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# Examination of Signals Involved in Dexamethasone Induced Apoptosis in Nb2 Lymphoma Cells

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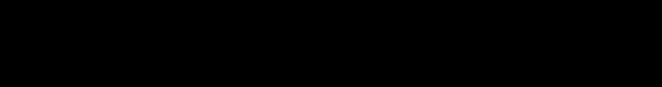
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Virginia Commonwealth University  
School of Medicine


This is to certify that the thesis prepared by Suhas Badarinath entitled "Examination of Signals Involved in Dexamethasone Induced Apoptosis in Nb2 Lymphoma Cells" has been approved by his committee as satisfactory completion of the thesis requirement for the degree of Master of Science.


  
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*July 22, 1999*

Examination of Signals Involved in Dexamethasone  
Induced Apoptosis in Nb2 Lymphoma Cells

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

by

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## **DEDICATION**

To my family

## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Raphael J. Witorsch, for the opportunity to work in this laboratory. His constant encouragement and guidance along with his sense of humor have allowed me to strive to become a true scholar and a gentleman. I would also like to acknowledge my friends and colleagues, Angelo Guanzon, Devang Patel, and Rhodaline Rebano. Furthermore, I would like to thank the members of my thesis committee, Dr. Mohammed Kalimi and Dr. Jennifer Stewart for their efforts on my behalf. Finally, I would like to thank my family for their unyielding love and support.

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## LIST OF ABBREVIATIONS

Apaf-1	Apoptosis Activating Factor-1
ABC	Avidin/Biotinylated Peroxidase Complex
BSA	Bovine Serum Albumin
°C	Degrees Celsius
DAB	Diaminobenzidine
Dex	Dexamethasone
ddH <sub>2</sub> O	Double Distilled Water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
FADD	Fas-associating protein with death domain
FCS	Fetal Calf Serum
FM	Fischer Medium
FMM	Fischer's Maintenance Medium
GR	Glucocorticoid Receptor
GST	Glutathione-S-Transferase
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
HS	Horse Serum
ICC	Immunocytochemistry
IGF1	Insulin Like Growth Factor-1
kDa	Kilodalton
M	Molar
µg	Microgram
µl	Microliter
ml	Milliliter
mM	Millimolar
ng	Nanogram
nM	Nanomolar
PBS	Phosphate Buffered Saline
Prl	Prolactin
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
SYN	Synthetic Medium
TIFF	Tagged Image File Format
TNF	Tumor Necrosis Factor/ Nerve Growth Factor
TUNEL	Tdt-Dependent dUTP-biotin Nick End Labeling

## ABSTRACT

### EXAMINATION OF SIGNALS INVOLVED IN DEXAMETHASONE INDUCED APOPTOSIS IN NB2 LYMPHOMA CELLS

By Suhas Badarinath, B.A.

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

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Consistent with previous studies, we demonstrated that dexamethasone (Dex) caused cytolysis/apoptosis in log phase Nb2 lymphoma cells, while prolactin (Prl) inhibited this effect. The Nb2 model was used to investigate the mechanisms of apoptosis control with the aid of immunocytochemistry (ICC). We established with absorption the specificity of staining due to Fas, Fas Ligand, Bcl-2, and Bax antibodies (the specificity of anti-p53 could not be verified). Dex-induced cytolysis/apoptosis was detected in synchronized ( $G_0/G_1$ ) cells after 6 and 8 hours of Dex exposure. A novel, computerized technique was used to quantitate the proportion of cells immunostained for the signals of interest in Nb2 cells in log phase and  $G_0/G_1$  after up to 8 hours of Dex exposure. We observed Fas, Fas Ligand, Bcl-2, Bax, and p53 in high proportions (72%-86%) of log phase Nb2 cells. Neither synchrony in  $G_0/G_1$  nor exposure of synchronized

cells to Dex for up to 8 hours altered the proportion of immunostained cells. This study has raised provocative issues regarding the resistance of Nb2 cells to Fas mediated apoptosis, the phenotype of the p53 protein in Nb2 cells, and the possible interaction of various signals that modulate apoptosis.

# Chapter 1

## Introduction

Cells mainly die by the processes of apoptosis and necrosis (Kieiss and Gallaher, 1998; Ameisen, 1996; Barinaga, 1996; Lavin and Watters, 1993; Mihich and Schimke, 1994; Milas et al., 1994; Sen, 1992; Steller, 1995; Thomson, 1995; Tomei and Cope, 1994). The word apoptosis was used by Wylie in 1980 to describe a stereotyped mode of cell death (Gottlieb and Babior, 1997; Wyllie et al., 1980). Currently, 'apoptosis' encompasses a distinct set of morphological and biochemical events that characterize a specific type of cell death (MacFarlane et al., 1996; Kerr et al., 1972; Wyllie et al., 1984; Arends et al., 1990).

Early morphological changes associated with apoptosis involve compaction of chromatin and its movement in segregated masses. The cytoplasm condenses as the cell surface and nuclear outline become convoluted (Kieiss and Gallaher, 1998; Ameisen, 1996; Lavin and Watters, 1993; Mihich and Schimke, 1994; Sen, 1992). Next, the nucleus fragments while protrusions, or blebs, form on the cell surface. These surface convolutions separate from the cell, become enveloped by membrane, and are released into the extracellular fluid. The membrane bound vesicles, named apoptotic bodies, contain fragmented DNA and intact cytoplasmic organelles. An increase in cell surface

phosphatidyl serine and integrin signals digestion of apoptotic bodies by mononuclear phagocytes. Ultimately, apoptosis kills the cell while keeping its contents separate from adjacent tissues (Gottlieb and Babior, 1997). Many structural changes related to apoptosis may be visualized. Nuclear DNA is fragmented into 200 base pair segments and appears as a ladder pattern when subjected to gel electrophoresis (Gottlieb and Babior, 1997). Although this nucleosomal pattern is common, apoptosis can occur devoid of DNA laddering (Gottlieb and Babior, 1997; Schulze-Osthoff et al., 1994). Apoptotic bodies may be viewed by light microscopy (Kiess and Gallaher, 1998; Sen, 1992; Tomei and Cope, 1994), whereas other morphological changes such as the condensation of chromatin and the compaction of cytoplasm require electron microscopy (Gavrieli et al., 1992).

Biochemical and molecular changes accompany the morphological changes involved in apoptosis. A change in  $\text{Ca}^{2+}$  concentration may be necessary for nuclease cleavage of DNA (Kiess and Gallaher, 1998; Nicotera and Rossi, 1994). Indeed, a  $\text{Ca}^{2+}$  -  $\text{Mg}^{2+}$  sensitive endonuclease was found to cleave double stranded DNA between nucleosomes (Kiess and Gallaher, 1998; Galli et al., 1995). The transcription and translation of certain genes into proteins is an additional molecular event involved in apoptosis (Kiess and Gallaher, 1998; Thompson, 1995; Tomei and Cope, 1994). Certain proteins have been shown to modulate elements of the apoptotic pathway in their regulation, catalysis, and degradation (Kiess and Gallaher, 1998). In fact, inhibition of protein synthesis was seen to halt or delay apoptosis (Kiess and Gallaher, 1998).

The killing of specific cells by apoptosis is central to many biological processes

since it allows for cell renewal and histological regulation (Kiess and Gallaher, 1998). Embryogenesis, for instance, relies upon targeted cell death for proper development. In mammals, apoptosis is crucial for the development of gut mucosa and retina, and for the regression of interdigital webs (Kiess and Gallaher, 1998; Haanen and Vermes, 1996). In immune tolerance development, B and T lymphocytes recognizing self-antigen are eliminated by apoptosis (Gottlieb and Babior, 1997; Pircher et al., 1992). During aging, lowered hormone levels lead to an atrophy of hormone sensitive tissue that is mediated by apoptosis (Gottlieb and Babior, 1997; Kiplesund et al., 1988). Cells containing DNA damage or viral infection are induced to die by apoptosis, the latter of which may be caused by interaction with cytotoxic T lymphocytes (Kiess and Gallaher, 1998; Le Deist et al., 1996; Migliorati et al., 1994). Finally, autoimmune diabetes and thyroid disease may be caused by the improper induction of pancreatic and thyroid cells into apoptosis (Kiess and Gallaher, Lavin and Watters, 1993; Mihich and Schimke, 1994; Tomei and Cope, 1994).

Regulation of apoptosis may be environmental or developmental (Desai and Gruber, 1999). Environmental agents that elicit apoptosis invariably cause nonspecific cellular damage. Examples of such stimuli include chemotherapeutic agents (Gottlieb and Babior, 1997; D'Amico and McKenna, 1994), ultraviolet radiation (Gottlieb and Babior, 1997; Gillardon et al., 1994), oxidizing agents (Gottlieb and Babior, 1997; McConkey et al., 1988), and reperfusion injury (Gottlieb and Babior, 1997, 1994). Developmental regulation of cell death involves genes whose protein products govern apoptosis and do not elicit an immune reaction. Once transcribed, these proteins may

promote cell survival or cause cell death (Desai and Gruber, 1999).

Cell death may also occur by necrosis. As mentioned earlier, the morphological characteristics of apoptosis include cell shrinkage, maintenance of intact organelles, and nuclear fragmentation (Thomson, 1998). Necrosis, however, entails the dilation of cells, destruction of most organelles, perforation of the plasma membrane, and relatively minor nuclear morphological changes (Thomson, 1998; Wyllie, 1981). Necrosis also occurs in groups of cells and produces inflammation (Levin, 1998). Whereas apoptosis is affected by environmental and physiological elements (Gottlieb and Babior, 1997), necrosis is provoked by a variety of non-physiological conditions, such as hypothermia, hypoxia, toxins, autolysis, and the inhibition of glycolysis and the tricarboxylic acid cycle (Wyllie, 1981).

There are instances where the morphological events of cell death fail to fit into the dichotomy of apoptosis and necrosis. Recently, it was suggested that 'oncosis' or cytoplasmic swelling and karyolysis, be instituted to characterize cell death. In this model, necrosis embodies all cell death and may be 'oncotic necrosis' if cells swell, or 'apoptotic necrosis' if cells shrink (Levin, 1998; Majno and Joris, 1996).

