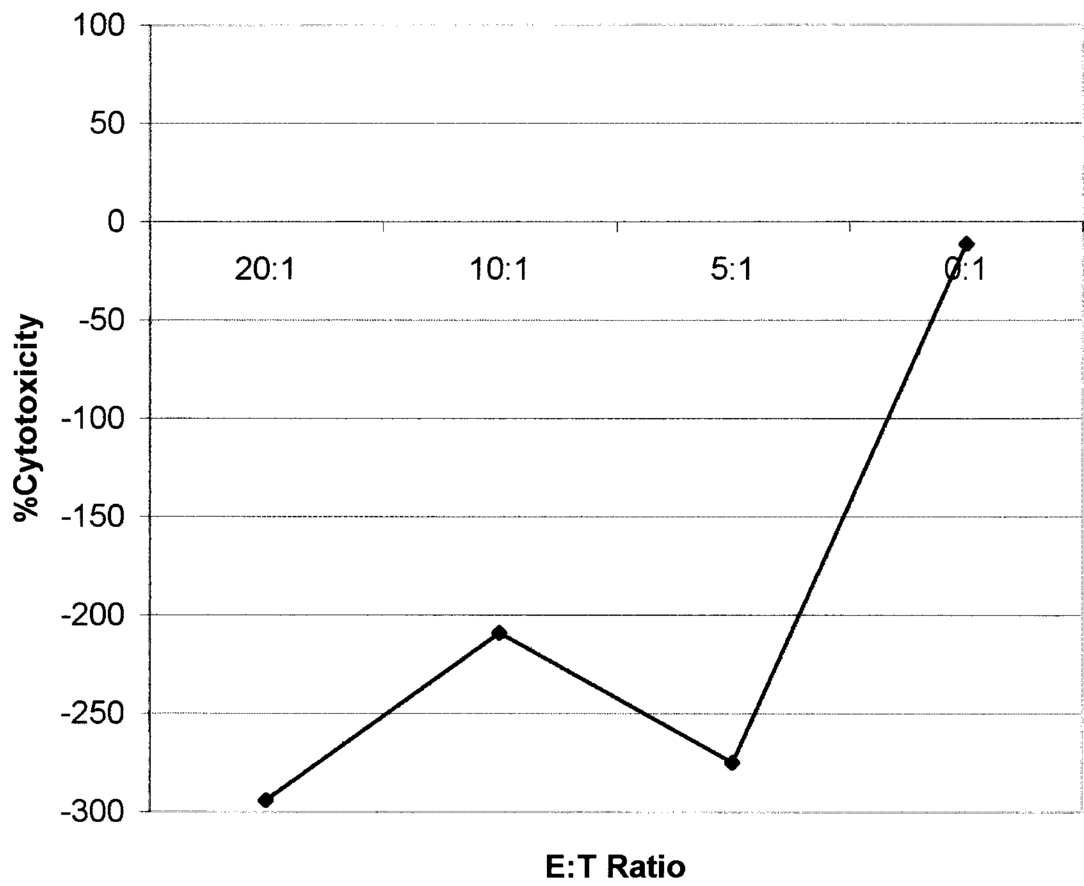
L1210 Assay #3

TS	*20:1*	*10:1*	*5:1*	*0:1*
0.046	***ES***	***ES***	***ES***	***ES***
0.053	0.062	0.010	0.009	0.043
0.259	0.007	0.017	0.005	0.041
	0.091	0.025	0.018	0.041
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.12	0.053	0.017	0.012	0.042
SD	***SD***	***SD***	***SD***	***SD***
±0.12	±0.043	±0.008	±0.007	±0.001
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.330	0.443	0.359	0.416	0.043
0.326	0.426	0.332	0.378	0.041
0.314	0.333	0.375	0.382	0.043
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.32	0.40	0.36	0.39	0.042
SD	***SD***	***SD***	***SD***	***SD***
±0.008	±0.06	±0.02	±0.02	±0.001

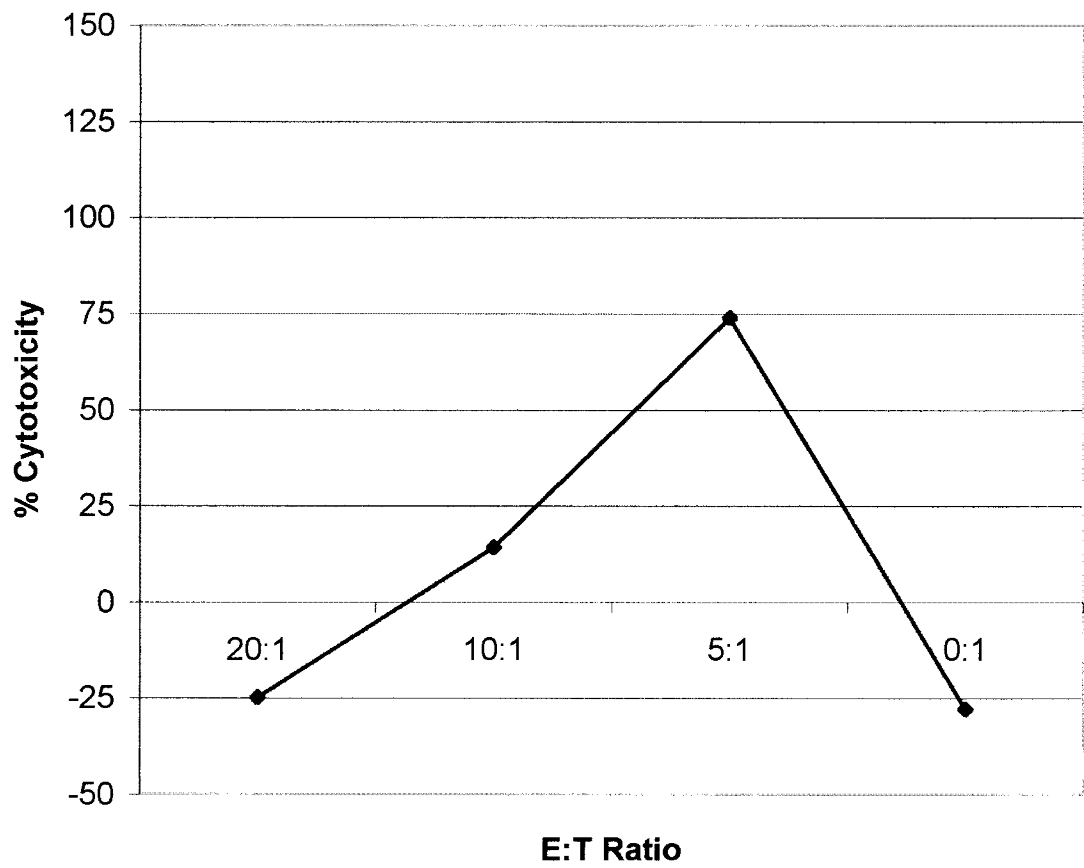
TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

