NON-RADIOACTIVE KILLER CELL CYTOTOXICITY ASSAY

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NON-RADIOACTIVE NATURAL KILLER CELL CYTOTOXICITY ASSAY

Nancy Lynn Milligan

MASTER of SCIENCE

YOUNGSTOWN STATE UNIVERSITY

ABSTRACT

Natural killer cells are lymphocytes that act as the first line of defense against tumors. Natural killer cells do not require previous exposure to target cells for activity. This allows them to control abnormal cell growth prior to the development of an Immune response. These unique immune system cells are able to kill target cells directly, or through antibody dependent cell cytotoxicity. The chromium release assay is the most common procedure used to evaluate natural killer cell lytic activity . This assay requires the use of radioactivity, and thus is expensive. Also, problems with biosafety and waste disposal make this a difficult procedure to establish in the laboratory. Many cells are also resistant to labeling with chromium. (Korzeniewski and Callewart, 1983).

Lactate dehydrogenase (LDH), a cytosolic enzyme found in most cells, is released when a cell is lysed. Using this

information it is possible to study and measure the cytotoxic activities of natural killer cells against different tumor cell lines. The LDH assay used in this study is a non-radioactive, colorimetric alternative to the sensitive chromium release assay. This study examines several tumor cell lines to determine if any contained high levels of LDH and had low spontaneous release of the enzyme.

In this study using the non-radioactive cytotoxicity assay, it was observed that, the cell line MPCII had a 3.9 fold higher total LDH release when compared to spontaneous LDH release. The cell line P815 had a 1.9 fold higher total LDH release when compared to spontaneous LDH release. The cell line YAC-1 had no detectable LDH released with addition of lysis buffer, suggesting insufficient levels of enzyme. Nulli-SCC had 39 fold higher total LDH release. Cell lines found to be useful using this assay were MPCII, P815 and NULLI-SCC. These cell lines were plated with natural killer cells and % cytotoxicity was measured. These studies demonstrate that the 96 TM cytotoxicity assay may be effectively used to measure natural killer cell mediated cytotoxicity using the MPCII, NULLI-SCC and P815 target cell lines.

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DEDICATION

This thesis is dedicated to my Lord and Savior, who is my rock, my strength, and my shield.

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ABBREVIATIONS

FCS	FETAL CALF SERUM
LDH	LACTATE DEHYDROGENASE
HAT	HYPOXANTHINE-AMINOPTERIN-THYMIDINE
HPRTase	HYPOXANTHINE-QUANINE PHOSPHORIBOSYL TRANSFER
HT	HYPOXANTHINE-THYMIDINE
P/S	PENICILLIN-STREPTOMYCIN
BSA	BOVINE SERUM ALBUMIN
IL-2	INTERLEUKIN-2
NK	NATURAL KILLER
DMSO	DIMETHYL-SULFOXIDE

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INTRODUCTION

The two most important immune cells required for prevention of tumor growth in the mammalian system are natural killer cells (NK) cells, and T cells. Natural Killer cells recognize and kill primitive or nonself cells, and T cells recognize and kill self cells in an altered state (Hogan and Basten, 1988). T cells require time after exposure to antigen before maturation to lytic cells. However, unlike T cell activity, natural killer cell activity is spontaneous. Natural Killer cells do not require previous exposure to target cells for activity. This allows them to control abnormal cell growth prior to the development of an immune response. While natural killer cells are the first line of defense against tumour cells (Trinchieri, 1988), this is not their only function. They also play very important roles in viral immunological defense. This type of non-adaptive, non-MHC restricted cell mediated cytotoxicity, in which the natural killer cells do not depend on the MHC for recognition of antigen, as T cells do, was defined as natural cytotoxicity, and the cells acting in this manner were then termed natural killer cells (Herberman and Ortaldo, 1981). Natural killer cells are non-adherant and non-phagocytic cells that have been found in most normal individuals and a wide variety of mammalian and bird species. Natural killer cells can react against a wide variety of cells of different species, and members of the same species.

Natural killer cells were discovered during studies of natural cell mediated cytotoxicity, in which the resistance against the growth of natural killer susceptible tumor cell lines in vivo was observed. This approach involves looking for correlations between resistance in vivo to the growth of implanted tumor cell lines and the levels of natural killer activity in the recipient animals. In a variety of situations, tumors have grown less well in recipients with high natural killer activity than in those with low activity (Herberman and Ortaldo, 1981).

The spleen is one of the major sources of natural killer cells in both human and animals. These resting, circulating natural killer cells, present at all times in all healthy individuals, can be triggered to lyse a target cell within minutes when confronted with the appropriate target structure (Trinchieri, 1988).

Natural killer cells binding to target cells prior to cell lysis is independent of both extrinsic antigen stimulation and the major histocompatibility complex (MHC) (Hogan and Basten, 1988). It is believed that this step is guided by several different receptors which collectively confer specificity upon the interaction. It is not certain, but it is believed that the natural killer cell binds to a glycoprotein, and the natural killer-target cell interaction is further enhanced by nonspecific binding of the target to specialized leukocyte function-associated molecules on the natural killer cell surface (Hogan and Basten, 1988). The ability of a natural killer cell to bind to a cell

line is necessary for cell lysis. Following binding, activation of the cytotoxic mechanism in natural killer cells, requires a second structure found on the target cells, possibly distinct from the one responsible for cell binding, that must trigger the effector cells (Natural killer cells). The target cells may also present molecules that can interact directly with funcitonal receptors on the natural killer cells surface, such as IgG antibodies. Natural killer cells bind to IgG molecules through the CD16 marker that has been found on the surface of the natural killer cells. The susceptibility of target cells to natural killer cell lysis appears to be dependent on components of the cell membrane. However, it is not certain yet why some target cells are more sensitive then others to natural killer cells at this time. It could be that sensitivity is due to synthesis, by the target cell, of high concentrations of interferon, a potent acitvator of natural killer cell cytotoxiciy.

Natural killer cell activity has also found to be enhanced by other cytokines. Cytokines are proteins that regulate the duration and intensity of an immune response by stimulating or inhibiting various immune cells, or their function (Kuby, 1988). Cytokines serve as messengers of the immune system. They also effect lymphocyte activation. Cytokines are made by effector cells such as T helper cells, and they act on target cells that have receptors for the cytokines. Examples of cytokines that have been found to be effective in increasing natural killer cell activity are, interferon (IFN), and Interleukin-2 (IL2).

A study done by (Pattengale et. al. 1983), showed that following incubation with IL-2, resting natural killer cells became activated, and within one to four hours could lyse both freshly isolated tumour cells and leukaemic blast cells. (Gately, et. al., 1992) also showed that there was an upregulation of purified natural killer cell activity by IL-2. Natural killer cells treated with IL-2 differ from natural killer cells not treated with IL-2, in that they have a greater proliferative activity, an increase in TNF (Tumour Necrosis Factor) production, and an increase in expression of certain cell surface receptors (Herberman and Ortaldo, 1981). This study will examine the effects of incubating natural killer cells in the presence of the cytokine IL-2, and how this cytokine influences natural killer cell cytotoxicity in the colorimetric assay.

The natural killer cell cytotoxic response can be divided into three phases. The first phase involves interaction of natural killer cells with the target cells or with immune complexes, and induces a rapid response resulting in cytotoxicity and the release of granule contents that are harmful to the target cells. These interactions along with stimulation by IL-2 induce the second phase. In this phase, genes encoding lymphokines and surface activation antigens are transcribed and expressed. In the presence of IL-2, the natural killer cells proceed into the third phase of the response, with blast formation, DNA synthesis and proliferation (Trinchieri, 1988).

Recent studies in our laboratory have looked at the effects of vitamin D on the regulation of natural killer cells. Vitamin D is a steroid hormone largely recognized for its role in the regulation of calcium homeostasis (Leung, 1989). More recently, vitamin D has also been recognized as a hormone capable of regulating immune responses (Reichel, et. al., 1989). In a study done by (Leung, 1989), vitamin D inhibited natural killer cell cytotoxicity and IL-2 production by natural killer cells and lymphokine activated killer cells. Exogenous IL-2 was able to reverse this inhibitory effect upon natural killer lysis. IL-2 as mentioned before, is a potent activator of cytotoxic natural killer cell activity, and is able to induce proliferation of cytotoxic cells. Thus, according to this study, the inhibition of IL-2 production by vitamin D, would in turn, result in decreased natural killer cell activity. Other studies, such as that done by (Manolagas, 1987), showed that vitamin D, when administered by injection to immune suppressed mice was found to supress the growth of human colonic cancer, indicating increased natural killer cell activity. The conflicting results of whether vitamin D has an inhibitory or activating effect on natural killer cells may be dependent upon the purity of the cell population. Many studies testing the effects of vitamin D on natural killer activity examined a cell population containing T cells and monocytes, with natural killer cells representing only 10% of the cells. A study done by (Fagan, et. al., 1988) looked at the effect of vitamin D on cytolytic activity for a purified natural killer cell population.

This purified natural killer cell population contained less then 1% monocytes, and was contaminated with 10-30% T cells. This study indicated that vitamin D might directly inhibit natural killer cell function. However, even with extensive purification of the natural killer cells, the contaminating T cell population was still strongly influencing the results of these experiments. A pure natural killer cell line is needed for vitamin D testing. Therefore, we have experimented with the production of natural killer cell hybridomas. Creating hybridomas involves the fusion of mouse spleen cells containing natural killer cells with mouse myeloma cells, a continuously dividing cell line. The procedure for the development and use of hybrid cell lines includes growing the parental myeloma cell line, conducting the cell fusion and selecting the resulting hybrids with hypoxanthine-aminopterin-thymidine (HAT) medium. (Harlow and Lane, 1988). In the selection process, unfused myeloma cells and myeloma-myeloma cell hybrids that lack the enzyme, hypoxanthine-quanine phosphoribosyl transferase (HPRTase), are eliminated in the presence of aminopterin since they lack the ability to utilize the salvage pathway for nucleotide synthesis. In a study done by (Suzuki, 1992), spleen cells from a BALB/c nude mouse were fused with an NS-1 mouse myeloma cell line. The purified natural killer cell hybridoma had cytotoxic activity, but had lost expression of functional IL-2 receptors . This loss of Il-2 responsiveness might be due to the fusion with partner cells which can grow continuously without addition of growth factor. However, this cell line may be

useful for analysis of function and differentiation of murine natural killer cells, since there are no other reports on the establishment of natural killer cell lines using the cell fusion technology. In these studies, we attempt to produce a hybrid natural killer cell line. This pure cell line could then be used in cytotoxicity assays, observing their activity against certain tumor cell lines, or it could be used to observe the effects of vitamin D on natural killer activity.

