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APOPTOSIS PREVENTION OF NON HODGKIN'S LYMPHOMA CELLS 
IN CULTURE BY HUMAN PLASMA 

by 

MARCELA VICTORIA GRONDA 

A thesis submitted in conformity with the requirements for the Degree of Master of Science, Department of Medical Biophysics University of Toronto

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ABSTRACT:

Non Hodgkin’s Lymphoma (NHL) is a disease characterized by the clonal expansion of malignant cells of B or T origin. A culture system was developed in our laboratory that allowed the establishment of 20 NHL cell lines from direct samples from patients with NHL. Their further characterization in terms of growth regulation and genetic changes provided the opportunity to examine pathogenic mechanisms.

I was able to demonstrate that human platelet poor plasma (PPP) is required by NHL cells for proliferation and survival. Its withdrawal induces programmed cell death in all tested cell lines except one.

Proliferative and survival signals provided by PPP could not be replaced by two fully defined media: one composed of bovine serum albumin, human transferrin and bovine insulin and a second, a commercially available preparation (Stem Pro 34®) containing proprietary reagents. The addition of cytokines to both serum replacement media did neither improve proliferation nor survival.
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INTRODUCTION:

ESTABLISHMENT OF NHL CELL LINES

Non Hodgkin's lymphoma (NHL) is a disease characterized by the clonal expansion of malignant cells of B or T origin. Patients with NHL are heterogeneous with respect to their clinical presentation, histologic appearance, immunophenotype, and response to therapy. Patients with bulky disease and high proliferative potential are of poor prognosis. Evaluation of growth promoting activities may provide novel insight into the pathogenesis of NHL. For this reason a culture system was developed in Dr. Messner's laboratory to identify and evaluate clonogenic NHL progenitor cells and to study their growth requirements. Samples of bone marrow, peripheral blood, lymph nodes, ascites, pleural and cerebrospinal fluid directly obtained from NHL patients were cultured in a semi-solid system containing human platelet poor plasma (PPP) and Phytohemagglutinin Stimulated Leukocyte Conditioned Medium (PHA-LCM) as sources of nutrients and growth factors. After 14 days in culture, primary lymphoma colonies were observed in approximately 40% of tested samples from patients with intermediate and high grade lymphoma. These colonies were replated and subsequently expanded in liquid suspension culture to generate cell lines (1). All samples that formed NHL colonies gave rise to cell lines. A total of 20 EBV negative cells lines (OCI-LY1 -20) were established under these conditions (2). It is important to note that not all cell lines required the addition of cytokines to grow. However, all samples required the presence of plasma to initiate and sustain growth. Attempts to replace PPP with Fetal Bovine Serum (FBS) failed.
GROWTH OF DISEASE PROPAGATING CELLS IN CULTURE

NHL cells propagated under these conditions were characterized and their properties compared with those of the original tumour cells. That cell lines reflect the disease in each patient was demonstrated by the following observations:

a) The same JH or TCR rearrangements were found in fresh samples and the respective derived cell lines. Cells of the lines raised in this culture system were subjected to clonality studies using appropriate probes for the JH region of the immunoglobulin heavy-chain locus or the constant region of the T cell receptor β-chain to determine the status of the immunoglobulin or T cell receptor gene. Unique immunoglobulin or T cell receptor rearrangement patterns were observed in all cell lines. The respective rearrangement was identical to that seen in the primary sample. Cell lines derived from samples obtained from two separate sites (bone marrow and cerebrospinal fluid) of the same patient also shared the same rearrangement. In addition, cell lines raised from the same patient at presentation and during relapse showed the same rearrangement pattern, demonstrating their origin from the same clone (3, 4).

b) The same genetic lesions were present in original samples and resulting cell lines. Samples obtained from different patients and cell lines derived from these specimens were evaluated for the status of p53 (gene associated with cell cycle arrest after injury), c-myc (gene associated with cell cycle regulation) and bcl-2 (one of the genes involved in prevention of apoptosis). The same alterations were found in original samples and respective cell lines (5). One of the cell lines (OCI-LY 18) showed multiple immunoglobulin gene rearrangements (by using Southern blot analysis) that were also observed in the original patient sample (6). These unusual rearrangements were found to be a stable property of the examined clonogenic cells and were maintained after expansion "in vitro" as well as after
transplantation into SCID mice (7).

c) Cell lines contain disease propagating cells that cause the development of lymphomas “in vivo”.
Two cell lines (OCI-LY 17 and 18) and a fresh tumour sample from a patient with end stage NHL were injected into SCID mice to test for the presence of tumour propagating cells. All three specimens caused formation of tumours with histological and immunological features consistent with the primary lymphoma. Injections of OCI-LY 18 in limiting dilutions indicated that as few as 1000 cells were sufficient to generate detectable tumours in all host animals. This suggests that disease propagating cells represent a rare subpopulation among cells comprising the cell line (7).

d) Growth in culture was associated with poor prognosis.
A series of 69 patients derived samples was studied with this culture system. Specimens of 16 patients formed colonies in culture. None of these patients survived for more than 12 months after initiation of the study. Their survival was significantly shorter compared to that of patients with the same histologic classification whose cells did not grow in culture. The behaviour in cell culture appeared to be of prognostic value, independent of other previously recognized prognostic indicators (2).
GROWTH CONTROL IN NHL

One of the essential components of the culture system is PPP (8). Growth is not observed in the absence of PPP (2). Cells obtained from some patients, however, require further addition of growth factors to promote their growth. These are routinely provided in the form of PHA-LCM as a source of multiple cytokines.

The cell lines were tested to determine whether or not growth of their clonogenic progenitors was dependent on cytokines and whether or not they were capable of producing cytokines endogenously (3). Four different patterns of factor requirement and production were observed:

a) factor dependence without endogenous factor production (OCI-LY 9 and 13.1)

b) factor dependence associated with endogenous factor production (OCI-LY 3; 12; 13.2 and 18)

c) factor independence without endogenous factor production (OCI-LY 1; 2; 4; 7; 8; 10)

d) factor independence with endogenous factor production (OCI-LY 17)

The cytokines produced by the various cell lines included IL 1; 4; 5; 6 and GM-CSF. IL 6 was identified as the predominant cytokine. These patterns may change over time as shown for 2 cell lines raised from the same patient. One line (OCI-LY 13.1) was initiated at diagnosis, the second (OCI-LY 13.2) during therapy resistant relapse. Colony formation by OCI-LY 13.1 was dependent on activities present in PHA-LCM without endogenous production of detectable growth promoting activities. In contrast, OCI-LY 13.2 formed colonies without the addition of factors and released activities into the supernatant that enhanced their own growth and provided stimulatory signals for OCI-LY 13.1. The activity has a molecular weight of 32 kD and has not yet been fully characterized. Studies were performed to determine whether or not the release of growth factors by lymphoma cells influenced the growth of cells with proliferative capability. In general cell lines dependent upon endogenous production of cytokines demonstrate a lag phase during the early culture
period when seeded at low cell density but not if cultures are initiated at high cell density (9, 10). The cell number will increase rapidly after accumulation of the required factor. The lag phase can be overcome by adding the respective factor at initiation of the culture. This concept was used to study two growth factor dependent cell lines OCI-LY 3 and OCI-LY 12. Both cell lines exhibited density-dependent cell growth when cultured at increasing cell numbers. The addition of IL 6 to both cell lines at initiation of cultures abolished the lag phase of cells seeded at low density, whereas the addition of polyclonal anti-IL 6 generated a lag phase for cells seeded at high density (9, 10).

The potential role of IL 6 in the clinical course of NHL was suggested by a study where blood levels of this cytokine were determined in 35 patients. 18 patients exhibited elevated levels of IL6 during active disease. Treatment resulted in a reduction of IL6 levels for patients with intermediate and high grade disease, but not for patients with low grade lymphoma (11).

One of the NHL-cell lines (OCI-LY 17) provided an example of cytokine production that did not influence tumour growth. This line was generated from a peripheral blood sample of a patient with NHL that presented with extensive eosinophilia of 80,000 per mm$^3$. Cells of this line elaborated IL5 and IL6. Both cytokines were biologically active when tested on normal clonogenic eosinophilic precursors (IL5) and on IL6 sensitive cells. However, the cell line itself did not show any increment in proliferation when cultured in the presence of either cytokine, nor was the rate of proliferation reduced when anti-IL 5 or anti-IL 6 antibodies were added (4). Cytokine production in this particular case gave rise to the paraneoplastic syndrome of eosinophilia.

Table I (page 34) summarizes the growth factor production and requirement of all OCI-LY cell lines, as determined with the technology available at the time of the study.
GENETIC EVENTS IN NHL

The marked clinicopathologic heterogeneity of NHL is reflected by various genetic events associated with the development of NHL such as activation of dominantly acting oncogenes (12), deletion and inactivation of tumour suppressor genes (12), viral infections (13, 14), deregulation of cytokine pathways (15, 16, 17) and chronic antigen activation (18, 19). Based on available information NHL can be defined as a progressive accumulation of malignant cells that originate from a single transformed lymphoid cell as a consequence of multiple, sequential genetic events (20). These genetic changes are likely responsible for the increased proliferation of disease propagating cells, their aberrant maturation profiles, and immortalization. The concept of sequential genetic events is suggested by a series of clinical observations and studies in animal models. There are several chromosomal alterations associated with NHL. In most of the cases these lesions involve oncogene loci such as c-myc in intermediate and high grade lymphoma (12, 21), bcl-1 (22, 23), bcl-2 (24, 25), bcl-3 (26, 27), bcl-6 (28, 29) in patients with low grade and p53 in patients with Burkitt’s lymphoma (high grade) (30). Nearly 90% of follicular B-cell lymphomas and 20% of large cell diffuse B-cell lymphomas are characterized by a 14;18 translocation [t(14;18)] which involves the immunoglobulin locus on chromosome 14 and bcl-2 locus in chromosome 18 (31, 32). The association of bcl-2 (33, 34) with the heavy chain locus results in over expression of bcl-2, which in turn will prevent or at least delay cells from undergoing programmed cell death (apoptosis). The prolonged survival of cells carrying the translocation may enhance the probability for secondary events to occur, such as the activation of c-myc. A similar cooperation can be observed in Burkitt’s lymphomas where a point mutation of the tumour suppressor gene p53 is frequently associated with changes in c-myc (30, 35).

These changes are corroborated by observations in NHL lines. As previously mentioned, alterations in c-myc, bcl-2 and p53 are frequently present. Analysis of 14 out of the 20 cell lines revealed that 10 contained changes in one or more of the above mentioned genes.
A correlation was observed between clinical outcome and c-myc alteration. Patients whose tumour cells gave rise to NHL lines with rearranged c-myc survived for a shorter time compared to patients with cell lines where c-myc was found to be in wild type status. These c-myc, bcl-2 and p53 alterations appear to evolve over time within patients. This was best demonstrated by studies on OCI-LY 13.1 and OCI-LY 13.2 (see above). OCI-LY 13.1 was found to carry wild type forms of these genes. In contrast OCI-LY 13.2 demonstrated rearrangements in c-myc, a deletion of one p53 allele and rearrangement of the other. These studies suggest that disease progression at least in this patient was associated with a series of genetic changes involving c-myc and p53 (36).

Several "in vivo" experiments have examined the biological role of c-myc and bcl-2 genes and the their possible interaction in the development of disease. EBV immortalized lymphoblasts become transformed after transfection with c-myc and produce tumours when injected into nude mice (37). Transgenic mice carrying c-myc fused to the Ig enhancer (Eu-myc) initially develop a polyclonal pre-B hyperplasia that progresses over time to a monoclonal malignant lymphoma (38). The observed delay in tumour development suggests that secondary genetic changes may be necessary. On the other hand, it has been reported that B cells expressing large quantities of bcl-2 protein are able to survive without exposure to their respective antigen. Mice carrying a bcl-2 transgene show a relatively normal primary response to T-dependent antigens, but demonstrate a remarkably prolonged secondary response (39). These mice uniformly developed initially a polyclonal follicular lymphoid hyperplasia resembling the proliferation of IgM/IgD expressing B cells. Over time these transgenic mice progress from indolent polyclonal follicular hyperplasia to monoclonal diffuse large cell immunoblastic lymphomas. Again the survival advantage of cells mediated by bcl-2 may increase the probability of secondary genetic events. These secondary events are suggested by the observation that about 50% of animals develop lymphomas that have c-myc translocated into the proximity of the Ig heavy chain locus (40).
LYMPHOID CELLS DEVELOPMENT:

Antigen-specific immune responses are mediated by lymphocytes. They recognize antigens through cell surface antigen receptors. For B cells the receptors are immunoglobulins (Ig) while for T cells, the receptors are T Cell Receptor complexes (TCR).

In postpartum mammals, cells committed to lymphoid differentiation arise in the bone marrow from multipotential stem cells. Whereas T cells undergo further differentiation in the thymus there is no similar organ that serves exclusively as site for the early phases of B-cell differentiation. The bone marrow is both a primary and secondary lymphoid organ. As a primary lymphoid organ it is responsible for the development of immunocompetent cells from their precursors. As a secondary lymphoid organ it is the site of immune responses by immunocompetent cells upon capture of foreign antigens. After B cells leave the marrow and T cells the thymus, they circulate into secondary lymphoid organs (spleen, lymph nodes and mucosal-associated lymphoid tissues) and return from these organs via the lymphatic system or bloodstream (41, 42, 43).

THE ORIGIN OF B-LYMPHOCYTES:

In human bone marrow, most haematopoietic progenitors can be identified by the expression of the CD34 antigen. It has been shown by single cell culture techniques, that bone marrow derived cells with CD34+, CD38-, HLA-DR- phenotype give rise to progeny representing all haematopoietic lineages including B-lymphocytes (44).

The stages in primary B cell development are defined by the sequential rearrangement and expression of heavy and light chain immunoglobulin genes (Figures 1, 2). The earliest cells committed to the B cell lineage are known as pro-B cells. They are progenitor cells with limited self-renewal capacity and are characterized by rearrangement of heavy-chain immunoglobulin gene segments. D_{H} to J_{H} joining at the early pro-B cell stage is followed by V_{H} to DJ_{H} joining at the late pro-B cell stage. Productive VDJ_{H} joining leads to the expression of an intact μ chain which is the hallmark of the pre-B cell stage. The μ chain in large pre-B cells is expressed transiently at the cell surface in combination
with a surrogate light chain as part of the pre-B cell receptor and permits the cells to divide further before giving rise to small pre-B cells. The pre-B cell receptor consists of a productively rearranged heavy $\mu$ chain, the surrogate light chains VpreB and $\lambda 5$ and the Ig$\alpha$ and Ig$\beta$ signal transduction units. This receptor is expressed on the cell surface. Its expression is required for further development. After activation of this receptor with its yet unknown ligand large pre-B cells enter into cell cycle, resulting in a significant expansion of all pre B cells with in-frame VDJ$_{H}$ joins. It also signals for heavy-chain rearrangement to cease, thus ensuring that each cell contains only a single rearranged heavy-chain gene. Proliferating large pre-B cells eventually give rise to non-dividing small pre-B cells characterized by intracellular $\mu$ chains. At this stage the pre-B cell receptor is no longer displayed and light chain gene rearrangements proceed. The cell is defined as immature B cell once a light chain gene is assembled and the complete IgM molecule is expressed on the cell surface. At this stage the cell is subjected to selection for self-tolerance and leaves the bone marrow to undergo alternative splicing of the heavy chain transcripts to generate mature B cells expressing surface IgM and IgD (B$\mu$D cells) (45, 46, 47, 48, 49, 50).

**B cell activation:**

Secondary lymphoid organs contain cellular elements that facilitate immune responses, including antibody formation of all immunoglobulin classes, inflammatory and killer T cell immunity, and regulation of the immune response. Activation of B cells in peripheral lymphoid organs leads to the production of progeny of plasma cells which will secrete antibodies (51).

