1999

Correlation of Visualized Glucocorticoid Receptor and Apoptosis In Individual Clones of Nb2 Cells

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August 10, 1999
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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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DEDICATION

To my family

ACKNOWLEDGEMENTS

I would be remiss if I did not first and foremost express my gratitude to my advisor Dr. Raphael J. Witorsch. His guidance, enthusiasm, and dedication fostered a desire to become a true gentleman and scholar. It was a sincere honor to have worked with him, and I will always appreciate and value the student-mentor relationship we developed during my tenure in his laboratory.

I would like to thank Rhodaline Rebano who was my colleague, collaborator, and friend for the past year. I would also like to thank Suhas Badarinath and Angelo Guanzon for their friendship and for their assistance in laboratory training.

Furthermore, I would like to thank the members of my thesis committee, Dr. Jack Grider and Dr. Joseph Liberti. In addition, I sincerely appreciate the advice and guidance of both Dr. James Poland and Dr. Linda Costanzo who I have consulted throughout my graduate school career.

Finally, I must express my deepest and most heart-felt thanks to my loving family. My parents and grandmother have given me unconditional love and support which has allowed me to pursue my dreams. My brother has given me the love and frienship that only a brother can provide. Thanks mom, dad, ba, and Vishal.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
DAB	Diaminobenzidene
Dex	Dexamethasone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
FCS	Fetal Calf Serum
FM	Fisher's Medium
FMM	Fisher's Maintenance Medium
GC	Glucocorticoid
GR	Glucocorticoid Receptor
HS	Horse Serum
ICC	Immunocytochemistry
JAK	Janus Associated Kinase
M	Molar
µg	Microgram
µl	Microliter
ml	Milliliter
mM	Millimolar
ng	Nanogram
nM	Nanomolar
PRL	Prolactin
SE	Standard Error
SYN	Synthetic Medium
TUNEL	TdT-dependent dUTP-biotin Nick End Labeling

ABSTRACT

CORRELATION OF VISUALIZED GLUCOCORTICOID RECEPTOR AND APOPTOSIS IN INDIVIDUAL CLONES OF NB2 CELLS

Devang Patel

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Virginia Commonwealth University, 1999

Director: Raphael J. Witorsch, Ph.D., Professor, Department of Physiology

The Nb2 lymphoma cell line is susceptible to glucocorticoid (GC)-induced apoptosis in the absence of mitogen and this effect is inhibited by the mitogen prolactin (PRL). This process is believed to be mediated by the glucocorticoid receptor (GR) and various downstream signals. The purpose of this study was to use the limiting dilution method to isolate subsets of Nb2 clones which expressed either highly enriched amounts of GR or an absence of GR and to relate the amount of GR to the magnitude of GC induced cytolysis. The presence of GR in these clones was determined by immunocytochemistry (ICC) using an affinity purified polyclonal rabbit antibody directed towards the GR. The presence of GR was then correlated to cytolysis in these cells produced by incubation with dexamethasone (Dex), a synthetic GC, in the presence and absence of PRL.

Cytolysis was measured by two different methods, Trypan Blue exclusion counts, to determine the percentage of dead and the TUNEL (TdT-dependent dUTP-biotin Nick End Labeling) assay used to label apoptotic cells. The limiting dilution produced 28 clones with varying amounts of GR and all were responsive to Dex and this response was inhibited by PRL. A significant correlation (r^2=0.419 and $p<0.0001$) was observed between the percentage of cells stained for GR and the % dead cells after Dex treatment. Comparison of mean values for percent dead and percent TUNEL labeled for DMSO, Dex±PRL, and PRL alone for clones and wild types (assayed along with clones) revealed that these two endpoints of apoptosis were in close agreement with one another. The mean values of the percentage of cells undergoing apoptosis agreed closely with the percentage of cells labeled for GR. Furthermore the mean values for all treatments of the clones were very similar to those obtained for the wild type cells. It is concluded that the cytolytic response to dexamethasone is representative of the apoptotic response and that the GR observed by ICC is a quantitative predictor of the apoptotic response to GC.

Chapter 1

INTRODUCTION

Apoptosis, also known as programmed cell death, is a vital process in the survival, renewal, and maintenance of many tissues (Wyllie, 1980; Gavrieli et al., 1992). This selective deletion of cells is responsible for many changes in the embryonic development of organisms such as the deletion of interdigital webbing and the proper formation of the retina (Hammar et al., 1971; Penfold et al., 1986; Gerschenson et al., 1992). Apoptosis has also been implicated in cytotoxic immunological reactions (Hasbold et al., 1990; Strasser et al., 1991; Gorczyca et al. 1993) and as the process of death for differentiated cells at the end of their life span (Appleby et al., 1977; Benedetti et al., 1988; Gorczyca et al. 1993). The involvement of apoptosis in these processes as well as other cellular deletion processes has increased interest in how tumor cells may respond to antitumor agents (Williams, 1991).

Apoptosis is a distinct, well-coordinated process by which cells commit suicide due to specific intracellular signals (Gerschenson, 1992). The morphological changes that cells undergo during programmed cell death led Kerr and Searle to name this phenomenon

"apoptosis" from the Greek "falling off" (Kerr et al., 1972; Gerschenson et al., 1992). This specific pattern of morphological and biochemical changes differentiates apoptosis from necrosis, the process of cell death induced by injury or trauma (Gerschenson et al., 1992).

Necrosis is the manner of cell death most often associated with disease, toxins and hypoxemia. Necrotic cells are identified by swelling and rupturing due to a loss of ion balance and osmoregulation in response to the trauma or injury just described. The ruptured contents of the necrotic cell are released into the extracellular matrix where intracellular proteases and toxic enzymes can cause necrotic death of neighboring cells and inflammation of the effected tissue (Ameisen, 1995). Necrotic cells also undergo a random breakdown of DNA in a single and double stranded manner. This random fragmentation of the DNA results in a smear when the DNA is subjected to gel electrophoresis (Gerschenson et al., 1992).

In contrast to necrotic cells, apoptotic cells shrink due to an active loss of intracellular fluid which is accompanied by blebbing of the cell surface due to fusion of the swollen endoplasmic reticulum with the cytoplasmic membrane. Concurrent with these cytoplasmic changes the nucleus compacts and begins to form discrete fragments. These cells then break into apoptotic bodies which are membrane bound fragments of the cell with cytoplasmic organelles and some nuclear material (Wyllie, 1980; Gerschenson et al., 1992). These apoptotic bodies are often phagocytized by adjacent cells or by

macrophages which means complete removal of the cell and its contents without exposing surrounding cells to any toxicity in the apoptotic cell. An important characteristic of apoptosis is the active nature of the process requiring gene regulation and RNA synthesis. Thus apoptosis involves many intracellular signals which participate in concert to establish an organized process of cell self-destruction (Williams, 1995).

Biochemically, the most characteristic property of apoptosis is the double strand cleavage of DNA in the linker regions between DNA. The fragmentation of the DNA in apoptosis results in irreparable damage that commits the cell to death (Wyllie, 1980; Arends et al., 1990; Gerschenson et al., 1992). During an apoptotic event, endogenous endonucleases are activated to cleave the DNA in the manner described (Wyllie, 1980). The doublestranded DNA is wrapped around a nucleosomal core of histone proteins twice (180 base pairs) with linker DNA connecting adjacent nucleosomes (McGhee et al., 1980). The Ca^{2+} and Mg^{2+} dependent endonucleases (Cohen et al., 1984) cleave at these internucleosomal regions and produce DNA fragments of approximately 180 base pairs or multiples of that number (Gerschenson et al., 1992). This fragmentation results in the characteristic DNA ladder pattern when the DNA from apoptotic cells is subjected to agarose gel electrophoresis (Kerr et al., 1972).

Apoptosis is believed to occur by activating specific cysteine proteases collectively known as caspases. Caspases exist as pro-enzymes which must be activated. The apoptotic triggers (oxidants, ceramide, caspases, etc.) are believed to cause a compromise of the mitochondrial membrane. This may occur either by matrix swelling which causes the outer membrane to rupture or by the opening of channels in the mitochondria. The mitochondria then releases cytochrome c which in turn binds to and activates a cytosolic protein known as apoptotic protease-activating factor 1 (Apaf-1). Apaf-1 activates caspase-9 which activates a cascade of other caspases (Green, et al., 1998). The activated caspases commit the cell to death by various actions. They will initiate degradation of nuclear lamins which are intermediate filament proteins which polymerize to form the rigid understructure of the nuclear membrane. This degradation results in nuclear membrane destruction/condensation. Caspases also activate depolymerization of cytosolic proteins such as gelsolin and actin leading to cytoplasmic blebbing. Other caspases inactivate inhibitors of proteins that promote apoptosis such as the cleavage of I^{CAD}. Caspase-activated deoxyribonuclease (CAD) is a nuclease responsible for DNA laddering and is normally bound to its inhibitor I^{CAD}, but this inhibitor is cleaved by caspases and the CAD translocates to function in the nucleus. Caspases are in a sense the machinery which carries out the destruction of the cell targeted for apoptosis (Thornberry et al., 1998).

Glucocorticoids have long been used clinically to suppress inflammation and the immune response although the mechanism of this was poorly understood (Auphan et al., 1995). Lymphocytes which are treated with glucocorticoids will show the characteristic apoptotic death described above, and this is often shown by using the synthetic glucocorticoid, dexamethasone (Wyllie, 1980). The glucocorticoid (GC) works by binding to the glucocorticoid receptor (GR) which then translocates to the nucleus where it acts as a nuclear transcription factor. The GR normally localizes in the cell cytoplasm waiting to be activated by GC as a ligand regulated transcription factor (Flomerfelt et al., 1994). The GR has a non-conserved amino terminus, but the highly conserved DNA binding domain contains two cysteine zinc fingers which are the DNA response element (Vedeckis, 1992). Exactly which genes are turned on and off by the GR is not completely known.

GCs have been shown to interfere with the function of the nuclear factor kappa B (NFκB) in lymphocytes and this is thought to be the pathway by which GCs mediate immunosupression (Scheinman et al., 1995). NFκB is believed to protect against apoptosis via its activity as a protein transcription factor and it is activated by cytokines, such as Tumor Necrosis Factor (TNF), to turn on genes involved in the immune response (Barinaga, 1996). NFκB is a heterodimer consisting of a 50-kD protein (p50) and a 65-kD protein (p65). In an unstimulated cell, NFκB is bound and repressed in the cytoplasm

by the 37-kD protein IκBα. IκBα binds to NFκB and prevents its translocation to the nucleus where it may regulate gene transcription (Israel, 1995). When factors such as TNF stimulate a cell, the IκBα is quickly degraded due to a phosphorylyation at the conserved amino acid residues Ser32 and Ser36. This releases NFκB and allows for its translocation to the nucleus (Brown et al., 1995). Thus, the activation and translocation of NFκB is important in protecting the cell from apoptosis. Glucocorticoid-induced apoptosis has been shown to occur by disrupting this process. The activated GR translocates to the nucleus where it binds the appropriate DNA response element to induce transcription of IκBα (Scheinman et al., 1995). This upregulation of IκBα synthesis will lead to more of the protein being available to bind NFκB and prevent its activation in the nucleus (Auphan et al., 1995). This lymphocyte immunosuppression effect of GR mediated by IκBα (Auphan et al., 1995) in effect induces apoptosis in the cell by negating the protective properties of NFκB (Beg et al., 1995).