In order to determine the success of our fusions, we must be able to assay the cytotoxic ability of fusion lines. The cytotoxicity 96TM assay, which was used in this study, is a colorimetric alternative to other methods used to quantitate natural killer cell cytotoxicity. The chromium release assay has been the most commonly used assay for monitoring the cytotoxic activity of natural killer cells (Korzeniewski and Callewart, 1983). In this method, target cells are labeled with radioactive chromium 51 that is released into the supernatent when the target cells are killed (Korzeniewski and Callewart, 1983). The amount of radioactivity released into the cell free supernatent is directly proportional to the percentage of target cells killed. Although it is widely used, the chromium release assay has numerous diadvantages. These include the use of a radioactive isotope and the relatively high spontaneous release seen with chromium 51. Also the exact effect of chromium 51 on the effector cells in not exactly known. Finally, certain types of cells are resistant to labeling with chromium, and, due to the use of radioisotopes, this

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procedure is expensive.

The 96TM cytotoxicity assay, is a non-radioactive assay. It is a colorimetric alternative to the chromium release cytotoxicity assay. This colorimetric assay quantitatively measures lactage dehydrogenase (LDH). Lactate dehydrogenase is a stable cytosiolic enzyme that is released when a cell is lysed (Korzeniewski and callewart, 1983). Thus, instead of measuring chromium, as would be done in the radioactive assay, this assay measures the release of LDH. The LDH released into culture supernatents is meaured by a coupled enzymatic assay. This enzymatic assay results in the conversion of tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Using the ELISA plate spectrophotometer, visible vavelength absorbance data is obtained at 490 nm.

This method has many advantages over the chromium release assay. Because no radiation is being used, data is easily obtained. Also, there is less cost involved because no special disoposal is needed for a radioactive isotope. However, there are some disadvatages to using this assay. Tissue culture media can contribute to background absorbance because of LDH from animal sera present in the media. Also, phenol red in the media will contribute to background absorbance (Korzeniewski and Callewart, 1983). Thus, serum free media and phenol red free media are used to reduce the background absorbance. The use of serum free media with 1% BSA will also eliminate the source of background.

Target cells contain various amounts of LDH, therefore preliminary experiments were done using the target cell population to determine the total amount of LDH release found in the target cells, as well as the level of spontaneous LDH release for that cell line. The target cells that have previously been used in cytotoxicity assays where lysis was detected by the LDH assay are the K562 target cells. This cell line was not examined in these studies as we were looking at lysis by mouse natural killer cells, and K562 cells are only lysed by human natural killer cells. No previous studies have been done to determine the usefulness of the cytotoxicity 96 TM assay when examining mouse natural killer activity. The cell lines YAC-1, MPC-II, X-63, and RLoi, have been found to be sensitive to mouse natural killer cell activity. Cell lines that have been considered insensitive to mouse natural killer cell cytotoxicity (negative controls), are P815, L12010, El-4, RBL-5, NULLI-SCC, and PVS-2. (Wigzell et. al., 1989). Some cell lines were not useful for these studies because they had a different MHC haploytpe than the natural killer cells being used in this study. If the cell line haplotype does not match the mouse haplotype, the target cells will be attacked by contaminating T cells that bind to foreign MHC antigens, rather than to tumor cell antigens. The remaining cell lines that were available for purchase from the American Culture Collection, were examined for use with the 96 TM method for detection of cytotoxic activity.

In summary, we have produced fusion cell lines to be examined for natural killer activity. To determine if these cell lines had

natural killer function, we first had to develop a non-radioactive cytotoxicity assay for use in the laboratory. A commercially prepared method for measuring LDH release from the cells was available but had not been tested for use with mouse natural killer cells. Therefore, we tested various cell lines for usefulness in this assay. The cell lines examined were YAC-1, and MPCII, positive controls, meaning they were susceptible to lysis by the natural killer cells. The other cell lines used were NU11-SCC, P815, and L1210, which were negative controls, meaning they were not susceptible to lysis by natural killer cells. The following materials and methods will discuss the data found using these different cell lines with the 96TM cytotoxicity assay.

CHAPTER II

MATERIALS AND METHODS

CELL LINES

The target cells used in this study were YAC-1, Nulli-SCC, MPCII, L1210 and P815. All of these cell lines are tumor cell lines derived from mice. The cell line used in the hybridoma fusions was P3X63. The cell lines were purchased from the American Type Culture Collection (Rockville, Maryland).

MATERIALS

The cytotoxicity reagents were obtained from the 96TM Cytotoxicity Assay Kit received from the Promega Coorporation, (Madison, Wisconsin). The cell culture media, fetal calf serum (FCS), penicillin/streptomycin (P/S), and L-Glutamine, were obtained from GIBCO BRL, Life Technologies, (Grand Island, New York). The mice used in this study, DBA/2, and C57/B16, were obtained from Charles Rivers, (Charleston, Va.). Sodium bicarbonate, amonium chloride, Dimethyl-sulfoxide, and the hybridoma Kit, were obtained from the Sigma Chemical Company, (St. Louis, MO.). Human recombinant Interleukin- 2 (IL-2), was obtained from the Boehringer Mannheim Co. (West, Germany).

METHODS

A: PREPARATION OF HYBRIDOMA CELLS:

A DBA/2 mouse was killed by CO_2 inhalation and the spleen was removed. The spleen was pressed through a sieve in order to release white blood cells and to remove large clumps. The spleen cells were then washed twice with sterile RPMI 1640 medium containing penicillin(100 ug/ml)/streptomycin(100 ug/ml)(P/S) and 0.29 mg/ml of L-glutamine, with no FCS. The spleen cells were then centrifuged at 250 X g for 8 minutes.

The P3X63 myeloma cells were harvested in the log phase and washed in 37° C RPMI 1640 medium containing antibiotics P/S and L-glutamine, without Fetal calf serum (FCS). The spleen cells and myeloma cells were then pelleted together in the proportion of 2-4 spleen cells:1 myeloma, (for example, 4 X 10^7 spleen cells: 1 X 10^7 myeloma cells). To this cell pellet, 50% PEG (polyethylene glycol), at 37° C, and neutral pH was added slowly over a period of five minutes. The PEG was diluted by slow addition of 20 ml of RPMI 1640 medium, without FCS, with swirling.

The cells were then centrifuged for 5 minutes, at 400 X g, and the supernatent was removed. Complete medium (RPMI 1640, 20% FCS, 50% spleen cell supernatent, L-glutamine and P/S), was then added to the cell suspension and the cells dispersed into a 24 well plate (1 ml/well). After overnight incubation, 1 ml of hypoxanthine-aminopterin-thymidine (HAT) medium, containing 50 ml

of RPMI 1640 plus 20% FCS, plus 1 ml of HAT), was added to all the wells. Every other day, 1 ml of HAT was removed, and another ml added. After 14 days, hypoxanthene-thymidine, (HT) medium containing 50 ml of RPMI 1640, plus 20% FCS, plus 1 ml of HT (1.5 M), was added to the wells. After two more weeks of every other day media replacement, complete medium (containing RPMI 1640 plus 20% FCS, P/S, and L-glutamine), was added to the wells. When confluent, the cells from one well were transferred to a T25 flask with 5 ml of medium. When the cells became close to being confluent in the T25 flask, they were ready to be tested for natural killer activity or a cell surface marker. Aliquots of these hybridomas were frozen down in media containing 10% FCS amd 10% DMSO, and were placed in a -70° C freezer for later studies.

B: CONTROL STUDIES

Target cells Yac-1, Nulli- SCC, MPCII, P815 or L1210, were washed three times using RPMI 1640 media containing 1% BSA and 2.0 g/L of sodium bicarbonate, with no FCS, or phenol red. The target cells were then counted using a hemocytometer and adjusted to a concentration of 1 x 10^6 per ml. Four determinations were done on each cell line. Spontaneous release of LDH was determined by incubation of target cells alone plus media. The cells were then centrifuged and the supernatent collected to determine the amount of LDH in the media. Background absorbance was measured on wells containing media alone. Total LDH found in the cells was determined by plating target cells plus lysis buffer in a 96 well

plastic microtiter plate. All incubations were performed in triplicate. The cells were centrifuged at 150 X g for five minutes, to assure contact between the cells, and the plate was then incubated for four hours at 37° C in the presence of 5% CO_2. After incubation the plate was centrifuged at 300 X g for five minutes to separate the cells from the supernatent.

The supernatent was removed and 50 ul transferred to a new plate. Substrate buffer(50 ul), was added to each well of the new plate. The substrate buffer was prepared as described in the cytotoxicity 96 TM assay kit. The buffer contains a tetrazolium salt that reacts with the LDH released from the cells to produce a red color change. The plate was then incubated in the dark for thirty minutes at room temperature. After thirty minutes, 50 ul of stop solution was added to all wells. The plate was then read on an ELISA reader from Beckman Instruments Inc. (Fullerton, Ca.) at 492 nm.

C: CYTOTOXICITY ASSAY

A mouse was killed by inhalation of CO_2 gas. The spleen was removed and pressed through a sieve to separate the white blood cells from large clumps. These effector spleen cells were then washed three times using RPMI plus 1% BSA and 2.0 g of sodium bicarbonate, without phenol red or FCS. Each wash was done at 150 X g for 5 minutes. The cells were then counted using a hemocytometer, and the concentration adjusted to 10^7 cells/ml.

Target cells were washed three times in RPMI 1640 media plus 1% BSA, and 2.0 g of sodium bicarbonate, with no FCS, or phenol red. The target cells were counted using a hemocytometer, and 10^6, 5 X 10^5, or 10^5 cells were added to each well. Experimental wells received a constant number of effector cells and various numbers of target cells, in order to test several effector-target cell ratios. The experimental wells received 10^6, 5 X 10^5, and 10^5 target cells plus 10^6 effector cells. This results in an effector/target cell ratio of 1:1, 2:1, or 10:1.

In studies that received interleukin-2 (IL-2), (Figures 10 and 11), spleen cells were split into four groups, then incubated with RPMI 1640 plus 10% FCS, containing 0, 1, 10 or 100u/ml of interleukin-2 overnight at 37° C. These cells were then added to the wells at the concentration for effector cells indicated above. Each experiment included all controls described in the control studies.

The plate was centrifuged at 250 X g for 5 minutes, then incubated for four hours at 37° C and 5% CO_2. After incubation, the plate was centrifuged at 250 X g for 5 minutes, then 50 ul of supernatent was removed and transferred to a new plate. To this new plate was added 50 ul of substrate mix. The substrate mix was prepared as described in the cytotoxicity 96TM Assay Kit. The plate was then incubated in the dark for 30 minutes. After 30 minutes, 50 ul of stop solution was added to each well and the absorbance of each well was read at 490 nm.

D: CALCULATIONS AND STATISTICS

The formula used to determine the percent cytotoxicity for each cell line at each effector/target cell ratio, was:

1. Subtract the average of absorbance values for the culture medium background from all absorbance values of A)Experimental, B) Target cell spontaneous LDH Release, and C) Effector cell spontaneous LDH release.

2. Subtract the average of the absorbance values for the volume correction control (lysis control) from the absorbance values obtained for target cell maxiumum LDH release.

3. Use the corrected values obtained in Steps 1 and 2 above in the following formula to compute percent cytotoxicity for each effector target cell ratio.

$$\%CYTOTOXICITY = \frac{(\text{Experimental-Effector spont.}) - \text{Target spont.}}{\text{Target Maximum- Target spontaneous}} X100$$

Each incubation was performed in triplicate. From each well a cytotoxicity value was obtained using the formula described above. These cytotoxicity values were averaged together to obtain a mean, and from this mean, standard error was calculated.

