The Use of Lactate Dehydrogenase for the Detection of Murine Natural Killer Cell Function

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Brian P. Schmidt

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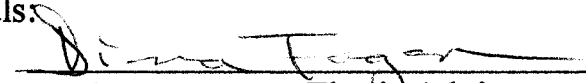

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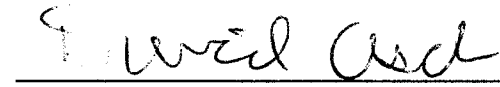

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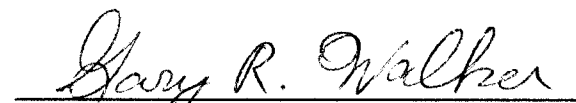

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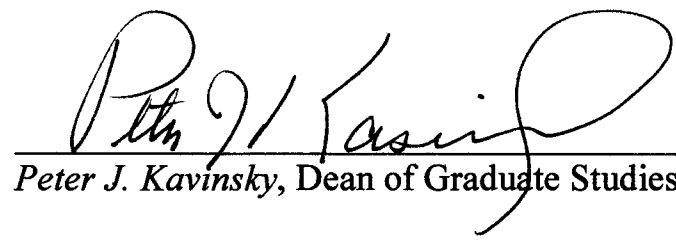

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ABSTRACT

Natural Killer (NK) cells are white blood cells that participate in the direct cellular elimination of tumor and virus-infected cells. NK cell function is determined through the use of cytotoxicity assays. Our study investigates the use of an LDH assay (Promega Cytotox 96® LDH assay) to characterize murine NK cells. Four target cell lines were tested for their effectiveness in this assay. Studies using ^{51}Cr release assays have shown that MPC II and YAC-1 cell lines are targeted for lysis by murine NK cells (positive controls) and that L1210 and P815 cell lines are not targeted for NK cell lysis (negative controls). The optimal number of target cells (total LDH two-fold higher than background) was 5,000 cells/100µl for P815 and YAC-1, 10,000 cells/100µl for L1210 and MPC II. The 4 and 24 spontaneous release values (percentage of the total LDH release) were as follows: P815: 0 and 46.651%, YAC-1: 0 28.985%, L1210: 0 and 12.661%, and MPC II: 13.490% and 41.542%. Experimental cytotoxicity assays were also performed, with the optimal number of target cells incubated with NK cells at various effector (NK) to target cell ratios. P815 experienced a low % cytotoxicity (0.369%) and was an effective negative control cell line. MPC II exhibited a high % cytotoxicity (25.8%) and was an effective positive control cell line. No specific LDH release was seen with YAC-1 target cells at the effector to target cell ratios used in these studies. The LDH cytotoxicity assay is useful in determining murine NK cell cytotoxicty. The spontaneous and total release data along with the results of the LDH cytotxicity assays obtained from these positive and negative control target cell lines will aid in the determination of the functional activity of an NK cell preparation.

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LIST OF ABBREVIATIONS

Ab	antibody
ADCC	antibody dependant cellular cytotoxicity
ANOVA	one way repeated measures analysis of variance
APC	antigen presenting cell
BrdU	bromodeoxyuridine
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
DMEM	Dulbeco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonulceic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GF-FAAS	graphite furnace flameless atomic absorption spectroscopy
GraB	granzyme B
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin

LFA	lymphocyte function associated antigen
MHC	major histocompatability complex
NK	natural killer
PBS	phosphate buffered saline
TCR	T cell receptor

INTRODUCTION

The vertebrate immune system is a survival mechanism that guards against invading pathogens such as bacteria and viruses, and against tumor forming tissue such as cancer. The immune system has four significant actions: recognition, response, elimination, and memory of pathogens. Foreign pathogens and tumor-containing tissue possess slight chemical differences from each other and from the body's own tissue. A functional immune system is able to recognize these slight differences and distinguish them from the host's normal tissue. When a foreign molecule, or antigen, is recognized, the immune system will activate an effector response. This effector response involves certain cells and molecules of the immune system, and will lead to the eventual neutralization or elimination of the antigen. Another important feature of the immune response is a memory response. During an immune response, certain effector cells differentiate into memory cells. Exposure to the same antigen at a later time will cause these memory cells to initiate a heightened immune response. This memory response is important in disease prevention because the pathogen is eliminated before it can induce harmful effects (1).

Two types of immunity exist in vertebrates: innate and acquired immunity. Innate immunity uses protective barriers for defending against infection. Acquired immunity is able to recognize specific antigens, and neutralize or eliminate them (1).

Innate immunity includes anatomic, physiologic, phagocytic/endocytic, and inflammatory responses that act as defense mechanisms. Anatomic barriers include the skin and the mucous membranes. The skin provides a protective covering that prevents the entry of foreign microorganisms. The sebaceous glands in the skin secrete sebum at a pH between 3 and 5 which further inhibits microorganism growth. Mucous membranes contain mucus secretions that surround foreign particles and allow their elimination by cilia or other means. These mucous membranes also contain nonpathogenic flora, which compete with other microorganisms for attachment sites and nutrients. Temperature, low pH, and chemical mediators make up the physiologic barriers. Normal body temperature can inhibit the growth of some microorganisms. An increased body temperature, which occurs during a fever, may also inactivate some pathogens. The acidic environment of the stomach is a physiologic barrier

which can inactivate most ingested microorganisms. Lysozyme, interferon, and complement are chemical mediators, which participate, in innate immunity. Lysozyme is an enzyme that cleaves the cell walls of bacteria. Interferon is a cytokine that causes non-infected cells to enter an anti-viral state. Complement may cause microorganism lysis or contribute to their phagocytosis. Endocytosis and phagocytosis may eliminate foreign macromolecules and whole microorganisms respectively. Various cell types may contribute to the endocytosis of macromolecules while monocytes, neutrophils, and macrophages are capable of the phagocytosis, killing, and digestion of whole microorganisms. Tissue damage and infection can cause an inflammatory response. This inflammatory response causes the migration of phagocytic cells into the infected area. It also causes the leakage of acute phase proteins into the inflamed area. These proteins have antibacterial activity (1).

Acquired immunity is usually being referred to in descriptions of the immune response. It is able to recognize and differentiate between billions of antigens, has the ability to respond to these antigens, can recognize self-molecules, and possesses a memory response. Lymphocytes are the main

effector cells of the acquired immune response while antigen presenting cells and other leukocytes have accessory roles. Cell-mediated and humoral are the two types of acquired immune responses (1).

B-lymphocytes generate the humoral immune response. B cells originate and mature in the bone marrow. These cells have synthesized glycoproteins, called Immunoglobulins (Ig), for a receptor. This Immunoglobulin-like receptor consists of two identical heavy polypeptide chains and two identical light polypeptide chains held together by a disulfide linkage. The Ig molecules also contain a highly variable antigen-binding region. When a B cell is exposed to its antigen it will divide and differentiate into a plasma cell and a memory cell. Plasma cells are effector cells that lack membrane-bound Immunoglobulin and secrete soluble Antibody (Ab). These soluble Ab's have a variety of important functions. They may cross-link foreign antigens, which causes large antigen-Ab clusters to form. These clusters are easily eliminated by phagocytic cells. When Ab's bind to antigens on intact microorganisms the complement system may become activated. Complement activation will cause the foreign cell to lyse. Ab's may also bind viral particles and toxins. This will

cause their neutralization and keep them from infecting host cells. Memory B cells possess the same membrane–bound Ig as the parent B cell, and possess a longer life span than the plasma cells. These cells remain after the initial immune response has finished, and are utilized in the memory immune response (1).

T-lymphocytes generate the cell-mediated branch of immunity. T cells originate in the bone marrow and mature in the thymus. They have a membrane bound T cell receptor (TCR). This TCR will only recognize a foreign antigen associated with a major histocompatability complex (MHC) molecule. Two populations of T cells exist: T helper cells (T_H cells) and T cytotoxic cells (T_C cells) (1).

T_H cells possess the glycoprotein CD4, and are activated when they interact with the proper antigen associated with an MHC class II molecule. These MHC class II molecules are present on antigen presenting cells (APC's) such as macrophages, B cells, and dendritic cells. APC's internalize antigen and display it in conjunction with the MHC class II molecule on their membrane surface. Upon contact with T_H cells, the APC's deliver a co-stimulatory signal that activates T_H cells. When T_H cells

becomes activated they secrete various cytokines (growth factors). Cytokines regulate B cells, T_C cells, macrophages, and other cells in the immune response. The types cytokines secreted determine the immune response that is generated. Thus, T_H cells are important regulators of both cell-mediated and humoral immunity (1).

T_C cells are another T lymphocyte important in cell-mediated immunity. They possess a CD8 glycoprotein, and interact with antigen associated with MHC class I molecules. MHC class I molecules are located on almost all nucleated cells. Following stimulation by cytokines released by T_H cells and interaction with an MHC-antigen complex, T_C cells differentiate into cytotoxic T cells (CTL's) and memory T_C cells. CTL's are able to eliminate cells containing intracellular pathogens and tumor cells (altered-self cells). Altered-self cells display foreign antigen with MHC class-I molecules on their membrane surface. When a CTL encounters an altered-self cell, it generates a cytotoxic response that destroys the target cell. Since almost all nucleated cells contain class I MHC molecules, a CTL response can act against virtually all types of infected cells. Memory T_C

cells have a longer life span than CTL's and function in the memory immune response (1).

The CTL response has two phases: T_C cell differentiation into a CTL and the CTL cytotoxic response. The T_C cell must first differentiate into a CTL. An antigen-class-I MHC molecule complex interacts with the T_C cell's TCR and causes signal transduction. Next, a T_C cell surface molecule, CD 28, binds to another marker on the target cell, B7. This interaction causes a co-stimulatory signal to be generated. Finally, the cytokine IL-2, which is released from activated T_H cells, binds the IL-2 receptor on the TC cell and causes it to differentiate into a CTL (1).

Now the CTL can recognize and cause the elimination of target cells. Programmed cell death, or apoptosis, is the mechanism by which CTL's cause target cell elimination. Apoptosis in CTL's may occur via two ways: with cytotoxic proteins or with membrane signals. Certain CTL's contain cytotoxic proteins such as perforin and granzymes. Perforin is a pore forming protein, and granzyme is a protease. When a CTL binds to a target cell, adhesion molecules on the CTL membrane (LFA-1) bind to adhesion molecules on the target cell (ICAM's). This causes a CTL-target cell

conjugate to become formed. Next, the Golgi stacks and storage granules containing the cytotoxic proteins reorient in the CTL to become close to the target cell junction. A Ca^{2+}-dependant, energy requiring step causes the release of the stored granules from the CTL. The perforin granules form cylindrical pores in the target cell membrane, and allow the entrance of granzymes and other cytotoxic molecules into the cytoplasm of the target cell. The granzymes cause the apoptosis mechanism to begin. The CTL dissociates from the target cell, and within 15 minutes to 3 hours the target cell undergoes cell death. CTL's lacking storage granules can also cause target cell destruction. CTL's which lack cytotoxic proteins cause target cell apoptosis by activating a transmembrane death-signaling receptor (FAS). The FAS receptor is located on the target cell membrane. The CTL possesses proteins that bind to and activate the FAS receptor on their membrane surface. Following target cell-CTL interaction, the FAS receptor is activated and apoptosis occurs in the target cell (1).

Natural killer (NK) cells are another type of lymphocyte that cause cell-mediated immunity. NK cells originate in the bone marrow, but do not require the thymus for maturation. In general, they comprise 5-10% of

lymphocytes and are found in individuals lacking B and T cells. Without previous sensitization to antigens, NK cells are capable of spontaneously lysing tumor and virus-infected target cells. This lytic activity may also be used against tumor cells and even some normal cells. Responding early in infection, NK cells control viral replication, while waiting for a T cell response to be generated (1). Various cytokines such as interleukins (IL) and interferons (IFN) alter NK cell activity. NK cells secrete IFN-γ in order to inhibit viral replication in uninfected cells (3). NK cells are stimulated to proliferate in response to interleukin (IL)-12, IL-1, IL-15 and IL-2. NK cells immediately show a response to antigen, with their peak levels occuring at about 3 days (1). CTL's exhibit activity around 2 days after infection, with peak levels occurring between 7 to 11 days (1). T cells and NK cells express common surface markers and may arise from a common progenitor cell. However, NK cells lack a T cell receptor (no recombination of TCR genes) and CD3 (found on all T cells) (5). Therefore, the method of antigen recognition differs in T cells and NK cells. In terms of location, NK cells are found primarily in the spleen and peripheral blood (1). They are

found in lower numbers in the lymph nodes and bone marrow, and are not found in the thymus at all.

Although T and B cells possess the ability to respond to a variety of antigens, they are functionally unique from NK cells. B cells are derived from the bone marrow and exhibit antibody production. In contrast to NK cells, they do not act as direct effector cells against intracellular infection and tumors. As previously noted, T cells display similar characteristics to NK cells, but also have significant differences. Originating in the bone marrow, T cells require the thymus for proper maturation and function. NK cells originate in the bone marrow, but mature independantly of the thymus (2). T cells also require the proper presentation of their target antigens on MHC molecules in order to generate an immune response. NK cells do not need antigen presented on MHC molecules, and exhibit spontaneous cytotoxic activity. T cells do not exhibit this spontaneous cytotoxic activity. As with NK cells, T cells proliferate in response to IL-2, but a latent period of 7-10 days is needed for T cells to become activated. Yet another difference between T and NK cells is the ability of T cells to generate

memory cells which, upon later exposure to an antigen, cause an increased and faster immune response (2).