When antigen is introduced into mice, it is captured and processed by professional antigen presenting cells (APC) especially the dendritic cells that are present in the T cell zones of secondary lymphoid tissues such as the paracortical area of lymph nodes and periarteriolar lymphoid sheath (PALS) in the spleen. Circulating naive T cells migrate into the proximity of dendritic cells and those rare T cells whose receptors bind peptides derived from antigens are trapped very efficiently and are activated (see "T cell activation"
and Figures 3, 4). B cells migrate from the periphery into the secondary lymphoid tissues and enter the T cell zones (52). Those rare B cells that are specific for the antigen responsible for T cell activation are trapped. The rest of the B cells move through this area to the B cell zone located in the outer cortex in lymph nodes and B cell corona of the spleen and return to the circulation. The binding of antigen to the antigen receptor in naive Bμδ cells triggers a cascade of intracellular reactions that eventually will lead to proliferation and differentiation of B cells but is often insufficient to activate B cells. Most antigens require additional signals to activate B cells; these are most commonly provided by T helper cells. In the absence of T cell signals these antigen-specific B cells die by apoptosis. B cells that have seen specific antigen and have been activated by T helper cells will proliferate in primary foci. Some of them will migrate after that to the medullary cords of lymph nodes and red pulp adjacent to the PALS in spleen where they develop into antibody secreting plasma cells (see Figures 1, 2). Others will move with the T cells that cause their activation into the primary follicles, proliferate and form secondary follicles (see Figure 2). The cellular and molecular factors that determine the differentiation pathways within germinal centers leading to memory B cells and plasma cells are poorly understood. However, it is known what factors enhance or prevent germinal center B cells from apoptosis.

The most obvious form of a secondary follicle is the germinal center (GC). Typically germinal centers are composed of activated B cells, follicular dendritic cells (FDCs), macrophages and T lymphocytes defining three areas: the dark zone (centroblasts), the light zone (centrocytes) and the mantle zone (plasmablasts and memory cells).

Activated Bμδ cells are considered to be almost certainly the precursors of germinal center blast cells (ref). Blast B cells in the dark zone also known as centroblasts, receive cytokine signals, such as IL 4 and IL 2, from T cells. They also interact via their CD40 receptor with CD40 ligand expressed by T helper cells (53, 54). It is believed that this interaction results in proliferation and somatic hypermutation within the IgV regions of centroblasts cells. While some centroblasts continue to proliferate and differentiate, others die by programmed cell death (PCD) if they fail to encounter T helper cells expressing
CD40 ligand in their membrane (55). It is also believed that some somatic mutations lead to higher affinity centroblasts. These higher affinity centroblasts interact with antigens more avidly and would continue to be antigen driven. Centroblasts that survive this selection stop proliferating, undergo immunoglobulin heavy chain class switching, and move to the light zone to differentiate into centrocytes expressing surface IgA, IgE or IgG (56). Centrocytes are presumably tested for antibody affinity by antigen bound to FDCs and are contacted by helper T cells bearing CD40 ligand. Centrocytes whose surface Ig have low or no affinity for antigen undergo apoptosis while those that survive the selection process leave the germinal center to become either memory B cells or antibody secreting plasma cells in the mantle zone (Figure 2). As a consequence of the extensive apoptosis taking place in germinal centers, they are characterized by the presence of phagocytes engulfing apoptotic cells, also called "tingible body macrophages" (57).

B-1 and B-2 cells:

Not all B cells conform to the developmental pathway described. A subset of B cells arises in mice and humans early in ontogeny that has distinctive receptor repertoires and functional properties. These cells were first identified by their display of surface IgM with little or no IgD. This property does not change even when they mature. These unconventional B cells are termed B-1 B cells, because their development precedes that of conventional B cells, sometimes called B-2 B cells. B-1 B cells are also known as CD5 B cells because they express CD5 antigen. Expression of CD5, however cannot be essential for their function since cells with characteristics typical for B-1 cells develop normally in mice that lack the CD5 gene. In addition, rat B-1 cells do not display CD5. Although present in relatively low frequency in lymph nodes and spleen, they are predominantly found in the peritoneal and pleural cavities. B-1 B cells appear to originate from immature stem cells that are most active during the prenatal period. During their development mice undergo a change so that from a certain time point on, only B-2 B cells are produced. In adult animals the population of B-1 B cells is maintained by continued
division in peripheral sites, this process apparently requires IL-10 (58).

As in the mouse, human B-1 B cells have been associated with autorreactivity. In humans, about 10-20% of all B cells in adult peripheral blood cells are CD 5+ (59).

B-1 B cell receptors and secreted antibodies tend to bind numerous different ligands at relatively low affinity; a property known as polyspecificity. They display preference for the binding of common bacterial polysaccharides.

THE ORIGIN OF T-LYMPHOCYTES:

While T cells arise in the bone marrow from pluripotent haematopoietic stem cells, the principal pathway for the development of T cells that express TCR complexes takes place in the thymus. TCRs are disulphide linked heterodimers that recognize small peptides (usually 9-11 amino acids long) contained within the cleft of either class I or class II major histocompatibility complex (MHC) expressing cells.

The first T cell specific developmental events occur after progenitors seed the thymus. These T cell progenitors are reported to express CD34 and CD7 antigens (60)(Figure 3). They mature to T cell precursors expressing gradually CD2, CD44 and CD25 antigens. The T cell precursors may undergo TCR \(\beta\) chain rearrangements and, at the same time, start to express CD44 at a low level. After successful rearrangement early cortical thymocytes express a TCR \(\beta\) chain in the membrane in association with a surrogate \(\alpha\) chain called pT\(\alpha\) (pre-T cell receptor \(\alpha\)) and CD 3 antigen. Expression of this complex leads to cell proliferation. The induction of proliferation, the arrest of \(\beta\) chain gene rearrangement, and the eventual induction of CD4 and CD8 expression all require the protein tyrosine kinase Lck, which later associates with the co-receptor proteins. When the cells start to proliferate, further \(\beta\) chain rearrangements are arrested by suppression of genes that regulate this process. Gene rearrangement takes place with the intervention of a complex of enzymes, some of which are already characterized. The machinery responsible for TCR rearrangements is essentially the same that participates in B cell receptor recombination. The cleavage of the genes that are going to be rearranged is
made by a specialized heterodimeric endonuclease formed by the products of two genes: RAG-1 and RAG-2 (Recombination Associated Genes). Other components of this complex are enzymes that usually help to repair double stranded breaks in DNA. They include at least 3 nuclear proteins and a DNA-dependent protein kinase. During the proliferative phase triggered by the expression of the pre-TCR, the RAG-1 and RAG-2 genes are repressed and RAG-2 protein is inactivated by phosphorylation. RAG-1 and RAG-2 mRNA degraded in the rapidly cycling cells. Hence, no rearrangement of α-chain genes occurs until the proliferative phase ends, allowing RAG-1 and RAG-2 mRNA and RAG-2 protein to accumulate again. This ensures that each successful rearrangement of a β-chain gene gives rise to many CD4+, CD8+ (double positive) cells, each of which can independently rearrange its α-chain genes once the cells stop dividing, so that a single β chain is associated with many different α chains in the resulting progeny. Once cells cease to proliferate and become small CD4+, CD8+ cells, the enzymes responsible for gene rearrangement are reactivated. Small double positive thymocytes express only low levels of TCR, and more than 95% are determined to die. These are the cells that express receptors that cannot recognize self MHC and then fail positive selection. Double-positive cells that recognize self MHC mature to express high levels of TCR and subsequently cease to express one or other of the coreceptor molecules, resulting either in CD4 or CD8 positive thymocytes. Thymocytes also undergo negative selection during the double-positive stage. T cells recognizing ubiquitous self peptides in the context of MHC, die presumably by apoptosis. Those that survive this dual screening mature to CD4 or CD8 positive naive T cells that are rapidly exported from the thymus to join the peripheral T cell repertoire (61, 62, 63, 64, 65) (Figure 4).

CD4, CD8 cells:

CD4 T cells are the population that includes the most important helper cell regulators of immune responses. They recognize peptides contained within the cleft of MHC class II molecules. CD8 T cells, also known as cytotoxic T cells, recognize peptides contained in MHC class I clefts and are mainly killer T cells. MHC class II molecules are
largely restricted in their expression to cells that are part of the immune system such as APCs and B cells, that interact with CD4 T cells, whereas MHC class I molecules are expressed on most cell types. The CD4 molecule functions as coreceptor for the MHC class II molecule. CD8 is a coreceptor for MHC class I. The binding of TCR and CD4 complex in the context of self class II MHC (and that of TCR and CD8 in the context of self class I MHC) plays a critical role in delivering signals to the T cell indicating that antigen has been recognized appropriately. Additional surface receptors on these T cells must be triggered either simultaneously or sequentially to cause proliferation and differentiation. Activated CD4 cells produce cytokines that initiate the generation of T and B effector cells (66, 67).

Two types of CD4 positive cells have been described: TH1 and TH2. The conditions that determine the differentiation pathway of a CD4 cell into TH1 or TH2 are not fully understood. Pathogens that accumulate in large numbers inside macrophage vesicles tend to stimulate differentiation of TH1 cells while extracellular antigens tend to stimulate production of TH2 cells. TH1 cells activate microbicidal properties of macrophages and induce B cells to produce IgG antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells. TH2 cells initiate the humoral immune response by activating naive antigen-specific B cells to produce IgM antibodies, and may subsequently stimulate the production of different isotypes (68, 69).

T cell activation:

T cells enter the bloodstream from which they migrate into the paracortex of lymph nodes, analogous structures in the spleen and Peyer's patches in the gastrointestinal tract. They recirculate until they encounter antigen (70). The crucial step in adaptive (long lasting and specific) immunity is the activation of naive antigen-specific T cells by APCs (71)(Figure 4). The most distinctive feature of APCs is the expression of co-stimulatory molecules of which the B7 (B7.1 and B7.2) family is the best characterized. Naive T cells will respond to antigen only when cells present both, specific antigen to the T cell receptor and B7 molecules to CD28, the receptor for B7 on the T cell (72, 73). The activation of T
cells by APCs leads to their proliferation and differentiation into armed effector T cells. The proliferation and differentiation of T cells depends on the production of cytokines such as the T cell growth factor IL2 and its binding to a high-affinity receptor on activated T cells (74). T cells whose antigen receptors are bound in the absence of costimulatory signals fail to produce IL2 and instead become anergic. This dual requirement for both receptor binding and co-stimulation helps to prevent naive T cells from responding to self antigens. Once an expanded clone of T cells achieves effector function, all its members can act on any target cell that displays the specific antigen on its surface.

αβ and γδ T cells:

Similarly to B-1 B cells and B-2 B cells that arise during B lymphopoiesis, T lymphopoiesis is characterized by the development of different TCRs: the most common is TCRαβ, the less common is TCRγδ. T cells that appear first during embryonic development carry γδ TCR. They are produced in discrete waves. The first wave of this population homes specifically to the epidermis where they are called dendritic epidermal T cells. The second wave homes to the epidermal layers of the reproductive and genital tract. Receptors expressed by these early waves of γδT cells are essentially homogeneous. Later in development, T cells expressing either αβ or γδTCR are produced continuously rather than in waves. T cells with αβTCR predominate. The γδ T cells produced at this stage are different from those generated during the early waves. They display considerably more diverse receptors. Most of these γδ T cells are found in peripheral lymphoid tissues. It is to be noted that most of the data available regarding γδT cells were obtained for mice and it is not known whether or not similar changes in the pattern of receptors expressed by γδT cells occur in humans. The γδT cells that home the skin of mice do not seem to have exact human counterparts although there are γδT cells associated with the human reproductive and gastrointestinal tracts. Human γδT cells do not express CD4 and most lack CD8 or express it only at low levels. In contrast to αβT cells, no common restriction element such as MHC is presently known for γδT cells. Many of γδT cell clones secrete IL-2 and IL-4 at lower or undetectable levels when compared
to their $\alpha\beta$ counterparts. The functional significance of the $\gamma\delta$T cells still remains unclear (75, 76, 77).
CYTOKINES INVOLVED IN B AND T CELL DEVELOPMENT

B lymphocyte activation, growth and differentiation in all stages are regulated by cytokines. In vitro studies as well as Northern blot analysis of cellular mRNA and receptor identification suggest that the antigen independent phase in the bone marrow requires SCF, IL 7, IL 11 and IGF produced by stromal cells. Studies in stromal cell lines showed that IL7 stimulates the preferential production of late pre-B cells from bone marrow and fetal liver progenitor cells (78). In addition to IL7, other cytokines presumably contribute to the proliferation and differentiation of B cell progenitors: SCF (Stem Cell Factor, also known as Mast Cell Growth Factor, Steel Factor or c-kit ligand) has been reported to play an important role in early B-lymphopoiesis. SCF synergises with IL7 in inducing proliferation in pro-B and pre-B cell progenitors irresponsive to IL7 alone (79, 43). Experimental data suggest that SCF promotes the transition of IL7 irresponsive progenitors to IL7 responsive cells (80, 43). Similarly, SCF in combination with IL7 can replace the requirement for stroma to induce pro-B cell proliferation but not differentiation into pre-B cells. In addition, SCF in combination with IL7 can stimulate stroma-independent B cell progenitor cell development from candidate murine stem cells or from bipotent macrophage-B cell progenitor cells (43, 81, 82). IL-11 , also a stromal derived cytokine induces differentiation of early B cell progenitors in combination with SCF and IL7 . The resulting cells are stromal cell independent (43). Early pro-B cells may differentiate into Cμ positive cells in the presence of IL7 and insulin like factor 1 (IGF-1) without SCF stimulation (83 ).

Once the B cell differentiates to a mature naive B lymphocyte the majority of cytokines which are important in the antigen-dependent phase are derived from helper T lymphocytes. IL 2 and likely also IL 6 are involved in the growth regulation of cycling B cells and enhancement of antibody secretion (84). During the secondary immune response, B cells undergo isotype switching. Two kinds of signals are required for this process: i) triggering of CD 40 via binding to its ligand ii) a cytokine signal. The isotype produced after switching is positively or negatively influenced by cytokines. In humans,
IL 4 promotes switching to IgE and IgG 1 isotypes while inhibiting switching to IgM, IgG3 and IgG2a. TGFβ promotes switching to IgG2b and IgA preventing switching to IgM, IgG3 and IgE. Switching to IgE is inhibited by IFNγ (ref) which induces IgG3 and IgG2a switching. IL5 has been reported to induce IgA production in cells that have already undergone switching (85).

The early stages of thymic colonization by T cell progenitor cells are still poorly understood. Several homing molecules have been described such as CD44, MCP-1 etc that might promote the seeding, proliferation and differentiation of T cell precursors in the thymus. Both SCF and IL7, which are produced by thymic stromal cells (86, 87), appear to play a role during these early proliferative phases as shown by markedly reduced thymocyte numbers in IL7− mice (88) and by antibody blocking of the receptor for SCF (89, 90). The relative involvement of these factors in the first and second proliferative wave remains to be clarified, although the pattern of c-kit expression suggests that SCF is primarily involved in the first wave (89, 90). IL 2 induces the proliferation and differentiation of antigen induced T cells through an autocrine loop.
CELL ADHESION MOLECULES IN LYMPHOPOIESIS:

Like other developmental processes, lymphopoiesis is highly dependent upon cell adhesion molecules (CAM). These molecules mediate direct cell contact with the microenvironment of stromal cells in bone marrow and thymus. Some of them play a key role in the egress of mature virgin B cells from the bone marrow and in the homing and migration of B and T cells to lymph nodes, mucosal-associated lymphoid tissues (MALT) and spleen (91). CAM not only function as anchors that bind precursors cells to stromal cells, extracellular matrix molecules or endothelium but also function as signal transducers that contribute to the regulation of B and T cell growth and differentiation (92).

Interactions of B cells with adhesive molecules appear to be important in preventing cells from entering apoptosis. Lymphohematopoietic progenitors have been seen to express β1 (Very Late Antigen-VLA-CD29) and β2 (LFA, CD18) integrins, which are members of the large family of integrin adhesion molecules. Integrins, consisting of a large α chain that pairs non-covalently with a smaller β chain, function in multiple cell-cell and cell-matrix interactions (93). Particularly, the β1 integrins α4β1 and α5β1 appear to have an important role in early B cell development. Integrin α4β1 is expressed on stem cells and lymphoid progenitors and interacts with the vascular cell adhesion molecule-1 (VCAM-1, CD106), a transmembrane molecule and member of the immunoglobulin superfamily, that is expressed on bone marrow stromal cells (94). In addition, lymphohematopoietic cells interact with fibronectin exposed by stromal cells or deposited in the extracellular matrix through both α4β1 and α5β1. This interaction results in cell adhesion as well as in migration (95).