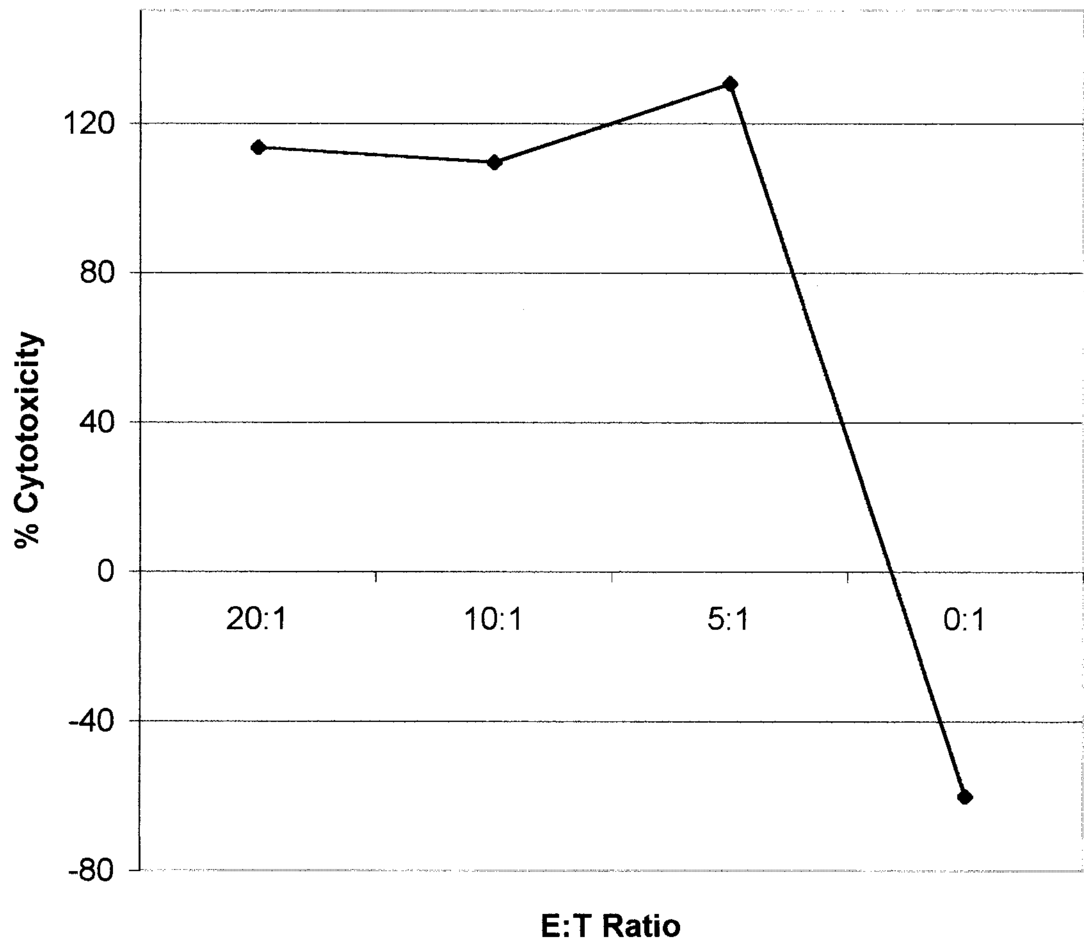
Table 9

L1210 Assay 1



L1210 Assay 2




L1210 Assay 3
% Cytotoxicity
120
80
40
0
-40
-80
20:1
10:1
5:1
0:1
E:T Ratio

As percent cytoxicity is not normally seen above 50 %, this assay was excluded from further consideration. To correct for the absence of effector cell spontaneous LDH release, the average effector spontaneous release was calculated using the data from assays 1 and 2. The adjusted percent cytotoxicity at 20:1 was –29.5%; 10:1 was –10%; 5:1 was 6%; and 0:1 was –70.5%. The mean percent cytotoxicity of the L1210 cell line was not calculated due to the inaccuracy of the original data. The adjusted values for assay 1 were calculated to determine if the inaccurate data was corrected if it would have a similar trend seen in assay 2. Corrected data from assay 1 and data from assay 2 demonstrate measurable NK cytotoxicity using this cell line. However, more experiments need to be performed on the L1210 cell line to confirm this trend.

P815

The data obtained from the three experimental trials is in Table 10-12. In assay 1, the percent cytotoxicity at 20:1 was 344%; 10:1 was 231%; 5:1 was 214%; and 0:1 was 6.25% (Figure 14). For this reason, assay 1 data was not included in the calculation of the mean percent cytotoxicity. In assay 2, the percent cytotoxicity at 20:1 was –14.5%; 10:1 was –34.5%; 5:1 was –7.2%; and 0:1 was –52% (Figure15). No cytotoxicity was seen in this assay. In assay 3, the percent cytotoxicity at 20:1 was 27%; 10:1 was 20%; 5:1 was –17%; 2.5:1 was –10%; 1.25:1 was –23%; and 0:1 was –30% (Figure 16). The maximum response was seen at the E:T ratio of 20:1. The mean percent cytotoxicity was