The purpose of this study is to examine specific regulatory elements associated with apoptosis in the Nb2 cell line by employing a morphological approach that implements immunocytochemistry. The Nb2 cell line was shown to be sensitive to both pro and anti-apoptotic hormonal signals (Fletcher-Chiappini et al., 1993; Witorsch et al., 1993; La Voie and Witorsch, 1995). This property allowed us to study the modulation of apoptosis.



The Nb2 cell line was obtained from lymphomas generated in estrogen treated male Nb rats. The cells were derived from thymocytes at an immature stage of development (Fleming et al., 1982). Thus, they share characteristics with the normal rat thymus, such as a large nucleus and nucleolus, sparse endoplasmic reticulum and Golgi bodies, and a higher volume of log phase cells than stationary cells (Fleming et al., 1982; Hwang et al., 1974). Nb2 cells appeared to be double positive (CD4/CD8), since they possessed early antigenic determinants of helper T cells and non-helper T cells. (Fleming et al., 1982). In T cell maturation, immature thymocytes express both markers, while differentiated thymic cells contain either marker. Accordingly, the double positive Nb2 cells were likely frozen in an early stage of development.

Nb2 cells are dependent on a lactogenic hormone, such as prolactin, for mitogenesis (Noble et al., 1985). Incubation in a lactogen free medium for 24 hours synchronizes approximately 90% of cells in G<sub>0</sub>/G<sub>1</sub> (Lavoie and Witorsch, 1995). Administering Prl to synchronized cells restores log phase growth (Krumenacker et al., 1998; Richards et al., 1982). Nb2 cells were also observed to be sensitive to glucocorticoids such as Dex. In the absence of mitogen, Dex caused apoptosis of log phase Nb2 cells after 12-24 hours of exposure. In the presence of Prl, Dex was antiproliferative although apoptosis did not occur (Fletcher-Chiappini et al., 1993). Further studies indicated that Prl inhibited Dex-induced apoptosis in a specific dose dependent manner (Fletcher-Chiappini et al., 1993).

The phenomenon of Dex-induced apoptosis in Nb2 cells and its inhibition by Prl provides a model for studying the signaling involved in the regulation of programmed

cell death. In 1995, Lavoie and Witorsch detected DNA fragmentation in synchronized Nb2 cells after 4 hours of Dex treatment. Recently, the Tdt-dependent dNTP-biotin Nick End Labeling (TUNEL) assay was implemented in our laboratory as a morphological, rather than a biochemical means to detect DNA fragmentation. Guanzon (1998) detected apoptosis after 6 or 8 hours of Dex treatment in synchronized Nb2 cells using the TUNEL assay. We intended to identify the signals involved in Dex mediated apoptosis of synchronized Nb2 cells by examining any changes in the levels or distribution of pro and anti-apoptotic proteins in Dex treated cells at time points within this 8 hour time interval. The fluctuation in the level of a protein prior to apoptosis would suggest that the signal may be involved in mediating cell death. Our experimental design consisted of an immunocytochemical examination of specific signals (Fas, Fas Ligand, Bcl-2, Bax, and p53) in synchronized Nb2 cells that had been exposed to Dex for a duration of 0, 1, 2, 4, 6, and 8 hours.

The cascade of events leading to apoptosis is highly conserved and involves all of the signaling proteins under consideration (Figure 1). In mammals, a pivotal event in the apoptotic cascade is the release of cytochrome c from the mitochondria. Cytochrome c activates apoptosis activating factor-1 (Apaf-1), which subsequently activates and recruits caspase-9 (Adams and Cory, 1998). In general terms, caspases are specific proteases that either regulate the activity of other caspases or proteins, or directly cause apoptosis by cleavage of specific proteins (ie. structural and regulatory proteins). Caspase-9 and Caspase-8 are thought to be involved in the regulation of other caspases that cause cell death (Thornberry and Lazebnik, 1998). Bcl-2 and Bax effect apoptosis

by regulating the release of cytochrome c and the activation of Apaf-1 and caspase-9. Occupation of the Fas receptor causes apoptosis mainly through its interaction with Fas-associated protein with death domain (FADD) and with caspase-8 (which appears to be independent of the caspase-9 pathway). Lastly, p53 may cause apoptosis by stimulating Bax or Fas, and by repressing Bcl-2. Elements of the apoptotic cascade integrate opposing pro-apoptotic and anti-apoptotic signals to determine if cell death is required (Adams and Cory, 1998).

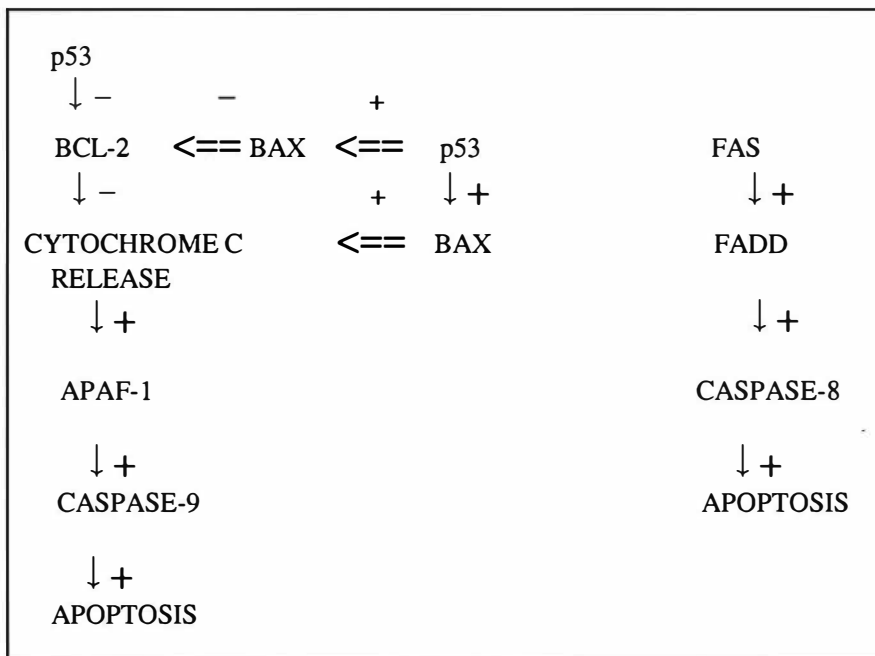


Figure 1. Apoptotic Pathway Involving Fas, Fas Ligand, Bcl-2, Bax, and p53.

Fas and Fas Ligand are involved in the regulation of apoptosis in many cell types.

Fas, or APO-1, is a member of the tumor necrosis factor/nerve growth factor receptor family (Laytragoon-Lewin, 1998; Ohm et al., 1992), and is found in the liver, kidney, heart, thymocytes, and B lymphocytes. The binding of the Fas receptor to Fas Ligand activates Fas, which induces cell death. The Fas protein is comprised of several specialized regions including extracellular, intracellular, transmembrane, and 'death' domains. The death domain consists of a conserved sequence of amino acids that plays a key role in evoking cell death. The initial event in Fas-mediated apoptosis is the association between Fas and Fas Ligand. This interaction causes the death domain of Fas to self-interact as a result of 'clustering', which enables Fas to activate FADD (Boldin et al., 1995). Subsequently, FADD activates caspase-8, which induces apoptosis (Adams and Cory, 1998; Gottlieb and Babior, 1997; Chappell and Restifo, 1998).

Fas Ligand is a 45kDa glycoprotein, and a member of the TNF family (Gottlieb and Babior, 1997). This protein is mainly expressed on activated T cells, natural killer cells, Sertoli cells of the testes, and in the eye (Chappell and Restifo, 1998; French et al., 1996; Griffith et al., 1995). In certain instances, metalloproteases cleave Fas Ligand into a 26 kDa soluble form of the protein (Chappell and Restifo, 1998; Kayagaki et al., 1995; Gottlieb and Babior, 1997).

The binding of Fas Ligand to Fas stimulates apoptosis in various cell types. In tolerance development of T cells, the adherence of an immature T cell receptor with self-antigen results in increased synthesis of both Fas and Fas ligand, inducing cell death (Ogasawara et al., 1995; Gottlieb and Babior, 1997). Indeed, when either Fas or Fas

Ligand is inactivated by mutation in mice, immature T cells are retained and autoimmune diseases may develop (Ogasawara et al., 1995). In addition, natural killer cells and cytotoxic T lymphocytes display the Fas Ligand and kill virally infected cells expressing Fas. Ironically, it is this upregulation of Fas by HIV infected CD4 cells that causes many lymphocytes to die, hastening the progression of AIDS (Gottlieb and Babior, 1997; Katsikis et al., 1995). Fas and Fas Ligand also evoke apoptosis as downstream effectors of other signals like c-Myc and p53 (Chappell and Restifo, 1998; French et al., 1996; Green, 1997; Hueber et al., 1997). For instance, cytotoxic drugs and irradiation activate p53, which in turn stimulates Fas mediated apoptosis (Chappell and Restifo, 1998; Friesen et al., 1996; Reap et al., 1997; Muller et al., 1997).

Bcl-2 and Bax, homologous members of the Bcl-2 family, also play an interactive role in mediating apoptosis. The Bcl-2 family is comprised of several proteins that function either as inducers, or as inhibitors of apoptosis (Gottlieb and Babior, 1997). The function of each member is dependent upon the conserved amino acid sequence, or domain, that they possess (Adams and Cory, 1998). Of the pro-apoptotic Bcl-2 family members, the presence of the Bcl-2 homology domain 3 is crucial for evoking cell death (Adams and Cory, 1998; Conradt et al., 1998; Chittenden et al., 1995). Anti-apoptotic proteins of the Bcl-2 family require the Bcl-2 homology domain 1 and the Bcl-2 homology domain 2 (Adams and Cory, 1998). The Bcl-2 protein proper is a 26 kDa protein associated with mitochondrial membrane, nuclear membrane, and endoplasmic reticulum (Gottlieb, et al., 1997). Bcl-2 protects against apoptosis, chiefly in lymphoid derived cells (Gottlieb and Babior, 1997). For example, an overexpression of Bcl-2 in

transgenic mice resulted in increased lymphoid follicles (Gottlieb and Babior, 1997; Sentman et al., 1991).

