DETECTION OF IL4 AND TNFα AND THEIR RECEPTORS IN CHRONIC LYMPHOCYTIC LEUKEMIA OF B LINEAGE

A Thesis Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the degree of

Master of Science

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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BY

Carmen Morales

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ABSTRACT

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Chronic lymphocytic leukemia of B cell lineage (B-CLL) is a common neoplasm of the elderly, in which rising lymphocytosis, in combination with a low proliferation rate, point to an abnormally long life span of the B-CLL lymphocytes. No etiologic cytogenetic abnormality has been detected thus far for B-CLL, suggesting that tumor environmental factors and intercellular signaling processes may be important in its pathogenesis.

Purpose: This study focuses in the role of tumor generated cytokines as modulators in the neoplastic cell biology. In particular, the potential of B-CLL lymphocytes to produce two cytokines previously implicated in cell survival and proliferation: IL4 and TNFα, as well as to express their surface receptors.

Method: Peripheral blood samples from 44 untreated B-CLL patients were analyzed to determine expression of intracellular IL4 and TNFα and their receptors in the B-CLL lymphocytes, using a standard flow cytometric technique. Levels were correlated with the clinical stage of the disease.

Results: In most cases of indolent B-CLL, the tumor cell populations showed a large proportion of IL4 receptor and intracellular IL4 positive cells, while intracellular TNFα and TNFα receptor were detected only in a small subset of tumor cells. In contrast, the aggressive stage cases showed decreased levels of intracellular IL4, and a slightly lower proportion of IL4 receptor positive cells. Intracellular TNFα and its receptor expression were increased in these high risk B-CLL populations. These findings parallel the traditional knowledge of population subsets in B-CLL: a majority of non-dividing, long lived cells, with a small proliferating pool in the low risk cases, changing to an increased proportion of proliferating cells in high risk B-CLL. This study suggests that B-CLL produced IL4 may support apoptosis resistance in the accumulating cells, while TNFα generated by some of the B-CLL cells could foster the proliferating subset.

LIST OF ABBREVIATIONS

B-CLL: B- Chronic Lymphocytic Leukemia

BCR: B cell receptor

CD: Cluster Designation

ECD: Phycoerythin-Texas^{R} Red-X

FITC: Fluorescein isothiocyanate

Ig: Immunoglobulin

IL: Interleukin

IL4R: Interleukine 4 receptor

IFN: Interferon

PBS: Phosphate buffered saline

PE: Phycoerythryn

PerCP: Peridinin Chlorophyll

RD1: Phycoerithryn (Beckman-Coulter)

TCR: T cell receptor

TGF: Tumor growth factor

TNF: Tumor necrosis factor

TNFαR: Tumor necrosis alpha receptor

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INTRODUCTION

INTRODUCTION

B-chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the western world. Its incidence approaches 50 per 100,000 after the age of seventy, and it is considered the prototype of cancer in the elderly. It has a documented familial tendency and occurs preferentially in Caucasians.[1,2] The leukemia usually has a protracted natural course of years and even decades, but eventually accelerates as the cells acquire sequential genetic defects.[1,2,3] Biologically, the disease has not only oncologic, but also immunological aspects[2] since patients develop immunodeficiency and autoimmune processes which cause cytopenias (autoantibodies).[1,2]

B-CLL leukemia differs from many other malignancies in that monoclonal B-CLL cells accumulate relentlessly, not because of unregulated proliferation—the majority of circulating cells are non-dividing—but due to an abnormally prolonged life span[4], which likely is a consequence of altered interactions between defective B-CLL cells and their environment.[2,3] Cytokines are essential factors in cell homeostasis and cell-cell dialogue, and are proposed to be critical in this B-CLL milieu.

BIOLOGY OF B CELL CHRONIC LYMPHOCYTIC LEUKEMIA: BRIEF REVIEW OF CURRENT KNOWLEDGE

I. PROPOSED CELL OF ORIGIN

B-CLL cells are phenotypically similar to the so-called B-1 fetal-cord blood or peritoneal cavity small lymphocytes, and to the compact mantle cell lymphocytes of secondary lymphoid follicles.[5 6 7]

They express CD5[5], and Ig-V regions denoting preferential use of certain V-genes, V1-6.[6-9] Ig production of B-CLL cells often mirrors that of normal CD5+ counterparts: polyreactive, low-affinity autoantibodies.[10] However, while 50% of B-CLL cases show no evidence of V-gene somatic point mutation, in half of the cases studied there was evidence of hypermutation, indicating a post-germinal center memory cell that had responded to antigen-related stimulation.[11-14] These findings seem to correlate with the proposed two ontogenetic pathways of B-1 lymphocytes: one would be an early self-replicating stem cell, with constitutive expression of CD5, ancestor to the subset without evidence of point mutations.[14 15] A bone marrow born B cell would give rise to the second tier of B-1 lymphocytes, which would express CD5 only after antigen exposure, affinity maturation, and relocation to the follicular mantle as a memory cell[5,16].

The same division of cell types appears to correspond also with disease behavior patterns. The naïve/unresponsive/anergic B-CLL type eventually requires intensive therapy, while memory-type B-CLL behavior is indolent, survival in these patients being equivalent to that of matched controls.[16-19] This new insight into the origin and evolution of B-CLL is a radical change from the original views of B-CLL lymphocytes as homogeneously naïve, unresponsive, immunologically incompetent cells.[4 20]

II. GENETIC DEFECTS

A common initial transforming event has not been found for B-CLL. There appears to be no causative relation to ionizing radiation or chemical exposure. Karyotypic abnormalities tend to increase in frequency and number during the course of the disease[1 3 20].

Chromosomal translocations, thought to occur mainly during the gene rearrangement process and common in other lymphoid malignancies, are rare in B-CLL. When translocations are found, they tend to result in genetic loss, rather than in the formation of a fusion gene or over-expression of an oncogene. These facts raise the pathogenetic possibility of missing tumor suppressor genes.

The most common genetic abnormalities in B-CLL are 13q deletions (50% of cases), 13q14 deletions (associated with an indolent course), trisomy 12 (12q13-15, with over-expression of MDM-2 oncoprotein which suppresses p53), and 11q22-q23 deletions (20% of cases).[21-25] 11q22-q23 deletions are associated with lower levels of adhesion molecules (CD11a/CD18, CD31/PECAM-1, CD48, and CD58/LFA-3), as well as of cell signaling molecule expression (CD45, CD6, CD35, CD39).[26-29] These deletions are found in the germline in some cases, suggesting a genetic predisposition to B-CLL development in some patients.[27-28]

III. FUNCTIONAL ABNORMALITIES

1. Abnormal expression of B-Cell Receptor and membrane molecules, resulting in an activation-refractory state:

B-CLL cells express surface molecules such as CD23 (low affinity receptor for IgE), CD25 (IL-2R α chain), and CD27 (co-stimulatory molecule), which in other

settings indicate a state of activation.[2 29] In contrast, other membrane molecules that amplify signaling via the B-cell receptor (BCR) such as CD22, are weakly expressed or undetectable in B-CLL[30 31].

Cap formation (polar redistribution of antibody-receptor complexes) is also reported as defective in B-CLL cells[32].

For decades it has been well noted that B-CLL cells are unresponsive to most B lymphocyte mitogens[2 7 33], and extremely difficult to maintain in culture[33-36].

It has been proposed that the cause of this frustrated state of activation is an abnormal BCR complex. This complex is formed by the sIg molecule, and the CD79a/CD79b heterodimer that transmits signaling from the BCR to the cell interior. In most cases of B-CLL, the BCR is weakly expressed and the extracellular Ig-like domain of CD79b is lacking.[37-39]. A CD79b truncated form arises by alternative splicing of the CD79b gene, and lacks exon-3, which encodes the extracellular domain. The absence of this extracellular domain appears to decrease the stability of the BCR on the cell surface. This spliced variant is present in human B cells and B cell lines, but appears to be the dominant form of CD79b in all cases of B-CLL tested, suggesting a role for the CD79b spliced variant in causing the reduced expression of BCR on B-CLL cells. This would account for the defective signal transduction, similar to that seen in non-neoplastic anergic or post-activation B cells[19].

The level of immunoglobulin expressed on the surface of the leukemic cells is lower than in normal cells. Half of B-CLL cases express IgM and IgD, one-fourth IgM, and less than 10% have Ig isotypes other than IgM. IgG expression is extremely rare. An excess of light chain production compared to heavy Ig chains has been reported[40 41 20].

Moreover, other evidence suggests that transmission of signals by the B cell receptor is impaired predominantly in the CD38 positive "naïve" B-CLL cell, but functional in the CD38 negative, post-germinal center hypermutated subset.[16 -18] Experimental ligation of IgM BCR appears to induce apoptosis in the CD38 positive leukemic cells, while activation through IgD promotes cell survival and

plasma cell differentiation. However, signaling through IgM negates the effects of IgD cross-linking.[16]

In addition, B-CLL cells appear unable to present soluble antigens and alloantigens, and express low levels of CD80 (B7-1, co-stimulatory molecule). CD38 expression was not investigated in this context.[42]

The refractoriness of B-CLL cells to respond normally to receptor mediated signals implies that a different mechanism must operate to rescue cells from apoptosis, and to induce proliferation in this disease.

2. Abnormal interaction with bone marrow stromal cells:

B-CLL cells typically infiltrate the bone marrow. It is not known whether they originate or home there, or both, depending on their ontogenetic subtype. Recent studies have demonstrated that they express functional CXCR4 receptors[43] for the chemokine stromal cell-derived factor-1 (SDF-1), and migrate towards SDF-1—secreting stromal bone marrow cells in an experimental system. SDF-1 ligand appears to be also part of a possible mechanism by which stromal "nursing cells" would support B-CLL survival in the bone marrow and perhaps in circulating blood[44]. This effect is not seen to occur for normal B cell counterparts. Further studies are needed to determine whether this mechanism is functional *in vivo*, and to what extent contributes to the biology of the disease.

3. Abnormal cell survival

The prolonged survival of B –CLL cells in vivo contrasts sharply with their rapid in vitro cell death.[34 36] This suggests humoral factors or cellular interactions which modify an otherwise preserved mechanism of programmed cell death.