P815 Assay #1

TS	*20:1*	*10:1*	*5:1*	*0:1*
0.010	***ES***	***ES***	***ES***	***ES***
0.001	0.061	0.021	0.002	0.043
0.006	0.063	0.022	0.003	0.040
	0.074	0.031	0.009	0.041
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.006	0.066	0.025	0.0047	0.041
SD	***SD***	***SD***	***SD***	***SD***
±0.005	±0.007	±0.006	±0.0038	±0.002
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.039	0.167	0.107	0.080	0.043
0.039	0.186	0.100	0.079	0.047
0.037	0.192	0.108	0.079	0.044
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.038	0.182	0.105	0.079	0.045
SD	***SD***	***SD***	***SD***	***SD***
±0.001	±0.013	±0.004	±0.0006	±0.002

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

Table 10

P815 Assay #2

TS	*20:1*	*10:1*	*5:1*	*0:1*
0.137	***ES***	***ES***	***ES***	***ES***
0.104	0.360	0.285	0.187	0.043
0.189	0.333	0.308	0.190	0.044
	0.352	0.303	0.162	0.043
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.143	0.348	0.30	0.18	0.043
SD	***SD***	***SD***	***SD***	***SD***
±0.043	±0.014	±0.012	±0.015	±0.0006
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.403	0.370	0.337	0.331	0.044
0.436	0.361	0.335	0.351	0.043
0.406	0.360	0.363	0.345	0.044
Mean	***Mean***	***Mean***	***Mean***	***Mean***
0.415	0.364	0.345	0.342	0.044
SD	***SD***	***SD***	***SD***	***SD***
±0.0182	±0.006	±0.016	±0.01	±0.0006

TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

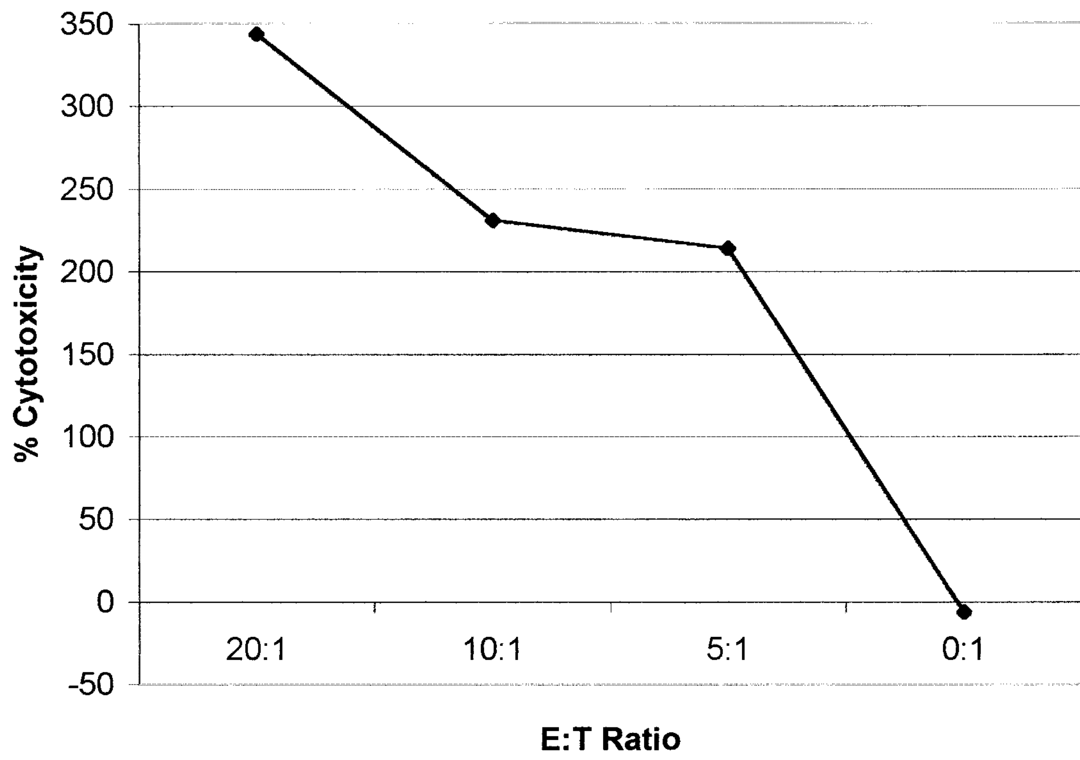
Table 11

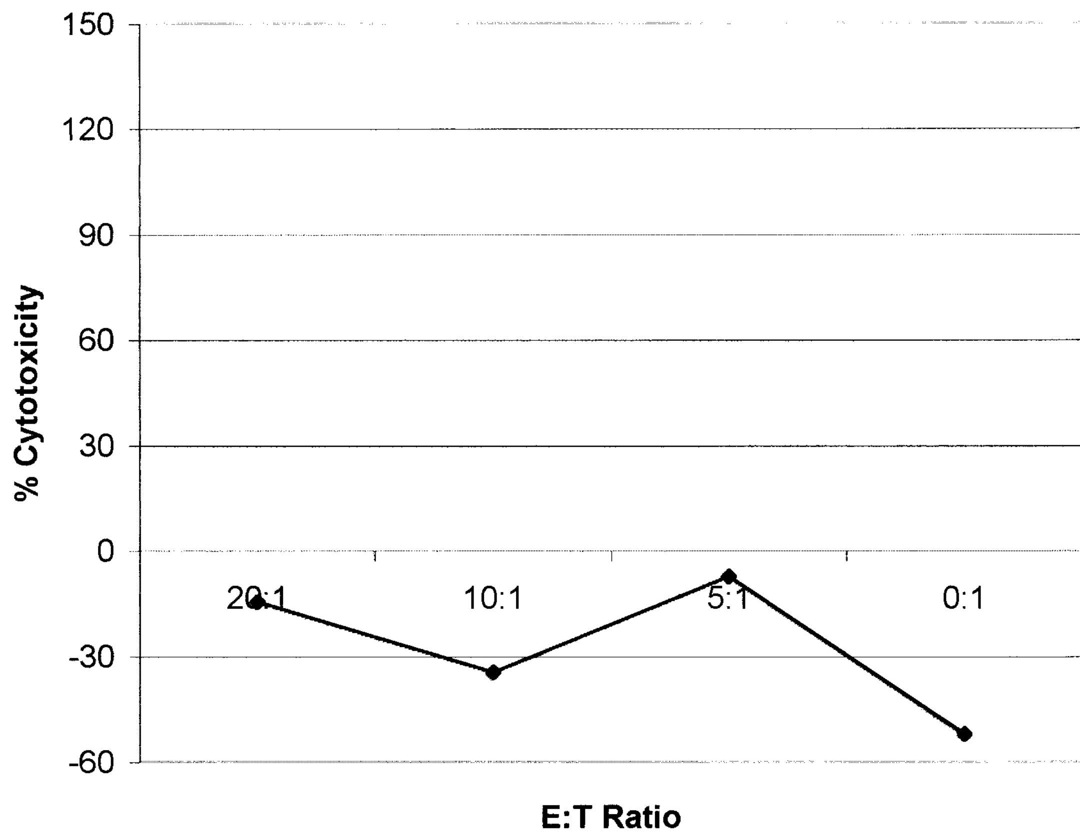
P815 Assay #3

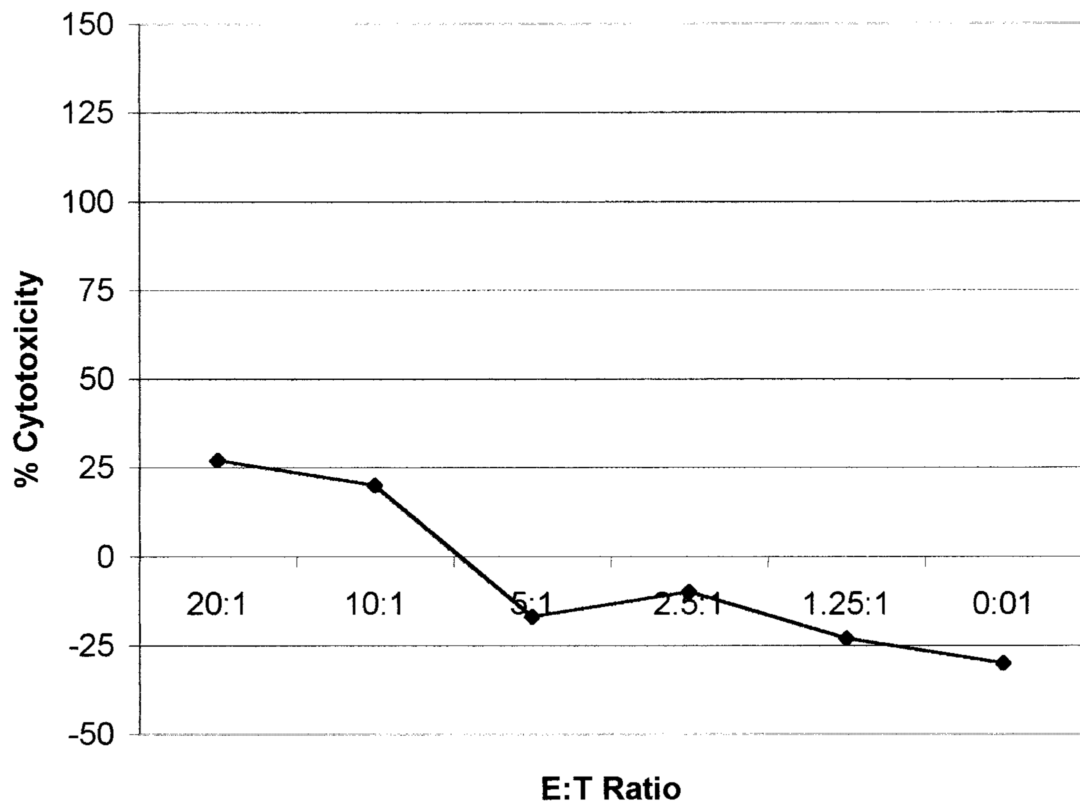
TS	*20:1*	*10:1*	*5:1*	*2.5:1*	*1.25:1*	*0:1*
-0.001	***ES***	***ES***	***ES***	***ES***	***ES***	***ES***
0.01	0.189	0.095	0.039	0.046	0.029	0.029
0.003	0.162	0.083	0.037	0.044	0.028	0.027
	0.162	0.070	0.039	0.043	0.028	0.023
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.004	0.171	0.083	0.038	0.044	0.028	0.026
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.006	±0.016	±0.013	±0.001	±0.002	±0.0006	±0.003
TM	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***	***Exp.***
0.011	0.163	0.078	0.049	0.051	0.041	0.035
0.006	0.169	0.081	0.045	0.05	0.038	0.041
0.009	0.169	0.085	0.048	0.052	0.039	0.041
Mean	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***	***Mean***
0.009	0.167	0.081	0.047	0.051	0.039	0.039
SD	***SD***	***SD***	***SD***	***SD***	***SD***	***SD***
±0.003	±0.003	±0.004	±0.002	±0.001	±0.002	±0.003

