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Carl J. Wust Ph.D., Major Professor

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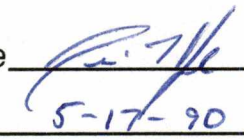
Signature 

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Induced Cell Death in the Leukemia Cell Line, K-562, by Specific Antibodies

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

James W. Hodge

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ABSTRACT

The detrimental effect of specific antibody on K-562 cells is evident in certain cellular processes. K-562 human leukemia cells grown in the presence of rabbit-anti-K-562 serum or derived gamma globulin die within 5 days without observable lysis. Within the first 24 hours, there is over a 90% decrease in DNA synthesis. These events occur in the absence of complement or effector cells.

To characterize the mechanism of this induced cell death using well-defined antigens, 9 monoclonal antibodies found to be reactive with K-562 cells by cytofluorography, could be categorized into three groups based on their effect on the uptake of ^{3}H-TdR into acid precipitable DNA. The groups are: no effect [IL-2R1(IgG2a), Leu-M1(IgG2b), Leu-M1(IgM), OK-M1(IgG2b)], increased ^{3}H-TdR uptake [BA-1(IgM), HLA-A,B,C(IgG2a)] and decreased ^{3}H-TdR uptake [OKT9(IgG1), OKB2(IgG1), and PM81(IgM)]. These categories are not correlated with antibody isotype, suggesting that a non-specific Fc binding event is not responsible for the antibody inducement of cell death. These findings suggested that antibody may induce a programmed cell death, or apoptosis. Since one of the main event markers for apoptosis is DNA fragmentation, this was assesed in the K-562 model. DNA fragmentation studied in most apoptotic systems has been done in the mouse and evidence of DNA degradation in human cells has been controversial. As expected, we did not find double strand fragmentation of DNA from K-562 cells treated with specific antiserum. Upon examination of DNA from K-562 cells treated with antiserum, extensive single strand nicking was observed after analysis of

strand separating agarose electrophoresis and centrifugation of purified DNA through an alkaline (pH 13) sucrose gradient. Interestingly, the DNA of control K-562 cells also showed evidence of single strand nicking albeit to a lesser extent than treated cells. Antibody induced cell death could not be prevented with cycloheximide, suggesting that protein synthesis is not required for DNA cleavage. Antibody induced cell death was prevented by the addition of aurintricarboxylic acid, an endonuclease inhibitor and general inhibitor of DNA binding proteins.

All of these results taken together support the conclusion that some surface receptors react with antibody to transduce signals which lead to a programmed cell death. Once the mechanisms of these transductions are understood, other reagents could be used to trigger the process. This capability to trigger death in unwanted cells could have a unique therapeutic advantage.

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CHAPTER I

INTRODUCTION

Many current leukemia therapies consist of treatments with chemicals or drugs that can induce immortal leukemia cells into terminal differentiation and normal death. In this report, the role of antibodies on the inducement of cellular suicide is examined.

The use of antibody to inhibit cellular growth has been shown in several different in- vivo systems. For example, Bernstein et al. (3) noted regression of mouse T cell leukemia after treatment with monoclonal antibodies against a thymus differentiation antigen. This group theorized that complement directly killed the tumor cells at the local and metastatic sites. Herlyn et al. (14) noted the inhibition of colorectal carcinoma in nude mice by the monoclonal antibody 1083-17-1A, while Latif et al. (22) found that human leukemia cells transplanted into nude mice were inhibited to grow by the injection of goat anti-K-562 serum even in mice that were given cobra venom factor to inhibit complement. Although the Herlyn and Latif groups formulated the hypothesis that killer cells were responsible for the loss of tumor cell viability, their results supported the alternative theory that antibody alone may have directly reduced cell viability.

In this report, the mechanism(s) of antibody induced cell death without complement, effector cells, or cytolysis was investigated. Rabbit-anti-K-562 serum or derived gamma globulin incubated with the human leukemia cell line, K-562, caused over 80% of the cells to die within 5 days. The mechanisms of specific inducement of leukemic cell death by antibody appear to resemble events associated with the phenomenon of apoptosis or programmed cell

death and, accordingly, the aim of this project was to begin to understand the mechanisms of this phenomenon. The concept of programmed cell death is not to teach unwanted cells to die, but to activate a sequence of metabolic events that lead to normal senescence. Once the sequence in apoptosis is understood, the use of antibody might be replaced by appropriate chemical reagents that can induce the same cascade. The capability to trigger unwanted cells into programmed cell death may have a therapeutic advantage in cancer patients.

CHAPTER II

SURVEY OF THE LITERATURE

I. Use of Polyclonal or Monoclonal Antibodies to Inhibit Cell Growth

The use of antibody to inhibit cellular growth has been shown in several different in vivo systems. For example, Bernstein et al. (3) noted regression of mouse T cell leukemia after treatment with monoclonal antibodies against a thymus differentiation antigen. The tumor suppressive effects of the monoclonal antibody, Thy1.1, were amplified by the administration of exogenous complement. This combined antibody-complement therapy resulted in the cure of a significant number of the C57/BL6 mice transplanted with ML.AKRSL2 cells. Bernstein et al. (3) theorized that the complement directly killed the tumor cells at the local and metastatic sites. Herlyn et al. (14) noted the inhibition of colorectal carcinoma in nude mice by the hybridoma derived monoclonal antibody 1083-17-1A. They concluded that the mechanism of direct lysis of the tumor cells was not due to complement activation since the monoclonal antibody did not cause cytolysis in in vitro assays. Latif et al. (22) found that the growth of human leukemia cells transplanted into nude mice was inhibited by the injection of goat anti-K-562 serum even in mice that were given cobra venom factor to inhibit complement. Although the Herlyn and Latif groups formulated the hypothesis that killer cells were responsible for the loss of tumor cell viability, their results supported the alternative theory that antibody alone may have directly reduced cell viability.

In invitro studies, antibody to certain tumor cells decreased cell viability. Johnson et al. (18) noted that IgG1 from antiserum, containing antibodies specific for the murine lymphoma 6C3HED, caused a decrease in viability and eventual cell death when incubated with a C3H derived lymphoma, 6C3HED, of thymic origin. Yang and Vas (59) reported that antibody specific for mouse leukemia L5178 cells inhibited the growth of the cells in vitro and cell death was preceded by a decline in the rates of DNA, RNA, and protein synthesis as measured by uptake of radioactive nucleic acid precursors or amino acids (60). Trauth et al. (50) reported that tumor regression by monoclonal antibody appeared to induce cell death analogous to apoptosis. From this laboratory, Green et al. (11,27) showed that goat antiserum, made against the human leukemia cell line, K-562, would inhibit macromolecular synthesis similar to the inhibitions observed by Yang and Vas in their system (59). In addition, certain monoclonal antibodies against K-562 cells have been found to accelerate the killing of the cells by large granular lymphocytes (40), although this was probably due to antibody-dependent cellular cytotoxicity.

As noted above, the effects of specific antisera on the growth of K-562 cells have been the subject of several studies and it was concluded that antibody alone could cause the death of K-562 cells in the absence of complement or effector cells (11,27,22,53,55). The mechanisms by which the antibodies cause cell death are currently unknown. Further, the declination of cell viability by antibody is not the only effect that may be initiated by antibody in this cell line. Ritz et al. (41) observed that monoclonal antibodies used in vitro could induce modulation of a specific leukemia antigen. Weissman et al. (52) noted that the

exposure of K-562 cell to the anti-transferrin receptor monoclonal antibody, OKT9, resulted in two major effects related to receptor dynamics: a re-distribution and a hyperdegradation of the transferrin receptor. On the basis of the above reports, the role of antibodies as passive reactant bystanders in immune reactions may need to be re-evaluated.

II. Apoptosis as a mechanism for cell death.

Apoptosis, or programmed cell death, is a Greek word used to describe the falling of leaves from trees, or petals from flowers (16). When used to describe cellular events, the term apoptosis refers to programmed cell suicide. During ontogeny, as certain cells are no longer needed (e.g., those on the tail of a developing tadpole), they undergo a physiological cell death. During embryogenesis, the human fetus develops web-forming tissue between the fingers of the hands which is lost during further development. It has been hypothesized that as development continues, the cells of that tissue undergo apoptosis and the webs are reabsorbed (16). Wyllie et al. (56) noted that the phenomenon of apoptosis in cells can be identified by several cellular events. In the earliest stages, the cell can be revealed by electron microscopy to show that a majority of the chromatin becomes aggregated into large compact granular masses. Next, after chromatin condensation, the cytoplasm usually begins to condense. At this point, DNA damage occurs in the form of double or single strand strand fragmentation and the nuclear membrane starts to exhibit extensive blebbing. Finally, the loss of volume homeostasis, irreversible damage to mitochondria and altered ionic concentrations within the cell are

associated with a collapse of all other vital functions, including macromolecular synthesis. Eventually, the cell dies (56); however, the mechanism by which they die appears to differ greatly from the mechanism described as necrosis. Unlike cells undergoing necrosis, apoptotic cells do not show any evidence of increased membrane permeability. The comparative cellular content of energy rich nucleotides also emphasize the differences between apoptosis and necrosis. Unlike necrosis, depletion of intracellular ATP or cAMP in apoptosis does not regularly precede the onset of the terminal morphological changes (57,58).

III. Processing of DNA in cells undergoing damage from various sources.

As stated above, DNA fragmentation is a marker event for apoptosis as well as in other mechanisms of cell death. Chang et al. (7) reported that extensive DNA cleavage preceded diphtheria toxin-induced cytolysis. Calcium-dependent DNA fragmentation has been shown to occur in immature thymocyte suspensions extracted from young rats in vitro (33) and cytotoxic T cells have been shown to induce significant DNA degradation (9). Wyllie et al. (57) has noted that chromatin damage and other apoptotic events can be induced in cells. For example, Cohen et al. (8) noted that corticosterone can kill mouse thymocytes as measured by eosin uptake after several hours of in-vitro incubation. These authors theorized that this killing required RNA and protein synthesis because it was inhibited by actinomycin D and cycloheximide, respectively. Furthermore, they postulated that a magnesium or calcium ion influx probably activates an endonuclease that causes DNA fragmentation,

especially in the linker regions between nucleosomes. They did not observe this effect in mature human T cells in vitro. Wyllie et al. (56) also noted a requirement for endonuclease in the glucocorticoid-induced thymocyte apoptotic system since the endonuclease inhibitor, aurintricarboxylic acid, blocked the final outcome.. Kizaki et al. (20) reported that calcium ionophores and phorbol esters could activate a suicide process similar to apoptosis in rat thymocytes. They theorized that the calcium ionophore, A23187, induced an influx of extracellular calcium, which then activated a calcium-dependent endonuclease that fragmented the genomic DNA. McConkey et al. (32) noted that a sustained increase in cytosolic calcium levels appeared to stimulate both endonuclease activation and cell death in thymocytes exposed to glucocorticoid hormones and anti-CD3 antibodies (32).

The DNA processing mentioned thus far has been described as double stranded fragmentation in murine systems. Apoptosis in human cells has been difficult to define because no double strand fragmentation has been observed with human cell models. For example, many authors have found that double strand fragmentation was not occurring in human target cells lysed by human effector lymphocytes, including cytotoxic T cells, natural killer cells, and killer cells; however, Gromkowski et al. (12) reexamined these systems and found that in many cases where double stranded fragmentation appeared to be absent, there was, in fact, extensive single strand nicking (12). This is important because apoptosis in human cell lines must be apprised for single strand DNA nicking since double strand fragmentation may not occur.

Endonuclease can be activated by mechanisms other than that of calcium or magnesium ion influx. Gromkowski et al. (12) proposed that DNA damage

could be triggered by any kind of event or substance that activated the enzymes of the cell, possibly by releasing enzymes and/or their activators from sequestering compartments.