The Nb2 lymphoma cell line is a useful model for studying the regulation of signals involved in GC-induced apoptosis. This particular cell line has been studied and characterized extensively by previous work in our laboratory. The cell line was isolated from an estrogen treated male Noble rat (Noble et al., 1985). Further studies showed that this cell line is of thymic origin at an intermediate stage of differentiation as determined by the presence of surface antigens expressed (Fleming et al., 1982). Nb2 cells require

prolactin (PRL) or other lactogenic hormones for proliferation (Noble et al., 1985). The amount of human, ovine, bovine, or rat PRL required for proliferation ranges from 10pg/ml to 1ng/ml making the Nb2 cells highly sensitive to lactogenic hormones. This specificity and sensitivity allows for the use of the Nb2 cell line as an in vitro bioassay system for measuring serum lactogens (Tanaka et al., 1980).

Knowing that GCs promote cytolysis in lymphocytes (Compton et al., 1986), specific properties of this relationship were examined in this laboratory using the Nb2 cell line. Using the synthetic GC, dexamethasone (Dex), Fletcher-Chiappini et al. (1993) showed that GCs induce apoptosis in the Nb2 cell in a receptor-mediated and dose dependent manner. The same work also revealed that Dex inhibits the mitogenic properties of PRL on the Nb2 in a dose-dependent manner and that PRL inhibits the Dex apoptotic effect, also in a dose-dependent manner. LaVoie further examined the anti-apoptotic action of PRL by using agonists and antagonists of signals implicated in prolactin induction of mitogenesis in Nb2 cells. The inhibition of tyrosine phosphatase in the presence of basal tyrosine kinase activity mimicked the anti-apoptotic action of PRL. Those studies suggested that tyrosine phosphorylation participation is involved in the anti-apoptotic effect of PRL (LaVoie and Witorsch, 1995).

Studies from other laboratories have also implicated the importance of tyrosine phosphorylation in PRL action. The action of PRL on lymphocytes is mediated by the

PRL receptor (PRL-R) and two of its principle signaling pathways, the JAK/STAT and MAPK protein kinase cascades. Both of these pathways involve multiple tyrosine phosphorylations for signal activation (Yu-Lee, 1997). The PRL-R is a member of the cytokine receptor family and does not exhibit intrinsic kinase or catalytic domains on its intracellular domains (Kelly, 1991). The PRL-R signaling occurs by signal transduction mediated by receptor-associated protein tyrosine kinases (PTK) such as Janus Associated Kinase 2 (JAK2). PRL binding to the receptor results in PRL-R dimerization (Elberg, 1990) which activates JAK2. JAK2 then phosophorylates two tyrosine residues on the PRL-R intracytoplasmic domain (Campbell, 1994), Y382 and Y309. These two tyrosine residues are particularly important for the proliferation of Nb2 cells (Yu-Lee, 1997). These phosphorylated tyrosines are believed to serve as docking sites for a group of latent cytoplasmic transcription factors called signal transducers and activators of transcription (STAT) (Stahl, 1995). STAT1, STAT5, and STAT3 have been shown to be PRL-inducible. JAK2 then phosphorylates the STATs activating them to form homodimers with other STATs via SH2 domain mediated by phosphorylated tyrosines. This activated STAT complex will translocate to the nucleus and bind to its corresponding DNA response element to regulate gene transcription (Yu-Lee, 1997).

In addition to the JAK/STAT pathway described above, the activation of other protein kinase cascades has been implicated in the mitogenic effect of PRL on the Nb2 cell. The activation of MAPK is required for the protein synthesis which occurs during

mitogenesis, but MAPK activation alone will not lead to PRL induced mitogenesis of the Nb2 cell (Carey et al., 1995; Carey et al., 1998). The MAPK pathway is usually associated with the growth factor kinase receptor (e.g. GRB2, SOS, RAS, Raf-1, MEK, MAPK) and also involves tyrosine phosphorylation at several sites, namely JAK autophosphorylation, PRL-R phosphorylation and tyrosine phosphorylation of MAPK (Camarillo et al., 1997).

Recent work in our laboratory has focused on a more morphological approach to studying the signals involved in hormonal control of apoptosis in Nb2 cells. Detection and characterization of apoptotic cells usually involves biochemical and molecular biological methods, such as measurements of DNA fragmentation by the diphenylamine assay and agarose gel electrophoresis, and quantitation of signals by Western blot analysis. All of these involve disrupted cell populations which prevents the detection of apoptotic activity and signaling in individual cells (Gavrieli et al., 1992).

In contrast to disrupted cell techniques, Guanzon utilized a morphological and immunocytochemical approach to examine the signals involved in Dex-induced apoptosis. This provided a view of the GC-induced apoptosis at the individual cellular level rather than as a population of disrupted cells (Guanzon, MS thesis, 1998). In 1992 Gavrieli designed the TUNEL assay as an immunological method of detecting cell-specific apoptosis. The assay capitalizes on the DNA fragmentation evident in

apoptotic cells by labeling these broken DNA strands with biotinylated dUTP using the enzyme terminal deoxynucleotidyl transferase (TdT). The signal is amplified by avidin-peroxidase and visualized with the insoluble chromaphore diaminobenzidene (DAB). With this methodology, individual cells in a population can be determined as having undergone apoptosis and the cellular morphology of those particular cells can also be visualized (Gavrieli et al., 1992). Tornusciolo took this concept one step further by proposing that immunocytochemistry (ICC) could be performed on these TUNEL labeled cells to determine the immunophenotype of apoptotic cells (Tornusciolo et al., 1995).

Guanzon used immunocytochemistry to examine several intracellular signals (GR, NFκB, IκBα, and STAT5b) in synchronized (G_0/G_1) Nb2 cells induced to undergo apoptosis by GC exposure. ICC analysis of the Nb2 cells revealed that approximately 40% of the cells displayed staining for GR, NFκB, IκBα as well as STAT5b and that number did not change upon exposure to Dex. Furthermore, no translocation of GR or NFκB from the cytoplasm to nucleus could be detected. TUNEL labeling of synchronized Nb2 cells was detectable 6-8 hours after addition of Dex to the cells which correlated with all death as measured by Trypan Blue exclusion. Previous studies using gel electrophoresis and the diphenylamine assay detected apoptotic activity in as little as four hours (Guanzon, M.S. thesis, 1998).

Guanzon's observations raised important questions about the intracellular signals involved in GC induced Nb2 cell apoptosis. First, was it merely coincidence that the same percentage of cells were immunostained for all the signals and that this percentage corresponded to how many cells underwent apoptosis by GC induction, or was this a single subset of cells containing all the signals examined? Secondly, did the relatively low proportion of cells containing GR limit the ability to detect changes resulting from GC induced apoptosis? Thus, the main objective of this study was to overcome the limitations mentioned above by trying to clone a GR enriched cell line using the limiting dilution method.

We hoped to use the limiting dilution to generate clones of the Nb2 cell in which 100% of the cells were GR positive or at least enriched in GR. The expectation was that vastly different clones would be generated, either very high or very low presence of GR. With this expectation, we intended to quantitate GR by ICC and then relate these levels to GC induced effects of apoptosis by Trypan Blue exclusion and the TUNEL assay. Other studies in the laboratory examined the other signals involved in apoptosis.

Chapter 2

MATERIALS AND METHODS

2.1 Maintenance of Nb2 Lymphoma Cells

The cell line used for all experiments was the wild-type Nb2 lymphoma cell line (Nb2-U17) provided by Dr. Peter Gout of the Cancer Control Agency of British Columbia. The cells were resurrected from frozen aliquots stored at -70°C and were then maintained in Fischer's Maintenance Medium (FMM). The FMM consists of Fischer's Medium (FM) supplemented with 10% horse serum (HS), 10% fetal bovine serum (FBS), 0.1mM β-mercaptoethanol, 50U/ml penicillin, 50μg/ml streptomycin, and 0.075% sodium bicarbonate (Tanaka et al., 1980). Cell cultures were seeded between 0.5×10^5 and 1.0×10^5 cells/ml and incubated in a water saturated atmosphere with 5% CO_2 and 95% room air at 37°C. These cultures were subcultured in fresh FMM (1:9 or 1:19) every 2nd or 3rd day to maintain a maximal cell concentration of about 1.0×10^6 cells/ml. The concentration and viability of cells was determined using the Trypan Blue exclusion test in which cells were counted with a hemocytometer. Trypan Blue, purchased from Gibco BRL, was added to the cells (0.1ml cells plus 0.9ml Trypan Blue) and three individual

aliquots from the mixture were counted for viability and concentration using a hemacytometer (triplicate counts). Triplicate counts were performed to attain accurate estimates of cell number and viability in a time-effective manner as determined by previous studies in this laboratory (Fletcher-Chiappini, 1993).

2.2 Limiting Dilution

The limiting dilution technique is used to isolate subsets of a population of cells that give a specific response (Miller, 1982). In this study, the objective was to obtain Nb2 clones which exhibited varying proportions of GR. In particular we were interested in obtaining clones that were homogenous in which 100% of the cells were GR positive while others would be 100% GR negative. The source of the cells used for the limiting dilution assay were log phase Nb2 wild type cells at a concentration of approximately 1.0 X 10^6cells/ml exhibiting a viability of 90% or greater. Four separate aliquots of these source cells were counted by Trypan Blue in triplicate (as described above) to ensure an accurate count for cell concentration and viability. An aliquot of the cell culture was adjusted to a concentration of 1.0 X 10^6cells/ml in FMM and then subjected to a serial dilution to reach a final concentration of 0.5 cells/ml. The 0.5 cells/ml dilution was then dispensed into a 24 well plate at a volume of 1ml per well. At this dilution of cells, one cells is likely to be plated in every other well which minimizes the possibility of heterogeneity. The 24 well plates were then placed in a 5% CO_2 incubator at 37°C for 10 days. On the tenth day, the contents of each well were transferred into 4ml of fresh FMM in six well plates

to allow further growth of the clone in fresh medium. After an additional 4-7 days when cells were visible in the wells, the concentration of the cells in the well was determined by a single count with Trypan Blue. If the concentration of clones was greater than 0.5 x 10^6cells/ml, the contents of the well were transferred to a 50ml flask containing 5ml of fresh FMM for a total volume of 10ml. When the concentration of the cells in these flasks reached 0.75-1.0 x 10^6cells/ml (2-4 days), the clones were processed for fixation and for exposure to Dex with and without PRL (cytolytic assay).