NK cells exhibit cell surface receptors and adhesion molecules that regulate target cell destruction (7). They are able to adhere to endothelium, extracellular matrix, stromal cells and target cells. The stimulation of NK cells, such as with IL-2, alters the expression of adhesion molecules. In order to respond to a target cell, the NK cell must first attach to it with adhesion molecules (7). The receptor molecules then determine if there is a cytolytic response. Examples of surface adhesion molecules located on NK cells are the following: CD-2, CD-16, and CD-11a. CD-2 is a β subunit of the IL-2 receptor, and possesses the ability to function as a signal-transducing molecule along with its adhesion properties. Another adhesion molecule, CD16 (FcγRIII), is a receptor for the F_c region of the antibody IgG, and is involved in antibody dependent cellular cytotoxicity (ADCC). CD11a is used to detect ICAM-2 that is redistributed in pathologic conditions (7).

NK cells contain a plethora of receptors, in addition to those just described, many of which have undetermined functions. Since this study

specifically deals with murine NK cells, only mouse NK receptors will be discussed. Two types of NK receptors, activating and inhibitory, exist in NK cells. NKRP-1 is a receptor which causes activation of the NK cell's cytotoxic mechanism, which is activated upon binding to carbohydrates expressed on virus-infected and tumor cells (1,4,5,6). Ly-49A, on the other hand, is an example of an inhibitory receptor. Binding to class I MHC molecules on target cells inactivates the cytotoxic response generated by NKRP-1 (4,5,6). While inhibition of cytotoxicity toward normal cells is the primary known effect of Ly-49A, there is evidence that Ly-49A may have a dual role, functioning as an inhibitor and activator of the NK response (6). Other receptor molecules on NK cells include NK 1.1, CD69, LFA-3, and CD16, which are all found on murine NK cells. As previously mentioned, CD16 is involved in ADCC and causes activation of NK cells. CD69 is absent on resting cells, and along with NK 1.1 and LFA-3 causes a positive cytotoxic response (4).

Constantly expressing granules in their cytoplasm, NK cells are considered to be constitutively cytotoxic (1). Cytotoxic ability is determined by the excitatory and/or inhibitory signals that are mediated by

receptors and costimulatory molecules, which occur when the NK cell interacts with a target cell (8,9). Upon adhesion to the target cell and the receptors making contact, the NK cell is stimulated and a signal cascade is activated. This allows the cytotoxic mechanism to be expressed. The electron-dense granules in NK cells consist of perforin and lymphocyte-specific granule esterase granzyme B (GraB). Both granules need to be released by the NK cell in order for programmed cell death to occur in the target (1). Perforin is a Ca^{2+}-dependant pore-forming molecule, and the GraB causes chromatin condensation and DNA fragmentation. The combination of perforin and GraB release from the NK cell causes GraB to migrate to the nucleus of the target cell and activate molecules of the caspace family. These effects result in target cell apoptosis.

This study characterizes a method for measuring murine NK cell cytotoxicity. Murine NK cells have not been studied as thoroughly as human NK cells. Therefore, the murine NK cell characteristics are not well established. Human and murine NK cells differ in their cell surface markers, cell receptors, and target cells. Our study will examine the murine NK target cells. K562 erythroleukemia and A375 melanoma cells are types

of human NK target cells. Examples of murine NK target cells are the YAC-1 lymphoma, Wehi 164 fibrosarcoma, P815 mastocytoma, MPC II myeloma, L1210 lymphocytic leukemia, and the Nulli-SCC teratocarcinoma cells (22). YAC-1, P815, MPC II, Nulli-SCC, and L1210 murine NK target cells are available to be studied in our laboratory. YAC-1, MPC II, and Nulli-SCC cells are known to be targeted for lysis by murine NK cells. L1210 and P815 cell lines are insensitive to lysis by murine NK cells and are used as negative controls. An assay that successfully measures the lysis, or lack of, in these target cells can be used to characterize cytotoxic activity in murine NK cells.

CYTOTOXICITY ASSAYS

There are a variety of methods to detect the cytotoxic effects of NK cells, although many of these cytotoxicity assays are not available for use in our laboratory due to lack of the necessary equipment. The pre-labeling of target cells with various agents and measuring their release into the supernatant is a common procedure in assays such as, time-resolved fluorometry (13,14,15,16), chromium (Cr) release (nonradioactive and radioactive) (17,18,19), and DNA fragmentation ELISA (20). Finally,

measuring the activity of cytosolic enzymes that are released upon target cell lysis is seen in the lactate dehydrogenase (LDH) assay. A general overview of each of these techniques will be addressed in the following paragraphs.

The most commonly used and widely accepted method for detecting cytotoxicity in the chromium (^{51}Cr) release assay. A radioactive isotope of chromium (^{51}Cr) is used to measure cytotoxicity. The target cells are pre-labeled with $Na_2{}^{51}CrO_4$. $Na_2{}^{51}CrO_4$ freely enters cells but becomes bound to cytoplasmic proteins, which prevents its release unless the cells are lysed. Following incubation with target cells, the supernatant is removed and analyzed using a liquid scintillation counter to determine the amount of ^{51}Cr released. This amount can be used to determine the percent lysis. The major drawback to this technique is the danger and inconvenience of handling radioactive materials (19).

Nonradioactive Cr in the form of Na_2CrO_4 is yet another way to detect NK cytotoxicity. Target cells are incubated and pre-labeled with Na_2CrO_4. Following exposure to and further incubation with effector cells, the Cr is released into the supernatant from lysed target cells. The

nonradioactive Cr present in the supernatant is measured using graphite furnace flameless atomic absorption spectroscopy (GF-FAAS), and used to determine percent lysis. This technique offers highly sensitive results at a limited cost. Chromium's ability to be stored for long periods and the absence of radioactivity are other advantages to this method. Although, the need for an atomic absorption spectrophotometer and the possible toxic effects of Cr metal on target cells limit its use (17,18).

Flow Cytometric analysis is a novel technique used to study NK cell cytotoxicity. It is a single cell assay that allows the distinction of target-effector cell conjugates, living and dead effector (NK cells), and living and dead target cells (cells killed by NK cells) (10). First, target and effector cells are pre-labeled with fluorescent dyes which specifically bind to each cell type and the side scatter (indicates granularity) is measured on the flow cytometer. Next, the target and effector cells are incubated together for the cytotoxicity assay. Propidium iodide is then added to the cell suspension prior to the end of incubation time. Propidium iodide is a nuclear dye that will counterstain dead cells. Finally, the resulting flow cytometer analysis will show the number of living and killed target and effector cells, with the

dead target cells corresponding to the amount of cytotoxicity exhibited by the NK cells (10,11,12). When NK cells and target cells become attached, conjugates are formed. These NK-target cell conjugates exhibit a type of fluorescence called autofluorescence that can be detected by the flow cytometer (10). The advantages to this technique are as follows: the labeling of target cells is not limited to any one phase of cell growth, long incubation times are permitted, radioactivity is unnecessary, the remaining viable target cells may be counted, and the amount of spontaneous release of materials by target and effector cells is not an issue as in other assays (12). Fluorescent dyes that may leak out of target cells and label effector cells pose a problem in this type of assay. Therefore, stable dyes that stay in the cell are advantageous (11). Although this method has its advantages, it can not be used in our laboratory, as we do not possess a flow cytometer.

Another fluorescent technique relevant to measuring cytotoxicity is time-resolved fluorometry. Target cells are pre-labeled with lanthanide diethylenetiaminopentaacetates (LaDTPA's) such as SmDTPA, TbDTPA, and EuDTPA (samarium, terbium, and europium). These lanthanide chelates are fluorochromes, and are incorporated into target cells using

dextran sulfate. Dextran sulfate causes increased membrane permeability, and allows entry of the fluorochromes into the target cells. Following effector and target cell incubation and target cell lysis, the lanthanides are released into the supernatant. An enhancer solution is added to the supernatant which causes each lanthanide (Sm^{3+}, Tb^{3+}, Eu^{3+}) to form a fluorescent complex which emits its own individual emission line (allowing for labeling of multiple target cell species). The fluorescence is then detected with a time-resolved fluorometer after a certain delay in which the background autofluorescence emitted by the media is allowed to decay. The lanthanide chelates exhibit a long decay time and are not affected by the delay. This feature allows for increased sensitivity due to the elimination of background noise caused by media (13,14,15,16). The resulting data may be used to determine percent lysis. LaDTPA's do not adhere to cellular proteins due to their inert properties and are able to diffuse rapidly through a damaged membrane. This offers an advantage to Cr release assays (mentioned later) because Cr is able to form ionic bonds with proteins in the cell which slow down its release upon membrane damage (15,16). The reduced interference due to media, the increased speed of dye release, and

the radioactive-free procedure are a few of the advantages to this procedure. Although, pre-labeling using dextran sulfate may cause a leaky cell membrane and lead to above normal levels of spontaneous release (13,14,15,16). This method requires a fluorometer, and is therefore excluded from research in our lab.

For the DNA fragmentation ELISA cytotoxicity assay, target cells are incubated with BrdU (bromodeoxyuridine), a thymine analog. BrdU is incorporated into the target cells DNA. When the target cells are incubated with effector cells and apoptosis (programmed cell death of the target cell) occurs, resulting in DNA fragmentation. The BrdU labeled DNA is released into the supernatant. The DNA released into the supernatant is bound to an anti-DNA plate, and denatured. The resulting DNA fragments are analyzed with a cellular DNA fragmentation ELISA kit which contains an enzyme bound anti-BrdU antibody. The antibody treated DNA plate is incubated with the substrate for the enzyme, and a colorimetric change results. This absorbance is measured and percent cytotoxicity is determined. This method is shown to be more sensitive than the ^{51}Cr release assay, but has the

disadvantages of using the toxic compound BrdU and the high price of the DNA plates (20).

Lastly, the lactate dehydrogenase (LDH) assay is an enzymatic release cytotoxicity assay which will be used in our laboratory. The LDH assay is a simple, convenient technique which does not require a pre-labeling step. LDH is a stable enzyme which is located in the cellular cytoplasm. Following exposure to effector cells, the LDH is released into the supernatant as target cells become lysed. The supernatant is incubated with a substrate that generates a coupled enzymatic reaction. The reaction results in the formation of a formazan product from a tetrazolium salt (INT). This red product can be measured on a standard 96 well microplate reader, with the absorbance being proportional to the number of lysed cells. Various factors such as, effector cell spontaneous LDH release, target cell spontaneous LDH release, target cell maximum LDH release, culture medium background, and volume controls need to be taken into account, and will be addressed later. Such factors are used in determining the percent lysis of target cells (Promega). The LDH assay has been compared to other methods of detecting cytotoxicity such as ^{51}Cr, and it has proven to

be comparable, showing lower levels of spontaneous release (22). In addition to detecting cell-mediated cytotoxicity, the LDH assay has been used to detect the toxic effects of certain substances such as carbamide peroxide (21) and amyloid β protein (26) on target cells. Some drawbacks to this assay are the background absorbance due to phenol red in the media, and the spontaneous release of LDH. The absence of radioactivity and pre-labeling steps make the LDH assay a safe convenient means to determine NK cell cytotoxicity. The presence of a microplate reader in the lab makes this an available method.

The purpose of this study was to determine if the murine target cells YAC-1, P815, MPC II, L1210, and Nulli-SCC could be used to measure NK cytotoxicity using the LDH assay. The LDH assay has been shown to be effective in measuring murine NK cytotoxicity using P815 and YAC-1 cells (22). The data from the previous study proved comparable to ^{51}Cr data using the same target cells. Our experiments examined the YAC-1 and P815 target cells in addition to the MPC II, L1210, and Nulli-SCC target cells. The LDH assay was used to measure spontaneous and total LDH released by these target cells. In addition, the LDH assay was also used to

measure the cytotoxic response when murine NK cells were incubated with the target cells. This data was used to determine if these target cells could be used for measuring murine NK cytotoxicity. If the LDH assay proves to be effective, it will be used to measure cytotoxicity in a murine NK cell hybridoma.

MATERIALS & METHODS

The Cytotox 96 assay kit (containing stop solution, substrate mix, assay buffer, and 10 X lysis buffer) was purchased from Promega Corporation (Madison, WI). Dulbeco's Modified Eagle Medium (DMEM high and low glucose), RPMI 1640, MEM powder with Earl's salts, fetal calf serum (FCS), L-glutamine, and gentamicin were purchased from Gibco BRL life Technologies (Grand Island, NY). Cryogenic vessels, Type A gelatin, $NaHCO_3$, 0.4% trypan blue solution, and cell culture tested dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Corporation (St. Louis, MO). YAC-1, L1210, Nulli-SCC, P815, and MPC II cell lines were purchased from American Type Culture Collection (Rockville, MD). Swiss Webster mice were purchased from Charles River (Wilmington, MA).