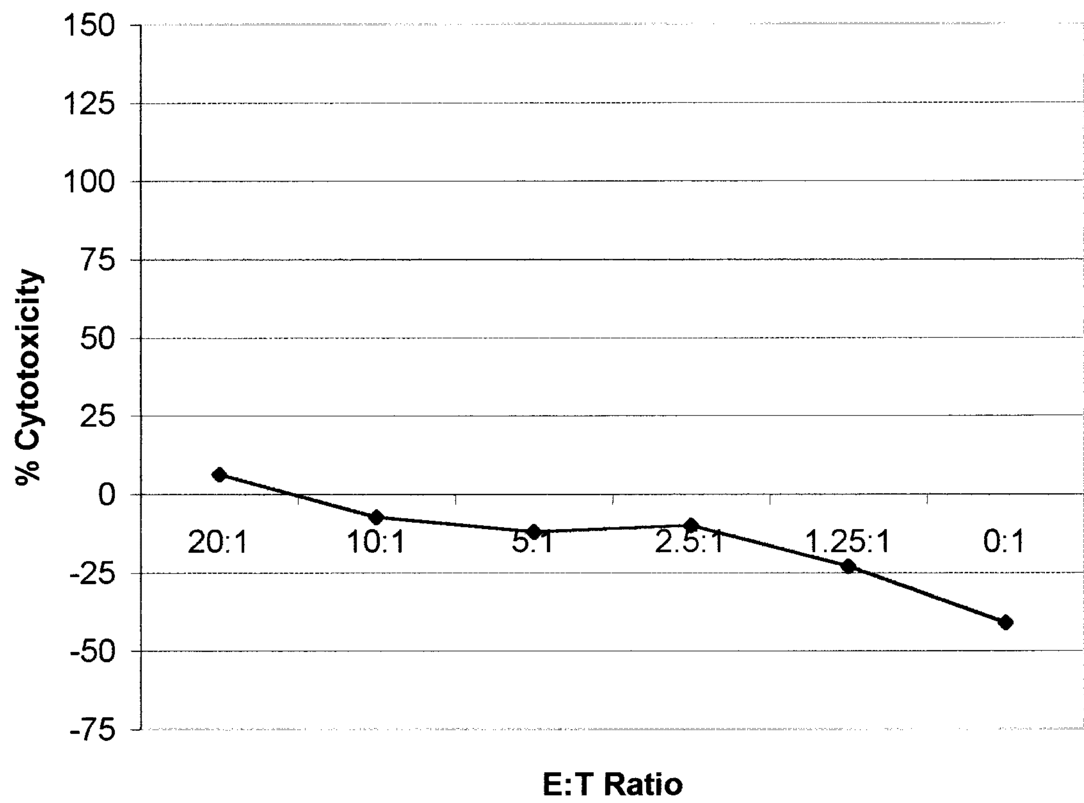
TS- Target spontaneous LDH release
TM- Target maximum LDH release
ES-Effector spontaneous LDH release
Exp.- Experimental LDH release
SD-Standard deviation

Table 12


P815 Assay 1
% Cytotoxicity
350
300
250
200
150
100
50
0
-50
20:1
10:1
5:1
0:1
E:T Ratio


P815 Assay 2
150
120
90
60
30
0
-30
-60
% Cytotoxicity
20:1
10:1
5:1
0:1
E:T Ratio


P815 Assay 3
150
125
100
75
50
25
0
-25
-50
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:01
E:T Ratio


P815 Mean % Cytototoxicity vs. E:T Ratio
150
125
100
75
50
25
0
-25
-50
-75
% Cytotoxicity
20:1
10:1
5:1
2.5:1
1.25:1
0:1
E:T Ratio

calculated for each E:T ratio from assays 2 and 3(Figure 17). The mean percent cytoxicity at 20:1 was 6.25% (± 29.3); 10:1 was –7.25% (± 38.5); 5:1 was –12.10% (± 6.9); 2.5:1 was –10% (± 0.0); 1.25:1 was –23% (± 0.0); and 0:1 was –41% (±14.8). The overall maximum response was at 20:1. A One Way Analysis of Variance showed that there is no statistical difference between the mean values at each E:T ratio (p = 0.795).

Cell Line Comparisons

A Two Way Analysis of Variance was performed to compare the statistical significance of the mean experimental trial data between the best positive and negative control target cell lines. The best positive cell line was MPC 11, and the best negative cell line was P815. A statistical difference between the E:T ratios of the MPC11 and P815 cell lines was observed. A statistical difference was observed at 20:1 ($p = 0.015$); 10:1 ($p = 0.049$); and 5:1 ($p = 0.016$). This indicates that these cell lines are adequate for use as positive and negative control target cell lines in murine NK cell studies.

Discussion

Lactate dehydrogenase is an intracellular enzyme that is found in all nucleated cells. When a target cell is lysed, LDH is released. The amount of natural killer cell cytoxicity can be measured using an assay to determine the amount of LDH that is released into the cellular supernatant. The supernatant containing the LDH is incubated with an enzymatic substrate (iodonitrotetrazolium chloride (INT)). The substrate will interact with the LDH and cause a colorimetric reaction to occur. This reaction is stopped with the addition of an inhibitor to the substrate enzyme (acetic acid), and the absorbance is read at 490 nm in a standard microplate reader. The amount of LDH in the supernatant is proportional to the amount of cytotoxicity. The purpose of this study was to use an LDH assay to measure natural killer cell cytoxicity towards the Yac-1, MPC 11, L1210, and P815 murine target cell lines and to determine if this is a useful model for measuring murine natural killer cell function.

The chromium release assay was the first cytotoxicity assay that has been used extensively to study human natural killer cell cytotoxicity. In the chromium release assay, target cells are labeled with chromium and incubated with effector cells. The amount of chromium released in to the supernatant is proportional to the amount of cytotoxicity (see Cytotoxicity Assays). Early examples comparing the use of the chromium and LDH assays are seen in Korenzeniewski and Callewaert, 1983 and in Decker and Lohmann-Matthes, 1988. Studies done by Korenzeniewski and Callewaert utilized the chromium assay to study human NK cell cytotoxicity towards human target cells (K562 erythtoleukemia cell) (Korenzeniewski and Callewaert 1983). Murine target cells were not tested in this study. Decker and Lohmann-Matthes also used the chromium assay to

study NK cytotoxicity (Decker and Lohmann-Matthes 1988). In all cell lines, high amounts of spontaneous chromium release was detected (7.9 %-11.3 %) (Decker and Lohmann-Matthes, 1988). In the Decker and Lohmann-Matthes study, human NK cells were incubated with K562 human target cells to measure the amount of killing based on the amount of chromium released into the supernatant by the target cells. The highest response seen with the K562 cell line was 31.8 % at the 20:1 E:T ratio (Decker and Lohmann-Matthes 1988). In these studies, the P815 cells were tested as target cell for human T cytotoxic cells but not for natural killer activity. Decker and Lohmann-Matthes also used murine NK cells and murine target cells in their study (Decker and Lohmann-Matthes 1988). Murine NK cell cytotoxicity was measured using only Yac-1 murine target cells. The maximum response of 17.6 % was observed at the 25:1 E:T ratio, indicating that the Yac-1 cells are targeted for murine NK lysis.