Bax is a 21 kDa protein that causes apoptosis (Gottlieb and Babior, 1997). The Bax protein is widely distributed throughout the body and exists in higher concentrations in cells with rapid turnover rates such as the colonic epithelium (Gottlieb and Babior, 1997). Upon self-dimerization, Bax induces apoptosis (Gottlieb and Babior, 1997) by releasing cytochrome c from the mitochondria (Chappell and Restifo, 1998). Bcl-2, however, separates the Bax homodimer and forms a Bcl-2:Bax dimer (Gottlieb and Babior, 1997). The Bcl-2 protein is thought to prevent apoptosis by inhibiting caspases by means of blocking cytochrome c release or binding to Apaf-1 (Lincz, 1998). The relative concentrations of Bax and Bcl-2 determine whether apoptosis is suppressed by Bcl-2 or promoted by the Bax homodimer.

In response to DNA damage, a cell may arrest growth, evoke cell death, or repair altered DNA (Evan and Littlewood, 1998). If a cell sustains significant DNA damage, it is often prudent to terminate the cell rather than risk its potential for neoplastic growth. The p53 protein is responsible for causing apoptosis or growth arrest in cells containing DNA damage. Cells with altered p53 genes have a diminished capacity to curtail cell growth, and they often proliferate uncontrollably as a neoplastic growth (Jensen et al., 1997). Hence, the loss of p53 function is associated with a large number of cancers (Evan and Littlewood, 1998).

In order for p53 to regulate cell proliferation, it must first be separated from its associated repressor protein, Mdm2. This separation is achieved through the

phosphorylation of p53 or Mdm-2 by a group of protein kinases (Evan and Littlewood, 1998). Once separated from Mdm2, p53 is active and capable of inducing apoptosis or growth arrest (Evan and Littlewood, 1998). Depending on the environment, cell type, and other signals, either growth arrest or apoptosis is favored (Evan and Littlewood, 1998). Oftentimes, as in the case of glucocorticoid mediated apoptosis, arrest in the G1 phase of the cell cycle precedes apoptosis (King and Cidlowksi, 1998).

It is thought that p53 causes apoptosis by repressing the anti-apoptotic protein Bcl-2, stimulating pro-apoptotic signals such as Bax and Fas (King and Cidlowksi, 1998; Miyashita et al., 1994; Owen-Schaub et al., 1995), or by interacting with IGF1 (Evan and Littlewood, 1998; Thornberry and Lazebnik, 1998). Alternatively, the process of growth arrest involves p53-induced transcription of the cyclin dependent kinase inhibitor p21, which arrests cells in the G1 phase (King and Cidlowksi, 1998; Dulic et al., 1994; El-Deiry et al., 1993).

In summary, the primary objective of this study has been to elucidate the roles of specific signaling proteins (Fas, Fas Ligand, Bcl-2, Bax, and p53) in apoptosis using a morphophysiological approach. Initially, we attempted to establish the existence and specificity of these cellular signals by immunostaining and immunoabsorption. Then, we attempted to quantitate these signals over an 8 hour period following Dex exposure. In doing so, we hoped to appraise the feasibility of a morphologically based approach for examining the physiological control of apoptosis.

## Chapter 2

### Materials And Methods

#### 2.1 Hormones, Antibodies, and Antigens

Dexamethasone (1,4 pregnadiene-9fluor-16 $\alpha$ -methyl-11 $\beta$ ,17 $\alpha$ ,21-triol-3,20-dione) was acquired from Sigma Chemical Co., St. Louis, MO. The Antibodies for Fas, Fas ligand, Bcl-2, Bax, and p53 were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Glutathione agarose, the p53 fusion protein, and antigens corresponding to the antibodies for Fas, Fas ligand, Bcl-2, Bax, and p53 were also acquired from Santa Cruz Biotechnology Incorporated. Ovine prolactin (oPrl s-15) was donated by the National Hormone and Pituitary Program.

Primary antibodies to Fas, Fas ligand, Bcl-2, Bax, and p53 (N-19) were affinity-purified polyclonal antibodies raised against a specific region of a protein. Anti-p53 (FL-393), however, was raised against the entire p53 antigen. According to specifications provided by the supplier, the antibodies to Fas Ligand, Bcl-2, Bax, and p53 (FL-393) reacted to antigens of rat, mouse, and human origin, whereas anti-Fas reacted to antigens of mouse and rat origin, and anti-p53 (N-19) reacted to p53 of human origin. Antibodies for the above proteins were diluted in PBS-.1%BSA to concentrations that exhibited optimal staining and absorption. In particular, anti-Fas, anti-Fas ligand, and anti-p53



were diluted to 2µg/ml, while anti-bax and anti-bcl-2 were diluted to 1µg/ml. Anti-Fas (M-20) is directed against a peptide corresponding to amino acids 308-327 of the carboxy terminus of the Fas precursor of mouse origin. Anti-Fas ligand (N-20) was raised against a peptide coinciding with amino acids 2-19 at the amino terminus of Fas ligand of rat origin. Anti-p53 (FL-393) is directed against a fusion protein corresponding to amino acids 1-393 of p53 protein of human origin (the precise location of the immunoreactive site is not specified). Anti-p53 (N-19) was raised against a protein coinciding with amino acids 2-20 of the amino terminus of p53 protein of human origin. Anti-Bcl-2 (N-19) is directed against a peptide corresponding to amino acids 4-21 at the amino terminus of Bcl-2 of human origin. Finally, anti Bax (I-19) binds to a protein coinciding with amino acids 80-98 at the carboxy terminus of Bax of human origin. (Santa Cruz Biotechnology Inc., 1997)

## **2.2 Maintenance of Nb2 Lymphoma Cells**

The cultured, prolactin dependent Nb2 cells were obtained from Dr. Robert Adler of the McGuire Veteran's Hospital. Dr. Peter Gout of the Department of Cancer Endocrinology of the British Columbia Cancer Agency was the initial supplier of the cells (Nb2 clone U-17). The Nb2 cells were resurrected from storage at -70<sup>0</sup>C and sustained in Fischer's Maintenance Media (FMM), consisting of Fischer's Media supplemented with 50 units/ml Penicillin, 50µg/ml Streptomycin, .075% NaHCO<sub>3</sub>, 10% Horse Serum, 10% Fetal Calf Serum, and 0.1mM β-Mercaptoethanol. Culture flasks

used in splitting and reculturing cells were Falcon brand 50 ml. polystyrene flasks with 0.2um vented blue plug seal caps.  $\beta$ -mercaptoethanol was obtained from the Sigma Chemical Company, while the remaining reagents were purchased from Gibco Life Technologies, Grand Island, NY. Cultures were incubated in a water-saturated atmosphere of 5% CO<sub>2</sub> and 95% room air at 37<sup>0</sup> C. The cell count was determined by Trypan Blue exclusion using a hemacytometer, and cells were recultured twice a week at a concentration of  $0.5 \times 10^5$  -  $1.0 \times 10^5$  cells/ml. Nb2 cells were periodically frozen (in FMM + 0.8% DMSO) when the cell viability was at least 90% and the concentration of cells reached  $1.0 \times 10^6$  cells/ml.

### **2.3 Dexamethasone Cytolytic Assay and Dex-Prl Coincubation Assay**

Assays were conducted to determine the cytolytic response of Nb2 cells to Dex treatment after 24 hours (chronic) and at discrete time points (acute). Furthermore, the effects of Prl on Nb2 Cells was examined by the Dex-Prl coincubation assay.

For the acute Dex cytolytic assay, cells were first synchronized in G<sub>0</sub>/G<sub>1</sub>. Cell synchronization was achieved in the following manner: when cells reached a concentration of approximately  $0.5$ - $0.75 \times 10^6$  cells/ml in FMM, they were centrifuged twice at 1000 rpm for 5 minutes at 8-10<sup>0</sup> C and washed twice in chemically defined, serum-free synthetic medium (SYN), consisting of .1mM  $\beta$ -mercaptoethanol, 50 units/ml Penicillin, 50  $\mu$ g/ml Streptomycin 0.15% (wt/vol), Bovine serum albumin, 4 $\mu$ g/ml Linoleic acid, 1mM Sodium pyruvate, 12  $\mu$ g/ml Transferrin, 15ng/ml Selenium, 1x

vitamins (diluted from a commercial stock of 100x), 0.33x amino acids (diluted from a commercial stock of 100x), 0.5mM CaCl<sub>2</sub>, and 0.15mM Hepes (dissolved in Fischer's medium). The cell concentration was then adjusted to 500,000 cells/ml in SYN, and cells were incubated at 37<sup>0</sup>C for 24 hours. After this 24 hour incubation in SYN, approximately 90% of cells enter into G<sub>0</sub>/G<sub>1</sub> (Lavoie and Witorsch, 1995). Synchronized cells were washed twice in Fischer's Medium and cells were counted with a hemacytometer. From the washed cells, cell concentration was adjusted to 2.0 x 10<sup>6</sup> cells/ml in SYN and plated at 3ml/well in Costar brand 6-well plates. Then, 1.25 µl/ml of DMSO and 80nM Dex in DMSO were added to the cell suspensions. Cells were incubated with Dex or vehicle for 0, 1, 2, 4, 6, and 8 hours. Following each time point, cells were harvested and percent viability per well was determined with a hemacytometer by counting living and dead cells (those cells that do not exclude Trypan Blue) in triplicate. Previous studies have indicated that three counts are required to achieve reliable cell quantitation (Fletcher-Chiappini et al., 1993). After each time point, cells were also fixed to slides for immunocytochemical studies.

In the chronic cytolytic assay, cells were tested for their responsiveness to Dex ± Prl following a 24 hour incubation period using a 4 well design. Log phase cells in FMM were centrifuged twice at 1000 rpm for 5 minutes at 8-10<sup>0</sup> C and washed twice in SYN. The cells were then reconstituted in SYN and counted by Trypan Blue exclusion using a hemacytometer. The concentration of cells was then adjusted to approximately 0.5 x 10<sup>6</sup> cells/ml (at a volume of 1.5ml in SYN) containing one of the following 4 treatments: Control (0.125% DMSO), Dex (100nM Dex in 0.125% DMSO), Dex + Prl

(100nM Dex in 0.125% DMSO, 1ng/ml oPrI S-15), and PrI (1ng/ml oPrI S-15, 0.125% DMSO). All incubations occurred at 37<sup>0</sup> C in a 5% CO<sub>2</sub> incubator for 24 hours, at which time percent viability per well was determined by Trypan Blue exclusion as described above.