Sydecan, a heparan sulfate proteoglycan (also a CAM) is expressed on pre-B cells but lost as these mature (96). There is evidence that this molecule is involved in cell adhesion and can immobilise regulatory cytokines. Expression of members of the selectin family as well as of heavily glycosylated proteins like CD34 and CD43, which may function as scaffolds for selectin ligands, is closely regulated during haematopoiesis and lymphopoiesis. These molecules may have a role in lymphopoietic stromal cell interactions
but certainly play a role in interaction with the vasculature during lymphocyte migration and homing. Furthermore, certain growth factors function as adhesion ligands either because they are made in both soluble and transmembrane forms or because they are immobilised by extracellular matrix components. For example, alternative splicing of transcripts for SCF results in secreted or transmembrane forms of the protein (GM-CSF, IL3 and IL7 can attach to the matrix, presumably through heparan sulfate proteoglycans (97). The immobilization of soluble mediators by proteoglycans on haematopoietic precursors and stromal cells, and on matrix components spatially limits the inductive microenvironment since only cells in the direct vicinity of the immobilized factors can respond to them.

Several studies have reported the presence of extracellular matrix (ECM) components in the thymus including type I and IV collagen, laminin, fibronectin, vimentin, and merosin (98, 99). Whether the compartmentalization of the ECM is important for T cell development is not clear. However, production of ECM in the thymus is mirrored by the expression of receptors for these molecules on developing thymocytes, suggesting that interactions between T cell precursors and ECM molecules may play a role in T cell maturation. Several studies show that ECM receptor expression (ie α6β4 integrin which binds laminin) is highest in CD4-CD8- precursors and gradually decreases during maturation.

CD44 is another member of CAM molecules expressed on both, haematopoietic progenitors and stromal cells. Antibodies against CD44 block the early phases of development of myeloid and lymphoid precursor cells in long term culture possibly by preventing interactions with hyaluronate, collagen and fibronectin known ligands of CD44 (100, 101). In the lymphoid compartment CD44 is found in T cell precursors as well as early cortical thymocytes. CD44 can undergo extensive alternative splicing and posttranslational modification and is converted to proteoglycan by certain tissues (101). Proteoglycan-bearing forms of CD44 might function by immobilizing cytokines (102, 97). Also heparan sulfate forms of CD44 might function this way (93).

As they migrate through the cortical region of the lymph node, naive T cells bind transiently to each antigen presenting cell they encounter. Professional APCs including
dendritic cells in particular bind naive T cells through interactions between lymphocyte function-associated antigen-1 (LFA-1), CD2 and intracellular adhesion molecules (ICAM) such as ICAM-3 on T cells and ICAM-1, ICAM-2, LFA-2 and LFA-3 on APCs. In those rare cases in which a naive T cell recognizes its specific peptide-MHC ligand, signalling through the TCR induces conformational changes in LFA-1 which greatly increases the affinity for ICAM-1 and ICAM-2. The change stabilizes more the interaction allowing the T cell to proliferate along several days and its progeny to differentiate into armed effector T cells (104, 105).
ROLE OF APOPTOSIS IN THE IMMUNE SYSTEM:

WHAT IS APOPTOSIS?

Apoptosis or programmed cell death (PCD), is a conserved terminal differentiation program that multicellular organisms employ to remove cells that are potentially harmful, or cells that are not needed. This process is an active and physiological mechanism in which cells initiate and execute a program of self destruction. In the immune system, apoptosis is used at several checkpoints, one of which is the elimination of potentially harmful lymphocytes, i.e. those with self-reactive receptors (106), or cells that are not fully capable of eliciting an efficient immune response, i.e. B cells expressing low affinity antigen receptors (107). Apoptosis is also used to regulate the extent and duration of an immune response (i.e. to preclude excessive proliferation and preserve lymphocyte homeostasis) (108). Impairment of the removal of self reactive cells may lead to autoimmune disorders and failure to eliminate redundant cells after completion of the immune response may cause lymphoproliferative diseases.

The process of apoptosis can be divided into at least three functionally distinct phases: initiation, effector and degradation (109). During the initiation phase, cells are exposed to a death-inducing stimulus such as the lack of obligatory survival factors, shortage of metabolites, ligation of death-signal-transmitting receptors, contradictory signal combinations or subnecrotic damage by toxins, heat or irradiation. Biochemical events that take place during the initiation phase of apoptosis constitute “private” pathways that are only activated through a death signal. It is only during the subsequent effector phase that these initiating events are followed by metabolic reactions and “the decision to die” is taken (110). Extensive work done in the nematode Caenorhabditis elegans has provided evidence for the role of a particular group of enzymes (caspases) which are considered the “core” of the effector phase (111) Thus, the ultimate fate of cells during the effector phase is subject to regulatory events. Beyond this stage, during the degradation phase, an increase in the overall entropy, including activation of catabolic enzymes, precludes further regulatory effects. During this last phase the morphology and characteristic
biochemistry of apoptosis, eg. DNA fragmentation, massive protein degradation, etc become apparent. All cells can undergo apoptosis by default in the presence of the protein synthesis inhibitor cycloheximide, suggesting that cells constitutively express all of the protein components required to execute the death program (112).

PHASES IN APOPTOSIS:
The induction phase:

The first important clues about the molecular basis of PCD came from genetic studies of the nematode *C. elegans*. Two genes ced-3 and ced-4 are needed for all PCD occurring during the worm's development whereas another, ced-9 suppresses the action of ced-3 and ced-4. Vertebrate homologues of ced-3 have been identified which encode a caspase (113). Also a ced-9 homologue gene was described: bcl2 (111). Caspases are proteases with cysteine in their active center that have specific proteolytic activity at aspartic acid residues. Caspases are synthesized as precursors forms, and an apoptotic signal converts the precursors to mature enzymes which subsequently cleave other caspasases that are downstream in the cascade.

These enzymes were initially described in vertebrates participating at least in the induction and degradation phases of apoptosis. Caspase-8 is one of the best characterized members of this family and its activity is specifically triggered by some receptors associated with apoptosis. These receptors belong to the Tumour Necrosis Factor (TNF) receptor superfamily.

TNF-R1 and Fas (also known as APO-1 or CD95) are cell surface receptors of the TNF superfamily that promote apoptosis when crosslinked by their ligands (TNF-α and Fas ligand respectively) (114). More recently, DR3 (TRAMP/wsl-1/APO-3/LARD/AIR) and DR4 (TRAIL-R1/APO-2) were also described as members of this superfamily of cell death receptors (115, 116, 117, 118) The induction of apoptosis by TNFα and Fas ligand does not require protein synthesis but does require the presence of a conserved 70 amino acid cytoplasmic region termed the death domain (DD). The DD couples the death receptors to
the apoptosis-inducing machinery. A number of signalling molecules carry a DD that directly binds to these receptors. These molecules, generally called adaptors, include FADD (Fas Associated Death Domain also known as MORT1), TRADD (TNF-R1-Associated Death Domain), RIP (Receptor Interacting Protein) RAIDD (RIP-Associated Ich-1/CED-3 homologous protein with a death domain, also known as CRADD) and MADD (119) (see Figure 5). Only some of them, however, have been detected as part of an in vivo complex with their receptors in a ligand-dependent fashion. FADD was found to be part of the Fas DISC (Death-Inducing Signalling Complex) and TRADD was found associated with TNF-R1 upon binding of TNFα. Caspase-8 (also known as FLICE/MACH/Mch5) is recruited to the Fas DISC by the adaptor FADD (Figure 5). At its N terminus FADD contains a death effector domain (DED), a motif that was also found in two copies at the N terminus of caspase-8. Caspase-8 is activated by binding to Fas DISC. The active subunits p10/p18 are released into the cytoplasm, likely cleaving several substrates that have to be yet identified (119).

The effector phase:

It has been shown in many cellular systems that different apoptosis-inducing pathways will culminate in the induction of permeability transition (PT). PT involves a sudden permeability increase of the inner mitochondrial membrane to solutes such as calcium, protons, glutathione, etc. Upon PT, apoptogenic factors are released from the mitochondrial intermembrane space and leak into the cytosol. Some of these factors have been already characterized: they include cytochrome c (also called Apaf-2, Apoptosis Activating Factor 2) (120) and AIF (Apoptosis Inducing Factor)(121).

Studies in C. Elegans suggested that ced-4 was required for ced-3 function (ced-3 is homologous to the mammalian caspase-3) whereas ced-9 (homologous to mammalian bcl-2) regulate apoptosis by preventing activation of the caspase encoded by ced-3 (122). Recent biochemical data support these concepts as ced-3 and ced-4 can physically interact, most likely by virtue of their N-terminal domains, which both contain a motif designated as caspase recruitment domain (CARD) (123). Therefore, it appears that ced-4
is an adaptor protein that can receive an apoptotic signal, bind to pro-ced-3, and cause it to release its activated proteolytic domain. By binding to ced-4, ced-9 prevents it from activating pro-ced-3 (122).

Bcl-2 is one member of a family comprising at least fifteen different proteins. The bcl-2 family in mammalian systems include bcl-2, bcl-x, mcl-1, a1, bcl-w, bax, bak, bad and nbk/bik. In viral systems the family includes asfv, lmv-5, ebv and others (124). Bcl-2 has been shown to inhibit apoptosis and extend cell survival in a variety of conditions (125). Bax, an antagonist of bcl-2, was characterized as a bcl-2 binding protein and shares significant homology with bcl-2 (126). Broadly the proteins in this family fall into two categories: the inhibitors of apoptosis like bcl-2, bcl-xL, bcl-w, mcl-1 and the promoters of apoptosis bax, bik, bak, bad, bcl-xs (127). The various bcl-2 members of the family can dimerise with one another, with one monomer antagonising or enhancing the function of the other. In this way, the ratio of inhibitors to activators in a cell may determine the propensity of the cell to undergo apoptosis (127). Bcl-2 and bcl-x are localized to the outer mitochondrial membranes and endoplasmic reticulum as well as nuclear membranes. Bcl-2 has been found to interact with calcineurin, p53 binding protein, NIP-1,-2 and -3, R-RAS, RAF-1, BAG-1 and galectin-3. Recently it was proposed that bcl-2 functions by forming pores to allow ions or small molecules to cross the outer mitochondrial membrane (128). It also has been suggested that bcl-2 proteins is to retain cytochrome c in the mitochondria and prevent the activation of down stream caspases (129). In vitro studies have shown that cytochrome c (known as Apaf-2), dATP, ced-4 (also known as Apaf-1) and Apaf-3 (unidentified yet) are sufficient to activate pro-caspase-3 (130).

A recent publication, however, showed that bcl-2 was incapable of preventing bax-induced cytochrome c release in cells over expressing bax. Nevertheless, caspase-3 activation was prevented by bcl-2 and nuclear fragmentation did not take place. The mechanism of this down stream effect of bcl-2 has not been proven yet, but it may be due to an interaction between bcl-2 and the cytochrome c receptor Apaf-1/ced-4, described as mediator of caspase-3 activation by cytochrome c (131).

It was also reported that ced-4 localizes to the same intracellular sites as ced-9
 Binding to ced-9 (or bcl-xL) thus may sequester ced-4 from the cytosol and inhibits its ability to activate caspases. There is also evidence suggesting that pro-apoptotic bcl-2 proteins disrupt the interaction between bcl-xL and ced-4 without themselves binding to ced-4. Presumably their binding to bcl-xL displaces ced-4.

It has been also reported that bcl-2 and bcl-xL may be down regulated by phosphorylation (133) and proteolytic cleavage (134). In addition, some pro-apoptotic members of the bcl-2 family may be down regulated by phosphorylation. IL3 might promote cell survival by phosphorylating bad. Bad lacks the hydrophobic domain that targets most of the bcl-2 family proteins to intracellular membranes. The non-phosphorylated bad antagonizes bcl-2 and bcl-xL by forming heterodimers with bcl-2 and bcl-xL, thereby promoting cell death. IL3 induced phosphorylation causes the dissociation of bad releasing bcl-xL protective function. (135) One of the candidates for this phosphorylative activity is Raf-1, a serine/threonine kinase which normally forms part of the mitogen-activated protein (MAP) kinase cascade.

Calcineurin is a Ca\(^{2+}\) dependent protein phosphatase that binds to bcl-2. Over expression of active calcineurin induces apoptosis that can be suppressed by bcl-2. How calcineurin induces apoptosis is unclear but it has been suggested that dephosphorylation of bad is one possibility (136).

The degradation phase:

Cellular disassembly is the final step in apoptotic cell death and results from the cleavage of substrates by caspases. The nuclear enzyme poly (ADP-ribose) polymerase (PARP) was identified as a substrate of caspase-1 (also known as ICE: interleukin 1\(\beta\) converting enzyme-like protease). PARP is involved in repairing damaged DNA, therefore its proteolytic inactivation accelerates apoptosis (137). A new DNase has been recently described that is activated by caspase-3 (Caspase Activated DNase, CAD) and digests DNA during apoptosis. CAD activity is negatively regulated by its inhibitor ICAD (138). The cascade of proteases cleaves a large number of substrates and therefore, induces alteration of cell morphology. The rounding of the cells and detachment from their support
may result from destruction of the cytoskeleton. Topoisomerase, lamin B, histone H1, protein kinase c, phospholipase A2 degradation have also been documented during apoptosis (139) (Figure 5).

Other genes involved in apoptosis:

p53 was described years ago as a tumour suppressor gene or antioncogene. Intrinsic to the function of p53 is its ability to induce apoptosis and to cause cell cycle arrest. P53 functions have been extensively investigated, however the mechanisms involved in p53 induction of apoptosis are not yet fully known. One of the known characteristics of p53 is its ability to function as transcription factor, but as it is was mentioned before, apoptosis does not require de novo transcription or translation. Thus p53 protein might induce apoptosis by binding to and directly activating various cellular proteins required for apoptosis. Alternatively, p53 might repress the transcription of survival factors which prevent cells from entering into apoptosis. This possibility is consistent with the ability of some growth and survival factors (such as IL-6 and SCF) to prevent p53 mediated apoptosis (140, 141). It is unclear whether or not p53-mediated apoptosis and p53 mediated G1/S cell cycle arrest are related. Apoptosis might be a consequence of G1/S arrest, but in those cells undergoing p53 mediated apoptosis, the ability to enter into apoptosis is independent of their position in the cell cycle (142). Moreover, the G1/S arrest involves de novo transcripts of GADD45 (member of the Growth Arrest and DNA Damage-Inducible family) and p21 (cell cycle arrest protein). In addition, it has been shown that it is possible to inhibit apoptosis without perturbing G1/S arrest (141). These observations are consistent with the idea that the G1/S arrest and apoptosis mediated by p53 represent divergent biological pathways.

An early functional study of bcl-2 showed that it can cooperate with the oncogene c-myc to immortalize pre-B cells. Subsequently, it was found that inappropriate c-my
expression under conditions such as heat shock in Chinese hamster ovary (CHO) cells or serum deprivation of Rat-1 fibroblasts lead to rapid onset of apoptosis (143, 144). Constitutive expression of bcl-2 inhibited myc-induced apoptosis, allowing immortalization to occur (143). This would suggest that c-myc induces a growth signal that results in proliferation in high serum conditions but, under serum starvation, cells are unable to proliferate and undergo apoptosis. More recently, cell culture experiments using inducible myc constructs in serum-deprived fibroblasts showed that expression of myc activated both proliferation and apoptosis and that survival of cells was dependent on survival factors (145). Factors such as IGF-1 suppress the inherent genetic apoptotic program. The induction of apoptosis and its inhibition by specific cytokines was not dependent on new protein synthesis. These observations support the view that c-myc induced apoptosis appears to be a normal physiological aspect of c-myc function whose execution is regulated by the availability of survival factors.