CELL CULTURE

Cell culture and the target cell lines L1210, YAC-1, Nulli-SCC, MPC II, and P815 were grown in the following media: L1210 cells in Dulbeco's Modified Eagle Medium (DMEM) (low glucose), 10% fetal calf serum (FCS), 29.2mg% L-glutamine, and 0.2g% $NaHCO_3$, YAC-1 cells in RPMI

1640 containing 0.45g% glucose, 10% FCS, 29.2 mg% L-glutamine, and 0.2 g% $NaHCO_3$, Nulli-SCC in DMEM (high glucose) with 0.45g% glucose, 10 % FCS, 29.2mg% L-glutamine, 10μg/ml gentamicin, and 0.37g% $NaHCO_3$, MPC II cells in MEM powder with Earls Salts, 20% FCS, 29.2mg % L-glutamine, and 0.22g% $NaHCO_3$, and P815 cells in DMEM (low glucose), 10% FCS, 29.2mg% L-glutamine, and 0.37g% $NaHCO_3$. For the LDH assays, the cells were washed with RPMI 1640 (no phenol red) containing 0.20g% $NaHCO_3$. The cells were maintained in a humidified chamber at 37°C and 5% CO_2 and fed every 24-72 hours under sterile condition.

Target cells were stored by freezing in cell culture media containing 10% dimethyl sulfoxide (DMSO). Target cells suspended in DMSO (1 x 10^6 cells) were added to a cryopreservation vial, enclosed in a styrofoam box, and placed at –70°C. After 24 hours, the vials were transferred to liquid nitrogen for permanent storage.

TOTAL AND SPONTANEOUS RELEASE ASSAYS

NK cytotoxicity was measured using the Cytotox 96 Assay (Promega, Madison, WI). Preliminary studies were performed to determine the

optimal number of cells for each target cell line that were used in the experimental LDH assay. The spontaneous LDH released from target cells after 4 and 24 hour incubations was determined. Total release, spontaneous release, and experimental assays were performed 24 hours after target cells were fed. Target cells were counted on a hemocytometer, and cells adjusted to a concentration of 2×10^5 cells/ml. Of these cells, 5ml were placed into a 15ml conical tube and centrifuged for 8 minutes at 400 x g. The supernatant was aspirated, and target cells were resuspended in 5 ml of RPMI 1640 (containing no phenol red, 0.20 g% $NaHCO_3$). This washing procedure was repeated two times. Following the final wash step, the cells were recounted on the hemocytometer, and the cells adjusted with media to achieve 5,000 cells/100µl, 10,000 cells/100µl, and 20,000 cells/100µl dilutions. A media background control containing 100µl (0 cells/100µl) of media only was also used (background control). The cell dilutions and background controls were pipetted in triplicate into a 96-well round-bottom microplate (Fig. 1). The final volume of each well was adjusted to 100µl. In addition, 10µl of 10X lysis solution was added to each of the total release wells to cause maximum target cell lysis. The microplate, containing both spontaneous

[a]4 Hour Spontaneous Release

[c]Total Release

	0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl			0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl	
	0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl			0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl	
	0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl			0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl	
	0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl							
	0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl							
	0cells/ 100µl	5000 cells/ 100µl	10000 cells/ 100µl	20000 cells/ 100µl							

[b]24 Hour Spontaneous Release

and total LDH release wells, was incubated with a lid for 4 hours in a humidified chamber containing 5% CO_2 and at a temperature of 37°C. Following the 4 hour incubation, the microplate was removed and centrifuged for 4 minutes at 250 x g. A 50µl aliquot of the supernatant from each of the 4 hour spontaneous and total release wells was transferred to a flat-bottom 96-well microplate in the same pattern as above. This microplate was frozen overnight. The original microplate containing the 24 hour spontaneous wells was again incubated for 20 hours in a humidified chamber containing 5% CO_2 and at a temperature of 37°C. Following the 20 hour incubation, the plate was centrifuged for 4 minutes at 250 x g. Aliquots of 50µl from each of the 24 hour spontaneous wells were transferred to the thawed flat-bottom 96-well microplate from above. Again, it was plated in the same pattern.

The substrate solution was prepared by thawing frozen assay buffer and substrate mix (Promega, Madison, WI) and adding 12ml of assay buffer to a bottle of substrate mix. The bottle was inverted and gently shaken, forming the reconstituted substrate mix. This reconstituted substrate mix (50µl) was then added to each of the wells to initiate the colorimetric

reaction. The plate was then incubated for 30 minutes at room temperature and hidden from light. After the incubation, 50µl of stop solution (Promega, Madison, WI) was added to each well to stop the reaction. The absorbance was read on the EL311 microplate reader at 490nm.

Triplicate observations were averaged to calculate a mean $\pm$ the standard error of the mean for spontaneous and total release for each group. The spontaneous release for each cell dilution was determined by subtracting the mean of the background wells from all wells containing cells. The average absorbance indicated the amount of LDH spontaneously released from 5,000 cells/100µl, 10,000 cells/100µl, and 20,000 cells/100µl. The optimal number of target cells to use in the experimental wells was calculated by dividing the average total release by the average background from each row. The optimal number was at least twice the absorbance of the background wells.

EXPERIMENTAL ASSAYS

The experimental assay involved incubating effector cells (NK cells) with target cells (cells to be killed). The mouse was anesthetized using either CO_2 or metaphane. Effector cells were taken from the spleen of a

mouse (splenocytes). This splenocyte population contained the mouse (murine) NK cells. Following respiratory failure, the diaphragm was cut, and the spleen removed. The spleen was placed in a petri dish containing RPMI 1640 media. The dish was kept on ice to prevent loss of NK activity. The spleen was then trimmed of excess fat. The splenocytes were removed by pressing the spleen through a metal sieve with a syringe plunger to disrupt the tissue. This was done over a petri dish to collect the cells in the media. The sieve and plunger were then rinsed with media over the petri dish to ensure maximum cell collection. The contents of the petri dish were transferred to a 50ml conical tube, and placed on ice to allow clumps to settle. After 5 minutes, all but the last 5-10ml of supernatant were removed and placed into another 50ml conical tube. The tube was centrifuged for 8 minutes at 400 x g at 4°C. The supernatant was removed and the pellet was resuspended in RPMI 1640 (no phenol red). This wash step was performed two more times to remove all phenol red. Following the final wash, the spleen cells were counted on a hemocytometer. A trypan blue solution was used to identify the live spleen cells. To 1ml of a 0.4% trypan blue solution, 5µl of acetic acid (lyses red blood cells) were added. This solution was

added 1 to 1 with a sample of spleen cells and the cells were counted. This method allowed the counting of only live effector cells due to the fact that dead cells appeared blue and were not counted. Once the cells were counted, they were suspended in media. The effector cells were then placed on ice until the plating step.

Target cells were counted on a hemocytometer to determine if enough cells were present for an assay (minimum of 2×10^5 cells/ml needed). Of these cells, 5ml were placed into a 15ml conical tube and centrifuged for 8 minutes at 400 x g. The supernatant was then aspirated, and the target cells were resuspended in 5ml of RPMI 1640 (no phenol red). This washing step was repeated two more times. Following the final wash step, the cells were recounted on the hemocytometer, and the cells adjusted with RPMI media (no phenol red) to achieve the optimal cell dilution (determined previously). The final target cell volume was 100μl. Along with the experimental wells, a number of control wells were used to determine the % target cell lysis, including target cell spontaneous release, target cell maximum release, culture medium background, volume correction control (media and 10 X lysis buffer), and effector cell spontaneous release. Cell lines known to be

sensitive (YAC-1, MPC II, Nulli-SCC) or insensitive (L1210, P815) to NK cell lysis were used as positive and negative controls, respectively. The experimental dilutions and controls were plated in triplicate in a 96-well round-bottom microplate (Fig. 2).

The target cells and effector cells were incubated together in the experimental wells at various effector cell to target cell ratios (E:T ratio). The E:T ratio indicated the number of effector cells per each target cell in the wells. The E:T ratios were as follows: 10:1, 5:1, 2.5:1, 1.25:1, 0.62:1, 0.31:1, 0.16:1, 0.08:1, 0.04:1, and 0.02:1. The microplate was incubated with a lid for 4 hours in a humidified chamber containing 5% CO_2 and at a temperature of 37°C. Following the 4 hour incubation, the plate was removed and centrifuged for 4 minutes at 250 x g. The supernatants from the wells were transferred to a 96-well flat-bottom microplate in the same pattern as above. This supernatant contained the LDH released from the target and effector cells. In order to test the supernatant for the presence of LDH, the reconstituted substrate mix was prepared as described above. Aliquots of 50µl of reconstituted substrate mix were added to each of the wells to initiate the colorimetric reaction. The plate was then incubated for

[a]T. S	[b]C . M . B .										
				[e]Effector Spontaneous							
[c]T. M	[d]V. C	10:1	5:1	2.5:1	1.25:1	.062:1	.31:1	.16:1	.08:1	.04:1	.02:1
		10:1	5:1	2.5:1	1.25:1	.062:1	.31:1	.16:1	.08:1	.04:1	.02:1
				[f]Experimental							
		10:1	5:1	2.5:1	1.25:1	.062:1	.31:1	.16:1	.08:1	.04:1	.02:1

30 minutes at room temperature and hidden from light. After the incubation, 50µl of stop solution was added to each well to stop the reaction. The absorbance of each well on the microplate was read on the EL311 microplate reader at 490nm. The triplicate wells were averaged, and the % target cell lysis was determined from the following formula:

$$\% \text{ cell lysis} = \frac{\text{experimental - effector spontaneous - target spontaneous}}{\text{target maximum - target spontaneous}} x100$$

RESULTS

The optimal number of target cells to be used in the experimental LDH assays was determined by measuring the total and spontaneous LDH release for each cell line. Spontaneous LDH release was measured after incubating each cell line for 4 or 24 hours at 37°C and 5% CO_2. Three trials were performed on each cell line with the exception of the L1210 24 hour spontaneous release group (only two trials were performed). Each trial contained triplicate wells with zero (blank), 5,000, 10,000, and 20,000 target cells/ 100µl. As previously noted, lysis buffer was used to cause total LDH release from the target cells. One Way Repeated Measures Analysis of Variance and the Student-Newman-Keuls Pairwise Multiple Comparison Procedure were used to determine significant differences.

TOTAL LDH RELEASE

For each cell line, the optimal number of target cells to be used in experimental assays is determined by comparing the average absorbance value for each cell concentration in the total LDH release wells to the average background absorbance (blank wells). The average absorbances for each cell concentration in each trial (performed in triplicate) were divided by

the average background absorbance. These values were averaged over three trials, and the cell concentration that gave a minimum value of two-fold higher absorbance than the background was determined to be the optimal cell concentration. The value which corresponds to the optimal cell number is described below and highlighted in red on each cell line total LDH release table (Tables 1, 2, 3, and 4).

MPC II

The data obtained from the three trials of MPC II total LDH release are shown in Table 1. A One Way Repeated Measures Analysis of Variance showed that the variation in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p<0.0001$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the absorbance values for 20,000 cells/ 100µl wells were significantly different from the 0, 5,000, and 10,000 cells/ 100µl wells ($p<0.05$). In addition, absorbance values for 10,000 cells/ 100µl wells were significantly different from the blank wells, but not the 5,000 cells/ 100µl wells. No significant difference was seen between the wells containing 5,000 cells and either the 10,000 cells/ 100µl

MPC II TOTAL LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.076	0.181	0.302	0.572
	0.074	0.238	0.304	0.589
	0.074	0.206	0.163	0.574
****Average Absorbance**	**0.075**	**0.208**	**0.256**	**0.578**
Average Absorbance/Background		**2.790**	**3.433**	**7.746**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.093	0.143	0.222	0.33
	0.091	0.145	0.105	0.332
	0.094	0.143	0.204	0.334
****Average Absorbance**	**0.093**	**0.144**	**0.177**	**0.332**
Average Absorbance/Background		**1.550**	**1.910**	**3.583**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.089	0.112	0.146	0.191
	0.085	0.11	0.12	0.194
	0.099	0.113	0.143	0.229
****Average Absorbance**	**0.091**	**0.112**	**0.136**	**0.205**
Average Absorbance/Background		**1.227**	**1.498**	**2.249**

3 Trial Average		**1.856**	**2.280**	**4.526**

* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm
red indicates optimal cell number

Table 1

wells or the blank wells. The three trial average absorbance/background for each cell concentration were 1.855 (± 0.028 SEM) for 5,000 cells/ 100µl, 2.280 (± 0.035 SEM) for 10,000 cells/ 100µl, and 4.525 (± 0.110 SEM) for 20,000 cells/ 100µl. The optimal cell number for MPC II was determined to be 10,000 cells/ 100µl.

YAC-1

The data obtained from the three trials of YAC-1 total LDH release are shown in Table 2. A One Way Repeated Measures Analysis of Variance showed that the variation in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p<0.0001$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 0, 5,000, 10,000, and 20,000 cells/ 100µl wells all showed significant differences from each other ($p<0.05$). The three trial average absorbance/background for each cell concentration were 2.111 (± 0.002 SEM) for 5,000 cells/ 100µl, 3.273 (± 0.004 SEM) for 10,000 cells/ 100µl, and 5.587 (± 0.005 SEM) for 20,000 cells/ 100µl. The optimal cell number for YAC-1 was determined to be 5,000 cells/ 100µl.