How can antibodies, defined to induce apoptosis, cause target cell DNA degradation ? McConkey et al. (32) noted that sustained calcium ion increases, such as those observed in response to antigens or mitogenic lectins, also mediate lymphoid cell proliferation. However, these signals are only mitogenic when they are accompanied by other independent signals, delivered by accessory cells. Since these independent signals can often be replaced by phorbol esters, which activate protein kinase C (PKC), they suggested that these second opposite signaling events, either for T cell proliferation or apoptosis, includes PKC activation. Interestingly, Muller et al. (37) reported that the phosphorylation of histone H1 was responsible for the normal chromatin condensation preceding cellular division in a Physarum polycephalum model. Since apoptosis is characterized by chromatin condensation preceding DNA fragmentation, the unregulated activation of a protein kinase could lead to the hyper/hypo phosphorylation of certain regulatory proteins such as histones. Antibody could also be mimicking the effects of growth factors on target cells such as anti-insulin receptor antibodies that activate the insulin receptor. The antibodies that induce apoptosis could resemble hormones in that they can bind to certain receptors and cause signal transduction, but they could be significantly different from hormones in their rate of degradation and the kinetics of receptor associations/dissociations. These differences could possibly induce unregulated metabolism and the eventual cascade of events leading to cell death. To support this notion, McConkey et al.

(35) noted that Interleukin-1 inhibited anti-T cell receptor monoclonal antibody mediated apoptosis in immature thymocytes and Koury et al. (21) found that erythropoietin retarded DNA degradation and prevented programmed cell death in erythroid progenitor cells. Hematopoietic colony stimulating factors were also found to promote cell survival by suppressing apoptosis (54).

IV. The K-562 cell line.

The K-562 cell line was established by Dr. Carmen B. Lozzio from a patient with chronic myelogenous leukemia in terminal blastic crisis (24).This cell line was found to be particularly sensitive to natural killer cell activity and is now used worldwide as a standard target in natural killer cell assays. The cell line has many distinctive properties including the persistence of the Philadelphia (Ph^1) chromosome. The Ph^1 chromosome is a reciprocal translocation [+(9:22)(q34:q11] in which the c-abl oncogene from chromosome 9 is translocated to chromosome 22 and the c-sis oncogene from chromosome 22 to 9. K-562 cells are pluripotent and are capable of differentiating into precursors of the granulocytic, monocytic, and erythrocytic cell lineages (24,25,26,28). Certain sublines of the K-562 cell line are also capable of continuous or modulation expression of hematopoietic cell antigens. Rowley et al. (43) noted that specific translocations are a prominent feature of many types of leukemia and lymphomas. Further, based on studies of subline karyotyping (C. Lozzio, personal communication) the K-562 cells could be continually undergoing translocations, wherein ligation and repair of the genomic DNA is occurring.

Associated with these translocation events in the K-562 cell line is the phosphorylation of various regulatory proteins by the oncogene BCR-ABL and the oncogene product p210, which is theorized to be responsible for the immortality of the tumor cell. Kipreos et al. (19) proposed that mitotic phosphorylation of histone H1 could cause chromatin condensation that may be involved in the normal chromatin condensation and nuclear breakdown. It is likely that if antibody can cause an imbalance in the cellular machinery, the genes responsible for normal division and senescence could be reactivated. The concept of programmed cell death is not to teach unwanted cells to die, but to activate a sequence of metabolic events that lead to normal senescence. Once the sequence in apoptosis is understood, the use of antibody might be replaced by appropriate chemical reagents that can induce the same cascade. The capability to trigger unwanted cells into programmed cell death may have a unique therapeutic advantage in cancer patients

CHAPTER III

MATERIALS AND METHODS

I. Animals

Female, 6 lb, New Zealand White rabbits were obtained from Myrtle's Rabbitry, Thompson Station, Tennessee. The rabbits were fed 1/2 cup food per day and water ad libitum.

II. Cell Cultures

The multipotent leukemia stem cell line, K-562, subline F-1-1 , was obtained from the laboratories of Dr. Carmen Lozzio at the University of Tennessee Medical Research Center where they were examined for karyotype and morphology.

The cells were maintained in suspension culture in Eagle's medium supplemented with nonessential amino acids (Flow) and 10% bovine calf serum (Hyclone) heat-inactivated at 56 C for 30 minutes. This growth medium will hereby be referred to as EM-10a. The K-562 cells were grown to a density not to exceed 5×10^6 cells/ml from a seed of 5×10^4 cells/ml. When maximum density was reached, the cultures were split 1:1 with fresh EM-10a.

In some experiments, The cells were grown in a serum-free medium in which LPSR-1 (Sigma) was substituted for bovine calf serum in the EM-10a medium. All cultures were grown at 37 C in a 5% CO_2 atmosphere.

III. Polyclonal Antisera Production

Initially, each rabbit was bled to obtain pre-immune serum and then injected with 10^7 K-562 cells in Complete Freund's Adjuvant (Gibco) of which 0.2 ml was given in both hind legs below the popliteal lymph node and 1.35 ml was given subcutaneously in the intrascapular region.

After two weeks, a second dose of 10^7 K-562 cells in Incomplete Freund's Adjuvant was administered at sites similar to the first immunization. The rabbits were fed 1/2 cup food per day and water ad libitum except for the 24 hours before bleeding when the food was withheld to reduce lipid content in the serum.

After 14 days, blood was obtained with a #21 gauge needle from the right central ear artery. Serum was separated from the blood clot and the gamma globulins fractionated by ammonium sulfate precipitation.

IV. Precipitation of Gamma Globulins from Whole Serum by Ammonium Sulfate

Serum was diluted 1:4 with water and one-third volume of saturated ammonium sulfate was then added dropwise. The ammonium sulfate was adjusted to pH 7.8 with ammonium hydroxide just prior to the precipitation of the gamma globulin. After the addition of the ammonium sulfate, the mixture was stirred for an additional 2-3 hours. The suspension was then centrifuged at room temperature for 30 minutes at 3000 rpm with an average rotating radius of 14 cm and the precipitate was dissolved in a volume of PBS equivalent to that of the original serum sample. The procedure was repeated twice more. After the third precipitation, the pellet was dissolved in PBS to a final volume of half the original serum volume and the solution was dialyzed for at least 2 days against a constant feed of PBS. Dialysis was considered complete when no precipitate formed with the addition of barium chloride to the dialysate (10).

V. Labeling of Cells with ^{51}Cr

K-562 cells ($8 \times 10^{6)}$ were centrifuged at 3000 rpm for 10 minutes at ambient temperature and the pellet was suspended in 1 ml of EM-10a, then 800 microCuries of ^{51}Cr (Na_2CrO_4, ICN) was added. The suspension was incubated in a 37 C water bath for 1 hour. The radiolabled cells were then centrifuged at 3000 rpm for 10 minutes. After centrifugation, the supernatant fluid was discarded and the cells suspended in 10 ml of EM-10a. This wash step was repeated five times to remove all non-internalized ^{51}Cr. After the last wash, the cells were suspended in 2 ml EM-10a and viability was assessed by the trypan blue dye exclusion.

VI. Analysis of Viability with Trypan Blue Dye Exclusion.

Live and dead cells were distinguished on the basis of their ability to exclude trypan blue dye (10). The membrane of a living cell is impermeable to this dye and the cell appears colorless in the presence of trypan blue. The samples to be tested were placed into 10 x 75 mm test tubes and one third the volume of 0.4% trypan blue stain (Gibco) was added. The samples were incubated at room temperature for 5 minutes to allow the dead cells to take up the dye. After incubation, 20 µl of sample were pipetted onto the stage of a hemocytometer and viable cells were quantified. The following formula was used to calculate viability:

$$\% \text{ Viable cells} = \frac{\text{\# Live cells}}{\text{Total \# cells counted}} \times 100$$

VII. Antibody Dependent Complement Mediated Cytotoxicity (ADCMC)

To determine the total releasable amount of ^{51}Cr in the cells, , 100 µl of 3% Triton-X-100 were added to 100 µl of cells in medium (100 µl). The spontaneous release labeled cells were incubated without further additions. Other controls consisted of cells with complement alone, or with antiserum or gamma globulin alone. Serum or gamma globulin was two-fold serially diluted in round bottom microtiter plates in a final volume of 50 µl to which was added 50 µl (10^4 cells) of ^{51}Cr-labeled K-562 cells.

Low-Tox-H Rabbit Complement (Cedarlane) was reconstituted with ice-cold EM-10a and 50 µl was added to each well except for the spontaneous release wells. Plates were incubated for 90 minutes at 37 C and then centrifuged at 700 x g for 7 minutes. Supernatant fluid (75 µl) was harvested from each well into a 12 x 75 mm borosilicate glass culture tube for determination of released radioactivity in a Beckman Gamma Spectrometer.
The formula used to determine specific Cytotoxicity was:

$$\% \text{ Specific Cytotoxicity} = \frac{\text{Sample CPM - Spontaneous Release CPM}}{\text{Total Releasable CPM}} \times 100$$

VII. Monoclonal Antibodies

The monoclonal antibodies used in this study were:

MAb	Source	Isotype	Antigen
LEU M-3	Becton Dickinson	IgG2b	CD14; found on monocytes; macrophages, granulocytes, and follicular reticular dendritic cells.
LEU M-1	Becton Dickinson	IgM	CD15; found on granulocytes and monocytes.
OK-T9	Coulter	IgG1	CD71; transferrin receptor found on proliferating cells and macrophages.
OK-M1	Coulter	IgG2b	CD11b; found on monocytes, granulocytes, and natural killer cells.
OK-B2	Coulter	IgG1	CD21; found on B cell subset.
IL2R1	Coulter	IgG2a	CD25; interleukin-2 receptor.
HLA-1	Dako	IgG2a	Class 1 common histocompatibility antigen.
BA-1	Boehringer Mannheim	IgM	B cell associated antigen.
PM-81	E. D. Ball. Dartmouth	IgM	Myeloid antigen

VIII. Statistical Treatment of the Data

All experiments were done and repeated in triplicate under identical conditions, and the data were statistically analyzed using the commercial statistical analysis package Statveiw (Abacus Concepts Inc.)

IX. Viability Assays (Thymidine uptake and Trypan Blue exclusion)

Polyclonal:

The antiserum was twofold serially diluted in EM-10a in round bottom microtoiter plates and 100 µl of K-562 cells, adjusted to 5 x 10^3 cells/100 µl, were added to each well. The plates were Incubated for various lengths of time, when 50 µCuries ^{3}H-thymidine (TdR) (ICN) was then added for a 10 hour incubation. ^{3}H-TdR uptake was determined with precipitation by trichloroacetic acid and collection on glass fiber filter paper. Cells were scored for viability by trypan blue dye exclusion using a hemocytometer. In Experiments where limiting dilutions of polyclonal rabbit anti-K-562 were used, a serial dilution greater than that required to elicit microscopic agglutination of the cells was used as the starting concentration from which the limiting dilutions were made.

Monoclonal:

Twofold serial dilutions of each monoclonal antibody in 50 µl samples were made in wells of round bottom microtiter plates. The K-562 cell concentration was adjusted to 5 x 10^3 cells/100 µl and 100 µl were added to each well.

The microtiter plates were incubated for various lengths of time, when 50 µCuries of ^{3}H-TdR were added. Cells were incubated with the radiolabel for 10 hours. ^{3}H-TdR uptake was measured with the filter paper method.