Double transgenic mice expressing both bcl-2 and myc exhibited hiperproliferation of pre-B and B cells and development of tumours of a haematolymphoid cell type at a markedly increased rate (146, 147). Synergy between these oncogenes of two different classes results in more potent transformation than by either oncogene alone.

APOPTOTIC SIGNALS IN B AND T CELLS:

Fas in T cell development: The importance of the Fas-Fas ligand interaction in mature T cell death was revealed by the discovery that a defect in the gene encoding either Fas or Fas ligand, was responsible for the development of lymphoproliferative disorders in lpr (148) or gld (149) mice respectively. Mature lpr or gld-T cells do not die after activation, and activated cells accumulate in lymph nodes and spleen of these mice (150). This indicates that Fas is involved in activation-induced cell death (AICD) of T cells. However, Fas is not involved in thymic clonal deletion because mice lacking functional CD95 or its ligand have normal thymic development (151). Recently, several patients with autoimmune lymphoproliferative syndrome, ALPS, have been characterized as carriers of mutations
in CD95 genes, confirming the biological importance of these molecules (152, 153).

During T cell activation a phenomenon takes place that has been the object of a large body of work: TCR ligation results in both lymphocyte activation and apoptosis. As mentioned before, two signals are crucial for efficient T cell activation: the interaction of the TCR with MHC+antigen and the crosslinking of the T cell molecule CD28 with B7 antigen exhibited by professional APCs. Costimulation with CD 28 delays the onset of apoptosis in T cells activated through TCR without preventing it. CD95 is up regulated in activated T cells and apoptosis would be expected. CD 28 costimulation does not decrease the amount of CD95, but it increases the production of bcl-xL (a member of the bcl-2 family with antiapoptotic activity). Thus, T cells are protected from cell death at least for several days while stimulation is provided. The protection however is transient and as expression of bcl-xL wanes, the CD95/CD95 ligand system resumes operation to initiate the apoptotic program. That means that once the offending antigen has been cleared and the TCR and CD28 are no longer engaged, bcl-xL levels diminish, the effectors of apoptosis are derepressed and the population of activated lymphocytes is eliminated (154).

Fas in B cell development: In the primary immune response, activated B cells differentiate into IgM secreting plasma cells in the presence of cytokines and other appropriate signals. In the absence of the proper signals, activated B cells undergo apoptosis (108). In addition to T cells, Fas deficient mice also accumulate B cells and have elevated levels of immunoglobulins, suggesting an involvement of the Fas system in the deletion of autoreactive B lymphocytes (155).

It has been shown that some of the activated cells rescued from programmed cell death enter GC and proliferate as centroblasts for about 24 hours. Centroblasts undergo spontaneous apoptosis unless stimulated by CD40 ligand of activated T cells. At this stage, Ig genes undergo somatic mutation without isotype switching. Several groups have demonstrated that centroblasts are resistant to Fas-induced apoptosis (156). Following CD40 stimulation, centroblasts differentiate into centrocytes which are susceptible to Fas-mediated killing by activated T cells in an antigen-nonspecific manner. However, GC B
cells can escape Fas-mediated killing if their Ig receptors are occupied by antigen complex on FDC, thus safeguarding antigen specific B cells with high affinity receptors (57) Fig 2.

**Bcl-2 in B cell development:** Although bcl-2 is known to be critical for maintaining the survival of memory B and T cells (156) bcl-xL appears to play an important role in activated lymphocytes (157).

When naive (bcl-xL negative) cells are activated by CD40 ligand, they express bcl-xL transiently when they proliferate. As the bcl-xL level decreases in CD40 ligand activated cells, cells become susceptible to apoptosis by anti-Fas antibodies. Anti-Ig stimulation protects Fas+ B cells from Fas-mediated killing by upregulating bcl-xL expression. This resistance to Fas-killing appears to be independent of other bcl-2 gene product since bcl-2, Bax and Mcl-1 expression remains unaffected (158).
OBJECTIVES AND EXPERIMENTAL DESIGN:

The culture system employed in the laboratory to study the behavior of NHL cells \textit{in vitro} is based on the utilization of PPP and PHA-LCM in semi solid medium as source of multiple biological activities. This system allowed the establishment of 20 NHL cell lines and their further characterization in terms of growth regulation and genetic changes that could explain the underlying processes of neoplastic development in this disease. Experiments carried out in the laboratory showed that PPP was an essential component of the culture system; without PPP none of these cell lines could have been established.

Early work suggested that NHL cell lines are heterogeneous with respect to their proliferative capacity. Semi solid culture studies of NHL cell lines demonstrated that only a small number of cells in each cell line were clonogenic and able to maintain a high proliferative potential while the majority showed limited or not growth. This suggested the existence of a hierarchy of cells with different proliferative potential as depicted in Figure 6. In this model we postulated the existence of few clonogenic cells with high proliferative capacity that function as disease propagating cells. These cells might eventually give rise to a subpopulation with restricted proliferative capacity that might undergo apoptosis after some divisions. It is also conceivable that cells with high proliferative potential may undergo programmed cell death under certain conditions.

The question remains whether or not PPP is a source of specific signals to NHL cells e.g. in the form of cytokines or provides solely nutritional support. It was the objective of my thesis, to understand the contribution of PPP to cell growth and death in NHL with the focus to evaluate if PPP withdrawal may induce apoptosis. This objective included the assessment of cytokines in the survival and proliferation of NHL cells. In addition, I became interested in the question whether or not apoptosis in NHL induced by culture conditions may be of interest in auto BMT as a form of purging. To address the questions mentioned above I focused my work on two NHL T cell lines, OCI-LY 13.1 and OCI-LY 13.2 and expanded it to seven additional B and T cell NHL lines. I was particularly interested in
studying these two cell lines, OCI-LY 13.1 and 13.2, which were generated from bone marrow samples from the same patient. OCI-LY 13.1 was created at diagnosis and OCI-LY 13.2 during therapy resistant relapse. Previous studies showed that OCI-LY 13.1 is a slow growing cell line that requires addition of growth promoting activities to the culture medium. p53, c-myc and bcl-2 genes (involved in cell cycle, growth and apoptosis regulation and whose mutations have been associated with neoplastic transformation) were found to be in wild type status. On the other hand, OCI-LY 13.2 grew rapidly and independently of external sources of cytokines, however the addition of PHA-LCM slightly increased the proliferation of clonogenic cells. Molecular studies revealed that p53 and c-myc genes have undergone mutation while bcl-2 remained in wild type status. Taking into account that both cell lines exhibit same pattern of TCR rearrangement, and are thus part of the same clone, we believe the biological changes observed in these two cell lines may mirror disease progression within the patient.

SPECIFIC AIMS:

Specific aim 1: to test the effect of PPP withdrawal on colony formation by clonogenic NHL cells.

Specific aim 2: to test the effect of PPP withdrawal on disease propagating NHL cells.

Specific aim 3: to test if bulk NHL cells require PPP for their proliferation in culture.

Specific aim 4: to test the effect of PPP withdrawal on programmed cell death (apoptosis) in bulk NHL cells by propidium iodide staining followed by FACScan and the development of DNA ladders.

Specific aim 5: to test the effect of two fully defined serum-free systems: a combination of bovine serum albumin, human transferrin and bovine insulin (BIT) and a commercially
available medium (Stem Pro34®)

Specific aim 6: to test the effect of single cytokines (IL 1, IL2, IL4, IL6, IL7, IL10, IL11 and SCF) or combinations on NHL cell proliferation under serum-free conditions.

Specific aim 7: to test the effect of cytokines on proliferation and survival in a sample directly taken from a patient with NHL.

Specific aim 8: to compare the response to PPP withdrawal in NHL cells and human normal haematopoietic cells by means of clonogenic assays and evaluate the possibility of using this approach as a purging method for autologous transplant in patients with NHL.
<table>
<thead>
<tr>
<th>Lineage</th>
<th>Histotype</th>
<th>CD20</th>
<th>Growth Factors</th>
<th>p53</th>
<th>c-myc</th>
<th>bcl-2</th>
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<td></td>
<td>mRNA Protein Requirement</td>
<td>receptors</td>
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<td>B7</td>
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<td>NO</td>
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<tr>
<td>OGLY-20</td>
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<td>B7</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
<td>IL-5, IL-6</td>
</tr>
</tbody>
</table>

DLC: Diffuse Large Cell Lymphoma; IMI: Immunoblastic Lymphoma; SLL: Small Lymphocytic Lymphoma; SNCCL: Small Non Cleaved Cell Lymphoma; DLCL: Diffuse Mixed Lymphoma; mut.: mutated; wt.: wild type (at least for the sequences analysed); (+): positive reaction with antibody that recognizes mutated and wild type protein, not confirmed by DNA sequencing; (**) rearrangement of one allele, deletion of the other; (***) deletion of one allele (cytogenetics). OGLY-14, 15 and 16 lost early in the establishment.
Figure 1. Generation of competent B cells
Figure 2. Generation of competent B cells (cont.)
Figure 3. Generation of competent T cells
Figure 4. Generation of competent T cells (cont)
Figure 5. Schematic depiction of the cell death pathway
Figure 6. Proliferation and death in NHL cells: a proposed model
MATERIAL AND METHODS:

MAINTENANCE OF CELL LINES:

Non Hodgkin’s Lymphoma (NHL) cell lines (OCI-LY 1-20) (1,2) were routinely grown in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco, Grand Island, NY) containing $5 \times 10^{-5}$ M 2-mercaptoethanol (2ME, Sigma, St. Louis), 10-20% normal human plasma prepared under platelet-poor conditions (PPP) and 2.5-5% of PHA-LCM (PHA, HA15; Wellcome Diagnostics, Dartford, England) or defined cytokines when required. The cells were incubated at 37°C in humidified atmosphere supplemented with 5% CO₂. Medium was completely changed at weekly intervals.

CULTURE OF A DIRECT SAMPLE FROM A PATIENT:

A sample of cerebrospinal fluid (CSF) obtained from a NHL patient with CNS involvement was centrifuged. The cells were resuspended in IMDM, centrifuged and cultured in the liquid suspension system described above.

CYTOKINES:

Multiple cytokines were tested on several NHL cell lines and fresh samples from patients with NHL. These included: IL-1β (R&D), recombinant human IL-2 (Hoffmann-La Roche), IL-4 (R&D Systems), IL-6 (R&D Systems), IL-7 (R&D), IL-10 (Schering), recombinant human IL-11 (Genetics Institute), SCF (AMGEN).
SERUM-FREE SYSTEMS:

Cells were cultured in IMDM containing 4.72 mg/ml Bovine Serum Albumin (BSA Sigma), 120 μg/ml human Transferrin (Behring) and 0.02 mg/ml bovine Insulin (Sigma) as PPP replacements (159, 160). Stem Pro34° Serum Free Medium (Gibco, Grand Island, NY) was also used in some experiments.

PROLIFERATION ASSAYS:

a) Clonogenic assay for NHL cells:

Cells were cultured in IMDM, 30% normal human plasma prepared under platelet-poor conditions, 5x10⁵ M 2ME and 0.8% methylcellulose (Dow Chemical Co, Midland, MI) in 35 mm Petri dishes. The cultures were incubated at 37°C in humidified atmosphere supplemented with 5% CO₂. Colonies were scored after 2 weeks in culture and reevaluated after 4 weeks of incubation (1, 2).

b) Tritiated thymidine uptake:

Freshly prepared cells were cultured in liquid suspension system in 96 microwell plates (Nunc) at a concentration of 5x10³ per well. Five wells were used per assay per time point. At each time point 20 μl of IMDM containing 1 μCi of 3(H) Thymidine (DuPont NEN, Thymidine, [Methyl-³H] 6.7 Ci/mM) were added in each well and cultures incubated for 16 hours at 37°C in humidified atmosphere supplemented with 5% CO₂ (161). Cultures were then harvested in a semiautomatic multiwell harvester (Cell Harvester, Cambridge Technology Inc.), filters placed into vials containing scintillation fluid (CitoScint ES, ICN) and counted in a Beckman Scintillation Counter.

c) Clonogenic assay for normal human haematopoietic progenitors:

Cells were cultured in IMDM, 30% normal human plasma prepared under platelet-
poor conditions, $5 \times 10^{-5}$ M 2ME, 50 ng/ml of SCF, 2U/ml EPO (human recombinant Erythropoietin, Ortho), 10% PHA-LCM and 0.8% methylcellulose (Dow Chemical Co, Midland, MI) in 35 mm Petri dishes. The cultures were incubated at 37°C in humidified atmosphere supplemented with 5% CO$_2$. CFU-GM, BFU-E, CFU-Mk, CFU-GEMM and CFU-BI were scored after 2 weeks in culture (162).

PLASMA WITHDRAWAL EXPERIMENTS:

The effect of PPP withdrawal was evaluated in most of the experiments on clonogenic cells that were incubated at a density of $4 \times 10^4$ cells/ml in PPP-containing or PPP-free medium and plated by volume (50 µl/ml per Petri dish). One set of experiments was performed by incubating cells in PPP-free and PPP-containing medium at densities of $4 \times 10^4$, $4 \times 10^5$ and $4 \times 10^6$ cells/ml. After 72 hours cells were diluted appropriately to adjust for the high concentration and plated by volume (50 µl/ml per Petri dish).

FLOW CYTOMETRY ANALYSIS OF DNA CONTENT:

Cells grown in liquid suspension culture were placed in 4 ml Falcon snap cap tubes, washed twice at room temperature in Ca$^{2+}$-Mg$^{2+}$-free PBS, fixed in 3 ml of ice-cold 80% ethanol and stored at -20°C from 1 hour to several days. 1 ml of cold Ca$^{2+}$-Mg$^{2+}$-free PBS was added to each tube after fixation. Cells were centrifuged at 1500 rpm for 10 minutes, washed once at room temperature in Ca$^{2+}$-Mg$^{2+}$-free PBS and resuspended in 3 ml of staining buffer (0.2% Triton X-100, 1 mM EDTA in Ca$^{2+}$-Mg$^{2+}$-free PBS) for 5 minutes at room temperature. Cells were centrifuged and 1 ml of staining buffer containing 50 µg/ml of propidium iodide (Sigma) and 50 µg/ml of DNase-free RNase A (Boehringer Mannheim) added to the resuspended pellet. Cells were stained over night in the dark and filtered through a 50 µm nylon mesh to remove large aggregates prior to flow cytometry. Each sample was analysed on a Becton Dickinson FacScan using FL2 channel at low rate. (163, 164)
DNA EXTRACTION AND GEL ELECTROPHORESIS OF OLIGONUCLEOSOMAL FRAGMENTS (DNA LADDER FORMATION):

Cells growing in liquid suspension culture were washed twice in Ca\(^{2+}\)-Mg\(^{2+}\)-free PBS and lysed in buffer containing 10 mM EDTA pH: 8, 50 mM Tris HCl pH: 7.5 and 0.5% sodium dodecyl sulfate (SDS) (165). 20 μg/ml of DNase-free RNase A were added to the lysed cells and incubated for 1 hour at 37°C. 100 μg/ml proteinase K (Gibco, Grand Island, NY) were added for 3 hours at 50°C. DNA was extracted with phenol chloroform isoamyl alcohol 25:24:1 (Sigma), and precipitated sequentially with isopropanol (Sigma) and 70% ethanol. DNA was diluted in buffer containing 10 mM Tris HCl pH: 7.5 and 1 mM EDTA pH: 8. DNA samples were run in 2% agarose gel in 0.5% TBE buffer containing ethidium bromide during 4 hours at 50 V. As a positive control for apoptosis, HL60 cells were incubated for 3 hours in IMDM containing 10% Fetal Calf Serum (FCS, Sigma) and 0.2 μg/ml Camptothecin (Sigma) (166).

NOD-SCID MOUSE EXPERIMENTS:

NOD SCID mice (167) were housed in sterile static microisolator cages, fed irradiator rodent chow and autoclaved water, changed and manipulated under a laminar flow change hood at the animal colony of the OCI.