YAC-1 TOTAL LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.078	0.148	0.228	0.37
	0.084	0.152	0.239	0.382
	0.077	0.154	0.234	0.392
****Average Absorbance**	**0.080**	**0.151**	**0.234**	**0.381**
Average Absorbance/Background		**1.900**	**2.933**	**4.787**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.078	0.197	0.299	0.502
	0.079	0.196	0.299	0.531
	0.082	0.198	0.314	0.545
****Average Absorbance**	**0.080**	**0.197**	**0.304**	**0.526**
Average Absorbance/Background		**2.473**	**3.816**	**6.603**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.08	0.156	0.241	0.396
	0.075	0.154	0.238	0.427
	0.084	0.159	0.255	0.461
****Average Absorbance**	**0.080**	**0.156**	**0.245**	**0.428**
Average Absorbance/Background		**1.962**	**3.071**	**5.372**

3 Trial Average		**2.112**	**3.273**	**5.587**

* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm
red indicates optimal cell number

Table 2

Table 3 indicates the data obtained from the three trials of P815 total LDH release. A One Way Repeated Measures Analysis of Variance showed that the variation in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p<0.0001$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 0, 5,000, 10,000, and 20,000 cells/ 100µl wells all showed significant differences from each other ($p<0.05$). The three trial average absorbance/background for each cell concentration were 2.204 for ($\pm$ 0.016 SEM) 5,000 cells/ 100µl, 3.291 ($\pm$ 0.042 SEM) for 10,000 cells/ 100µl, and 4.827 ($\pm$ 0.066 SEM) for 20,000 cells/ 100µl. The optimal cell number for P815 was determined to be 5,000 cells/ 100µl.

L1210

The data obtained from the three trials of L1210 total LDH release are shown in Table 4. A One Way Repeated Measures Analysis of Variance showed that the variation in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p = 0.0205$). The Student –Newman

P815 TOTAL LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.114	0.298	0.486	0.661
	0.116	0.295	0.46	0.747
	0.114	0.269	0.462	0.654
****Average Absorbance**	**0.115**	**0.287**	**0.469**	**0.687**
Average Absorbance/Background		**2.506**	**4.093**	**5.994**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.117	0.218	0.33	0.46
	0.123	0.221	0.331	0.403
	0.124	0.252	0.347	0.518
****Average Absorbance**	**0.121**	**0.230**	**0.336**	**0.460**
Average Absorbance/Background		**1.898**	**2.769**	**3.794**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.112	0.229	0.303	0.502
	0.122	0.267	0.381	0.58
	0.117	0.279	0.373	0.566
****Average Absorbance**	**0.117**	**0.258**	**0.352**	**0.549**
Average Absorbance/Background		**2.208**	**3.011**	**4.695**

3 Trial Average		**2.204**	**3.291**	**4.828**

* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm
red indicates optimal cell number

Table 3

L1210 TOTAL LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.191	0.246	0.278	0.29
	0.189	0.259	0.26	0.33
	0.17	0.175	0.225	0.368
****Average Absorbance**	**0.183**	**0.227**	**0.254**	**0.329**
Average Absorbance/Background		**1.236**	**1.387**	**1.796**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.095	0.195	0.313	0.623
	0.085	0.175	0.21	0.595
	0.088	0.189	0.301	0.611
****Average Absorbance**	**0.089**	**0.186**	**0.275**	**0.610**
Average Absorbance/Background		**2.086**	**3.075**	**6.825**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.155	0.434	0.35	0.425
	0.107	0.281	0.283	0.367
	0.134	0.279	0.238	0.415
****Average Absorbance**	**0.132**	**0.331**	**0.290**	**0.402**
Average Absorbance/Background		**2.510**	**2.199**	**3.048**

3 Trial Average		**1.944**	**2.220**	**3.890**

* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm
red indicates optimal cell number

Table 4

Keuls Multiple Comparison Procedure reported that the 20,000 cells/ 100µl wells were significantly different from the 0, 5,000, and 10,000 cells/ 100µl wells ($p<0.05$). In addition, 10,000 cells/ 100µl wells were not significantly different from the blank wells. No significant difference was seen between the absorbance values for the 5,000 wells and either the 10,000 cells/ 100µl wells or the blank wells. The three trial average absorbance/background for each cell concentration were 1.944 for ($\pm$ 0.043 SEM) 5,000 cells/ 100µl, 2.220 ($\pm$ 0.010 SEM) for 10,000 cells/ 100µl, and 3.889 ($\pm$ 0.084 SEM) for 20,000 cells/ 100µl. The optimal cell number for L1210 was determined to be 10,000 cells/ 100µl.

4 & 24 HOUR SPONTANEOUS LDH RELEASE

The 4 hour and 24 hour spontaneous release trials were used to characterize the amount of LDH released from the target cell lines in the absence of cell death. For each cell line, the average background absorbance (blank wells) was subtracted from the average absorbance (n=3) of the supernatant from the cell-containing wells. This data was used to determine the spontaneous LDH released over 4 hour and 24 hour incubation periods. The averages of the total, 4 hour spontaneous, and 24 hour spontaneous

LDH data from each cell line were plotted together (see Figures 3-6).

MPC II

The 4 hour spontaneous release data for MPC II is seen in Table 5. The three trial averages for the average absorbance-background were 0.005 (± 0.002 SEM) for 5,000 cells/ 100µl, 0.014 (± 0.010 SEM) for 10,000 cells/ 100µl, and 0.043 (± 0.038 SEM) for 20,000 cells/ 100µl. A One Way Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p = 0.0168$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 20,000 cells/ 100µl wells were significantly different from the 0, 5,000, and 10,000 cells/ 100µl wells ($p<0.05$). The 10,000 and 5,000 cells/ 100µl wells showed no significant difference from each other or the blank wells.

The 24 hour spontaneous release data for MPC II is shown in Table 6. The three trial averages for the average absorbance-background were 1.350 (± 0.006 SEM) for 5,000 cells/ 100µl, 1.588 (± 0.017 SEM) for 10,000 cells/ 100µl, and 2.591 (± 0.069 SEM) for 20,000 cells/ 100µl. A One Way

MPC II 4HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.076	0.095	0.128	0.208
	0.067	0.09	0.107	0.176
	0.069	0.088	0.111	0.214
****Average Absorbance**	**0.071**	**0.091**	**0.115**	**0.199**
Average Absorbance-Background		**0.020**	**0.045**	**0.129**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.085	0.085	0.084	0.089
	0.088	0.085	0.084	0.087
	0.087	0.083	0.084	0.08
****Average Absorbance**	**0.087**	**0.084**	**0.084**	**0.085**
Average Absorbance-Background		**-0.002**	**-0.003**	**-0.001**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.086	0.084	0.086	0.091
	0.084	0.084	0.087	0.086
	0.085	0.085	0.085	0.087
****Average Absorbance**	**0.085**	**0.084**	**0.086**	**0.088**
Average Absorbance-Background		**-0.001**	**0.001**	**0.003**

3 Trial Average		**0.006**	**0.014**	**0.043**

* 100µl media and 10µl lysis buffer

** Absorbance read at 490nm

Table 5

MPC II 24HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.069	0.123	0.163	0.304
	0.071	0.116	0.147	0.315
	0.066	0.115	0.159	0.378
****Average Absorbance**	**0.069**	**0.118**	**0.156**	**0.332**
Average Absorbance-Background		**0.049**	**0.088**	**0.264**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.087	0.099	0.113	0.133
	0.084	0.094	0.115	0.13
	0.083	0.097	0.11	0.144
****Average Absorbance**	**0.085**	**0.097**	**0.113**	**0.136**
Average Absorbance-Background		**0.012**	**0.028**	**0.051**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.087	0.132	0.103	0.116
	0.086	0.09	0.099	0.115
	0.088	0.089	0.1	0.117
****Average Absorbance**	**0.087**	**0.104**	**0.101**	**0.116**
Average Absorbance-Background		**0.017**	**0.014**	**0.029**

3 Trial Average		**0.026**	**0.043**	**0.115**

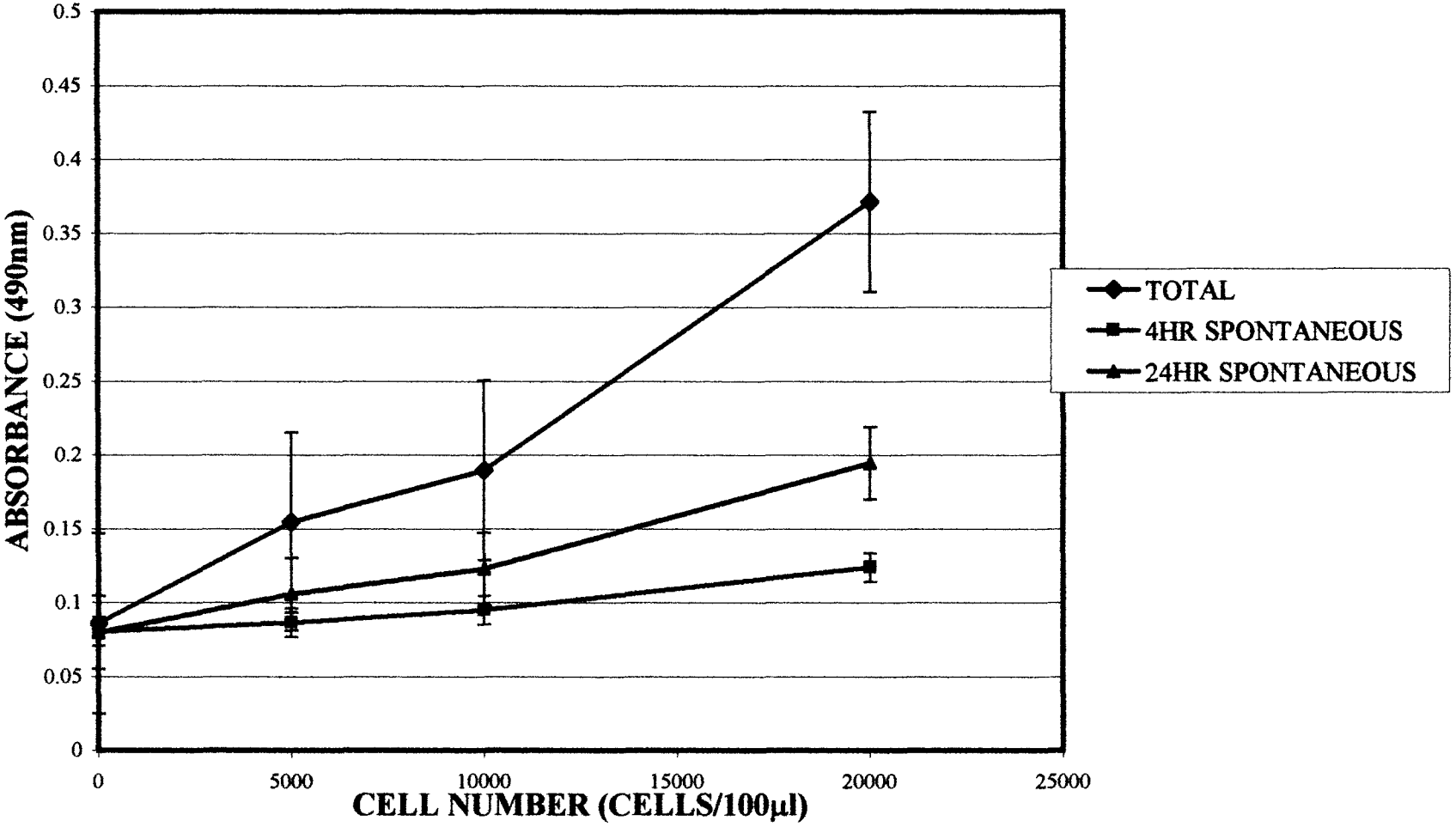
* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm

Table 6

Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p<0.001$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 20,000 cells/ 100µl wells were significantly different from the 0, 5,000, and 10,000 cells/ 100µl wells ($p<0.05$). The absorbance values for the 10,000 and 5,000 cells/ 100µl wells showed no significant difference from each other or the blank wells. Figure 3 shows the three trial average absorbance values for total, 4 hour, and 24 hour spontaneous LDH release.

YAC-1

The 4 hour spontaneous release data for YAC-1 is shown in Table 7. The three trial averages for the average absorbance-background were –0.001 ($\pm$ 0.002 SEM) for 5,000 cells/ 100µl, -0.002 ($\pm$ 0.004 SEM) for 10,000 cells/ 100µl, and –0.004 ($\pm$ 0.005 SEM) for 20,000 cells/ 100µl. A One Way Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p = 0.00607$). The Student –Newman-Keuls Multiple Comparison


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ABSORBANCE (490nm)
0
5000
10000
15000
20000
25000
CELL NUMBER (CELLS/100µl)
TOTAL
4HR SPONTANEOUS
24HR SPONTANEOUS

YAC-1 4HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.076	0.074	0.076	0.08
	0.077	0.077	0.078	0.078
	0.076	0.075	0.082	0.08
****Average Absorbance**	**0.076**	**0.075**	**0.079**	**0.079**
Average Absorbance-Background		**-0.001**	**0.002**	**0.003**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.077	0.076	0.076	0.087
	0.086	0.08	0.081	0.092
	0.079	0.082	0.08	0.092
****Average Absorbance**	**0.081**	**0.079**	**0.079**	**0.090**
Average Absorbance-Background		**-0.001**	**-0.002**	**0.010**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.082	0.073	0.069	0.081
	0.071	0.077	0.067	0.068
	0.072	0.068	0.064	0.075
****Average Absorbance**	**0.075**	**0.073**	**0.067**	**0.075**
Average Absorbance-Background		**-0.002**	**-0.008**	**0.000**

3 Trial Average		**-0.002**	**-0.003**	**0.004**

* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm

Table 7

Procedure reported that the 20,000 cells/ 100μl wells were significantly different from the 0, 5,000, and 10,000 cells/ 100μl wells ($p<0.05$). The 10,000 and 5,000 cells/ 100μl wells showed no significant difference from each other or the blank wells.