2.3 Fixation of Cells for GR Staining and TUNEL Assay

Once the clones were determined to be of sufficient concentration for the cytolytic assay, samples of the clone cells were fixed for immunocytochemistry for visualization of the glucocorticoid receptor and TUNEL assay. For the fixation, a 750µl aliquot of the pre-counted cells was harvested and added to an equal volume of 7.4% Formaldehyde (Fisher Brand) in 0.01M PBS in an Eppendorf centrifuge tube. The mixture was allowed to incubate at room temperature for 10 minutes and was then centrifuged at 1000 rpm for 5 minutes. The supernatant was then removed and discarded and a volume of 0.01M PBS was added to the pellet so that the suspension would have a concentration of approximately 3.0 x 10^6 cells/ml. Ten micro liters of the fixed cells were then spotted onto Fischer Brand Super Frost Plus microscope slides on which circles of 5mm diameter had been etched. These spotted cells were allowed to dry onto the slides for at least 24

hours before any immunocytochemistry was conducted on these samples. This method of fixation was designated as the "quick fix" method and differed from the method previously used in this laboratory (Guanzon, M.S. thesis, 1998). The former method of fixation involved pelleting (1000 rpm) of cells and removal of supernatant before the addition of 3.7% formaldehyde to fix the cells.

2.4 Cytolytic Assay

The cytolytic assay was used to determine the cytolytic response to dexamethasone and the protective effect of PRL on the wildtype Nb2 cells and each of the Nb2 clones generated by the limiting dilution. Fletcher-Chiappini et al. (1993) and LaVoie (1994) had previously established protocols for the assays used in this experiment which we employed with some minor modifications. For the cytolytic assay, cells were harvested from the clone samples at a concentration of 0.5-1.0 x 10^6 cells/ml in FMM with a viability of 90% or higher. During each cytolytic assay, a sample of wild type (WT) cells was treated in the same manner. After centrifugation for 5 minutes at 1000 rpm at 8-10°C, the FMM was aspirated and the cells were resuspended in FM as a washing step. The cells were re-centrifuged as before and the FM was aspirated. This pellet was resuspended in a chemically defined serum free medium designated as synthetic medium (SYN). SYN consists of FM containing 0.1mM b-mercaptoethanol, 50U/ml penicillin, 50µg/ml streptomycin, 15mM Hepes, 0.15% bovine serum albumin (BSA), 4µg/ml

linoleic acid, 1mM pyruvate, 12μg/ml transferrin, 1.5ng/ml selenium, 1X vitamins, 0.33X amino acids, 100μM spermidine and 0.5mM $CaCl_2$ at a pH of 7.4 (Walker, 1987).

The concentration and viability of these cells in SYN was then determined using Trypan Blue exclusion. Clones and WT controls exhibiting less than 85% viability at this point were discarded from further study. For each sample, the cell concentration was adjusted by adding SYN so that the final plated volume of 1ml in SYN had a cell concentration of 0.5 x 10^6 cells/ml. The cells were plated in 24 well plates with the following 4-well design for each sample:

1: 0.25% Dimethyl Sulfoxide (DMSO), the vehicular control and diluent for Dex

2: 100nM Dex

3: 100nM Dex + 1ng/ml oPRL

4: 1ng/ml oPRL

Dexamethasone (1,4 pregnadiene-9fluor-16α-methyl-11β,17α,21-triol-3,20-dione) was acquired from Sigma Chemical Co. (St. Louis, MO) and the ovine prolactin (oPRL s-15) was donated by the National Hormone and Pituitary Program. The ovine prolactin was diluted in SYN from a frozen stock of 10ng/ml oPRL in 0.01N NaOH. The cells were then incubated for 24 hours at 37°C in a 5% CO_2 incubator. After 24 hours the concentration and viability of the cells was again determined in triplicate by Trypan Blue

exclusion. Samples from the wells were then taken and subjected to the quick fix protocol for the TUNEL assay.

2.5 TUNEL (Tdt-dependent dUTP-biotin Nick End Labeling) Assay

The TUNEL assay used was purchased as a kit from Trevigen Inc., Maryland. The protocol provided by Trevigen was followed with minor modifications (Guanzon, MS thesis, 1998). The TUNEL assay uses the DNA ends of fragmented apoptotic DNA as a substrate for the enzyme Terminal deoxynucleotidyl Transferase (TdT). TdT catalyzes the formation of complexes of covalently bound biotinylated nucleotides at the DNA ends generated by apoptosis, and these biotinylated nucleotides can be visualized using streptavidin-horseradish peroxidase conjugate. The streptavidin conjugate reacts with diaminobenzidine (DAB) to form a visible insoluble colored substrate at the site of apoptotic fragmentation (Trevigen, Inc., 1996). The spotted slides were immersed in 0.01M PBS for 10 minutes at room temperature to hydrate the cells fixed on the slides. Cells were then treated with Cytopore detergent at room temperature for 20 minutes to permeabilize the cells. After washing the cells in 0.01M PBS, the exogenous peroxidase activity was quenched by immersion of slides in a 2% hydrogen peroxide solution in double distilled H_2O for five minutes at room temperature. After another wash in 0.01M PBS, the cells were equilibrated in 1X labeling buffer while the labeling mixture was prepared.

The labeling mixture consisted of 1µl of Tdt dNTP mix, 1µl of 50X Co^{2+}, and 1µl of Tdt enzyme for every 50µl of 1X labeling mix. This labeling mixture was added in 15µl aliquots to each spot and the slides were placed in a humidified chamber for a 1 hour incubation at 37°C. After the incubation period, the slides were immersed in 1X Stop Buffer for 5 minutes at room temperature. The cells were washed in 0.01M PBS and then treated with streptavidin-horseradish peroxidase for 10 minutes at room temperature. After another wash in 0.01M PBS, the slides were immersed in a DAB solution of 0.25ml of DAB stock solution and 50µl of 30% H_2O_2 in 50ml of 0.01M PBS for 10 minutes at room temperature. The slides were carefully rinsed in water and dehydrated with sequential immersion in 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol, and finally Xylene. The slides were then coverslipped using Protex mounting medium.

2.6 Immunocytochemistry

For the immunocytochemistry, an antibody against the glucocorticoid receptor was obtained from Santa Cruz Biotechnology. The affinity purified polyclonal rabbit antibody was directed against the amino acids 750-769 at the carboxy terminus of an alpha human glucocorticoid receptor and arrived in an initial concentration of 200µg/ml. The GR (P-20) was then diluted to a working concentration of 4µg/ml in 0.01M PBS/0.1% bovine serum albumin (BSA).

The Vectastain ABC anti-rabbit kit by Vector (Burlingame, CA) was used to reveal the binding of the antibody to the receptors. Vectastain reagents (blocking serum, biotinylated antibody, and ABC reagent) were prepared as stock solutions according to the protocol outlined by Vector. The cells used in immunocytochemistry were fixed as described above. The slides were immersed in 0.01M PBS at room temperature for 5 minutes to hydrate the cells. The slides were then removed from the PBS and excess fluid was removed from the slides while not allowing the cells to dessicate. Between each step, the slides were washed with 10ml of 0.01M PBS using a syringe, immersed in PBS, and dried in the manner just described. Reagents were applied using a 1ml syringe to form a bubble of fluid on the spotted cells unless otherwise noted. The cells were permeabilized in Cytopore (20µl/spot applied by micropipet) for 30 minutes at room temperature. This incubation and all subsequent incubations were carried out in an airtight humidified chamber.

Next, a blocking solution of 0.1% immunohistochemical-grade BSA in 0.01M PBS (pH 7.4) was applied to each spot for 20 minutes at room temperature to prevent non-specific binding of immunoreagents. This blocking solution is also used as the biotinylated antibody vehicle (BAV). The glucocorticoid receptor antibody was then added to each spot for a 3 hour incubation at room temperature. An internal control of PBS/BSA was applied to one spot in each experiment as a negative control to show that the secondary antibody bound specifically to the aGR. After this incubation, the secondary antibody was added to each spot. This

Vectastain biotinylated antibody was a 12.5% dilution in BAV of the standard working stock and was applied for 30 minutes at room temperature. The slides were treated with a 20% dilution of the standard working stock of Vectastain ABC reagent in 0.01M PBS (pH 7.4) for 30 minutes at room temperature.

The slides were washed in PBS and then immersed in a DAB solution of 12.5mg DAB in 50ml of 0.05M Tris HCL (pH 7.6) with 50µl of 3% hydrogen peroxide. After this 10 minute incubation at room temperature, the slides were dehydrated by sequential exposure to ddH_2O, 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol, and then Xylene. Following the Xylene exposure, the slides were coverslipped with Protex mounting medium.

2.7 Determining Polyclonal Rabbit Antibody Specificity by Antibody Absorption

The specificity of the GR polyclonal rabbit antibody (aGR) was determined by incubation of the antibody with a ten fold concentration of its complement peptide which absorbs the antibody. For this antibody absorption, 4µg of the GR peptide in 10µl of 0.1M PBS/0.1%BSA was added to 100µl of aGR (0.4µg/100ml). The negative control consisted of 10µl of PBS/0.02%BSA added to 100µl of aGR (0.4µg/100ml). These samples were incubated overnight at 4°C. These samples were then used as primary antibodies in an ICC on Nb2 cells.

The specificity of the antibody was validated if the absorption eliminated staining (Witorsch, 1980).

2.8 Photography and Staining Quantification

Photomicrographs were taken using a Nikon Eclipse E800 Microscope fitted with a DKC-5000 Sony Digital PhotoCamera. The images captured by the digital camera were imported into Adobe Photoshop as a TIFF Graphics file. To obtain cell samplings representative of the entire field, photomicrographs were taken of four fields at positions analogous to 3, 6, 9, and 12 o'clock in the etched circle. If a larger sample size was needed (i.e. number of cells), additional photomicrographs were taken at 1, 4, 7, and 11 o'clock on the etched circle. Scion Image, an image analysis program (Scion Corp, Frederick, Maryland) was then used to quantify stained versus unstained cells in the photomicrographs. The photomicrograph was opened as a black and white image and the total cells were counted by adjusting the threshold of the image so that only the outlines of the cells were visible. The "Analyze Particles" option was then used to count the visible cell outlines. To count stained cells, the threshold of the black and white image was lowered so that only the stained inclusions of the cells were visible rather than the cell outlines. This eliminates the unstained cells from the visible field which was again counted using the "analyze particles" function.

2.9 Statistics

Linear regression analysis was performed on the clonal and wild type data for comparisons of cytolytic data, TUNEL data, and GR staining data (all as percent cells). The correlation coefficients generated by a linear regression are indicative of the relationship between two populations (Daniel, 1999). Also, mean values of the four treatments of the cytolytic assay for clonal and wild type data were generated and the treatments were compared by analysis of variance (ANOVA) with a post-hoc test (Duncan's multiple range tests). All statistical analysis was performed using the computer software Sigma Stat (Version 2.0). The minimum level of significance used was $P \leq 0.05$.

Chapter 3

RESULTS

3.1 Generation of Clones

A total of 28 Nb2 clones were obtained from 4 different limiting dilutions (series 3-6) as shown in Table 1. These clones were examined for GR presence and their response to Dex ± PRL via TUNEL and Trypan Blue exclusion. A total of eight wild type Nb2 cell samples were also examined with each limiting dilution.

3.2 Immunocytochemistry and Antibody Absorption for GR

Immunocytochemical testing revealed specific immunostaining of GR in all wild type Nb2 cells and clones generated by limiting dilution and this specificity was established by immunoabsorption. Figure 1 (A) shows heterogeneous staining of a wild type Nb2 cells for GR typical of both WT and clones of Nb2 cells. As reported previously (Guanzon, M.S. thesis, 1998) the staining appears primarily in the periphery of the cells and is typical of Nb2 log phase cells. Figure 1 (B) shows immunospecificity where

immunostaining is eliminated when the antibody was incubated with a 10X concentration of its antigen for 24 hours prior to use in the ICC.