NHL cells were washed in IMDM containing 0.1 mg/ml DNase I (DNase I, Boehringer Mannheim) and diluted appropriately in 0.5 ml of the same medium. Cells were injected subcutaneously into anaesthetized, 8 weeks old NOD-SCID mice. Atravet (Acepromazine maleate, Ayerst) and Ketalean (MTC) were diluted 1/10, mixed 1:1 and administered intra peritoneally 5-10 minutes before the procedure in order to anaesthetize the mice.

Mice were checked three to four times weekly for the development of tumours and signs of morbidity. Once the tumours developed, mice were checked daily. When tumours
reached a size of approximately 1 cm in diameter, mice were euthanized by carbon monoxide inhalation or cervical dislocation.

All animal procedures were carried out according to study protocol and the general guidelines of the Animal Colony and the Canadian Commission of Animal Care.
RESULTS:

PPP is an essential component of the culture system for NHL cells. It was the purpose of my studies to determine whether or not PPP functions predominantly as a source of nonspecific nutrients or influences survival or death of NHL cells through specific, possibly cytokine mediated interactions. I made use of the available resource of well characterized NHL-cell lines raised in the laboratory. I primarily focused my interest on two T cell lines, OCI-LY 13.1 and 13.2. These NHL cell lines were derived from samples obtained from the same patient at diagnosis and during therapy resistant relapse (see page 4, Introduction). Previous studies showed that OCI-LY 13.1 is a slow growing cell line that requires addition of growth promoting activities. The p53, c-myc and bcl-2 genes of OCI-LY 13.1 were found to be in wild type status. In contrast, OCI-LY 13.2 grew rapidly and independently of external sources of cytokines. The addition of PHA-LCM as a source of exogenous growth factors resulted only in a slight increase of colonies. Molecular studies revealed that the p53 and c-myc genes had undergone mutations while bcl-2 remained in wild type status. Taking into account that both cell lines have the same antigenic phenotype and exhibit the same TCR rearrangement pattern, it is likely that the biological changes observed in these two cell lines mirror disease progression within the same original clone.

I also extended the study to include seven additional B and T NHL cell lines to test whether or not observations made on OCI-LY 13.1 and 13.2 were cell line specific or of more general importance for NHL. More recently I also examined one fresh NHL sample. Seven specific aims were defined in order to address the questions mentioned above.
Specific aim 1: to test the effect of PPP withdrawal on colony formation by clonogenic NHL cells.

Previous studies in this laboratory have identified within NHL lines a subpopulation of cells with extensive proliferative potential. These cells form colonies when cultured in semi-solid medium, and propagate the disease when injected into immunocompromised animals. Knowing that PPP was essential for the establishment of NHL cell lines, my first objective was to examine the effect of PPP withdrawal on clonogenic NHL cells. OCI-LY 13.1 and 13.2 were used as a model for exogenous factor dependent and independent growth. The study was extended to include four additional B cell NHL lines with different biological features as outlined in Tables II and III. Belonging to the same malignant clone, OCI-LY 13.1 and OCI-LY 13.2 exhibit different biological characteristics. Medium conditioned by OCI-LY 13.2 contains growth factors that stimulate proliferation of OCI-LY 13.1 and other NHL cell lines as shown previously in the laboratory. The nature of the OCI-LY 13.2 derived growth promoting activity has not been characterized. In addition, the p53, c-myc and bcl-2 status differs in these two cell lines as shown in Table II. I was interested in determining whether or not the growth requirements and/or genetic background of these cell lines could influence their response to PPP withdrawal.

Table II. Biological characteristics of OCI-LY 13.1 and 13.2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lineage</th>
<th>p53 status</th>
<th>c-myc status</th>
<th>bcl-2 status</th>
<th>Growth Factors (GF)</th>
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<tbody>
<tr>
<td>OCI-LY 13.1</td>
<td>T</td>
<td>?</td>
<td>wt</td>
<td>wt</td>
<td>required/not produced</td>
</tr>
<tr>
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<td>T</td>
<td>mutated(*)</td>
<td>mutated</td>
<td>wt</td>
<td>required/produced</td>
</tr>
</tbody>
</table>

?: p53 protein reactive with antibody that recognizes mutated form, gene sequencing did not confirm the presence of mutations. (*): one allele rearranged, the second deleted
OCI-LY 13.1 and 13.2 cells were incubated for 0, 3, 24 and 48 hours at a concentration of $4 \times 10^4$ cells/ml in PPP-containing and PPP-free IMDM after being thoroughly washed (3 times) in IMDM. After the incubation period, cells were plated by volume (50 μl/plate) under optimal conditions in semi-solid medium containing PPP to evaluate the presence and frequency of clonogenic cells.

The number of colony forming cells was significantly reduced under plasma-free conditions after the first 24 hours of incubation (to 9% for OCI-LY 13.1 and 23% for OCI-LY 13.2, see Figure 7). Only few colonies were observed 48 hours after PPP withdrawal (6.5% and 2.9% for OCI-LY 13.1 and 13.2 respectively). In contrast, plasma containing medium promoted the expected increase in clonogenic cells for both OCI-LY 13.1 and 13.2 (115 and 185% respectively).

This set of experiments clearly shows that PPP deprivation reduces the frequency of clonogenic cells in these two NHL T cell lines independently of their requirement for cytokines or their p53 and bcl-2 genes status.

Figure 7. Colony formation by OCI-LY 13.1 and 13.2 after incubation in PPP-containing and PPP-free medium. Experiment done in one replicate.
The study was expanded to test four additional NHL B-cell lines (OCI-LY 7, 8, 10 and 18). Their properties are listed in Table III. Three of the cell lines grew without the addition of exogenous cytokines (OCI-LY 7, 8 and 10). The fourth (OCI-LY 18) appears to produce endogenously, yet unidentified growth promoting activities.

**Table III. Biological characteristics of OCI-LY 7, 8, 10 and 18**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lineage</th>
<th>p53</th>
<th>c-myc</th>
<th>bcl-2</th>
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<td>wt</td>
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<tr>
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<td>mutated(*)</td>
<td>mutated</td>
<td>mutated</td>
<td>required/produced</td>
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</tbody>
</table>

(*): deletion of one allele.

OCI-LY 7, 8, 10 and 18 cells were incubated for 0, 3, 6 and 24 hours at a concentration of $4 \times 10^4$ cells/ml in PPP-containing and PPP-free IMDM after being thoroughly washed (3 times) in IMDM. After the incubation period, cells were plated by volume (50 µl/plate) under optimal conditions in semi-solid medium containing PPP to evaluate the presence of clonogenic cells. Figure 8 shows the effect of PPP withdrawal in these four cell lines in comparison to PPP containing cultures. OCI-LY 7, 8 and 18 gave rise to few colonies after 3 hours of incubation in PPP free medium. As expected, they formed an increased number of colonies when incubated in PPP-containing medium. OCI-LY 10 was the least affected cell line. After incubation in PPP free medium, 38 percent of colony forming cells were recovered after 3 hours of PPP deprivation. After 24 hours the percentage of clonogenic cells dropped to almost 0. The growth for this cell line in PPP-containing medium suggests a lower rate of proliferation when compared to OCI-LY 7, 8 and 18 and that could be the reason for the delayed effect after PPP-deprivation.
I further investigated whether or not the sensitivity of NHL clonogenic cells to PPP withdrawal may be dependent on cell concentration. OCI-LY 3 cells, that were previously characterized as producers of IL-6 and require this cytokine for their own growth, were thoroughly washed and incubated in IMDM with and without PPP at concentrations of 4x10^4, 4x10^5 and 4x10^6 cells/ml. After 72 hours of incubation cells were plated by volume in semi-solid medium containing 30% PPP. Colonies were counted after 10 days. OCI-LY 3 clonogenic cells did not form colonies at 4x10^4 cells/ml (concentration used in the experiments performed with OCI-LY 13.1, 13.2, 7, 8, 10 and 18). However, some colony formation was observed when cells were incubated at higher densities (data not shown).

These initial studies demonstrated that clonogenic cells of T and B NHL are highly sensitive to PPP withdrawal. The effect as early as 3 hours after PPP withdrawal appears to be irreversible for the majority of clonogenic NHL cells as shown when preincubated
samples were subsequently grown in plasma containing optimal conditions. In addition, at least in one cell line, the sensitivity to PPP withdrawal seems to vary with cell concentration, the lower the concentration of cells, the higher the sensitivity.

These results raised two questions: 1) Is this sensitivity to PPP withdrawal only typical for the small subpopulation of clonogenic NHL cells or could it be also observed in the remaining NHL cells characterized by a restricted proliferative activity? 2) Is the impact on proliferative potential following PPP withdrawal associated with programmed cell death? Unfortunately, clonogenic (highly proliferative cells) and low proliferative NHL cells do not display defined antigenic profiles that may be used for their selection and separate assessment. I have therefore evaluated the effect of PPP on the proliferative capacity of NHL cell lines by H\textsuperscript{3}TdR uptake (Specific Aim 3).
Specific aim 2: to test the effect of PPP withdrawal on disease propagating NHL cells.

In specific aim 1, I determined that PPP withdrawal results in a dramatic decrease of clonogenic NHL cells.

One of the cell lines, OCI-LY 3, was previously evaluated in NOD-SCID mice for the presence of disease propagating cells. I was able to generate tumours after subcutaneous inoculation of cells in all mice injected with $10^6$, $10^7$ and $10^8$ cells/mouse. In order to investigate the consequences of PPP withdrawal on disease propagating cells, $10^7$ cells were incubated for 72 hours in PPP containing and PPP-free medium at a concentration of $4 \times 10^4$ cells/ml. After culture the cells were subcutaneously inoculated into 8 weeks old NOD-SCID mice. The mice were inspected three to four times weekly for the development of tumours and signs of morbidity. All mice injected with PPP-incubated cells had to be killed within 23 days because of tumours developing in the site of inoculation. More than 50% of animals inoculated with cells grown under PPP-free condition were alive at the time when the last animal of the control group inoculated with cells grown in PPP-containing medium died.

At the moment, more than two months after inoculation, 2 out of 10 mice injected with IMDM-incubated cells are still alive and disease free. Figure 9 shows Kaplan Meier survival curves (plotted using GraphPad software) comparing both populations.
The test revealed that the survival of both populations is significantly different with a p value of 0.0013 as determined by log ranks statistics.

The samples injected into the mice were also cultured in semi-solid medium to quantify the number of clonogenic cells. The number of colonies ranged from 80 to 137 per dish. This was a rather unexpected finding taking into account that previous experiments (Specific aim 1) had indicated that clonogenic cells did not survive 72 hours of PPP withdrawal. The presence of clonogenic cells still able to proliferate after PPP withdrawal may suggest that the method of scaling up has to be optimized. Data obtained under specific aim 1 already showed that PPP withdrawal sensitivity, at least in OCI-LY 3 is dependent on cell concentration. Nevertheless, the number of clonogenic cells detected after incubation in PPP-free medium was 6 times smaller than the number of clonogenic cells detected after incubation of cells in PPP-containing medium (number of colonies varying from 554 to 682 per dish).

This represents a very preliminary experiment and has to be repeated under optimal conditions. The significant difference in survival between the two groups of mice may
suggest that PPP withdrawal affects disease propagating cells. In addition, the fact that one of the two surviving disease-free mice received the same number of clonogenic cells as animals that developed tumours (in the group injected with IMDM incubated cells) could indicate that not all clonogenic cells represent disease propagating cells.
Specific aim 3: to test if bulk NHL cells require PPP for their proliferation in culture.

In order to test the effect of PPP on the proliferation of NHL cells in liquid suspension culture, I chose a group of T and B NHL cell lines, that represented different patterns of growth factor requirement/production as outlined in Table IV.

The reason for this choice was to evaluate whether or not the requirement for PPP differed in lines that need and/or produce cytokines compared to growth factor independent cell lines. Of particular interest is the comparison between OCI-LY 13.1 and 13.2.

Table IV. Biological characteristics of OCI-LY 13.1, 13.2, 2 and 7

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lineage</th>
<th>p53</th>
<th>c-myc</th>
<th>bcl-2</th>
<th>Growth Factors (GF)</th>
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<tr>
<td>OCI-LY 13.1</td>
<td>T</td>
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<tr>
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<td>B</td>
<td>mutated</td>
<td>wt</td>
<td>wt</td>
<td>not required/not produced</td>
</tr>
<tr>
<td>OCI-LY 17</td>
<td>T</td>
<td>mutated</td>
<td>wt</td>
<td>wt</td>
<td>not required/produced</td>
</tr>
</tbody>
</table>

?: p53 protein reactive with antibody that recognizes mutated form, gene sequencing did not confirm the presence of mutations. (*): one allele rearranged, the second deleted.

OCI-LY 13.1, 13.2, 2 and 17 were cultured at different PPP concentrations. Cultures were evaluated by tritiated thymidine uptake at time 0, and after 3 and 5 days of culture. The 4 panels in Figure 10 show the data obtained with and without PPP during the 5 days of culture. The optimal concentration of PPP ranged from 10 to 20%. In the case of OCI-LY 13.1 (panel A) an external source of growth factors was used (conditioned medium of OCI-LY 13.2) in addition to PPP. The increase of PPP from 5 to 20% resulted in a significant concentration dependent increase in H^9TdT uptake in this cell line. The addition of
conditioned medium from cultures of OCI-Ly 13.2 had marginal effect compared to the impact of PPP (Figure 11). OCI-LY 13.2 and the growth factor independent cell line OCI-LY 2 did not show a significant difference in H³TdR uptake (Figure 10, panels B and C) when grown with different concentrations of PPP. OCI-LY 17, a growth factor independent cell line that releases IL-5 and IL 6 (panel D), showed optimal growth in 5% of PPP and did not increase H³TdR uptake with higher concentrations. However, none of the cell lines demonstrated H³TdR uptake in the absence of PPP.

Figure 10. H³TdR uptake by OCI-LY 13.1, 13.2, 2 and 7 in liquid suspension cultures initiated with PPP-containing and PPP-free medium. Mean ± SEM
Replacement of PPP by human serum prepared from the same donors resulted in similar level of H\textsuperscript{3}TdR uptake as that observed with PPP for the four cell lines (data not shown). Cells cultured without serum ceased to incorporate H\textsuperscript{3}TdR in all tested cell lines.

In summary, the experimental data obtained from the study of OCI-LY 13.1 and 13.2 plus five B and one T NHL cell lines confirms that PPP provides essential signals for the growth of NHL cells. Once PPP is removed from the culture medium, cells stop growing. This effect is independent of the patterns of growth factor production and/or dependence, presence of cytokines, lineage association and genetic status of p53, c-myc and bcl-2.

The dependence on PPP and irreversibility of the effect of its withdrawal was confirmed in additional experiments carried out in liquid culture with OCI-LY 7, 8 and 18 where cells were initially incubated in PPP free conditions and PPP was added after a delay of 48 and 96 hours by the addition of PPP. Proliferation was assessed by H\textsuperscript{3}TdR uptake (data not shown). Cells could only be rescued by adding PPP at the beginning of the incubation period. OCI-LY 2 was the only tested cell line demonstrating some H\textsuperscript{3}TdR uptake when PPP addition was delayed for 2 days. This suggests that highly proliferative clonogenic
cells and cells with more restricted proliferative potential that can be detected in liquid suspension culture share the response to PPP withdrawal. The delayed addition of PPP to cultures, or the plating of clonogenic cells in PPP containing methylcellulose could not reverse the effect of PPP withdrawal. This was observed in B and T cell lines independently of their growth factor requirement, and p53, c-myc or bcl-2 status and is reminiscent of cell removal by apoptosis.

I therefore proceeded to test the hypothesis that highly proliferative clonogenic cells and cells with more restricted proliferative potential belonging to the NHL cell population undergo apoptosis after PPP deprivation. As it was mentioned before, these two subpopulation cannot be easily separated and the experiments were therefore designed to include the whole population of each NHL line (Specific Aim 4).
Specific aim 4: To test the effect of PPP withdrawal on programmed cell death (apoptosis) in bulk NHL cells by propidium iodide staining followed by FACScan analysis and the development of DNA ladders.

