2000

Functional Characterization of Mitogen-Dependent and Self-Perpetuating Nb2 Lymphoma Cell Lines

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December 11, 2008

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Functional Characterization of Mitogen-Dependent and Self-Perpetuating Nb2 Lymphoma Cell Lines

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 all those who have directly and indirectly given
me guidance and help in writing this thesis.

ACKNOWLEDGEMENTS

First, I would to thank my advisor and friend, Dr. Raphael J. Witorsch, for his encouragement, understanding, and sense of humor. His exuberant personality and fascination with science were infectious in the laboratory. Next, I would like to thank my co-worker, Beth Gannon, for her friendship and cooperation in the laboratory and throughout the writing process. In addition, I would to like to acknowledge the members of my thesis committee, Dr. Shobha Ghosh and Dr. John DeSimone, whom I hold in the highest regard as scientists and am honored to have on my committee. Lastly, I would like to thank my family and friends, especially TJ, for their patience, constant encouragement, and love during this stressful and time-consuming process.

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LIST OF ABBREVIATIONS

Full Name	Abbreviation
Analysis of Variance	ANOVA
Bovine Serum Albumin	BSA
Carbon Dioxide	CO_2
Cobalt	Co^{2+}
Dexamethasone	DEX
Diaminobenzidine	DAB
Dimethylsulfoxide	DMSO
Distilled Water	dH_2O
Ethanol	EtOH
Fetal Calf Serum	FCS
Fischer's Maintenance Media	FMM
Fischer's Media	FM
Fischer's Stationary Media	FSM
Gram	g
Glucocoticoid	GC
Glucocorticoid Receptor	GR
Horse Serum	HS
Immunocytochemistry	ICC
Inhibitor kappa B alpha	IκBα
Micro (10^{-6})	μ
Milli (10^{-3})	m
Molar	M
Nano (10^{-9})	n
Noble	Nb
Nuclear Factor kappa B	NFκB
Phosphate Buffered Saline	PBS
Prolactin	PRL
Signal Transducer and Activator of Transcription 5b	STAT 5b
Synthetic Media	SYN
TDT-Mediated dUTP-Biotin Nick End-Labeling	TUNEL
Units	U
Volume	vol
Weight	wt

ABSTRACT

FUNCTIONAL CHARACTERIZATION OF MITOGEN-DEPENDENT AND SELF-PERPETUATING NB2 LYMPHOMA CELL LINES

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

Virginia Commonwealth University, 2000

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This investigation involved the functional characterization of four Nb2 sublines (Nb2-U-17, Nb2-11, SFJCD1, and D5) for their mitogenic and apoptotic responses to DEX ± PRL treatment. Many protein factors have been implicated to be crucial in controlling the apoptotic and mitogenic pathways. In conjunction with functional studies, an immunocytochemical investigation of four protein signals (NFκB, IκBα, STAT 5b, and the GR) was undertaken in an attempt to establish a relationship between the functional responsiveness of a given cell line and the concentration of a particular protein marker. Comparison of the functional data obtained for the four lines reveals marked differences in behavior in response to DEX ± PRL. The Nb2-11 and U-17 sublines were dependent upon prolactin for proliferation and were sensitive to DEX induced cytolysis/apoptosis in the absence of PRL. Co-incubation of DEX and PRL resulted in both an inhibition of

apoptosis by PRL and an inhibition of mitogenesis by DEX. On the other hand, the SFJCD1 (SF) and D5 sublines proliferated in the absence of prolactin and were resistant to the anti-mitogenic and cytolytic/apoptotic effects of DEX. The differences in functional behavior between the mitogen-dependent and –independent cell lines could not be correlated with the expression of a particular protein marker.

CHAPTER ONE

INTRODUCTION

1.1 Background

Apoptosis is a natural process whereby a single cell or groups of cells are programmed to commit suicide in an orderly fashion; for this reason, apoptosis is interchangeably referred to as programmed-cell death (Donner, et al. 1999) . Apoptosis occurs during many physiological processes such as, embryogenesis, t-cell tolerance, metamorphosis, and adjustment of cell number including tumor cell and infected cell elimination (Lang, et al. 1999). In addition to the beneficial and crucial physiological processes that rely on apoptosis, there are a multitude of environmental and internal factors that result in the stimulation of unnecessary apoptosis, which can be harmful to an organism (Lang, et al. 1999). Some of these factors include, bacterial toxins, radiation, gross increase in extracellular osmolarity, oxidative stress, *c-myc* over-expression, growth factor depletion, stimulation of certain membrane receptors (TNF and CD-95/Fas/APO-1 receptor), ischemia, and glucocoticoid hormones (GCH) (Cifone, et al. 1999; Lang, et al. 1999). Cells are programmed to die when the delicate balance between survival factors and death factors is upset (Mann, et al. 2000). Excessive apoptosis can cause neurodegeneration and immunodeficiency, while inadequate apoptosis may lead to tumor

development or excessive functions such as, enhanced hormone production or autoimmune disease (Lang, et al. 1999).

Apoptosis is an energy-dependent process (Kerr, et al. 1972; Wyllie, et al. 1980; Kerr and Searle 1980; Whitfield, et al. 1968) that is easily recognizable and distinguishable from necrosis by certain hallmark events. The process of apoptosis is initiated by caspase activation, thought to be the central mediator of the apoptotic phenotype (Mann, et al. 2000). Morphologically, apoptosis is recognizable by the appearance of cellular shrinkage thought to be induced by the loss of intracellular potassium ions (Bortner, et al. 1997; Bortner, et al. 1997). In addition, nuclear condensation and translocation of phosphatidylserine residues to the extracellular surface occurs, which aids in recognition of the dying cell by local cells and the immune system (Mann, et al.2000; Lang, et al. 1999). Later in the onset of apoptosis, the chromatin of the cell condenses and the DNA is cleaved by endonucleases (Wyllie, et al. 1980; Mann, et al. 2000). Ultimately, the cell is broken down into small particles called apoptotic bodies (Lang, et al. 1999), which are quickly engulfed by resident tissue cells and digested within a few hours by phagolysosomes (Searle, et al. 1982). Throughout the process of apoptotic cell break down the cell membrane is never breached, thus avoiding intracellular protein release and consequent inflammation (Lang, et al. 1999).

In contrast to apoptosis, necrosis is a less organized cell death process that ultimately results in an immune response and inflammation of the surrounding tissues (Wyllie, et al.

1980). Necrosis can be caused by a number of factors including physical damage, hyperthermia, inhibition of oxidative phosphorylation, glycolysis or the citric acid cycle, autolysis, hypoxia, complement, and a variety of toxins (Lang, et al. 1999; Thompson 1998). Unlike apoptosis, which usually affects single cells or scattered cells, necrosis usually affects groups of contiguous cells (Wyllie, et al. 1980), or tracts of tissue (as in an infarct) (Searle, et al. 1982). Affected cells dilate, their mitochondria swell, other organelles dissolve, the plasma membrane ruptures, the nucleus dilates, and the chromatin disperses (Wyllie, et al. 1980; Searle, et al. 1982). When the membrane bursts intracellular proteins leak out into the surrounding extracellular fluid, activating an immune response, which results in inflammation and tissue damage (Lang, et al. 1999). Specialized phagocytic cells (Wyllie, et al. 1980) eventually ingest the debris produced from the rupture of a necrotic cell, but dead cells may persist in a histologically recognizable form for days to weeks (Searle, et al. 1982).

The focus of our laboratory has been on glucocorticoid-induced apoptosis in the rat Nb2 lymphoma cell line. Glucocorticoids (GCs) have been proven to activate apoptosis in thymocytes and neoplastic lymphocytes (Wyllie 1980; Cohen and Duke 1984; Bansal, et al. 1991; Baxter, et al. 1971), including Nb2 cells (Fletcher-Chiappini, et al. 1993), making GCs an effective treatment for lymphoid cancers (Feinman, et al. 1999). Cifone et al. (1999) have recently proposed a pathway through which GC-induced apoptosis occurs. The first step involved is the binding of hormone or in this case dexamethasone (DEX), a synthetic glucocoticoid, to the glucocorticoid receptor (GR). This leads to

phosphoinositol-phospholipase C (PI-PLC) activation, which consequently initiates diacylglycerol (DAG) release. DAG release leads to acidic sphingomyelinase (aSMase) activation, which cleaves ceramide thereby releasing it to activate the caspase cascade. Activation of the caspase cascade that leads to transcription and protein synthesis and ultimately results in apoptosis (Cifone, et al. 1999). Other investigators have proposed that transcription is the initial event leading to the apoptosis cascade (Cohen and Duke 1984; Nicholson, et al. 1978; Helmberg, et al. 1995), however apoptosis induced by exogenous ceramide is prevented by inhibiting mRNA and protein synthesis, suggesting that transcription is a downstream event (Cifone, et al. 1999).

It is apparent that one or more regulatory pathways must exist to keep the deadly action of glucocorticoids to some cell types in check. Jamieson et al. (2000) were able to demonstrate the existence of a regulatory crosstalk pathway between the GR and the T-cell receptor (TCR), and were able to show that the GR inhibits TCR/CD3 activation-induced death via MEK (mitogen-activated protein kinase kinase) in primary splenic T cells (Jamieson and Yamamoto 2000). Furthermore, other investigators have shown that the GR inhibits TCR-induced apoptosis via repression of FasL transcription (Yang, et al.1995). *In vivo* GC levels are regulated in response to diurnal cycling and stress levels (Jamieson and Yamamoto 2000). It is possible to spéculate that this GR/TCR crosstalk pathway may also be responsible for preventing GC-induced apoptosis of T cells combating inflammation or infection (Jamieson and Yamamoto 2000) making it a reciprocal pathway. Jamieson's group of investigators also found that Ras signaling

during TCR signal transduction in a thymoma cell line prevented GC-induced apoptosis (Jamieson and Yamamoto 2000). This implies that the same crosstalk pathway may also exist in thymocytes (Jamieson and Yamamoto 2000).

The Nb2 lymphoma cell line used by our laboratory is a pre-T cell (thymocyte) suspension culture derived from the lymph node of an estrogen-treated male Noble (Nb) rat (Noble, et al. 1985; Gout, et al. 1980; Fleming, et al. 1982). Although these cells were taken from the lymph node, they are not mature B or T lymphocytes (Fleming, et al. 1982). The surface antigens expressed or not expressed by Nb2 cells reveal that these cells are not B cells, mainly due to the lack of immunoglobulin expression (Fleming, et al. 1982). On the other hand, it is their expression of both W3/25-HLK (CD4) and OX8-HL (CD8) antigens, which are expressed on different subsets of mature T cells, that indicate that they are too immature to be defined as T cells (Fleming, et al. 1982). In fact, their surface antigens suggest that the Nb2 lymphoma cell line arose from a thymocyte at some intermediate stage of development that is not clearly defined (Fleming, et al. 1982). The first experimental use of the Nb2 cell line was as a bioassay for lactogenic hormones (e.g. human growth hormone and human PRL hormone) (Tanaka, et al. 1980). Nb2 cells are profoundly sensitive to lactogenic hormones from a variety of species and can be used to quantitate the concentration of a given hormone (Tanaka, et al. 1980).

Our investigation involved the use of the following four unique Nb2 sublines: Nb2-U-17, Nb2-11, SFJCD1, and D5. The Nb2-U-17 (wild-type) and Nb2-11 lines are critically

dependent upon prolactin (PRL) or other lactogen (e.g. human growth hormone or placental lactogen) for proliferation (Gout, et al. 1980; Gout, et al. 1994), and the SFJCD1 (SF) and D5 cultures proliferate in the absence of PRL or other mitogen (Gout, et al. 1994). The lactogen-independent lines were developed by the withdrawal of lactogenic hormone from PRL-dependent cultures, and subsequent propagation of the surviving cells in media devoid of lactogens (Gout 1987). The autonomous nature of the SF cell prompted an investigation into whether these cells were producing their own PRL-like substance. Assays testing for the presence of autonomous mitogens (endogenous lactogenic hormones) or growth factors produced by these cells were negative (Witorsch, et al. 1993; Gout 1987).