The expression and association of several proteins tightly regulate the process of apoptosis. The relative balance of these proteins controls cell life span. Genes responsible for this system include the BCL-2 family, the tumor necrosis factor-receptor and genes such as myc and p53. All the death pathways promoted by these genes appear to have a common "demolition" cascade, represented by the protease family of the caspases.[46-49] B-CLL cells consistently express high levels of products of the anti-apoptosis members of the BCL-2 family (bcl-2, bcl-x_L, bax), while the bcl-2 function inhibitor bcl-x_s is markedly reduced.[2 47] The mechanism involved in over-expression of bcl-2 is currently unclear. The leukemic cells of B-CLL are negative or weakly positive for Fas. They generally remain resistant to anti-Fas antibody mediated death even after stimulation-induced Fas expression. In rare sensitive cases, cell death occurs independently of bcl-2 expression by a mechanism still uncharacterized.[50]

It would appear that BCL-2 over-expression and the Fas pathway are mechanisms involved in the pathophysiology of B-CLL but not necessarily critical causative events. Mediators including cytokines are likely to link the initial etiologic factor with the terminal pathways of apoptosis.

4. Altered kinetic properties

Most B-CLL cells are the in Go phase of the cell cycle,[2 33-36] and can not be induced to enter the proliferative phase by conventional methods such as concanavalin-A, phorbol esters, or receptor cross-linking, which induce the proliferation of normal B lymphocytes. Only a small subset of cells appear to enlarge the clonal population in response to an unknown promoting signal[34 36]. Proliferation promoting cytokines may provide this stimulus *in vivo*.

5. Altered Nuclear Factor Binding

Studies have shown that there is constitutive nuclear translocation of NF-ATp, a member of the NF-AT family of factors, as well as of NF-kB2/p52, in B-CLL cells but not in normal CD5 positive resting B lymphocytes.[51]

Constitutive binding of other transcription factors to DNA (Jun D, Fos B) was detected in fewer B-CLL cases.[51] This finding suggests again an abnormal "switched-on" state in the absence of appropriate stimulation. The mechanisms that result in this "pre-activation" state and their detailed downstream consequences remain unknown.

IV. B-CLL ENVIRONMENT

B-CLL cells accumulate at the expense of the normal B cell pool. Total T cells on the other hand, are usually increased. The bone marrow T lymphocytes are predominantly CD4+ cells as seen in autoimmune disorders such as rheumatoid arthritis and sarcoidosis. There is frequently a Th2 predominant cytokine phenotype in peripheral blood. Abnormalities in TCR repertoire have been reported also[2 3].

Reports indicate that T lymphocytes and stromal cells may have a key role in supporting an environment capable of perpetuating the life span of the B-CLL cells.[46 52-54] Both, the malignant cells and their T cell entourage express a variety of surface molecules and their receptors: CD5 and its ligand CD72, CD27 and CD70.[5] These findings open various possibilities of mutual interaction which could result directly or indirectly (cytokines) in cell self-preservation. Such lengthy survival would, in turn, increase chances for accumulation of gene

mutations and genetic instability,[55] which favors disease progression through dysregulation of cell cycle check-points, and resistance to cytotoxic therapy.[56-58]

V. CYTOKINES AND B-CLL BIOLOGY

The role of cytokines in the maintenance of homeostasis in organisms, and their participation in disease processes has been the object of intense study for the past decade. Cytokines have been reported as mediators in all hemapoietic systems, and cited as ultimate regulators of health and disease.

Study of cytokine involvement in any process is inherently difficult, since cytokines exist as part of complex networks. Dissection and isolation of network loops for investigation has been accepted, given our limitations in reproducing physiologic conditions.

1. Cytokine effects in B-CLL cells

As previously mentionned, B-CLL results from massive accumulation of neoplastic cells. Only a minor pool within this population (the so-called prolymphocytes) exhibit proliferative activity. The majority of the population accumulates as a result of longevity. Two mechanisms are then required to perpetuate this leukemia: one would support the small reproductive subset, and the other would mediate the survival of the non-dividing cell majority. Since no recurrent genetic event has proven to be responsible for this pathology, researchers have turned to humoral and/or cell-cell interactions for an explanation. The symbiotic interaction between B-CLL cells and their environment is almost certainly mediated by the secretion of cytokines and modulated by adhesion molecules.[2]

Investigation of cytokine involvement in B-CLL has generated a substantial body of data supporting or disproving various cytokines as mediators of proliferation and/or prolonged life span in this leukemia (Fig. 1, page 24 for summary)).

a) Cell Survival

Isolated B-CLL cells in culture undergo spontaneous apoptosis. Rescue from programmed cell death occurs when IL4, Interferon γ or β are added to the culture medium.[59-62] This is probably mediated by up-regulation of BCL-2 protein levels.[63]

Studies have demonstrated IFN-γ production in B-CLL culture supernatant, as well as mRNA signal in the cells. Positive results have not been reproduced by all authors for IL4 and IFN-α. Various other cytokines (IL1, IL2, IL3, IL5, IL6, IL7, IL8, IL10, IL15, Interferons, and Tumor Necrosis Factor) have generated conflicting reports in support of or against their role in inhibition of B-CLL apoptosis.[64-77]

Regarding *in vivo* effects, some studies show that Transforming Growth Factor β fails to produce apoptosis in the leukemic cells.[78-82] On the other hand, IL10 has been shown to increase *in vivo* apoptosis of B-CLL cells in early stage patients.[83] Moreover, IL10 production by B-CLL cells (mRNA) decreases and serum levels drop as the disease becomes more aggressive.[84] [85] IL6 production appears to diminish also in advanced stages.[73]

See Figure 1.

b) Cell Proliferation

In general, reported findings indicate lack of proliferative response of B-CLL cells to most cytokines. Exceptions would be IL2 and TNFα, both tested *in vitro*. Various experiments have shown TNF-α mediated increase in DNA synthesis when added to B-CLL cultures.[75 76]

IL6 was reported to inhibit B-CLL proliferation induced by TNF α or β,[74] and IL4 counteracts IL2 mediated proliferation.[60]

See Figure 1.

2. Constitutive Cytokine Production in B-CLL

Cytokine production investigations have demonstrated reverse-transcription polymerase chain reaction signals for IL1, IL2, IL3, IL4, IL5, IL7, TNFβ, IL6, and TNFα[86]. These findings have been contradicted by other studies which showed negative results for IL4, IL3 and IL6. In contrast, TGFβ secretion has been shown in normal B-1 lymphocytes, as well as IL10 secretion[87].

No other cytokine production has been reported to be constitutive for these cells.

Two cytokines appear to be consistently shown to influence cell dynamics in B-CLL: IL4 as a longevity factor and TNFα as a promoter of cell division.

VI. TUMOR NECROSIS FACTOR ALPHA

Normally, TNFα is produced mostly by monocyte/macrophages, but also by some activated T and B cells, after exposure to bacterial toxins, viruses, mycobacteria, fungi, parasites, activated complement, antigen-antibody complexes, and other cytokines. TNFα has a 17KD form (secreted), and a 26KD

form (membrane bound). TNFα, also known as a cachectin, has various biological effects on normal cell populations: stimulation of tissue factor expression on endothelial cells and monocytes, induction of neutrophil activation, suppression of hematopoietic progenitors, enhancement of NK cell cytotoxicity, and of monocyte and eosinophil cytotoxicity, and direct cytotoxic effect in some tumors.[88-90]

VII. TNFα RECEPTORS

Type I (TNFR-55) and Type II (TNFR-75) are present in the membranes of all normal cells except mature erythrocytes. The two receptors share structural homology in the extracellular binding domains. They cluster upon ligation, and initiate separate cytoplasmic signaling pathways.[90]

VIII. TNFα AND B-CLL CELLS

TNFα promotes survival and proliferation of B-CLL cells in vitro.[75] [76] The presence of TNFα receptors has been reported by some authors, while others found no receptor expression in minimally processed B-CLL cells.[89] Serum levels of TNFα increase proportionally with advanced disease stage and progression.[85] Soluble TNFR in the serum of patients may play a role in regulating the effects of the cytokine in the tumor biology, at least during the indolent phase of the disease (see Figure 1).[91]

There is mixed evidence as to the nature of the TNFα-producing cell which would provide the B-CLL environment with the cytokine.[75] T cells and monocytes have

been proposed. The B-CLL cells are also candidates, since TNFα mRNA was detected in these cells, and TNFα protein in their culture supernatant (see Figure 1).

IX. INTERLEUKIN 4

Human IL4 is a member of the hematopoietic family of cytokines. It is a glycoprotein which exists in molecular weight forms between 15000 and 19000 Daltons. Reduction or alkylation destroys its biologic activity.[92] IL4 plays an important role in the regulation of B cell proliferation/activation and expression of membrane antigens such as increase in the numbers of MHC Class II molecules. It acts as a switch factor for IgE and IgG1 production. IL4 also mediates T cell and mast cell growth and survival. It has an inhibitory effect in macrophage activation, as well as for hemopoietic cell growth, through inhibition of stromal cell support for colony formation.[92 93] IL4 is the prototype TH2 cytokine, produced mainly by CD4 T cells driving humoral immune responses. Production has been demonstrated also in mast cells.[52 53 92]

Low levels of IL4 mRNA have been detected in some normal B cells in vitro, and in increased levels in B-CLL cells.[64 93]