The 24 hour spontaneous release data for YAC-1 is shown in Table 8. The three trial averages for the average absorbance-background were 0.025 ($\pm$ 0.015 SEM) for 5,000 cells/ 100μl, 0.035 ($\pm$ 0.009 SEM) for 10,000 cells/ 100μl, and 0.078 ($\pm$ 0.022 SEM) for 20,000 cells/ 100μl. A One Way Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p<0.0001$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 20,000 cells/ 100μl wells were significantly different from the 0, 5,000, and 10,000 cells/ 100μl wells ($p<0.05$). The 10,000 cells/ 100μl wells were significantly different from the blank wells ($p<0.05$). The 10,000 cells/ 100μl wells and 5,000 cells/ 100μl wells showed no significant difference from each other, and the 5,000 cells/ 100μl showed no significant difference from the blank wells. Figure 4 shows the three trial average

YAC-1 24HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.077	0.109	0.113	0.131
	0.084	0.166	0.114	0.137
	0.076	0.112	0.123	0.139
****Average Absorbance**	**0.079**	**0.129**	**0.117**	**0.136**
Average Absorbance-Background		**0.050**	**0.038**	**0.057**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.078	0.109	0.136	0.213
	0.081	0.11	0.123	0.208
	0.111	0.123	0.14	0.194
****Average Absorbance**	**0.090**	**0.114**	**0.133**	**0.205**
Average Absorbance-Background		**0.024**	**0.043**	**0.115**

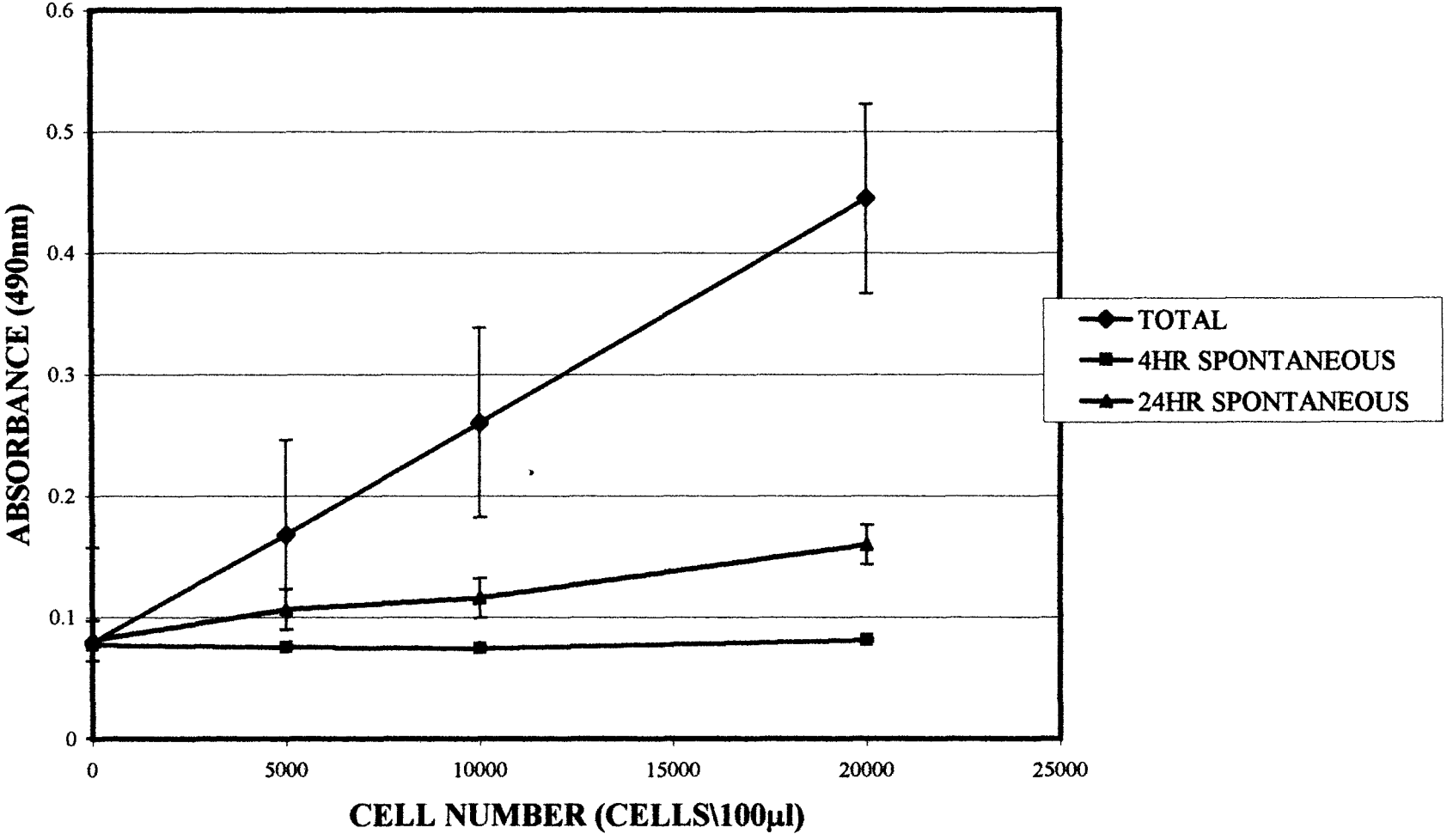
TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.07	0.075	0.098	0.142
	0.073	0.08	0.106	0.129
	0.082	0.079	0.094	0.149
****Average Absorbance**	**0.075**	**0.078**	**0.099**	**0.140**
Average Absorbance-Background		**0.003**	**0.024**	**0.065**

3 Trial Average		**0.026**	**0.035**	**0.079**

* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm

Table 8


0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (490nm)
0
5000
10000
15000
20000
25000
CELL NUMBER (CELLS\100µl)
TOTAL
4HR SPONTANEOUS
24HR SPONTANEOUS

absorbance values for total, 4 hour, and 24 hour spontaneous LDH release.

P815

The 4 hour spontaneous release data for P815 is shown in Table 9. The three trial averages for the average absorbance-background were –0.001 (± 0.008 SEM) for 5,000 cells/ 100µl, 0.013 (± 0.012 SEM) for 10,000 cells/ 100µl, and 0.039 (± 0.023 SEM) for 20,000 cells/ 100µl. A One Way Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p<0.0001$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 20,000 cells/ 100µl wells were significantly different from the 0, 5,000, and 10,000 cells/ 100µl wells ($p<0.05$). The 10,000 and 5,000 cells/ 100µl wells showed no significant difference from each other or the blank wells.

The 24 hour spontaneous release for P815 is shown in Table 10. The three trial averages for the average absorbance-background were 0.065 (± 0.009 SEM) for 5,000 cells/ 100µl, 0.246 (± 0.039 SEM) for 10,000 cells/ 100µl, and 0.459 (± 0.019 SEM) for 20,000 cells/ 100µl. A One Way

P815 4HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.09	0.077	0.183	0.177
	0.087	0.088	0.094	0.15
	0.093	0.084	0.095	0.137
****Average Absorbance**	**0.090**	**0.083**	**0.124**	**0.155**
Average Absorbance-Background		**-0.007**	**0.034**	**0.065**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.107	0.11	0.122	0.168
	0.105	0.108	0.11	0.142
	0.107	0.107	0.116	0.165
****Average Absorbance**	**0.106**	**0.108**	**0.116**	**0.158**
Average Absorbance-Background		**0.002**	**0.010**	**0.052**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.084	0.087	0.084	0.084
	0.087	0.085	0.083	0.092
	0.087	0.084	0.08	0.089
****Average Absorbance**	**0.086**	**0.085**	**0.082**	**0.088**
Average Absorbance-Background		**-0.001**	**-0.004**	**0.002**

3 Trial Average		**-0.002**	**0.013**	**0.040**

* 100µl media and 10µl lysis buffer

** Absorbance read at 490nm

Table 9

P815 24HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.09	0.184	0.348	0.492
	0.091	0.153	0.495	0.514
	0.085	0.194	0.405	0.539
****Average Absorbance**	**0.089**	**0.177**	**0.416**	**0.515**
Average Absorbance-Background		**0.088**	**0.327**	**0.426**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.101	0.138	0.305	0.528
	0.104	0.145	0.315	0.516
	0.107	0.15	0.242	0.654
****Average Absorbance**	**0.104**	**0.144**	**0.287**	**0.566**
Average Absorbance-Background		**0.040**	**0.183**	**0.462**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.088	0.159	0.228	0.604
	0.086	0.138	0.316	0.534
	0.084	0.167	0.402	0.592
****Average Absorbance**	**0.086**	**0.155**	**0.315**	**0.577**
Average Absorbance-Background		**0.069**	**0.229**	**0.491**

3 Trial Average		**0.066**	**0.247**	**0.460**

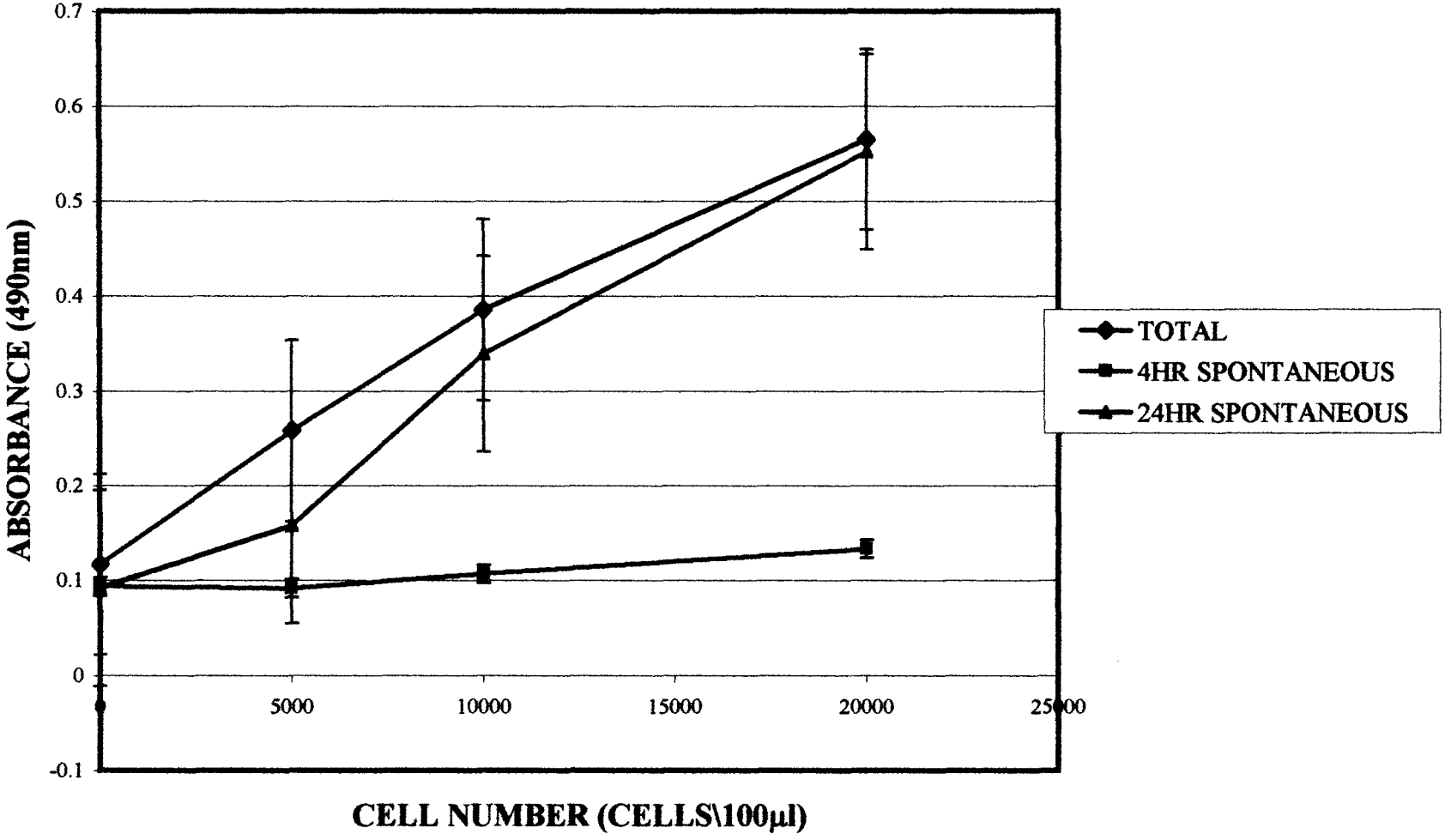
* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm

Table 10

Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p < .0001$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 0, 5,000, 10,000, and 20,000 cells/ 100µl wells all showed significant differences from each other ($p<0.05$). Figure 5 shows the three trial average absorbance values for total, 4 hour, and 24 hour spontaneous LDH release. The high amount of spontaneous LDH release observed in the 24 hour incubation group indicated that the P815 cell line could not be used in long term assays.