Our laboratory has investigated the functional behavior of both PRL-dependent and – independent Nb2 lines in response to DEX ± PRL treatment. We have observed the following: 1) DEX initiates apoptosis in a concentration dependent manner via the GR in the PRL-dependent Nb2 clone 2 line (Fletcher-Chiappini, et al. 1993), 2) At very low concentrations (1ng/mL) PRL effectively inhibits DEX-induced apoptosis in PRL-dependent Nb2 lines (Fletcher-Chiappini, et al. 1993), 3) 100nM DEX is anti-mitogenic to the PRL-independent SFJCD1 (SF) (Witorsch, Day, et al. 1993), and 4) Extracellular Ca^{2+} is not required for PRL or DEX action (LaVoie and Witorsch 1995). In addition to the anti-apoptotic action of PRL, a reciprocal effect could be demonstrated whereby DEX was able to prevent PRL-induced mitogenesis of Nb2 cells (Fletcher-Chiappini, et al. 1993). Due to the divergent nature of the signal transduction pathways leading to

mitogenesis and apoptosis, it has been suggested that the opposite inhibitory actions caused by DEX on mitogenesis and PRL on apoptosis involve a complex interplay between the post-receptor mechanisms of the PRL and glucocoticoid receptors (Fletcher-Chiappini, et al. 1993).

The immunocytochemical (ICC) approach has been employed by our laboratory to study the signaling associated with apoptosis control in Nb2 lymphoma cells. Using ICC we have demonstrated the existence of the following protein markers in Nb2 cells: NFκB, IκBα, STAT 5b, GR, Bcl-2, Bax, Fas, FasL, and p53 (Gaunzon 1998; Badarinath 1999). With the exception of p53, the specificity of immunostaining the preceding antibodies has been validated by immunoabsorption (Gaunzon 1998; Bardarinath 1999). Preliminary studies were unable to establish the existence of a dynamic relationship between the level of expression of a given signal before and after DEX treatment (Gaunzon 1998; Badarinath 1999).

More recently our laboratory has focused on the following four protein markers: NFκb, IκBα, STAT 5b, and GR. The first marker mentioned, NFκB or nuclear factor kappa B, is the most frequently occurring heterodimeric complex (p65/p50) of the NFκB family found in the cell. However, there are actually five proteins in the NFκB family including, NFκB1 (p50/p100), NFκb2 (p522/p100), RelA (p65), c-Rel and RelB (De Bosscher, et al. 2000). In the inactivated state, NFκB is bound to an inhibitor, IκBα, in the cytoplasm of the cell (Hettmann, et al. 1999). Upon activation NFκB is released from IκBα and

NFκB dimers translocate to the nucleus where they bind to various κb DNA binding sites and regulate the transcription of certain genes (Hettmann, et al. 1999). NFκB can promote apoptosis or cell survival depending upon the cell type and context (Feinman, et al. 1999). However, most studies indicate that the Rel/NFκB family of transcription factors play an important role in the protection of cells from apoptosis either by constitutive expression of NFκB or by its induction (Sonenshein 1997). In a study performed by Ivanov and Nikolic-Zugic (1998) on double positive thymocytes (as are Nb2 cells), GCs were shown to induce a moderate upregulation of RelA-p50 (NFκB) relatively late in the apoptosis cascade after caspase-dependent degradation of several nuclear proteins. This up-regulation of NFκB was associated with the activation of the proteosome and the consequent degradation of IκBα (Ivanov and Nikolic-Zugic 1998). In addition, it has been shown by another group of investigators that after NFκB has translocated to the nucleus, the GR interferes with the transactivating domain of the p65 subunit thereby repressing transcription of p65 mediated gene products (De Bosscher, et al. 2000). Therefore, although NFκb is upregulated by glucocorticoids late during the apoptosis cascade, subsequent downregulation in the nucleus ultimately results in a repression of κB gene transcription and the protective effects of the gene products. This evidence is supported by the findings of Sen and Sen, who found that following the injection of mice with GCs there was a drop in NFκB DNA-binding activity in immature $CD8^+/CD4^+$ lymphocytes preceding apoptosis (Sonenshein 1997). These investigations suggest a possible crucial role for NFκb in determining cell survival.

IκBα or inhibitor kappa B alpha, mentioned briefly above, is bound to inactivated NFκB in the cytoplasm of the cell (Hettmann, et al. 1999), functioning as an inhibitor by sequestering NFκB in the cytoplasm. IκBα contains six copies of a structural motif called an ankryrin repeat, which is necessary for the interaction with NFκB (Whiteside and Israël 1997). Ankyrin repeats sequester NFκB in the cytoplasm by masking the nuclear localization sequence situated in the C-terminal region of the Rel homology domain (Beg, et al.1992; Ganchi, et al. 1992). Additional structural attributes include an N terminal pair of serine residues and a C-terminal region rich in proline, glutamate, aspartate, serine and threonine residues (PEST domain) implicated in regulating the stability of IκBα, as well as playing a role in the ability of Iκbα to inhibit DNA-binding by Rel/NFκB complexes (Whiteside and Israël 1997). When the cell is activated by any number of apoptotic stimuli, IκBα is phosphorylated and subsequently degraded resulting in the release of NFκB (Hettmann, et al. 1999). Upon release from IκBα, NFκB dimers translocate to the nucleus where they bind to various κB promoter sites and regulate the transcription of genes that have protective effects on the cell (Hettmann, et al. 1999; Sonenshein 1997). After degradation of IκBα it is then resynthesized in an NFκB-dependent manner (Whiteside and Israël 1997). In the case of glucocorticoid treatment, the induced synthesis of IκBα by NFκB leads to translocation of IκBα to the nucleus where it can inhibit NFκB by binding to its target sites (Arenzana-Seisdedos, et al. 1995). Furthermore, translocation of NFκB to the nucleus is inhibited by mutating IκBα to a

super-repressor form (Wang, et al. 1996). Translocation to the nucleus can also be prevented by mutations to the serine-specific kinases that phosphorylate Iκbα or to the proteosome responsible for degradation of IκBα (Whiteside and Israël 1997; Brown, et al. 1995). These investigations suggest that the integrity and concentration of the IκBα protein play a critical role in determining cell survival, and that IκBα is a mobile protein capable of inhibiting NFκB in both the cytoplasm and the nucleus.

Signal Transducer and Activator of Transcription 5B or STAT 5B may also play a role in controlling apoptosis. The JAK/STAT pathway is activated as a result of PRL binding to the PRL receptor and it is implicated in transducing the mitogenic signal to the nucleus of the cell (Rui, et al. 1998). JAKs are constitutively associated with the cytoplasmic domain of cytokine receptors (Starr, et al. 1999). Binding of ligand to its receptor induces dimerization of the receptor chains, bringing together two JAK kinases that are activated by transphosphorylation (Starr, et al. 1999). In the case of PRL binding, Jak2 is activated. Jak2 in turn phosphorylates STAT 5A, 5B, 1, and 3 in addition to the serine kinases ERK 1 and 2 (Starr, et al. 1999; Rui, et al. 1998). Upon activation STATs dimerize and migrate to the nucleus where they activate the transcription of genes that mediate the cytokine-induced biological response (Starr, et al. 1999). It may be postulated then that over-expression of STATs, particularly STAT 5B, in Nb2 cells may afford protection from apoptosis. In support of this hypothesis it has been documented that STAT 5 represses the GC response (Pfiztner, et al. 1998). Therefore, an over-

expressed STAT 5B protein in Nb2 lymphoma cells may afford protection from GC-induced apoptosis.

A fourth protein that may play a role in producing either glucocorticoid resistance or sensitivity is the GR itself. It has been postulated that mutation or downregulation of the GR may be the cause of glucocorticoid hormone insensitivity. In 1988 Rosewicz et al. (1988) found that downregulation of the GR could be found for up to 72 hours after GC treatment (Rosewicz, et al. 1988). However, they also found that the synthetic glucocoticoid dexamethasone (DEX) had no effect on the level of GR expressed, and studies performed by Oldenburg et al. in 1997 support this evidence. In addition, Oldenburg and his group found that DEX-insensitive thymocytes possessed fully functional GRs, and they found that the molecular weight and abundance of the receptor does not change after DEX treament, indicating that the GR is neither downregulated nor mutated (Oldenburg, et al. 1997), which is consistent with earlier findings in our laboratory (Witorsch, et al. 1993). Oldenburg's findings suggest that the GR is not responsible for the apoptosis-resistant phenotype, and that there must be other factors that confer apoptosis resistance or sensitivity.

The over- or under-expression of one or more of these proteins could have an obvious effect on apoptosis regulation. The initial studies by Guanzon (1998) and Badarinath (1999) failed to demonstrate any acute changes between a given signal and DEX treatment. More recently we have attempted to correlate the functionality of Nb2 cells

with the level of expression of these markers. 26 sublines were generated from a single Nb2-11 cell using a limiting dilution technique. Some of these sublines demonstrated distinct levels of sensitivity to DEX-induced cytolysis/apoptosis as measured by trypan blue exclusion and TdT-mediated dUTP-biotin nick end labeling (TUNEL) (Patel 1999; Rebano 1999). In addition, the degree of sensitivity of a given subline to DEX treatment was statistically correlated to the percentage of cells immunostained for NFκB, IκBα, STAT 5b, and the GR (Patel 1999; Rebano 1999). This suggested that all of the markers were localized within a single population of cells, which was confirmed by co-localization of the four markers discussed using a modification of Immunocytochemistry methodology (Rebano 1999).

1.2 Objective

To date, immunocytochemistry methodology has provided little insight into the mechanisms involved in control of apoptosis and mitogenesis in Nb2 lymphoma cells. Through the use of ICC we have been unable to observe changes in the expression of signals associated with physiologic manipulation of cells (e.g. treatment with DEX or removal of mitogen) (Gaunzon 1998; Badarinath 1999). The limiting dilution approach whereby sublines were generated for the characterization of signaling in apoptosis control was found to be of limited value for two main reasons. First, the generation of the sublines through limiting dilution is not a quantitative process because there is no way to confirm that a given cell line arises from only one cell. Therefore, the homogeneity of a given subline is assumed. Second, the functional variation between sublines was narrow

making differences difficult to correlate statistically. On the other hand, the results of this approach suggested that a correlation did exist between functional behavior and the existence of certain markers.

Our current strategy was to determine if differences in signal expression as measured by ICC could be detected in sublines of Nb2 cells that exhibit marked functional differences. The Nb2-11 and U-17 (wild-type) lines are dependent upon PRL for mitogenesis, and it has been documented that the former undergoes apoptosis in response to nanomolar concentrations of DEX (Fletcher-Chiappini 1993). In contrast, the SFJCD1 (SF) and D5 lines are PRL-independent with regard to mitogenesis and the former has been shown to be resistant to DEX-induced apoptosis. If the signals of interest are involved in the apoptosis pathway, than differences in their level of expression may be demonstrable by ICC.

The purpose of this thesis was to functionally characterize the hormonal control of mitogenesis and apoptosis in the SF, D5, Nb2-11, and U-17 cell lines. Functionality was examined by determining the cytolytic/apoptotic and mitogenic responses to DEX ± PRL treatment. ICC was used to determine if we could detect differences in immunostaining of the four signals of primary interest (NFκB, IκBα, STAT 5b, and GR) in the four cell lines.

In addition to the four lines described above, we have generated two additional phenotypic variants in our laboratory. One line (named LTI for long-term culture) was

generated by the prolonged incubation of Nb2-11 cells with subculturing (dilutions of 1:9 or 1:19 every two to three days) for an extended period of time (one year or more). This resulted in a phenotype that was less sensitive to DEX-induced cytolysis and had the capacity to self-proliferate. A second subline (named PI for prolactin-independent) was generated by subculturing the Nb2-11 in media devoid of mitogen (fetal bovine serum). The PI cells appeared to be mitogen-independent and also had a decreased sensitivity to DEX-induced cytolysis (Gannon 2000, unpublished results).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Maintenance of Nb2 Lymphoma Cells

Four Nb2 lymphoma cell lines were obtained from Dr. Peter Gout of the Cancer Control Agency in Vancouver, British Columbia in February of 2000. Mitogen dependent cell lines (Nb2-U-17 and Nb2-11) were maintained in Fischer's Maintenance Medium (FMM), containing chemically defined Fischer's Medium (Sigma), 50U/mL penicillin, 50μg/mL streptomycin, 0.1mM β-mercaptoethanol, 0.075% sodium bicarbonate, 10% horse serum (HS) and 10% fetal calf serum (FCS). Mitogen independent cell lines (SFJCD1 and D5) were maintained in Fisher's Stationary Medium (FSM), which is equivalent to FMM with the exception that it contains no FCS. Fischer's Medium and β-mercaptoethanol were purchased from the Sigma Chemical Company, and all other media components were purchased from Gibco BRL/ Life Technologies, Inc.