3.3 Effect of Dex ± PRL on WT and Clone Nb2 cells

The responsiveness of Nb2 cells (WT and clones) to hormones was evaluated in using two endpoints - percent dead cells as determined by Trypan Blue exclusion and TUNEL labeling. Figure 2 provides an example of TUNEL labeling of Wild Type Nb2 cells following exposure to Dex ± PRL. The cells in Figure 2 (A) were treated with DMSO and exhibit little TUNEL labeling. The Dex treated cells in Figure 2 (B) show a marked increase of TUNEL labeling which decreases in the Dex treated cells co-incubated with PRL (Figure 2 (C)). Figure 2 (D) displays the PRL treated cells which are virtually unlabeled. These data are consistent with previous observations (Guanzon, M.S. thesis, 1998) and similar trends in TUNEL labeling were observed for the clones when subjected to the cytolytic assay.

The quantitative responses of individual clones and wild types to Dex ± PRL are shown in Table 1. In both WT and clones, variability was observed in the cytolytic and TUNEL response to Dex. For example the Dex-induced WT percent increase in dead cells above DMSO control ranged from 45% to 78% for WT cells. The TUNEL labeling showed a change from 40% to 68% above DMSO control for the WT cells. The clones showed a change in percent dead ranging from 37-95% and a change of 31-91% for the TUNEL

labeling. In addition the WT cells showed a variation in GR staining from 48-83% and the clone GR staining ranged from 52-87%. The PRL protective effect was seen in every WT and clone sample treated with Dex. In 4 of the 7 WTs, PRL brought the percent dead back to the baseline values, while the other 3 WTs exhibited a partial PRL inhibitory effect. In the clones there also was a variability in the magnitude of the PRL inhibitory effect on Dex induced apoptosis. Fourteen out of twenty-three clones showed a complete inhibition of cytolysis while the remaining nine clones showed partial inhibition by PRL. Similarly eleven of twenty-two clones showed inhibition of apoptosis as measured by TUNEL labeling and eleven showed partial inhibition. In the absence of DEX, percent dead or TUNEL labeling after PRL was at the baseline DMSO level or less in all clones and WTs.

3.4 Correlations Between Trypan Blue Staining, TUNEL Labeling, and GR Staining

Linear regressions were conducted between individual values of clones and wild samples for GR, percent dead, and percent TUNEL labeled cells. As shown in Figure 3 a statistically significant ($P<0.0001$) correlation was observed between GR staining and percent dead after Dex treatment. Figure 4 shows that a statistically significant correlation existed between percentage of cells stained for GR and increase in percentage of cells dead from DMSO after Dex treatment. Figures 5 and 6 similarly compare GR staining with TUNEL counts. Unlike Trypan Blue counts, a statistically significant correlation could not be demonstrated between individual TUNEL values and GR values.

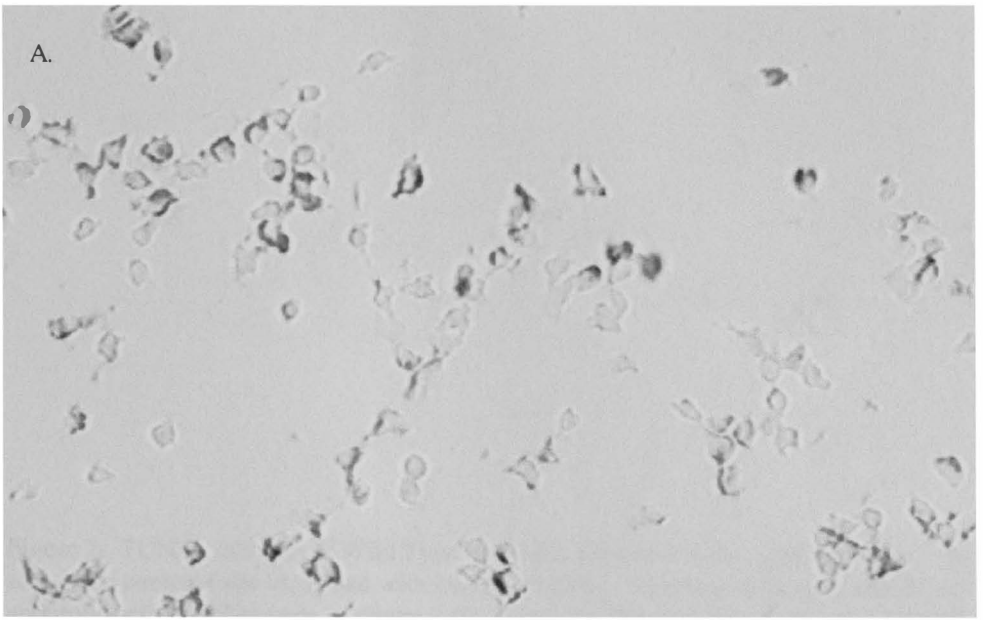
In Figures 7 and 8, the relationship between TUNEL labeling and percent dead counts was examined when cells were treated with Dex. Neither comparison, percent TUNEL labeled after Dex versus percent dead after Dex (Figure 7) nor increase in TUNEL labeling after Dex treatment versus increase in percent dead after Dex (Figure 8) showed significant correlations. In Figure 9 TUNEL and percent dead values were correlated for all four treatments of the cytolytic assay. Here TUNEL and cytolysis are highly correlated ($P<0.0001$).

3.5 Comparison of Means of Clone and WT Data for TUNEL, Trypan Blue, and GR

Figure 10 compares the mean values of TUNEL labeling and percent dead cells for the four treatments of the cytolytic assay on the Nb2 Wild Type cells. The mean for GR staining counts is also included for comparison with the Dex treatment results. A response to Dex and its inhibition by co-incubation with PRL is clearly seen for both endpoints. Furthermore, cytolytic and TUNEL mean values are very close to one another for each treatment. In addition, the percent GR staining corresponds quite closely to percent dead and percent TUNEL labeled values for the Dex treatment. Figure 11 similarly examines the mean values of TUNEL labeling and percent dead cells for the clones. The clones again display a definite response to Dex which is inhibited by co-incubation with PRL as seen by TUNEL labeling and percent dead cells. The TUNEL mean values are very close to the percent dead mean values for each treatment and the percent GR staining is very close to the Dex mean values for both endpoints. Also the

mean values of the clones are very similar to the mean values of the WT cells for each of the treatments (Figure 10 compared Figure 11).

Figure 1. (A) shows WT Nb2 cells stained with an antibody to GR at a concentration of 4μg/ml. (B) demonstrates the disappearance of this staining when the aGR is absorbed out by incubation with the antigen peptide concentration at 40μg/ml. (20X objective magnification).


A.

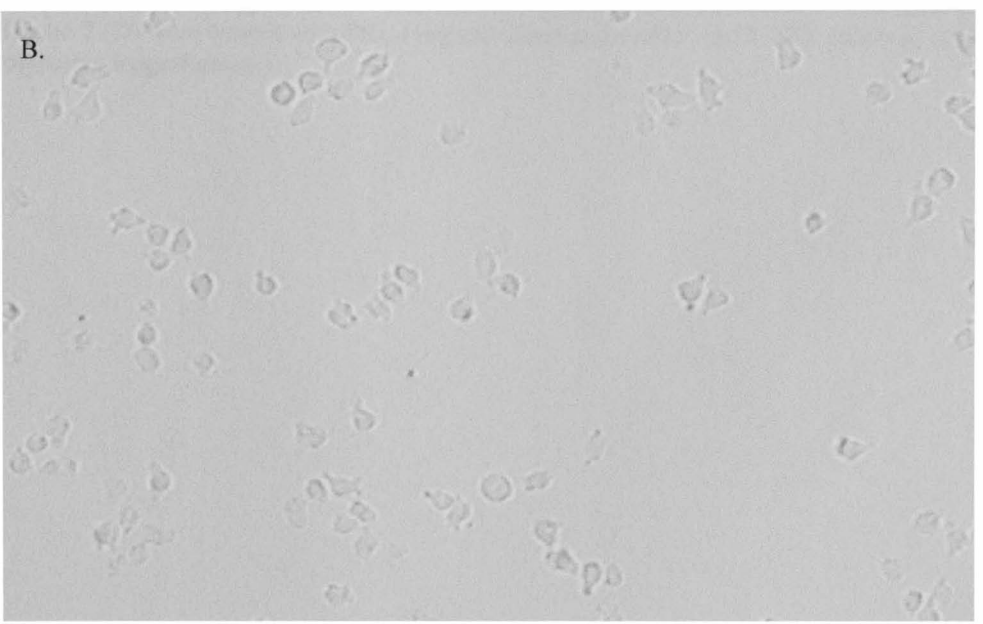
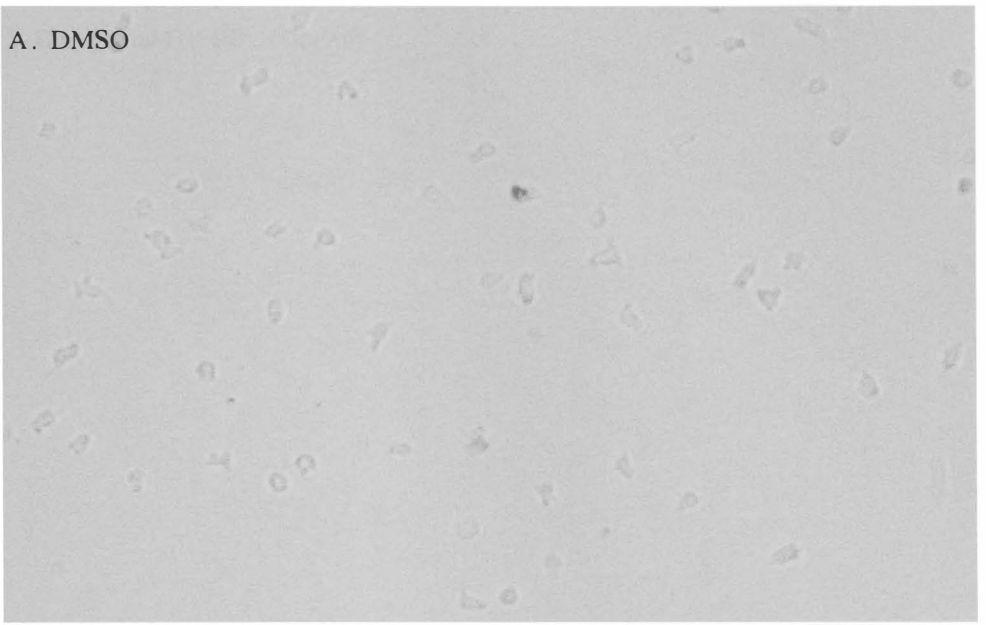
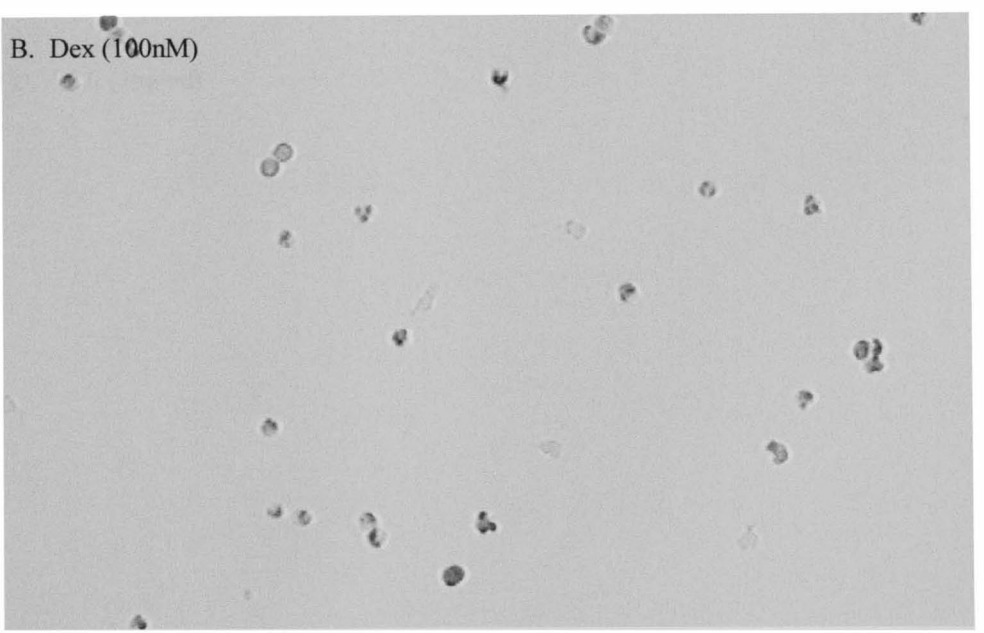