It is well known that during the degradation phase of apoptosis several catabolic enzymes are activated and cells undergo extensive DNA fragmentation and protein degradation accompanied by alteration in cell morphology. I evaluated the possibility that apoptosis is induced by PPP withdrawal using 2 techniques designed to reveal changes in DNA content and integrity. In addition, I observed the morphological changes of the cells. The first is the staining of cellular DNA with propidium iodide followed by flow cytometry analysis. Cells undergoing apoptosis are treated with detergents or prefixation with alcohol to permeabilize the cellular membrane. This treatment leads to the loss of low molecular weight fragments of DNA generated during apoptosis which leak out of the cells during subsequent rinsing and staining. As a consequence, apoptotic cells have reduced DNA content and therefore can be recognized, following staining of cellular DNA, as cells with low DNA stainability located in the sub G0/G1 peak (168). An example of cell accumulation in the sub G0/G1 peak is displayed in Figure 7, panels A and B. The degree of DNA degradation varies depending on the stage of apoptosis, cell type and nature of apoptosis-inducing agents (169). The sub G0/G1 peak may represent, in addition to apoptotic cells, mechanically damaged cells, cells with lower DNA content (e.g. in a sample containing cell populations with different DNA indices) or cells with different chromatin structure (e.g. cells undergoing erythroid differentiation) (170). Therefore, apoptosis should be confirmed by other tests, such as changes in membrane permeability, changes in mitochondrial transmembrane potential, morphology, etc. One of the advantages of flow cytometry is that provides a quantitative measure of populations undergoing apoptosis.

The second method I applied to evaluate apoptosis is the demonstration of internucleosomal DNA ladders by gel electrophoresis. It has been reported that apoptosis
is frequently associated with the production of high molecular weight DNA fragments in early stages of macromolecular degradation and internucleosomal fragments in later stages. DNA cleavage can be detected by gel electrophoresis, either in form of fragments of 50-150 kb or internucleosomal ladders of DNA with bands being multiples of 180 bp (171, 172). Even though apoptosis has been commonly associated with DNA fragmentation, in some systems programmed cell death may not be accompanied by DNA fragmentation (173). In addition, few researchers have reported that internucleosomal DNA fragmentation is not necessarily indicative of apoptosis and some cases of accidental cell death (or necrosis) have been reported to show DNA ladder formation. High molecular weight DNA fragmentation was described in cells without the stimuli of inducers of apoptosis (174, 175).

I evaluated NHL cells after PPP withdrawal with both flow cytometry analysis and gel electrophoresis of DNA. In addition, I evaluated morphological changes by microscopical examination of the cells. Flow cytometric studies were performed on multiple occasions during the incubation period of the cells under evaluation. The profiles obtained during the early time points and data from PPP containing cultures were also used as controls for mechanically damaged cells that could render a sub G0/G1 peak. The combined use of both methodologies gives the opportunity to validate the observations.

4.1 Visual inspection:

All NHL cell lines cultured in PPP containing and PPP free conditions were subjected to daily inspection by inverted microcopy. In some cases, cells were stained and observed by light microscopy. Figure 12 shows pictures of slides prepared from OCI-LY 3. Cells were cultured for 4 days in PPP (panel A) and IMDM (panel B) and stained with May Grünwald-Giemsa. Cells in panel A show the typical appearance of viable lymphoma cells with intact morphology. Cells in panel B, exhibit certain characteristics of apoptosis: such as fragmentation of chromatin ("pulverization") in most of the cells; chromatin condensation and formation of micronuclei. The cell membranes seem to remain unchanged.
Figure 12. May Grünwald-Giemsa staining of OCI-LY 3 cells after 4 days of incubation in PPP-containing (panel A) and PPP-free medium (panel B). Large arrows in panel B show cells with chromatin fragmentation, small arrows indicate micronuclei.
4.2 Propidium iodide staining and flow cytometry:

In order to investigate the effect of PPP withdrawal on apoptosis in the bulk population of NHL cell lines, seven cell lines were screened. OCI-LY 13.1, 13.2, 2, 3, 7, 8 and 18 were analysed by flow cytometry following DNA staining with propidium iodide after incubation in PPP-free and PPP-containing medium. Growth of these cell lines was dependent on PPP (Specific Aims 1 and 3).

As mentioned earlier in this specific aim, one cannot assume that all cells located in the sub G0/G1 peak are exclusively apoptotic; necrotic cells and cells mechanically damaged can also contribute to signals observed in this region. Nevertheless, apoptosis was confirmed by visual inspection of the cells and the formation of DNA ladders.

OCI-LY 13.1 was the only tested cell line that exhibited a noticeable sub G0/G1 area even under optimal conditions (PPP-containing cultures) suggesting that this cell line undergoes spontaneous apoptosis. This was in accordance with the high level of dead cells detected by vital stainings such as eosin.

When PPP was withdrawn from the culture system, all tested cell lines except OCI-LY 18 exhibited a more prominent sub G0/G1 area with time after starvation. Unfortunately proper quantitation was not possible due to the disintegration of cell membranes generating an area of apoptotic DNA in the profiles in place of a clear peak. Panels A and B of figure 13 show as example the DNA profile of OCI-LY 8 after 4 days in PPP-free and PPP-containing culture where M1 indicates the sub G0/G1 area.

OCI-LY 7 was the only tested cell line where no live cells were detected after 96 hours of PPP withdrawal suggesting that this cell line exhibits the highest sensitivity to this condition.

The cell lines were heterogeneous in terms of kinetics for the development of apoptosis following PPP withdrawal. OCI-LY 13.1, 3 and 7 showed sub G0/G1 areas increasing from time 0 while OCI-LY 13.2, 2 and 8 displayed no sub G0/G1 areas until 24, 48 and 72 hours respectively.
4.3 DNA gel electrophoresis:

DNA ladder development after PPP withdrawal was observed for OCI-LY 13.1 and 13.2, 2, 3 and 7. As a representative example Figure 14 shows a picture of internucleosomal DNA ladders developed over time in OCI-LY 13.2. Cells were cultured for increasing time intervals in PPP-free or PPP-containing medium. The data suggest that oligonucleosomal fragments of DNA are not detected at the beginning of the culture and for a period of up to 2 days. By the 3rd day some degree of ladder formation is observed in PPP-free cultures. The intensity increases by the 4th and 5th day of culture. By day 4 and 5 nucleosomal fragments of DNA are also observed in PPP-containing cultures (although in a small cell population as indicated by the very light bands) suggesting that even under most favorable culture conditions, DNA fragmentation takes place to a certain extent. When OCI-LY 13.1 was tested, internucleosomal DNA fragmentation was detected from the beginning of the culture period in PPP free and also PPP containing medium (data not shown). For OCI-LY 2, 3 and 7, DNA ladders were detected in PPP-free medium.
and not in PPP-containing cultures (data not shown). DNA ladders were not observed for OCI-LY 18, confirming the flow cytometry data and morphological examination of the cells.

![Figure 14. Internucleosomal DNA ladder formation by OCI-LY 13.2 in liquid suspension culture. Lane M: 100bp ladder; lanes 1, 4, 7, 10, 13: DNA from PPP cultures; lanes 2, 5, 8, 11, 14: DNA from IMDM cultures; lanes 3, 6, 9, 12, 15: DNA from BIT cultures. Lanes 16-17: OCI-LY 2 (IMDM culture) and HL-60 (CAM containing culture) DNA samples as positive control. Arrow: 600 bp band.]

There is no certain explanation as to why DNA ladder formation was not reproducibly detected in OCI-LY 8 considering that the percentage of cells in sub G0/G1 was quite high and that I was able to detect DNA ladders in cell lines with lower percentages of cells in sub G0/G1. Sample associated technical problems may account for this result.

Table V summarizes the data obtained after DNA analysis by flow cytometry and gel electrophoresis in all tested NHL cell lines.
Table V. p53, c-myc, bcl-2 gene status in OCI-LY 2, 3, 7, 8, 13.1, 13.2, and 18. Presence of apoptotic DNA (sub G0/G1 area) and DNA ladder formation in PPP-free and PPP-containing medium.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>gene status</th>
<th>PPP</th>
<th>no PPP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>p53</td>
<td>c-myc</td>
<td>bcl-2</td>
</tr>
<tr>
<td>OCI-LY 2</td>
<td>mutated</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>OCI-LY 3</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>OCI-LY 7</td>
<td>mutated</td>
<td>mutated</td>
<td>wt</td>
</tr>
<tr>
<td>OCI-LY 8</td>
<td>mutated</td>
<td>mutated</td>
<td>mutated</td>
</tr>
<tr>
<td>OCI-LY 13.1</td>
<td>?</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>OCI-LY 13.2</td>
<td>mutated(#)</td>
<td>mutated</td>
<td>wt</td>
</tr>
<tr>
<td>OCI-LY 18</td>
<td>mutated(##)</td>
<td>mutated</td>
<td>mutated</td>
</tr>
</tbody>
</table>

#: p53 protein reactive with antibody that recognizes mutated form, gene sequencing did not confirm the presence of mutations. (#): one allele rearranged, the second deleted (##): deletion of one allele. Data evaluated at (*): 3 days, (**) : 4 days.

The detection of a sub G0/G1 area and observation of DNA ladders in OCI-LY 13.1 under optimal growth conditions (that is in the presence of PPP and the addition of growth factors) was of particular interest. Comparing OCI-LY 13.1 and 13.2, one can see that a high proportion of OCI-LY 13.1 cells undergo spontaneous and PPP-withdrawal-induced apoptosis. This may be related to the fact that as the disease progresses the malignant clone accumulated mutations in genes necessary for cell cycle and growth control as seen for p53 and c-myc. As a consequence of these changes, the malignant clone may become resistant to therapy.

The data obtained by flow cytometric analysis of cellular DNA content, electrophoresis of internucleosomal DNA fragments and morphological examination of cells incubated in PPP-free medium, are indicators that cells engage in programmed cell death following PPP withdrawal. This response to PPP withdrawal is independent of
growth factor pattern and genetic status for p53, c-myc and bcl-2.

The significance of PPP induced apoptosis and its relationship with the genetic background and other biological characteristics of the cell lines will be analysed in detail in the Discussion.
Specific aim 5: To test the effect of two fully defined serum-free systems: a combination of bovine serum albumin, human transferrin and bovine insulin (BIT) and a commercially available medium (Stem Pro 34°).

As it was mentioned earlier, it was also my objective to evaluate if any nutritional component, single cytokines or combinations of cytokines could provide the same level of survival and growth support to NHL cells as PPP. For this purpose I tested two serum free systems known to support growth of normal hemopoietic progenitors. The experiments were performed with and without cytokines.

At the time these studies were initiated, no reliable commercial serum replacements were available. I decided therefore, to test a serum replacement system that had been used at the OCI to grow lympho-hematopoietic cells. This serum replacement is based on the use of albumin, insulin and transferrin. For freshly explanted cells it is advisable to include a source of lipids, but established cell lines usually show little dependence on exogenous lipids. Albumin is usually employed in the form of Bovine Serum Albumin (BSA). Albumin is the major plasma protein, which has the capacity to bind different molecules such as fatty acids, hormones, bilirubin, several amino acids, etc. It is the principal substance to maintain the osmotic pressure of blood. Unbound fatty acids are generally toxic to cells in culture except at concentrations too low to satisfy the requirements of growing cells for any length of time (159). Albumin provides to the culture system a convenient source of fatty acids at acceptable levels. Although this is probably the major function of albumin in culture, albumin is also able to bind other hydrophobic substances as well as metal ions (including Ca++) and might play a role in adjusting their free concentrations.

Insulin has been shown to exhibit growth promoting activities in several cell types. It is believed that these activities are mediated by IGF-1 receptor (176, 177).

Transferrin is the major ion transport protein in plasma. It is required by most cells for optimal growth in serum-free conditions. There is evidence to suggest that its role involves
iron delivery (159)

More recently some companies have produced serum-free media that support the growth of hematopoietic cells. Stem Pro 34\(^{\circledast}\) (Gibco), for instance, is a recently marketed serum-free medium for hematopoietic progenitor cells. This medium was our second choice to test NHL cell lines. Major details about its composition are not available to the customer but, the company revealed that this medium contains human albumin, human insulin and human transferrin. The disadvantage of a commercial product is that it cannot be modified to adjust to specific needs.

5.1. OPTIMIZATION OF BSA, INSULIN AND TRANSFERRIN FOR SERUM FREE CONDITIONS:

The essential components of a defined serum free culture system for hematopoietic cells include albumin, transferrin and insulin (160). In order to establish optimal concentrations of BSA, insulin (also bovine) and transferrin (human) to be utilized for NHL cells, I tested these components in increasing concentrations on two NHL cell lines using \(H^3\text{TdR}\) uptake as read out. The reported concentrations for hematopoietic cells were included as reference. The optimal concentrations of BSA, insulin and transferrin for NHL cells (4.72 mg/ml, 0.02 mg/ml 120 \(\mu\)g/ml respectively) were in the same range as previously reported for normal hematopoietic progenitors (159). This combination is referred to as BIT.

5.2. GROWTH PROMOTING ACTIVITY IN BIT

Several NHL cell lines (OCI-LY 13.1; 13.2 2; 3; 7; 8; 12; 17 and 18) were cultured in BIT and Stem Pro 34\(^{\circledast}\) and their growth evaluated by \(H^3\text{TdR}\) uptake. These cell lines are either of B or T cell origin, they present different patterns of growth factor production/requirement and differ in their p53, c-my and bcl-2 status. Therefore, they can
be considered to represent the various NHL cell lines generated in the laboratory.

The effect of BIT and Stem Pro34® upon proliferation measured by H³TdR in two cell lines: OCI-LY 13.1 and 13.2 is shown in Figure 15 and Table VI.

<table>
<thead>
<tr>
<th>1*: PPP 20% + CM 2.5%</th>
<th>3*: BIT</th>
<th>5*: PPP 10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2*: IMDM + CM 2.5%</td>
<td>4*: Stem Pro 34®</td>
<td>6*: IMDM</td>
</tr>
</tbody>
</table>

Figure 15. H³TdR uptake by OCI-LY 13.1 and 13.2 in BIT and Stem Pro 34® containing cultures. PPP and IMDM were used as positive and negative controls. Mean ± SEM

OCI-LY 13.1 exhibited the highest level of H³TdR incorporation in PPP-containing cultures and the lowest in IMDM, in spite of the fact that cytokines were present. BIT did not stimulate significantly proliferation during the incubation period of 5 days (about 5% of the counts observed in PPP). Stem Pro 34® did stimulate growth without the addition of growth factors. Considering the amount of counts in controls with PPP, Stem Pro 34® provided stimulation equivalent to 76% of that of PPP. OCI-LY 13.2, the clone previously described as independent of exogenous cytokines, exhibited a higher proliferation index compared to OCI-LY 13.1 when cultured in the presence of BIT (about 25% of the counts in PPP). Stem Pro 34® provided stimulation equivalent to 51% of that of PPP.
The effect of BIT and Stem Pro 34® for the remaining cell lines on H³TdR uptake was heterogeneous (see Table VI). OCI-LY 7 grew almost as well in Stem Pro 34® as in PPP-containing medium. A marginal effect was observed with BIT and no effect in IMDM (72, 3 and 0.01% respectively). In contrast, OCI-LY 18 showed no significant increase in H³TdR uptake when incubated in IMDM and Stem Pro 34® and moderate growth with BIT when compared to the optimal conditions provided by PPP (0.14, 1, 24% respectively). OCI-LY 12 was the only cell line, apart from OCI-LY 18, which did not proliferate in the presence of Stem Pro 34®. This cell line also failed to grow in BIT. OCI-LY 3 and 8 showed a higher level of H³TdR uptake in Stem Pro 34® compared to PPP. One of the possible reasons for this effect is that PPP, at the concentrations used, seems to be less effective in keeping the medium from extreme acidification. For OCI-LY 2 and 17, the percentage of H³TdR uptake in the presence of Stem Pro 34® in comparison to PPP was 70, and 89 respectively. The H³TdR uptake induced by BIT was low (0.08 and 6.2% respectively).