All cell lines were cultured in ventilated polystyrene culture flasks in a humidified incubator that was maintained at 37°C and 5% CO_2/95% room air. Cell culture concentration and viability were determined by a cell count using a hemacytometer and Trypan Blue (Gibco BRL, Life Technologies, and Inc.) exclusion (0.1mL cells + 0.9mL

trypan blue). Cell subculturing and assays were performed in a sterile environment and cell cultures were not diluted to less than 0.05 X 10^6 cells/mL when subcultured.

2.2 Freezing and Resurrecting Cells

Cells were routinely frozen every couple of weeks for later resurrection as needed. First, an aliquot of cells was centrifuged at 1000 rpm for five minutes. The cells were washed one time with FM, which involved resuspension in FM, centrifugation at 1000 rpm for five minutes, and aspiration of the supernatant. After the wash step, the cells were resuspended in 1 mL of freezing medium, transferred to 2 mL biofreeze vials (Corning Costar Corp.), and finally stored at –70°C. The freezing medium composition differed according to whether the cell line was mitogen-dependent or -independent. For mitogen-dependent sublines (U-17 and Nb2-11) the freezing medium consisted of FMM/8% DMSO (Sigma). The freezing solution for mitogen independent sublines (SFJCD1 and D5) consisted of FSM/8% DMSO.

Cells to be resurrected were first thawed and the contents of the biofreeze vial were placed in a centrifuge tube of warmed pre-gassed (5% CO_2) FMM or FSM depending upon the cell line. The cells were then placed in a humidified, 37°C incubator for ten minutes with the cap loosened to allow the DMSO to escape from the cells. The cells were then centrifuged at 1000 rpm for five minutes, washed with FM and resuspended in warmed, pre-gassed FMM or FSM. Cell viability and concentration were determined by

Trypan Blue exclusion. The cells were cultured for at least a week post-resurrection before using them for experiments.

2.3 Cell Fixation and Spotting

The procedure for fixing and spotting cells was identical for all cell cycle phases and/or cell treatments. First, an aliquot of cells was fixed with an equal volume of fixative (0.01M PBS/7.4% formaldehyde prepared by diluting stock formaldehyde, 37% solution, obtained from J.T. Baker Chemicals with 0.01M PBS at 7.0 pH) at room temperature and allowed to stand for ten minutes. The fixed cells were then centrifuged for five minutes at 1000 rpm. The supernatant was removed and the cells were resuspended in enough 0.01M PBS to give a cell concentration of 3.0-3.5 X 10^6 cells/mL. Approximately 20μL of fixed cells were spotted into etched circles of approximately 5mm in diameter on Fischer Superfrost/Plus slides. The spotted slides were then covered and allowed to dry at room temperature overnight before use in experiments.

2.4 Cytolytic Assay

The cytolytic assay is a four well assay used to test the cells responsiveness to DEX ± ovine prolactin (oPRL). An aliquot of cells were centrifuged at 1000 rpm for five minutes, washed once with FM and finally resuspended in chemically defined serum-free synthetic medium (SYN). SYN consists of 0.1mM β-mercaptoethanol, 50U/mL penicillin, 50μg/mL streptomycin 0.15% (wt/vol.), bovine serum albumin (0.15%), 4 μg/mL linoleic acid, 1mM sodium pyruvate, 12μg/mL transferrin, 15ng/mL selenium, 1X

vitamins (diluted from 100X stock), 0.33X amino acids (diluted from stock 100X), 0.5mM $CaCl_2$ and hepes dissolved in FM. The cells were then counted in triplicate using a hemacytometer and Trypan Blue exclusion, and diluted with SYN to obtain a concentration of 0.56 X 10^6 cells/mL.

Four wells were set up on a 24 well plate as follows: 1) 2.5 µl DMSO, 0.1mL SYN and 0.9 mL cells (control), 2) 1.25 µL DMSO, 1.25 µL DEX, 0.1 mL SYN and 0.9mL cells (DEX), 3) 1.25 µL DMSO, 1.25 µL DEX, 0.1 mL oPRL (DEX + PRL), and 0.9 mL cells, 4) 2.5 µL DMSO, 0.1mL oPRL and 0.9mL cells (PRL). The assay was incubated at 37°C and 5% CO_2 for 24 hours. After incubation each well was counted in triplicate using a hemacytometer and Trypan Blue exclusion to determine the cell viability (percent living and dead) and concentration (number of cells/mL).

2.5 Mitogenic Assay

The objective of the mitogenic assay was to determine the proliferation of a given cell line when exposed to oPRL ± DEX over a 72-hour period. Prior to cell treatment the cells were "cooled down" or arrested in G1 phase of the cell cycle by incubating the cells for 24 hours in FSM/1% FCS (Buckley, et al., 1996; Gilks, et al., 1995). This was done by first centrifuging an aliquot of cells at 1000 rpm for five minutes, washing two times with FM and finally resuspending in FSM/1%FCS. The cells were then counted one time using Trypan Blue exclusion to determine concentration and viability, and diluted to 0.50 X 10^6 cells/mL. The diluted cells were incubated in a humidified, 37°C and 5.0% CO_2

incubator for 24 hours. The same protocol was followed for the mitogen -independent cell lines with the exception that they were resuspended and diluted with FSM. However, these cells were not expected to "cool down" because they are mitogen independent, but they were subjected to the same procedure to maintain experimental consistency.

After incubating the cells for 24 hours an aliquot of the "cooled down" cells was removed, centrifuged, washed two times with FM and resuspended in SYN. The cells were then counted in triplicate using a hemacytometer and Trypan Blue exclusion, and diluted to a concentration of 0.11×10^6 with SYN.

The four well set up for the assay was identical to that described for the cytolytic assay above. The cells were then incubated for 72 hours in a humidified, 37°C and 5.0 % CO_2 incubator. At end of the incubation period each well was counted in triplicate using a hemacytometer and Trypan Blue exclusion.

2.6 Immunocytochemistry (ICC) and Immunoabsorption

Immunocytochemical assays were performed to test for the presence and abundance of four protein antigens presumably associated with hormonal control of apoptosis and mitogenesis. Working stock solutions of each polyclonal rabbit IgG antibody were prepared from concentrated antibody solutions obtained from Santa Cruz Biotechnology. Anti-GR (P-20) and anti-NFκB p65 (C-20) were diluted from a concentration 200 μg/ml

to a working concentration of 4μg/ml with 0.01M PBS/0.1% BSA, and anti-IκBα (C-21) and anti-STAT 5B (C-17) were diluted from 100μg/mL to 2μg/mL with PBS/BSA (Sigma).

ICCs were performed using the Vectastain kit obtained from Vector Laboratories, which contained blocking serum, secondary biotinylated antibody and tertiary ABC antibody. Solutions in the Vectastain kit were prepared according to the kit instructions, and further modifications as described below. After each treatment, the slides were incubated in a humidified chamber at room temperature. Between each treatment the slides were washed with 10mL of 0.01M PBS and immersed in a Coplin jar containing 0.01M PBS to maintain hydration.

First, slides that had been spotted with the four Nb2 sublines (in log phase) were hydrated for ten minutes in 0.01M PBS. Cytopore (Trevigen) was then added to each spot to permeabilize the cells, and the slides were incubated for thirty minutes. Second, blocking serum was added from the Vectastain kit to minimize non-specific staining, and the slides were incubated for twenty minutes. Next, the primary working antibody solutions (working stock solutions prepared above) were added to the spotted cells and a negative control slide was treated with 0.01M PBS/0.1% BSA (Sigma). The slides were then incubated for three hours. Next, a secondary biotinylated antibody (Vectastain) was added at a diluted concentration (diluted 1:7 with 0.01M PBS) to each spot, and incubated for thirty minutes. Subsequently, tertiary ABC antibody was added after being

diluted 1:4 with 0.01M PBS, and incubated for thirty minutes. The slides were then placed in a coplin jar for eight minutes containing 12.5 mg Diaminobenzidine (Aldrich), 50mL TRIS-HCl at 7.6 pH and 50μL of 3.0% hydrogen peroxide, and subsequently immersed in and washed with tap water. The slides were then run through a dehydration series in the following order: dH_2O, 50% ethanol (EtOH), 70% EtOH, 95% EtOH, 100% EtOH, 100% EtOH and a series of four xylenes. Lastly, the slides were coverslipped using Pro-Texx mounting medium (Scientific Products).

Immunocytochemical assays were used to test for the presence and abundance of four protein markers: glucocorticoid receptor (GR), nuclear factor kappa B (NFκB), Signal Tranducer and Activator of Transcription 5B (STAT 5B) and Inhibitor κBα (IκBα). Polyclonal rabbit IgG antibodies (Santa Cruz Biotechnology) directed against these four protein antigens were generated in a rabbit against a 17-21 amino acid length peptide corresponding to the amino or carboxyl terminus. Prior to performing immunocytochemical assays, we validated antibody specificity by immunoabsorption. To test for antibody specificity, working stock solutions of anti-NFκB p65 (4 μg/mL), anti-IκBα (2 μg/mL), anti-STAT 5B (2 μg/ml) and anti-GR (4 μg/mL) were incubated with their respective peptide antigens for 24 hours at 4°C at a ratio of 1:10 (antibody to antigen on a mass basis), as recommended by the supplier. After the incubation period, the absorbed antibody solution along with a negative control solution consisting of PBS/0.02% BSA and of the respective working stock antibody were run through an ICC using slides spotted with log phase Nb2 cells. We were able to confirm the specificity of

the antibody through the absence of staining in cells treated with the absorbed antibody solutions.

2.7 TDT-Mediated dUTP-Biotin Nick End-Labeling (TUNEL) Method

The TUNEL method was used as a qualitative confirmation of DEX-induced apoptosis. Fischer Superfrost/Plus slides spotted with cells from the DMSO (control) and DEX treated wells of cytolytic assays were used for TUNEL assays. An apoptosis detection kit obtained from Trevigen was used for all TUNEL assays, and the following solutions were used from the kit: 10X TdT Labeling Buffer, 10X Stop Buffer, Tdt dNTP, Tdt , 50X Co^{2+}, Strepavidin Horseradish Peroxidase (Strep-HRP) and Diaminobenzidine (DAB) staining solution. After each treatment and subsequent incubation, the slides were washed with at least 10ml of 0.01M PBS.

First, the slides were hydrated in 0.01M PBS for ten minutes. Next, the cells were permeabilized using the detergent Cytopore (Trevigen) for thirty minutes. The slides were then subjected to five minutes of peroxidase quenching in a coplin jar at room temperature. The peroxidase solution consisted of 3.3ml 30% H_2O_2 and 47.7mL ddH_2O to give a solution of 2% H_2O_2. 1X labeling buffer was then applied to each circle to equilibrate the cells to the buffer solution. Then the buffer was removed with filter paper and 18μL of labeling mix was applied to each spot. The labeling mix was prepared as follows: 1μL Tdt, 1μL Tdt dNTP, 1μL Co^{2+} and 50μL of 1X labeling buffer prepared for every two spots. After applying the labeling mix, slides were placed in a petri dish lined

with moist filter paper. The petri dish containing the slide was then placed in a 37°C, humidified incubator with an atmosphere composed of 5% CO_2/95% room air for one hour. After one hour the slides were placed in a 1X Stop Buffer solution (5mL 10X Stop Buffer + 45ml ddH_2O) for five minutes. Next, the slides were treated with Strep-HRP and allowed to incubate for ten minutes at room temperature. The slides were then placed in a DAB staining solution for ten minutes and thereafter washed with tap water in a coplin jar. Lastly, the slides were run through a dehydration series in the following sequence dH_2O, 50% ethanol (EtOH), 70% EtOH, 95% EtOH, 100% EtOH, 100% EtOH and four xylenes, and coverslipped using Pro-Texx mounting medium.