L1210

The 4 hour spontaneous release data for L1210 is shown in Table 11. The three trial averages for the average absorbance-background were –0.003 (± 0.002 SEM) for 5,000 cells/ 100µl, -0.003 (± 0.000 SEM) for 10,000 cells/ 100µl, and 0.000 (± 0.006 SEM) for 20,000 cells/ 100µl. A One Way Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were not great enough to exclude the possibility that the difference was due to random


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
ABSORBANCE (490nm)
5000
10000
15000
20000
25000
CELL NUMBER (CELLS\100µl)
TOTAL
4HR SPONTANEOUS
24HR SPONTANEOUS

L1210 4HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.091	0.09	0.092	0.081
	0.096	0.092	0.098	0.094
	0.093	0.095	0.09	0.098
****Average Absorbance**	**0.093**	**0.092**	**0.093**	**0.091**
Average Absorbance-Background		**-0.001**	**0.000**	**-0.002**

TRIAL 2

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.091	0.09	0.092	0.081
	0.096	0.092	0.098	0.094
	0.093	0.095	0.09	0.098
****Average Absorbance**	**0.093**	**0.092**	**0.093**	**0.091**
Average Absorbance-Background		**-0.001**	**0.000**	**-0.002**

TRIAL 3

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.128	0.098	0.101	0.109
	0.098	0.098	0.095	0.118
	0.088	0.098	0.089	0.102
****Average Absorbance**	**0.105**	**0.098**	**0.095**	**0.110**
Average Absorbance-Background		**-0.007**	**-0.010**	**0.005**

3 Trial Average		**-0.003**	**-0.003**	**0.000**

* 100µl media and 10µl lysis buffer
** Absorbance read at 490nm

Table 11

sampling variability. There was not a statistically significant difference ($p = 0.630$).

The 24 hour spontaneous release data for L1210 is shown in Table 12. The two trial averages for the average absorbance-background were 0.016 ($\pm$ 0.017 SEM) for 5,000 cells/ 100µl, 0.0175 ($\pm$ 0.014 SEM) for 10,000 cells/ 100µl, and 0.040667 ($\pm$ 0.039 SEM) for 20,000 cells/ 100µl. A One Way Repeated Measures Analysis of Variance showed that the differences in the mean absorbance values among the different cell concentrations were greater than would be expected by chance, with a statistically significant difference ($p<0.05$). The Student –Newman-Keuls Multiple Comparison Procedure reported that the 20,000 cells/ 100µl wells were significantly different only from the blank wells ($p<0.05$). No other groups compared exhibited significant differences. Again, this group of data only contained only two trials, which may have contributed to this lack of significant difference. Figure 6 shows the three trial average absorbance values for total, 4 hour, and 24 hour spontaneous LDH release.

L1210 24HR SPONTANEOUS LDH RELEASE

TRIAL 1

Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.091	0.085	0.098	0.095
	0.09	0.093	0.092	0.095
	0.098	0.096	0.097	0.094
****Average Absorbance**	**0.093**	**0.091**	**0.096**	**0.095**
Average Absorbance-Background		**-0.002**	**0.003**	**0.002**

TRIAL 2

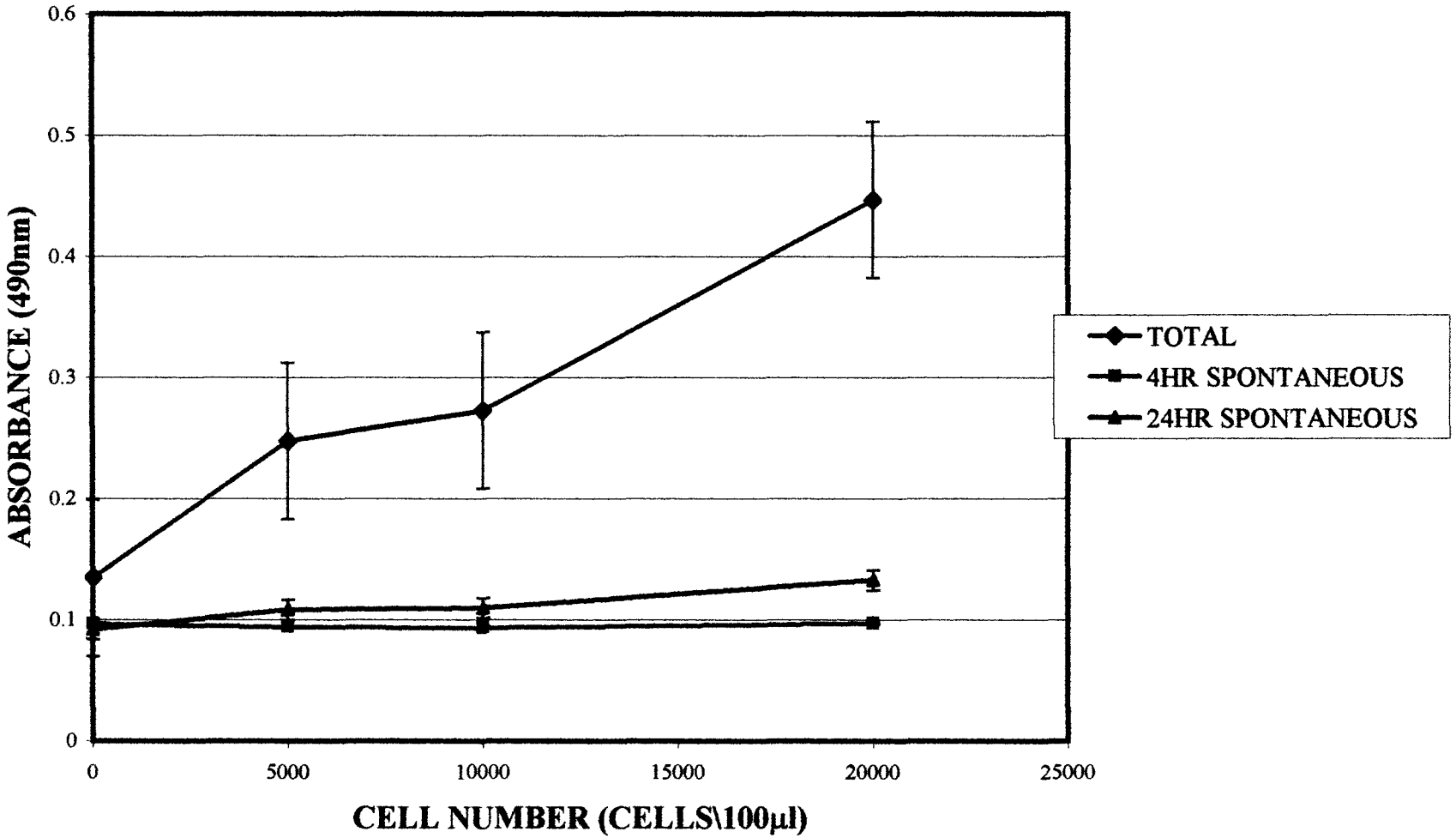
Cell Number (Cells/100µl)	*BLANK	5,000	10,000	20,000
	0.089	0.12	0.111	0.165
	0.093	0.125	0.124	0.167
	0.094	0.132	0.138	0.183
****Average Absorbance**	**0.092**	**0.126**	**0.124**	**0.172**
Average Absorbance-Background		**0.034**	**0.032**	**0.080**

2 Trial Average		**0.016**	**0.018**	**0.041**

* 100µl media and 10µl lysis buffer

** Absorbance read at 490nm

Table 12


0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (490nm)
0
5000
10000
15000
20000
25000
CELL NUMBER (CELLS\100µl)
TOTAL
4HR SPONTANEOUS
24HR SPONTANEOUS

EXPERIMENTAL ASSAYS

Experimental assays involved incubating murine NK cells (effector cells) along with target cells and measuring the amount of LDH released from the target cells using the Cytotox 96 LDH assay. These experimental assays were performed to determine if the LDH assay could be used to measure murine NK cell cytotoxicity. Experimental assays were performed on three cell lines: P815, MPC II, and YAC-1. As previously stated, the MPC II and YAC-1 cell lines are positive controls and the P815 cell line is a negative control cell line. The positive control cell lines are specifically targeted for destruction by murine NK cells, while the negative control cell lines are not targeted for lysis. Because of the high amount of background interference, only the highest three E:T ratios were used (although ten E:T ratios were actually performed in each experiment).

The absorbance values and calculated percent lysis for P815 are shown in Table 13. From the data, only one E:T ratio (10:1) resulted in detectable % cytotoxicity (0.370). Table 14 shows the data from the MPC II experimental assay. This also has only one E:T ratio that resulted in detectable % cytotoxicity (25.792). Finally, two experiments were performed on the YAC-1 cell line. Table 15 shows the data from one of

P815 Experimental Assay (5,000 cells/ 100µl)

E:T Ratio									
10:1	**5:1**	**2.5:1**	**1.25:1**	**.62:1**	**.31:1**	**.16:1**	**.08:1**	**.04:1**	**.02:1**
E.S.									
0.097	0.083	0.080	0.076	0.074	0.074	0.070	0.073	0.070	0.074
0.097	0.084	0.078	0.074	0.071	0.073	0.072	0.071	0.072	0.072
0.100	0.084	0.077	0.075	0.071	0.073	0.070	0.072	0.072	0.071
AVG.									
0.098	**0.084**	**0.078**	**0.075**	**0.072**	**0.073**	**0.071**	**0.072**	**0.071**	**0.072**
E.S.-BACK.									
0.024	**0.010**	**0.005**	**0.001**	**-0.002**	**0.000**	**-0.003**	**-0.002**	**-0.002**	**-0.001**
EXP.									
0.135	0.113	0.110	0.103	0.111	0.104	0.103	0.105	0.103	0.109
0.136	0.119	0.111	0.112	0.107	0.106	0.106	0.109	0.107	0.113
0.138	0.116	0.114	0.109	0.107	0.104	0.105	0.110	0.106	0.107
AVG.									
0.136	**0.116**	**0.112**	**0.108**	**0.108**	**0.105**	**0.105**	**0.108**	**0.105**	**0.110**
EXP.-BACK.									
0.063	**0.042**	**0.038**	**0.034**	**0.035**	**0.031**	**0.031**	**0.034**	**0.032**	**0.036**

E:T Ratio	% LYSIS	T.M.	BACK.	V.C.
10:1	**0.370**	0.301	0.075	0.082
5:1	**-2.957**	0.299	0.072	0.080
2.5:1	**-2.403**	0.297	0.074	0.081
1.25:1	**-2.588**	**0.299**	**0.074**	**0.081**
0.62:1	**-0.739**			
0.31:1	**-3.512**	T.S.	T.S.-BACK.	T.M.-V.C.
0.16:1	**-2.033**	0.113	0.038	0.218
0.08:1	**-0.924**	0.108		
0.04:1	**-2.033**	0.113		
0.02:1	**-0.185**	**0.111**		

T.S. Target Cell Spontaneous Release Wells
T.M. Target Cell Maximum Release Wells
Back. Background Wells
V.C. Volume Control Wells
E.S. Effector Spontaneous Wells
EXP. Experimental Wells
AVG. Average
E:T Ratio # Effector Cells per Target Cell
% Lysis Calculated from formula in materials and methods
red indicates the E:T ratios used in analyses

Table 13

MPCII Experimental Assay (10,000 cells/ 100µl)

E:T Ratio									
10:1	**5:1**	**2.5:1**	**1.25:1**	**.62:1**	**.31:1**	**.16:1**	**.08:1**	**.04:1**	**.02:1**
E.S.									
0.100	0.070	0.066	0.067	0.068	0.067	0.061	0.066	0.071	0.066
0.102	0.070	0.063	0.064	0.067	0.066	0.062	0.066	0.068	0.067
0.108	0.070	0.063	0.063	0.065	0.063	0.065	0.063	0.066	0.062
AVG.									
0.103	**0.070**	**0.064**	**0.065**	**0.067**	**0.065**	**0.063**	**0.065**	**0.068**	**0.065**
E.S.-BACK.									
0.039	**0.006**	**0.000**	**0.001**	**0.003**	**0.001**	**-0.001**	**0.001**	**0.004**	**0.001**
EXP.									
0.143	0.076	0.064	0.065	0.065	0.065	0.072	0.071	0.069	0.067
0.130	0.083	0.069	0.066	0.069	0.071	0.066	0.074	0.073	0.071
0.142	0.088	0.075	0.076	0.074	0.071	0.073	0.078	0.114	0.078
AVG.									
0.138	**0.082**	**0.069**	**0.069**	**0.069**	**0.069**	**0.070**	**0.074**	**0.085**	**0.072**
EXP.-BACK.									
0.074	**0.018**	**0.005**	**0.005**	**0.005**	**0.005**	**0.006**	**0.010**	**0.021**	**0.008**

E:T Ratio	% LYSIS	T.M.	BACK.	V.C.
10:1	**25.792**	0.158	0.064	0.072
5:1	**-4.977**	0.167	0.064	0.067
2.5:1	**-14.480**	0.154	0.064	0.071
1.25:1	**-15.837**	**0.160**	**0.064**	**0.070**
0.62:1	**-18.100**			
0.31:1	**-16.742**	T.S.	T.S.-BACK.	T.M.-V.C.
0.16:1	**-11.312**	0.066	0.016	0.090
0.08:1	**-9.050**	0.067		
0.04:1	**1.357**	0.107		
0.02:1	**-12.217**	**0.080**		