Total LDH release can be seen for each cell line in figures 1-6. Total LDH release was calculated by using the formula: (total - background). Spontaneous LDH release can also be seen in figures 1-6, and was calculated by using the formula: (spontaneous release - background).

Availablae LDH remaining after spontaneous release, and available for release following a specific cytotoxic response, is shown in Figure 7 and was calculated by using the formula:

$$\frac{(\text{Total - lysis control}) - (\text{Spontaneous - media control})}{(\text{Total release - lysis control})} \times 100$$

CHAPTER III

RESULTS

NATURAL KILLER CELL CYTOTOXICITY AGAINST DIFFERENT TUMOR CELL LINES:

In these studies we examined the cell lines P815, L1210, YAC-1, Nulli-SCC, and MPCII to observe the amount of LDH released when these cell lines were lysed by effector cells. This gave us the % cytotoxicity of effector cells against these different tumor cell lines. These cell lines were chosen because they had compatible major histocompatibility antigen (MHC) haplotypes with the mice we were using and are known to be negative or positive targets for mouse natural killer cells (Wigzell, <u>et</u>. <u>al</u>., 1989). The cell lines P815 and Nulli-SCC are negative target cells, and the remaining cell lines are the positive target cells.

SPONTANEOUS AND TOTAL LDH RELEASE

The spontaneous LDH released from the cell lines was sudied using an LDH assay. The amount of spontaneous LDH release was determined by plating the target cells alone for four hours and testing the supernatent for the presence of LDH, a stable cytosolic enzyme. Spontaneous LDH release was then calculated by subtracting the background from the spontaneous release, and spontaneous release was then expressed as absorbance as seen in

figures 1-6. The cell line with the least spontaneous release was the L1210 cell line. (Figure 1, absorbance measured at 490 nm for 10^6 cells/ml = .03, for 5 X 10^5 cells/ml = .07, and for 10^5 (5) cells/ml = .03. However, the total LDH found in L1210 cells was also very low, as discussed in the next paragraph. The YAC-1 cell line also had very low levels of spontaneous release (Figure 2, absorbance measured at 490 nm for 10^6 cells/ml = .148, for 5 X 10^5 cells/ml = .078, and for 10^5 cells/ml = 0. Spontaneous release by the P815 cell line (Figure 3, absorbance measured at 490 nm for 10^6 cells/ml = 1.37, for 5 X 10^5 = 1.07, and for 10^5 cells/ml, absorbance = .32). Spontaneous release by the Nulli-SCC cell line (Figure 4, absorbance measured at 490 nm = .017 for 10^6 cells/ml, .087 for 5 X 10^5 cells/ml, and 0 for 10^5 cells/ml. Spontaneous release by the MPCII cell line EXP.1 (Figure 5, absorbance read at 490 nm = 1.09 for 10^6 cells/ml, .83 for 5 X 10^5 cells/ml, and .32 for 10^5 cells/ml. Spontaneous release by the MPCII cell line EXP.2, (Figure 6, absorbance at 490 nm = .255 for 10^6 cells/ml, .225 for 5 X 10^5 cells/ml and .195 for 10^5 cell/ml.

The maximum amount of LDH released from a particular target cell line was determined by plating the target cell plus lysis buffer. When the target cell is lysed by the lysis buffer, the LDH inside the cell is released, giving the maximum amount of LDH that can be released by the cell. Total LDH release was calculated by subtracting the background from the total LDH release. The L1210 and YAC-1 cell lines contained very low levels of total LDH (Figures 1 and 2). L1210 LDH levels were not significantly

different from the media control (absorbance at 10^6 cells = .15 above background). LDH levels slightly above that in the media control were seen with YAC-1 cells when 10^6 cells were lysed (absorbance = .15 above background). The cell line with the highest total LDH release was the MPCII cell line, (Figure 5 absorbance read at 490 nm = 2.04 for 10^6 cells, 1.15 for 5 X 10^5 cells/ml, and .635 for 10^5 cells/ml). The P815 cell line and the Nulli-SCC cell lines also had detectable levels of total LDH with absorbace values of 9.76 and 2.51, respectively for 10^6 cells (Figures 3 and 4). Specific cytotoxic release from the cells is shown in Figure 7 and represents (total LDH - lysis control) - (spontaneous LDH - media control) / (total LDH - lysis control) X 100.

The amount of LDH remaining after spontaneous release, and availbable for release following a specific cytotoxic response, is shown in Figure 7 for all of the cell lines. In the following discussion, this shall be called available LDH. NULLI-SCC had 99% available LDH at 10^6 cells/ml (78.4 fold higher total LDH than spontaneous release at 10^6 cells/ml). The P815 cell line had 94% available LDH at 10^6 cells/ml (18.0 fold higher total LDH than spontaneous release). The YAC-1 cell line had 37% available LDH at 10^6 cells/ml (1.6 fold higher total LDH than spontaneous release). The L1210 cell line had 70% available LDH at 10^6 cells/ml (3.3 fold higher total LDH than spontaneous release). The MPCII cell line in experiment 1 had 99% available LDH at 10^6 cells/ml, (91.2 fold higher total LDH than spontneous release). In experiment 2,

the MPCII cells had 95% available LDH at 10^6 cells/ml (22.2 fold higher total LDH than spontaneous release).

NATURAL KILLER CELL CYTOTOXICITY AGAINST TUMOR CELL LINES:

We next examined natural killer cell cytotoxicity against different tumor cell lines using the assay for detection of cytosolic LDH release. Various concentrations of target cells were incubated with effector cells, and cytotoxicity was calculated by measuring the amount of LDH released using the 96 TM cytotoxicity kit, as described in methods. Both positive (mouse natural killer sensitive cell line, MPCII) and negative (mouse natural killer resistant cell lines, P815 and Nulli-SCC) controls were studied. The L1210 and YAC-1 cell lines were not used due to low total LDH.

NEGATIVE CONTROLS

P815

The P815 cells were plated with natural killer cells, as described in methods, and LDH released as a result of specific cytotoxicity was measured using the 96 TM cytotoxicity assay kit. As shown in Figure 8, at the effector/target cell ratio of 10:1 (10^6 effector cells plus 10^5 target cells), 10% of the P815 cells

were lysed. At the effector/target cell ratio of 2:1 (10^6 effector cells plus 5 X 10^5 target cells), 12% of the P815 cells were lysed. And at a 1:1 effector/target cell ratio (10^6 effector cells plus 10^6 target cells), 17% of the P815 cells were lysed.

NULLI-SCC

The NULLI-SCC cell line, when plated with natural killer cells showed relatively low cytotoxicity values (Figure 9). The highest cytotoxicity value of 13% occured at 5 X 10^5 target cells (effector/target cell ratio of 2:1). At the effector/target cell ratio of 10:1 (10^6 effector cells plus 10^5 target cells), the NULLI-SCC cell line had a cytotoxicity value of 7.6%. At the effector/target cell ratio of 1:1 (10^6 effector cells plus 10^6 target cells), there was no cytotoxicity observed.

POSITIVE CONTROLS

MPCII plus IL-2

In both studies done with the MPCII cell line, cytotoxicity against the MPCII target cells increased with treatment of the effector cells with IL-2. Figure 10 shows the results of

experiment 1 using natural killer cells from C57/B16 mice. At the effector/target cell ratio of 1:1 with no IL-2 treatment, (10^6 effector cells plus 10^6 target cells), 27% cytotoxicity was observed. At the effector/target cell ratio of 2:1 with no IL-2 treatment (10^6 effector cells plus 5 X 10^5 target cells), and at the effector/target cell ratio of 10:1 with no IL-2 treatment (10^6 effector cells plus 10^5 target cells), no cytotoxicity was observed. At the effector/target ratio of 1:1 (10^6 effector cells plus 10^6 target cells), with 1 U/ml IL-2 treatment, cytotxicity was 45%. At the effector/target cell ratio of 2:1 (10^6 effector cells plus 5 X 10^5 target cells) and 10:1 (10^6 effector cells plus 10^5 target cells), with 1 U/ml of IL-2 treatment, cytotoxicity was 0%. At the effector/target cell ratio of 1:1 (10^6 effector cell plus 10^6 target cells), with 10 U/ml of IL-2 treatment, cytotoxicity was 62%. At the effector/target cell ratio of 2:1 (10^6 effector cells plus 5 X 10^5 target cells), with 10 U/ml of IL-2 treatment, cytotoxicity was 33%. At the effector/target cell ratio of 10:1 (10^6 effector cells plus 10^5 target cells) with 10 U/ml of IL-2 treatment, cytotoxicity was 0%. At the effector/target cell ratio of 1:1 (10^6 effector cells plus 10^6 target cells) treated with 100 U/ml of IL-2, cytotoxicity was 57%. At the effector/target cell ratio of 2:1 (10^6 effector cells plus 5 X 10^5 target cells) with 100 U/ml of IL-2 treatment, cytotoxicity was 95%. At the effector/target cell ratio of 10:1 (10^6 effector cells plus 10^5 target cells), with 100 U/ml of IL-2 treatment, cytotoxicity was

9.6%.

The second study using MPCII target cells was done with mouse natural killer cells from DAB/2 mice (Figure 11). At the effector target ratio of 1:1 (10^6 effector cells plus 10^6 target cells), 2:1 (10^6 effector cells plus 5 X 10^5 target cells), and 10:1 (10^6 effector cells plus 10^5 target cells), with no IL-2 treatment, no cytotoxicity was observed. At the effector/target cell ratio of 1:1, 2:1, and 10:1, with 10 U/ml of IL-2 treatment, no cytotoxicity was observed. At the effector/target cell ratio of 1:1, 2:1, and 10:1, with 1 U/ml of IL-2 treatment, no cytotoxicity was observed. At the effector/target cell ratio of 1:1, with 100 U/ml of IL-2 treatment, cytotoxicity was 44%, at the effector/target cell ratio of 2:1, with 100 U/ml of IL-2 treatment, cytotoxicity was 100%. And at the effector/target cell ratio of 10:1, with 100 U/ml of IL-2 treatment, cytotoxicity was 0%.

FIGURE 1

SPONTANEOUS AND TOTAL LDH RELEASE BY THE L1210 CELL LINE

This graph shows the results of spontaneous and total LDH release from the cell line L1210. Target cells were washed three times using RPMI 1640 medium containing 1% BSA, without phenol red or fetal calf serum, and were plated in a plastic 96 well microtiter plate using various concentrations of target cells (10^6, 5 X 10^5, or 10^5) with or without lysis buffer to determine total and spontaneous LDH release. Released LDH is measured by a coupled enzymatic assay (96 TM Cytotoxicity Assay, Promega Coorporation). Visible wavelength absorbance data is read at 490 nm. Spontaneous LDH release is indicated by circles, total LDH released is indicated by squares. These results represent the mean and standard error of triplicate observations from the L1210 cell line.