Table VI. H³TdR incorporation in OCI-LY 13.1, 13.2, 2, 3, 7, 8, 12, 17 and 18 in BIT and Stem Pro 34® as PPP replacements.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Growth Factor</th>
<th>IMDM(*)</th>
<th>PPP(*)</th>
<th>BIT(*)</th>
<th>Stem Pro 34®(*)</th>
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<td>53683</td>
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<tr>
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<td>65020</td>
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<tr>
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<td>288</td>
<td>242744</td>
<td>189</td>
<td>170364</td>
</tr>
<tr>
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<td>60744(*)</td>
<td>4591</td>
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<tr>
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<td>11953(**)</td>
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<tr>
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<td>45986</td>
<td>668</td>
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<td>88944</td>
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<td>376</td>
<td>263437</td>
<td>73669</td>
<td>2734</td>
</tr>
</tbody>
</table>

(*): H³TdR uptake cpm at day 5 of culture (**) : values are low due to low pH in the cultures. At day 4, cpm values for PPP-containing cultures of OCI-LY 3 and 8 were 183879 and 351545 respectively. (#): OCI-LY 13.1 was cultured with 2.5% of medium conditioned by OCI-LY 13.2.
5.3. APOPTOSIS PREVENTION USING BIT:

I tested the effect of BIT on PPP-withdrawal-induced apoptosis by propidium iodide staining followed by FACScan and gel electrophoresis of DNA. OCI-LY 2, 3, 7, 8, 12, 13.1, 13.2 and 18 were cultured for 4 days in BIT containing cultures using PPP and IMDM as controls. BIT provided some form of protection against apoptosis after PPP withdrawal in OCI-LY 8 and OCI-LY 7. BIT had no effect on the behavior of OCI-LY 18, 2; 3 and 13.2 while it had a moderate effect on OCI-LY 12 and 13.1.

When apoptosis was analysed by DNA gel electrophoresis on OCI-LY 13.2 (see Figure 14), oligonucleosomal DNA fragments were not observed in the BIT-containing system during the first 3 days of culture; on day 3 in IMDM cultures and on day 4 and 5 in both PPP- and BIT-containing cultures, DNA ladder formation was observed suggesting the onset of apoptosis. Similar data were obtained for OCI-LY 7. OCI-LY 2 showed indications of apoptosis by day 3 in IMDM and BIT but not in PPP-containing cultures (data not shown).

Summarizing the data obtained in the experiments described under this specific aim, I conclude that OCI-LY 13.1 and 13.2 show a different response to the replacement of PPP by BIT and Stem Pro 34® in terms of proliferation and prevention of apoptosis after PPP withdrawal. BIT permits proliferation of OCI-LY 13.2 but has very little effect on OCI-LY 13.1. There is no apparent correlation between this behavior and the pattern of growth factor requirement, or status for p53, c-myc and bcl-2. Stem Pro 34® seems to promote proliferation more consistently. All tested cell lines except OCI-LY 12 and 18 exhibited levels of H³TdR comparable to 50% and higher of the counts seen in PPP containing cultures. This may support the view that human sources for plasma replacements are more favorable to promote proliferation compared to albumin and insulin of bovine sources.
Specific aim 6: To test the effect of single cytokines (IL 1, IL 2, IL 4, IL6, IL7, IL 10, IL 11 and SCF) or combinations, on NHL cell proliferation under serum-free conditions.

6.1. INFLUENCE OF CYTOKINES IN THE GROWTH OF NHL CELL LINES UNDER BIT-SERUM-FREE CONDITIONS:

6.1.1. Individual cytokines

I used OCI-LY 13.1 and 13.2 to test if defined cytokines with reported biological activity "in vitro" on lymphoid cells may replace some of the activities observed in PPP. Cells were cultured with a panel of individual cytokines that included: IL 1; IL 2; IL 4; IL 6; IL 6; IL 10 and IL 11. The cytokines were tested at concentrations usually employed in "in vitro" assays. Tritiated thymidine uptake was assessed on days 0, 2 and 5 of culture in medium containing BIT. Cultures established under PPP-free and PPP-containing conditions were added as controls. As an example, Figure 16 shows the data obtained for OCI-LY 13.1.
Figure 16. Effect of different cytokines in BIT containing serum-free cultures using OCI-LY 13.1 as test cell line. Mean ± SEM.
None of the cytokines was able to increase the level of \( H^3TdT \) uptake above basal levels observed with BIT alone. IL-1 exhibited a rather inhibitory effect at concentrations of 10-100 ng/ml in OCI-LY 13.1 and 13.2 (data not shown) in these particular experiments. This effect was not observed when other batch of the same cytokine was tested suggesting batch to batch variations.

The same evaluation was carried out for 6 additional NHL cell lines (OCI-LY 2, 3, 7, 8, 17 and 18) that represent different patterns of growth factor production and requirement. The results were very similar to the ones obtained when studying OCI-LY 13.1 and 13.2. None of the cytokines increased \( H^3TdT \) uptake above that observed with BIT alone (data not shown).

In addition, OCI-LY 2 and 17 (B and T cell lines respectively) were tested at low concentration of cytokines (ranging from 10000 to 1000 times less), to rule out the possibility of an inhibitory effect of usually employed concentrations. Lower concentrations did not improve \( H^3TdT \) uptake (see Table VII for OCI-LY 2 data. Data not shown for OCI-LY 17).
Table VII. Effect of low concentration cytokines in BIT-serum free culture on OCI-LY 2 proliferation.

<table>
<thead>
<tr>
<th></th>
<th>OCI-LY 2</th>
<th></th>
<th>OCI-LY 2</th>
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<tr>
<td></td>
<td>day 0</td>
<td>day 2</td>
<td>day 5</td>
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<tr>
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<td>293959</td>
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<td>5409</td>
<td>3815</td>
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<td>6732</td>
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<td>1633</td>
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<td>BIT+IL1 0.001 pg/ml</td>
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<td>BIT+IL7 0.001 pg/ml</td>
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<td>3206</td>
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<td>BIT+IL1 0.4 pg/ml</td>
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<td>3519</td>
<td>756</td>
<td>BIT+IL7 0.4 pg/ml</td>
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<tr>
<td>BIT+IL2 2.3x10^4 U/ml</td>
<td>5907</td>
<td>3681</td>
<td>963</td>
<td>BIT+IL10 0.001 pg/ml</td>
</tr>
<tr>
<td>BIT+IL2 1.25x10^4 U/ml</td>
<td>5746</td>
<td>3373</td>
<td>1173</td>
<td>BIT+IL10 0.05 pg/ml</td>
</tr>
<tr>
<td>BIT+IL2 2.5x10^4 U/ml</td>
<td>5602</td>
<td>3467</td>
<td>827</td>
<td>BIT+IL10 0.1 pg/ml</td>
</tr>
<tr>
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<tr>
<td>BIT+IL4 0.01 pg/ml</td>
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<td>2202</td>
<td>169</td>
<td>BIT+IL11 0.001 pg/ml</td>
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<tr>
<td>BIT+IL4 0.05 pg/ml</td>
<td>5940</td>
<td>2177</td>
<td>273</td>
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<tr>
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<td>6055</td>
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<tr>
<td>BIT+IL4 0.8 pg/ml</td>
<td>5971</td>
<td>3074</td>
<td>892</td>
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</tr>
<tr>
<td>BIT+IL6 0.001 pg/ml</td>
<td>5481</td>
<td>2862</td>
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<td>BIT+SCF 0.1 pg/ml</td>
</tr>
<tr>
<td>BIT+IL6 0.05 pg/ml</td>
<td>5840</td>
<td>3402</td>
<td>657</td>
<td>BIT+SCF 10 pg/ml</td>
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<tr>
<td>BIT+IL6 0.1 pg/ml</td>
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<td>6051</td>
<td>3746</td>
<td>1002</td>
<td>BIT+SCF 150 mg/ml</td>
</tr>
</tbody>
</table>

* Numbers denote cpm determined by H^3TdT uptake
6.1.2 Combination of cytokines

I considered the possibility that individual cytokines are not enough to provide the necessary signals for NHL proliferation. At the time these experiments were done, the only cytokines tested under this specific aim that are detected in normal human plasma were IL 6, IL 7 and SCF (178). I evaluated the effect of the combination of IL 6, and SCF plus BIT, at concentrations normally detected in human PPP and in concentrations that were 1000 fold higher on OCI-LY 13.1 and 13.2 as well as OCI-LY 2 and 17. Figure 17 shows the experimental data. None of the combinations showed any stimulatory activity over the basal level of BIT in the tested NHL lines.
Figure 17. Effect of combinations of IL 6, IL 7 and SCF in IMDM and BIT containing cultures of OCI-LY 13.1, 13.2, 2 and 17. Mean ± SEM.
6.1.3. Effect of IL 1, IL 2, IL 4, IL 6, IL 7 and IL 11 upon cell survival.

Several experiments were performed to test whether or not cytokines could delay or prevent apoptosis on NHL cells cultured under plasma free conditions (BIT). The delayed addition of PPP was used to test the proliferative response of these cells as evaluated by H\(^3\)TdR uptake. OCI-LY 2 and 7 (growth factor independent cell lines) were cultured with BIT in the presence of cytokines with reported activity on lymphoid cells such as IL 1, IL 2, IL 4, IL 6, IL 7 and IL 11 at concentrations usually employed "in vitro". PPP was added at days 0, 2, 4 at a concentration of 5% and H\(^3\)TdR uptake performed at day 7.

Figure 18 shows data obtained for OCI-LY 2. Until day 2 of culture in BIT containing medium, IL 2 (5-10 U/ml), IL 4 (5 and 50 ng/ml) and to some extent IL 11 (50 ng/ml) were able to maintain the responsiveness of cells to PPP. After 4 days in BIT containing medium the cells were not able to proliferate after the addition of PPP. Comparable data were obtained with OCI-LY 7 (data not shown). The proportion of PPP responsive cells in this line after 4 days in BIT containing medium was higher than that in OCI-LY 2. In contrast, for OCI-LY 7 IL 2 (5-50 U/ml), IL 6, IL 7 and IL 11 (5-50 U/ml) contributed to the survival of cells that were able to mount a proliferative response to PPP.
These studies were expanded to three additional NHL cell lines in order to see if this was a general phenomenon. OCI-LY 3, 8 and 18 were cultured in BIT containing medium with the individual addition of IL 1, IL 2, IL 4, IL 6, IL 7 and IL 11. At day 4 of culture PPP was added at a final concentration of 5% and H³TdR uptake performed on day 7. Figure 19 shows the effect of BIT combined with cytokines on survival of OCI-LY 3, 8 and 18 (the
first, growth factor independent and the latter, growth factor dependent cell lines). The presence of BIT in the culture system increased the survival of cells able to respond to PPP added on day 4 by 50 to 90% IMDM (% based on cpm). For OCI-LY 3 and 8, IL 6 (10 ng/ml) seems to have an additional minor protective effect on the cells.

**Figure 19.** Effect of different cytokines in BIT containing serum-free cultures on survival of OCI-LY 3, 8 and 18. Mean ± SEM.

In summary, the data generated in these experiments suggest that proliferating cells of at least some NHL cell lines may show improved survival after exposure to specific cytokines. Whether or not they represent agents that may inhibit apoptosis has to be elucidated in the future.
6.2. INFLUENCE OF CYTOKINES IN THE GROWTH OF NHL CELL LINES UNDER Stem Pro 34\textsuperscript{®}-SERUM-FREE CONDITIONS

6.2.1. Individual cytokines

In order to test if defined cytokines with reported activity on lymphoid cells may replace some of the activities observed in PPP when added to Stem Pro 34\textsuperscript{®}, OCI-LY 13.2 and 17 cell lines were cultured with a panel of individual cytokines. IL 1; IL 2; IL 4; IL 6; IL 7; IL 10; IL 11 and SCF were available at the time of these experiments for these studies. No effect was observed with most of the cytokines over the basal level of Stem Pro 34\textsuperscript{®}. Only IL 7 and SCF showed some moderate stimulatory activity on OCI-LY 17 (Table VIII) and IL 4; IL 6 and IL 7 on OCI-LY 13.2 (data not shown).
Table VIII. Effect of different cytokines in Stem Pro 34® serum-free culture on OCI-LY 17 proliferation

<table>
<thead>
<tr>
<th>OCI-LY 17 (cpm)</th>
<th>OCI-LY 17 (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0</td>
</tr>
<tr>
<td>10% PPP</td>
<td>8238*</td>
</tr>
<tr>
<td>IMDM</td>
<td>1696</td>
</tr>
<tr>
<td>BIT</td>
<td>6562</td>
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<tr>
<td>PRO.34</td>
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<tr>
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<tr>
<td>BIT+IL 1 10ng/ml</td>
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<td>PRO.34+IL 1 10ng/ml</td>
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</tr>
<tr>
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</tr>
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<td>PRO.34+IL6 50 ng/ml</td>
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</table>
* Numbers denote cpm determined by H³TdR uptake

6.2.2. Combination of cytokines

Stem Pro 34® was tested on OCI-LY 13.1, 13.2, 2 and 17 with a combination of IL 6, IL 7 and SCF at concentrations normally detected in human PPP and in concentrations that were 1000 fold higher. None of the above mentioned combinations showed any stimulatory activity over the basal level of Stem Pro 34® (Figure 20).
Figure 20. Effect of combinations of IL-6, IL-7 and SCF on Stem Pro 34® containing serum-free cultures of OCI-LY 13.1, 13.2, 2 and 17. Mean ± SEM.

COMBO 1 = IL-6 1 pg/ml + IL-7 0.8 pg/ml + SCF 145 pg/ml
COMBO 2 = IL-6 10 ng/ml + IL-7 10 ng/ml + SCF 100 ng/ml
The experiments carried out under this specific aim suggest that none of the tested cytokines added to serum-free medium (either BIT or Stem Pro 34®) can replace the proliferative activities seen in PPP. IL 1, IL 2, IL 4, IL 6, IL 7, IL 11, or SCF added individually do not support proliferation when added at the concentrations reported to have biological activity "in vitro". Reported data indicate that IL 6, IL 7 and SCF are present in normal human plasma at measurable amounts. When a combination of these cytokines was tested in BIT containing cultures, no effect was observed on H³TdR uptake by NHL cells. The same observation was made for Stem Pro 34®.
Specific aim 7: To test the effect of cytokines over proliferation and survival in a sample directly taken from a patient with NHL.

The possibility was considered that the behavior of fresh NHL cells may be different from cell lines. The cellular composition of samples directly taken from patients is usually more heterogeneous than that of cell lines where successive passages enrich for tumour cells with proliferative potential. Direct NHL samples may contain accessory cells that may provide signals to tumour propagating cells as was reported in cultures of AML cells (179). Accessory cells may respond to cytokines that made a minor contribution in serum free conditions for NHL. I had the opportunity to study one fresh sample directly taken from a patient with EBV negative Burkitt's Lymphoma. Cells from this sample were cultured in quadruplicates in micro well plates under different conditions: PPP, BIT, IMDM and BIT plus single cytokines (IL 1, IL 2, IL 4, IL 6, IL 7 and IL 11). The cultures were daily inspected by inverted microscopy. After 6 days of culture, 5% PPP was added to half the microwells and cultures were maintained for 5 additional days. At the end of the culture period photographic record was taken of the different wells. Figure 21 shows a composite of several pictures. Panels A, C, E, and G show cells cultured in BIT containing medium, panels B, D, F and H the same conditions after delayed addition of PPP. Only IL 2 (panel E) and IL 6 (not shown) exerted some degree of stimulation on cell proliferation above the level seen with BIT alone. On the other hand, IL 1 (panel C); IL 4 (panel G); IL 7 (not shown) and IL 11 (not shown) did not exhibit any growth promoting activities. After PPP was added, cells in cultures containing IL 1 (panel D), IL 2 (panel F) and IL6 (not shown) demonstrated growth. I concluded from this experiment that IL 1, IL 2 and IL6 maintained cells with proliferative potential in a state that they could respond to the delayed addition of PPP. More direct samples have to be tested in order to confirm these observations and to determine that this function is associated with an antiapoptotic activity.
Panels A: BIT, B: BIT + PPP rescue, C: BIT + IL-1, D: BIT + IL-1 + PPP rescue, E: BIT + IL-2, F: BIT + IL-2 + PPP rescue, G: BIT + IL-4, H: BIT + IL-4 + PPP rescue

Figure 21. Effect of different cytokines on BIT containing serum-free cultures on a direct sample from a NHL patient.
The sample from this patient gave rise to a new cell line not included into the published 20 NHL cell lines and thus provided the opportunity to examine whether or not the cytokine effect was sustained.