## **2.4 Cell Fixation**

Cell fixation methods remained constant for synchronized, log phase, treated, and untreated cells. A total of 3 x10<sup>6</sup> cells were obtained from a cell suspension by determining the cell concentration and acquiring the necessary volume. These cells were centrifuged at 1000 rpm for 5 minutes in an Eppendorf microcentrifuge at room temperature. Following aspiration of supernatant, the cells were resuspended in 3.7% formaldehyde in .01M PBS (pH 7.1) for ten minutes at room temperature. The stock formaldehyde (37%) was diluted in .01M PBS to a concentration of 3.7%, and was allowed to reach room temperature prior to cell fixation. After fixation, cells were recentrifuged at 1000 rpm for 5 minutes, the supernatant was aspirated, and the remaining pellet was resuspended in 750 µl .01M PBS (pH 7.0). Approximately 12 µl of fixed cells were added to an etched circle (5mm in diameter) on Fischer Super-Frost Plus slides, and allowed to dry at room temperature.

## **2.5 Immunocytochemistry (ICC)**

ICC was used to localize antigens within cells with primary antibodies directed against signaling proteins. In this process, samples on slides were first hydrated in .01M PBS for 5 minutes. Fixed cells were hydrated directly by immersion of the slide in PBS. Paraffin sections, however, were deparaffinized by immersion in xylene, a series of alcohols (100% Ethanol, 95% Ethanol, 70% Ethanol, 50% Ethanol), and finally ddH<sub>2</sub>O. Once deparaffinized, the section was hydrated as above. Pituitary sections embedded in paraffin were used as positive controls in ICC experiments. The antibody added to control sections was rabbit anti-canine Prl at concentrations of 1:1600, 1:3200, 1:6400, and 1:12,800.

Following hydration of samples, specific reagents were added sequentially as drops, and then incubated in an airtight, humidified chamber at room temperature for specified time periods. In between the addition of each reagent, slides were flooded with 10ml of 0.01M PBS. The first reagent added was 0.2% Triton + PBS for 30 minutes, which permeabilized the section. Blocking solution was then added for 20 minutes. Subsequently, primary antibody was added and allowed to incubate for 3 hours (as mentioned earlier, dilutions of antibodies ranged from 1-2 µg/ml, and were based on the staining observed in pilot experiments). Then, biotinylated secondary antibody (12.5% solution) was added for 30 minutes. Next, ABC solution (20%) was added to the sample for 30 minutes. The slides were then inserted into a coplin jar filled with Aldrich brand Diaminobenzidine (DAB) in solution (12.5 mg DAB/50ml of .05M tris HCl at pH 7.6) plus 50µl of pharmaceutical grade 3% H<sub>2</sub>O<sub>2</sub> for a duration of 10 minutes. Following this incubation, the slides were immersed in tap water and dehydrated by insertion into

ddH<sub>2</sub>O, a series of alcohols (50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol), and lastly, xylene. Finally, coverslips were applied to slides using Pro-Tex mounting medium.

The Vectastain Universal Elite ABC kit was utilized to localize antibody bound to antigen (this kit recognized antibodies generated in rabbits and mice). In this procedure, the chromagenic substrate (DAB) binds to an avidin/biotinylated peroxidase complex (ABC), which itself is attached to a biotinylated antibody:primary antibody:protein complex. (Vector Laboratories, Inc., 1996). Vectastain solutions (blocking serum, biotinylated antibody, and ABC) were prepared as recommended in the kit instructions.

## **2.6 Antibody Absorption**

The specificity of antibody-induced staining in cells was examined through immunoabsorption. This involved creating mixtures of antibody with the antigen that was used for its generation. In most cases, the antigen used for absorption was a peptide of about 20 amino acids corresponding either to the N-terminus or C-terminus of the molecule. The rationale for immunoabsorption is that if the antigen binds to the immunoreactive site on the antibody, or epitope, it would prevent binding of the antibody to the antigen within the fixed specimen, hence precluding staining. In the immunoabsorption of Fas, Fas ligand, Bcl-2, and Bax, antibodies were incubated with their respective antigens at a ratio of 10:1 (peptide to antibody), which was the ratio recommended by the supplier, for 24 hours at 4<sup>0</sup> C. To achieve a 10:1 ratio of peptide to

antibody, a volume of 10  $\mu$ l of peptide (200  $\mu$ g/ml) was added to 100  $\mu$ l of working stock antibody (2  $\mu$ g/ml). The control consisted of 10  $\mu$ l of PBS -.02% BSA added to 100  $\mu$ l working stock antibody (2  $\mu$ g/ml) incubated as above. Absorbed and control solutions were applied to slides of fixed log phase cells. The absence of staining after exposure to the antibody/peptide solution signified absorption of that antibody by peptide, and hence verified specificity of immunostaining (Witorsch, 1980). As presented in the results, we were able to conclusively demonstrate immunoabsorption for anti-Fas, anti-Fas Ligand, anti-Bcl-2, and anti-Bax.

While the absorption of anti-Fas, anti-Fas Ligand, anti-Bcl2, and anti-Bax were straightforward, establishing the specificity of p53 immunostaining was more complex. To our knowledge, a rabbit antibody directed against the N or C terminus of p53 was not available with its corresponding antigen. However, goat antibody directed against the N terminus (amino acids 2-20) or the C terminus (373-391) of p53 was available with its respective peptide from Santa Cruz Biotechnology. Unfortunately, we were unable to obtain successful immunostaining using an antibody generated in a goat (N-19) while employing a Santa Cruz ABC kit capable of detecting goat antibodies (the Vector Universal ABC kit detects rabbit and mouse antibodies). A rabbit antibody directed against the entire human p53 molecule (FL-393) was available from Santa Cruz, which yielded excellent staining (intense staining with low background) using the Vector Universal ABC kit. The mixture of this antibody with peptides directed against the C terminus (amino acids 2-20) and N terminus (amino acids 373-391) together failed to diminish staining of anti p53 (FL-393). Other attempts at absorption involved using a

glutathione-S-transferase (GST) fusion protein containing the entire human p53 molecule (amino acids 1-393) as the absorption agent. This protein, which was the original antigen used to generate FL-393, consisted of p53 (53kDa) and GST (27 kDa). When the weight of the GST is factored into the calculation, the ratio of antigen to antibody is 15:1 in order to obtain the appropriate ratio of antigen to antibody of 10:1. The mixture of anti-p53 (FL-393) with the fusion protein (I-393) failed to establish immunospecificity of p53 immunostaining, even when the N-terminus (N-19) and C-terminus (R-19) were also added. Finally, attempts at removing the anti-p53/p53 GST complex from solution by the addition of glutathione agarose (Santa Cruz) followed by centrifugation also failed to satisfactorily establish immunospecificity.

## **2.7 Photomicrography**

Photomicrography of cells of ICC experiments was achieved by using a Nikon Optiphot photomicrography system and Kodak Ektachrome ISO 100 slide film. To attain an accurate representation of cells stained, four fields of cells in each etched circle were photographed at approximately 2, 4, 8, and 10 o'clock relative to the circle. Objective magnification was at either 20X or 40X for best visualization of immunostaining. Less intensely stained cells and sparse cell fields called for the higher magnification. Photographs were taken toward the center of the circle since central cells demonstrated more representative staining than peripheral cells, and were less subject to high background staining, or 'edge artifact'. Fields containing significant cell clumping, debris, or other artifacts were avoided.



## **2.8 Data Processing**

Photomicrography as described above yielded approximately 100 cells per field. Processing of these cell fields was accomplished by slide scanning, cell quantitation, and statistical analysis. The developed Ektachrome slides were first scanned into TIFF files by one of three methods: Sprint Scan, Nikon SF-200, or Artec Scanrom 4E. Cell quantitation was primarily achieved by use of Scion Image software (Scion Corp, Frederick, Maryland), which was downloaded from the Internet at [www.scioncorp.com](http://www.scioncorp.com). This software allowed for computerized counting of total cells and stained cells in digital images. Each scanned image was opened in greyscale and the total number of cells was determined by altering the threshold value of the image so that only the boundaries of cells were visible. By choosing the "Analyze Particles" function, cellular outlines were counted. To determine the number of stained cells, the threshold of each image was lowered to the point where cellular outlines disappeared and stained inclusions remained. In this case, the "Analyze Particles" function counted only the visible inclusions of stained cells. Some manual data processing was required due to incompatibilities of certain images with Scion Image software. In this case, the number of total cells and stained cells were counted visually from an image opened on Corel Photo Paint-8. Additionally, for the p53 ICC experiments, nuclear and cytoplasmic localization was examined visually.

ICC experiments on the proteins of interest were performed in triplicate. Data from these three experiments were used for statistical analysis using Sigma Stat 2.0

software. First, means and standard errors were calculated. Then, Kruskal-Wallis one way ANOVA on ranks compared differences between treatments over the 8 hour time period. Cytolytic data were compared by the Duncan's multiple range tests.

## Chapter 3

### Results

#### **3.1 Effect of Dex and Prl on cell viability in non-synchronized Nb2 lymphoma cells.**

Figure 2 depicts a typical cell viability response of log phase Nb2 cells after 24 hours of exposure to Dex ± Prl. Trypan Blue exclusion was employed to quantitate cell viability. Dex causes a 7-fold increase in the percentage of dead cells compared to DMSO treatment, bringing the proportion of dead cells to about 50 percent. Coincubation with Prl protects cells against Dex induced cytotoxicity.

#### **3.2 Effects of Dex on viability of synchronized Nb2 lymphoma cells.**

Table 1 shows the percentages of dead cells after Dex treatment for 0, 1, 2, 4, 6, and 8 hours in synchronized ( $G_0/G_1$ ) Nb2 cells. A significant increase in dead cells was observed after 6 and 8 hours of Dex exposure. Cytolytic data also suggested the possibility of spontaneous cell death since the percentage of dead cells after 8 hours of DMSO exposure (14.7%) was significantly different from the proportions of dead cells at -24 hours (2% dead) and at 0 hours (4% dead). The percentages of apoptotic cells were

measured in the same Nb2 cells by the TUNEL assay (Guanzon, 1998). There is a significant increase in the proportion of apoptotic cells after 8 hours of Dex treatment.