B.

Figure 2. TUNEL labeling of Wild Type Nb2 cells subjected to the cytolytic assay. (A) shows the control cells incubated with DMSO (0.25%). Very few cells were labeled for apoptotic activity. The cells in Figure 2 (B) reveal the effect of Dex treatment (100nM) as an increase in TUNEL labeling is readily apparent. Figure 2 (C) demonstrates the inhibitory effect of PRL (1ng/ml) co-incubation with Dex treated (100nM) cells. The frequency of observing apoptotic cells decreases from the Dex treated cells. The cells in Figure 2 (D) were treated with PRL (1ng/ml) alone and exhibit no TUNEL labeling. (20X objective magnification)

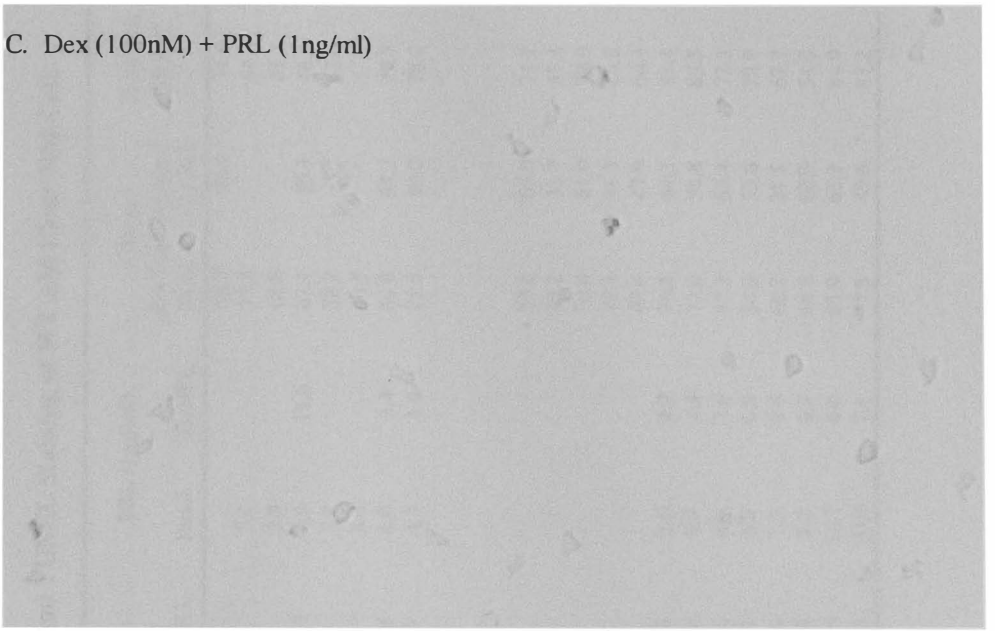
A. DMSO



B. Dex (100nM)



C. Dex (100nM) + PRL (1ng/ml)



D. PRL (1ng/ml)

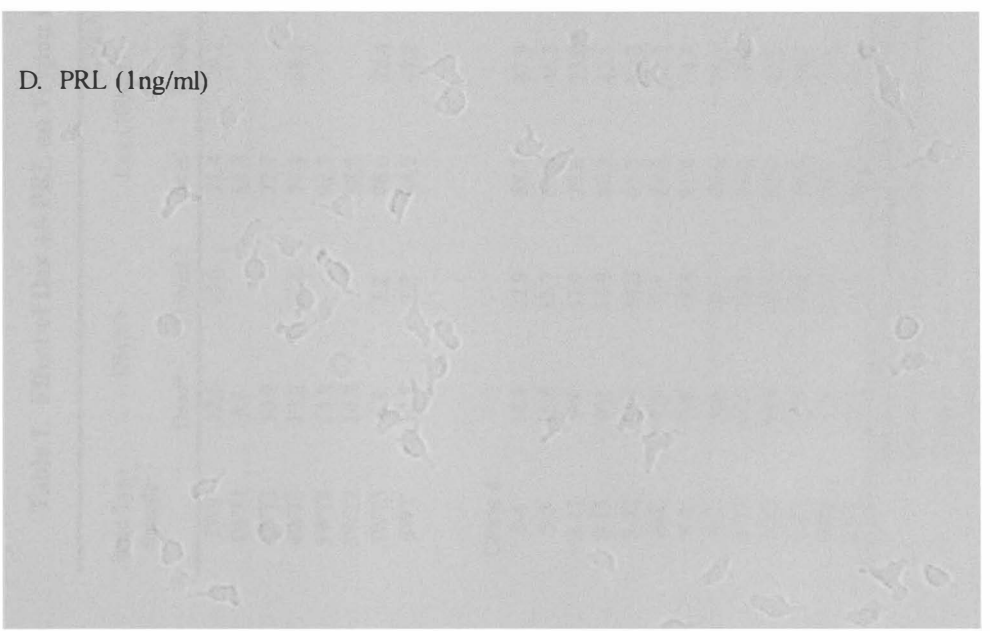


Table 1. Effect of Dex +/- PRL on Trypan Blue Exclusion and TUNEL Staining of WT and Clone Nb2 Cells

	TREATMENT										
Wild Type Sample	DMSO		Dex (100nM)		Dex (100nM) + PRL (1ng/ml)		PRL (1ng/ml)		Change after Dex treatment[c]		% of cells GR (+)[d]
	Dead[a]	TUNEL[b]	Dead	TUNEL	Dead	TUNEL	Dead	TUNEL	Dead	TUNEL	
3WT	12.5	10.0	71.4	78.2					58.9	68.3	71.8
4WT1	8.6		84.2		7.0		5.1		75.6		64.4
4WT2	10.9		77.7		15.6		3.9		66.8		61.3
4WT3	13.2	13.1	74.3	68.1	16.3	26.7	4.6	11.6	61.1	55.1	48.1
5WT1	14.5		92.7		35.9		3.8		78.2		82.6
5WT2	14.2		58.6		11.1		8.8		44.5		
5WT3	6.8	5.2	76.6	72.4	30.2	27.6	8.0	3.4	69.8	67.2	79.7
6WT	12.8	7.2	84.2	47.2	20.5	9.9	4.7	1.6	71.5	40.0	78.0
Clone #											
3-4	18.1	22.9	88.7	87.9					70.6	65.0	71.2
3-5	16.8	12.7	66.0	46.3					49.2	33.7	57.4
3-11	9.4	11.9	79.8	73.0					70.4	61.0	73.0
3-15	9.2	11.9	56.3	43.1					47.1	31.3	66.2
3-20	13.1	18.3	61.5	66.2					48.4	47.9	66.4
4-2	7.7	7.4	84.5	51.7	5.9	17.8	2.6	4.3	76.8	44.3	83.4
4-5	9.4	15.8	81.4	74.7	4.6	15.4	8.0	4.4	72.0	58.8	83.5
4-7	4.4	9.7	91.6	75.0	33.9	21.6	4.1	7.6	87.2	65.4	72.3
4-14	14.6	25.8	69.5	79.4	20.5	25.6	6.8	6.9	54.9	53.6	72.9
4-16	10.5	16.0	79.1	47.5	17.7	18.9	7.3	8.6	68.7	31.5	67.7
4-21	13.7	16.2	78.5	76.1	24.7	11.7	7.2	6.2	64.8	60.0	54.8
4-22	8.2	13.8	94.1	77.2	39.2	38.5	15.7	8.0	85.9	63.3	84.9
4-23	15.3	16.0	90.5	59.6	16.0	10.6	20.9	7.4	75.3	43.6	87.2

[a]percent dead cells as determined by Trypan Blue exclusion

[b]percent cells stained for apoptosis by TUNEL labeleing

[c]increase in % above DMSO (control)

[d]cells stained for GR using ICC

Table 1 Continued

Clone #	TREATMENT										% of cells GR (+)[d]
	DMSO		Dex (100nM)		Dex (100nM) + PRL (1ng/ml)		PRL (1ng/ml)		Change after Dex treatment[c]		
	Dead[a]	TUNEL[b]	Dead	TUNEL	Dead	TUNEL	Dead	TUNEL	Dead	TUNEL	
5-4	5.4		100.0		30.1		4.4		94.6		87.2
5-8	10.5	7.5	66.7	73.1	13.4	17.5	4.5	3.5	56.2	65.6	57.5
5-10	9.0	4.6	62.6	69.6	22.1	13.3	1.1	3.3	53.6	65.0	65.2
5-11	15.0	11.0	52.4	81.6	14.9	18.6	4.1	12.0	37.4	70.6	59.2
5-13	6.3	5.3	53.4	50.2	4.0	3.3	2.2	0.7	47.1	44.9	59.3
5-17	16.7	12.5	69.1	73.1	12.0	14.4	2.8	8.9	52.4	60.6	52.5
5-23	15.2	18.3	74.6	72.1	13.2	29.3	3.9	7.8	59.4	53.9	58.6
6-2	11.6		82.9		47.0		7.6		71.3		81.5
6-3	16.6	12.5	67.7	61.3	6.2	5.1	3.5	2.1	51.1	48.8	75.5
6-7	19.5	15.6	92.3	88.0	23.2	38.7	10.3	3.8	72.8	72.4	72.0
6-11	13.3	9.9	67.1	84.3	17.2	21.5	10.8	6.5	53.8	74.5	65.2
6-14	12.0	14.6	98.4	75.6	53.3	49.9	12.3		86.4	61.0	79.5
6-16	12.2	4.1	95.9	94.7					83.6	90.6	73.1
6-20	12.7	8.1	89.3	74.6	35.6	26.5	8.9	4.7	76.5	66.5	83.0
6-22	16.2	11.7	73.4	87.5	8.2	22.6	5.0	2.3	57.1	75.8	76.7