X. TCA Filter Method for Detecting ^{3}H-TdR Uptake

Hand-labeled glass microfiber filter disks (Whatman) were suspended on a pin inserted into a styrofoam board and 100 µl of samples were pipetted onto a series of filters. The pins hold the disks off of the support and hold the disks apart during the wash steps. After 10 minutes, the disks were placed in ice cold 10% trichloroacetic acid (TCA) (Sigma), using 3 ml TCA solution per disk. The disks were washed by agitation in an ice bath for 10 minutes, the liquid was then discarded and the disks washed two more times with 5% TCA. Following the last 5% TCA wash, the disks were washed in ethyl alcohol, 5 ml/disk for 15 minutes, two 50:50 ether/alcohol washes, and finally 100% ether, 5 ml/disk for 10 minutes. The liquid was decanted and the disks were allowed to dry. The disks were removed from the pins and each placed in a 4 ml scintillation vial (Wheaton) with 3 ml scintillation cocktail (Ecolume, ICN). The amount of ^{3}H-TdR incorporated by K-562 cells was determined in a Beckman LS7000 spectrometer (31).

XI. DNA Quantification Methods

Diphenylamine reagent was prepared by dissolving 1 gram diphenylamine (Sigma) in 100 ml glacial acetic acid (Fisher) and adding 2.75 ml concentrated sulfuric acid (Mallinckrodt). Standard DNA solutions were prepared by making serial dilutions of calf thymus DNA (Schwartz/Mann). A 1 ml sample of standard or unknown solutions was added to 2 ml diphenylamine reagent and boiled for 10 minutes. The absorbance of each sample was read at 595 and 650 Angstroms (A) against a blank of distilled water and diphenylamine reagent. The reading at 650A was subtracted from those at 595A to correct for the green color produced by other sugars. A standard curve was plotted for known concentrations of DNA and the absorbance of the unknown sample was compared to the standard curve to determine the amount of DNA.
DNA determinations were also done with the commercial DNA quantification kit,

DNA Dipstick (Invitrogen).

XII. Analysis of Double Stranded Fragmentation

K-562 cells were radiolabled with ^{3}H-TdR (ICN), 1 μCurie/10^{3} cells for 15 hours. The radiolabeled K-562 cells were then grown in the presence or absence of rabbit anti-K-562 gamma globulin at a dilution that brought about agglutination. In the assay, 5 x 10^{6} cells done in triplicate per time point, were used. At 6, 12, and 27 hours post antibody addition, the cells were washed with medium EM-10a by gentle centrifugation, 150 x g for 10 min, and suspended in 150 mM NaCl buffered with sodium bicarbonate to pH 7.0 at a cell concentration of 5 x 10^{7}/600 μl, which were then lysed by adding 6 ml of 25 mM acetate buffer, pH 6.6. The lysates were centrifuged at 27,000 x g for 20 minutes at ambient temperature. The DNA in the supernatant fluids, pellets, and aliquots of the uncentrifuged lysates was quantified by the diphenylamine reaction, the paper filter method and with the DNA Dipstick Kit (Invitrogen). The proportion of DNA in the 27,000 x g supernatant fluids was expressed as a percentage of the uncentrifuged lysates (56).

XIII. DNA Extraction Method

K-562 cells (5 ml, 5 x 10^{5}/ml) were grown in the presence or absence of rabbit anti-K-562 antiserum or gamma globulin and lysed by the addition of an equal volume of 10mM Tris pH 7.5 (Bio-Rad), 10mM EDTA (Sigma), 100 mM NaCl, and 0.2% sodium dodecyl sulfate (SDS; Bio-Rad). The cell lysates were gently shaken by inversion for 10 minutes to insure complete lysis and the volume adjusted to 62.5 ml per sample by the addition of TEN (10 mM Tris pH 7.5, 10mM EDTA, and 100 mM NaCl). Nucleic acids were extracted at ambient temperature from the lysates by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) saturated TEN, followed by gentle

inversion for 1 hour. Samples were centrifuged for 10 minutes at 25,000 x g and the aqueous phase of each sample was transferred to a second tube. Nucleic acids were precipitated from the aqueous phase by the addition of 2 volumes of ice cold isopropyl alcohol and stored at -80 C for 10 minutes followed by a 1 hour incubation at room temperature.

Samples were then centrifuged at 25,000 x g for 10 minutes and the tubes were inverted and drained for 5 minutes under vacuum. The pellets were rinsed twice by the addition of 10 ml cold 70% ethyl alcohol, 200 mM NaOAc, pH 5.5, centrifuged for 10 minutes at 25,000 x g, drained and dried.

The pelleted nucleic acids were suspended in 0.8 ml TEN and were transferred gently to 1.5 ml Eppendorf tubes. Bovine pancreatic RNAse (Sigma) was added to a final concentration of 120 µg/ml and the samples were incubated in a 37 C water bath for 30 minutes. DNA was precipitated by the addition of 500 µl 3M NaOAc and 4 ml ice cold isopropanol, stored at -80 C for 20 minutes followed by a 1 hour incubation at ambient temperature. Precipitates were collected by centrifugation at 27,000 x g for 30 minutes, rinsed once more in 70% ethanol and finally dissolved in 1 ml of TE (10mM Tris, 1mM EDTA, pH 7.4) (14).

DNA was also extracted using a commercially available isolation kit, the A.S.A.P. Genomic DNA Isolation Kit (Boehringer Mannheim), to minimize the degradative effects of the phenol treatment. Briefly, the cells were treated with a lysis buffer, proteinase K, and RNAse to isolate the DNA. The digested cell lysate was passed through an equilibrated proprietary column to separate the DNA from the other cellular components. This method of DNA isolation was considerably more gentle than standard phenol extraction methods.

XIV. Analysis of Double Stranded Fragmentation in Agarose Gels

DNA samples (20 µl) were added to 7.5 µl of tracking dye mix (10M urea (Bio-Rad), 50 mM Tris pH 7.5, 100 mM EDTA, and 0.05% bromphenol blue) and loaded onto a 1% submarine agarose gel prepared by melting 1 gram agarose

(Bio-Rad) in 100 ml TBE buffer (120 mM Tris, 90 mM boric acid, and 0.75 mM EDTA pH 8.0) in a microwave for 2.5 minutes. The gel was run at ambient temperature for 3 hours at 50 volts, 60 milliamps, and then stained with 50 ml ethidium bromide solution, 0.5 mg/ml (Sigma), and visualized by transillumination with an ultraviolet light box. The gel was photographed through a Kodak u.v. filter (2).

XV. Analysis of Single Strand Nicking by Alkaline Sucrose Gradient

Solutions of 5, 20, and 60% sucrose in 0.1 M NaOH, 0.1 M NaCl and 0.01 M EDTA were prepared. Solutions of 8, 11, 14 and 17% sucrose were prepared by mixing 4, 3, 2, and 1 parts of 5% sucrose with 1, 2, 3, and 4 parts of 20% sucrose, respectively, generating a total of seven alkaline sucrose solutions.

Linear alkaline (pH 13) sucrose gradients of 5 to 20% sucrose were prepared by placing 0.1 ml of 60% sucrose in a 5 ml 0.5 x 2.0 inch polyallomer centrifuge tube (Beckman) as a cushion, and layering 0.65 ml of each of the other six alkaline sucrose gradients, in order of decreasing density. Formed gradients were precentrifuged in a Beckman 50.1 swinging-bucket rotor for 1 hour at 30,000 rpm at ambient temperature. The gradients were then overlaid with 0.4 ml unwinding buffer (0.2 M NaOH, 0.01 M EDTA, 2% sodium lauryl sulfate). ^{3}H-TdR labled DNA (200 µl) extracted from K-562 cells that had been incubated for 24 hours, with or without antibody, was loaded onto the top of the gradient. Loaded gradients were incubated at ambient temperature to allow unwinding of the DS DNA and then centrifuged for 100 minutes at 30,000 rpm at ambient temperature in a 50.1 rotor. Gradients were fractionated with an ISCO Density Gradient Fractionator using glycerol as a pusher solution. A total of 20 fractions were collected from each gradient directly into 4 ml scintillation vials (Wheaton). The alkalinity of each fraction was neutralized by the addition of 2 drops of glacial acetic acid in order to maximize the efficiency of the scintillation cocktail. Samples were thoroughly mixed with 3.5 ml of scintillation

cocktail (Ecolume, ICN) and radioactivity in each sample was determined in a Beckman LS7000 spectrometer.

In a variation of this technique, DNA was also irradiated with 1500 rads before loading onto the gradient. This irradiation induces uniform single strand nicks and facilitates unwinding of the DS DNA (12).

XVI. Analysis of Single Strand Nicking on Strand Separating Agarose Gels

Radiolabeled DNA samples(20 µl) were added to 7.5 µl of tracking dye mix (10M urea (Bio-Rad), 50 mM Tris pH 7.5, 100 mM EDTA, and 0.05% bromphenol blue) and loaded onto a 1% submarine agarose gel prepared by melting 1 gram agarose (Bio-Rad) in 100 ml TBE buffer (120 mM Tris, 90 mM boric acid, and 0.75 mM EDTA pH 8.0) in a microwave for 2.5 minutes. The gel was run at ambient temperature for 3 hours at 50 volts, 60 milliamps. The gel was stained with 50 ml ethidium bromide solution, 0.5 mg/ml (Sigma), and visualized by transillumination with an ultraviolet light box. Strips of agarose containing DNA were cut from this preparative agarose gel and immersed in 0.3 M NaOH for 30 minutes at room temperature. The strips were then rinsed in several changes of ice-cold distilled water. A 1% submarine strand separating gel was formed by melting 1 gram agarose (Bio-Rad) in separation buffer (36 mM Tris-Cl pH 7.7, 30 mM sodium phosphate, and 1 mM EDTA). Slots were then cut in the separating gel to the correct size to accommodate the strips cut from the preparative gel. The preparative strips were loaded into the slots and gaps were filled with melted 1% separating agarose. Tracking dye mix (50 µl) was added to any empty slot. The gel was run at ambient temperature for 3 hours at 50 volts, 60 milliamps, and stained with 50 ml ethidium bromide solution, 0.5 mg/ml (Sigma) and visualized by transillumination with an ultraviolet light box. The gel was photographed through a Kodak u.v. filter.

XVII. Quantification of DNA in Strand Separating Gels

The lanes from the strand separating gel were cut into 1 mm slices and each slice was placed directly into a 10 ml borosilicate scintillation vial containing 1 ml 1N HCL. The vials were incubated in a 95 C water bath for 30 minutes in order to dissolve the gel slices and 8 ml of scintillation cocktail (Ecolume, ICN) was added to each vial. Radioactivity was quantified in a Beckman LS7000 spectrometer, making adjustment for variations in quenching (30).

XVIII. Effect of Cycloheximide on ^{3}H-TdR Uptake Suppression by K-562 Cells Treated with Specific Antiserum

Cycloheximide was twofold serially diluted in 50 µl quantities of EM-10a in wells of a round bottom microtiter plate. K-562 cells, adjusted to 5 x 10^{3} cells/100 µl, were added to each well at 100 µl. The samples were incubated for various lengths of time, when 50 µl ^{3}H-TdR (ICN) was added at a concentration of 1 µCurie/50 µl to each sample. Cells were incubated with the radiolabel for 10 hours and then ^{3}H-TdR uptake was measured with the filter paper method.

XIX. Effect of Aurintricarboxylic Acid on ^{3}H-TdR Uptake Suppression byK-562 Cells Treated with Specific Antiserum

Aurintricarboxylic acid (Sigma) was twofold serially diluted in 50 µl quantities of EM-10a in wells of a round bottom microtiter plate. K-562 cells, adjusted to 5 x 10^{3} cells/100 µl, were added to each well at 100 µl. The samples were incubated for various lengths of time, when 50 µl ^{3}H-TdR (ICN) was added at a concentration of 1 µCurie/50 µl to each sample. Cells were

Incubated with the radiolabel for 10 hours and then ^{3}H-TdR uptake was measured with the filter paper method.