T.S. Target Cell Spontaneous Release Wells
T.M. Target Cell Maximum Release Wells
Back. Background Wells
V.C. Volume Control Wells
E.S. Effector Spontaneous Wells
EXP. Experimental Wells
AVG. Average
E:T Ratio # Effector Cells per Target Cell
% Lysis Calculated from formula in materials and methods
red indicates the E:T ratios used in analyses

Table 14

YAC-1 Experimental Assay (10,000 cells/ 100µl)

E:T Ratio	10:1	5:1	2.5:1	1.25:1	.62:1	.31:1	.16:1	.08:1	.04:1	.02:1
	E.S.									
	0.185	0.131	0.108	0.094	0.09	0.081	0.073	0.07	0.068	0.075
	0.192	0.125	0.104	0.095	0.088	0.08	0.075	0.068	0.07	0.063
	0.181	0.126	0.102	0.091	0.087	0.084	0.111	0.065	0.062	0.066
AVG.	**0.186**	**0.127333**	**0.104667**	**0.093333**	**0.0883333**	**0.081667**	**0.086333**	**0.067667**	**0.066667**	**0.068**
	E.S.-BACK.									
	0.109666667	**0.051**	**0.028333**	**0.017**	**0.012**	**0.005333**	**0.01**	**-0.00867**	**-0.00967**	**-0.00833**
	EXP.									
	0.204	0.146	0.12	0.106	0.105	0.102	0.097	0.087	0.088	0.093
	0.21	0.152	0.115	0.108	0.108	0.106	0.081	0.088	0.083	0.099
	0.211	0.153	0.115	0.108	0.105	0.1	0.094	0.094	0.092	0.094
AVG.	**0.208333333**	**0.150333**	**0.116667**	**0.107333**	**0.106**	**0.102667**	**0.090667**	**0.089667**	**0.087667**	**0.095333**
	EXP.-BACK.									
	0.132	**0.074**	**0.040333**	**0.031**	**0.0296667**	**0.026333**	**0.014333**	**0.013333**	**0.011333**	**0.019**

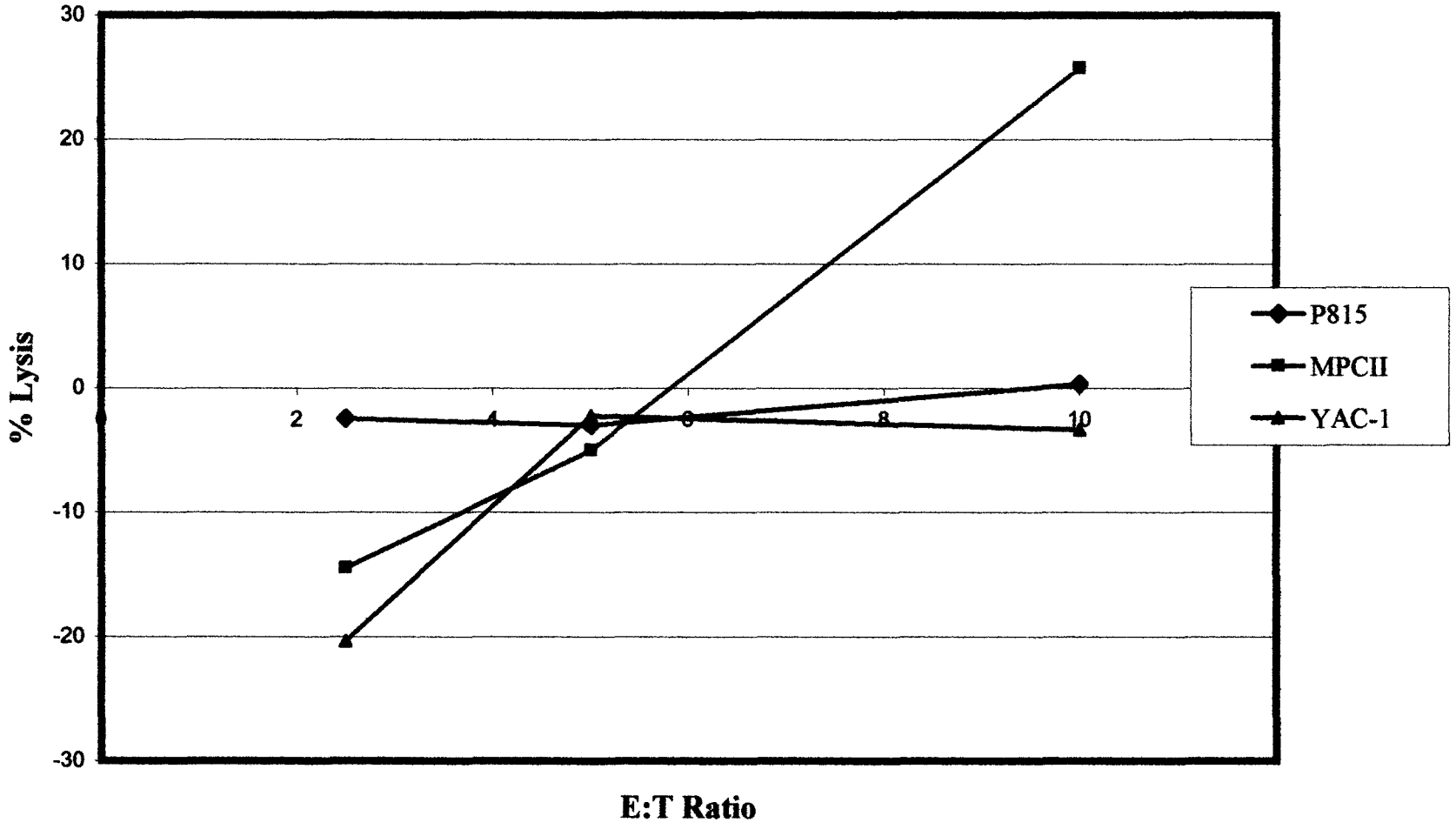
E:T Ratio	% LYSIS	T.M.	BACK.	V.C.
10:01	-3.2967	0.17	0.079	0.084
5:01	-2.1978	0.173	0.075	0.082
2.5:1	-20.3297	0.165	0.075	0.087
1.25:1	-17.033	0.169333	0.0763333	0.084333
0.62:1	-10.989			
0.31:1	-5.49451	T.S.	T.S.-BACK.	T.M.-V.C.
0.16:1	-32.967	0.097	0.0243333	0.085
0.08:1	-3.84615	0.102		
0.04:1	-5.49451	0.103		
0.02:1	4.945055	0.100667		

T.S. Target Cell Spontaneous Release Wells
T.M. Target Cell Maximum Release Wells
Back. Background Wells
V.C. Volume Control Wells
E.S. Effector Spontaneous Wells
E:T Ratio # Effector Cells per Target Cell
% Lysis Calculated from formula in materials and methods
red indicates the E:T ratios used in analyses

Table 15

these experiments. Neither experiment had E:T ratios with a detectable % cytotoxicity. Figure 7 presents the graphical representation (absorbance vs. E:T ratio) of the highest three E:T ratios from the MPC II, YAC-1, and P815 cell lines.

Two Way Analyses of Variance were performed to compare the statistical significance of two of the experimental assay data of the cell lines. When comparing P815 vs. YAC-1, MPC II vs. YAC-1, and P815 vs. MPC II, in all cases the difference in the mean values among the different levels of cell types were not great enough to exclude the possibility that the differences were just due to random sampling variability after allowing for the effects of differences in E:T ratios. There was no significant differences ($p = 0.542$). Also, the differences in the mean values among the different levels of E:T ratios were not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in cell types. There was no significant differences ($p = 0.203$).


30
20
10
0
-10
-20
-30
% Lysis
2
4
6
8
10
E:T Ratio
P815
MPCII
YAC-1

DISCUSSION

Natural killer cells are a type of lymphocyte that causes cell-mediated immunity. NK cells originate in the bone marrow but, unlike T cells, do not require the thymus for maturation. These NK cells are capable of spontaneously lysing tumor and virus-infected cells without previous sensitization to antigens. This makes them dissimilar from B and T cells. NK cells are constitutively cytotoxic, as they constantly express cytotoxic granules in their cytoplasm, allowing NK cells to immediately lyse specific target cells. Because they can recognize altered-self cells, NK cells are able to eliminate tumor and virus-infected cells prior to the development of a T cell response. NK cells are the first lines of defense against virus-infected cells while a T cell response is being generated.

NK cells possess an array of receptors, costimulatory molecules, and adhesion molecules that enable them to distinguish between normal and altered self-cells. Upon adhering to a target cell, the receptors and costimulatory molecules regulate the cytotoxic response of the NK cell. Their activity is also regulated by the presence of various cytokines such as interleukins and interferons. In addition to regulating NK cells, cytokines

cause T cells and lymphokine activated killer (LAK) cells to alter their normal basal functions.

Humans and mice both possess immune systems that contain NK cells, although mouse (murine) NK cells have not been as thoroughly studied as human NK cells. While human and murine NK cells have similarities, they differ in their cell receptors, surface markers, and target cells (cells they will kill). Human NK cell target cells include the K562 erythroleukemia and A375 melanoma cell lines. Murine NK target cells include the Nulli-SCC teratocarcinoma, L1210 lymphocytic leukemia, P815 mastocytoma, Wehi 164 fibrosarcoma, MPC II myeloma, and YAC-1 lymphoma cell lines. Of these mouse NK targets, some are targeted for and some are insensitive to NK cell lysis. The L1210 and P815 cell lines are insensitive to lysis by murine NK cells whereas the YAC-1, MPC II, and Nulli-SCC cell lines are targeted for lysis.

In order to determine if NK cells are exhibiting a lytic response towards target cells, cytotoxicity assays must be used. A cytotoxicity assay will determine if effector cells, in our case NK cells, are causing the destruction of particular target cells. A variety of these cytotoxicity assays exist. Time-resolved fluorometry, Cr-release, and DNA fragmentation

ELISA cytotoxicity assays involve pre-labeling target cells and measuring the labeled substance's release into the supernatant upon target cell lysis.

Another method, which is used in this study, is the lactate dehydrogenase (LDH) assay. The LDH assay is an enzymatic release cytotoxicity assay. LDH is a stable cytosolic enzyme. When target cells become lysed, the LDH contained in their cytoplasm is released into the cellular supernatant. The supernatant is then incubated with a substrate for the enzyme, which causes a red formazan product to be formed. The colorimetric reaction is then stopped with an enzyme inhibitor and the absorbance is read at 490nm on a standard 96-well microplate reader. The absorbance reading is proportional to the number of lysed cells. The advantage of the LDH assay is that it does not use harmful reagents or radioactive materials and is a safe and convenient means to measure cell-mediated cytotoxicity.

When performing an LDH assay, certain factors must be taken into account for determining the amount of target cell lysis. The media used to grow target cells may contain LDH and the effector and target cells may spontaneously release LDH. Control measurements performed to assess these factors include: effector cell spontaneous LDH release, target cell spontaneous LDH release, target cell maximum LDH release, culture

medium background, and volume correction controls. These control measurements, along with the absorbance obtained from incubating target and effector cells, can be used to determine the percent target cell lysis.

Although the LDH assay is a convenient and safe cytotoxicity assay, it has not been used significantly in measuring NK cell cytotoxicity. In an article published in the *Journal of Immunological Methods* in 1983, Korzeniewski and Callewaert first demonstrated the use of an LDH assay to measure NK cell cytotoxicity. They compared the LDH assay to a standard ^{51}Cr release assay for measuring NK cell cytotoxicity. They used human NK cells isolated from heparinized peripheral blood. The NK cells were washed three times in medium 199 (25mM Hepes buffer, 50μg/ml gentamicin, and 2% bovine serum albumin) that lacked phenol red and were resuspended at 5 x 10^6 cells/ml in the same media. K562, HSB-2, and CEM cells were maintained in RPMI 1640 (25mM Hepes buffer, 50μg/ml gentamicin, and 10% FCS) at 37° C and 5% CO_2. For the LDH assays, the desired target cells were washed three times in 199-albumin to remove the LDH in the FCS, the viable cells were counted using trypan blue, and the cells were diluted to 10 x 10^5, 8 x 10^5, 6 x 10^5, and 4 x 10^5 viable cells/ml. Of the target cell dilution, 0.1 ml of the cells and 0.1 ml of the NK cells was added to round bottom plates. The plates were centrifuged 150 x g, 5 min.

and the plates were incubated for 2 hours at 37° C and 5% CO_2. Following incubation, 50ml of cold medium 199 was added and the plate was centrifuged at 300 x g, 5 min. To a flat bottom microplate, 0.1 ml of the supernatant was added. Next, 0.1ml of lactate dehydrogenase substrate mix was added to each well at 3 second intervals. Finally, a microplate was used to measure the absorbance at 490nm in 3-5 minute intervals. The LDH activity was expressed as the change in absorbance per minute and was calculated by computer. The % cytotoxicity was determined by subtracting the target spontaneous LDH release wells from the experimental wells and dividing this amount by the maximum release wells minus the target spontaneous release wells. The maximum release was determined by sonification. The results of this study showed that the ^{51}Cr release assay has higher spontaneous release values than the LDH assay. In addition, the % cytotoxicity values from both the ^{51}Cr release assay and the LDH assay at 5.0, 6.2, 8.3, and 12.5:1 E:T ratios were comparable within standard deviation (25).