[a]percent dead cells as determined by Trypan Blue exclusion

[b]percent cells stained for apoptosis by TUNEL labeleing

[c]increase in % above DMSO (control)

[d]cells stained for GR using ICC

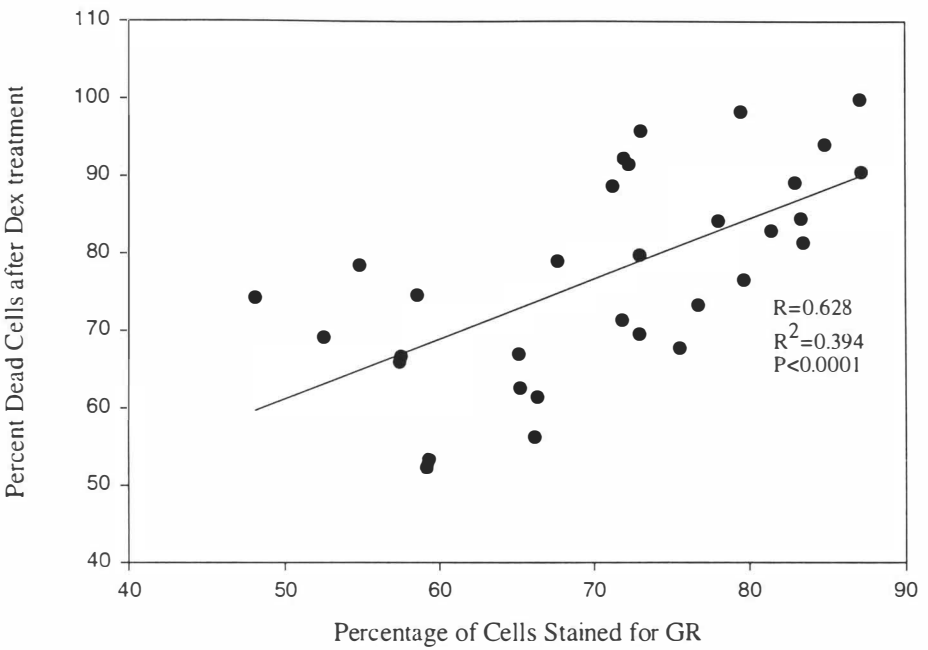


Fig. 3. Statistically significant correlation between cells stained for GR versus the % dead cells after Dex treatment. This figure incorporates all clone and wild type data.

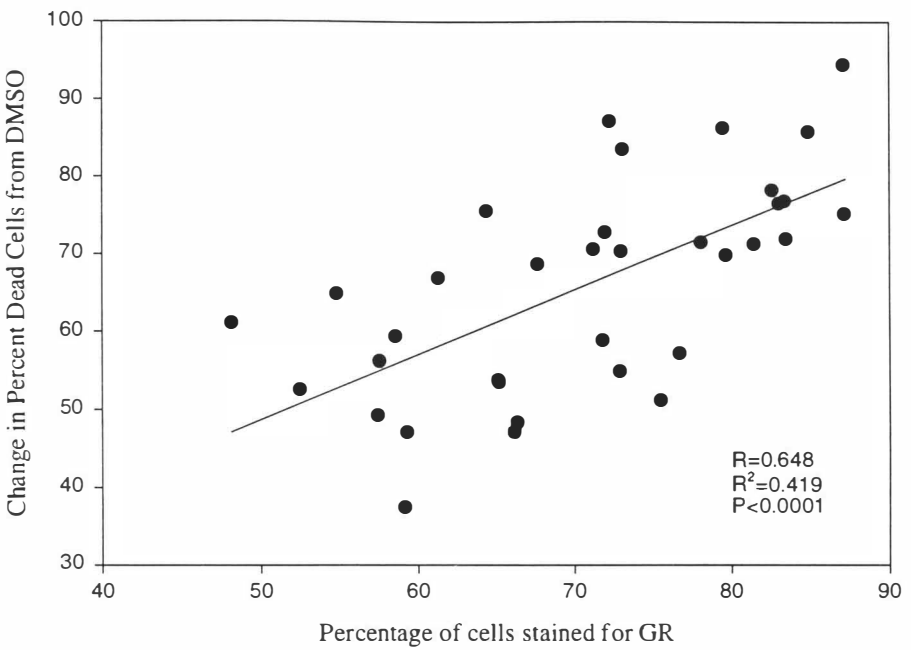


Fig. 4. Statistically significant correlation between cells stained for GR and the change in % dead cells from control values after Dex treatment. All clone and wild type data for Dex treatment included.

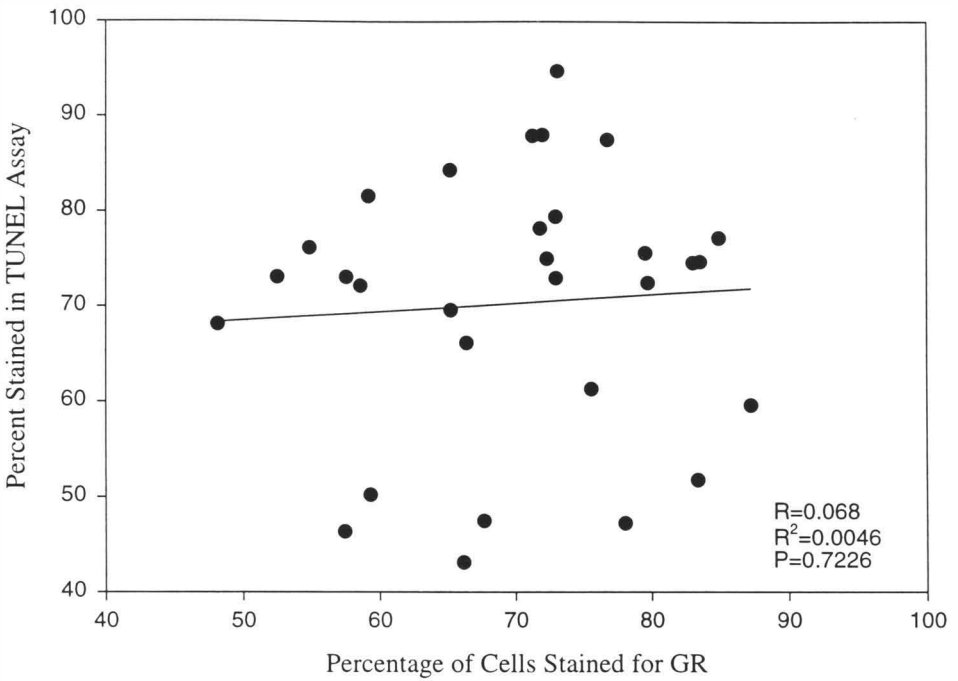


Fig. 5. Percentage of cells stained for GR versus percentage of cells labeled by TUNEL after Dex treatment. Correlation does not show a statistically significant relationship.

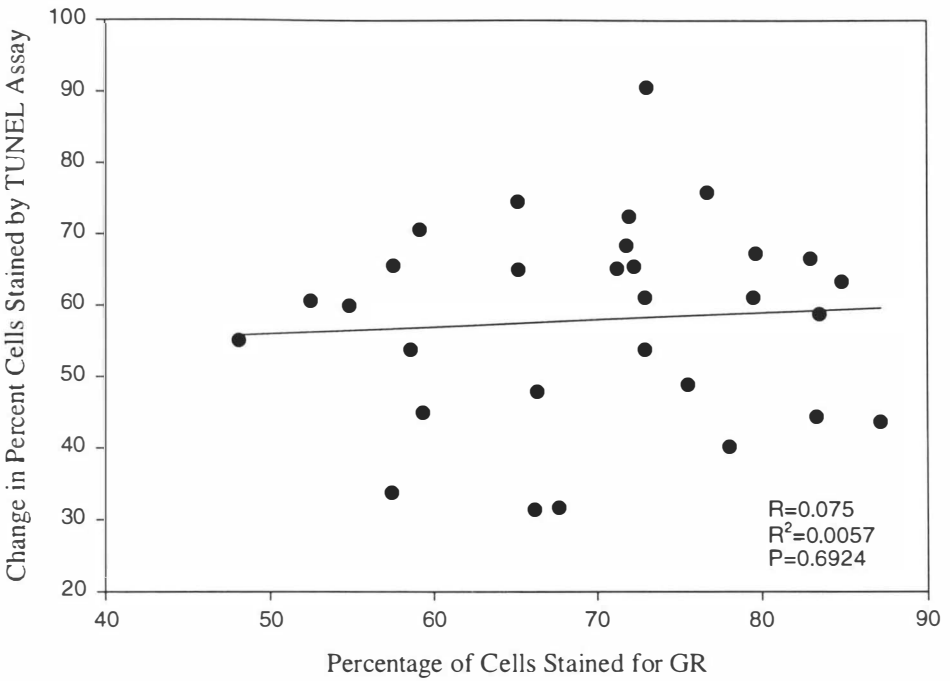


Fig. 6. Percentage of cells stained for GR versus change from DMSO in percentage of cells labeled by TUNEL after Dex treatment. Correlation does not show a statistically significant relationship.

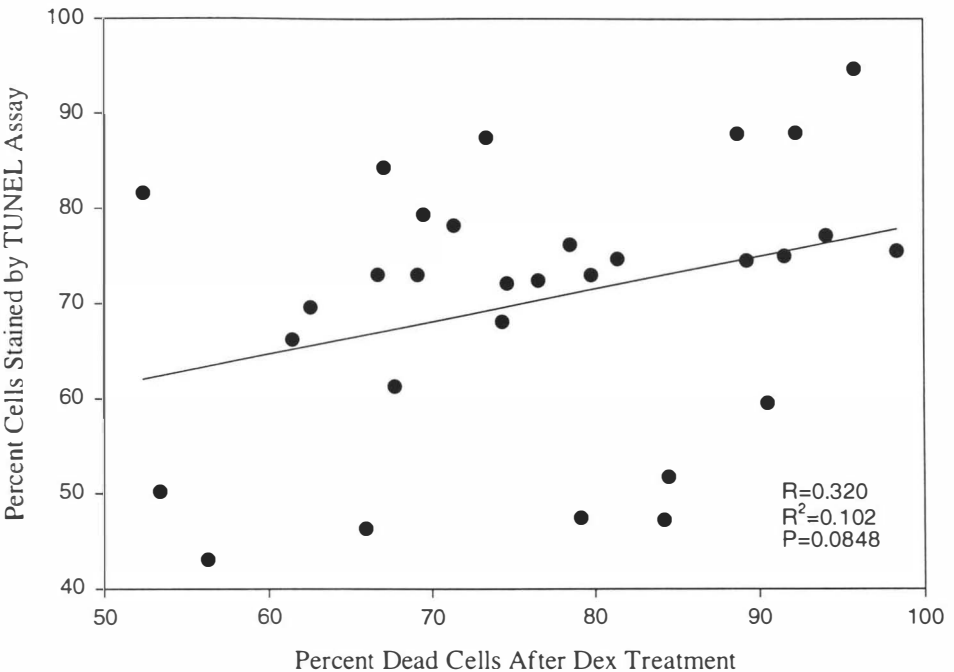


Fig. 7. Percentage of cells dead after Dex treatment versus percentage of cells stained by TUNEL assay after Dex treatment This is not a statistically significant relationship.