CHAPTER IV

RESULTS

I. Production of Specific K-562 Antiserum

To verify previous observations from this laboratory that specific antiserum made against K-562 cells could cause cell death, New Zealand White Rabbits were sensitized with K-562 cells and the resulting antiserum assayed for specificity and titer by the antibody dependent complement mediated cytotoxicity assay. Figure 1 indicates that the antisera obtained from rabbit D1 reacted with K-562 cells. Furthermore, fifty percent of the cells were lysed within 90 min at a serum dilution of 1:19 as detected by ^{51}Cr release.

II. Inhibition of Cell Growth and DNA Synthesis by Rabbit-Anti-K-562 serum.

It has been previously reported that K-562 cells incubated with specific antisera would cause a cessation of protein and RNA synthesis, and eventual cell death in the absence of complement or effector cells (11,28,23,54,56). The criterion of trypan blue dye uptake was used in initial studies, but there were many limitations with this marker of cell viability. ^{3}H-TdR uptake was also used to quantify relative DNA synthetic rates in K-562 cells treated with or without antiserum.

The specific antiserum, D1, was used undiluted in initial experiments to

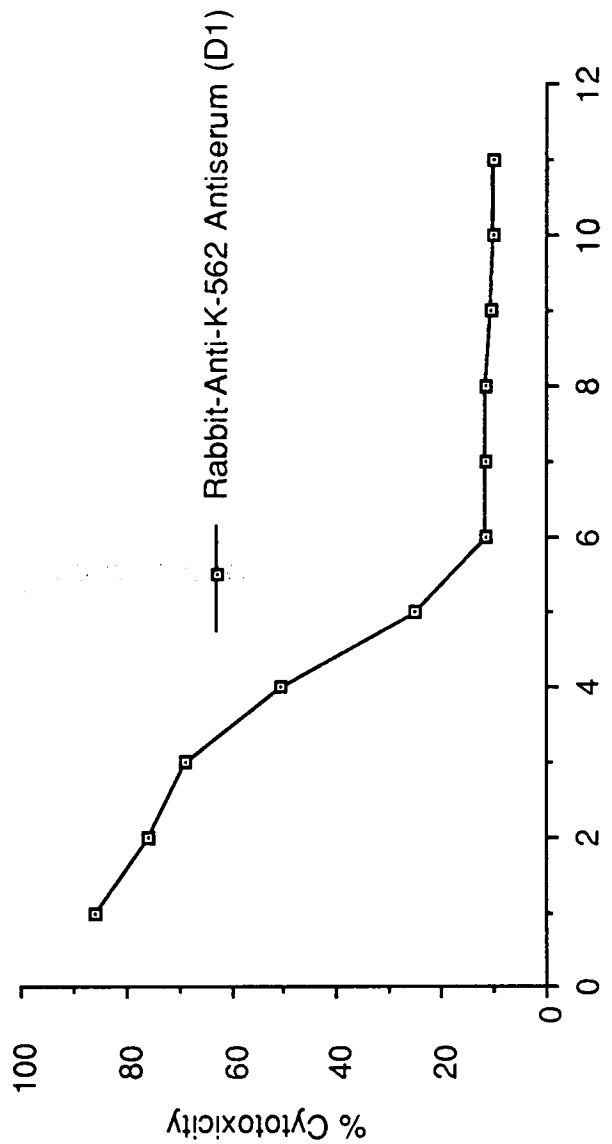


Figure 1. Antibody Dependent Complement Mediated Cytotoxicity by Rabbit Antisera

determine if ^{3}H-TdR uptake suppression and subsequent cell death would be observed. Figure 2 compares the time course of viability of K-562 cells treated with undiluted antiserum with the relative incorporation of ^{3}H-TdR. The undiluted antiserum caused an initial decline in the number of viable cells. This decline continued in a linear fashion until the end of the study at 5 days. At the end of the time period, only 23% of the K-562 cells remained viable. The cells that were determined to be dead by trypan blue dye exclusion showed no evidence of lysis when examined microscopically. The undiluted antiserum also caused an initial dramatic decrease in DNA synthesis as measured by ^{3}H-TdR uptake. The DNA synthesis was decreased by more than 90% within the first 24 hours, and the cells did not recover from this within the 5 days of the assay. This type of cell death in the absence of complement or effector cells within 5 days without lysis is not characteristic of a typical immunological reaction.

III. Inhibition and Recovery of K-562 Cell Growth and DNA Synthesis in Limiting Dilutions of Rabbit-Anti-K-562 Serum.

In an effort to better understand the mechanism(s) of cell death induced by specific antiserum, efforts have been made to save K-562 cells from antiserum induced death. Green et al. (11) found that glucose, succinate, and malate could not reverse the cytotoxicity of antiserum on K-562 cells. In the present set of experiments, limiting dilutions of antiserum were found to impair cell growth

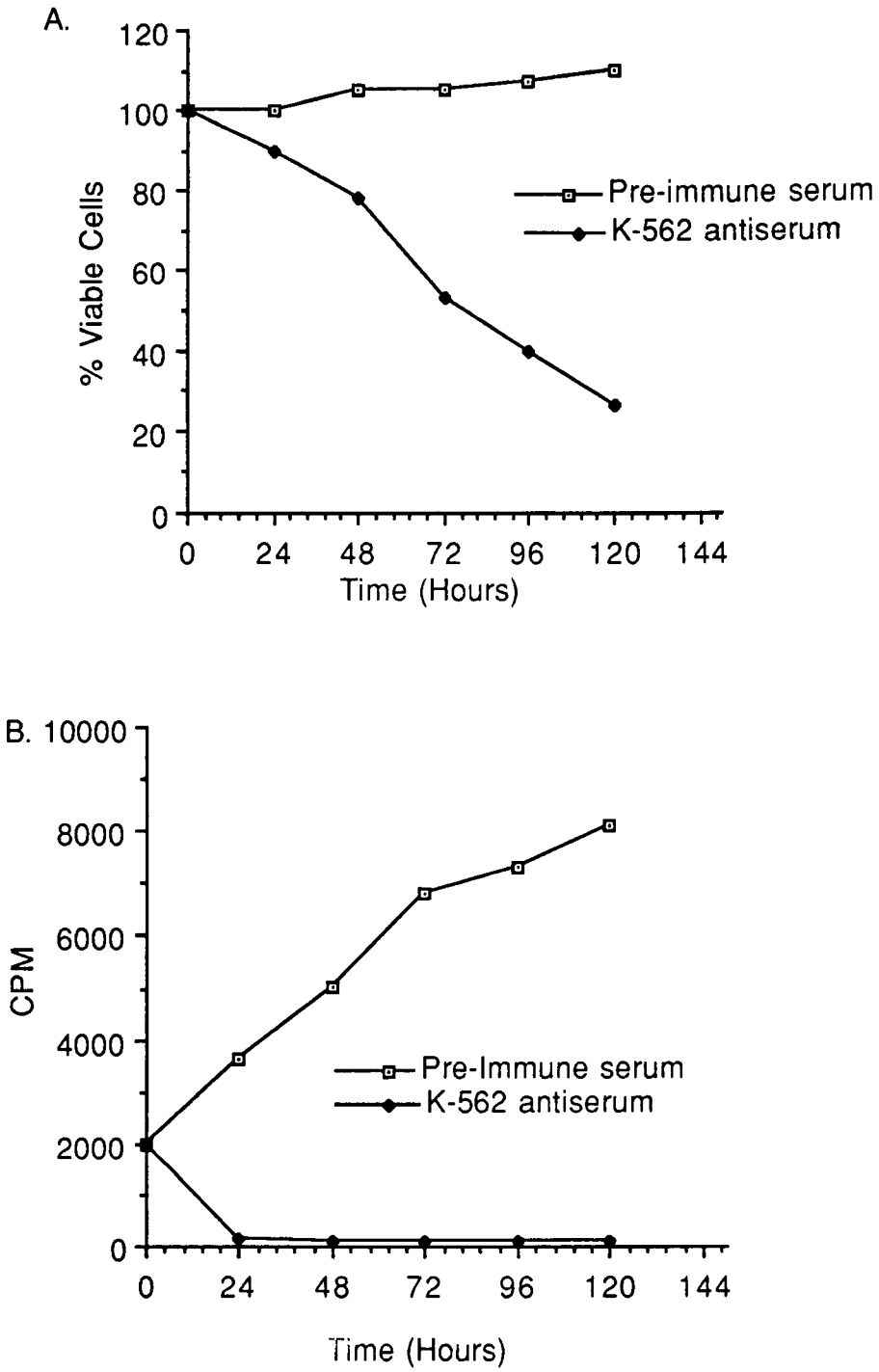


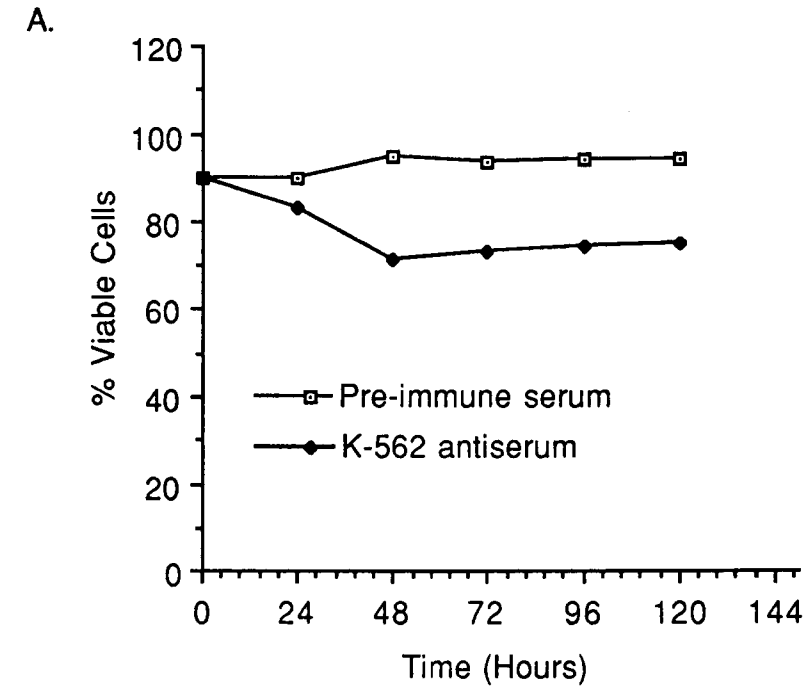
Figure 2. Effect of a lethal hit dilution of rabbit ploYclonal antiserum (1:16 of D1) on the growth and TdR uptake of K-562 cells

but the effect was reversed with continuous culture of the cells.

Figure 3 compares the growth and ^{3}H-TdR uptake over a period of time when K-562 cells were treated with a limiting dilution of antiserum on K-562 cells. The antiserum from rabbit D1 was diluted 1:48 with EM-10a, which was a dilution that gave a negative result in the ADCMC assay (see figure 1) and that was one twofold dilution beyond one causing any microscopic agglutination of K-562 cells. This limiting dilution caused a linear loss of cell viability for 2 days followed by an apparent recovery of cultures to a growth rate similar to that found with pre-immune serum. DNA synthesis was inhibited by over 50% in the first 24 hours followed by a rate of increase comparable to that observed with pre-immune treatment. These results could be interpreted to indicate a recovery of cells affected by antiserum or the selection of an antibody resistant subpopulation.