2.8 Statistical Analysis

The SigmaStat 2.0 computer program for Windows was used to statistically analyze raw data from mitogenic and cytolytic assays. Raw data obtained from cell counts was entered into a spreadsheet, subjected to normality and equal variance tests, and one-way analysis of variance (ANOVA) (a minimum of three individual assays (n=3) run at different times were used for statistical analysis). For the data with a $p<0.05$ a Tukey test was run, which involves a multiple pairwise comparison of the generated means. The means calculated from each treatment and each cell line were then used to create graphs using SigmaPlot 2000. The error bars on the graphs (Figures 2-5) represent the standard error of the mean (SEM) for a given treatment.

2.9 Photomicrography

Photomicrographs were taken of the TUNEL and ICC assays using a Nikon Polaroid camera with a landpack attachment mounted on a Nikon Optiphot microscope. Color polaroid type 669 film was used at 80X magnification. A representative field of cells was photographed from each spot on each slide. The photographs were then developed and scanned into the Corel Photo-Paint 8.0 computer program.

CHAPTER THREE

RESULTS

3.1 Nb2 Lymphoma Cell Morphology

Figure 1 represents Nb2 cells stained with Wright's Geimsa stain and illustrates the differences between the four Nb2 lines studied. Distinct morphological differences are clearly evident between the PRL-dependent and PRL-independent Nb2 lines. The SF and D5 cell lines appear to be larger and more dynamic. They also have numerous cytoplasmic projections, which are not seen as frequently in the PRL-dependent lines. The wild-type (U-17) and Nb2-11 lines display a smaller, more rounded appearance and they lack the dynamic appearance of the PRL-independent lines. Even though there are discrete differences in morphology between the PRL-dependent and –independent lines, all four cell lines display the high nucleus to cytoplasm ratio typical of neoplastic lymphoma cells (Fleming, et al., 1982).

3.2 Synchronized Nb2 Cell Responsiveness to 72-Hour PRL ± DEX Treatment (Mitogenic Assay)

Figure 2 shows the mitogenic responses of the Nb2-11 and U-17 sublines after synchronization in G1 phase, plating at 1 X 10^5 cells/mL in SYN, and incubation for 72 hours under the following conditions: no hormone (control), PRL (1ng/mL), DEX

(100nM), and PRL (1ng/mL) ± DEX (100nM). In the absence of PRL and DEX, there was little or no increase in cell number above the plating concentration. There was a significant increase in cell number for both lines after treatment with PRL illustrating the dependence of these sublines upon PRL for mitogenesis. The magnitude of the response to PRL was much greater for the Nb2-11 line, which showed a 13-fold increase in cell number compared to a 4.3-fold increase for the U-17 line. Co-incubation of PRL + DEX resulted in a significant inhibition of mitogenesis in both sublines.

Figure 3 illustrates the mitogenic assay results for the SF and D5 lines. Both sublines exhibited pronounced hormone-independent proliferation after 72 hours with the SF cell concentration increasing almost 19-fold and D5 cells increasing by almost 16-fold above the plating concentration. Incubation with PRL resulted in no further significant increase in cell concentration of either cell line. Incubation with DEX produced no significant effect in either cell line in the presence or absence of PRL. However, it is noteworthy to mention that the number of SF cells incubated with DEX in the absence of PRL was about half that of the SF cells in the control group.

3.3 Log Phase Nb2 Cell Responsiveness to 24-Hour Treatment with DEX ± PRL (Cytolytic Assay)

Figure 4 illustrates similar cytolytic effects of DEX ± PRL for the U-17 and Nb2-11 lines. Both lines showed an increase in the percentage of cell death after being subjected

to a 24-hour treatment with DEX (100nM). The U-17 subline exhibited a 2.3-fold increase in the percentage of dead cells after DEX treatment, whereas the Nb2-11 subline exhibited a 3.6-fold increase in the percentage of dead cells under the same conditions. Co-incubation of DEX with 1ng/mL PRL blocked DEX-induced cytolysis/apoptosis both cell lines.

Figure 5 shows the functional responses of the SF and D5 lines after a 24-hour treatment with DEX ± PRL. The SF and D5 lines exhibited no significant change in cell death percentages in response to DEX, indicating that the cells are resistant to DEX-induced cytolysis/apoptosis. Co-incubation of DEX with 1ng/mL PRL had no significant effects.

The cytolytic data collected for the D5 subline was split into two subsets ("D5" and "Resurrected D5") and analyzed separately. The D5 cells were split into two subsets due to the appearance of different functional behavior between batches of cells that had been cultured for more than two weeks called "D5" and those cultured less than two weeks post-resurrection from -70°C called "resurrected D5". Although statistical analysis revealed no significant differences in the treatments for the "resurrected D5" cells, these cells appeared to be functionally different from those perpetuated for over two weeks ("D5"). The resurrected cells have a much higher basal cell death percentage than the latter, and they appear to have some sensitivity to DEX-induced cytolysis, as indicated by a 23.9 % increase in cell death percentage from the control death percentage. In addition,

the PRL appears to inhibit the cytolytic effects of DEX in the newly resurrected cells, as indicated by death percentages comparable to the control.

3.4 TUNEL Method Performed on Control (DMSO) and 24- Hour DEX Treated Cells

Figure 6 is a photomicrograph of the representative TUNEL staining observed in the control and DEX treatments of cytolytic assays for the Nb2-11 and U-17 cell lines. In both cell lines, DEX exposure increases TUNEL labeling confirming that DEX-induced cytolysis reflects apoptosis, which is consistent with previous reports from this laboratory (Fletcher-Chiappini, et al., 1993; LaVoie and Witorsch 1995; Gaunzon 1998; Patel 1999; Rebano 1999). In contrast to that observed for the Nb2-11 and U-17, DEX failed to increase TUNEL labeling in the SF and D5 lines (Figure 7) consistent with the cytolytic assay results.

3.5 Immuncytochemical Assays Performed on Log Phase Nb2 Cells

Figures 8 through 12 are photomicrographs taken of the representative staining produced for the control (vehicle 0.1% BSA) and each marker (NFκB, IκBα, STAT 5b, and GR) in all four cell lines. These figures indicate no striking differences in location or intensity of staining between any of the markers among the four lines.

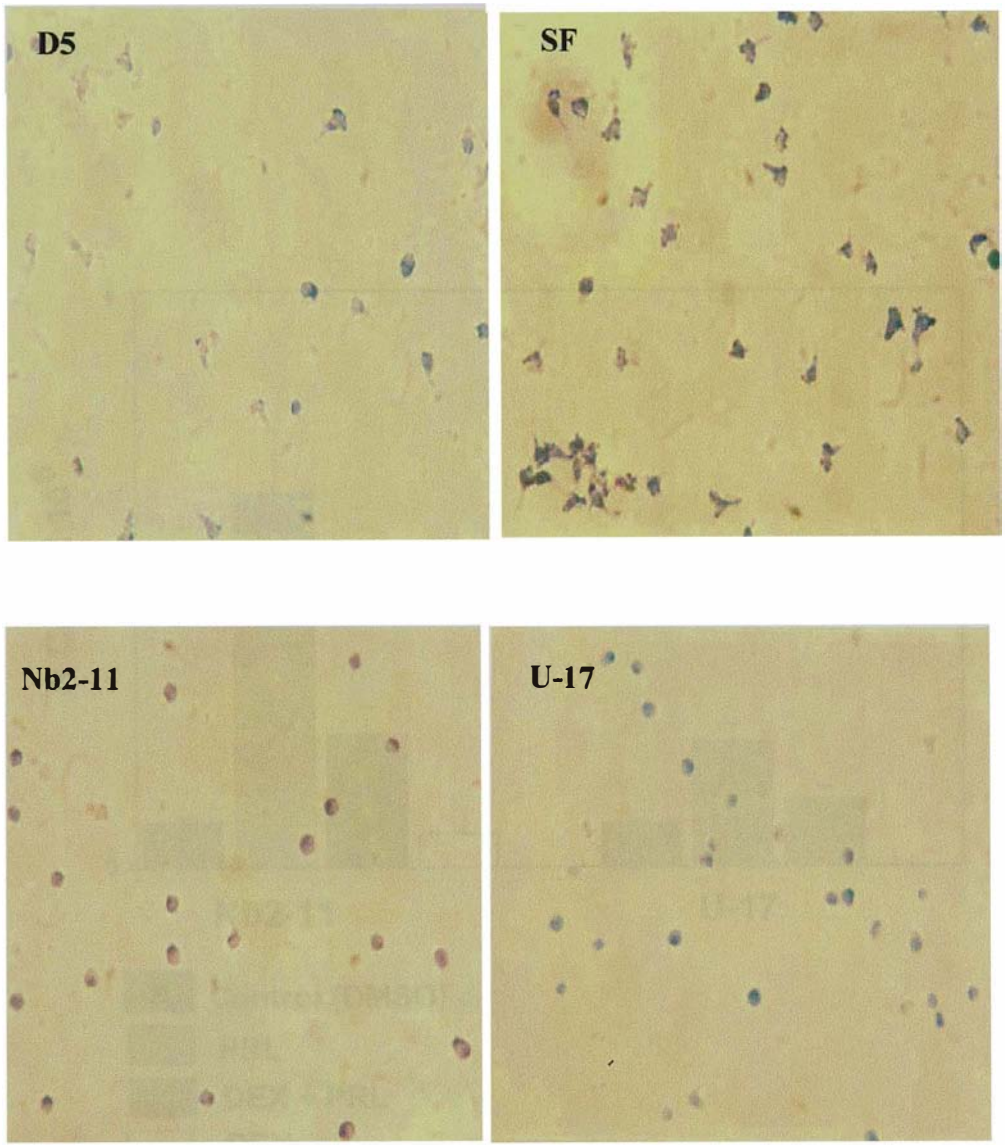

FIGURE 1. Wright's Geimsa stain applied to log phase Nb2 cells. Morphological differences between PRL-dependent (Nb2-11 and U-17) and -independent (D5 and SF) sublines are illustrated above (80X).

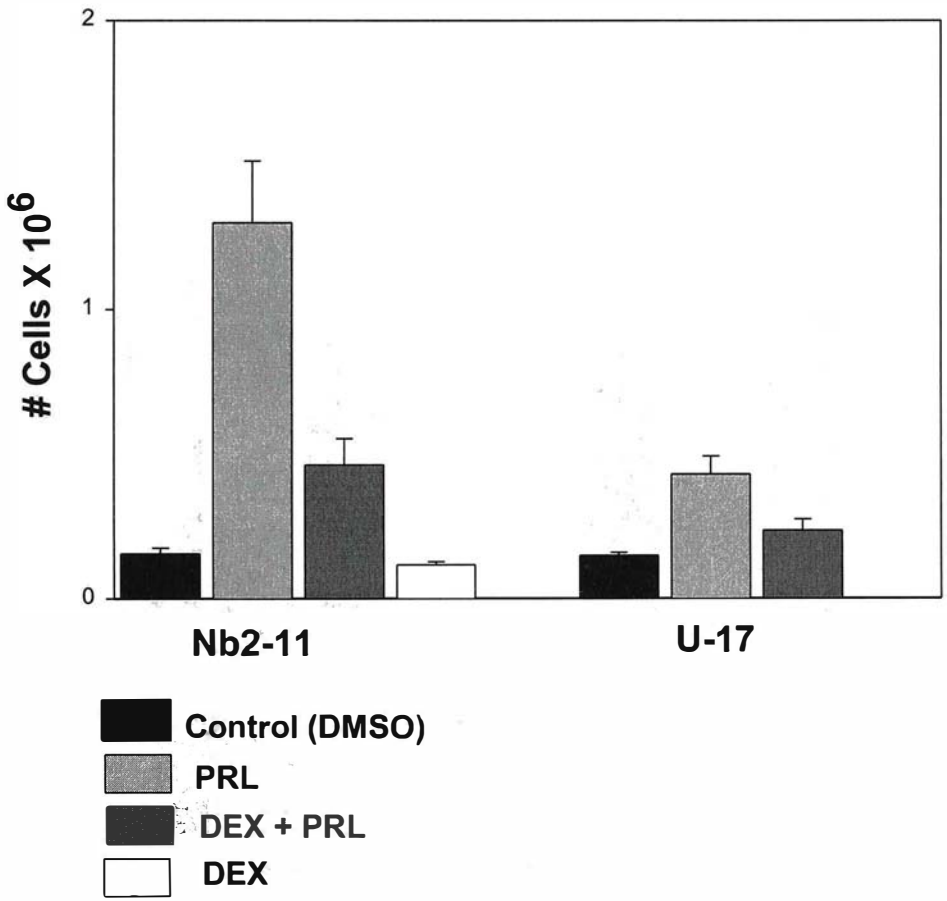

FIGURE 2. Effect of DEX +/- PRL on concentration of Nb2-11 and U-17 cells. Both cell lines show significant increase ($p<0.05$, $n=5$) in cell concentration after 72-hour PRL (1ng/mL) treatment. PRL-induced mitogenesis was prevented by co-incubation with DEX (100nM).