L1210 Spontaneous and Total LDH Release

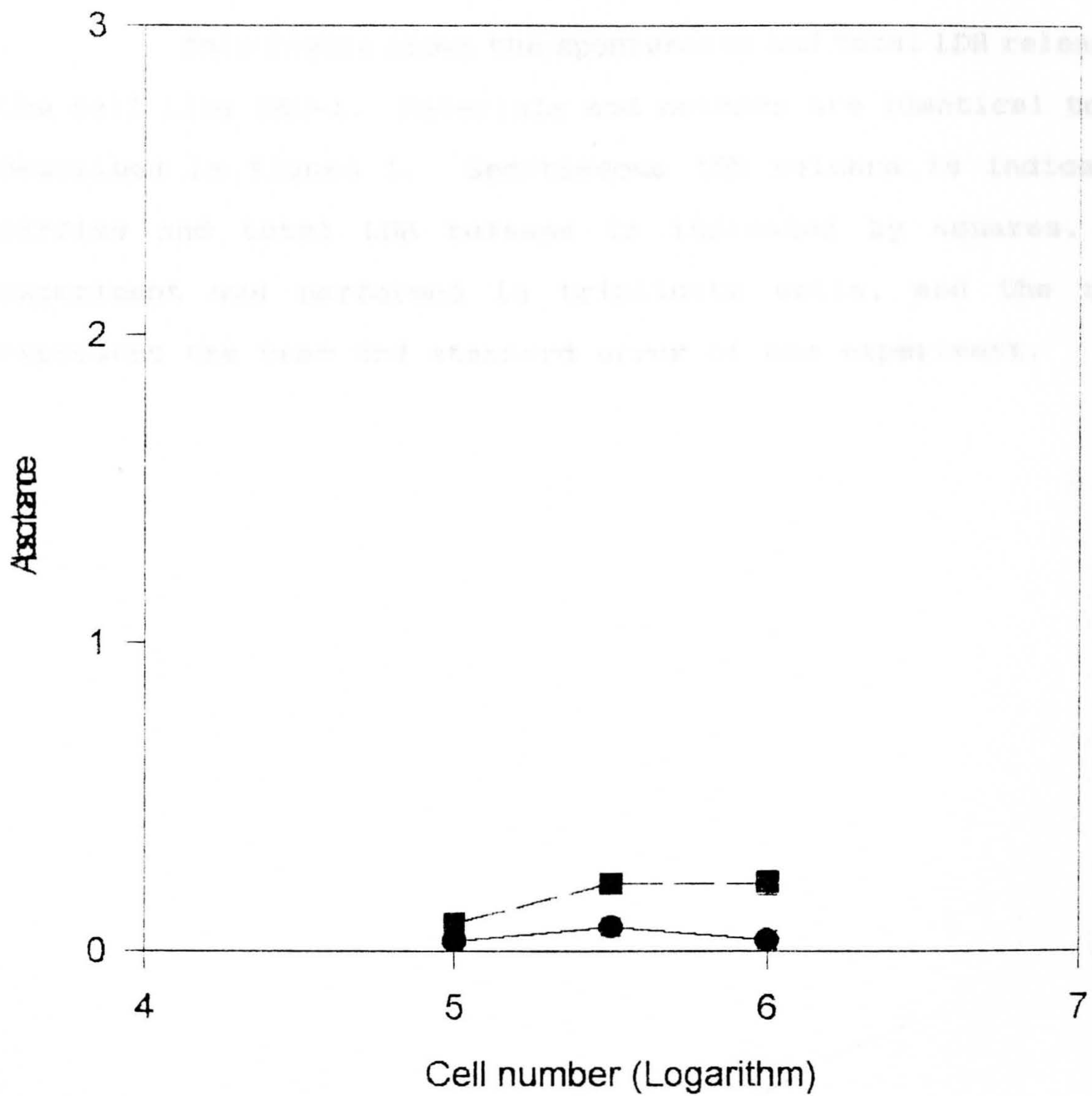


FIGURE 2

SPONTANEOUS AND TOTAL LDH RELEASE BY THE YAC-1 TARGET CELLS

This figure shows the spontaneous and total LDH release from the cell line YAC-1. Materials and methods are identical to those described in Figure 1. Spontaneous LDH release is indicated by circles and total LDH release is indicated by squares. This experiment was performed in triplicate wells, and the results represent the mean and standard error of one experiment.

YAC-1 Spontaneous and Total LDH Release

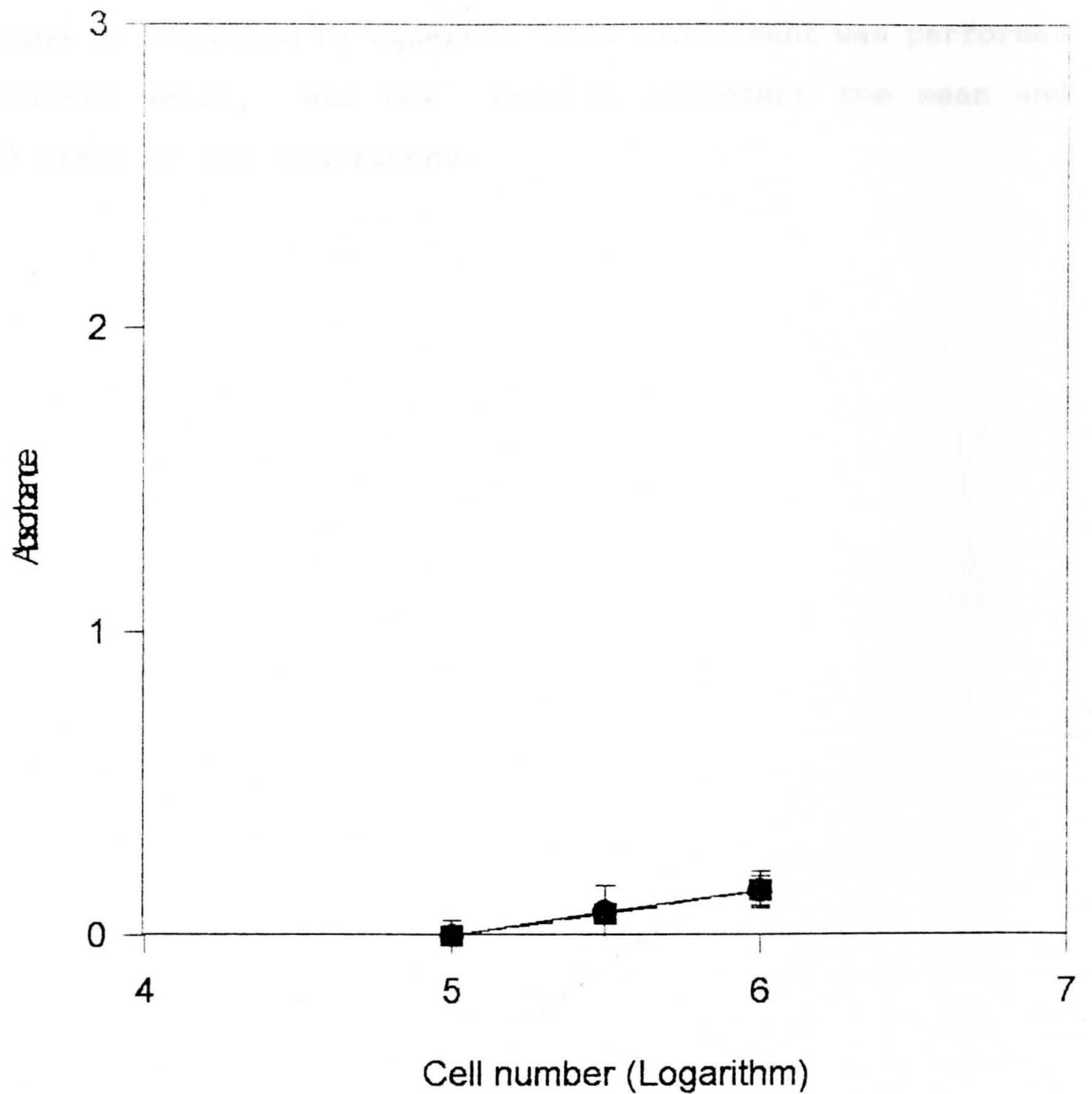


FIGURE 3

SPONTANEOUS AND TOTAL LDH RELEASE BY THE P815 TARGET CELLS

Figure 3 shows the total and spontaneous LDH release from the P815 cell line. The procedure is identical to that detailed in figure 1. Spontanous LDH release is indicated by circles and total LDH release is indicated by squares. This experiment was performed in triplicate wells, and the results represent the mean and standard error of one experiment.