IV. Effect of Selected Monoclonal Antibodies on DNA Synthesis

Polyclonal antisera can be composed of numerous isotypes of antibodies of varying specificity and reactivity, thus, it may be difficult to ascertain a single mechanism by which polyclonal antiserum can kill K-562 cells. To narrow the number of possible mechanisms that might be involved, a panel of nine monoclonal antibodies, reactive with K-562 cells by cytofluorogrphy, were used to treat the cells in culture (Figure 4). Three grouping of the monoclonal antibodies could be made on the basis of the effect of DNA synthesis as measured by ^{3}H-TdR uptake: those that enhance ^{3}H-TdR uptake (BA-1 and



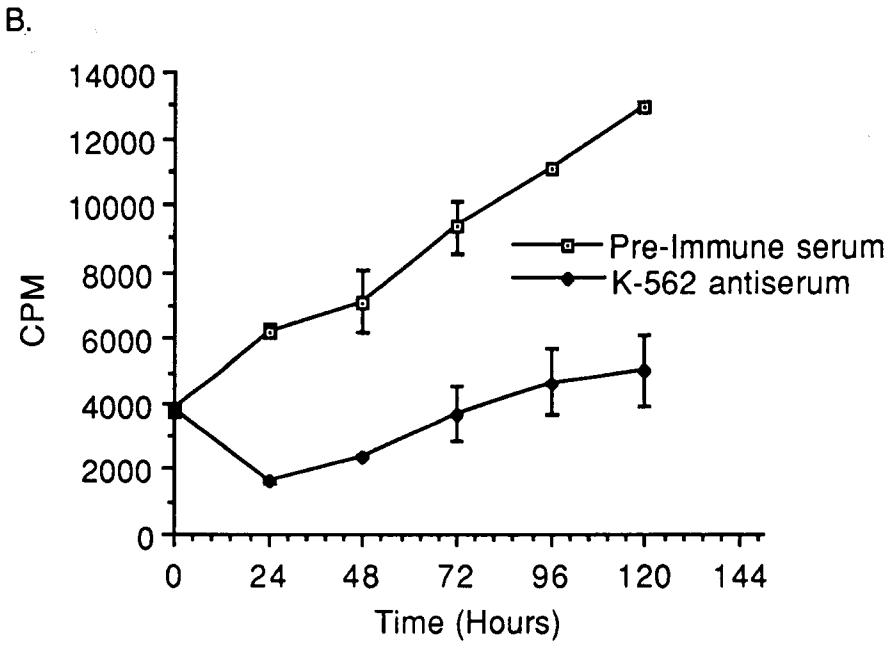


Figure 3. Effect of a limiting dilution (1:48 of D1) on the growth and TdR uptake by K-562 cells

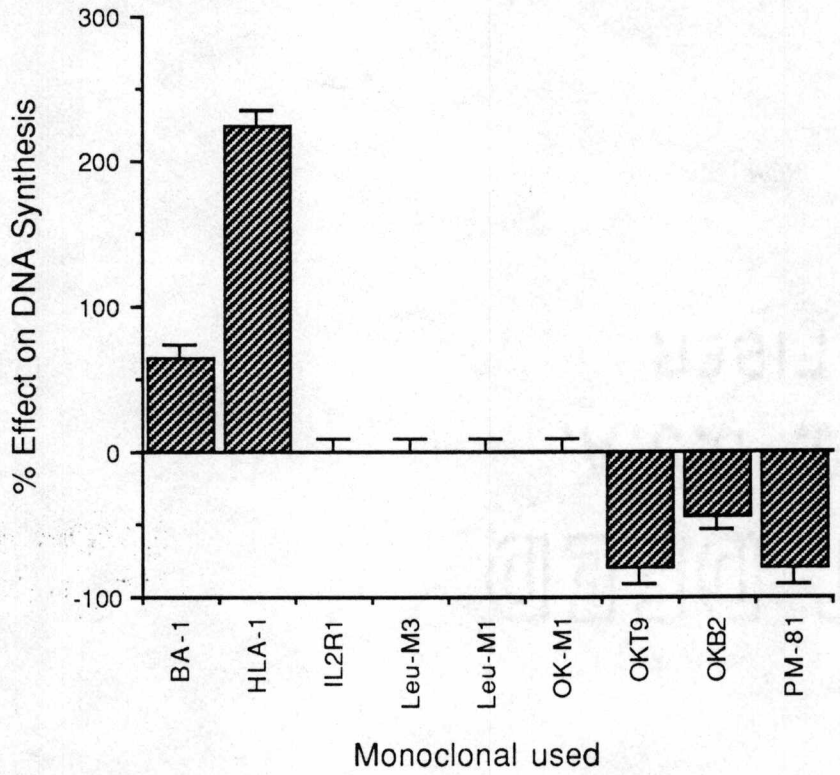


Figure 4. Summary of effects of monoclonal antibodies on the incorporation of radiolabeled thymidine into K-562 cells as compared to normal K-562 cells.

HLA-1); those that had no effect (IL2R1, LEU M-3, LEU M-1 and OK-M1); and those that inhibited uptake (OKT9, OKB2, and PM81). Each grouping has more than one isotype, therefore the effects do not appear to be a non-specific Fc receptor binding event. One example of the experimental results from each group is depicted in figures 5, 6, and 7 to clarify how the data from Figure 4 was obtained.

V. Failure to Detect Double Strand DNA Nicking in K-562 Cells Incubated with Rabbit Antiserum.

Based on the previous experiments which indicated that antiserum causes cell death within 5 days without complement, effector cells, or detectable cell lysis, we postulated that death was initially induced by antibody reacting with a cell surface receptor. This reaction, in turn, would transduce a sequence of cellular events similar to those described for apoptosis.

Apoptosis, or programmed cell death, is thought to be a normal and important process during embyogenesis and postnatal life. The destruction of cells that are no longer needed occurs normally as part of the developmental sequence in vertebrates and invertebrates (16). The recognizable events occurring in cells undergoing apoptosis are: membrane blebbing, chromatin condensation, DNA fragmentation and eventual cell death (58). Since double strand DNA degradation has been found thus far only in murine systems, and not in human cells, we did not expect double strand nicking in K-562 cells. K-562 cells were examined for the possibility of double strand degradation using two techniques, namely agarose gel electrophoresis and lack of acid

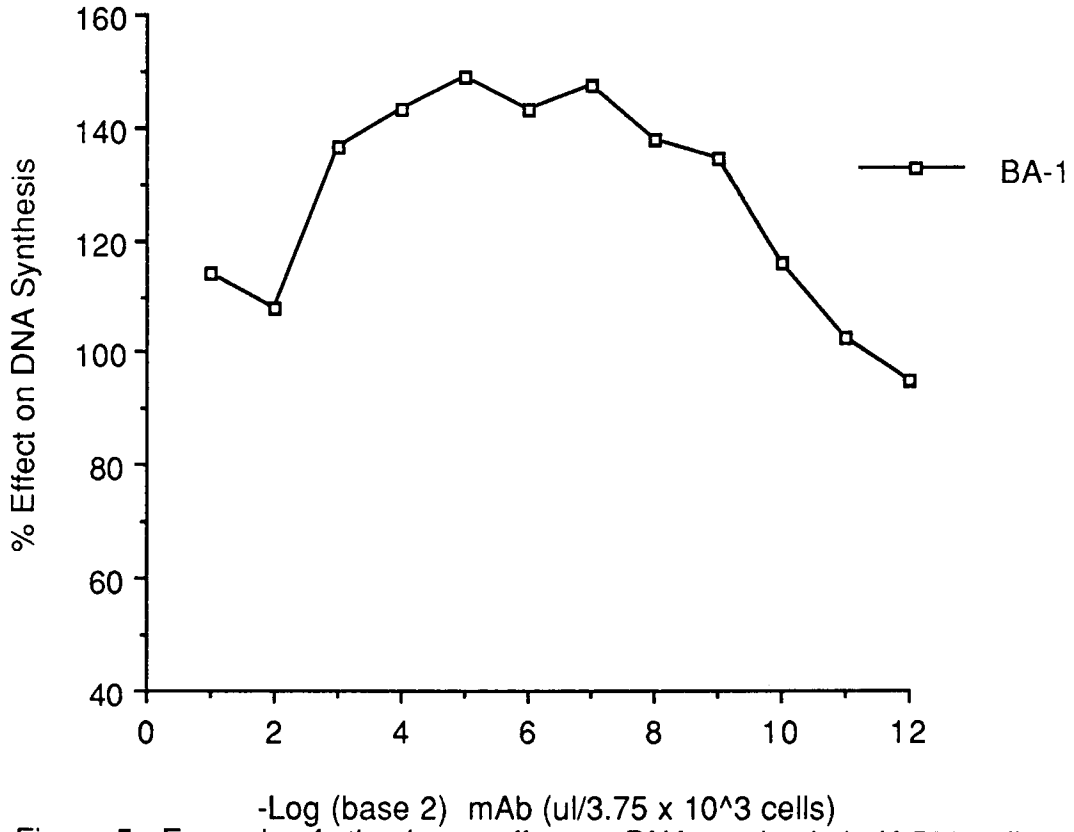


Figure 5. Example of stimulatory effect on DNA synthesis in K-562 cells treated with BA-1.

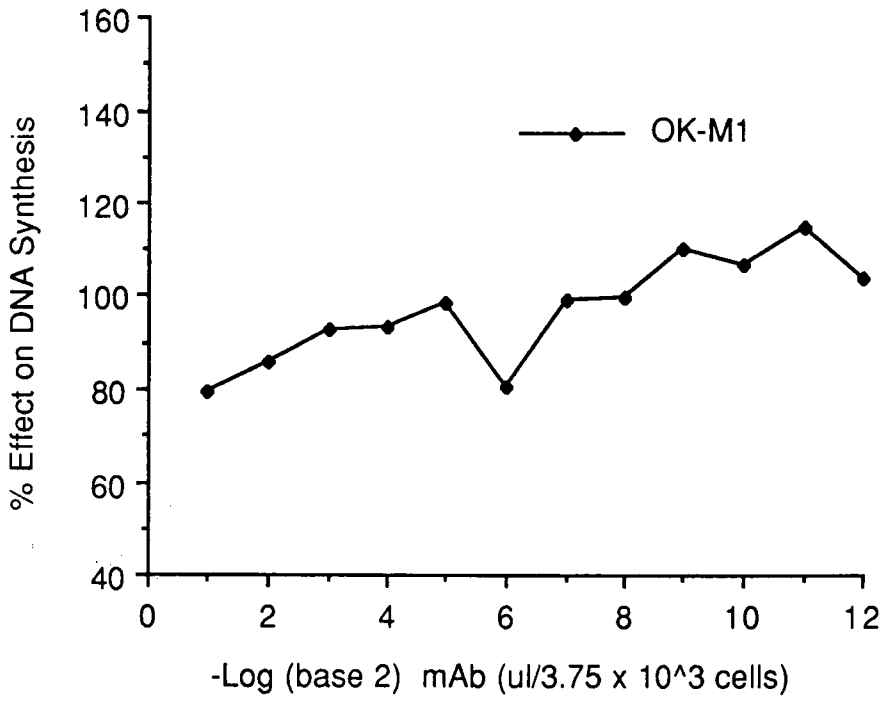


Figure 6. Example of no effect on DNA synthesis in K-562 cells treated with OK-M1.