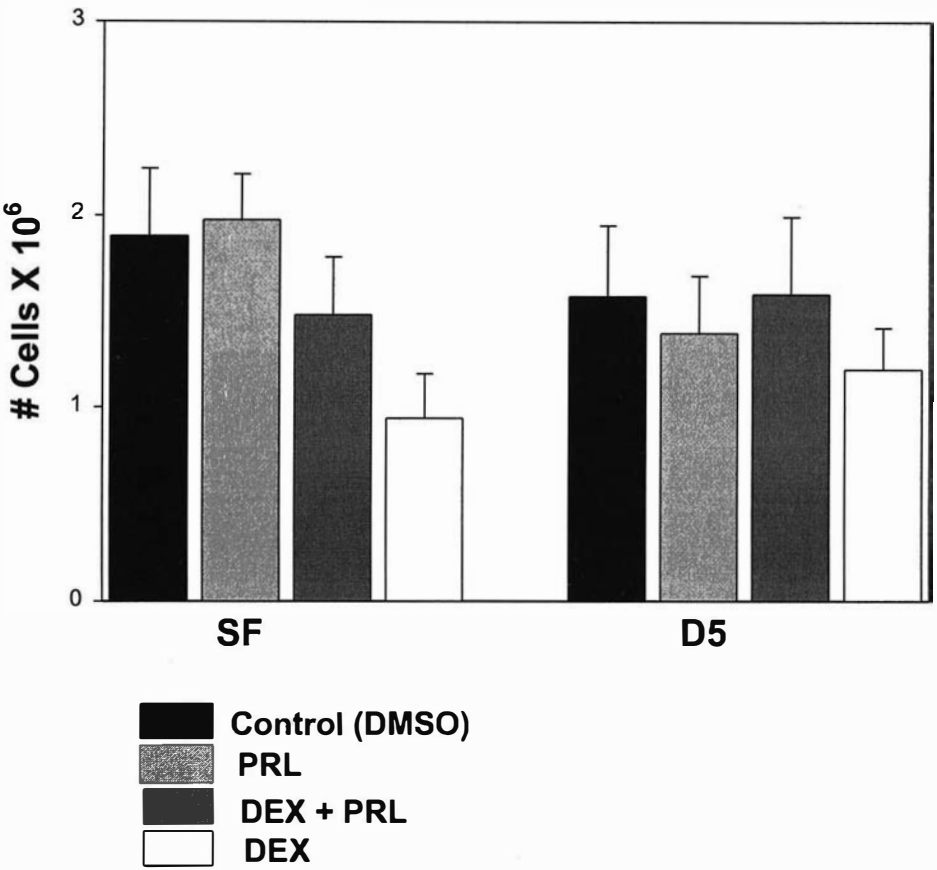

FIGURE 3. Effect DEX +/- PRL on concentration of SF (n=5) and D5 (n=4) cells. No significant differences ($p>0.05$) were found between any of the treatments. However, 72-hour DEX treatment appears to inhibit mitogenesis in the SF line.

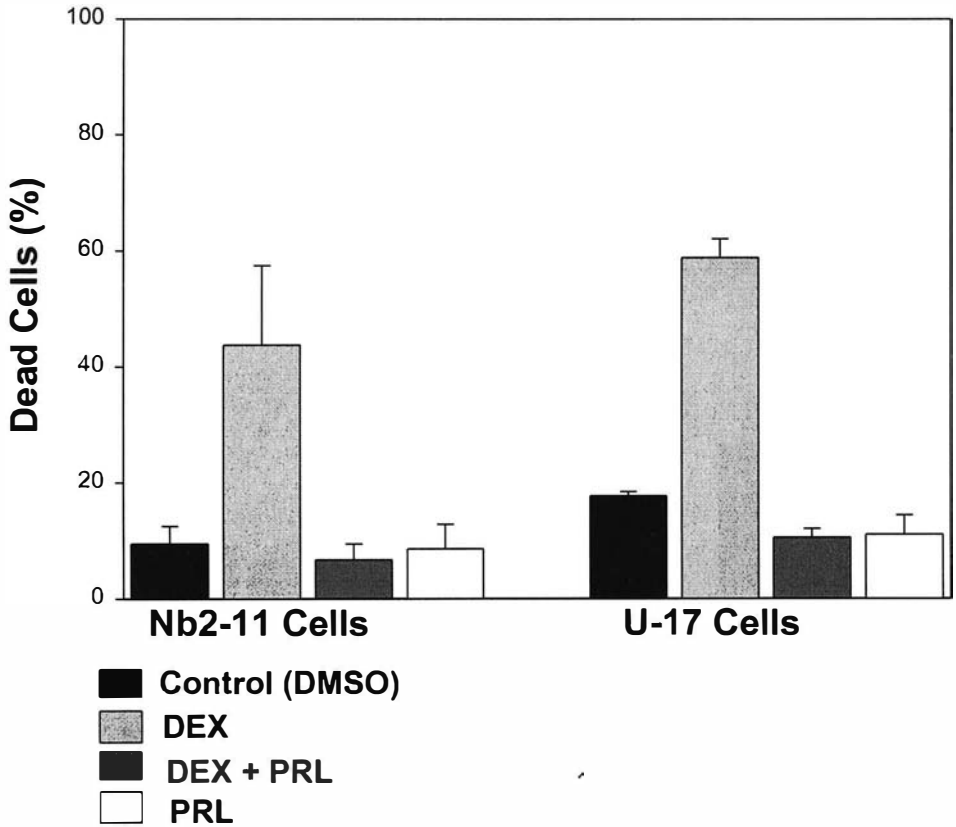

FIGURE 4. Effect of DEX +/- PRL on viability of Nb2-11 and U-17 cells. Both cell lines show significant increase ($p<0.05$, $n=5$) in cytolysis after 24-hour DEX (100nM) treatment. DEX-induced cytolysis is inhibited by co-incubation of PRL (1ng/mL) with 100nM DEX.

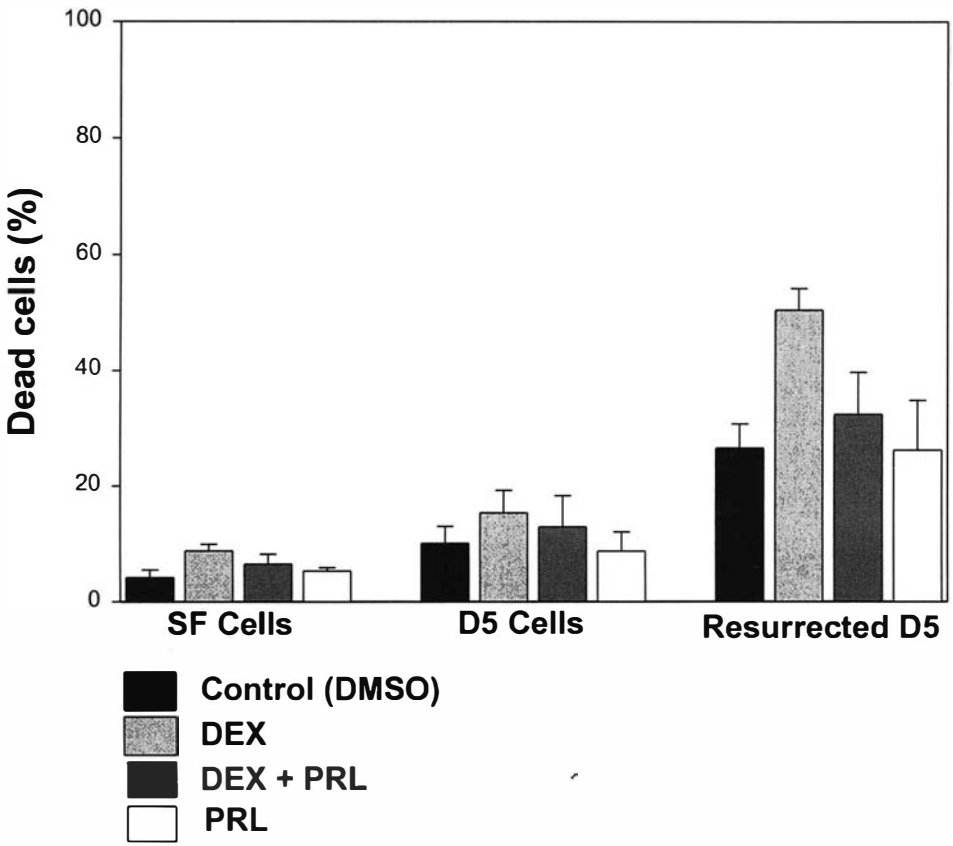

FIGURE 5. Effect of DEX +/- PRL on concentration of SF (n=5), D5 (n=4), and resurrected D5 (n=3) cells. No significant increases ($p>0.05$) were found for any of the cell groups. However, D5 resurrected group appeared to respond to 24-hour DEX treatment, and appeared to exhibit inhibition of cytolysis when co-incubated with PRL and DEX.

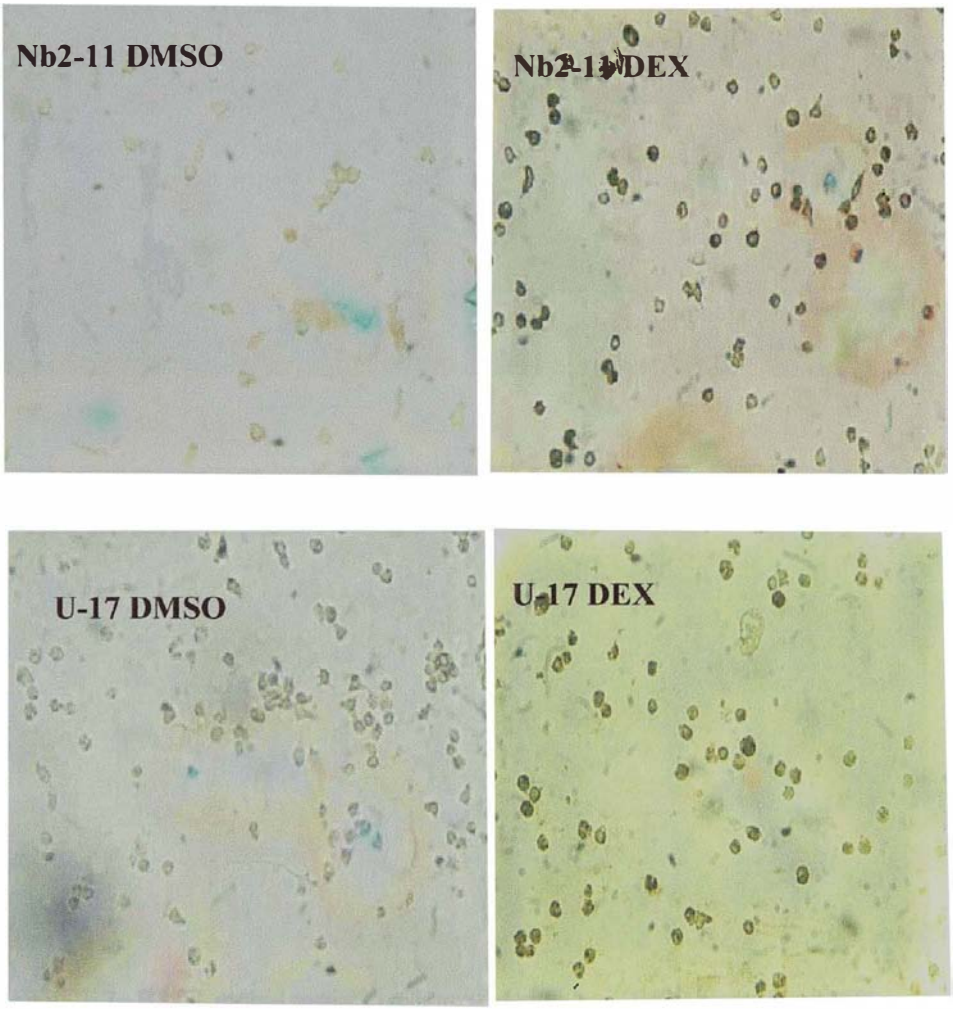

FIGURE 6. Both sublines show increased labeling of cells after 24-hour DEX exposure consistent with DEX-induced apoptosis as measured by trypan blue exclusion (80X).