When the derived cell line from this sample was tested with the same cytokines at the same concentrations, cell proliferation was not increased in the presence of IL 1, IL-2 or IL6. PPP rescue after 5 days in culture induced a significant increment in cell proliferation in cultures containing IL6 and to a lesser extent IL 11 and IL 7 (Figure 22). This observation suggests that some biological changes had taken place during the culture period that led to the establishment of the cell line. It is conceivable that the composition of the original cell suspension may have been more heterogeneous than the cells propagated as cell line and may have included non malignant cells with supportive function. This observation is clearly of importance since behavior in vivo may be a result of disease propagating NHL cells in the context of their regulatory signals. I do not have a specific answer to this question but the system permits to examine these possibilities prospectively.

**Figure 22.** Effect of different cytokines on proliferation and survival on the NHL cell line derived from the direct sample. Mean ± SEM.
**Specific aim 8:** To compare the response to PPP withdrawal in NHL cells and human normal hematopoietic cells by means of clonogenic assay and evaluate the possibility of using this approach as a purging method for autologous bone marrow transplant in patients with NHL.

As shown in previously reported work and the experiments described under Specific Aims 1 and 2 of this thesis, PPP is an essential part of the culture system for NHL cells. Withdrawal of PPP induces apoptosis in all but one of the tested cell lines. This observation could be of importance clinically if normal hematopoietic progenitors would be less affected by PPP withdrawal. A more sensitive differential effect on disease propagating NHL cells might facilitate the development of a novel purging approach in autologous bone marrow transplants for patients with NHL. We designed a series of experiments to assess the effect of PPP withdrawal on clonogenic NHL cells in comparison to normal hematopoietic progenitors. Figure 23 shows colony formation by mononuclear cells obtained from a normal bone marrow sample incubated in liquid suspension culture either with or without PPP for a period of up to 7 days followed by plating in semi solid medium containing hematopoietic growth factors.
Formation of colonies was observed during the first two days of incubation without PPP. By day 4, the number of CFU-GEMM (Colony Forming Units of Granulocytic, Erythroid, Monocytic and Megakaryocytic cells), BFU-E (Burst Forming Units of erythroid cells) and CFU-Mk (Colony Forming Units of Megakaryocytic cells) dropped to about 50% in PPP free cultures. By day 7, the number CFU-GM (Colony Forming Unit of Granulocytic and Monocytic cells) decreased to one half of the starting frequency.

Bone marrow and lymphoma cells (OCI-LY 18, chosen because of its sensitivity to PPP withdrawal in clonogenic assay, see Specific Aim 1) were initially incubated separately and then plated in the same culture dish (Figure 24) to compare the effect of PPP withdrawal in normal vs neoplastic clonogenic cells. The data confirm that normal clonogenic cells were able to survive after PPP withdrawal up to 7 days (22% CFU-GM, 2% BFU-E and 16.5% CFU-GEMM colonies) but lymphoma cells did not form colonies after 2 days of incubation in PPP-free medium.
Further analysis has to be done to test whether or not this effect extends to direct patient's samples, its dependence on cell concentration and reproducibility in vivo.

These experiments were performed on 6 NHL cell lines. In principle all cell lines demonstrated a similar behavior. Clonogenic NHL cells demonstrated a rapid decline in the absence of PPP while clonogenic cells from normal bone marrow survived for significantly longer period of time under plasma free conditions. This differential effect might be of clinical importance as a purging strategy if disease propagating cells demonstrate the same increased sensitivity to PPP withdrawal.
DISCUSSION:

The culture system employed in the laboratory to study the behavior of NHL cells in vitro is based on the utilization of PPP and PHA-LCM in semi solid or liquid medium as sources of multiple biological activities. This system allowed the establishment of 20 NHL cell lines. Their further characterization in terms of growth regulation and genetic changes provided the opportunity to examine pathogenetic mechanisms.

Experiments carried out in the laboratory showed that PPP was an essential component of the culture system. However, it was not clear whether or not PPP served merely as a source of nutritional support or whether it afforded regulatory controls of NHL cells. The growth patterns of these NHL cell lines revealed a marked heterogeneity with respect to the proliferative capacity of individual cells, thus discriminating cells with high proliferative potential from cells with limited proliferative activity and cells that had stopped growing. These differences were depicted in the Introduction as a model in Figure 1.

I took advantage of the availability of these cell lines to investigate the effect of PPP withdrawal on NHL cells and to determine whether or not the survival and proliferation promoting activities previously seen in PPP could be replaced by defined culture conditions or cytokines. I also had the opportunity to contrast some of the information obtained from NHL cell lines with data on one fresh sample directly taken from a patient.

Knowing the importance of having PPP in the culture system, I first decided to investigate the effect of its withdrawal on NHL cells at three different levels: clonogenic cells (colony forming cells in semisolid medium), disease propagating cells (cells that cause tumor formation in animal hosts) and the "bulk" of the cell lines (cells in liquid suspension culture). The data obtained under specific aims 1, 2 and 3 in this thesis demonstrate that neither NHL clonogenic progenitors nor the bulk population of all tested NHL cell lines proliferate when PPP is withdrawn from the culture system. This effect is irreversible for clonogenic cells after 72 hours of incubation in PPP-free medium and for
the bulk population of each cell line after 48-96 hours of plasma free cultures. The tested cell lines of B and T origin represented all four patterns of growth factor production and requirement suggesting that the mechanism associated with the decline of growth may be the result of other causes than a lack of cytokines. In addition, there was no apparent correlation between the response to PPP withdrawal and the status of p53, c-myc and bcl-2. Cell lines with wild type genes (OCI-LY 13.1 and 10) had the same response as cell lines with mutations of p53 (OCI-LY 2 and 17), p53 and c-myc (OCI-LY 13.2 and 7) or p53, c-myc and bcl-2 (OCI-LY 8 and 18).

The effect of PPP withdrawal on proliferation was dependent on cell concentration as demonstrated for OCI-LY 3 suggesting that some protective signals may be generated and perhaps released by a small minority population or by cell-cell interaction requiring a limiting cell density. This cell line was previously characterized as being a producer of IL 6 and dependent on this cytokine in PPP-containing medium (3). The possibility exists that IL 6 released from these cells afford protection from the effects of PPP withdrawal. I tested this hypothesis by adding IL 6 to cultures of cells seeded at low concentrations. The experiments did not confirm a protective effect by IL 6.

Although I was unable to identify a specific cytokine to be responsible for the effect, the system can be used as a model to study whether or not the protective effect of higher cell concentrations is dependent upon direct cell-to-cell contact or soluble long range signals are generated. The latter might result in the generation of molecules in culture to be identified in culture supernatants.

The virtual elimination of clonogenic cells in culture after PPP withdrawal provided the opportunity to test whether or not clonogenic cells indeed encompass disease propagating cells. In order to test this question, in vitro cultures had to be significantly scaled up to generate sufficient cells for in vivo studies. The same concentration of cells as in the small test tube was used for these experiments. I observed a 5-6 fold reduction but not complete elimination of clonogenic cells. The cells cultured in PPP-free medium were injected into NOD-SCID hosts. Survival of the experimental group injected with cells cultured in PPP-free medium was significantly longer than observed for animals inoculated
with cells grown in PPP containing medium. However, as expected the majority of animals eventually developed tumors. It is of note that one of the animals injected with cell suspensions containing a similar number of colony forming cells as animals that did not survive remains free of disease. This differential effect on survival, suggests that not all clonogenic cells may represent disease propagating cells, again confirming a model in which the malignant clone comprises cells with differing proliferative potential.

The evidence provided by these studies of clonogenic, disease propagating cells and bulk population of NHL cell lines indicates that PPP provides signals for survival and proliferation and that PPP withdrawal renders cells non-proliferative.

The sensitivity of cells to PPP withdrawal could be explored further as a purging technique provided normal haematopoietic progenitor cells prove to be more resistant to PPP withdrawal compared to NHL cells. When normal human haematopoietic cells were tested in plasma free cultures over a period of 7 days, a significant proportion of progenitor cells of different lineages survived the exposure. This information may suggest that normal haematopoietic progenitors are indeed less sensitive to PPP withdrawal. Alternatively normal bone marrow cell populations may contain cells that produce protective cytokines.

These questions can be addressed by experiments in which NHL cell lines are admixed with normal bone marrow samples where they would be exposed to the same endogenously provided cytokines. In addition, one may face a problem when the culture conditions are scaled up and significantly higher numbers of NHL cells are present. In spite of these limitations further explorations are indicated since the method would be inexpensive and may add a different mechanism of purging to remove NHL cells from an autograph.

After I had determined that NHL cell lines do not proliferate in the absence of PPP, I investigated the question of whether or not programmed cell death may have been the reason. Morphological examination, flow cytometry of DNA-stained cells and gel
electrophoresis of DNA, consistently indicated that all but one of the tested cell lines underwent apoptosis after PPP withdrawal.

The cell lines OCI-LY 13.1 and 13.2, were used as a model in this part of my thesis because they reflect disease progression in the same original NHL clone. The previously described differences were complemented by observations relating to apoptosis. A significant number of OCI-LY 13.1 cells exhibited apoptosis even under optimal conditions provided by PPP suggesting that a significant number of cells undergo spontaneous apoptosis. As expected, the level of apoptosis was further increased by PPP withdrawal. OCI-LY 13.2, on the other hand, showed no spontaneous apoptosis. These observations were further expanded by one of my colleagues who demonstrated a significant difference in radiation sensitivity. As expected OCI-LY 13.2 was significantly more radioresistant. This behavior is not unexpected for cells with p53 and c-myc mutations. One would expect cells with wild type p53 and c-myc to be more sensitive to signals that induce apoptosis. My studies indicated that this is not necessarily the case. For instance OCI-LY 3 cells are not more sensitive to PPP withdrawal than OCI-LY 7 or 8 (which both carry mutations in p53 and c-myc genes). The inconsistency is further supported by OCI-LY 7. This cell line exhibits the highest extent of apoptosis of all tested cell lines in spite of rearrangements in both p53 and c-myc. The involvement of p53 and c-myc in the promotion of apoptosis has been well established but there is also an increasing body of evidence suggesting that apoptosis may also take place independently of p53 activities (180, 181, 182), and thus may indicate the existence of multiple pathways for programmed cell death.

The fact that NHL cells can undergo apoptosis provides a model to study apoptosis related mechanisms of cell death in NHL cells and answer, for instance the following questions:

1) Are Fas, TNF ligands and their receptors involved in this process?

FAS and TNF receptors have been reported as being involved in the initiation of apoptosis. The possibility exists that NHL cells under this condition may produce Fas ligand or TNFα by initiating a cascade of events leading to apoptosis. Alternatively, Fas
or TNF receptors can undergo oligomerization that could potentially induce the transduction of death signals.

2) Are other death signals such as TRAIL (183) involved?

DR4 (Death Receptor 4) and DR5 have been recently characterized as death receptors that belong to the TNF superfamily. Their ligand, TRAIL (Apo2 ligand) induces the transduction of intracellular signals that cause the activation of caspases and finally leads to programmed cell death. As seen with other members of this superfamily, oligomerization of these receptors can lead to activation of signaling in the absence of a specific ligand (184, 184). It is of note that DR4 and DR5 are present in both, normal and neoplastic cells, but apparently only the latter are sensitive to TRAIL mediated apoptosis. A decoy receptor for TRAIL, (DcR1, TRID) was described in normal tissues that may be responsible for the resistance of normal cells to TRAIL induced apoptosis (184, 185).

3) What is the role of bcl-2?

Considering the effect observed in cells carrying wild type or mutated forms of the bcl-2 gene one has to raise the question whether or not alternative pathways of apoptosis may bypass the protective action of the bcl-2 protein in NHL.

4) Are caspases activated?

Caspases, are proteolytic enzymes involved in initiation, effector and degradation phases of apoptosis. They have been studied in TNF/Fas associated death pathways and other inductors of apoptosis. At least 10 caspases are expressed in human cells which have a wide variety of targets, many of them responsible for the morphological characteristics of apoptotic cells.

The technology to examine these questions is now readily available. The expression of genes encoding members of the TNF superfamily or their ligands can be further evaluated in NHL cells after PPP withdrawal following different approaches. Conditioned medium of NHL cells cultured in PPP-free medium can be tested for the presence of FAS ligand, TNFα or TRIAL by using ELISA and or Western blot technology. The expression of their receptors can be analysed by using FACScan analysis with
suitable antibodies and Western blotting. If needed, restriction enzyme analysis and sequencing may be employed to study the status of the genes and Northern blot analysis may add some information about their respective mRNAs. Caspase activities can be detected by using specific inhibitors and colorimetric substrates of these enzymes that are commercially available nowadays.

Once it was established that NHL cells engage in programmed cell death after PPP withdrawal, I decided to test whether or not defined nutrients could replace at least in part some of the activities described in PPP. I chose a combination of BSA, insulin and transferrin (BIT) that has been successfully used in Dr Iscove's laboratory (159, 160) to grow normal hematopoietic progenitor cells under low concentration of fetal calf serum to evaluate the effect of specific cytokines. This defined serum-free medium has the advantage that the composition is fully known and thus permits testing of various concentrations of each component in culture. In addition, I tested a commercially available serum replacement: Stem Pro 34® (from Gibco) that has the drawback of an undisclosed composition. I demonstrated that replacement of PPP by a defined combination of nutrients such as BIT seems to reduce PPP-withdrawal induced apoptosis in several cell lines. The effect was more complete with Stem Pro 34®. With neither system however, were we able to demonstrate a significant and consistent effect of cytokines used either alone or in combination. It has to be noted, that none of these serum replacements was able to replace completely PPP in terms of its growth promoting activity.

The abscence of a significant effect of defined cytokines on the proliferation and survival of NHL cells in the abscence of PPP was rather unexpected. These data did not reflect previous observations in PPP-containing cultures using two IL6 producing cell lines (OCI-LY 3 and 12). The addition of IL 6 eliminated the lag phase observed when no IL 6 or PHA-LCM was added to the culture system (9). One possibility for this different behavior is that substances in PPP served as cofactor in conjunction with IL 6. Alternatively, cell lines may have changed during successive passages and exhibit a different expression of cytokines and or their receptors compared to the profile at the time of the initial studies.
Therefore, I proceeded to examine the effects of cytokines in a fresh sample obtained from a patient with EBV negative Burkitt’s lymphoma with the objective to compare these data with information obtained at later time point after generation of a cell line. IL 2 and IL 6 in combination with BIT induced some level of proliferation in the fresh sample. None of the tested cytokines supported proliferation in the derived cell line. IL 1, IL 2 and IL 6 did maintain cell survival in the fresh sample compared to BIT alone but only IL 6, IL 7 and IL11 had this effect when tested on the cell line. One possible explanation for this phenomenon would be that accessory cells present in the fresh sample may have been responsible for the production of some survival signals under the stimulation of IL 1, IL 2 and IL 6. During passaging to establish the cell line, these accessory cells may have been lost. This would explain why some of the cytokines that supported survival/proliferation in the original sample are not active anymore in the derived cell line. Alternatively the cells that were responsive to cytokines for survival and proliferation in the fresh sample may have undergone changes such as loss or down regulation of cytokine receptors, autocrine stimulation or they may have disappeared during serial passaging. Other subclones may have become predominant with ability to respond to different cytokines, produce their own or be independent of growth factors. These possibilities require further exploration of fresh samples and subsequently established lines.

The model in Figure 6 (see Introduction) provides a minimalistic depiction of properties displayed by NHL subpopulations. Since accessory cells such as T-cells, monocytes, stromal cells, etc may be involved in the generation of growth controlling molecules, it might be more appropriate to embed the model into a network of cells with regulating function as suggested in Figure 25 to reflect the complexity of the system.
Figure 25. Proliferation and death in NHL cells. A more complex scenario
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