### **3.3 Immunocytochemistry and antibody absorption**

Figures 3-7 illustrate immunocytochemical staining for Fas, Fas-ligand, Bcl-2, Bax, and p53. All of the antibodies mentioned produced characteristic staining in log phase Nb2 lymphoma cells (Fig. 3A-6A, 7). In particular, Fas appeared to be localized to the cell surface and periphery of most cells (Figure 3A). Fas Ligand staining was most evident in the periphery of cells, although some cells appeared centrally stained (Figure 4A). Staining for Bcl-2 was most apparent toward the periphery of cells, while some cells were centrally stained (Figure 5A). Bax staining was characterized by both peripherally and centrally stained cells (Figure 6A). Finally, p53 exhibited predominantly peripheral staining (Figure 7). The immunospecificity of Fas, Fas Ligand, Bcl-2, and Bax staining was verified by their disappearance when the antibody was absorbed by admixture with its corresponding peptide antigen (3B-6B). As discussed in the methods section, the specificity of anti-p53 immunostaining by immunoabsorption could not be verified.

### **3.4 Examination of antibody staining in response to DMSO and DEX treatments of various time periods.**

Tables 2-6 display the percentages of cells stained for Fas, Fas Ligand, Bcl-2, Bax, and p53 after DMSO and DEX treatment for 0, 1, 2, 4, 6, and 8 hours. Since p53 has been implicated in nuclear translocation, nuclear localization was also examined for this protein. For each of the antibodies under consideration, the percentages of log phase cells that were stained ranged from 72% to 86%. Among these antibodies, there were no statistically significant differences in the proportions of cells stained between log phase cells and synchronized cells. In addition, we failed to detect any statistically significant changes in the percentages of stained cells between Dex and DMSO treatments over 8 hours. No obvious redistribution in staining (periphery to central) was evident for p53.

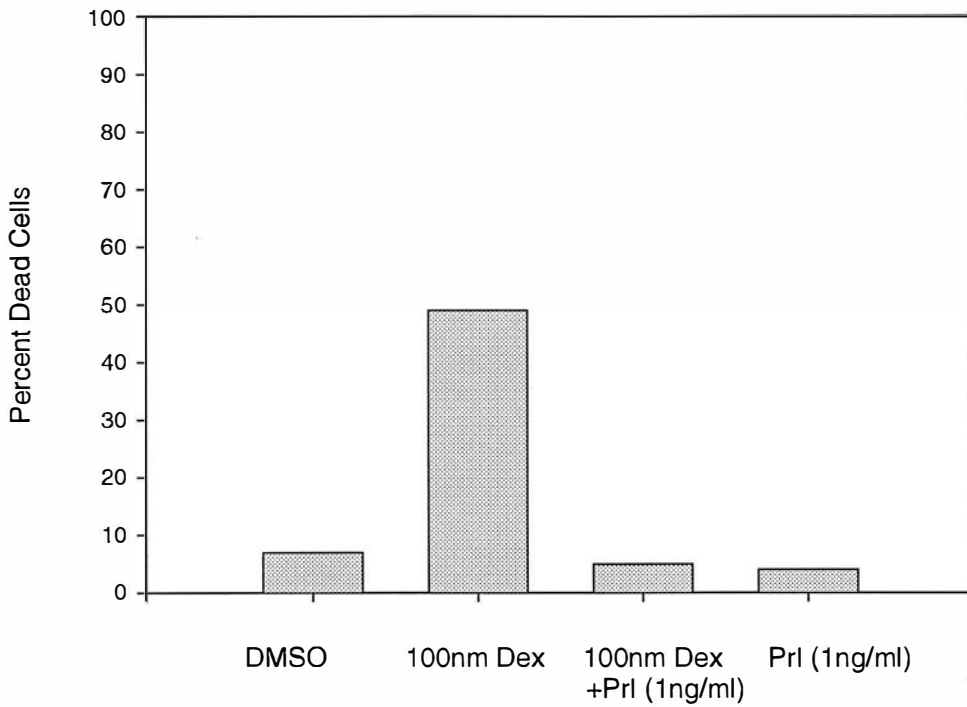


Fig 2. Cell viability of non-synchronized Nb2 lymphoma cells following 24 hour Dex  $\pm$  Prl co-incubation.

Table 1. Response of Synchronized Nb2 Lymphoma Cells to DMSO and DEX exposure after 0, 1, 2, 4, 6, and 8 Hour Time Periods.

Time (Hours)	Treatment	Percent Dead Cells as Measured by Trypan Blue Exclusion	Percent Apoptotic Cells as Measured by TUNEL Assay <sup>d</sup>
-24	Log phase	2.0±1.0	5.7±1.5
0	Synchronized	4.0±0.6	11.0±3.2
1	DMSO	8.2±1.6	10.7±3.2
	DEX	9.9±1.6	12.0±4.5
2	DMSO	7.7±2.3	6.0±1.7
	DEX	10.7±3.5	8.0±1.5
4	DMSO	10.7±0.3	9.3±4.1
	DEX	10.2±0.9	11.3±3.5
6	DMSO	10.0±0.6	10.3±1.2
	DEX	17.7±0.9 <sup>b</sup>	15.0±1.2
8	DMSO	14.7±1.5 <sup>c</sup>	10.0±2.3
	DEX	21.0±2.8 <sup>a</sup>	20.0±2.1 <sup>a</sup>

Values are Mean ± SEM; n=3

<sup>a</sup> Difference is significant at  $p \leq 0.05$  vs. 0 hour, all DMSO controls and 1, 2, 4, and 6 hour Dex treatments.

<sup>b</sup> Difference is significant at  $p \leq 0.05$  vs. 0 hour, 1, 2, 4, and 6 hour DMSO controls and 1, 2, and 4 hour Dex treatments.

<sup>c</sup> Difference is significant at  $p \leq 0.05$  vs. 0 hour, -24 hour (log phase), all DMSO controls, and 1, 2, 4, and 6 hour Dex treatments.

<sup>d</sup> Data obtained by Guanzon (1998).

Table 2. Proportion of Cells Stained for Anti-Fas in ICC Experiments in Nb2 Lymphoma Cells after DMSO and Dex Treatment of Various Time Periods.

Time (Hours)	Treatment	Percent Cells Stained for Anti-Fas (Mean $\pm$ SEM, n=3)
-24	Log Phase	71.7 $\pm$ 5.7
0	Synchronized	70.0 $\pm$ 5.0
1	DMSO	71.3 $\pm$ 6.7
	Dex	82.0 $\pm$ 3.2
2	DMSO	75.3 $\pm$ 2.0
	Dex	80.3 $\pm$ 5.0
4	DMSO	76.0 $\pm$ 4.2
	Dex	76.0 $\pm$ 4.1
6	DMSO	72.3 $\pm$ 2.9
	Dex	77.7 $\pm$ 4.3
8	DMSO	70.5 $\pm$ 1.5
	Dex	78.3 $\pm$ 3.8



Table 3. Proportion of Cells Stained for Anti-Fas Ligand in ICC Experiments in Nb2 Lymphoma Cells after DMSO and Dex Treatment of Various Time Periods.

Time (Hours)	Treatment	Percent Cells Stained for Anti-Fas Ligand (Mean $\pm$ SEM)
-24	Log Phase	79.0 $\pm$ 7.6
0	Synchronized	80.0 $\pm$ 2.9
1	DMSO	84.3 $\pm$ 0.9
	Dex	88.0 $\pm$ 3.8
2	DMSO	84.7 $\pm$ 1.5
	Dex	84.0 $\pm$ 2.3
4	DMSO	81.0 $\pm$ 3.2
	Dex	85.3 $\pm$ 1.9
6	DMSO	82.7 $\pm$ 2.9
	Dex	85.3 $\pm$ 3.3
8	DMSO	75.7 $\pm$ 1.9
	Dex	83.0 $\pm$ 2.7

Table 4. Proportion of Cells Stained for Anti-Bcl-2 in ICC Experiments in Nb2 Lymphoma Cells after DMSO and Dex Treatment of Various Time Periods.

Time (Hours)	Treatment	Percent Cells Stained for Anti-Bcl-2 (Mean $\pm$ SEM, n=3)
-24	Log Phase	76.3 $\pm$ 3.0
0	Synchronized	74.7 $\pm$ 2.2
1	DMSO	76.0 $\pm$ 3.2
	Dex	80.0 $\pm$ 1.0
2	DMSO	70.7 $\pm$ 5.4
	Dex	78.7 $\pm$ 5.0
4	DMSO	72.3 $\pm$ 1.5
	Dex	73.7 $\pm$ 1.8
6	DMSO	55.7 $\pm$ 10.9
	Dex	64.7 $\pm$ 15.4
8	DMSO	56.0 $\pm$ 15.3
	Dex	53.7 $\pm$ 13.1

Table 5. Proportion of Cells Stained for Anti-Bax in ICC Experiments in Nb2 Lymphoma Cells after DMSO and Dex Treatment of Various Time Periods.

Time (Hours)	Treatment	Percent Cells Stained for Anti-Bax (Mean $\pm$ SEM, n=3)
-24	Log Phase	86.3 $\pm$ 3.0
0	Synchronized	73.7 $\pm$ 6.6
1	DMSO	80.3 $\pm$ 2.3
	Dex	72.3 $\pm$ 10.0
2	DMSO	84.0 $\pm$ 1.0
	Dex	78.0 $\pm$ 6.5
4	DMSO	85.3 $\pm$ 1.8
	Dex	82.7 $\pm$ 3.5
6	DMSO	86.3 $\pm$ 1.3
	Dex	82.3 $\pm$ 2.3
8	DMSO	81.7 $\pm$ 2.9
	Dex	80.7 $\pm$ .9

Table 6. Proportion of Cells Stained for Anti-p53 in ICC Experiments in Nb2 Lymphoma Cells after DMSO and Dex Treatment of Various Time Periods.