This study differed from ours in a variety of ways. Minor differences were seen in the experimental method. For example, the Korzeniewski study used medium 199 with Hepes buffer, BSA, and gentamicin. Our study used RPMI 1640, MEM powder, and DMEM media with FCS, and our

experiments did not use gentamicin. There were also differences in the plating procedures. In our study, the target cell number remained constant for experimental assays and the NK cell number was varied to obtain the proper E:T ratio (see Konjevic, et al., below). The Korzeniewski study varied the target cell number and the NK cell number remained constant in the experimental assays. There was also a difference in the calculation of % cytotoxicity. The Korzeniewski study did not use the effector cell spontaneous release measurement in determining % cytotoxicity. This factor was used in our study to correctly determine target cell lysis (see Decker and Loman-Mtthes, below). More importantly, our study dealt with murine NK cells while theirs dealt with human NK cells. Because the Korzeniewski study used human NK cells, they used K562 human NK target cells. Our study used murine NK target cells (YAC-1, MPC II, L1210, and P815). Our study also used cell lines that were targeted for lysis by NK cells (positive controls) and cell lines that were not targeted for lysis by NK cells (negative controls) to ensure that NK cells, not another cell type such as LAK cells or T cells, were participating in the target cell destruction. The Korzeniewski study used only positive control target cells (lysed by NK cells) (25).

The results from the Korzeniewski study were somewhat similar to our study in measuring % cytotoxicity in a positive control cell line. The 12.5:1 E:T ratio in the Korzeniewski study showed a % cytotoxicity of about 29%. In our study, the MPC II positive control cell line showed a % cytotoxicity of 25.8% for an E:T of 10:1. The lower % cytotoxicity in our study could be because the Korzeniewski study used a different method to calculate the % cytotoxicity.

Significant modifications on the LDH cytotoxicity assay were described in the *Journal of Immunological Methods* in 1988 by Decker and Lohmann-Matthes. These studies simplified the LDH assay by introducing an enzyme inhibitor to terminate the reaction. The Decker study also compared the LDH cytotoxicity assay to a ^{51}Cr release assay, but used a variety of cell types. These included NK cells, macrophages, and cytotoxic T lymphocytes from both mouse and human donors. In order to compare their study to ours, only the murine NK cell cytotoxicity experiments will be discussed. YAC-1 lymphoma cells were used as NK targets, and were grown in RPMI 1640 supplemented with 3% FCS for the cells used in the LDH assays and 10% FCS for cells used in ^{51}Cr release assays. Murine NK cells were obtained by nylon wool passage of spleen cells obtained from Charles River mice. These effector cells were placed into round- or flat-

bottom well microtiter plates in 100μl of RPMI 1640 and 3% FCS. For the experimental assays, the YAC-1 cells in RPMI 1640 and 3% FCS were added in 50μl amounts to the effector cells. The plates were incubated at 37° and 5% CO_2 for an 8 hour incubation period. Following incubation, the cell suspension was allowed to cool and the supernatants were removed. To the supernatants, 20μl of lactate solution (36 mg/ml of 10mM Tris buffer, pH 8.5) were added along with 20μl of INT solution (an enzyme substrate, 2 mg/ml of PBS). By adding 20μl of a solution containing NAD^+ and diaphorase (NAD^+ 3 mg/ml, diaphorase 13.5 U/ml), the enzymatic reaction was started. This reaction was stopped after 20 minutes by adding the enzyme inhibitor oxamate. The % specific lysis was determined by subtracting the target cell spontaneous LDH release wells from the experimental release wells and dividing by total LDH release wells. The total LDH released from targets was determined by either freeze-thawing or Triton X-100 treatment. Spontaneous release assays were also performed on target (YAC-1 cells) and effector cells (murine NK cells) for 8, 24, and 36 hour incubation times. For these assays, the respective cell types were incubated, in media only, for various time periods and the assays were used to detect the spontaneous release of either LDH or ^{51}Cr (10).

The results from the Decker study showed that the spontaneous release from the LDH assay was less than from the ^{51}Cr assay. In addition, the Decker study showed the % cytotoxicity for YAC-1 targets to be 33.3, 25.3, and 19.6 for E:T ratios of 25:1, 12:1, and 6:1 respectively.

The Decker study differed from our study in a number of ways. The Decker study used only the YAC-1 target cells. Our study used YAC-1, MPC II, P815, and L1210 target cells that resulted in a panel of positive and negative control cell lines. Next, the target cells used in the Decker study were not washed before the LDH assay was performed (although, target cells in the Decker study were grown with reduced amounts of FCS). In our study, three wash steps were performed with media lacking FCS to remove any LDH that may have been present in the media. Another difference was that the Decker study used nylon wool passage of spleen cells to harvest NK cells. This step would remove macrophages, and may have caused an increase in the percentage of NK cells found in the effector cell population. Our study used just a spleen cell fraction to be utilized as NK cells. Finally, the effector cell spontaneous release was not factored into determining % cytotoxicity which would have made their % cytotoxicity seem higher than ours. This was correctly done in our study.

The correction of the cytotoxicity assay was addressed in a 1996 article in the *Journal of Immunological Methods* by Konjevic and Spuzic. This study also compared LDH and ^{51}Cr release assays using the human NK target K562. They compared the results of a ^{51}Cr release cytotoxicity assay to the results of an LDH assay, but determined the % cytotoxicity in the LDH assay two ways: without the effector spontaneous LDH release factored in and with it factored in. The Konjevic study used the same procedure as the Korzeniewski study but with some important changes. The Konjevic article observed that the NK population, not the target cell population, should be varied to create the correct E:T ratio. This was properly done in our study. The Konjevic study used E:T ratios of 80:1, 40:1, and 20:1 (26).

The results from the Konjevic study showed that the LDH assay yielded results closer to the ^{51}Cr release assay when the effector spontaneous LDH release control was factored in. They concluded that the correct way to determine % cytotoxicity involved factoring in the LDH spontaneously released from effector cells. This was not done in the Korzeniewski study, but was done in our study. The Konjevic study used E:T ratios of 80:1, 40:1, and 20:1. The largest E:T ratio in our study was 10:1 and our % cytotoxicity values could not be compared to their observed values.

The results of our study have determined the optimal number of cells to be used in cytotoxicity assays for the YAC-1 (5,000 cells/100µl), MPC II (10,000 cells/100µl), L1210 (10,000 cells/100µl), and P815 (5,000 cells/100µl) cell lines. In addition, the spontaneous LDH released from these cell lines for 4 and 24 hour incubation times was measured. The 24 hour spontaneous LDH released from P815 cells was almost as high as the total LDH release values. This would exclude P815 from being used in long term LDH assays. Experimental assays were performed with MPC II, P815, and YAC-1 target cells and murine NK cells. The results from the MPC II cells' experimental assay showed that they might be useful as a positive cell line with a % cytotoxicity of 25.8 at an E:T ratio of 10:1. The P815 cells' experimental assay determined that they might be useful as a negative cell line with a % cytotoxicity of only 0.369 at an E:T ratio of 10:1.

Experimental assays were not performed on L1210 cells. The experimental assays performed on the YAC-1 positive cell line did not show any % cytotoxicity in the positive range. This may have been due to high background absorbance because of LDH in the media or because of poor conditions during cell preparation. Therefore, this cell line was not useful as a positive control for detecting NK activity. The Nulli-SCC cell line also could not be used in the LDH assays. Both treating the Nulli-SCC cells with

gentamicin and coating them with gelatin failed to keep the cells from agglutinating during the washing steps. It was because of this agglutination that these cells could not be used.

Using target cells that are either destroyed (positive controls) or not destroyed (negative controls) by NK cells will ensure that the cell lysis that occurs in the cytotoxicity assays is due to NK cells and not some other type of cells such as LAK cells or activated T cells. If positive control NK target cells are destroyed by a population of spleen cells, it is indicative of NK cell cytotoxicity. If that same population of spleen cells also fails to destroy negative control NK target cells, that is also indicative of NK cell cytotoxicity. More experimental assays need to be performed on the L1210, MPC II, P815, and YAC-1 cell lines to determine if they will be useful as positive and negative controls for detecting murine NK cytotoxicity.

The results obtained from these experiments can be used for a variety of future projects. The spontaneous release data can be compared to a standard ^{51}Cr release assay using identical cell lines. This will determine the effectiveness of the LDH assay using these murine NK target cells. In addition, if the aforementioned positive and negative control target cells can be used properly with the LDH assay, they can be used to detect NK cell cytotoxicity in murine NK hybridoma cells.

REFERENCES:

1. Kuby, Janis. Immunology. W.H. Freeman and Company, New York 1997.

2. Weir, D.M. Cellular Immunology. Blackwell Scientific Publications, Oxford 1986. Vol. 2 of Handbook of Experimental Immunology in Four Volumes.

3. Yokoyama, Wayne M. "Natural killer cell receptors specific for major histocompatibility complex class I molecules." Proc. Natl. Acad. Sci. USA 92: 3081-3085 (1995).

4. Herberman, Ronald B. and John R. Orlando "Natural Killer Cells: Their Role in Defenses Against Disease." Science 214 No. 2: 24-30 (1981).

5. Helanders, T.S., and T. Timonen "Adhesion in NK Cell Function." Current Topics in Microbiology and Immunology 230: 89-99 (1998).

6. Yokoyama, Wayne M. "Natural killer cell receptors." Current Opinion in Immunology 7: 110-120 (1995).

7. Yokoyama, Wayne M. "Natural killer cell receptors." Current Opinion in Immunology 10: 298-305 (1998).

8. Moretta, Alessandro "Molecular Mechanisms in Cell-Mediated Cytotoxicity." Cell 90: 13-18 (1997).

9. Chambers, B.J., et al. "Triggering of Natural Killer Cell Mediated Cytotoxicity by Costimulatory Molecules." Current Topics in Microbiology and Immunology 230: 53-61 (1998).

10. Decker, Thomas and Marie-Luise Lohmann-Matthes "A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity." Journal of Immunological Methods 15: 61-69 (1988).

11. Blomberg, Kaj "Simultaneous measurement of natural killer cell cytotoxicity against each of three different target cell lines." Journal of Immunological Methods 168: 267-273 (1994).

12. Lovgren, Janita and Kaj Blomberg "Simultaneous measurement of NK cell cytotoxicity against two target cell lines labeled with fluorescent lanthanide chelates." Journal of Immunological Methods 173: 119-125 (1994).

13. Von Zons, Peter, et al. "Comparison of Europium and Chromium Release Assays: Cytotoxicity in Healthy Individuals and Patients with Cervical Carcinoma." Clinical and Diagnostic Laboratory Immunology 4, No. 2: 202-27 (1997).

14. Nagao, Fumiko, et al. "Application of Non-Radioactive Europium (Eu^{3+}) Release Assay to a Measurement of Human Natural Killer Activity of Healthy and Patient Populations." Immunological Investigations 25, No. 5&6: 507-518 (1996).

15. Borella, Paola, et al. "Use of flameless atomic absorption spectroscopy in immune cytolysis for nonradioactive determination of killer cell activity." 42, No. 2: 319-325 (1996).

16. Borella, Paola, et al. "The use of non-radioactive chromium as an alternative to ^{51}Cr in NK assay." Journal of Immunological Methods 186: 101-110 (1995).

17. Bromelow, K.V., et al. "A highly sensitive whole blood natural killer cell assay." Journal of Immunological Methods 217: 177-184 (1998).

18. Ito, Masahiro, et al. "Non-radioactive assay of natural killer cell-mediated cytotoxicity against cytomegalovirus-infected fibroblasts by DNA fragmentation ELISA." Journal of Virological Methods 56: 77-84 (1996).

19. Papa, Stefano and Massimo Valentini "Functional NK Assays Using Flow Cytometry." Methods in Cell Biology 42: 193-207 (1994).

20. Johann, Sabine, et al. "A versatile flow cytometry-based assay for the determination of short- and long-term natural killer cell activity." Journal of Immunological Methods 185: 209-216 (1995).

21. Flieger, Dimitri, et al. "A novel non-radioactive cellular cytotoxicity test based on the differential assessment of living and killed target and effector cells." Journal of Immunological Methods 180: 1-13 (1995).

22. Promega Corporation. CytoTox 96® Non-Radioactive Cytotoxicity Assay; Technical Bulletin No. 163; Madison, WI, 1997.

23. Sinesky, M.C., Leiser A.L. and H. Babich "Oxidative stress aspects of the cytotoxicity of carbamide peroxide: invitro studies." Toxicology Letters 75: 101-109 (1995).

24. Behl, C., et al. "Hydrogen Peroxide Mediates Amyloid β Protein Toxicity." Cell 77: 817-827 (1994).

25. Kozeniewski, Carol and Denis M. Callewaert "An Enzyme-Release Assay for Natural Cytotoxicity." Journal of Immunological Methods 64: 313-320 (1983).

26. Konjevic, G., Jurisic, V. and I. Spuzic "Corrections to the original lactate dehydrogenase (LDH) release assay for the evaluation of NK cell cytotoxicity." Journal of Immunological Methods 200: 199-201 (1996).