X. IL4 RECEPTOR

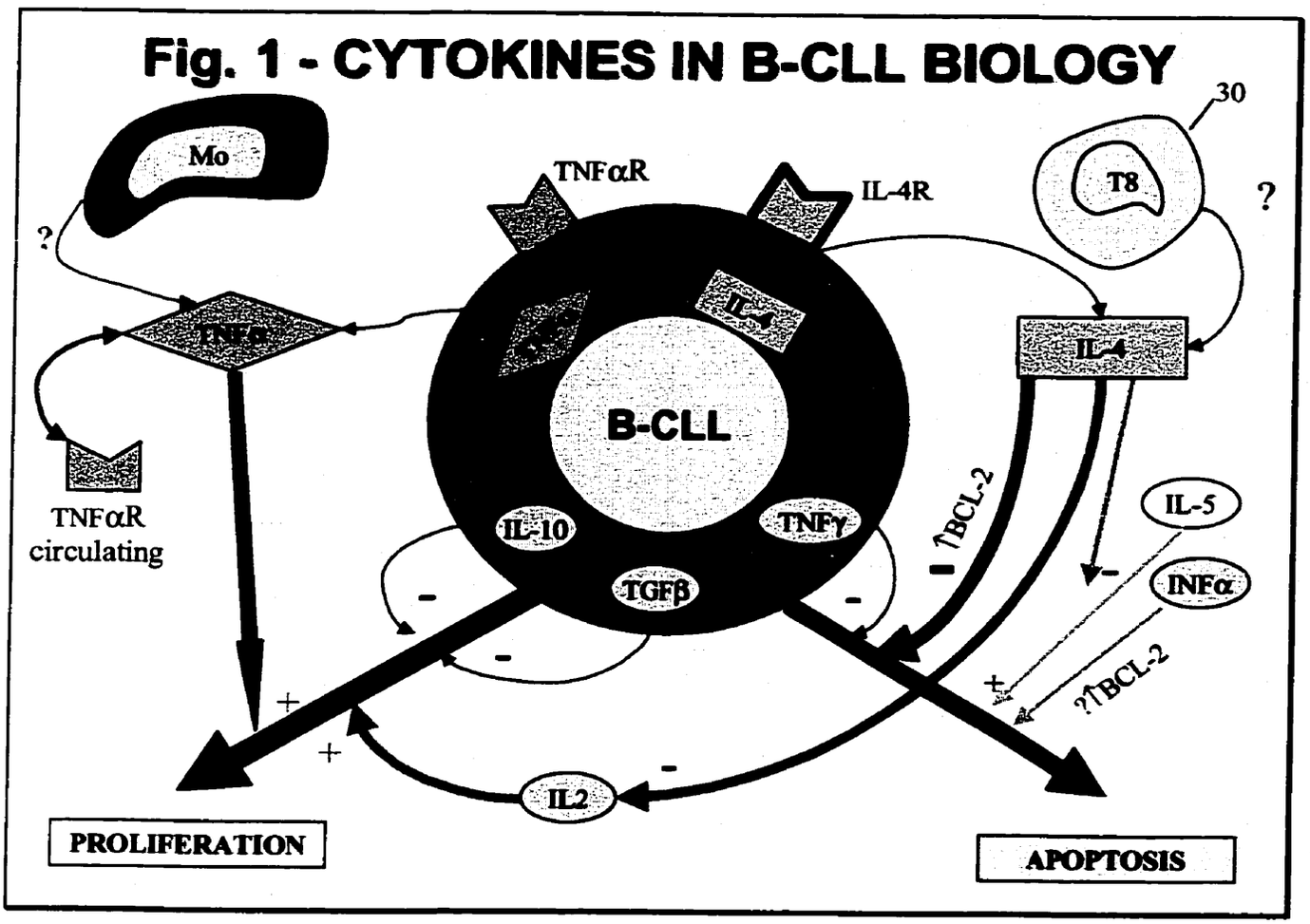
IL4 receptors with high affinity are expressed in relatively low numbers in normal cells. Structurally similar to other receptors in the hematopoietin family, it shares a β chain, and a common γ chain. It is found in B and T lymphocytes,

macrophages, other hemopoietic cells, fibroblasts, and stromal cells. Activation of T and B cells results in upregulation of receptor expression.[92]

XI. IL4 AND B-CLL CELLS

IL4 prevents spontaneous apoptosis of B-CLL cells in vitro, directly, and also by inhibiting IL5 promotion of cell death.[62] Production of IL4 has been reported in the CD30+, CD8+ T cell subset that forms part of the B-CLL entourage. In addition, mRNA was found in B-CLL cell isolates by others (see Figure 1).[52 53 61] It has been shown to sensitize the neoplastic cells to some in vitro drug toxicity (Melphalan).[62] Protection from apoptosis is mediated at least in part by upregulation, or inhibition of downregulation of BCL-2.[45 65]

Review of the literature supports a significant role for IL4 in the maintenance of B-CLL long survival, and of TNFα as a tempered proliferative stimulus. Various sources for these cytokines have been postulated, and production by tumor and background cells investigated by different methods, which do not include demonstration of direct intracellular protein secretion. See Figure 1, page 24.



+: induction

- : inhibition

Mo: monocyte/macrophage

HYPOTHESIS

B Cell Chronic Lymphocytic Leukemia cells are able to produce cytokines (TNFα, IL4) that in an autocrine and/or paracrine mechanism may contribute to the tumor environment which enables their abnormal survival in vivo.

PURPOSE OF THE STUDY

1. To demonstrate intracellular IL4 and TNFα synthesis in B-CLL cells by directly detecting intracellular production.

2. To demonstrate the expression of their corresponding membrane receptors on fresh, unstimulated B-CLL cells.

3. To determine whether there is a relationship between production of these cytokines by B-CLL cells and clinical stage of the disease (See tables 1a and 1b, next page).

Tables 1a and 1b

1a. RAI Classification System for B-CLL*

STAGE	Modified Stage	Description	Median Survival
0	Low risk	Lymphocytosis	>10 years
I	Intermediate risk	Lymphocytosis, Lymphadenopathy	>8 years
II	Intermediate risk	Lymphocytosis +splenomegaly +/-lymphadenopathy	6 years
III	High risk	Lymphocytosis +anemia +/-lymphadenopathy or splenomegaly	2 years
IV	High risk	Lymphocytosis +thrombocytopenia +/-anemia +/-splenomegaly +/-lymphadenopathy	2 years

*Adapted from Rai KR et al:Clinical staging of Chronic Lymphocytic Leukemia. Blood 46:219-227,1975

1b. BINET Prognostic Classification for B-CLL*

Stage	Clinical findings	frequency	Median survival
A	Lymphocytosis >40% lymphocytes in Bone marrow no anemia, thrombocytopenia <3 involved sites (lymphnodes, spleen, liver)	55%	Comparable with sex and age matched population
B	Stage A plus >3 involved areas	30%	7 years
C	Stage A plus >3 involved areas, Hemoglobin <10 g/L Platelet count <100,000/µL	15%	2 years

*Adapted from Binet J-L et al: Chronic Lymphocytic Leukemia: Proposals for a revised prognostic staging system. Report from the International Workshop on CLL. The writing Committee. British Journal of Haematology 48:365-378,1981.

MATERIALS AND METHODS

MATERIALS AND METHODS

I. SAMPLES

Peripheral blood (5 mL) was obtained from patients with known Chronic Lymphoid Leukemia. The patients were untreated or off-treatment at the time of collection.

Whole blood was processed to isolate mononuclear cells by Histopaque (Sigma Diagnostics Inc). The mononuclear cell layer was removed and washed twice with cold phosphate buffered saline (PBS) (Beckman-Coulter). After removing the supernatant, PBS was added to a final volume of 2 ml. Further, a 1:3 dilution in newborn calf serum (GibcoBRL) was made and cell count determined in an automated cell counter (MAXM, Beckman-Coulter, California).

The cell suspension was then aliquoted for basic immunophenotyping, surface cytokine receptor, and intracellular cytokine determination.

II. IMMUNOPHENOTYPING

Complete immunophenotyping of the leukemic cells was performed at the time of diagnosis. All cases met morphologic and immunophenotypic criteria for B-CLL, as required by the Revised European-American Classification of Lymphoid Neoplasms and the World Health Organization Classification [94 95].

Immunophenotyping of the mononuclear cell isolate:

The cell suspension obtained previously was adjusted to 4x 10^6/ml with PBS (phosphate buffered saline). From it, three tubes were set up containing 100µL each, and one of the following monoclonal antibody combinations: anti-CD2 RD1(Phycoerythin), 10µL of a 1:5 reagent dilution, and anti-CD19 FITC (fluorescein isothiocyanate), 25µL (both purchased from Beckman-Coulter), anti-CD4 RD1, 5µL of a 1:20 reagent dilution, and anti-CD8 FITC, 5µL of a 1:20 reagent dilution (both purchased from Beckman-Coulter), anti-CD3 ECD (phycoerythrine-Texas Red^R-X), 10µL, and anti CD14 FITC, 10µL (both purchased from Immunotech).

The three tubes were incubated for 20 minutes at room temperature, washed once at 4° C with PBS, centrifuged and the supernatant removed. Then, 500µL of PBS was added to each tube, and each analyzed in a Coulter XL flow cytometer[98].

The percentage of B cells, CD4 and CD8 T cells, and monocytes present in the sample were determined. No attempt was made to remove non B-CLL populations, since these are considered to be essential players in the tumor microenvironment, and can be easily distinguished from B-CLL during testing by immunophenotype and light scatter properties.

CYTOKINE SURFACE RECEPTOR TESTING

Commercial kits, Biotinylated Fluorokine Human IL4, and Human TNFα (R+D Systems) were used.

Sample staining

An aliquot of the initial mononuclear cell suspension was counted and adjusted to 4x10^6/ml with PBS.

A total volume of 250µL of cell suspension including 25µL of anti-CD22 phycoerythrin, or anti-CD19 PerCP (peridinin chlorophyll) 20µL and 10µL of CD5 RD1 monoclonal antibody reagents (Beckman-Coulter) was prepared, incubated for 20 minutes at room temperature, washed once with the buffer provided by the kit (RDF1 at 1:10 dilution in sterile distilled water as instructed).

The supernatant was removed and the cell suspension reconstituted with buffer back to 250µL.

See Figure 2.

Test tube:

25µL of anti CD22 or CD5/CD19 labeled cells and 10µL of biotin labeled cytokine.

Negative control:

25µL of anti CD22 or CD5/CD19 labeled cells and 10µL of biotinylated negative control provided by the kit (soybean protein).

Specificity testing:

CD 22 or CD5/CD19 pre-labeled cells are treated with purified human IgG (10µL of 15mg/mL/106 cells) for 15 minutes at room temperature, in order to block Fc-mediated interactions.

A separate tube was set up with 20µL of anti-human cytokine (IL4 or TNFα) blocking antibody mixed with 10µL of biotin labeled cytokine, and incubated for 15 minutes at room temperature.

After incubation, 1 x 10^5 Fc-blocked cells (in a volume up to 25µL) are added to the tube containing the anti-human cytokine blocking antibody and the labeled cytokine mixture.

Fluorochrome labeling and preparation for flow cytometry:

Each test, negative control, and specificity testing tubes were incubated for 60 minutes at 4°C. See Figure 2 in page 34.

To each tube, 10µL of avidin-FITC reagent was added without pre-washing.

The mixtures were incubated further for 30 minutes at 4°C in the dark, and then washed twice with 2mL of the kit buffer to remove unbound avidin-FITC .

The cells were then re-suspended in approximately 0.2mL of the buffer for analysis. The three tubes were examined by flow cytometry, using multiparameter analysis, which included light scatter and CD22 or CD5/CD19 combined gating.

III. INTRACELLULAR CYTOKINE DETECTION:

1. Sample preparation:

An aliquot of the original mononuclear cell suspension was adjusted to $2x10^6$/ml, using RPMI medium (Canadian Life Technologies Inc.) with 10% newborn calf serum (GIBCO).

See figure 3.

2. Cell activation and incubation:

Non-specific activation was provided with 25ng/ml of Phorbol 12-myristate 13- acetate (PMA) (Sigma , St. Louis, MO), and 1µg/ml of Ionomycin (Sigma).

Twenty µL each of Pen G and Streptomycin (Sigma) were also added to prevent bacterial growth.

The cells were incubated in this mixture for 20-23 hours, at 37°C in a 7% CO2 incubator.