P815 Spontaneous and Total LDH Release

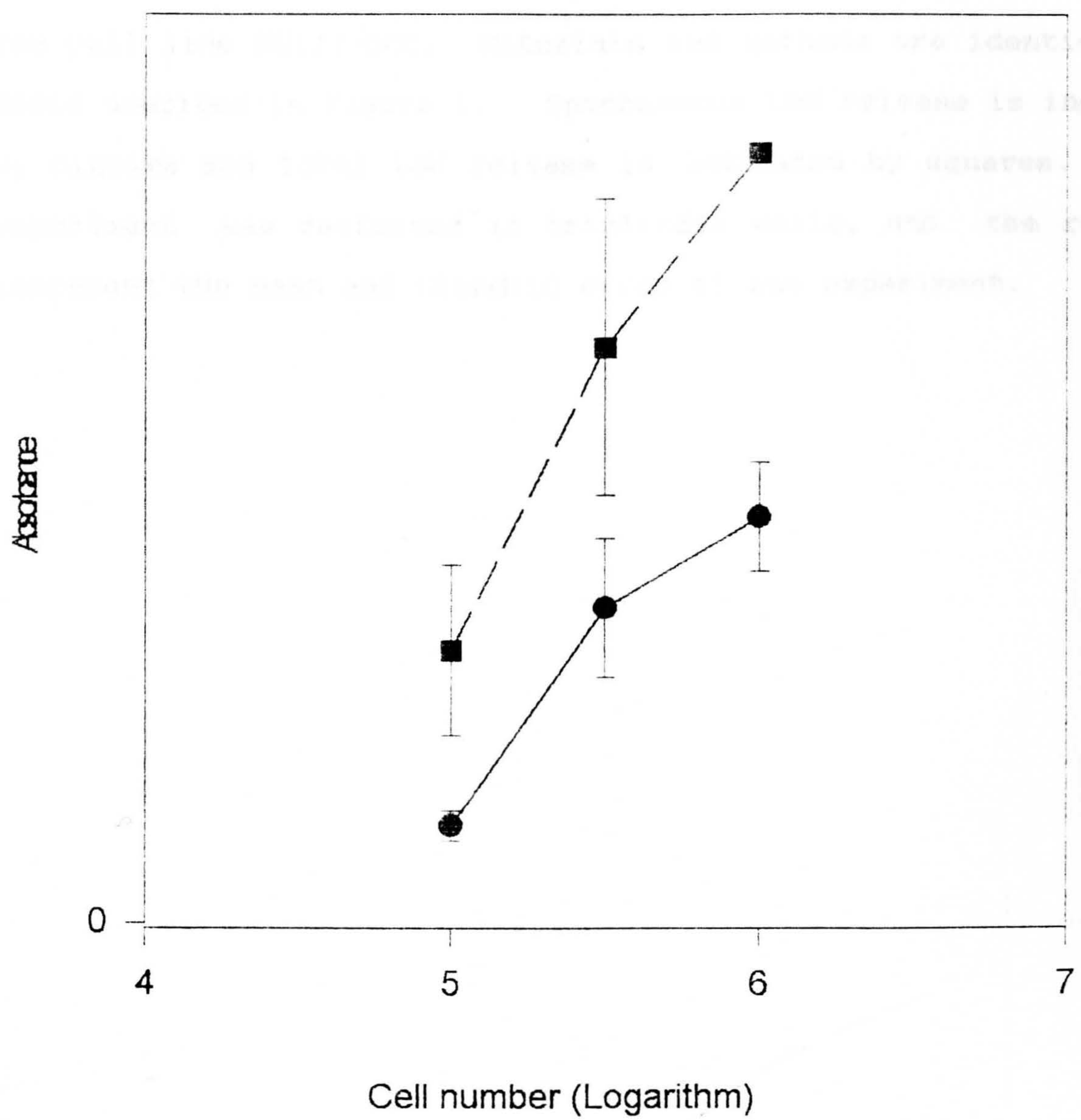


FIGURE 4

SPONTANEOUS AND TOTAL LDH RELEASE BY NULLI-SCC CELL LINE

This figure shows the spontaneous asnd total LDH release from the cell line NULII-SCC. Materials and methods are identical to those desribed in Figure 1. Spontaneous LDH release is indicatd by circles and total LDH release is indicated by squares. This experiment was performed in triplicate wells, and the results represent the mean and standard error of one experiment.

NULLI-SCC Spontaneous and Total LDH Release

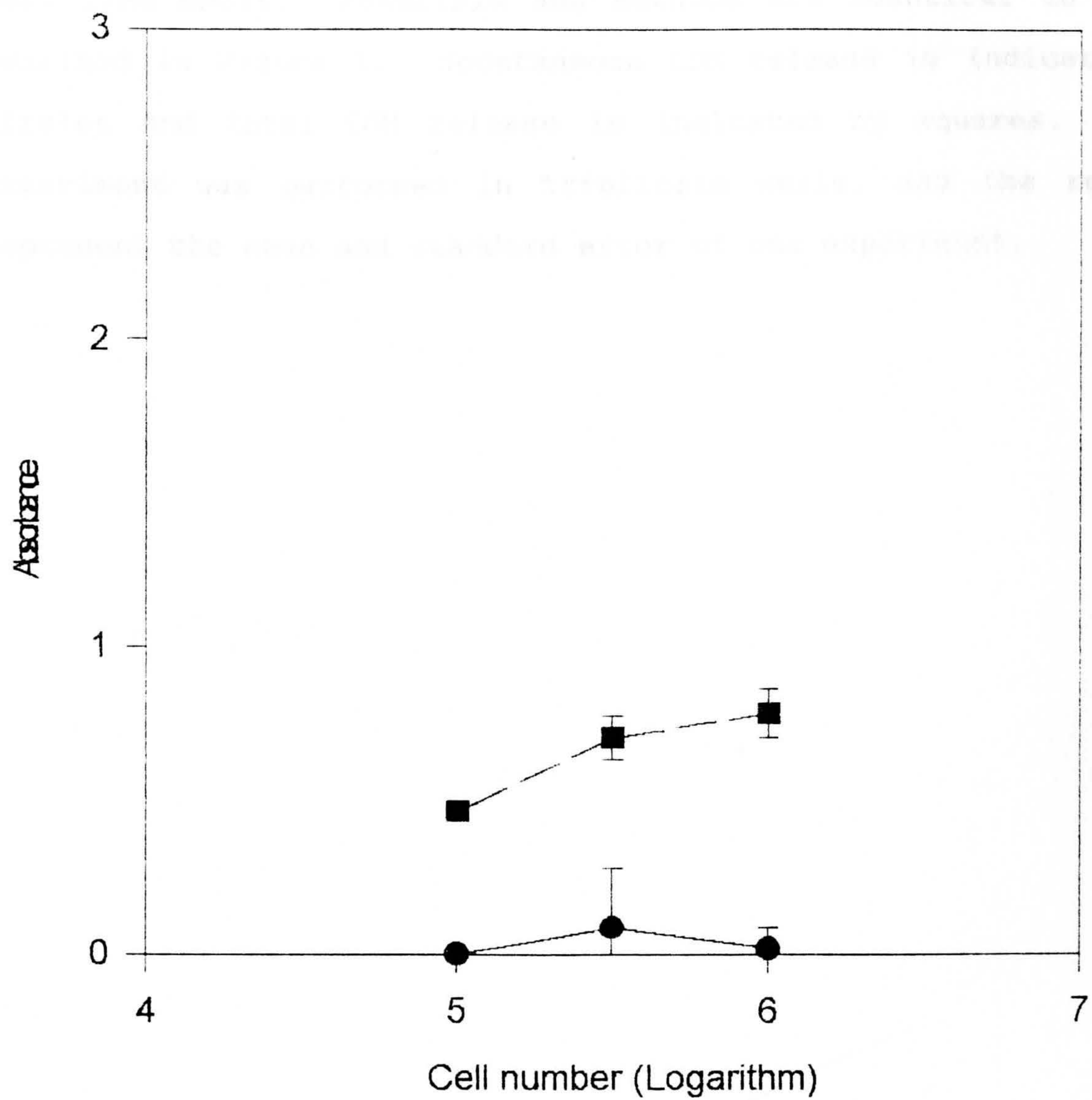


FIGURE 5

SPONTANEOUS AND TOTAL LDH RELEASE BY MPCII CELL LINE, EXP.1

Figure 5 shows the spontaneous and total LDH release from the cell line MPCII. Materials and methods are identical to those desribed in Figure 1. Spontaneous LDH release is indicated by circles and total LDH release is indicated by squares. This experiment was performed in triplicate wells, and the results represent the mean and standard error of one experiment.

EXP. 1, MPCII Spontaneous and Total LDH Release

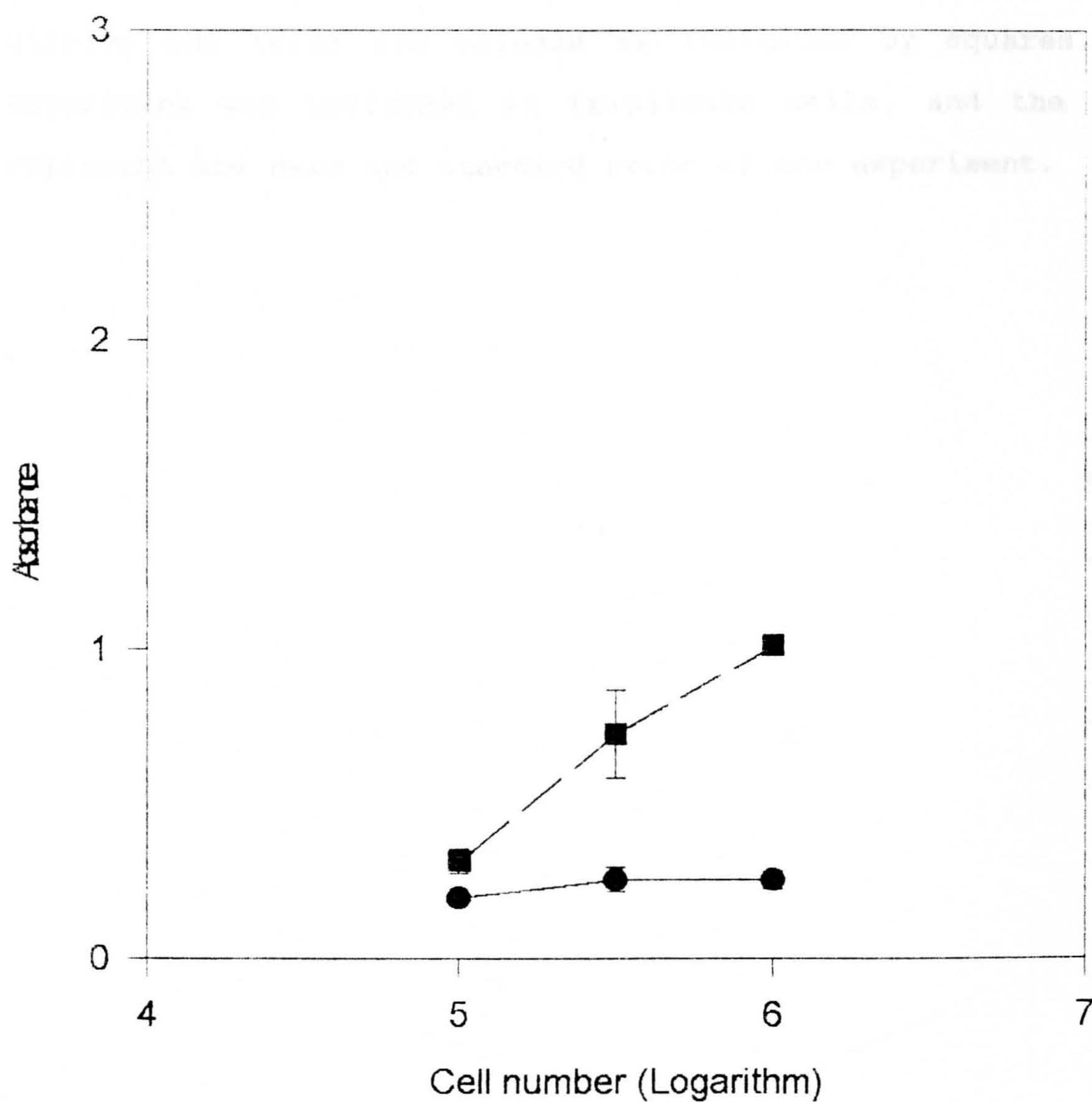


FIGURE 6

SPONTANEOUS AND TOTAL LDH RELEASE BY MPCII CELL LINE, EXP.2

This figure shows the spontaneous and total LDH release from the cell line MPCII. Materials and methods are identical to those desribed in Figure 1. Spontaneous LDH release is indicated by circles and total LDH release is indicated by squares. This experiment was performed in triplicate wells, and the results represent the mean and standard error of one experiment.