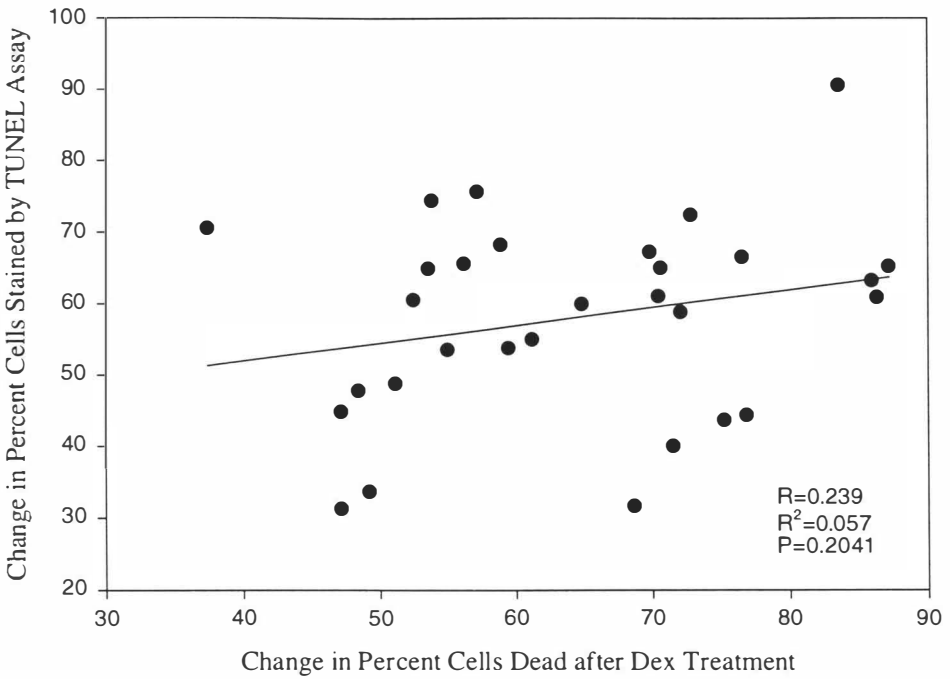


Fig. 8. Change from DMSO in percent dead cells after Dex treatment versus their the change from DMSO in percentage of cells stained by TUNEL. This is not a statistically signigicant relationship.

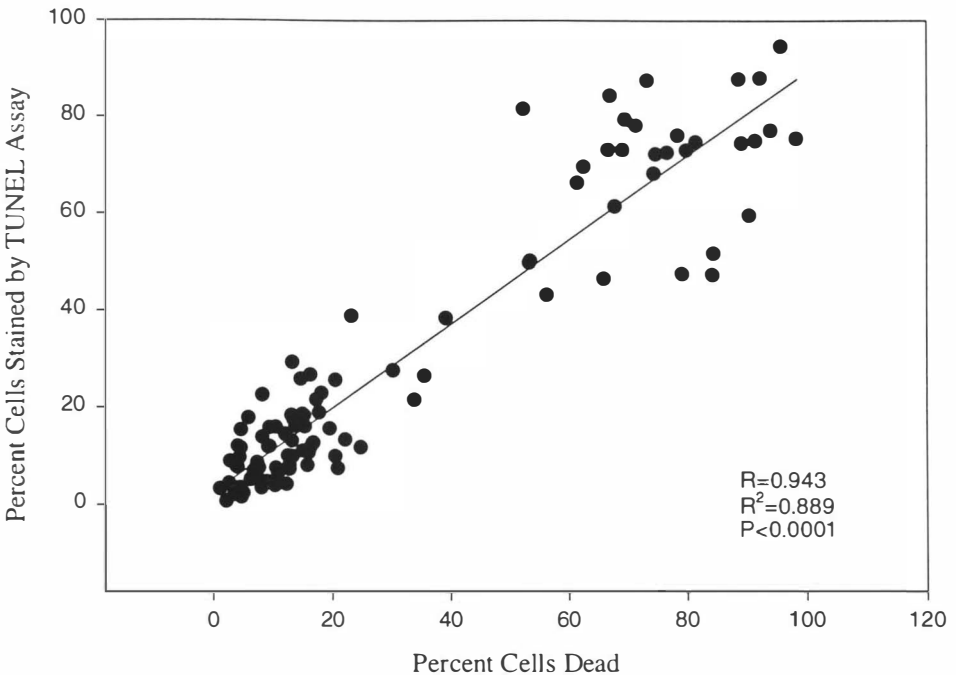


Fig. 9. Percentage of cells dead in all four treatments of cytolytic assay (DMSO, Dex, Dex+PRL, and PRL) versus the corresponding percentage of cells stained by TUNEL assay for clones and wild type.

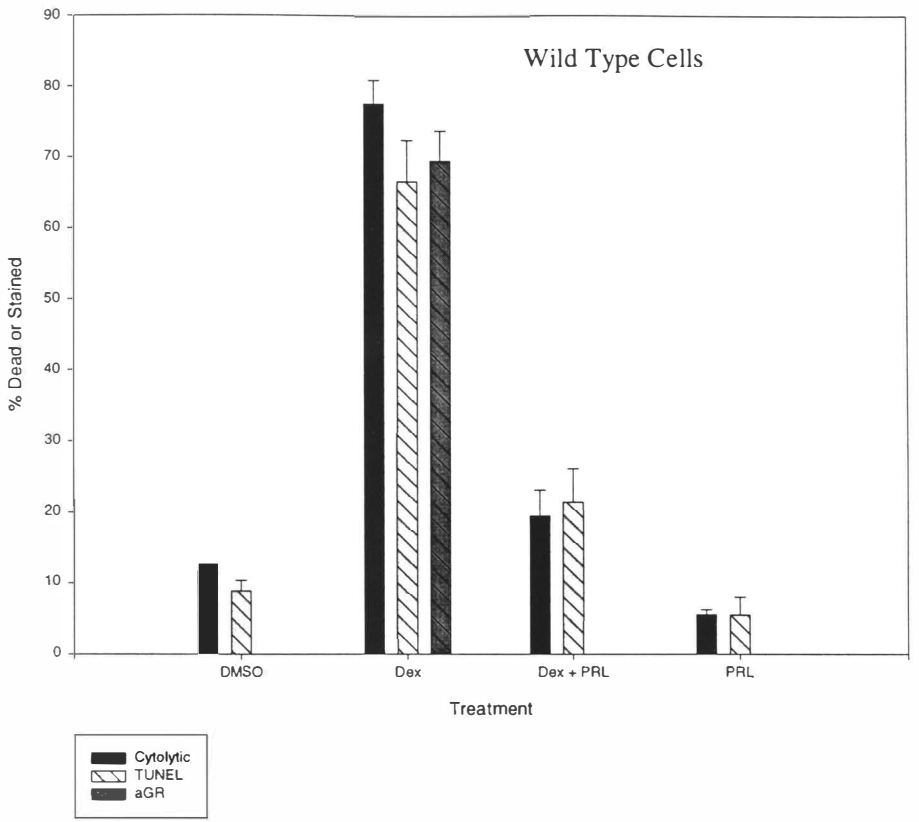


Fig. 10. Comparison of means (+/- SE) for TUNEL labeling, % dead, and % GR staining for all WTs. The four treatments are DMSO (0.25%), Dex (100nM), Dex (100nM) plus PRL (1ng/ml), and PRL (1ng/ml) alone. The Dex means differ in a statistically significant ($P<0.05$) increase in TUNEL labeling or % dead cells but the other treatments are not significantly different from the control.

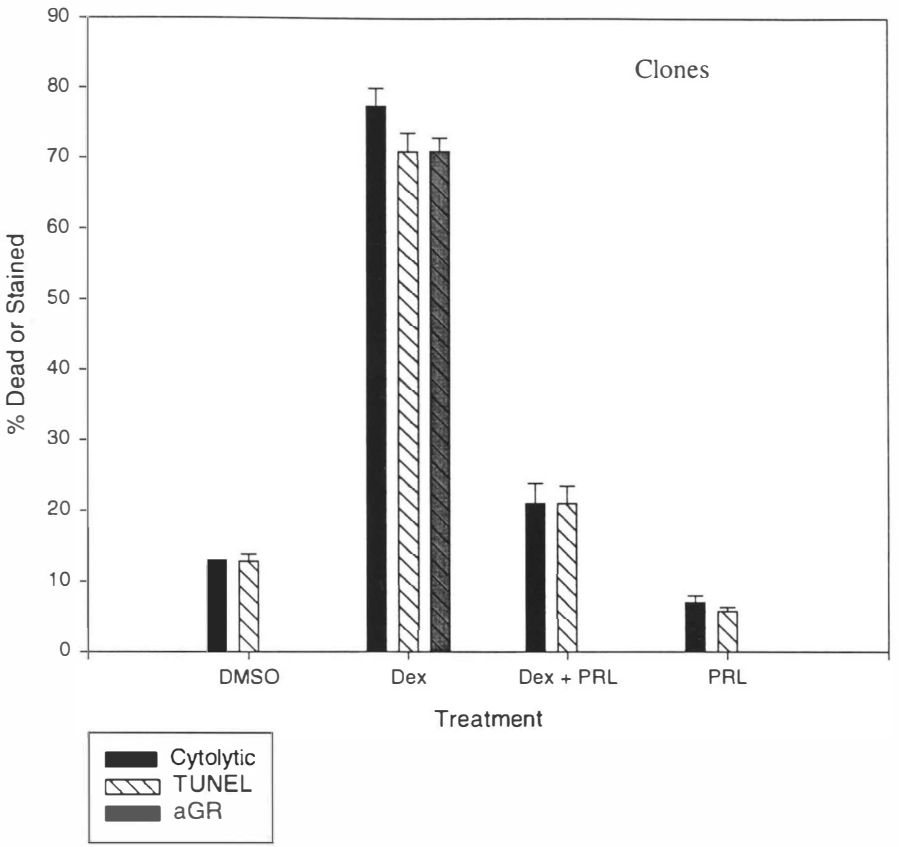


Fig. 11. Comparison of means (+/- SE) for TUNEL labeling, % dead, and % GR staining for all clones. The four treatments are DMSO (0.25%), Dex (100nM), Dex (100nM) plus PRL (1ng/ml), and PRL (1ng/ml) alone. The Dex means differ in a statistically significant ($P<0.05$) increase in TUNEL labeling or % dead cells but the other treatments are not significantly different from the control. The Dex+PRL treatment does differ from the PRL alone treatment.