Time (Hours)	Treatment	Percent Cells Stained for Anti-p53 (Mean $\pm$ SEM, n=3)
-24	Log Phase	73.3 $\pm$ 3.7
0	Synchronized	79.3 $\pm$ 1.3
1	DMSO	79.7 $\pm$ 2.9
	Dex	82.3 $\pm$ 1.5
2	DMSO	77.0 $\pm$ 7.1
	Dex	81.7 $\pm$ 3.5
4	DMSO	79.0 $\pm$ 5.0
	Dex	79.0 $\pm$ 2.5
6	DMSO	81.7 $\pm$ 2.0
	Dex	79.7 $\pm$ 2.9
8	DMSO	71.0 $\pm$ 4.4
	Dex	70.0 $\pm$ 5.5

Figure 3. A illustrates immunocytochemical staining for Fas in log phase Nb2 lymphoma cells. Antibody was at a concentration of  $2\mu\text{g/ml}$ . B reveals the disappearance of staining following absorption of antibody with its antigen. Antigen concentration was at  $20\mu\text{g/ml}$ . Magnification  $\times 354$ .

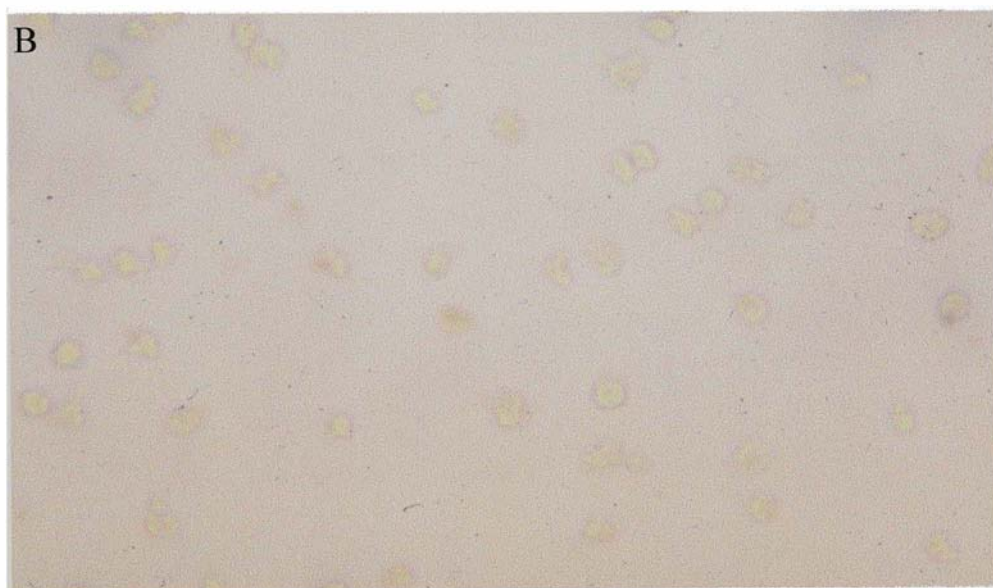
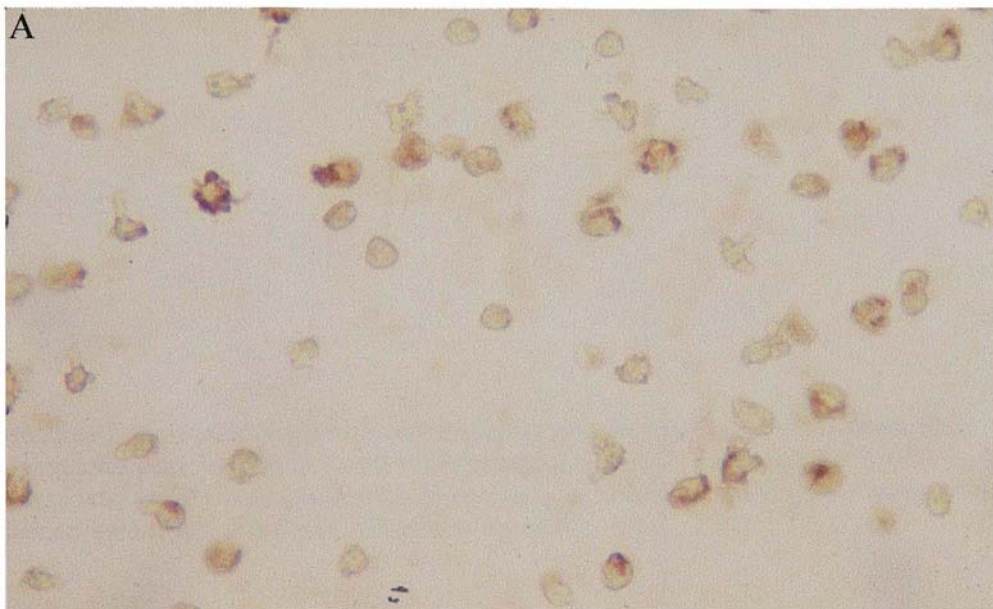


Figure 4. A illustrates immunocytochemical staining for Fas Ligand in log phase Nb2 lymphoma cells. Antibody was at a concentration of 2 $\mu$ g/ml. B reveals the disappearance of staining following absorption of antibody with its antigen. Antigen concentration was at 20 $\mu$ g/ml. Magnification x354.

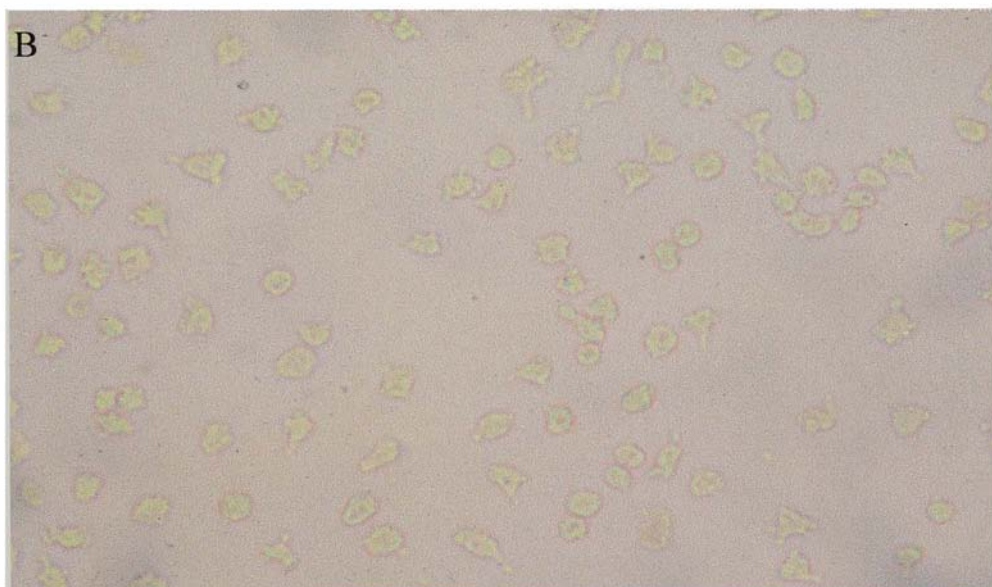
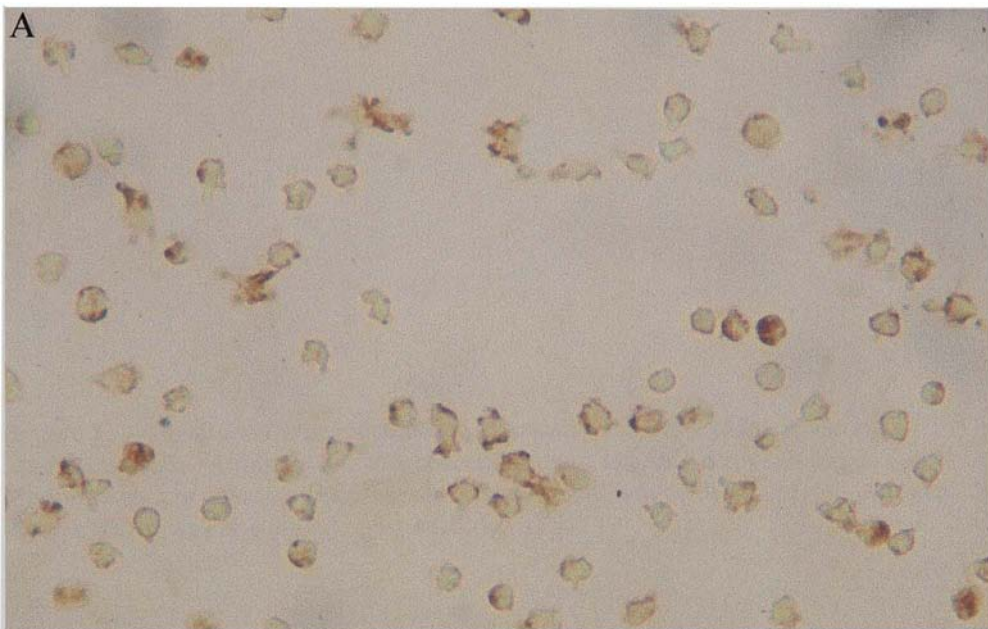




Figure 5. A illustrates immunocytochemical staining for Bcl-2 in log phase Nb2 lymphoma cells. Antibody was at a concentration of 1 $\mu$ g/ml. B reveals the disappearance of staining following absorption of antibody with its antigen. Antigen concentration was at 10 $\mu$ g/ml. Magnification x354.