The lactate dehydrogenase assay was first studied by Korzeniewski and Callewaert (1983). In this study, human effector and target cells were used. The results of LDH assay were compared to the chromium release assay. The lymphocytes were collected from healthy donors for use as the effector cells, and the target cells were the K-562, HSB-2, and CEM human cell lines (Korzeniewski and Callewaert 1983). The cells were washed in RPMI 1640 supplemented with Hepes buffer, gentamicin, and fetal calf serum. The use of fetal calf serum in cytotoxicity assays can alter the results because fetal calf serum contains LDH, causing an increase in the background levels. Viable cells were counted using trypan blue dye. The target cells were diluted in Hepes buffer and 199-albumin and added to a 96-well round bottom assay plate. 199- albumin was used to remove the LDH that was present in the fetal calf serum. Effector cells were added to the

plate at a concentration of 5 X 10^5 cells/0.1 ml. In this study, the target cell concentration was changed while the effector cell concentration remained constant. The E:T ratios used were 1:12, 1:8.3; 1:6.2; 1:5. The plate was centrifuged and incubated at 37°C for 2 or 4 hours. Following incubation, the plate was centrifuged again and 0.1 ml of the supernatant was transferred to a flat bottom 96-well assay plate and substrate solution was added to each well at 3-second intervals. The plate was read at 490 nm in a microplate reader at 3-5 minute intervals. The LDH activity was expressed in absorbance/minute and was calculated by computer. The percent cytotoxicity was calculated by subtracting the target cell spontaneous LDH release from the experimental LDH release. This value was divided by the maximum LDH release subtracted by the target cell spontaneous LDH release (Korzeniewski and Callewaert, 1983). This calculation differs from the calculation used in our study in that the calculation used by Korenzeniewski and Callewaert did not account for the effector cell spontaneous LDH release. Not accounting for the effector cell spontaneous LDH release causes the percent cytotoxicity values to appear higher.

Decker and Lohmann-Matthes made significant changes to the Korzeniewski and Callewaert procedure (1988). In the Decker study, murine and human target and effector cell were used. The murine cells used were the Yac-1, Wehi 164, and P815 lines. The human K562 and A375 target cell lines were also used. The use of murine target and effector cells became important to test because it allowed for easier studies of NK cell cytotoxicity. The effector cells were harvested from the peritoneal cavity of a mouse and the cells were obtained by passage over a nylon wool sheath, which will separate the effector cells from macrophages. For the LDH assay procedure, effector cells were

placed into a round 96-well assay plate in 100 ul of RPMI and 3 % FCS media. The target cells contained in 50 ul of RPMI and 3 % FCS were also added. The plate was incubated at 37°C in 5 % CO_2. Following incubation, the cellular supernatant was transferred to a flat-bottom 96-well plate. Lactate solution (20 ul) and iodonitrotetrazolium chloride (INT) solution (20 ul) were added to each well. Next, 20 ul of a solution containing NAD+ and diaphorase was added to start the colorimetric reaction. This was allowed to run for 20 minutes. The enzymatic reaction was stopped by the addition of 20 ul of an LDH inhibitor oxamate. The absorbance was read at 490 nm in a standard 96-well microplate reader (Decker and Lohmann-Matthes, 1988). The assay used in the Decker and Lohmann-Matthes study was the same as the original methodology given in the Korzeniewski and Callewaert study. Our study did not use the same methodology, as we used the CytoTox 96 kit. The method for the CytoTox 96 kit indicates that only 10 ul of lysis solution (Triton X-100) should be added to the target maximum and volume correction control wells (see figure 2). The previous studies did not add lysis solution to any of the wells to determine the target maximum LDH release or to correct for differences in volume between each well. Another difference between the CytoTox 96 kit and the previous experiments is the incubation time. For the CytoTox 96 kit the enzymatic reaction occurs for 30 minutes and then the stop solution is added, and the plate was read one time at 490 nm. In the previous procedures, the substrate was added in 3-second intervals and the plate was read at 490 nm every 5 minutes. The substrate mix in the previous studies was lactic acid dehydrogenase substrate mixture containing lactate, tetrazolium chloride, methosulfate, and Tris buffer. The substrate mix in the CytoTox 96 kit is INT tetrazolium salt.

Konjevic and Spuzic modified the original LDH assay studied by Korzeniewski and Callewaert and the Decker and Lohmann-Matthes studies (Konjevic and Spuzic, 1997). The original LDH assay did not account for the effector cell spontaneous LDH release when calculating the percent cytotoxicity. In the Konjevic study, as in our study, the effector cell concentration was varied, and the target cell concentration remained constant. The remaining LDH assay procedure followed the Korzeniewski procedure as previously described. The E:T ratios used were 80:1; 40:1; and 20:1, and the effector and target cells were human cell lines.. At the 80:1 ratio, the LDH release was 58.85% before the amount of human effector cell spontaneous release was accounted for. After subtracting the human effector cell spontaneous release, the percent cytotoxicity at 80:1 was 39.41%. The chromium assay was used as a standard to test the LDH results. At the 80:1 ratio, the percent cytotoxicity using the chromium assay was 39.94. The percent cytotoxicity at 40:1 for the LDH assay was 28.39 and the percent cytotoxicity for the chromium assay was 21.51. At the 20:1 ratio the cytotoxicity for the LDH assay was 19.5 and for the chromium assay it was 16.33. The adjusted LDH values coincide with the chromium release percent cytotoxicity results they observed (Konjevic and Spuzic, 1997). As a result of the Konjevic study, the percent cytotoxicity calculation was changed to account for the effector spontaneous release and is the same as the calculation listed in the methods section of this study (Konjevic and Spuzic, 1997).