3. Protein transport inhibition:

At 18-19 hours of incubation, 10μg of Brefeldin A (Sigma) were added to inhibit secretion of potential cytokine production from the incubating cells, by interfering with vesicular transport from the rough endoplasmic reticulum to the Golgi complex.

4. Cell labeling for subset identification:

At completion of the incubation period, the cells were washed once in PBS, and surface labeled with anti CD5 Cychrome (Pharmingen) 20μL/100μL, and anti CD19 ECD (Coulter) 10μL /100μL of cell suspension, to identify the B-CLL population. Cells and antibodies were incubated for 20 minutes at room temperature and washed once with PBS.

5. Cell fixation and permeabilization:

100μL of CD5/CD19 labeled cell suspension were treated with 250μL Cytofix-Cytoperm solution (Pharmingen) to fix and permeabilize cells for intracellular staining, incubated for 20 minutes at 4°C, followed by two washes with a saponin -containing buffer at 1:10 dilution (Perm/Wash, PharMingen), and reconstitution to 100μL.

6. Intracellular staining:

a) Intracellular cytokine production detection:

Staining for intracellular cytokines was performed by adding 5μL of anti-human IL4 or TNFα PE labeled antibody (PharMingen).

b) Isotypic control:

An isotypic control tube was also set up with 5µL of mouse IgG1 (ParMingen) instead of the specific anti cytokine antibodies.

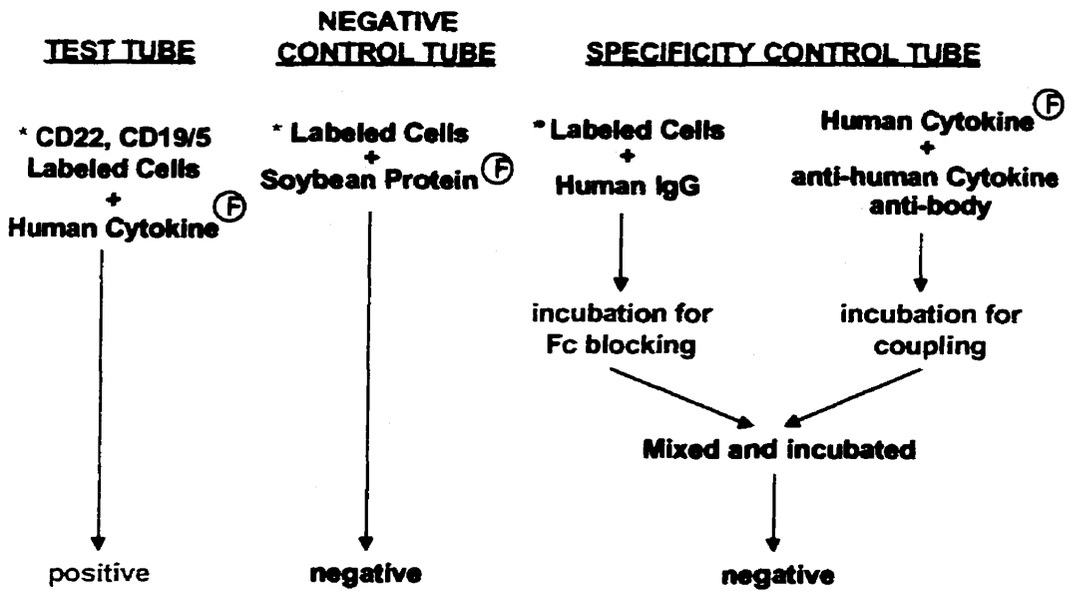
c) Specificity control:

Control for specificity was provided by a tube containing 100µL of the prepared CD5/CD19 labeled cell suspension, 10µL (15 mg/mL) of Human Gammaglobulin Fraction II (Nides Laboratories, Elkhart, IN) for Fc blocking, and 10µL of unlabeled anti-human cytokine (IL4 or TNFα) antibody, which was incubated for 15 minutes at room temperature. The latter step blocks intracellular cytokine tagging by the specific, PE labeled anti-human cytokine antibody, which was added at the same concentration as in the test tube.

Test and control tubes were incubated at 4°C for 20 minutes in the dark as per standard method for flow cytometric analysis, washed once with Perm/Wash buffer, and once with PBS, and examined by multiparameter flow cytometry, including gating events by light scatter and CD5/CD19 co-expression.

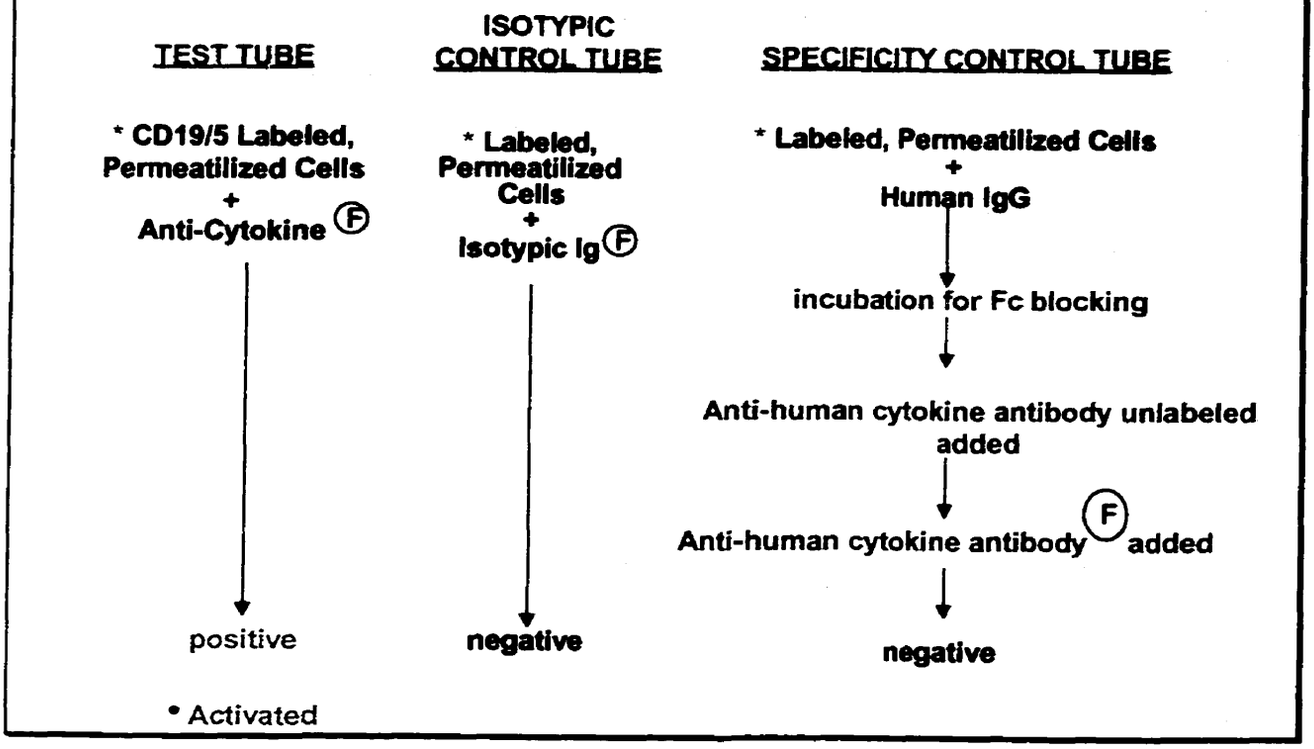
See Figure 3 in page 35.

Fig. 2 SURFACE RECEPTOR DETECTION



* Fresh, no in-vitro activation

Fig. 3 INTRACELLULAR CYTOKINE DETECTION



RESULTS

RESULTS

Peripheral blood samples from 44 B-CLL patients were tested for IL4 and TNFα receptor expression, as well as for secretion of the respective cytokine. Sample integrity was determined by side scatter comparison between unmanipulated and processed samples. Integrity was over 95% for unstimulated samples (receptor analysis), and varied between 70-95% for stimulated cells (cytokine measurements). Cases with less than 70% integrity were excluded (2).

Known positive controls

Commercial Peripheral blood mononuclear cells (Hick-1,Hick-2, PharMingen) were used to test cytoplasmic positivity for IL4 and TNFα cytokine content respectively. Performance under the described test conditions was as expected by manufacturer specifications (more than 20% positive cells) for both cell lines and both cytokines.

Normal cell counterpart analysis

Two control peripheral blood samples and two tonsil specimens from young subjects were tested in the same manner as the B-CLL patient samples. Cytokine secretion detection for IL4 and TNFα was less than 10%, as was TNFα receptor expression. IL4 receptor was expressed in a small subset of cells. These results are similar to some of the findings reported by others for receptor expression and cytokine mRNA studies.

Immunophenotyping of B-CLL cells in peripheral blood

The typical phenotypic flow cytometric analysis of B Chronic Lymphocytic leukemia is illustrated in the next page (Figure 4).

Figure 4. B-CLL IMMUNOPHENOTYPE

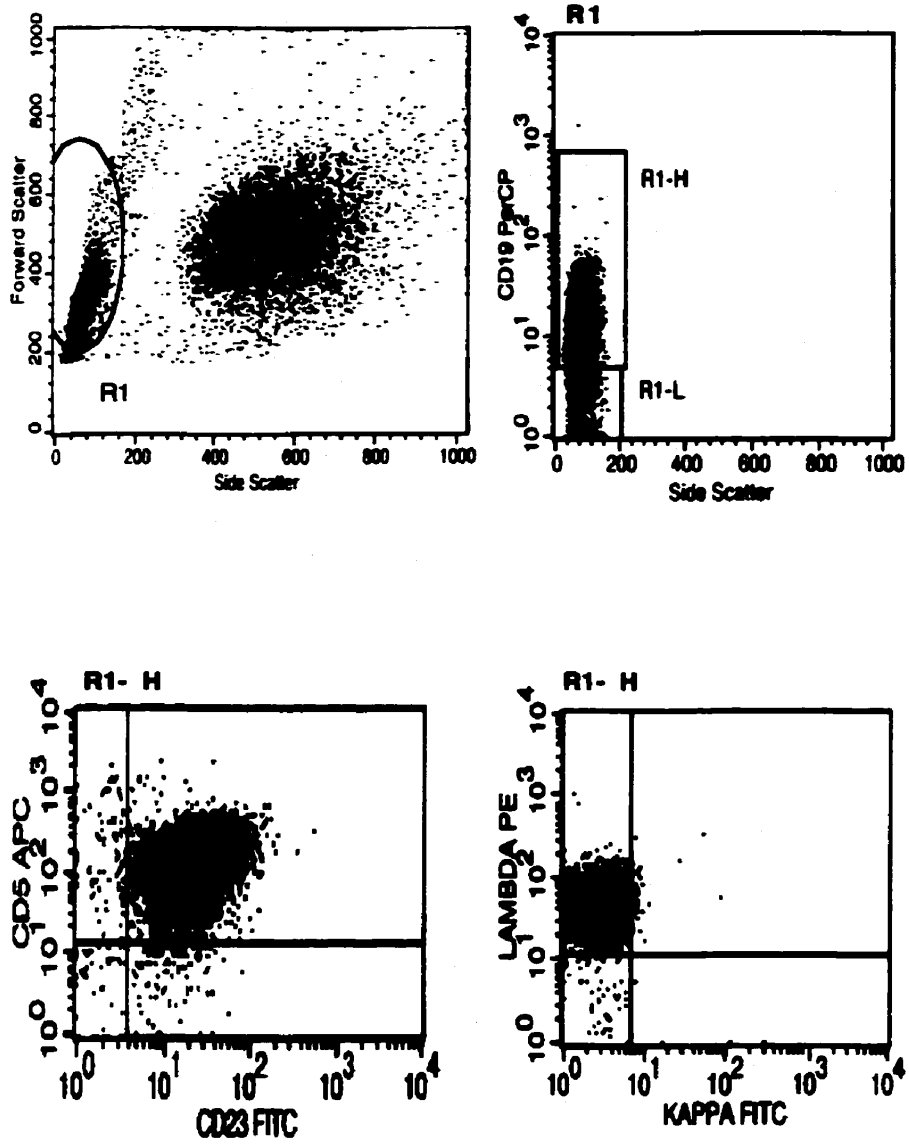
Top left: Lymphoid events are enclosed by Gate R1.