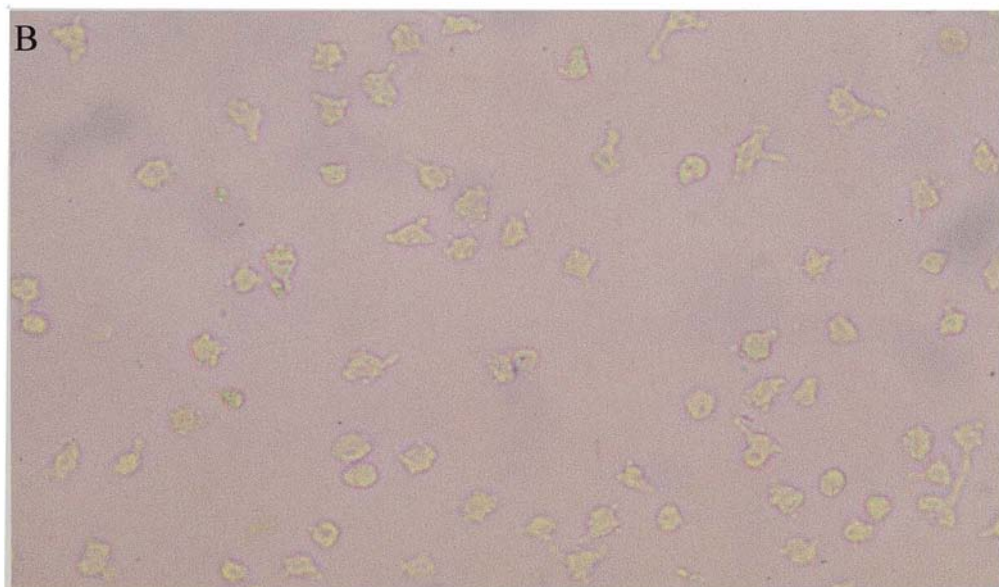
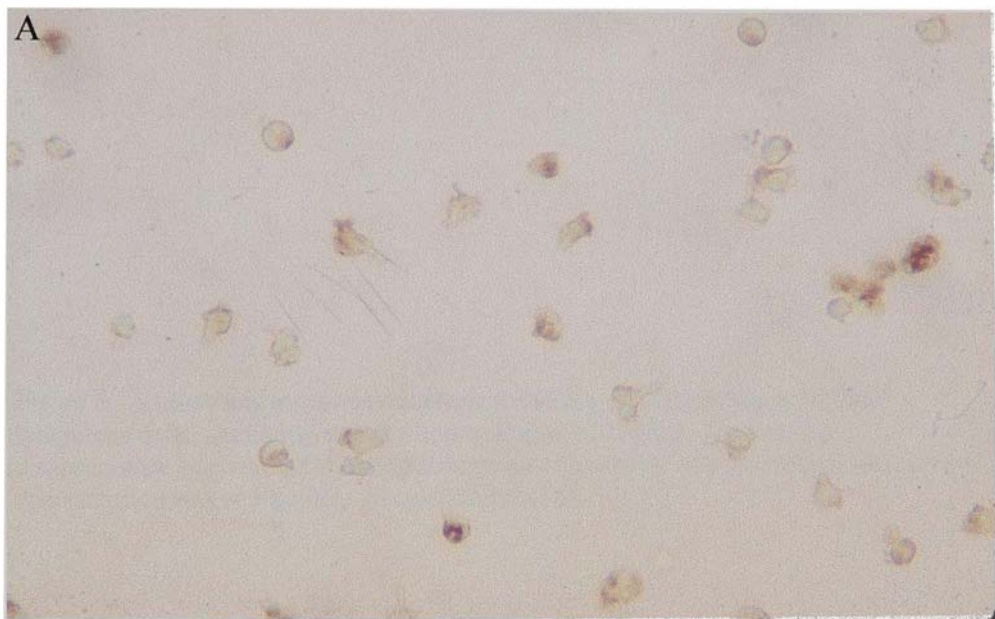


Figure 6. A illustrates immunocytochemical staining for Bax in log phase Nb2 lymphoma cells. Antibody was at a concentration of 1  $\mu\text{g/ml}$ . B reveals the disappearance of staining following absorption of antibody with its antigen. Antigen concentration was at 10  $\mu\text{g/ml}$ . Magnification x354.

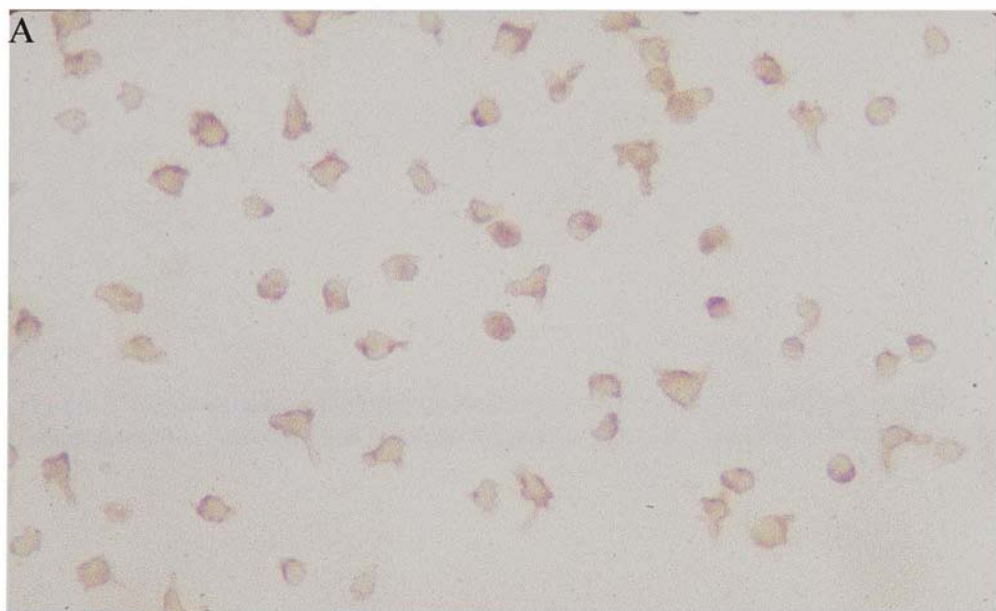
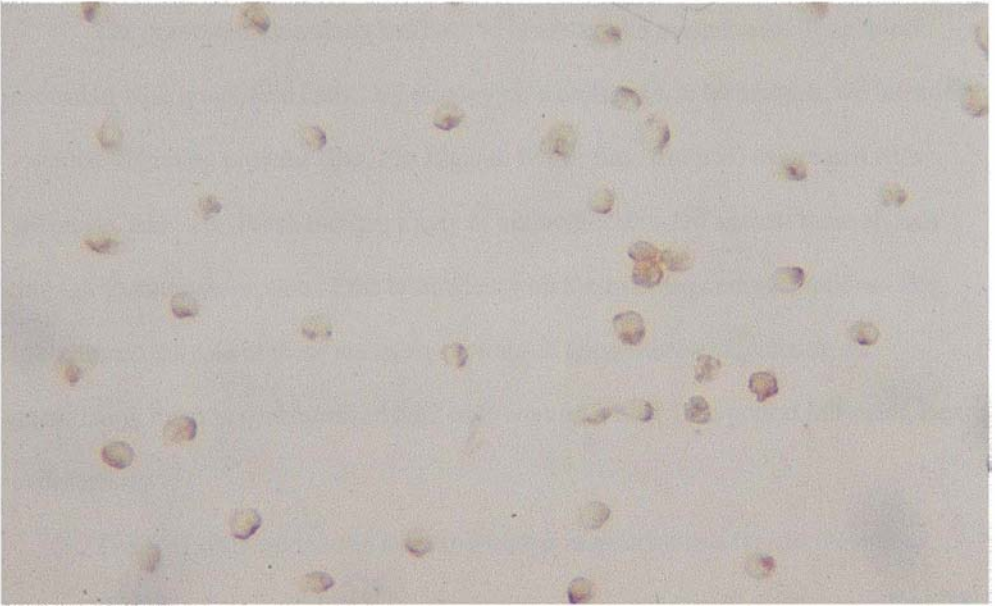


Figure 7. Demonstration of immunocytochemical staining for p53 in log phase Nb2 lymphoma cells. Antibody was at a concentration of 2 $\mu$ g/ml. Magnification x354.



## **Chapter 4**

### **Discussion**

The purpose of this study has been to elucidate the mechanisms of apoptosis control in Nb2 lymphoma cells. By employing morphological techniques, we intended to visualize signaling proteins (Fas, Fas Ligand, Bcl-2, Bax, and p53) associated with apoptosis, and to establish the specificity of antibodies directed against these signals through immunoabsorption of the antibodies with their corresponding peptides. We investigated the role of these signaling proteins in apoptosis by visualizing and quantitating them in synchronized Nb2 cells over an 8 hour time period following Dex exposure.

The Nb2 cells we used in our experiments responded in a typical manner to Dex±Prl exposure; Dex caused an increase in the percentage of dead cells over DMSO levels, and coincubation of Prl with Dex protected cells against Dex-induced cytolysis. This response is concordant with the results obtained from previous studies by Fletcher-Chiappini et al. (1993) and by Guanzon (1998). Since this typical response provides a model for studying the signaling involved in apoptosis, we were able to utilize these cells for further studies.

By using ICC, we intended to determine the feasibility of a morphological

approach for investigating apoptosis control. This approach was selected because it appeared to confer numerous advantages over the traditional biochemical techniques of Western blots and agarose gel electrophoresis. Biochemical methods require a homogenate of the cell population to extract pooled protein or DNA. Through immunohistochemistry, however, one can analyze single cells with preserved cellular architecture (Gavrielli et al., 1992). Furthermore, a maintained cell structure affords the opportunity to observe localization of staining within cells, and the ability to compare staining under varying treatments and at different time points. Examining qualitative aspects of cells in this manner would appear to offer much insight into the role of signaling proteins involved in apoptosis. In addition, ICC on intact cells may allow for simultaneous localization of signaling proteins.

Through ICC, we were able to localize Fas, Fas ligand, Bcl-2, Bax, and p53 in Nb2 cells. The antibodies for Fas, Fas ligand, Bcl-2, and Bax were affinity-purified rabbit polyclonal antibodies raised against a specific amino acid sequence at the N-terminus or C-terminus of the protein. The specificity of immunostaining for these 4 antibodies was verified by their disappearance when the antibodies were mixed with the peptides that were used to initially generate the antibodies.