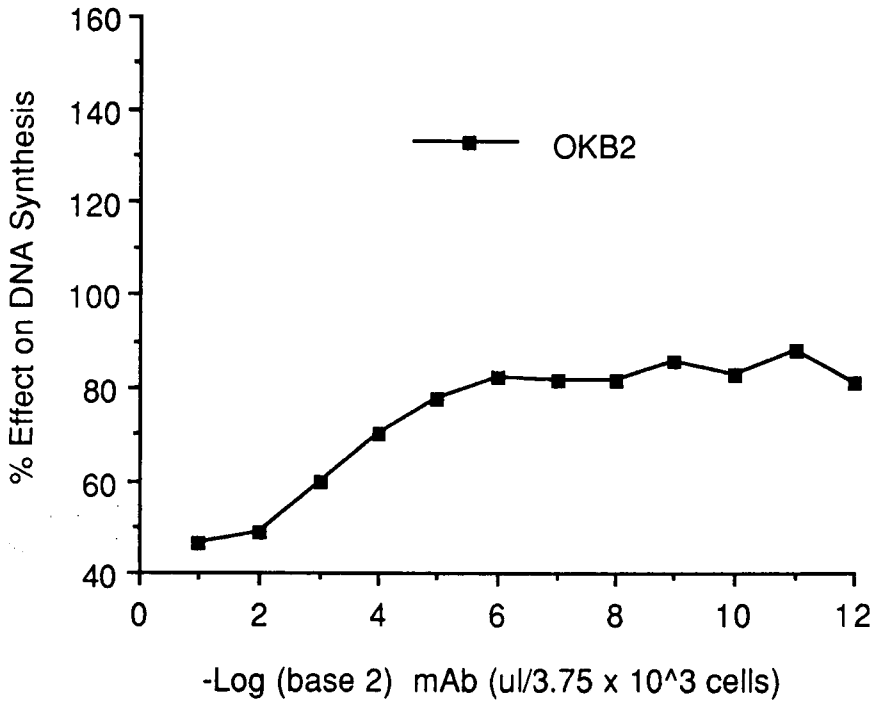


Figure 7. Example of inhibitory effect on DNA synthesis in K-562 cells treated with OKB2.

precipitability of ^{3}H-TdR labeled DNA over a period of time.

In Figure 8, lanes 1,2, and 3 contain DNA from K-562 cells that had been treated with pre-immune serum and lanes 5,6,7,and 8 contain DNA from cells treated with specific antiserum. Lanes A and B contain lambda DNA cleaved with the enzymes HIND III and EcoR1, as DNA molecular weight markers. A 1% agarose gel was used to increase the probability of recognizing small DNA fragments resulting from double strand degradation. The K-562 DNA from pre-immune treated cells migrated only slightly into the 1% gel indicating high molecular weight double stranded DNA. The DNA from antiserum treated cells migrated similarly to pre-immune DNA suggesting that no double stranded degradation had occurred.

If DNA is being degraded into double strand fragments, the small fragments should be detectable in the supernatant fluid of a cell lysate, Thus, intact double strand DNA would be acid-precipitable wheras small fragments would remain in the supernatant fluid. The precipitation of radiolabled DNA with trichloroacetic acid is given in Figure 9. The amount of radiolabled DNA was relatively constant in cells treated with pre-immune, immune, or no serum. The amount of DNA found in the supernatant fluids was very small and this could be attributed to the background in the assay system. The amount of DNA shown in Figure 9 also correlated with the relative amounts of DNA quantified with the diphenylamine reaction (data not shown). These results indicate that there is no breakdown of high molecular weight DNA into soluble fragments.

On the basis of these two techniques, I conclude that there is no double strand degradation induced by specific antiserum.

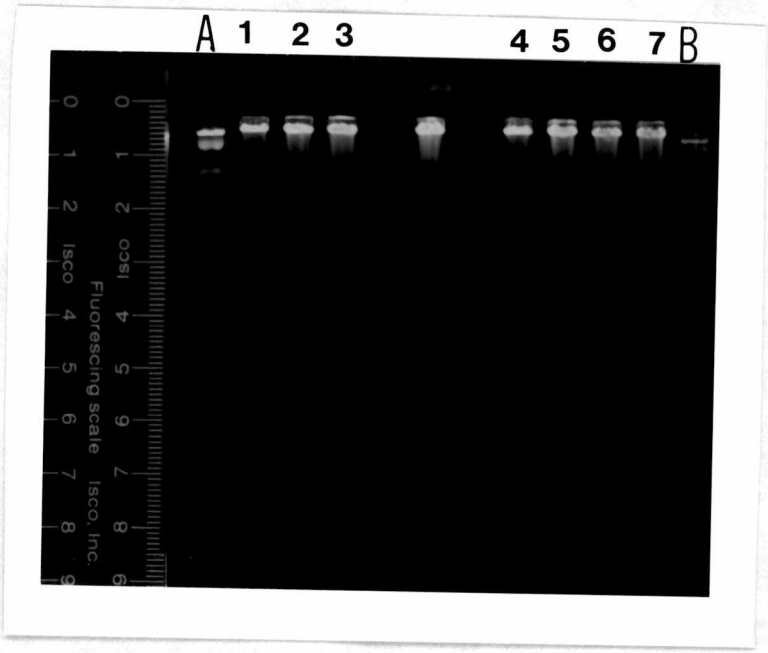


Figure 8. Gel analysis of radiolabeled DNA from K-562 cells treated (lanes 2-5) or not treated (lanes 6-9) with rabbit polyclonal antiserum. The 1% submarine gel was run at ambient temperature for 3 hours at 50 volts, 60 milliamps. Lanes A and B contain Lambda DNA molecular weight markers.

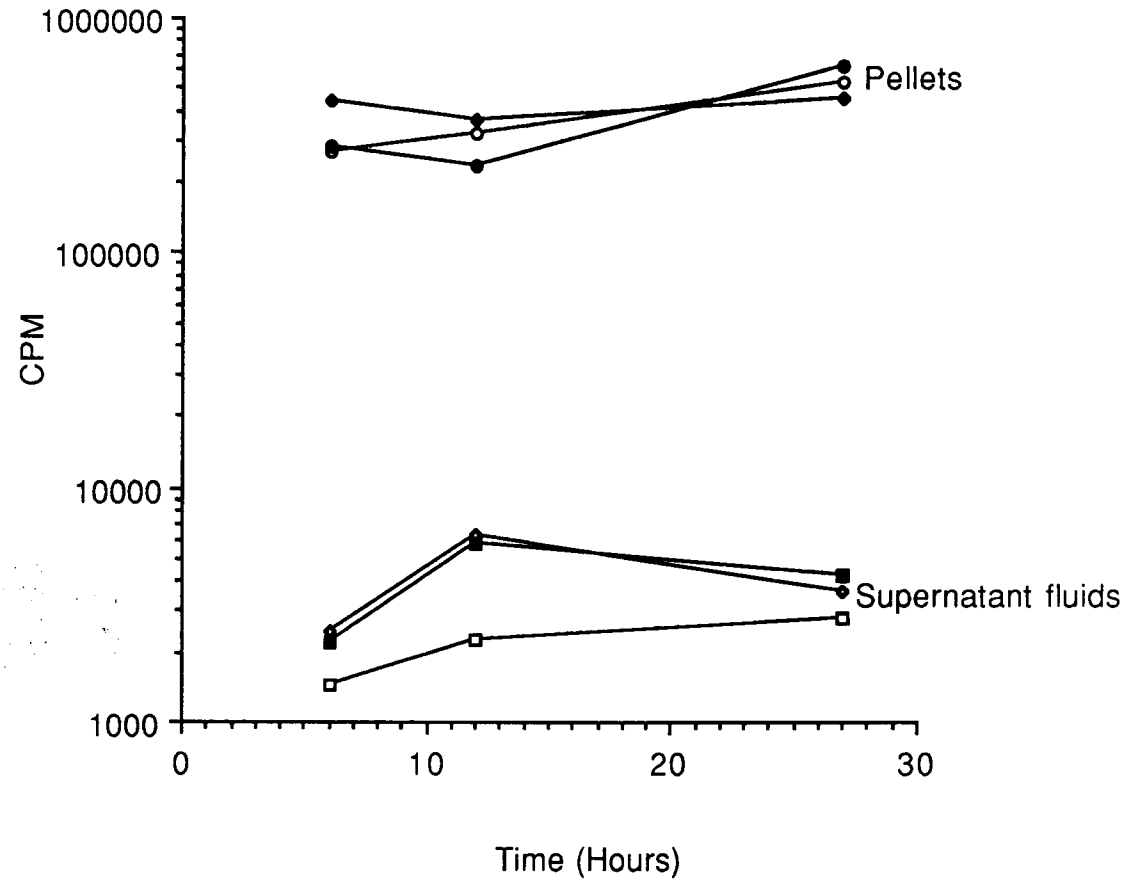


Figure 9. Trichloroacetic acid precipitation of ^{3}H-TdR labeled DNA from K-562 cells treated (closed circle, open square) or not treated (open circle, open diamond) with rabbit antiserum or K-562 cells treated with pre-immune serum (closed diamond, closed square).

VI. Positive Evidence of Single Strand Nicking of DNA in K-562 Cells Treated or not Treated with Antiserum.

As stated earlier, no human cell line has been shown to undergo double strand nicking but this criterion alone could be misleading. In many systems where double stranded nicking was absent, there was, in fact, extensive single strand nicking (12).

Cell death induced by a number of effector cells or agents is usually preceded by single strand DNA fragmentation in human cells (12). To analyze for the possibility that K-562 cells were undergoing single strand nicking , K-562 cells were radiolabled with ^{3}H-TdR, treated with immune serum or gamma globulin and the DNA isolated. The DNA was then analyzed by strand separating agarose gel electrophoresis and by centrifugation through an alkaline sucrose gradient.

In the electrophoresis experiments, the DNA was first electrophoresed on 1% agarose and visualized with ethidium bromide. The bands, similar to those in Figure 8, were cut from the gel and treated with sodium hydroxide to denature the double strand DNA. When DNA is denatured in agarose, the opportunity for the renaturation of complementary strands is minimized. The cut segments of the gel were fitted into custom cut slots on a DNA strand separating gel (30). The DNA was then electrophoresed and visualized with ethidium bromide. Each DNA sample usually gave rise to 2 or 3 bands in the strand separating gels. One of these bands was assumed to be native, double strand DNA, which forms by the renaturation of the 2 single strands during the loading

of the gel. If the denaturation procedure is complete, this band will not form at the top of the gel. The other two bands, usually much fainter and fuzzier than the first are the two separated strands. Figure 10 is a representation of the strand separating gel. Lanes 1 and 2 contain DNA from cells treated with pre-immune serum and lanes 3 and 4 contain DNA from cells treated with antiserum. Note the band at the top of lane 1, which is renatured DS DNA. The 2 other bands of DNA from either pre-immune or antiserum treated cells are blended into each other. The resolution of the gel was not sufficient to make a judgment as to the size of the fragments by visualization alone. Owing to the strand separating nature of this gel, molecular weight markers were not available.

To better understand the distribution of the single strands of DNA in the strand separating gel, the agarose was cut into 1 mm slices and each slice was assayed for radioactivity in a Beckman spectrometer. These results are depicted in Figure 11. The results indicate that DNA from cells treated with antiserum does undergo single strand nicking; however, the DNA from K-562 cells treated with pre-immune serum also showed evidence of single strand nicking albeit to a significantly lesser extent than that from cells treated with antiserum.

In a second set of experiments, the radiolabled double strand DNA was denatured with sodium hydroxide and then centrifuged through a 5% to 20% alkaline (pH 13) sucrose gradient. The denaturing process should unwind the double strand DNA. Intact double strand DNA remains at the top of the gradient, while intact single strands migrate into the gradient and nicked single strands migrate even further down the gradient. After centrifugation, the

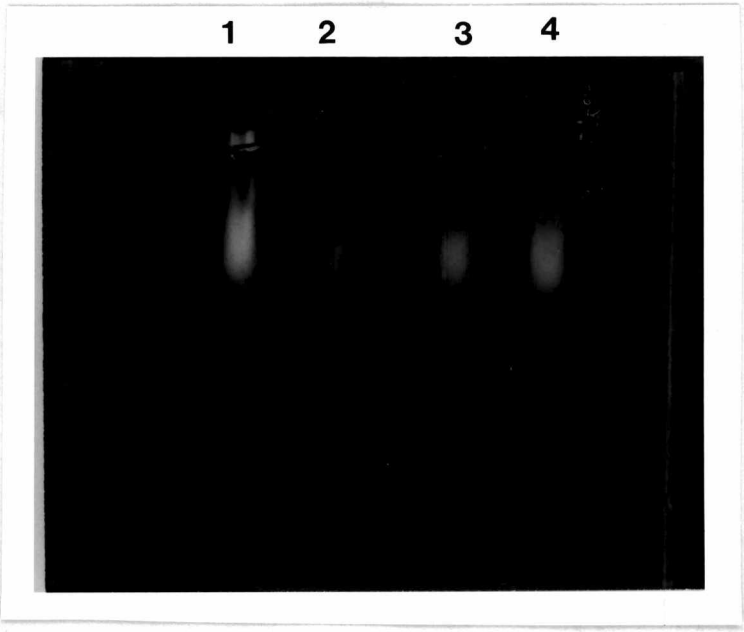


Figure 10. Strand separating gel analysis of radiolabeled DNA from K-562 cells treated (lanes 1 and 2) or not treated (lanes 3 and 4) with rabbit polyclonal antiserum.