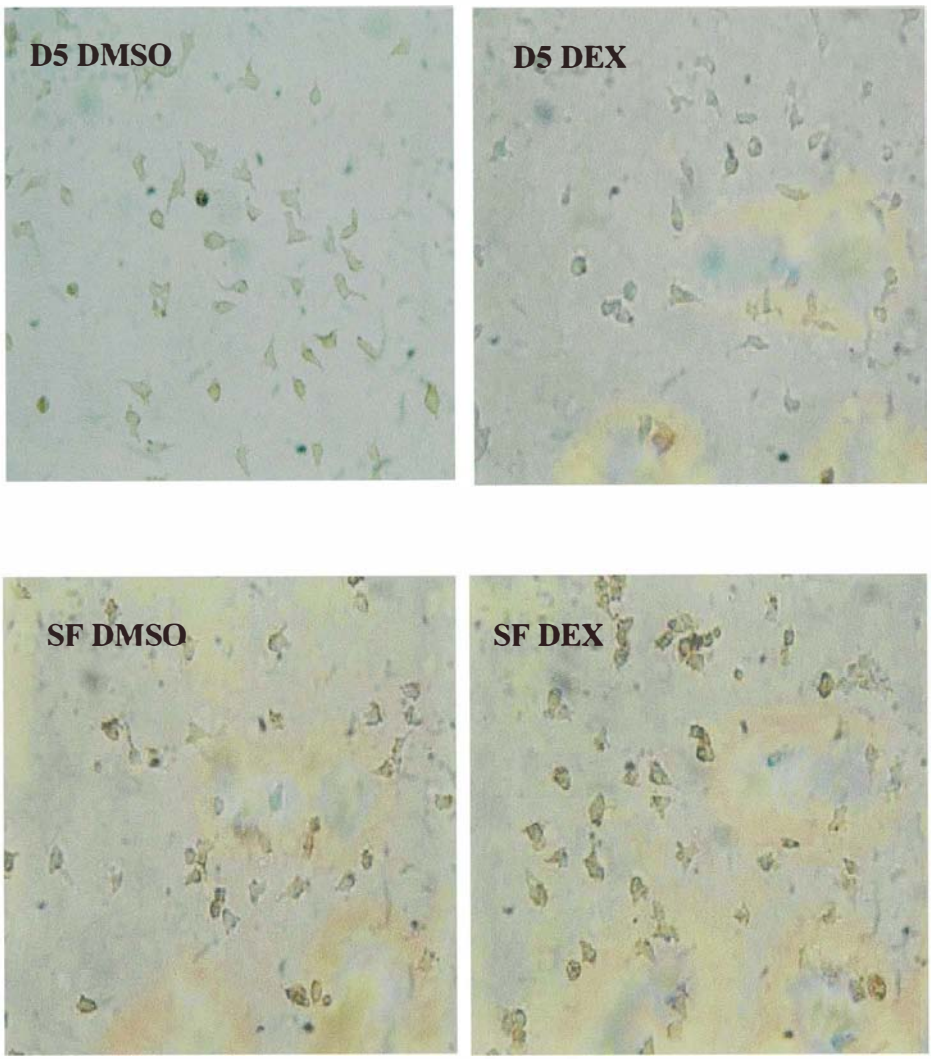

FIGURE 7. TUNEL method applied to cells exposed to 24-hour DMSO (control) and DEX treatments. Both sublines show little evidence of staining consistent with cytolytic assay results as measured by trypan blue exclusion (80X).

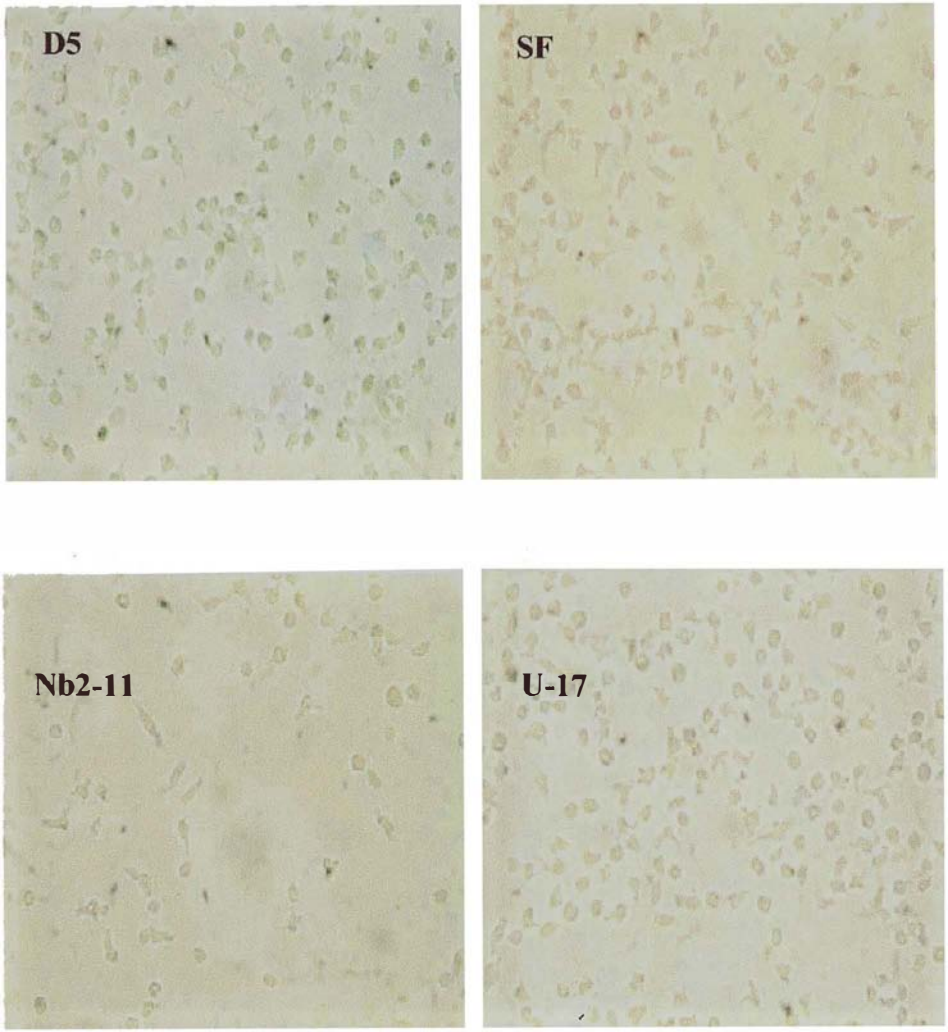

FIGURE 8. Negative immunocytochemical control treatments of log phase Nb2 cells illustrate an absence of staining when cells are treated with 0.1% BSA instead of primary antibody (80X).

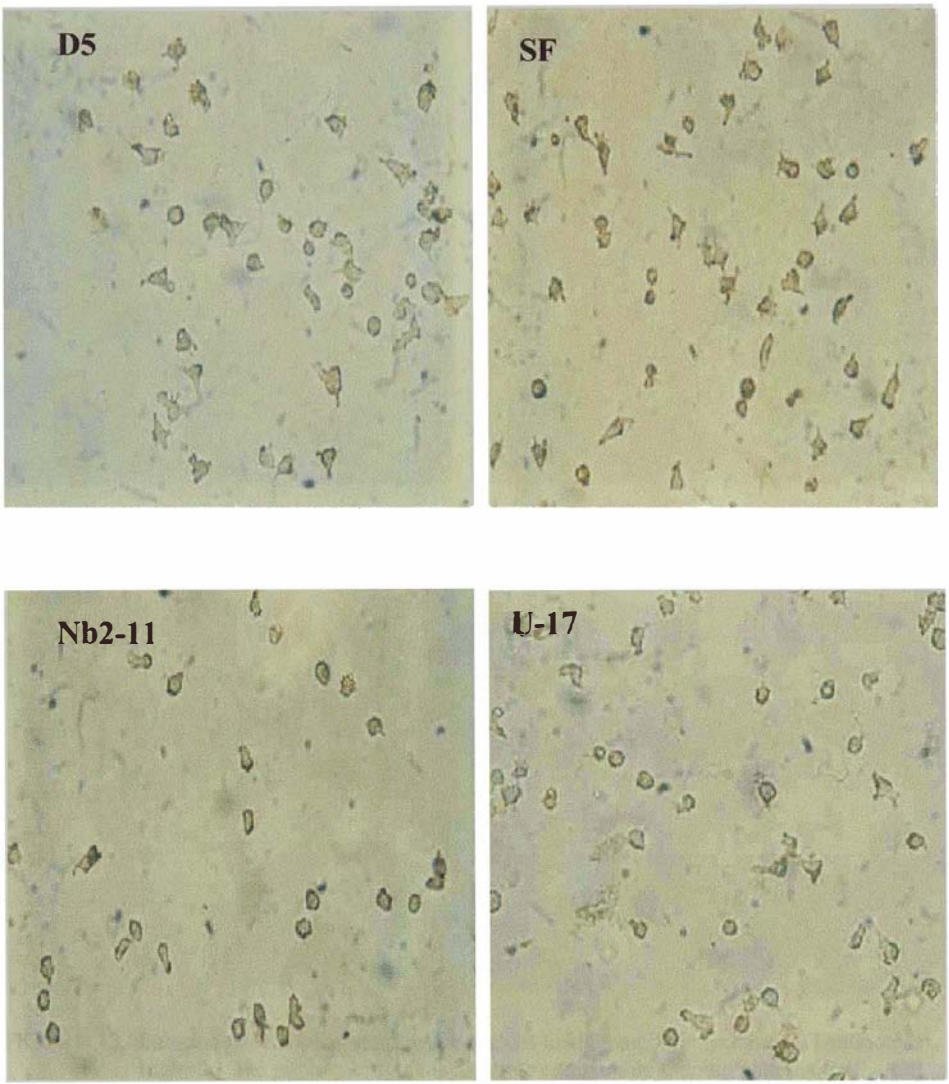

FIGURE 9. Immunocytochemical staining for NFkappaB using a rabbit polyclonal IgG anitbody at 4ug/mL. The pictures illustrate comparable patterns of staining in all four cell sublines (80X).