Exp. 2, MPCII Spontaneous and Total LDH Release

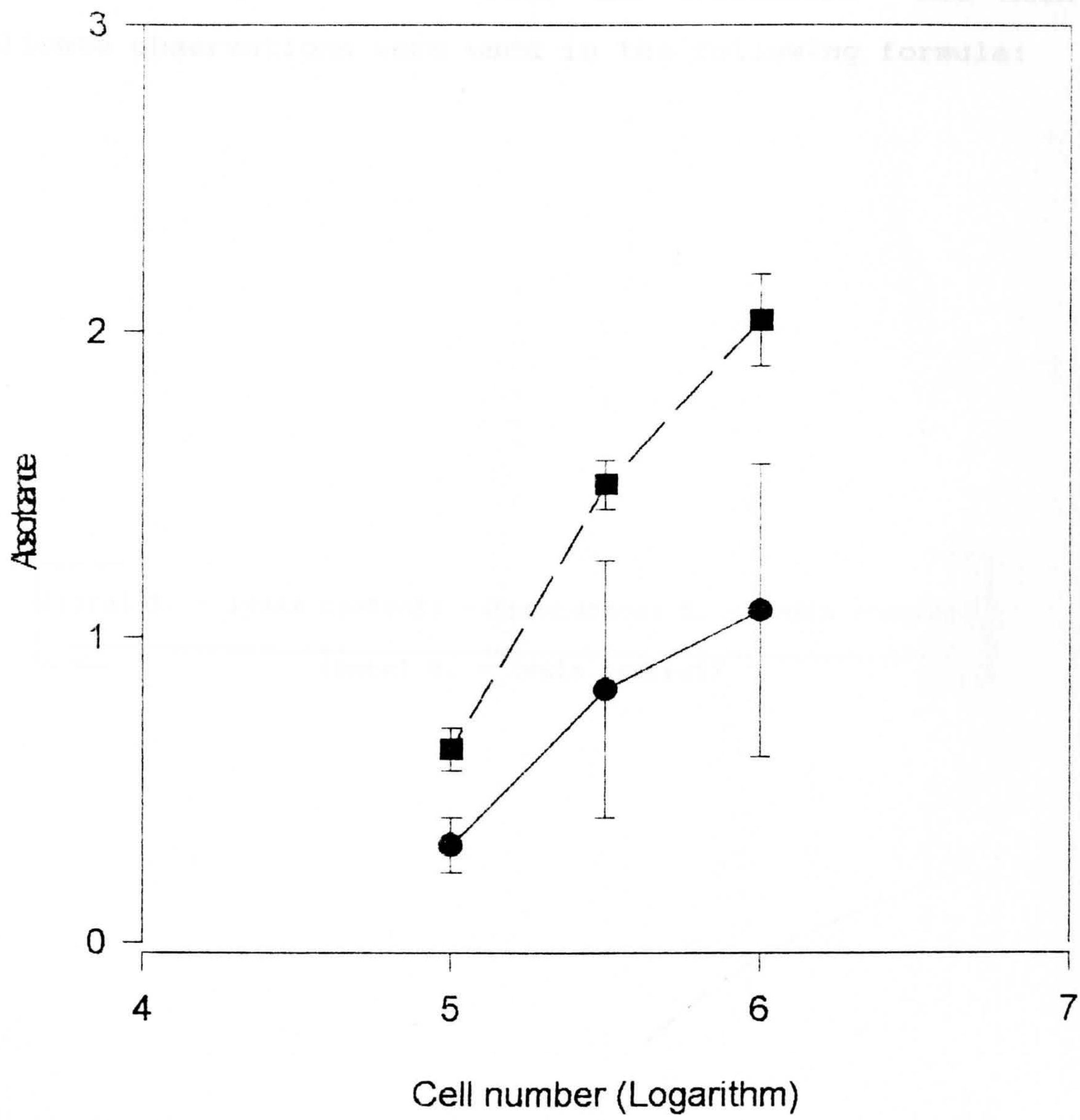


FIGURE 7

SUMMARY OF SPONTANEOUS AND TOTAL LDH RELEASE

Figure 7 is a summary of the spontaneous release and total LDH release from all of the cell lines with detectable LDH levels. The procedure followed is the same as that detailed in figure 1. LDH available for release, following a specific cytotoxic reaction is indicated as a percent of total LDH available. The mean of triplicate observations were used in the following formula:

$$\left[\frac{(\text{total R.} - \text{lysis control}) - (\text{Spontaneous R.} - \text{media control})}{(\text{total R.} - \text{lysis control})}\right]$$

Percent of Total LDH After Spontaneous Release

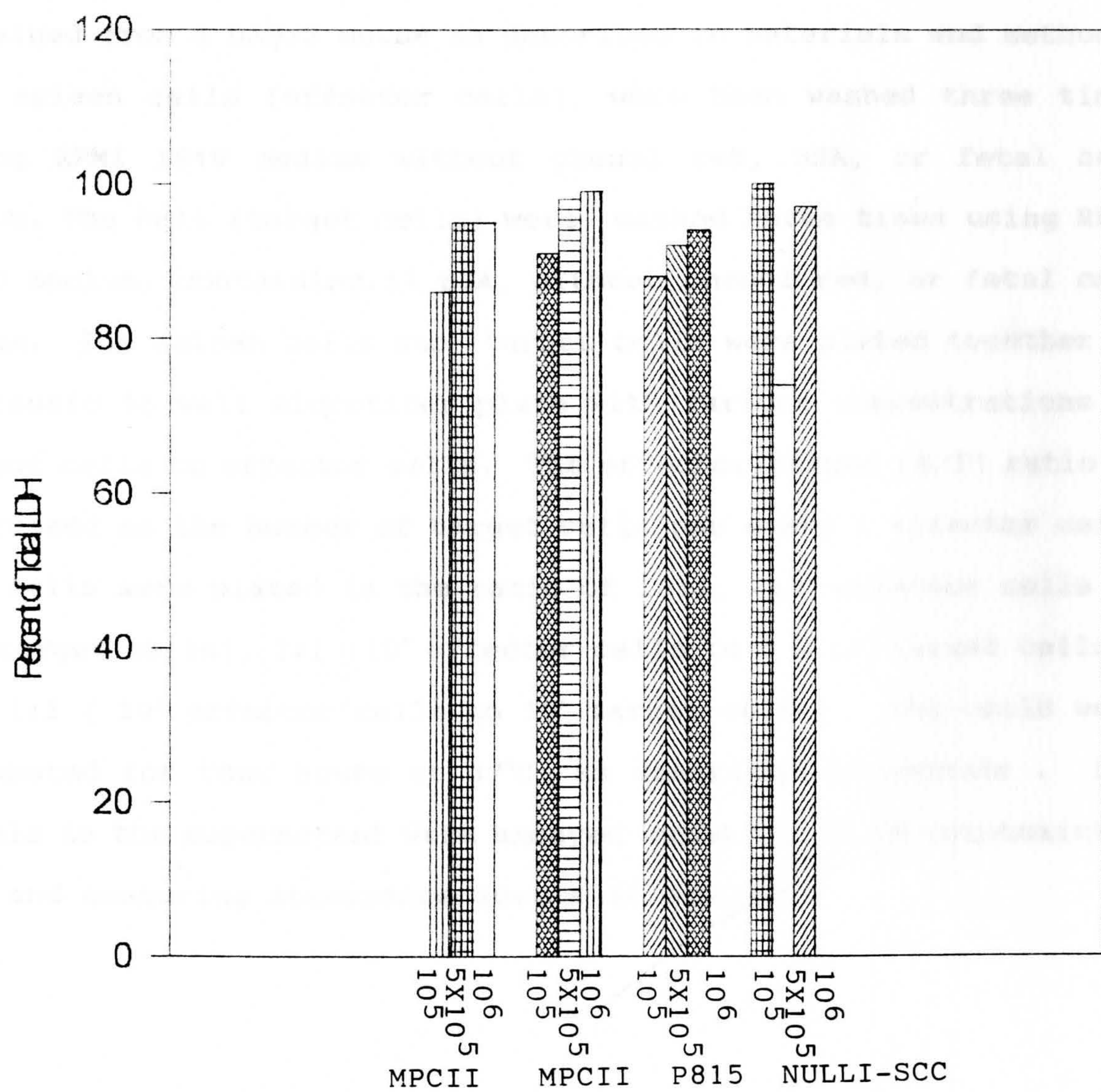


FIGURE 8

KILLER CELL LYSIS OF P815 TARGET CELLS

Figure 8 shows the percent cytotoxicity when P815 target cells were plated with effector cells from the spleen of the mouse haplotype DAB/2. All numbers represent the mean and standard error of triplicate observation from one experiment. Spleen cells were obtained from a DAB/2 mouse as described in materials and methods. The spleen cells (effector cells), were then washed three times using RPMI 1640 medium without phenol red, BSA, or fetal calf serum. The P815 (target cells) were washed three times using RPMI 1640 medium, containing 1% BSA, without phenol red, or fetal calf serum. The spleen cells and target cells were plated together in a plastic 96 well microtiter plate with various concentrations of target cells to effector cells. The effector/target (E/T) ratio is expressed as the number of target cells per every 1 effector cell. The cells were plated in the ratio of 10:1, (10^6 effector cells to 10^5 target cells), 2:1 (10^6 effector cells to 5 X 10^5 target cells), and 1:1 (10^6 effector cells to 10^6 target cells). The cells were incubated for four hours at 37°C, as described in methods . LDH levels in the supernatant were assayed using the 96 TM cytotoxicity kit and measuring absorbance levels at 490 nm.