Top right: B cell events (CD19 positive) are selected in Gate H-1 from all lymphocytes. Note the typically dim intensity of CD19 in B-CLL cells, overlapping with CD19 negative events. Gating above the very dim area ensures that only B cells will be included.

Bottom left: CD 19 positive cells from gate H-1 show CD5 and CD23 coexpression.

Bottom right: B cells express only lambda surface light chain, indicating monoclonality.

FIGURE . 4


R1
Forward Scatter
Side Scatter
R1
R1
CD19 PerCP
R1-H
R1-L
Side Scatter
R1- H
CD5 APC
CD23 FITC
R1- H
LAMBDA PE
KAPPA FITC

IL4 and TNFα Receptor and Intracellular Cytokine analysis

Representative examples of receptor expression and intracellular cytokine detection tests are shown in Figures 5 (next page) and 6 (page 39).

Figure 5. CYTOKINE RECEPTOR EXPRESSION MEASUREMENT

Top left: The forward vs. side scatter histogram is used to select the lymphoid population (Gate R1).

Top middle: Coexpression of CD5 and CD19 is plotted for the selected Gate R1 population. A second gate (B) is defined to limit analysis to the CD5+/CD19+ B-CLL cells.

Top right: Double gated events (R1 and B) are interrogated for non-specific uptake with the isotypic control (labeled soybean protein).

Middle left: IL4 Receptor Test. IL4 receptor expression in the selected population is detected by fluorescein - labeled cytokine.. Positive cell events occupy the top-right quadrant of the histogram.

Middle right: IL4 Receptor Specificity Control. Specific FITC-labeled cytokine ligation by receptor has been blocked by the unlabeled anti-cytokine antibody.

Bottom left: TNFα Receptor Test.

Bottom right: TNFα Receptor Specificity Control.

FIGURE. 5

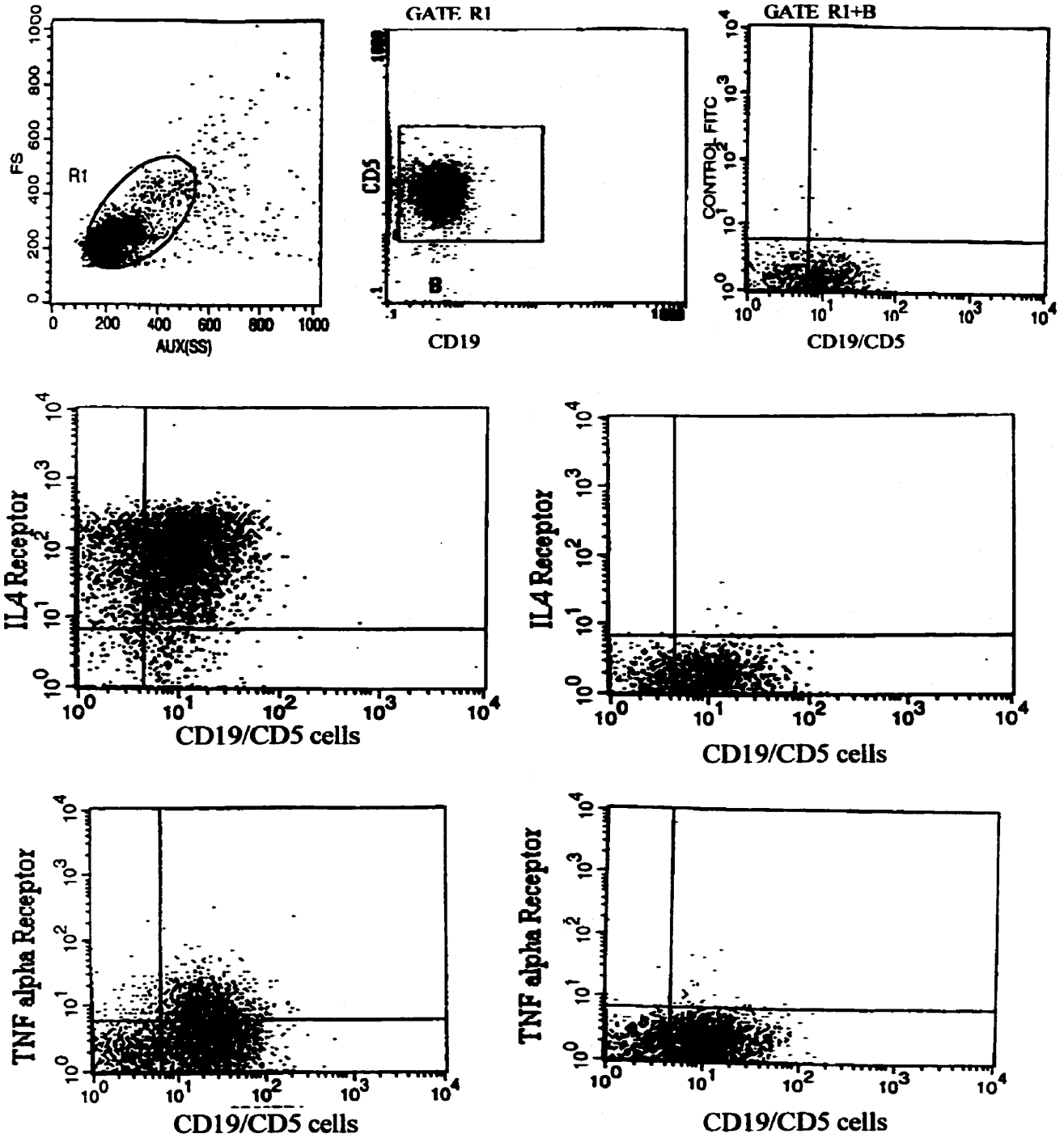

FS
AUX(SS)
R1
GATE R1
CD5
CD19
B
GATE R1+B
CONTROL FITC
CD19/CD5
IL4 Receptor
CD19/CD5 cells
IL4 Receptor
CD19/CD5 cells
TNF alpha Receptor
CD19/CD5 cells
TNF alpha Receptor
CD19/CD5 cells

Figure 6. INTRACELLULAR CYTOKINE MEASUREMENTS

Top left: Gate R1 contains lymphoid cell events.

Top middle: Gate R1 events are selected for CD19 and CD5 coexpression (Gate B).

Top right: CD19+/CD5+ B-CLL cells baseline phycoerythrine (PE) fluorescence with the isotypic control (GRD1) is determined.

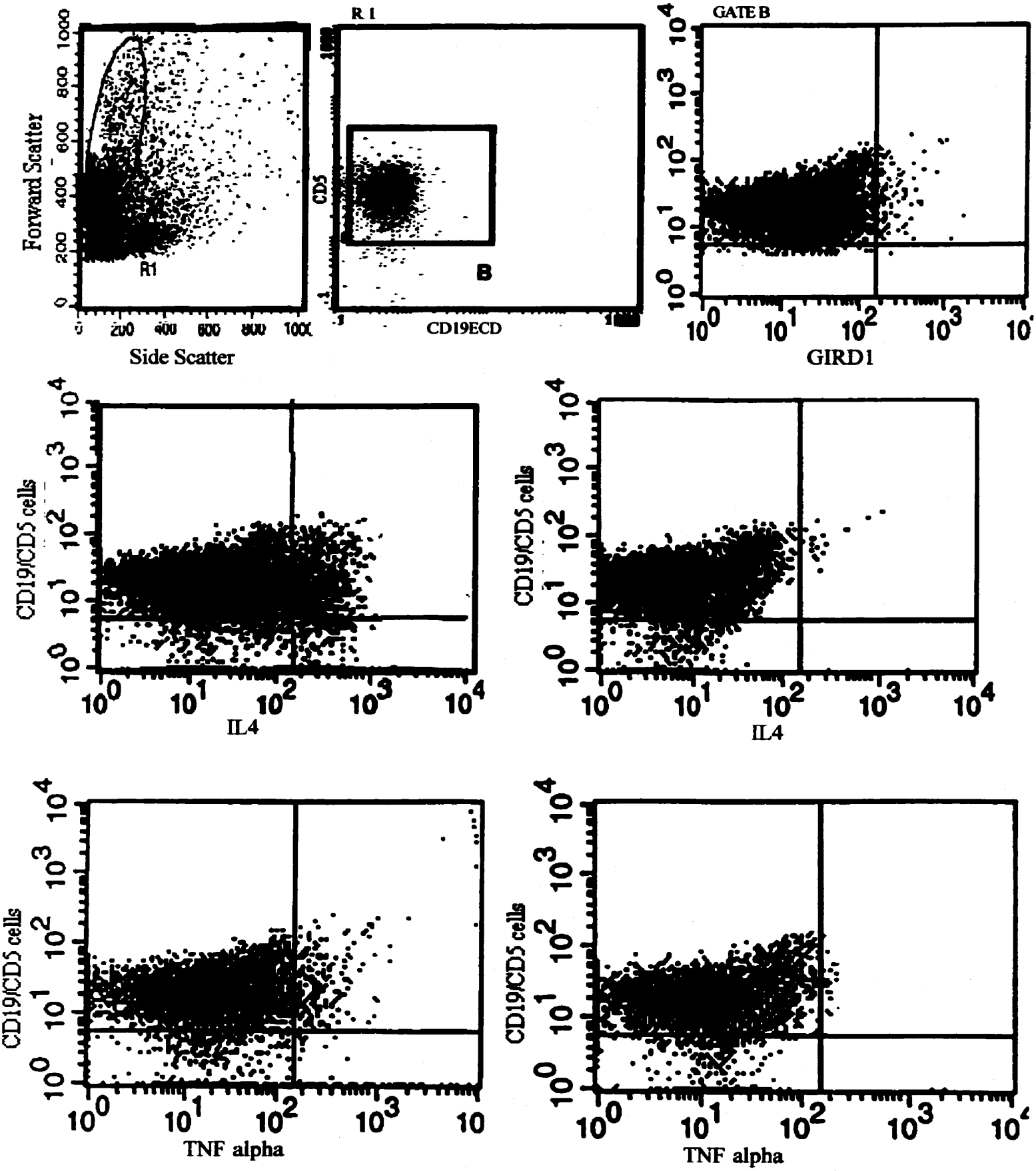
Middle left: Intracellular IL4 Test. Cell events positive for intracellular IL4 PE move to the top right quadrant of the histogram.