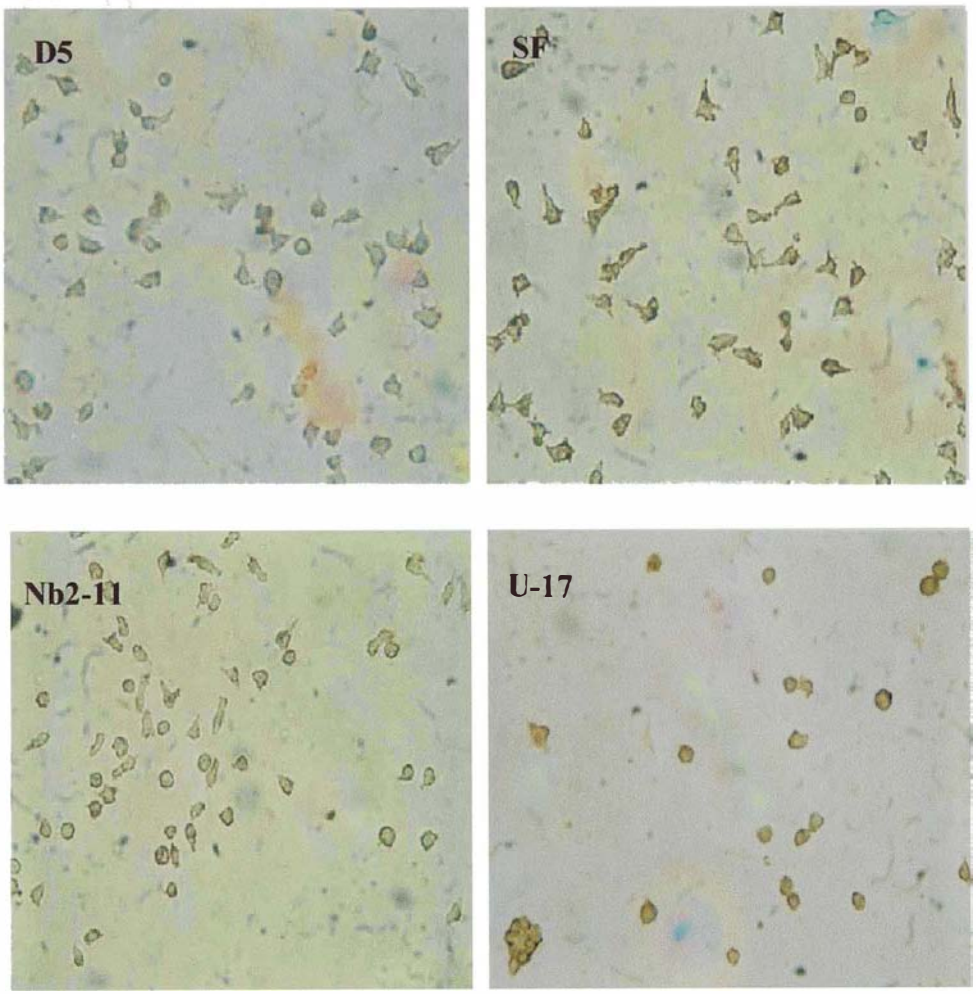

FIGURE 10. Immunocytochemical staining for the GR using a rabbit polyclonal lgG antibody at 4ug/mL. The photomicrographs illustrate comparable staining patterns for the GR in all four sublines (80X).

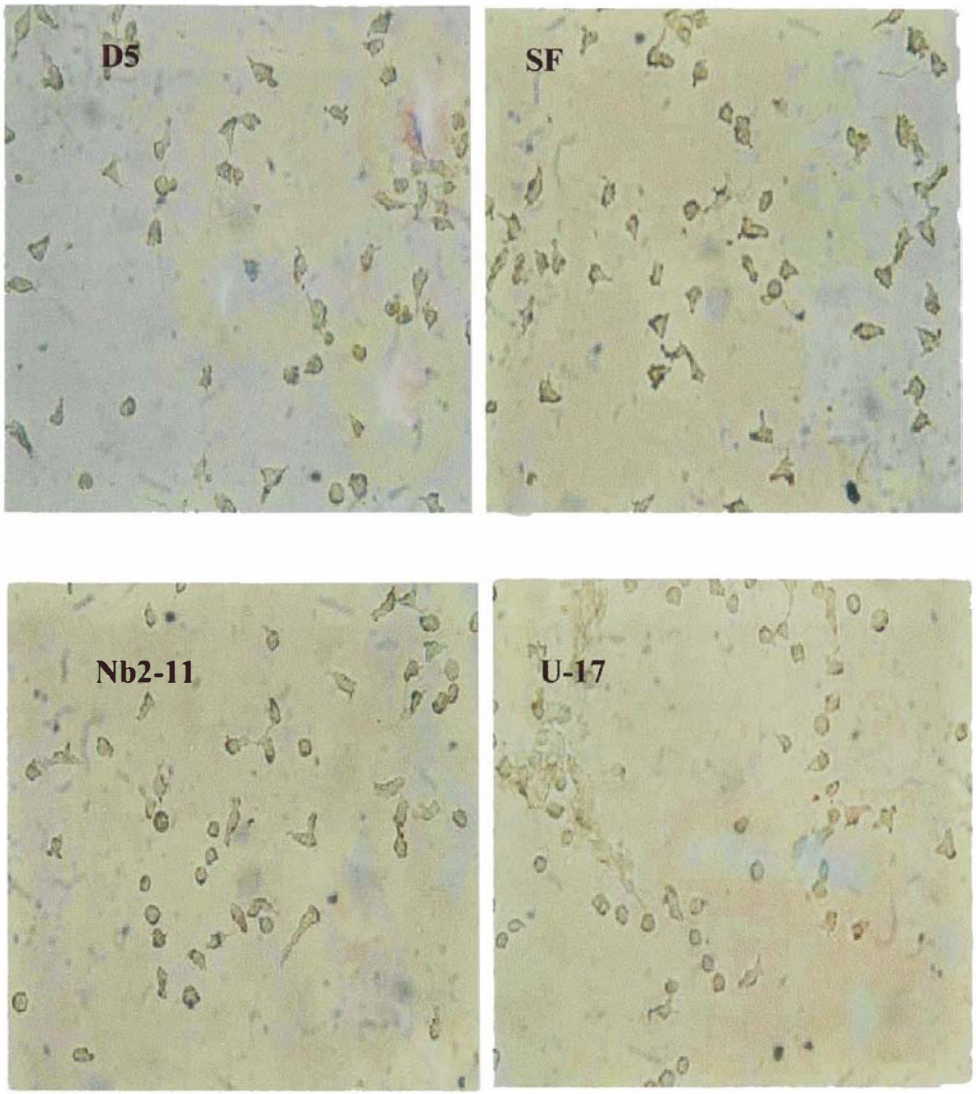

FIGURE 11. Immunocytochemical staining for IkappaBalpha using a rabbit polyclonal IgG antibody at 2ug/mL. The photomicrographs illustrate comparable staining for IkappaBalpha in all four sublines(80X).

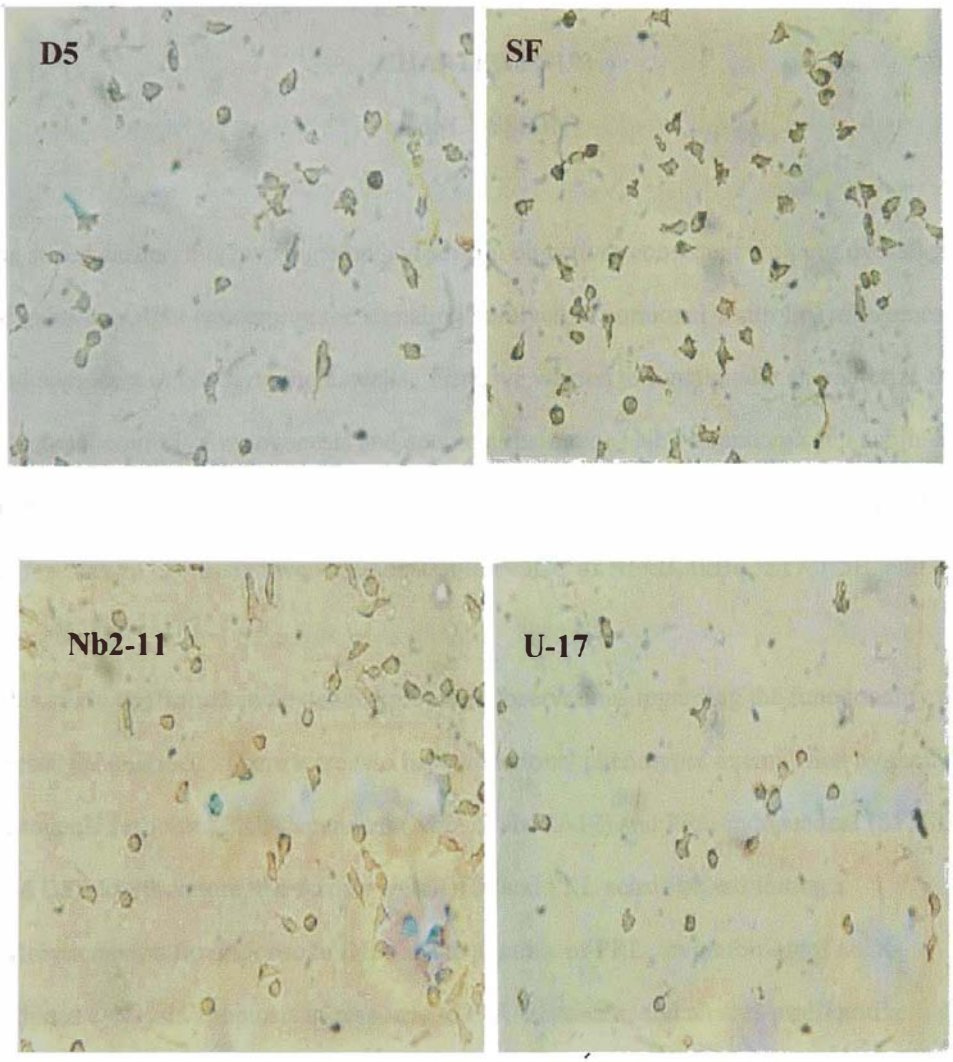

FIGURE 12. Immunocytochemical staining for STAT5B using a rabbit polyclonal IgG antibody at 2ug/mL. The photomicrographs illustrate comparable staining patterns for STAT5B in all four sublines (80X).

CHAPTER FOUR

DISCUSSION

As stated earlier, this investigation had several objectives consistent with our overall goal of gaining insight concerning the signaling involved in hormonal control of mitogenesis and apoptosis of Nb2 lymphoma cells. First, we wanted to functionally characterize the hormonal control of mitogenesis and apoptosis using four Nb2 lymphoma cell sublines as models (U-17, Nb2-11, SFJCD1, and D5). Secondly, we hoped to determine whether any differences in expression were demonstrable by ICC of NFκB, IκBα, STAT 5B, and GR.

This study confirmed and extended previous observations regarding the functionality of these Nb2 sublines. There were two basic functional phenotypes exemplified by their mitogenic response, PRL-dependent (Nb2-11 and U-17) and PRL-independent (SFJCD1 and D5). Furthermore, the former were DEX and PRL sensitive, exhibiting a cytolytic/apoptotic response to DEX in the absence of PRL, an inhibition of DEX-induced cytolysis/apoptosis in response to PRL treatment, and an anti-proliferative response when DEX is co-incubated with PRL. These data are entirely consistent with previous studies in Nb2-11-related sublines (Fletcher-Chiappini, et al., 1993; LaVoie and Witorsch 1995; Gaunzon 1998; Patel 1999; Rebano 1999). To the best of our knowledge

the data on DEX responsiveness and anti-apoptotic effects of PRL for the U-17 subline have not been previously reported.

In contrast to the U-17 and Nb2-11, the SF (SFJCD1) and D5 sublines proliferated robustly in the absence of PRL and in our hands, appeared to be PRL-insensitive. Previously, Gout et al. documented that the SF subline exhibited a slight sensitivity to PRL and the D5 was more sensitive than the SF (Gout 1987; Gout, et al., 1994; Gout, et al., 1997). The reason for the discrepancy between Gout's findings and those in our laboratory is unknown. Perhaps the differences are methodological or are due to the adaptive nature of cancer cells themselves. Minute differences may exist between different batches of cells that are as of yet undocumented. In contrast to PRL-dependent cells, the SF and D5 sublines exhibited DEX-resistance. No significant DEX-induced anti-proliferation was observed nor was there any indication of DEX-induced cytolysis/apoptosis in either line. PRL-independent proliferation is well established for these two lines (Witorsch, et al., 1993; Gout 1987; Gout, et al. 1994; Gout, et al., 1997); however, the lack of DEX sensitivity exhibited by the SF is inconsistent with a previous report from this laboratory. Witorsch et al. (1993) reported that DEX significantly inhibited PRL-independent mitogenesis of SF cells. It is noteworthy, however, that while no statistically significant effect of DEX on SF cell proliferation was demonstrable, the mean number of cells found in SF cells exposed to DEX for 72 hours was about 50% of the control. The lack of statistical significance under these conditions could reflect a "false negative" or type II or beta error resulting from an imprecision in the assay due to

inadequate sample size (n=5) or high variability in the results. Alternatively, the lack of statistical significance could indicate a change in DEX-sensitivity over time (i.e. current batch of SF cells versus those examined previously). As shown in other work in this laboratory (discussed below, Gannon 2000) hormone sensitivity of the Nb2 lymphoma can change with long-term culturing of the cells.