P815 plus KILLER CELLS

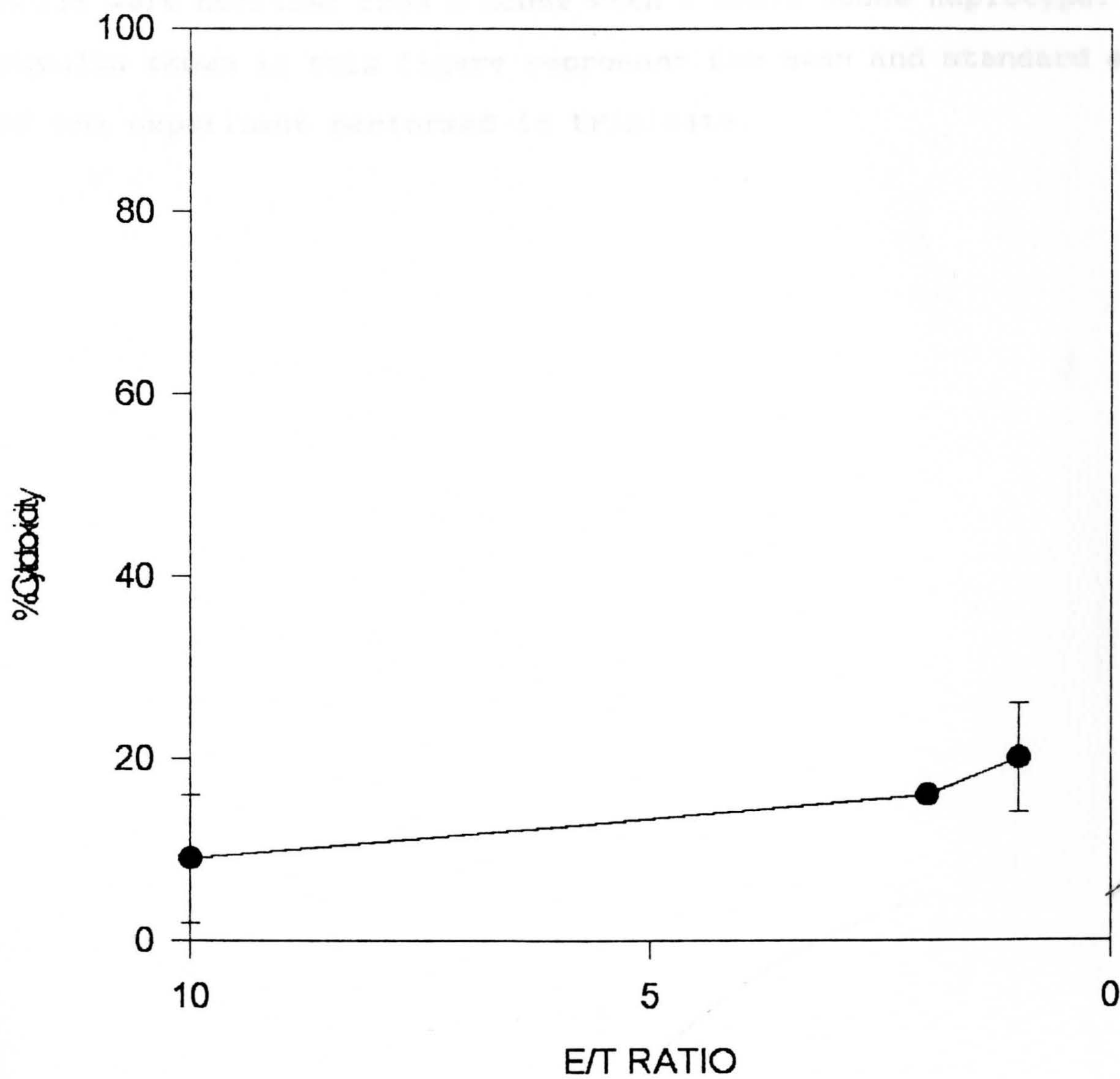


FIGURE 9

NATURAL KILLER CELL LYSIS OF NULLI-SCC TARGET CELLS

Figure 9 shows the percent cytotoxicity when Nulli-SCC target cells were plated with effector cells (spleen cells).The procedure followed is the same one that is detailed in figure 8. Spleen cells were obtained from a mouse with a DAB/2 mouse haplotype. The results shown in this figure represent the mean and standard error of one experiment performed in triplcate.

NULLI SCC plus KILLER CELLS

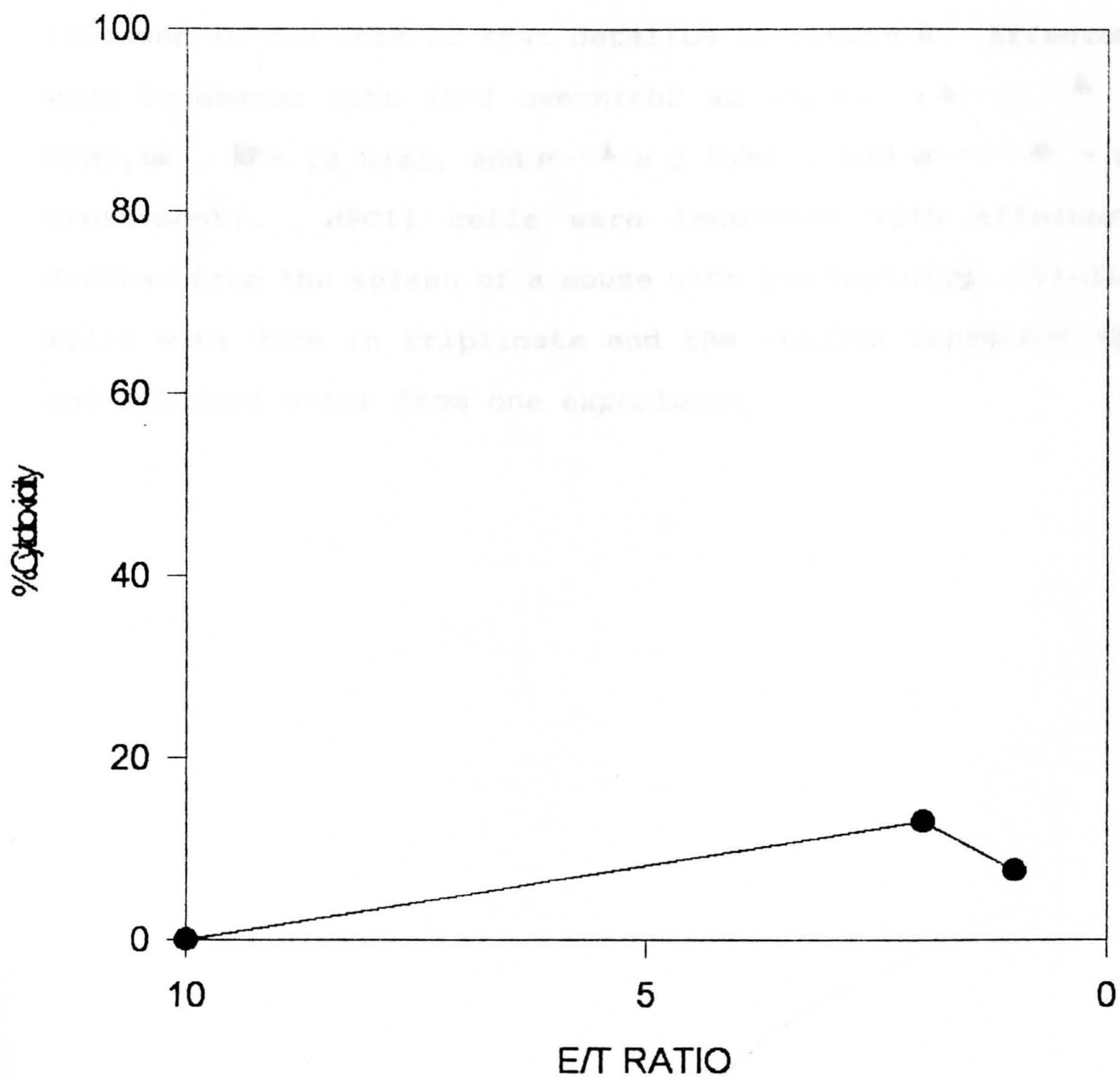


FIGURE 10

KILLER CELL LYSIS OF MPCII TARGET CELLS, EXP.1

Figure 10 shows the percent cytotoxicity when MPCII target cells were plated with effector cells (spleen cells). The procedure followed is the same as that detailed in figure 8. Effector cells were incubated with IL-2 overnight at 37₀ C. (▲ ---- ▲ = 100 U/ml, ■ -■= 10 U/ml, and ● --▲ = 1 U/ml , and ● --- ● = no IL-2 treatement). MPCII cells were incubated with effector cells derived from the spleen of a mouse with the haplotype C57/J16. All wells were done in triplicate and the results represent the mean and standard error from one experiment.