Chapter 4

DISCUSSION

The objective of this study had been to isolate clones of the Nb2 lymphoma cell line that were glucocorticoid receptor enriched and deprived of these receptors and to use these clones to determine whether GR is predictive of cytolysis as measured by Trypan Blue exclusion and apoptosis as measured by the TUNEL assay. In actuality no completely enriched GR clones were obtained nor were there two distinct groups of primarily GR enriched and primarily GR reduced clones. A statistically significant relationship was found between the percentage of cells stained for GR and cytolysis after Dex treatment as measured by Trypan Blue exclusion (Fig. 3 and 4). A statistically significant correlation between TUNEL labeling after Dex treatment and GR staining was not found (Fig. 5 and 6). Furthermore, the correlation between TUNEL labeling and cytolysis after Dex treatment was not found to be statistically significant (Fig. 7 and 8). However, when the TUNEL and cytolysis data for all treatments of cells (DMSO, Dex, Dex + PRL, and PRL) were regressed, a very high, statistically significant relationship was found (Fig. 9). When analyzing the mean values of the clones and wild types for each treatment, the TUNEL and percent dead mean values were very close to one another (Fig. 10 and 11).

In addition, the Dex effect could clearly be seen and the mean percent GR staining values were very similar to the Dex response values as measured by Trypan Blue or TUNEL.

The wild type cells and clones also exhibited mean values for all treatments which were very close to one another (Fig. 10 and 11).

The limiting dilution method in this particular study was modified and practiced twice before implementation in order to refine the method for our purposes. We used the limiting dilution method in preference to the transfection methods because it would appear to be a much more natural process of obtaining cells with specific characteristics, such as GR. Transfection also does not ensure the presence of other signals associated with GR that may be necessary for whatever processes GR mediates. Presumably this cascade of signals would be found in GR possessing cells. One of the above-mentioned refinements to the method we employed was to dilute the cells to a concentration of 0.5 cells/ml before plating at a final volume of 1ml. At this dilution of cells, one cell is likely to be plated in every other well which minimizes the possibility of heterogeneity. The clones were also tested at the earliest possible time point in order to limit any cellular changes that may occur due to prolonged growth in the media. The clones were also not "split" at any time in order to keep the entire clone population intact as the cells proliferated.

The results of the immunoabsorption study verified the specificity of the GR antibody used in the ICC experiments (Witorsch, 1980). This eliminated the possibility of non-specific staining and thus the ICC staining should be a valid visualization of GR presence in the various clone Nb2 cell lines. We expected two populations of cells – GR positive and GR negative – but obtained neither. This may be a reflection of the natural way in

which neoplastic cells behave. Neoplastic cells in culture differ from normal cells in a variety of ways. Neoplastic cells do not exhibit contact inhibition nor do they have a reliance on cell adhesion. Neoplastic cells also are known to exist at an intermediate level of differentiation. Differentiated cells do not usually proliferate so a neoplastic cell is usually at an earlier stage of differentiation. Neoplastic cells are also thought to dedifferentiate. These cells do not actually revert to a more primitive, undifferentiated precursor of that cell line but rather lose normal characteristics and function with tumor progression. Dedifferentiation can also be referred to anaplasia and may be the reason why we were unable to obtain GR enriched clones. Even though the cells were harvested at the earliest timepoint possible, they may have still undergone proliferation during which this dedifferentiation could occur (Braun, 1974; Franks, 1986).

The absence of GR positvie cells may have been due to the fact that the WTs were GR enriched to begin with. Guanzon showed lower GR staining and GC response than what we found. The Nb2 cells used in this work were from a line of cells provided by Dr. Peter Gout at the Cancer Control Agency of British Columbia and were representative of the original wild type Nb2 cell line. The Nb2 cells used in our previous ICC studies (Guanzon, M.S. thesis, 1998) were originally provided by Dr. Gout but had been at another laboratory at our institution prior to being made available to us. In contrast to the cells used in this study, the cells in Guanzon's study had undergone many more passages before being used in experiments. This may have been the reason why those cells showed approximately a 40%-50% response to Dex while the wild type Nb2 cells in the present study showed on average a 70%-80% response to Dex. In addition 70% of the cells in

our study stained positive for GR while Guanzon showed only about 40% staining for GR in those Nb2 cells. The wild type Nb2 cells were thus already an enriched GR cell line and that may have prevented the isolation of an even higher GR enriched clone. This however, does not explain why a GR devoid clone was not isolated. An alternate method for isolating a GR negative clone may be necessary. One possible method would be to incubate the wild type Nb2 cells in a cytolytic concentration (100nM) of Dexamethasone. This would presumably induce apoptosis in the cells with glucocorticoid receptors but not in GR negative cells. The GC resistant cells would then proliferate establishing a GR negative clone of Nb2 cells. However evidence does exist for the possibility of GC resistance lymphocytes with fully functional glucocorticoid receptors (Witorsch et al., 1993; Oldenburg et al., 1997). Alternatively, starting with Nb2 cells with lower percentage of GR positive cells may lead to better isolation of GR enriched and deprived clones. Preliminary studies in our lab show that the GC response of Nb2 cells decreases with time.

The correlations performed on the TUNEL labeling, cytolysis counting, and GR staining data presented some meaningful insights. The cytolysis induced by Dex treatment, presumably via apoptosis, showed a statistically significant relationship with the percentage of cells stained for GR. This relationship suggests that the apoptotic effect of glucocorticoid on the Nb2 cell can be predicted by the proportion of cells stained for the glucocorticoid receptor in the population. The TUNEL labeling did not show this same expected correlation nor did the TUNEL labeling correlate with the percent cells dead after Dex treatment. The high statistically significant correlation between TUNEL labeling and cytolysis for data from all 4 treatments of the cytolytic assay shows that these

two endpoints for apoptosis do in fact describe the same phenomenon over a broad range of values. In this combined correlation, the TUNEL labeling values and percent dead values ranged from nearly 0% to well over 90% which easily separated high, intermediate, and low response values from one another. If the limiting dilution had produced clones in the expected highly enriched and devoid subsets, the Dex response data may have given this wide distribution of values needed for correlating TUNEL labeling and cytolysis.

After having conducted statistical analysis on the data generated from the 28 clones and 8 individual experiments involving wild type cells, the limitations of the methodology were more apparent. Perhaps the most apparent limitation of the methodology was the imprecision of the immunocytochemical approach to the data. For the Trypan Blue exclusion values obtained in this study, each clone sample was counted in triplicate. This meant that three different aliquots of cells were taken from each treatment well and examined via hemocytometer counts of Trypan Blue exclusion. Previous work in our laboratory had shown that a single Trypan Blue count of a cell population can produce as much as a 40% variation from any other count taken of that same sample. Triplicate counts ensured precision and accuracy of the cell while being time and cost effective. When twelve counts were taken from the same sample, any three counts of that twelve would give a mean that was within 10% of the mean for the twelve counts. The quantitation of the TUNEL and GR staining may not have had this desired precision and accuracy. For the TUNEL and ICC assays, a 10µl sample of fixed cells was taken from each treatment of the cytolytic assay and spotted onto a slide. The cells on the slides were then subjected to ICC or TUNEL labeling and visualized using a microscope. The

quantitation of staining involved taking 4-8 pictures of each treatment for each clone. The pictures for a given treatment were taken at regular intervals in the field to give a representative view of the entire field of cells as described in the Methods section. The minimum number of cells counted for any treatment was about 60-62 cells with an average of 205-208 cells counted for all clones and wild types for all treatments. The multiple photographs and number of cells counted were steps taken to eliminate variation in the sample which could skew the data. However, the methodology did not account for variability which could occur when only one aliquot is examined from a given fixed cell population. In order to better emulate the hemacytometer counting, a minimum of three aliquots of fixed cells should have been spotted for every treatment of every clone. This inherent methodological variability likely prevented a more accurate correlation of the data. Even the correlation between the Dex response (as measured by cytolysis) and the GR staining could have been higher if the data for the GR had been measured in a manner closer to the hemacytometer counting. This would have held true for the GR versus TUNEL labeling correlations as well. The TUNEL labeling versus percent dead cells for all treatments showed a very high correlation, but the wide range of values probably minimized the effect of the variability in the TUNEL samples. With more accurate counting of TUNEL labeling, the Dex-induced response may have correlated with the percent dead cells by itself (i.e. without pooling all treatments). This possible limitation in the methodology was not anticipated but could be overcome in future experiments.

The mean values of the clones and wild types for the four treatments also revealed some interesting relationships. The mean values for TUNEL labeling and cytolysis were very similar to one another for both the clones and the wild type cells. This is another

indication that these two end points are essentially detecting the same phenomenon (apoptosis). By using the mean values of a relatively large population for each treatment, the possible variability of the individual clones made less of an impact on the results. In addition the mean values of GR staining were similar to the mean values of cytolysis and TUNEL labeling after Dex treatment. This again demonstrates the relationship between GR and GC-induced apoptosis which may not have been as clear with the correlations of the individual clone samples. The clone and wild type mean values were very close to one another for all four treatments as well as for the GR and maybe be a further indication that the individual clones eventually dedifferentiate to exhibit the same phenotype as the wild type cells.

The Dex response of the wild type and clone Nb2 cells, as well as the inhibition of this response with PRL co-incubation, concurs with previous findings in our laboratory (Fletcher-Chiappini et al., 1993; Guanzon, M.S. thesis, 1998). The data also show that cells that have GR will be induced into apoptosis by Dex treatment. Although this might be expected, there is evidence of lymphocyte cell lines which have functional GR yet cannot be induced into apoptosis by GC. The SFJCD1 cell line, a clone of the Nb2 cell line which proliferates independently of exogenous PRL, is one such example. This cell line demonstrated antiproliferative effects when incubated with Dex demonstrating functional GR action, but the cells did not undergo apoptosis when treated with Dex alone (Witorsch et al., 1993). Work with human leukemia and lymphomas has also shown that "the lack of response...to steroid therapy cannot be attributed to the absence of glucocorticoid binding or presence of altered receptors." (Stevens et al., 1985) More recently Oldenburg et al. (1997) have demonstrated rat thymocytes with functional

glucocorticoid receptors which are glucocorticoid resistant *in Vivo* but not *in Vitro*. With these studies in mind, the present work is significant in showing that GR presence in the Nb2 cell line is predictive of the apoptotic response.

The correlation between cytolysis and GR also reveals another interesting characteristic of apoptotic cells. If Dex had induced necrotic death in the Nb2 cells, the necrosis would have caused the release of toxins from dead cells which would in turn cause further cell death. In effect the stimulation of one GR would have caused the death of cells devoid of GR in a necrotic fashion. The experiments in this study actually show a direct relationship between cytolysis and the presence of GR which is more indicative of apoptosis where individual cells are selectively chosen to undergo death processes.

Although the desired GR enriched and deprived Nb2 cell lines were not obtained, the findings are useful to future work in generating and examining Nb2 clones expressing specific proteins or signals. Perhaps most importantly, the method of immunocytochemical quantitation would have to be refined further to provide for more accurate estimation of cellular markers. This study only examined apoptosis in the Nb2 cell as related to the GR, but other studies in our laboratory are examining other intracellular signals involved in apoptosis. Perhaps the combination of the information gained in this study with the information on signals such as STAT5b, IκBα, and NFκB will provide a better overall understanding of apoptosis and the intracellular signals that mediate those processes in Nb2 cells.

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