Previous studies determined that the Yac-1 and MPC 11 cell lines are targeted by natural killer cells and the P815 and L1210 cell lines are not targeted by natural killer cells (Decker and Lohmann-Matthes, 1988) (Ting *et al.*, 1982) (Ezaki *et al.*, 1983) (Vujanovic *et al.*, 1988) (Ortaldo *et al.*, 1986) (Kumagai *et al.*, 1982). The E:T ratios

used in the Decker study were 25:1, 12:1, and 6:1. The percent cytotoxicity for the Yac-1 target cells at 25:1 was 19.1 %; 12:1 was 15.8 %; 6:1 was 9.9 % (Decker and Lohmann-Matthes, 1988) indicating that the Yac-1 cells are susceptible to NK lysis. The results in our study indicate that the Yac-1 are susceptible to natural killer cell lysis, however the amount of cytotoxicity was lower than previous research has indicated. In our study, the E:T ratios were 20:1, 10:1, 5:1, 2.5:1, 1.25:1, and 0:1. For the Yac-1 target cell line, we did not observe high amounts of cytoxicity at the 20:1 or 10:1 E:T ratios. The highest percent cytotoxicity observed was 19.4 % at an E:T ratio of 2.5:1. There were, however, low levels of cytotoxicity observed at the lower E:T ratios. We did observe the same amount of maximum cytotoxicity as the Decker study, however the values were observed at differing E:T ratios. In our study the maximum cytotoxicity seen was 19.4% (2.5:1) and in the Decker study it was 19.1% (25:1). The reason our maximum cytotoxicity was observed at a low E:T ratio was because of the high amount of effector cell spontaneous release from the murine NK cells. The main difference between our study and the Decker and Lohmann-Matthes study is that the Cytotox 96 kit was used in our study. The Decker and Lohmann-Matthes study used the reagents as described in the original LDH assay done by Korzeniewski and Callewaert (1983). The most important difference between the studies that may account for differences in the percent cytotoxicity towards Yac-1 cells is that the Decker study used the original calculation for percent cytotoxicity. Our study used the corrected equation which is why we did not see cytotoxicity at the higher E:T ratios. However, in our study, we saw extremely high amounts of spontaneous effector (NK cell) LDH release. The high values seen at the higher E:T ratios may mask the true amount of NK cytotoxicity. At the lower E:T ratios, high amounts of

cytotoxicity were seen. This may be due to the decrease in effector cell spontaneous release as the effector cell numbers decline.

Ting *et al.* and Ezaki *et al.* studies also used Yac-1 (Ting *et al.*, 1982) (Ezaki *et al.*, 1983). In the studies by Ting and coworkers, the effector cells used were murine spleen cells. The results indicate that the NK cytotoxicity towards the Yac-1 cell was 29% (E:T ratio of 100:1) and 20% (E:T ratio of 30:1). In the studies by Ezaki *et al.*, the effector cells used were spleen cells from a CBA/J mouse. The results show that the cytotoxicity towards the Yac-1 cells at an E:T ratio of 200:1 was 25 % (Ezaki *et al.*, 1983). The methodology for this study followed the original LDH assay method by Korzeniewski and Callewaert (Korzeniewski and Callewaert 1983). Again, there was more cytotoxicity observed in the Ezaki study than in our study, which may be attributed to the previously described differences in methodology as well as the high E:T ratio used in the Ezaki study (200:1).

Vujanovic *et al.* used the Yac-1 cell line to test murine NK cell cytotoxicity (Vujanovic *et al.*, 1988). They used the standard chromium release assay to measure the amount of lysis. Their results indicate that murine NK cells target Yac-1 cell line. The amount of cytotoxicity observed was 40 %. Unlike our study, the Vujanovic study did not use specific E:T ratios. They used 2 X 10^{-6} cell/ml of the effector cells and 10^{7} cells/ml of the target cells. The Vujanovic study did not use the adjusted calculation for percent cytotoxicity. This may be a reason why they observed higher amounts of cytotoxicity. The Yac-1 cell line was also used in a study done by Ortaldo *et al.*, for studying mouse natural killer cells to mouse natural cytotoxic (NC) cells (Ortaldo *et al.*, 1986). The standard chromium release assay was used to measure the amount of

cytotoxicity. The Yac-1 cell line was used as a target cell for NK cells not NC cells. The highest amount of cytotoxicity observed towards the Yac-1 cell line an E:T ratio of 50:1 was 34.7 % (Ortaldo *et al.,* 1986). Again, the calculation used to determine the amount of cytotoxicity was not the adjusted calculation, which accounts for the amount of effector cell spontaneous release.

In summary, our study of the Yac-1 cell line showed the highest amount of cytotoxicity at the 2.5:1 E:T ratio. This is similar to what was seen in the Decker study. The highest amount of cytotoxicity was 19.1% at the 25:1 E:T ratio (Decker and Lohmann-Matthes 1988). The same amount of cytotoxicity was observed, however, it was at different ratios. Our study utilized the corrected equation for determining percent cytotoxicity, whereas the Decker study did not. Also in our study high amounts of effector cell spontaneous LDH was seen which may have masked the cytotoxicity at the higher E:T ratios.

Previous studies indicate that the P815 cell line is not targeted for lysis by NK cells. Results from the Ting *et al.* study showed that the cytotoxicity towards the P815 cells was 17 % (E:T ratio of 10:1) (1Ting *et al.,* 1982). The Ezaki *et al.* study showed that the cytotoxicity towards the P815 cells was 0.5 % at an E:T ratio of 200:1 (Ezaki *et al.,* 1983). Kumagai *et al.,* used the P815 cell line with murine effector cells to study NK cytotoxicity (Kumagai *et al.,* 1982). The chromium release assay, as previously described, was used to measure the amount of cytotoxicity. In their study, the percent lysis for P815 cells was 0.3% at an E:T ratio of 100:1. They did not use the adjusted calculation, which accounts for the effector cell spontaneous release, when determining the percent cytotoxicity. Vujanovic et al. also tested the P815 cell line, using murine

effector cells and the chromium release assay (as previously described) (Vujanovic *et al.*, 1988). They were not looking directly at NK cytotoxicity rather they were studying LAK cytotoxicity. They used the P815 cell line as an indicator of LAK activity. Their results indicate that the P815 cells were not targeted by the LAK cells (0% cytotoxicity) (Vujanovic *et al.*, 1988).

We also saw that the P815 cells were not targeted by murine natural killer cell lysis. However, in assay 1 abnormally high amounts of cytotoxicity was observed. As stated previously, this may be due to T cell activation in the mouse, as we saw elevated numbers of spleen cell when performing this assay. An increase in spleen cells may be a sign of infection. If infection had occurred in the mouse before the assay was performed, T cells or LAK may be responsible for lysing the target cells. This assay was excluded from the calculation of the mean cytoxicity. In our study, the highest amount of cytotoxicity observed was 6.25 % at an E:T ratio of 20:1. Our mean data showed no significant difference from the background and good negative results were observed. The results from our P815 studies coincide with the results seen in these previous studies, confirming that murine NK cells do not target P815 cells.