EXP.1

MPC II PLUS IL-2 STIMULATED KILLER CELLS

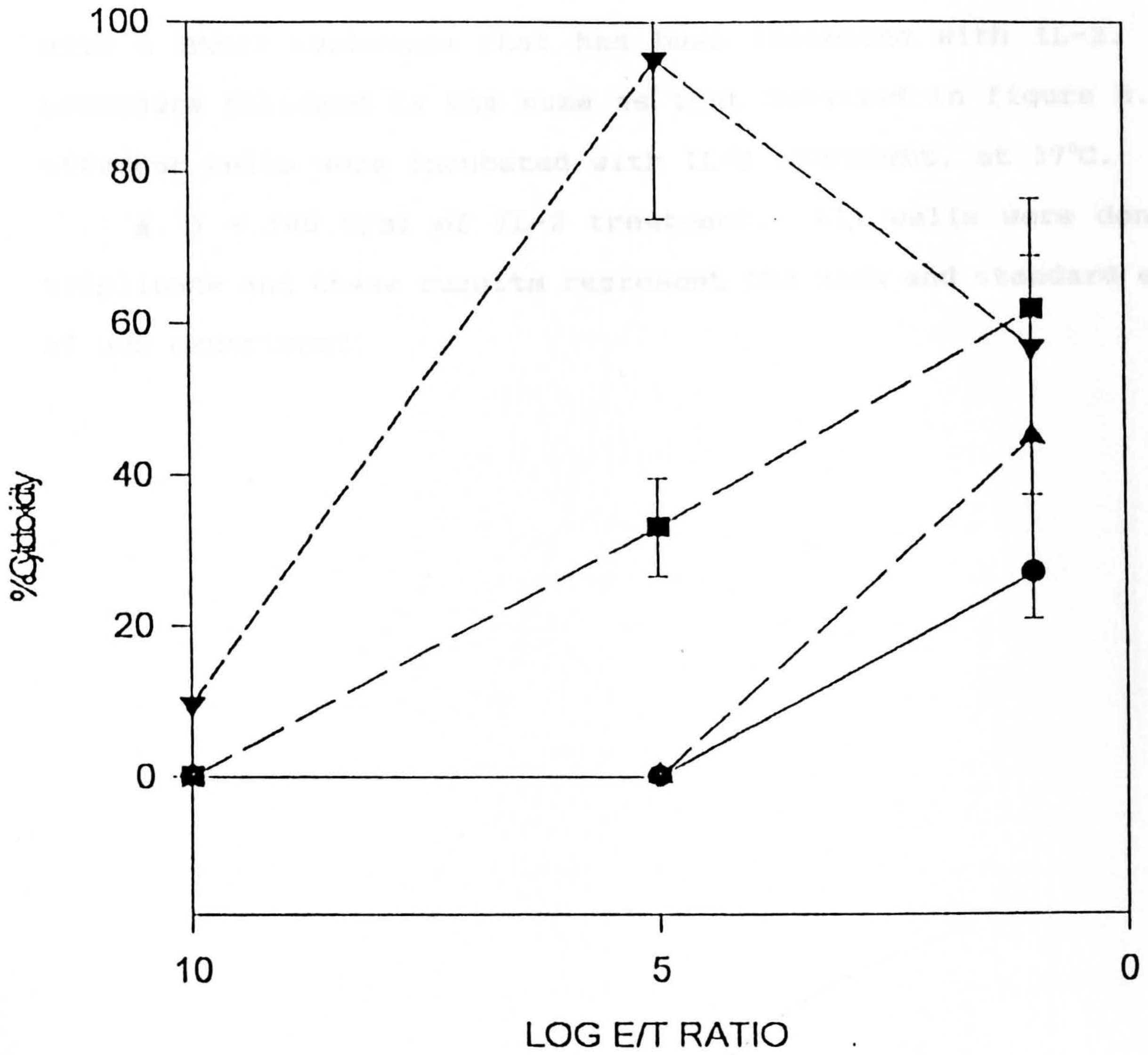


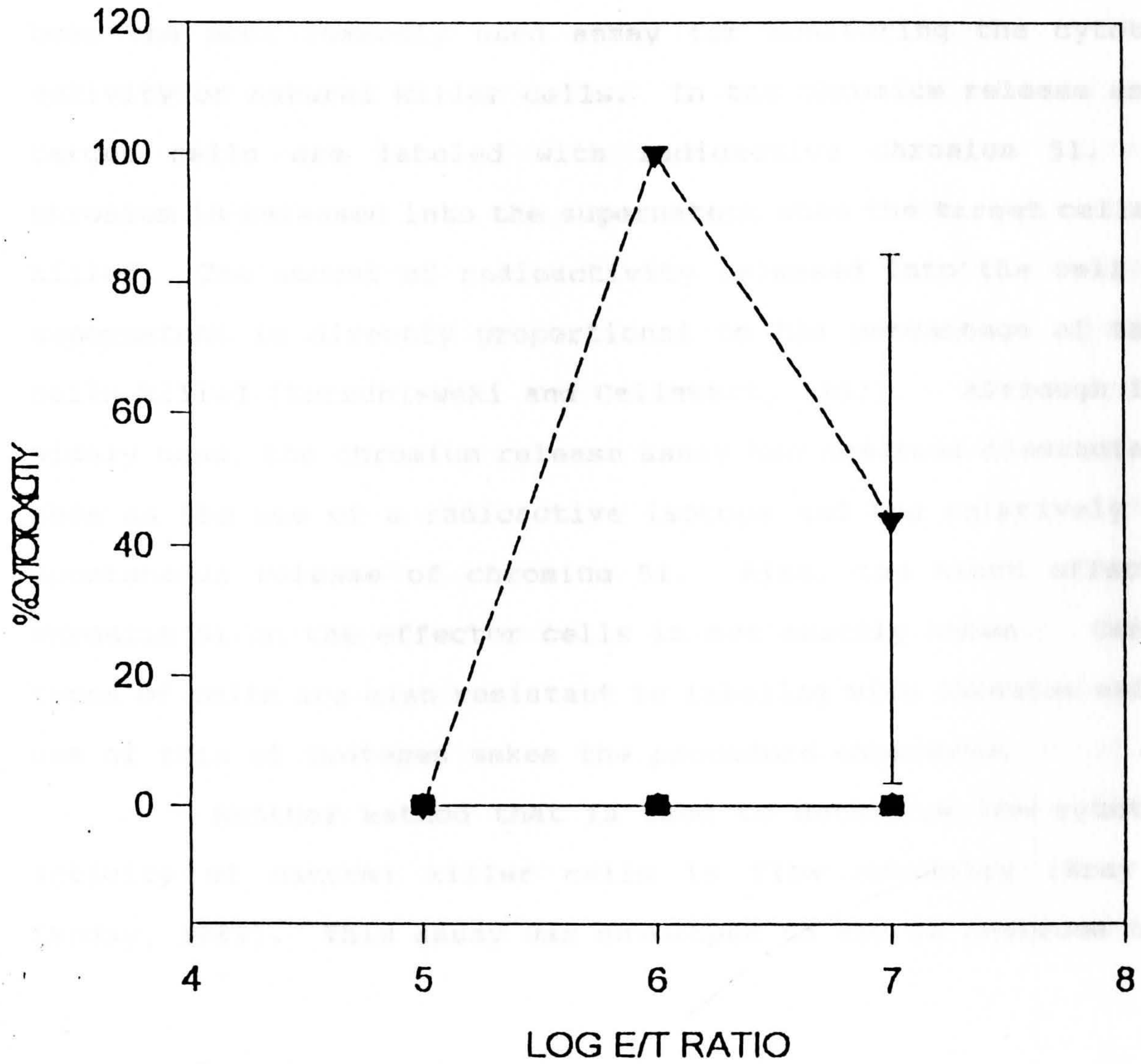
FIGURE 11

KILLER CELL LYSIS OF MPCII TARGET CELLS, EXP. 2

Figure 11 shows cytotoxicity values derived when MPCII target cells were incubated with effector cells from the spleen of a mouse with a DAB/2 haplotype that had been incubated with IL-2. The procedure followed is the same as that detailed in figure 8. The effector cells were incubated with IL-2 overnight, at 37°C. (▲ - - - ▲) = 100 U/ml of IL-2 treatment. All wells were done in triplicate and these results represent the mean and standard error of one experiment.

EXP.2

MPCII PLUS IL-2 STIMULATED KILLER CELLS



CHAPTER IV

DISCUSSION

The cytotoxicity 96 TM assay used in this sudy, is a colorimetric alternative to other methods used to quantitate natural killer cell cytotoxicity. The chromium release assay has been the most commonly used assay for monitoring the cytotoxic activity of natural killer cells. In the chromium release assay, target cells are labeled with radioactive chromium 51. The chromium is released into the supernatent when the target cells are killed. The amount of radioactivity released into the cell free supernatent is directly proportional to the percentage of target cells killed (Korzeniewski and Callewart, 1983). Although it is widely used, the chromium release assay has numerous diadvantages, such as the use of a radioactive isotope and the relatively high spontaneous release of chromium 51. Also, the exact effect of chromium 51 on the effector cells is not exactly known. Certain types of cells are also resistant to labeling with chromium and the use of this of isotopes makes the procedure expensive.

Another method that is used to determine the cytotoxic activity of natural killer cells is flow cytometry (Bray and Landay, 1989). This assay was developed to try to overcome the

disadvantages of using radioactive isotopes. Flow cytometers are instruments capable of analyzing properties of single cells as they pass through an opening at high velocity. Examples of measurements that can be made include physical characteristics such as size, volume, refractive index, viscosity and chemical features such as content of DNA and RNA, proteins, and enzymes. With the aid of a flow cytometer, a single cell suspension may be analyzed for various measurements simultaneously at the rate of five thousand cells per second. Flow cytometry can also be used to separate various classes of lymphoid cells through sorting by size or antibody marker, or it can be used to separate live or dead cells.

The most commonly encountered problems in flow cytometric cytotoxicity assays are the overlaps between the light scatter signals of the effector and target cells, and the spontaneous leaking of the fluorescent marker, used to determine cytotoxic activity, out of the cells (Radosevic, et. al., 1990). The use of flow cytometric cytotoxicity assays is not feasible for this project as it requires highly specialized and expensive equipment.

Another method used to study the cytotoxic activity of natural killer cells is the colorimetric microassay (Frey et. al., 1987). In this assay, natural killer cells are separated from other lympnocytes by flow cytometry, and further identified by morphology and staining. The natural killer cells are stained with a fluorescent antibody, then the natural killer cells are

incubated with target cells. After incubation, MTT dye is added and the plates are read at 600 nm on a computer. The MTT dye serves as an indicator of target cell survival for assessment of tumour cytotoxicity. However, this MTT dye leaks out of the cells, causing high background and low sensitivity. This assay is primarily used for identification of toxic compounds that kill large numbers of target cells and does not appear to be sensitive enough for natural killer cell cytotoxicity assays (Frey, et. al., 1987).

The single cell assay is another method used to look at the cytotoxic activity of natural killer cells. Using this method it is possible to define the phenotypes of the conjugate forming cells responsible for the natural killer cell function. In this method, total white blood cell counts are determined on a coulter counter (Schuit et. al., 1989). The target cells are incubated together with the effector cells. After incubation, they are centrifuged, part of the supernatent is removed, and the tubes are placed in a water bath and melted agarose is added. The cell pellet is then resuspended and an aliquot is placed onto a coverslip. On this slide conjugate forming cells are counted. These cells are defined as the proportion of natural killer cells binding to target cells. The proportion of effector cells capable of lysing a target cell is scored by counting at least one hundred conjugate forming cells (Schuit et. al., 1989). The natural killer cells are separated from other lymphocytes by monoclonal antibody binding. A second fluorescent antibody is then added that will bind to the first

antibody. This assay allows the study of binding patterns of natural killer cells to target cells. The absolute number of active natural killer cells can be calculated from this assay. This procedure is very tedious and time consuming, and therefore, not suitable for testing hybridoma cell lines, where possibly hundreds of fused cells will be examined.

The use of carboxyfluorescein diacetate is another way to study the cytotoxic activity of natural killer cells. This is an automated fluorometric assay used for the detection of natural killer cell activity (Suzuki, et. al., 1991). The lysis of target cells by natural killer cells is measured by the amount of carboxyfluroescein diacetate that is released into the supernatent of the culture wells. This method proved more accurate and rapid than the chromium 51 release assay. However, this method yields high spontaneous release and requires access to a fluorometer.

The methods discussed thus far, except for the chromium release assay, have been employed to avoid the use of radioactivity. We tested the 96TM cytotoxicity assay as it was the most convenient and suitable to our budget. Also we had the equipment necessary to use this assay. This assay is a colorimetric alternative to the chromium release assay. This colorimetric assay quantitatively measures lactate dehydrogenase. Lactate dehydrogenase is a stable cytosolic enzyme that is released when a cell is lysed. Thus, instead of measuring chromium, LDH released from the lysed cells is measured. This method involves less cost, but serum free medium and phenol red free medium must be used to

reduce background absorbance.

This study investigated possibile cell lines that could be used in the 96 TM cytotoxicity assay, since other studies using the 96 TM cytotoxicity assay have been done only with human cell lines, or cell lines with an inappropriate haploytpe. Thus, using this assay we examined the mouse myeloma cell lines YAC-1, P815, MPCII, NULLI-SCC, and L1210. Using the 96 TM cytotoxicity LDH assay, we determined the spontaneous and total release of LDH for each cell line by plating the cells alone and with lysis buffer. In this study, the YAC-1 and L1210 target cells did not contain sufficient amounts of LDH to be used in this assay, so that the cytotoxicity of natural killer cells against them could not be measured. The MPCII, P815, and NULLI-SCC cell lines did contain sufficient amounts of LDH necessary to observe the cytotxicity of natural killer cells against these tumor cell lines, and had relatively low levels of spontaneous LDH release in order to observe the cytotoxicity of natural killer cell aganist these tumor cell lines. We were able to determine % cytotoxicity using P815, NULLI-SCC, and MPCII cells, by measuring LDH released when these mouse myeloma cells were lysed by natural killer cells from mice.

In the cytotoxicity assay, Nulli-Scc cells were shown to act as expected for a negative controlative control. They were not lysed by the natural killer cells. THe P815 cells released higher amounts of LDH upon lysis, than was expected from a negative control. The MPCII cells were chosen as a positive control, because, using the Chromium 51 cytotoxicity assay as a detection

method, they are lysed by natural killer cell activity. The cytotoxicity assay involving MPCII cells worked well only when the MPCII target cells were incubated with IL-2-treated effector cells. It was observed that the greater the concentration of IL-2, the greater the cytotoxicity. These results suggest that the assay may not be sensitive enough to detect lysis by resting natural killer cells. However, similar problems may also be seen when using the Chromium 51 cytotoxicity assay.

In summary, the tumouricidal activity of natural killer cells against five different tumor cell lines, YAC-1, P815, NULLI-SCC, MPCII, and L1210 was observed in this study using the 96TM colorimetric cytotoxicity assay. We have demonstrated that the 96 TM cytotoxicity assay can be utilized to measure mouse natural killer cell activity. We can now use this assay to select a pure natural killer cell line from our hybridomas. With a pure natural killer cell line we can investigate the effect of vitamin D on natural killer cells without the problem of contaminating T cells or monocytes.

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