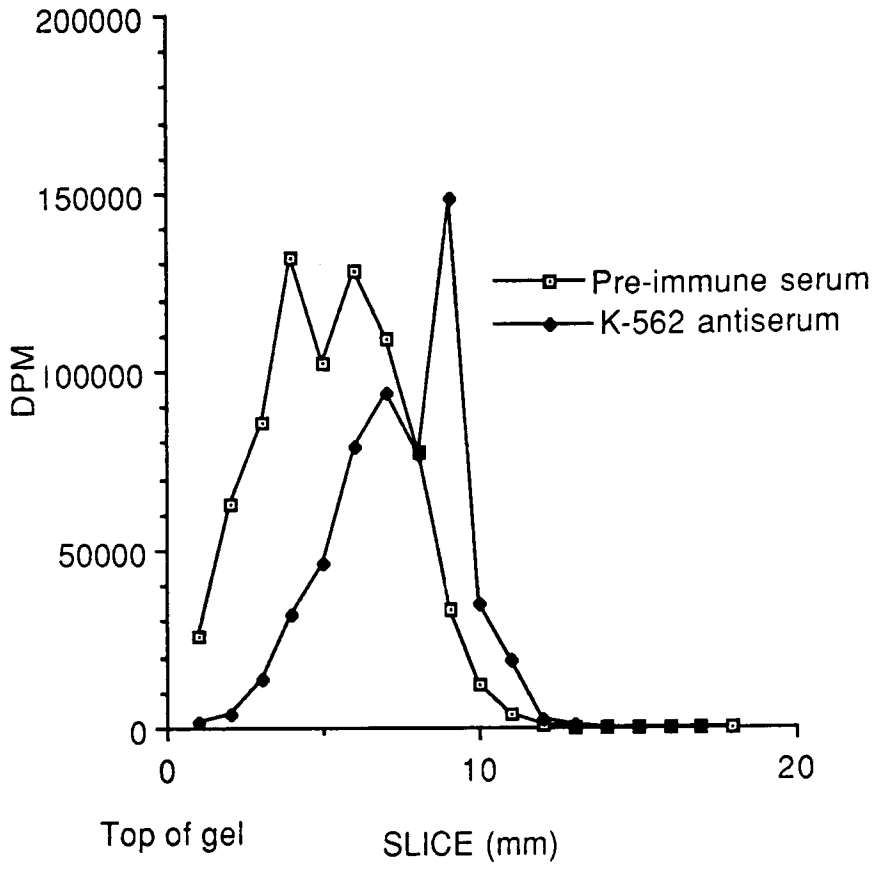


Figure 11. Analysis of radioactivity of agarose fractions after electrophoresis of ^{3}H-TdR labeled DNA isolated from specific antiserum treated K-562 cells.

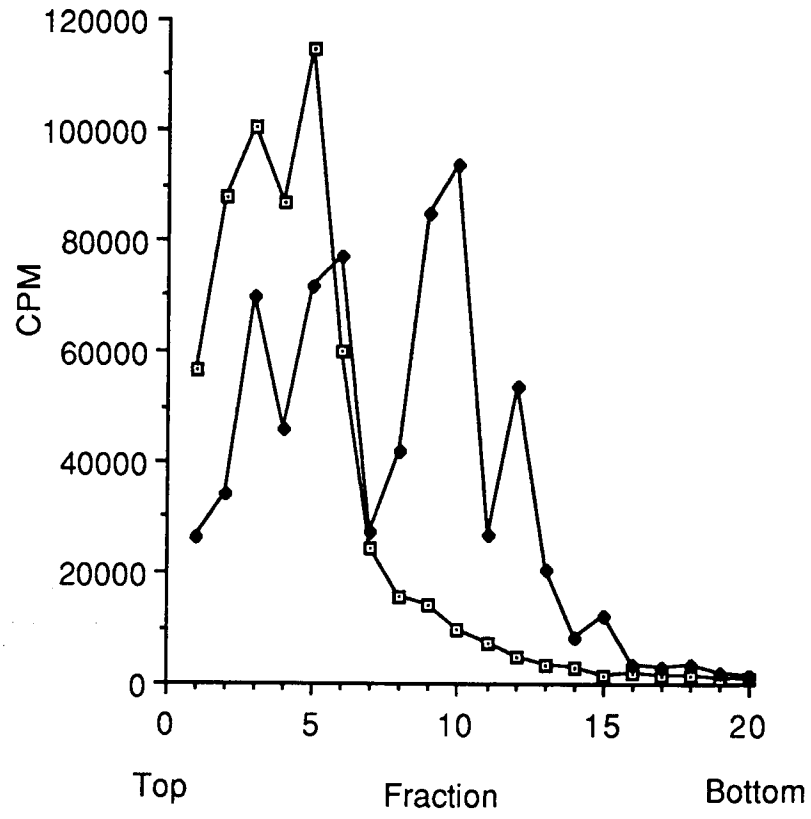


Figure 12. Analysis of DNA from K-562 cells treated (open squares) or not treated (closed diamonds) with polyclonal anti-K-562 gamma globulin labeled with ^{3}H-TdR and centrifuged through an alkaline (pH 13) sucrose gradient.

gradient was fractionated and radioactivity was quantified with a beta spectrometer. Figure 12 shows the relative radioactivity of the fractions of the gradient after centrifugation and supports the conclusion that single strand breaks occurs in both the DNA from cells treated with pre-immune or immune serum, but it is more extensive in the DNA from antibody-treated cells.

VII. Failure to Protect K-562 Cells from Antibody Induced DNA Synthesis Suppression with Cycloheximide.

Many systems for the study of apoptosis suggest the involvement of an endonuclease(s) in the DNA fragmentation event (7,33,9,57,8,56,32). In these systems, a surface acting agent or cell such as a cytotoxic T cell, induces protein synthesis, presumably for the synthesis of the endonuclease(s) that causes single or double strand DNA degradation. When protein synthesis was inhibited by various agents, DNA did not fragment and cell death was avoided.

Cycloheximide, an inhibitor of protein synthesis, was used in a series of experiments to test whether protein synthesis is required in K-562 cells treated with antiserum. Cycloheximide, at various concentrations, was added to antibody treated cultures of K-562 cells, and it was evident that the cells were not protected from DNA synthesis suppression, as we had expected. (Figure 13). As the concentration of the drug was increased, DNA synthesis decreased proportionately in both antibody treated and control cultures. These results indicate that protein synthesis is not needed in DNA fragmentation in this system.

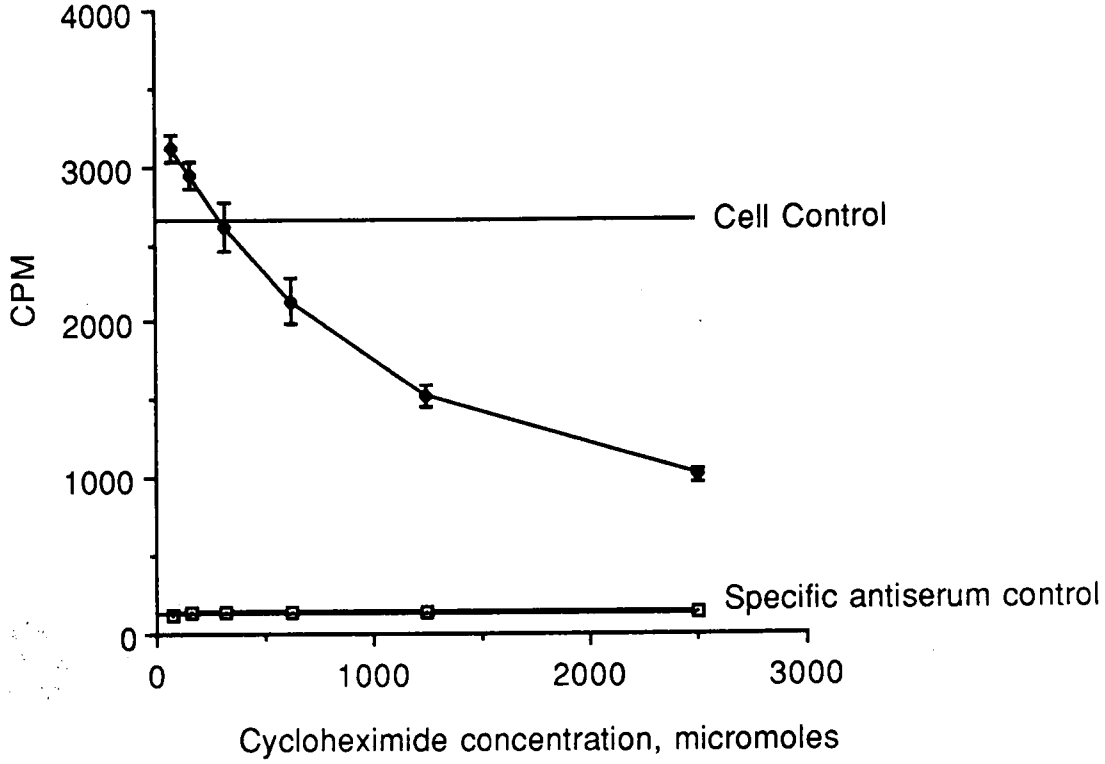


Figure 13. Effect of cycloheximide on ^{3}H-TdR uptake by K-562 cells treated (open circles) or not treated (closed circles) with polyclonal rabbit anti-K-562 gamma globulin. Cycloheximide (50 µl) was added to 3 x 10^{3} cells (50 µl) in triplicate wells of a round bottom microtiter plate that also contained 50 µl of a dilution of gamma globulin (200µg) or of medium. 1 µCurie of ^{3}H-TdR was added to each well after 14 hours of incubation and radioactivity of DNA was measured by the TCA filter method after a 12 hour incorporation.