In the current study, the L1210 results were not as expected. Cytotoxicity was observed in low amounts in assay 1 and high amounts in assay 3. Kiyohara *et al.* used the L1210 to measure NK cell cytotoxicity. In the Kiyohara study, the target cells were incubated with and without Meth A to induce cytotoxicity (Kiyohara *et al.*, 1982). The results show that the cytotoxicity towards the L1210 was 0 % at an E:T ratio of 100:1, but cytotoxicity could be induced to 49 % with the addition of Meth A. One difference between our study and this study is that in the Kiyohara study, the spleen cells were

cultured with penicillin and streptomycin. The spleen cells in our study were not cultured with antibiotics. In the Kiyohara studies, the spleen cells were harvested from a C3H/He mouse, which is a mouse that is genetically altered to express a gene that causes retinal degeneration. The mice used in our study were not genetically altered in any way. Our results do not coincide with previous studies; however, the L1210 cell line has not been used extensively in conjunction with murine effector cells. There is still a need to further test the L1210 cell line for use as a potential positive or negative control for NK targeting.

The MPC 11 cell line has not been used in many NK cytotoxicity studies; however, the results from this study indicate that this cell line is also targeted for killing by murine natural killer cells. An abundant amount of cytotoxicity was observed at the 5:1 E:T ratio. It would be expected that this value should be lower than the 20:1 and 10:1 E:T ratios. A possible reason for this may be the high amount of effector cell spontaneous LDH release at the higher E:T ratios, which may have masked the amount of cytotoxicity that was occurring. Our studies indicate that the MPC 11 is an ideal positive control for use in murine cytotoxicity studies using the LDH assay.

Natural killer cells, NC, NK-T, and CTL cell share similarities in cytotoxic abilities and need to be distinguished in these assays. Natural killer cells and natural cytotoxic cells target syngenic, allogenic and xenogenic tumor cells (Ortaldo 1986). NC cells do not possess the same surface markers as the NK cells. NC cells also require a longer incubation period in order to target and lysis the target cells. It has been shown that NC cells need an incubation of 18 hours or more to elicit a response (Ortaldo *et al.*, 1986). NK cells on the other hand, react quickly to eliminate target cells (4 hours)

(Ortaldo *et al.*, 1986). NC cells seem to be regulated by different agents than the NK cells are (Ortaldo 1986). Various cytokines stimulate the NC cells to target cells that the NK cells do not target. It has been shown that IFN increases murine NK cytolytic activity (Ortaldo 1986). IL-3 affects murine NC cells to increase cytolytic activities (Ortaldo 1986) (Ortaldo *et al.*, 1986), and IL-1 enhances both murine NK and NC cytotoxic abilities.

Another cell type that is similar to the NK cell is the LAK cells (lymphokine-activated killer cells), which are generated from high doses of IL-2 that can cause the LAK cells to lyse NK resistant tumor cells (Kuby, 1997) (Ribeiro-Dias *et al.*, 2000). NK-T cells are also similar in nature to NK cells. NK-T cells express NK inhibitory and activating receptors. Mainly NK-T cells express NK1.1 and receptors in the Ly-49 family. NK-T cells, unlike NK cells, need to be exposed to an MHC molecule to be activated to kill. Our study focused on determining positive and negative controls, as well as determining if the LDH assay was useful to test murine NK cell cytotoxicity. Previous studies only tested murine positive target and human negative targets. The importance of a negative murine target cell line is that is proves that NK cells and not other cytotoxic chemicals from other cells are killing the target cells. Also, many cell lines that are not killed by NK cells will be killed by LAK, NK-T or activated T cells. This can be used to prove that the activity that is seen is actually NK in origin. However, if the negative target cell is lysed, a cell other than the NK cell may have killed it. It is known that NK and NK-T cells both target the Yac-1 murine cell line for lysis. LAK cells also target Yac-1, however, IL-2 needs to be present for this to occur in a

measurable amount (Ribeiro-Dias, 2000). Of the cell lines we tested in this study, only the Yac-1 cell a line has been extensively used in NK-T and LAK cytotoxicity assays.

The results from the current study indicate that the MPC 11 cell line is targeted by murine NK cells and is a useful positive control for measuring murine NK cell cytotoxicity using the LDH assay. The results also indicate that the P815 cell line is not targeted for lysis by murine NK cells. This cell line would be useful as a negative control in murine NK cell cytoxicity studies. The Yac-1 cell line was shown to be targeted by murine NK cells. The L1210 results did not coincide with corroborating results previously seen in other laboratories. In our study, cytotoxicity was observed with the L1210 cells, indicating that this cell line is targeted for lysis by murine NK cells. As these results are in contrast with previous reports, additional tests are required to confirm these results. While these studies have shown that the LDH assay can be used to measure murine NK cell function, the assay is not effective at all effector cell concentrations. The cytotoxicity at the higher E:T ratios was masked by the high amount of murine NK cell spontaneous LDH release. The high levels of spontaneous LDH release requires that careful titration of effector cells be performed when measuring murine NK cell cytotoxicity using the LDH assay.

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Appendix

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October 24, 2000

Dr. Diana Fagan
Department of Biological Sciences
UNIVERSITY

RE: Submission of Protocol # 02-01

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the protocol you submitted (Protocol # 02-01) titled "Hybridoma Preparation," and determined it should be unconditionally approved.

This Protocol is approved for a period of three years; however, it must be Updated annually via the submission of an Update form **prior** to the expiration date of 10/25/00. You must adhere to the procedures described in your approved request. The Institutional Animal Care and Use Committee must first authorize any modification to the project.

Sincerely,

Peter J. Kasvinsky (cc)

Dr. Peter J. Kasvinsky
Dean of Graduate Studies

PJK:cc

c: Dr. Paul Peterson, Chair
Department of Biological Sciences