The localization of p53 by ICC required the use of a rabbit polyclonal antibody directed against the entire p53 protein (in fact, the original antigen was a p53-Glutathione-S-transferase fusion protein). We were unable to eliminate immunostaining produced by this antibody despite using several distinct approaches. For example, the addition of the p53 GST fusion protein and the peptide sequences corresponding to the



N-terminus (amino acids 2-20) and C-terminus (373-391) of p53 failed to eliminate immunostaining. Additionally, attempts at removing the anti-p53/p53GST complex by adsorption to agarose beads containing glutathione failed to prevent immunostaining. The failure to verify the immunospecificity of p53 may be due to several reasons. First of all, cell fixation may have altered the immunoreactive site of cellular p53, causing it to have a higher affinity for the anti-p53 antibody than the exogenous protein added during absorption. Secondly, the configuration of the immunoreactive site(s) on p53 may have been modified by its fusion to GST, consequently making it (them) inaccessible to the antibody. In addition, to our knowledge, the p53 GST fusion protein has never been used as an immunoabsorbant (according to consultation with Santa Cruz Biotechnology), and thus may act in an atypical manner. Third, the anti-p53 we used may not react with the C or N-terminus of p53 protein. Fourth, anti-p53 may be binding in a non-specific manner to another antigen, cellular protein, or an artifact of the cellular preparation. In other words, the staining may not be specific for p53. The failure to demonstrate immunospecificity of p53 signifies that its visualization can not be affirmed with certainty. Although immunoabsorption of an antibody with its antigen measures antibody specificity and antigen localization (Witorsch, 1980), many researchers fail to absorb their antibodies, and they may be visualizing something other than their targeted protein. In fact, numerous studies have reported to stain for p53 by ICC without using absorption controls, such as the visualization of p53 in breast carcinomas (Jensen et al., 1997), the localization of p53 in mammary epithelial cells (Delmolino et al., 1993), and the staining of p53 in tumors derived from transgenic mice (Hall et al., 1998).

Previously, immunocytochemical data that were generated in our laboratory were quantitated manually. The visual counting of hundreds of fields of cells became the rate determining step of data analysis. In the present study, we have implemented a novel, computerized means to quantitate the total number of cells, as well as the number of stained cells per field, using Scion Image software. This methodology offers a more objective, consistent, and expeditious means of cell quantitation versus non-computerized quantitation. Currently, we have been able to utilize the image analysis software to count the proportion of stained cells in a given field. We may potentially be able to use this software to determine the distribution of cell staining (ie. nuclear, cytoplasmic, or cell surface localization) and to measure the intensity of cellular staining.

While computer-assisted cell quantitation has yielded more accurate cell counts, we have become increasingly aware of sources of imprecision in our methodology. When cells were counted in a hemacytometer by Trypan Blue exclusion, a single cell count could vary as much as 40% from another cell count (unpublished observations in our laboratory). After empirical testing, we came to the conclusion that multiple (at least 3) aliquots of the same cell suspension should be utilized for quantitation in order to achieve a cell count within 10% of the actual cell concentration (from unpublished observations in our laboratory). By photographing and counting 4 fields of cells per etched circle, we quantitated staining for a *single* aliquot of a cell suspension. To minimize this variability in cell quantitation (in accordance with our hemacytometer findings), at least 3 aliquots of each cell suspension would have to each be photographed and counted as described above.

The proportions of log phase cells that were stained for Fas, Fas Ligand, Bcl-2, Bax, and p53 ranged from 72% to 86%. Using the same Nb2 cells, Guanzon (1998) found approximately 40% of log phase cells to be positively stained for Glucocorticoid receptor (GR), IkBA, NFkB, and STAT-5b. The discrepancy in the proportions of stained cells for different signals in Nb2 cells suggests the following: High percentages (72-86%) of cells stained for Fas, Fas Ligand, Bcl-2, Bax, and p53 indicate that the majority of Nb2 cells contain all of these signals. A lower percentage (40%) of cells stained for GR, IkBA, NFkB, and STAT-5b suggests that there may be a single subset of cells positive for all 4 signals, or several subsets of cells that each contain one or more of these proteins. To resolve these issues, we are currently examining the simultaneous localization of these signals in Nb2 cells.

Immunocytochemical experiments staining for Fas and Fas Ligand in log phase Nb2 cells revealed that 72% of cells contained Fas, while 79% of cells expressed Fas Ligand, indicating that both proteins are within the same cell in the majority of the cell population. Generally, the binding of Fas Ligand to Fas receptor induces cell death by apoptosis (Laytragoon-Lewin, 1998). It is intriguing that although Fas and Fas ligand are both present in Nb2 cells, Fas mediated apoptosis is absent, indicated by a continually high (>90%) cell viability. There are several possible explanations for the resistance of Nb2 cells to Fas mediated apoptosis. First, Fas and Fas ligand association is not always fatal, and depends on the presence of specific elements that regulate the cell's response; these elements cause significant variability of cellular responses to Fas and Fas ligand association. (Gottlieb and Babior, 1997). For example, while both macrophages and

endothelial cells express Fas, only macrophages undergo apoptosis in response to Fas ligand (Gottlieb and Babior, 1997; Richardson et al., 1994). In some instances, Fas occupation may even cause T cell proliferation (Gottlieb and Babior, 1997; Agarwal et al., 1995).

Next, the stage of development of T cells determines the cellular response to Fas and Fas Ligand binding. In T lymphocyte development, immature cells possess both the CD4 (helper T cell) marker and the CD8 (cytotoxic T lymphocyte) marker. As cells mature, T cells express only the CD4 or CD8 marker. Double positive, immature T cells are sensitive to Fas mediated apoptosis, whereas more mature, single positive T cells are resistant to apoptosis (Ogasawara et al., 1995). The Nb2 lymphoma cell line was shown to be positive for both markers, suggesting that they would be susceptible to Fas induced cell death (Fleming et al., 1982). The resistance to Fas mediated apoptosis may have been mediated by discrete signals downstream from Fas and Fas Ligand binding. Furthermore, the Nb2 cells that we utilized may have changed through time from the initial clone, resulting in a single positive (CD4 or CD8) phenotype that has become resistant to Fas induced apoptosis. Additionally, studies from Fleming et al. (1982) suggested that Nb2 cells were double positive (CD4/CD8), because monoclonal antibodies (W3/25-HLK and OX8-HL) bound to early antigenic determinants of helper T cells and nonhelper T cells. It may be prudent to reevaluate the markers in Nb2 cells using more current methodology.

Finally, overexpression of the transcription factor Bcl-2 has been found to partially block Fas mediated apoptosis (Nagata et al., 1995; Itoh et al., 1993). Since we

observed most log phase cells (76%) to be positive for Bcl-2, overexpressed Bcl-2 may explain the low occurrence of cell death in Fas and Fas ligand positive Nb2 cells. Furthermore, when Bcl-2 associates with its binding protein, BAG-1, Fas mediated apoptosis is completely inhibited (Nagata et al., 1995; Takayama et al., 1995). Further studies testing for the BAG-1 protein would verify or disqualify this phenomenon in Nb2 cells.

The p53 protein is usually found in low levels due to its association with Mdm2, which inactivates p53 (Admas et al., 1998). However, we observed p53 to be detectable by ICC in a majority (73%) of untreated log phase cells. The elevated p53 levels may be due to the presence of mutated p53 or upregulated, wild type p53. Since the viability of Nb2 cells remained over 90%, the p53 was probably not the wild type, which would evoke cell death and decrease this viability substantially. Hence, the p53 we localized by ICC in Nb2 cells was most likely in its mutated (inactive) form.

It was observed that glucocorticoids induce apoptosis in immature thymocytes by p53 independent mechanisms (Macfarlane et al., 1996). If this is accurate in Nb2 lymphoma cells, then Dex treatment would not effect p53 levels. The independence of p53 and Dex induction of apoptosis may explain why we observed no statistically significant changes in the percentages of stained cells for p53 over the 8 hour Dex time course assay.

Using immunoblot analysis, Krumenacker et al. (1998) demonstrated that there were no changes in the levels of Bcl-2 or Bax protein in stationary Nb2 cells that had been treated with Dex for 12 hours. Our results have corroborated this finding; we found

no statistically significant changes in the percentages of stained cells for Bcl-2 or for Bax over the 8 hour Dex time point assay.

Cytolysis was observed in synchronized Nb2 cells after 6 and 8 hours of Dex exposure using Trypan Blue exclusion counts. This cytolytic response was correlated to the occurrence of apoptosis at 8 hours, as measured by the TUNEL assay (Guanzon, M.S. thesis, 1998). Presumably, Dex-induced cytolysis was caused by apoptosis. There was also a possibility of spontaneous cytolysis, since the percentages of dead cells rose from 2% at -24 hours and 4 % at 0 hours to 14.7% after 8 hours of DMSO exposure (this type of cell death was not validated by the TUNEL assay, however). In previous studies (Lavoie and Witorsch, 1995), DNA fragmentation caused by apoptosis was observed in synchronized Nb2 cells after 4 hours of Dex exposure by the Diphenylamine assay and agarose gel electrophoresis. The morphologically based TUNEL assay and Trypan Blue exclusion counts appeared to have detected apoptosis at a later time than biochemical means detected apoptosis for the following reasons: The Nb2 subline we were using may have changed through time from the original cells after multiple generations. Consequently, these modified cells may have responded to Dex in a delayed fashion. On the other hand, our results indicate that log phase cells responded to Dex±Prl in the typical fashion, suggesting that these cells were not modified. Alternatively, Guanzon (1998) found that a relatively small proportion (40%) of cells expressed GR and underwent apoptosis due to Dex exposure. Hence, detectibility of the response may have been diminished by the high proportion of non-responsive cells. Finally, the possible occurrence of spontaneous cytolysis in Nb2 cells could delay the observation of the Dex

response.

Since the immunostaining for all antibodies except p53 was found to be specific, the lack of any significant changes in the proportion of stained cells would suggest the independence of these signals in response to synchrony and Dex treatment. In the case of Bcl-2 and Bax, this is consistent with the finding of Krumenacker et al. (1998), that no changes in the levels of bcl-2 or bax protein were observed (using immunoblot analysis) in response to Dex in Nb2 cells. Additionally, our findings appear to be consistent with those of MacFarlane et al. (1996), that glucocorticoid induction of apoptosis in immature thymocytes does not involve p53. Alternatively, the inability to demonstrate changes in signals may reflect limitations in the methodology in its present stage.

As indicated previously, there may be more variability in the quantitation of ICC experiments than we anticipated. While we performed ICC experiments in triplicate and photographed 4 fields per treatment, we utilized only one aliquot of cells per time point when spotting cells for the Dex time course assay. Quantitation of 3 aliquots per time point would have reduced this variability. Furthermore, refinements may have to be implemented in order to visualize the changes in intensity of immunostaining (ie. titration of antibody) and the possible intracellular redistribution of signals (ie. counterstaining or ultrastructural analysis).

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## Vita