Middle right: Intracellular IL4 Specificity Control. Specific labeling of intracellular IL4 is blocked by the non-fluorescent anti-IL4 antibody.

Bottom left: Intracellular TNFα Test.

Bottom right: Intracellular TNFα Specificity Control.

FIGURE. 6


Forward Scatter
Side Scatter
R1
R 1
CD5
CD19ECD
B
GATE B
GIRD1
CD19/CD5 cells
IL4
CD19/CD5 cells
IL4
CD19/CD5 cells
TNF alpha
CD19/CD5 cells
TNF alpha

Receptor detection and Intracellular cytokine testing results

Summarized results from the IL4 receptor detection and intracellular IL4 secretion tests are depicted in Figure 7 (page 45). Results for TNFα receptor and intracellular TNFα measurements are shown in Figure 8 (page 46).

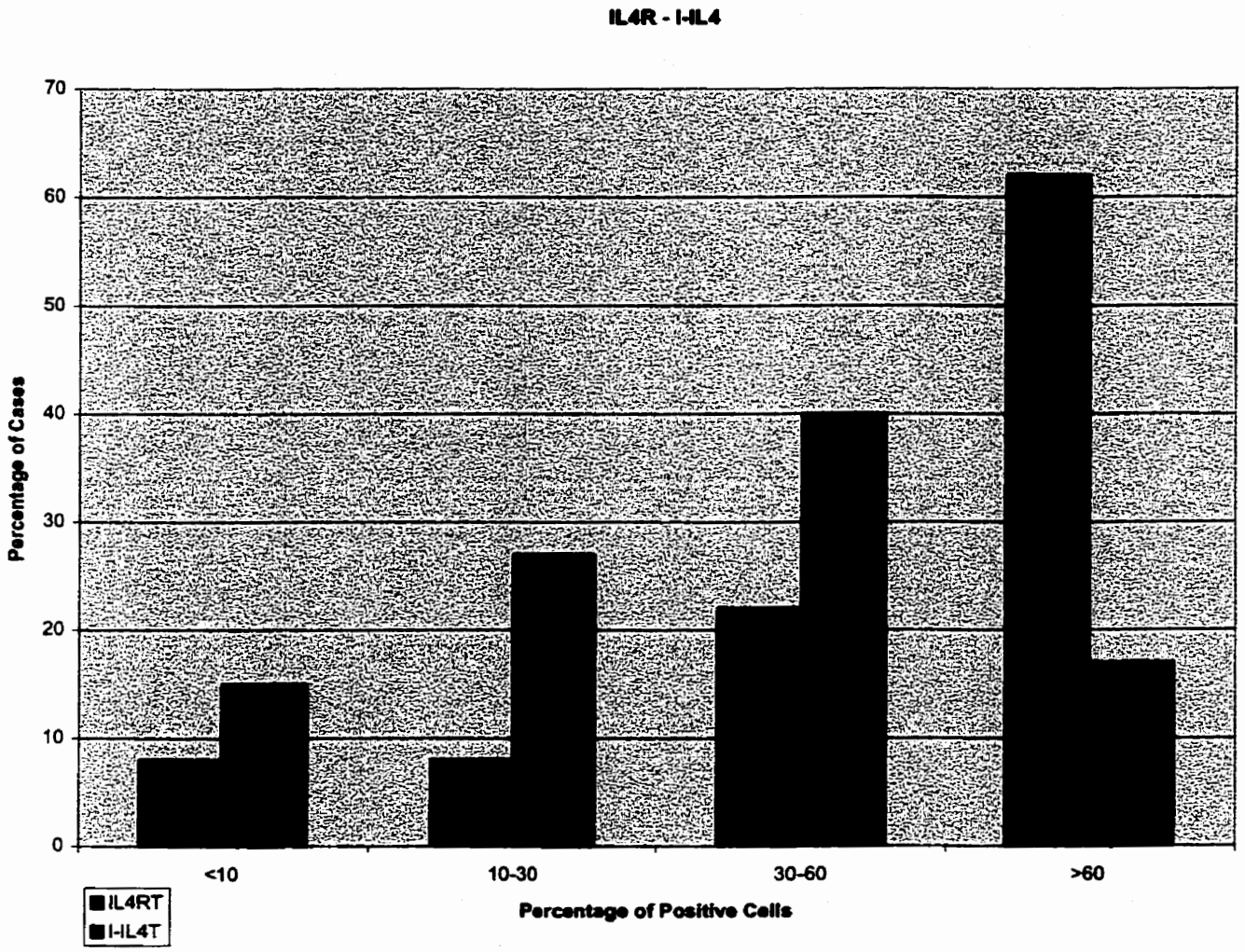


Figure 7

IL4 receptor expression results are correlated with cytokine production. Presence of receptor for IL-4 was demonstrated in the great majority of cases. In 84% of cases, the IL-4 receptor was noted in >30% of B-CLL cells. IL-4 production also was detected in all cases. 57% of B-CLL cell populations had over 30% secreting cells.

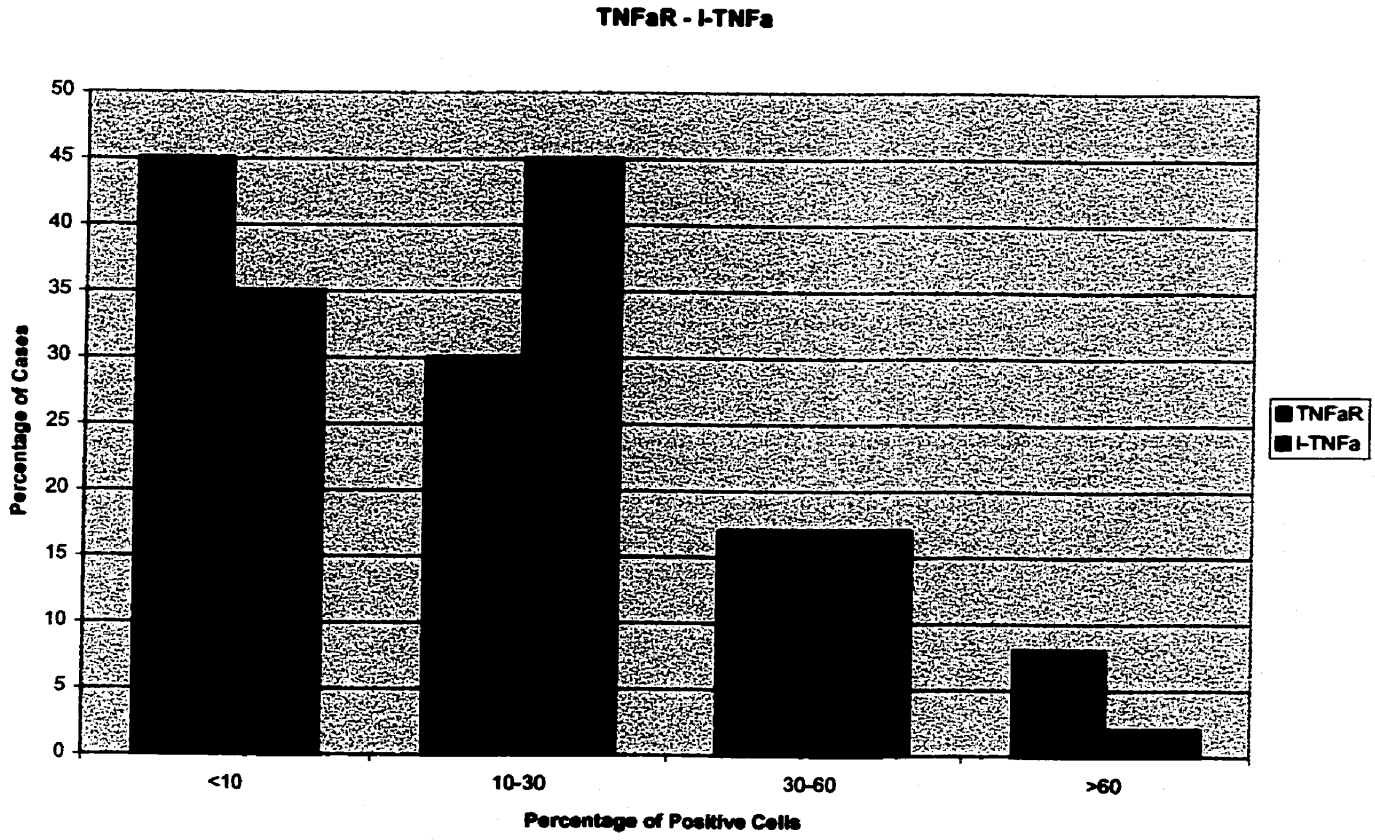


Figure 8

TNFα receptor expression levels are compared to TNFα production. Nearly half of the cases studied showed negligible receptor expression. 50% of cases did express receptors in at least 10% of cells. Cytokine secretion was detected in most cases.

Correlation of experimental data with clinical stage

A combination of the two most commonly used B-CLL staging classifications was used (see tables 1a and 1b)[96 97].

The patients were initially classified by the Rai system, commonly used in north american institutions. However, the Binet system consolidates survival groups in well differentiated, fewer categories. For the purpose of the study, Rai's stages 0 and I , and Binet's stage A were considered low risk, and Rai's stages II-VI, and Binet's stages B and C, high risk.

Each set of receptor/intracellular cytokine data is correlated first to the low risk stages in Figures 9 and 10 for IL4 and TNFα respectively (pages 48 and 49).

Similarly, the results for the high risk patients are compared in Figures 11 and 12 (pages 50 and 51).