Cytolytic assay data suggest a change occurs over a short period of time after resurrection in the D5 subline. In our previous experience, no such changes have been observed after resurrection in other lines. Freshly resurrected D5 cells appeared to behave like PRL-dependent cells exhibiting sensitivity to DEX-induced cytolysis and anti-apoptosis in response to PRL treatment, in addition to high basal cell death. While it is noteworthy that no statistical significance was observed (again reflecting a possible type II error; n=3), this apparent difference shortly after resurrection, as compared to behavior after two weeks of culture post-resurrection, could suggest some transformation that this subline might undergo, or that this subline is unusually compromised by freezing or needs time to assume its full potential after resurrection. In support of the possibility that these cells may have undergone transformation, Buckley et al. demonstrated that treating the SF line with a differentiating agent (sodium butyrate) results in a transient reversion back to PRL-dependence and a decrease in the level of *pim-1* expressed, and most importantly, the SF line becomes sensitive to DEX-induced apoptosis (Buckley, et al., 1997). Buckley's work suggests that PRL-dependence and DEX sensitivity are acquired through cell dedifferentiation, which may be what we are observing in the D5 line as

evidenced by their DEX responsiveness shortly after resurrection and their loss of responsiveness after approximately two weeks.

Cancer cells are known to change their phenotype with time, which usually correlates with malignant progression (Gout, et al., 1994). Phenotypic changes include the loss of growth requirements of specific hormones or growth factors, increases in growth rate, development of drug resistance, tissue invasiveness, metastatic ability, and drug resistance (Nowell 1986; Nicolson 1987). It has been specifically documented that lymphoid cancers that are initially responsive to GC treatment develop a resistance to GC's after prolonged treatment (Feinman, et al., 1999; Klumper, et al., 1995). We may be witnessing some of these phenotypic changes in the D5 line after resurrection. Gout *et al.* documented the change in growth and size over a 2.3-year period of an Nb2 lymphoma line (called Nb2-PRA) after splitting the line into two independent cultures (Gout, et al., 1994). Initially both cultures showed a gradual decrease in doubling time. Then one of the cultures spontaneously increased its doubling time in conjunction with the appearance of substantially larger cells, which eventually became the predominant cell type. In contrast, this larger cell type never developed in the other culture and the doubling time eventually stabilized at 13 hours (Gout, et al., 1994). Although the changes we witnessed in the D5 line resulted in DEX resistance, the cells were not cultured with DEX, and therefore their resistance to DEX-induced apoptosis cannot be attributed to prolonged exposure to DEX. Perhaps like the changes in the Nb2-PRA line, the D5 is undergoing a non-induced change in phenotype related to the progression of

that particular type of cancer cell. This phenotypic change consequently affords the D5 line with DEX resistance. The patterns observed in D5 and resurrected D5 (Figure 5) is an extremely rapid occurrence (less than two weeks), suggesting that cancer cells have the ability to change their functional behavior very rapidly.

The current investigation was paralleled by another investigation in the laboratory that studied the effects of long-term culture on an Nb2-11 line, similar to the investigation Gout et al. undertook with the Nb2-PRA line. Unlike the Gout's investigation, no obvious morphological changes were documented in the cells. However, phenotypically an Nb2-11 subline cultured for a year or more (named LTI for long-term incubation) became autonomous and less sensitive to DEX-induced apoptosis, suggesting that with time the Nb2-11 is undergoing malignant progression with long-term culture. This study also revealed a relatively rapid transformation of cells deprived of mitogen (FCS) (named PI for prolactin-independent). In addition to a loss of DEX sensitivity, both the LTI and PI exhibited hormone-independent proliferation (Gannon 2000). Thus, the sublines recently generated in our laboratory are similar functionally to the SF and D5 lines.

In contrast to the similar cytolytic assay results for the Nb2-11 and U-17 lines (Figure 4), the mitogenic assay results showed some variation in PRL responsiveness between the two lines. The Nb2-11 line gave a much more robust response to PRL producing a 13-fold increase in growth from baseline, while the U-17 only showed a 4.3-fold increase in cell number (Figure 2). In fact, the Nb2-11 line has been found to have a faster growth

rate when perpetuated in culture than the U-17 (unpublished results). Gout et al. documented a 14-hour doubling time for the Nb2-11 (Gout, et al., 1994; Gout, et al., 1997), which is in agreement with our records. This is significantly faster than the 17-24-hour doubling time for the U-17 (unpublished results), which may account for the more robust response of the Nb2-11 to PRL.

The inhibition of cell proliferation by DEX observed in the PRL exposed Nb2-11 and U-17 sublines, and possibly the autonomous SF subline in the mitogenic assay, may be due to cell cycle control mechanisms. King and Cidlowski found that glucocorticoid treatment of lymphoid cell lines expressing markers of immature thymocytes results in G1 arrest, and in most cases, growth inhibition is followed by cell death (King and Cidlowski 1998). Perhaps the observed inhibition of mitogenesis is actually an arrest in the G1 phase of the cell cycle.

In addition to the previously documented phenotypic differences among the Nb2 sublines supported by these studies, certain other functional and genotypic characteristics are worth noting. The U-17, Nb2-11, and SF lines all induce the growth of tumors when implanted subcutaneously in Noble rats, but only the Nb2-11 and SF lines metastasize to other sites in the animal (Gout, et al., 1994). Karyotyping of these three lines has shown the U-17 and Nb2-11 lines to be identical with both displaying five additional changes with respect to the normal Noble rat karyotype, and the SF line has six additional changes with respect to the U-17 karyotype (Horsman, et al., 1991). The identical nature of the

U-17 and Nb2-11 makes the differences in functional behavior between these two lines all the more intriguing.

The D5 line has not been studied as extensively as the U-17, Nb2-11 or SF lines. The functional results obtained from our investigation suggest that the D5 is probably closer to the SF in karyotype than the parent line. However, karyotyping of the D5 line has revealed that this line has no greater than two additional chromosomal changes compared to the parent line (Gout, et al., 1997). These two or fewer changes then must be responsible for a radically different phenotype compared to the parent line. The chromosomal and subsequent behavioral alterations associated with malignant progression in the Nb2 line suggest that the resistance to treatment seen in the SF and D5 lines is probably attributable to many cooperative changes.

The phenotypic differences observed when comparing the U-17 and Nb2-11 to the D5 and SF may reflect differences in intracellular signaling. While numerous signals could be implicated in self-proliferation and resistance to DEX-induced cytolysis/apoptosis this study considered the GR, STAT 5B, NFκB, and IκBα. As reviewed in the introduction, the GR is critical to initiate DEX-induced apoptosis. STAT 5B, a component of the JAK/STAT cascade mediates PRL-induced effects. ÍκBα, which sequesters NFκB in the cytoplasm of the cell, is involved in GC-mediated effects and prevents NFκB from translocating to the nucleus (Hettman, et al., 1999; Whiteside and Israël 1997) making it a pro-apoptotic factor. One would suspect, therefore, that a self-proliferating, DEX-

resistant cell line might exhibit increased expression of STAT 5B and NFκB, and reduced expression of the GR and IκBα, or a combination thereof relative to the Nb2-11 and/or U-17 cells. In support of this hypothesis it has been documented that STAT 5 represses the GC response (Pfiztner, et al., 1998), supporting the hypothesis that increased STAT 5B could prevent GC-induced apoptosis. However, our preliminary ICC studies reveal no striking differences in the immunostaining of the four signals of interest among the four Nb2 sublines. These negative findings must be interpreted cautiously. As it is currently performed, our ICC methodology may be inadequate to detect changes in the expression of proteins. Our current method is designed to produce maximal staining intensity, and this may be insensitive to modest changes in signal expression. Hence, we are only able to differentiate between cells that express antigen and those that do not (Gaunzon 1998; Badarinath 1999; Patel 1999; Rebano 1999). In other words, we might detect a change in the proportion of cells stained under varying conditions. However, the assay will have to be modified in order to detect changes in the levels of antigen expression (i.e. staining intensity differences on an individual cell basis). This requires titering of the primary antibody to the dose-response curve relating protein content to staining intensity. Such modification in ICC methodology is currently under development in our laboratory.

In addition, changes in antigen expression may not be a function of the physiologic phenotype, but of the physiologic status of the cells (i.e. the state of hormonal manipulation). In the current ICC studies, assays are performed on cells in log phase growth. The Nb2-11 and U-17 cells proliferate in response to lactogenic medium

(medium supplemented with FCS), whereas the SF and D5 are self-proliferating sublines. Changes in signal expression may, therefore, require such changes as removal of mitogen or exposure to DEX prior to the ICC assay.

Alternatively, other signals may be implicated in the phenotypic differences or the mechanisms involved may be of increased complexity. It has been previously observed that the SF line has the ability to increase glutathione production in response to oxidative stress (Meyer, et al., 1998). Therefore, perhaps pretreatment with DEX ± PRL may be required to produce noticeable changes in the concentrations of NFκB, IκBα, STAT 5b, and the GR. It been reported that levels of cysteine and glutathione in a human T cell line can modulate the activity of NFκB (Gout, et al. 1994). L-cystine or rather its reduced form cysteine is an essential amino acid required for mammalian lymphocytes, and a number of malignant human and animal cell lines of lymphoid origin, which lack the ability to synthesize the amino acid (Inglhart, et al., 1977; Ishii, et al., 1981). The Nb2-11, U-17, and D5 lines must be grown in media supplemented with 2-mercaptoethanol (2-ME) in order to synthesize cysteine (Gout, et al. 1997). On the contrary, the SF line has an enhanced cystine uptake capability affording it with 2-ME independence (Gout, et al. 1997). When the cells are subjected to an experiment involving DEX ± PRL treatment, they are first removed from their respective growth media and placed in serum-free, chemically defined media devoid of 2-ME. The SF is unaffected by the change in media. However, the Nb2-11, U-17, and D5 may undergo changes in NFκB concentration due to a change in media; the activity of NFκB

apparently depends upon optimal levels of cysteine and glutathione (Gout, et al., 1997). Placing the U-17, Nb2-11, and D5 cells in media devoid of 2-ME would inhibit cysteine production, which would in turn lower NFκB levels and make the cells more susceptible to DEX-induced apoptosis. This explanation is consistent with the functional response of the U-17 and Nb2-11, but it is inconsistent with the DEX-insensitivity we see in the D5 line. Therefore, although cysteine levels may play a role in the sensitivity to DEX-induced apoptosis, cysteine levels alone do not dictate functional behavior.

It has been reported that DEX-insensitive thymocytes possess fully functional GRs, and the molecular weight and abundance of the receptor does not change after DEX treatment, indicating that the GR is neither down-regulated nor mutated (Oldenburg, et al., 1997). Interestingly though, treatment with non-synthetic glucocorticoids has been reported to cause down-regulation of the GR for up to 72 hours after treatment (Rosewicz, et al., 1988). The reason for this discrepancy is unknown. The immunocytochemical staining intensity and concentration of the GR was similar for all four lines, but again, the ICC studies were performed on log phase cells. Therefore, future studies should involve comparison of log phase versus DEX treated cells in order to ascertain the integrity and concentration of the GR, and to confirm the studies performed by Oldenburg.

In the SF line $p21^{ras}$ and mitogen activated protein kinase (MAPK) are both constitutively active, but in hormone-dependent cultures such as the Nb2-11, PRL is necessary to

activate this signaling pathway in conjunction with the JAK2/STAT cascade (Buckley, et al., 1997). This suggests that the enhanced Ras and MAPK expression found in SF cells may be responsible for PRL-independence and cooperative in preventing apoptosis (Buckley, et al., 1997).

In conclusion, our findings indicate that major differences in functional behavior exist between the PRL-dependent and PRL-independent lines, as evidenced by cytolytic and mitogenic assay results. These differences in behavior are probably not attributable to any one protein signal or chromosomal change, but are most likely due to progressive changes in phenotype that accompany malignant progression (Nowell 1986). Future studies will involve a more focused immunocytochemical investigation of protein markers.

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