VIII. Aurintricarboxylic Acid Protects K-562 Cells from Antibody Induced DNA Synthesis Suppression.

The results thus far suggested that antibody signals an increased level of endonuclease activity since no new synthesis is required. We expected that the addition of aurintricarboxylic acid, a drug which inhibits many enzymes, including endonucleases and polymerases (4,5,33,15), to antibody-treated cultures would protect the DNA from breakage and subsequent cell death. Figure 14 shows the effect of the drug on antibody-treated cells. This experiment was repeated several times with similar results and shows that at concentrations of 0.1 to 0.25 mM with 5.5×10^3 cells, not only was there apparent protection of the DNA but also an actual enhancement of DNA synthesis 1.8-fold over untreated control levels. Further, at these concentrations, the drug was markedly inhibitory for DNA synthesis when added to untreated cells. Cells treated with immune gamma globulin, in contrast to whole antiserum, were also protected with similar levels of the drug; however, the level was only equal to that of the control. Since these results were unexpected, the drug was also used in cultures treated with monoclonal antibodies that caused decreased DNA synthesis. Figure 15 shows that aurintricarboxylic acid failed to conserve the DNA synthesis of both the monoclonal antibody-treated cultures. These findings suggest that both the polyclonal antisera and the monoclonal antibodies signal for decreased DNA

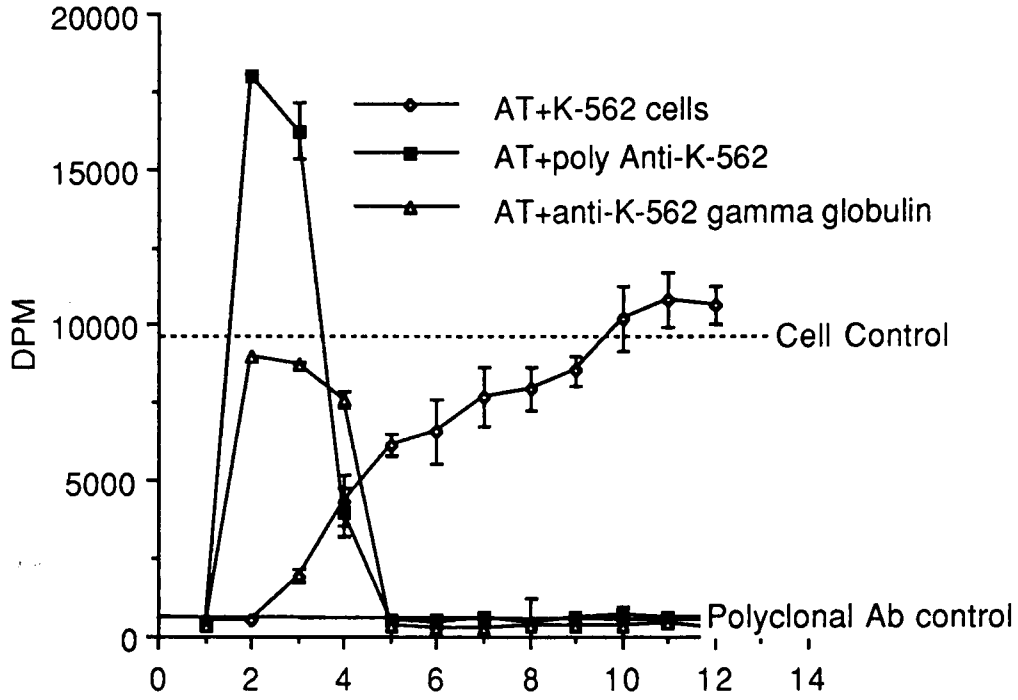


Figure 14. Effects of aurintricarboxylic acid on the DNA synthesis suppression induced by specific antiserum.

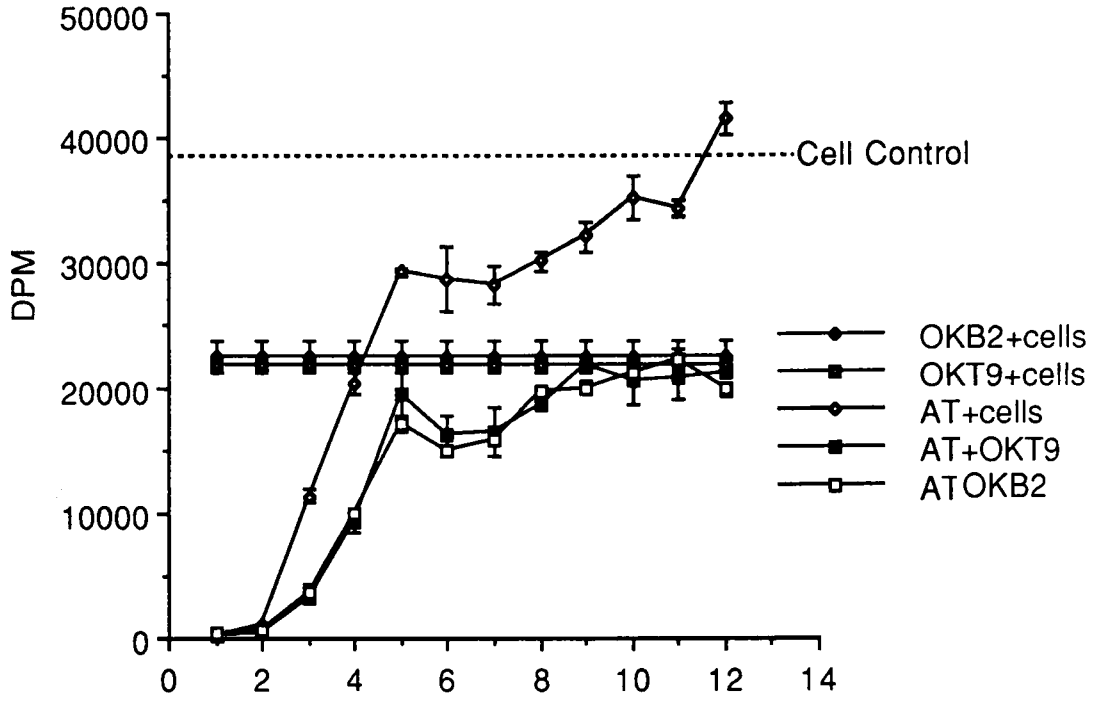


Figure 15. Effects of aurintricarboxylic acid on the DNA synthesis suppression induced by monoclonal antibodies.

synthesis but the polyclonal effect could be reversed by aurintricarboxylic acid and the monoclonal effects could not be reversed. We conclude that the mechanism being utilized to induce DNA synthesis suppression with immune polyclonal serum or gamma globulin may be different from that used by these particular monoclonal antibodies.

CHAPTER V

DISCUSSION AND CONCLUSIONS

In this report, we have demonstrated that rabbit anti-K-562 serum, when incubated with K-562 cells, causes a marked reduction in cell viability as well as an almost total decline in the rate of DNA synthesis within the first 24 hours. The capacity of the cells to synthesize DNA is not evident in surviving cells over 5 days of culture. At least 80% of the cells are non-viable at 5 days, and no cytolysis of dead cells is observed. The death of the cells occurred in the absence of added complement or effector cells. These results are consistent with previous studies involving K-562 cells and specific goat antiserum (11,22,27,28,55), but as yet no mechanism(s) have been elucidated for antibody-induced cell death.

In efforts to define a mechanism for antibody-induced cell death, attempts have been made to alter the outcome with reagents of known function. We have used the suppression of ^{3}H-TdR incorporation as indicating an early step leading to cell death. With limiting dilutions of antiserum, this suppression appears to be reversible, although one interpretation may be the selection of an antibody-resistant subpopulation. This explanation does not seem to be valid since every culture of cells used has been susceptible to antibody-induced death. It has been proposed in other systems, that when a cell is exposed to different lectins, hormones or antibody, the cellular metabolic machinery may be somehow overloaded and the cell goes into unbalanced growth and eventual cell death (12,16,21,32,35,54). The loss of cell viability preceded by the suppression of DNA synthesis in a dose-related fashion with antiserum

suggests that this explanation may be the case with the K-562 model. If cells dying with limiting dilutions of antiserum represent an antibody-sensitive subpopulation then the antibody-resistant subpopulation would be selected. One could speculate that the resistant population may have modulated surface antigens (17,49,52) to be unresponsive rather than being genetically resistant. This possibility must be considered when antibody is used in possible immune therapies.

To further attempt to define antibody induced cell death mechanistically, a panel of 9 monoclonal antibodies, reactive with K-562 cells as detected by cytofluorometry, were examined for their effects on K-562 cells. It was found that the antibodies could be categorized into three groups based on the effect of ^{3}H-TdR uptake into acid precipitable DNA. The groups are: no effect [IL-2R1(IgG2a), Leu-M1(IgG2b0, Leu-M1(IgM), OK-M1(IgG2b)], increased ^{3}H-TdR uptake [BA-1(IgM), HLA-A,B,C(IgG2a)], and decreased ^{3}H-TdR uptake [OKT9(IgG1), OKB2(IgG1), and PM81(IgM)]. The categories are not correlated with a particular antibody isotype, and this fact suggests that a non-specific Fc binding event is not responsible for the antibody inducement of cell death. The observation that different monoclonal antibodies in the decreased uptake group suppressed DNA synthesis to different levels might indicate that different pathways are being affected by different monoclonal antibodies. Possibly, the differences may be due to antigen density on the K-562 cell.

The hypothesis of apoptosis as a possible mechanism for antibody-induced cell death was explored. Although DNA fragmentation is a major event in

apoptosis (9,16,32,33,34,35,56,57,58), no double strand degradation was observed in K-562 cells treated with polyclonal antiserum using electrophoresis or acid precipitability of the DNA. This finding agrees with other studies in that double strand fragmentation is observed with apoptotic murine systems and not in human cell models. For example, it was reported that no double strand DNA degradation was found with effector cells, such as cytotoxic T cells, natural killer cells and killer cells (12). Thus, DNA cleavage was not considered prerequisite for cell death. However, it was later reported that in those same systems, there was significant single strand nicking (12). In the present study, single strand nicking was shown to be occurring in antibody-treated K-562 cells by strand separating gel electrophoresis and by centrifugation of the DNA through an alkaline (pH 13) sucrose gradient. It was surprising to find significant single strand nicking in K-562 cells treated with pre-immune serum or no serum. We postulate that the single strand breakage may be an ongoing event in these cells which consistantly show numerous translocations and other such chromosome abnormalities. A much increased level of single strand breakage was noted with DNA from antiserum-treated cells. One important consideration for the effect of antibody on the cells may be that the DNA ligation/repair mechanism(s) could be the target metabolic sequence.

Many studies of apoptosis suggest the involvement of an endonuclease(s) in DNA fragmentation. In these studies, a surface acting agent, or an effector cell such as a cytotoxic T cell, induces protein synthesis, presumably for the synthesis of the endonuclease that cleaves single (human) or double strand (murine) DNA. When protein synthesis was inhibited by specific reagents, DNA did not fragment and cell death was avoided. In the present study,

cycloheximide, an inhibitor of protein synthesis, did not protect K-562 cells cultured with antiserum from having suppressed DNA synthesis and undergoing subsequent cell death. These findings suggest that protein synthesis is not a prerequisite for DNA degradation in the K-562 system. This may indicate that a normally present precursor endonuclease may be activated or made less specific by the antibody binding event.

Aurintricarboxylic acid, used in many systems and DNA isolation procedures to inhibit endonucleases (4,5,15), protected K-562 cells from DNA synthesis suppression at certain concentrations. This observation suggests that an endonuclease(s) is involved although aurintricarboxylic acid also is known to bind many other DNA binding proteins. Further complicating an interpretation is the finding that the effect of aurintricarboxylic acid on K-562 cells treated with whole antiserum is different from that found with gamma globulin. Cells treated with gamma globulin and aurintricarboxylic acid showed a DNA synthesis level similar to that of control cells. However, cells treated with whole antiserum and aurintricarboxylic acid had a rate of DNA synthesis significantly enhanced over control levels. This effect cannot be readily explained, although a growth enhancing factor dependent on antibody may be normally present in whole serum, which is removed during fractionation.

Owing to the success of aurintricarboxylic acid in protecting K-562 cells from antiserum-induced cell death, the panel of inhibitory monoclonal antibodies were tested with the drug. Aurintricarboxylic acid did not protect the K-562 cells from monoclonal antibody-induced death. These findings suggest that these particular monoclonal antibodies may induce cell death by different

mechanisms than that of polyclonal antiserum, although polyclonal antiserum should contain a plethora of antibodies and the effect of antiserum would represent a composite of individual antibody mechanisms.

The use of antibody to induce programmed cell death is mainly a convenient one to study intracellular events. Once these events are understood, it may be possible to trigger the event with something other than antibody. The capability to trigger unwanted cells into programmed cell death may have a unique therapeutic advantage in cancer patients.

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