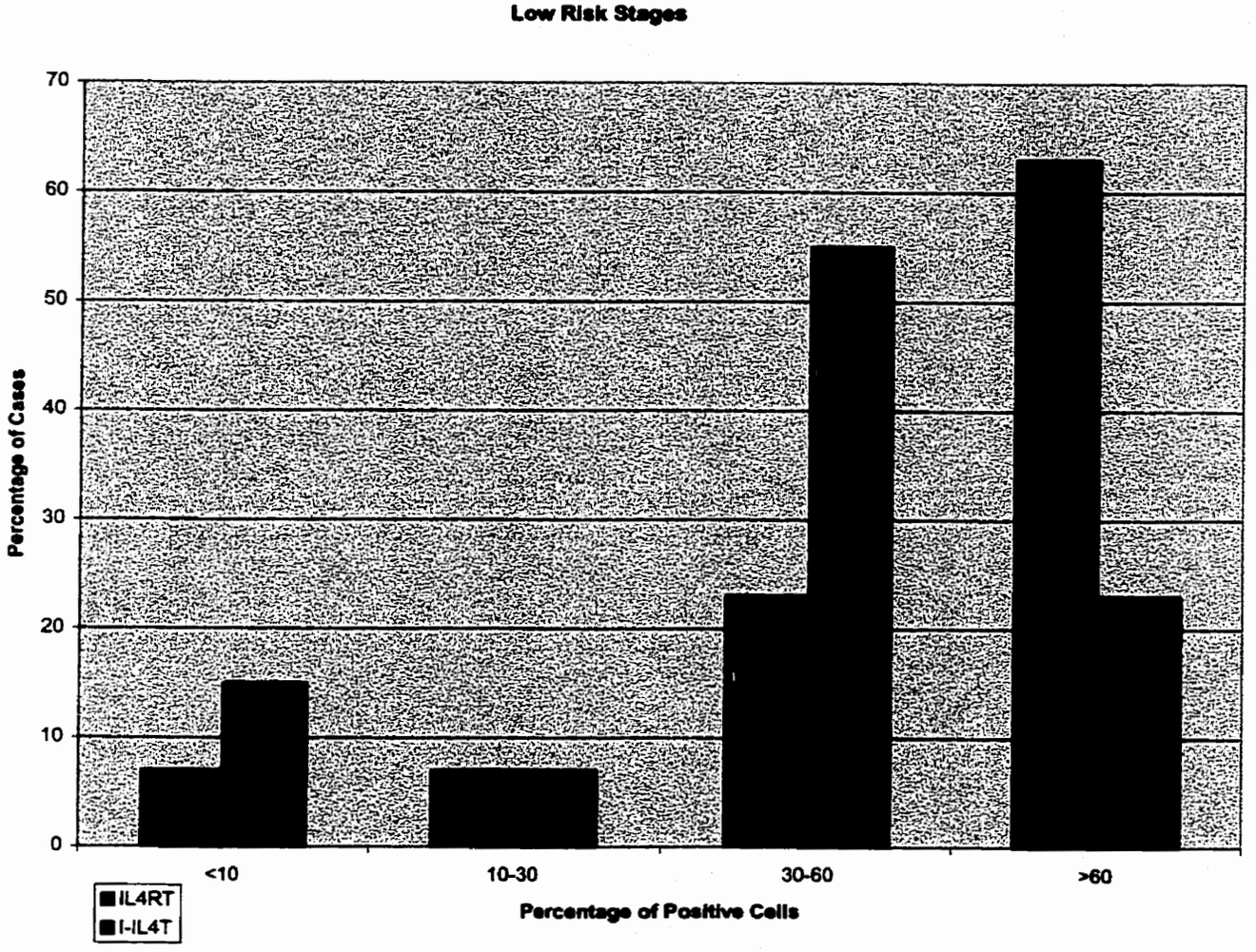


Figure 9

Low risk stages are correlated with IL4 receptor expression and IL-4 secretion. Most cases show sizable populations expressing both surface receptor and intracellular cytokine.

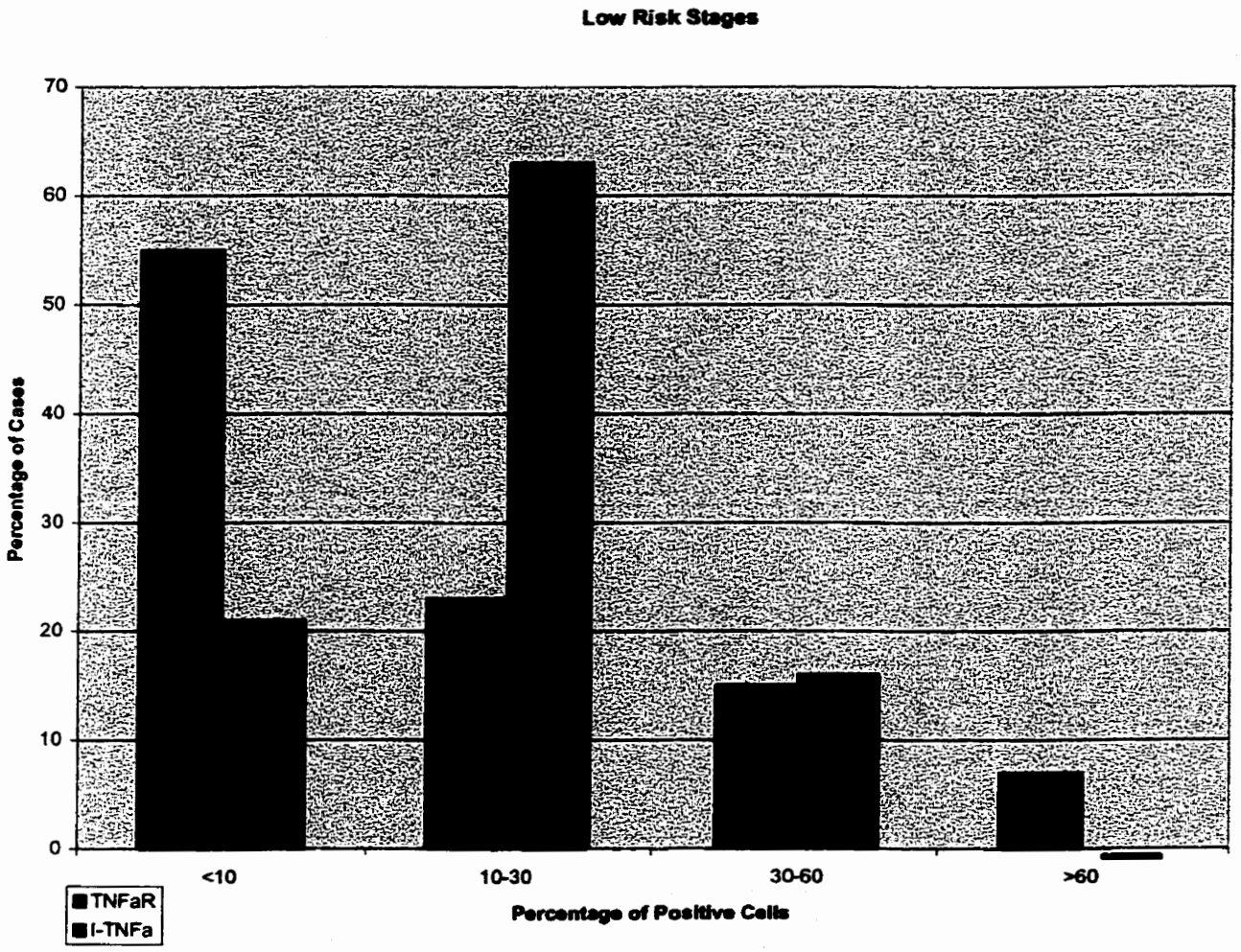


Figure 10

Low risk stages are correlated with TNFα receptor expression and cytokine production. Most cases show receptor expression and/or cytokine secretion only in a minor population of leukemic cells.

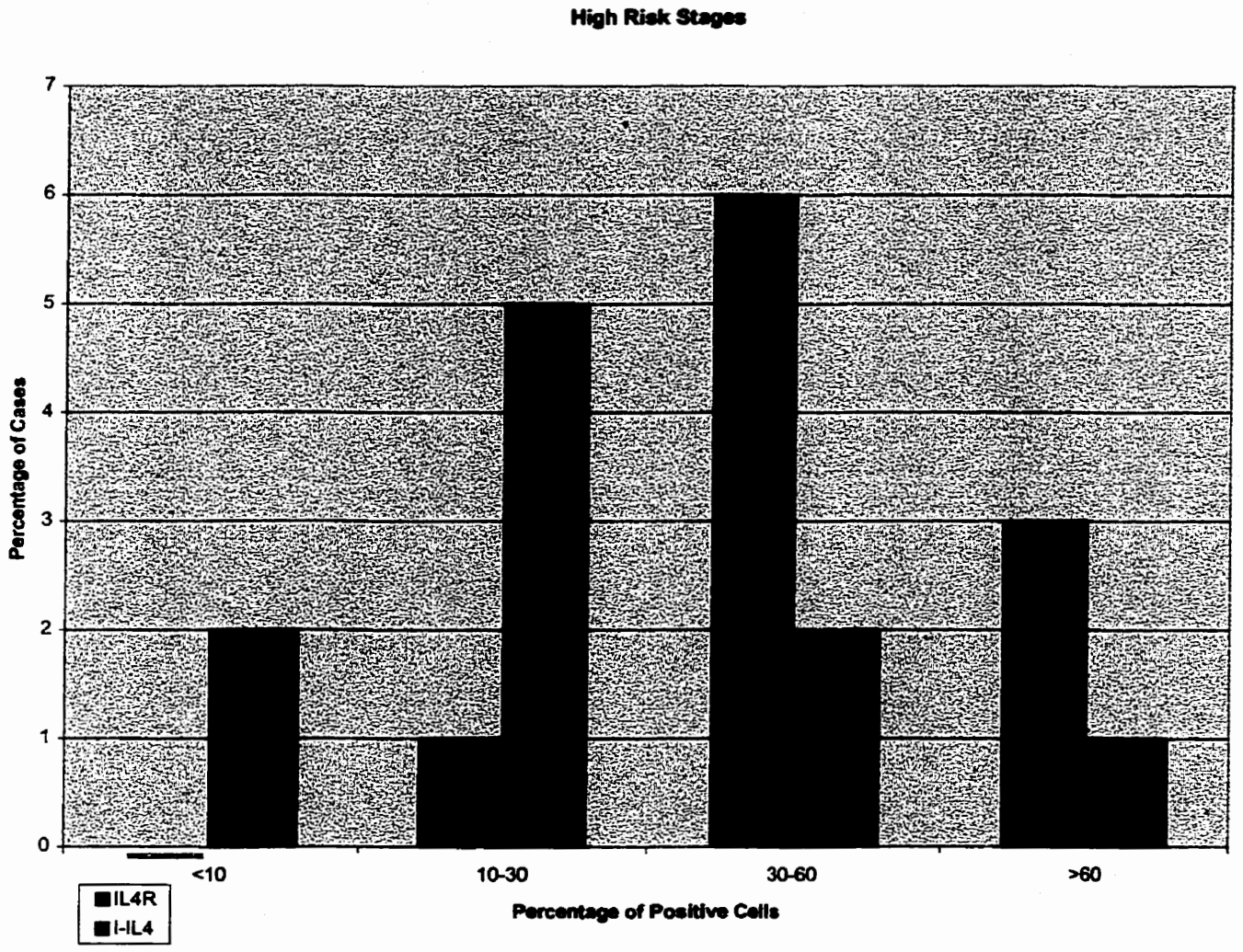


Figure 11

High risk stage: in contrast with the indolent cases, IL4R expression is detected in a slightly lower proportion of cells. There is a more pronounced negative difference in cytokine secretion.

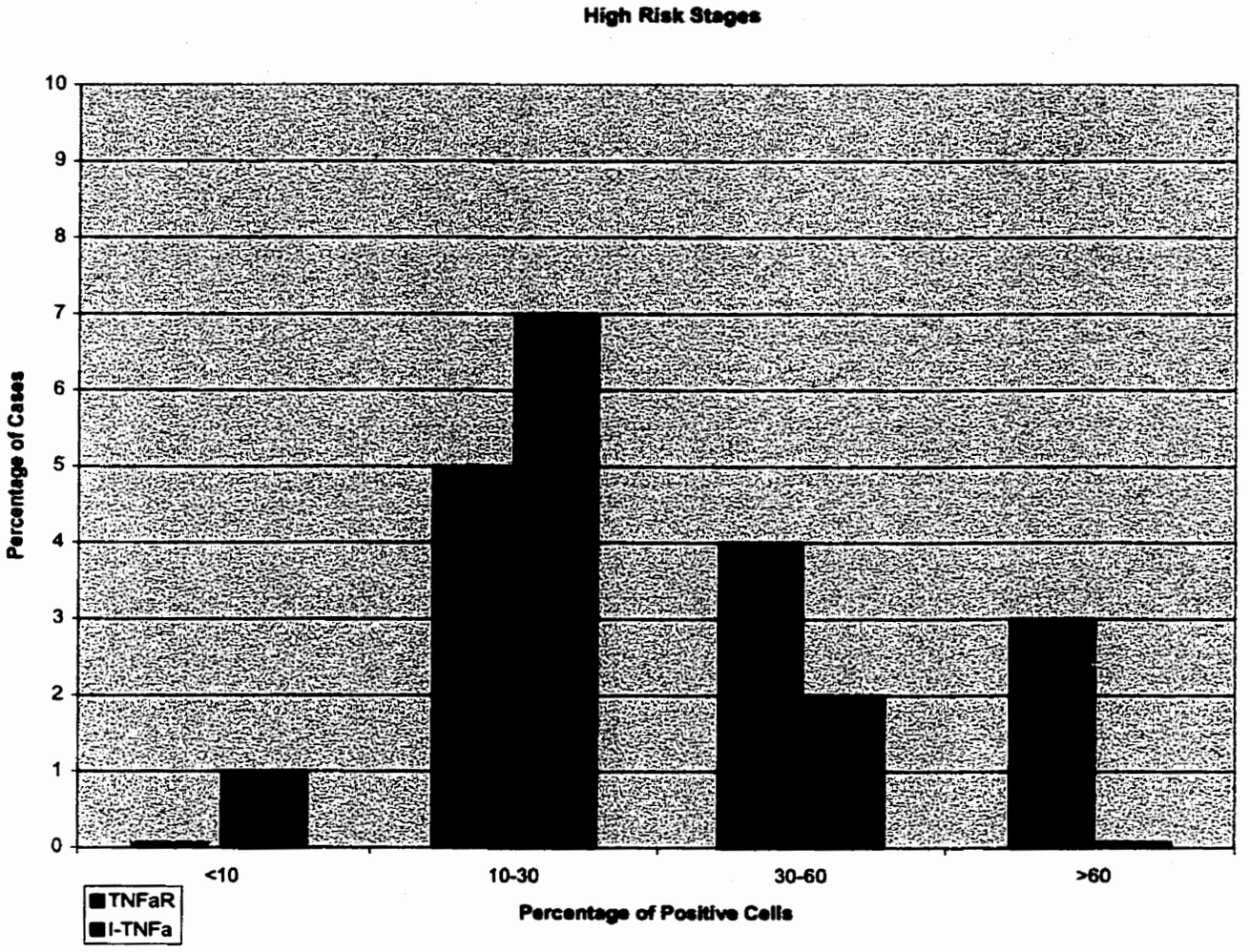


Figure 12

High risk stage cases are correlated with their TNRα receptor expression and cytokine production. Most of these cases show receptor expression and cytokine secretion in a sizable number of leukemic cells.

DISCUSSION

DISCUSSION

Chronic Lymphoid Leukemia of B lineage has been well characterized clinically for many decades. Its impact in the aging population is significant as the most common leukemia for that age group[1]. While most cases of B-CLL exhibit an indolent course, the disease takes a toll in the life of the patient through side effects such as cytopenias and infections[1-3]. Fewer patients with a more aggressive form of the disease are often younger, and have limited response to therapy. [99 101]

In the nearly forty years passed since the first descriptions of this disease, the scientific community has defined clinical and staging criteria, as well as reproducible laboratory features[1 96 97]. The latter were radically improved with the addition of a more complete immunophenotype[101 94], which allows distinction of B-CLL from other small B cell lymphoproliferative disorders[94 95]. Our current ability to better classify these entities has already favored development in our understanding of this process.

The pathophysiology of B-CLL has been studied by many[2 3 6 7 20]. Reports dissecting multiple aspects of its cell biology have given a piecemeal insight into the mechanisms and potential pathways that lead to disease behavior. However, to this time, the etiology and the blueprint that will allow proper fitting of this complicated puzzle have challenged investigators and clinicians.

Nearly every aspect of the disease, from genetics[23-28] to immunedisregulation[6 11 32 42] have been partially explored and/or explained. Elegant studies such as those on the peculiar B cell receptor features of B-CLL cells[38 39], and their relationship with bone marrow stromal cells[43 44] illustrate potential mechanisms for the leukemic process, but the connection among them is still elusive.

In summary, no single defect described to date for B-CLL can fully explain the biology of the disease, but it is likely that many of the findings reported will be shown to integrate sensibly in the future.

A possible link, and a potential mode of therapeutic intervention could be provided by defining the cytokine milieu in which these cells survive and prosper. Just as interleukins and growth factors are essential in normal lymphocyte development, function, and environmental interaction[59 60 62 64], it is plausible that they also have an important role in the dynamics of this leukemia.

The role of various cytokines on B-CLL cells has been explored and described in recent years[59-91]. Two cytokines appear to intervene in the main pathologic milestones of the leukemia: cell accumulation and clone maintenance.

IL4[59 60 62 64] and to some extent TNFα[65] have been reported to protect B-CLL cells from death, and encourage their survival in-vitro. TNFα was shown to foster in-vitro B-CLL proliferation in several studies[75 76 85]. Previous reports focus in the effects of these messengers mostly, while their source remained largely undefined. When measuring serum levels, other investigators have found these two interleukins present, even increased compared to normal subjects[59 75], but there is limited correlation with disease context.

The possibility of B-CLL cell self sufficiency to maintain a steady cytokine environment promoting leukemic survival was investigated in the experiment described earlier. The results demonstrate the ability of B-CLL cells to produce both IL4 and TNFα, and also the presence of receptors for them on the cell membrane. Therefore, autocrine and paracrine loops within the leukemic cell population can be an important link between pathologic events in this tumor, as it was previously speculated[59 62 75].

In this study, the B-CLL lymphocyte is proven positive for the intracellular presence of both cytokines, indicating that these cells are in fact capable to

translate into protein the RNA message that was previously demonstrated for IL4[86], and for both, IL4 and TNFα, the B-CLL lymphocyte confirmed as one source for the elevated cytokine levels found in B-CLL patients[74 85 86]. Demonstration of IL4 receptor in B-CLL cells concurs with positive findings by others[64 66]. The finding of TNFα receptor in the unstimulated tumor cells does contradict at least one previous study, which found the receptor present only after *in vitro* stimulation[90].

The overall results of the study are positive for the various tests performed, indicating the capacity of the cells to generate and bind these mediators, however, the findings for each cytokine and receptor are noticeably different in terms of distribution within the B-CLL population. These differences have not been explored or described before.

IL4 receptor was widely found in the B-CLL population of nearly every case tested, as opposed to TNFα receptor, which was only detected in 50% of the cases, and appears restricted to a minority of cells in the clone.

Over half of the cases tested were found to have large IL4 secreting populations, compared to TNFa production, which was detected in fewer cases and in much smaller proportion of cells.

The differences appear to be consistent beyond what could be attributed to test sensitivity and variation issues for both receptor and intracellular cytokine detection experiments.

For most cases, the finding of a majority of IL4 secreting, IL4 receptor expressing cells was accompanied by TNFa receptor in slightly lower numbers of cells, and a much smaller TNFa producing cell subset. The opposite was seen in a minority of the leukemias analyzed.

The comparison study gives additional interesting connections between the two cytokines and disease status. Although clinical stage[96 97] information could not be obtained for all cases (only for 26 out of 42), case distribution mirrors disease demographics: two-thirds of these 26 patients were classified in the low risk stages, and only one-third in the high risk category .The numbers are too small to draw definitive conclusions. However, it would appear that there is correlation between stage and both receptor expression and the ability to produce the cytokine, for both IL4 and TNFα.

With few exceptions, in indolent B chronic lymphoid leukemias the population at large was capable of producing and binding IL4, while only a low percentage of cells were TNFα secretors. Also, binding of TNFα was restricted to a smaller subset of the clone then IL4 binding. This dichotomy reflects well the generally accepted facts on apoptosis and cell proliferation in this leukemia[2 4 33-36]. I propose that B-CLL cells in the resting phase of the disease would accumulate through anti-apoptotic mechanisms, favored, at least in part, by a self-generated IL4-rich environment. During this phase, only a minor population of B-CLL cells would be able to supply and respond to the proliferative signal provided by TNFα.

In high risk cases, the biological dynamics would be altered by expansion of the TNFα-responsive proliferative pool[100], supported by a larger number of TNFα secreting cells. The influence of IL4 in maintaining the cells long life span is still likely at play, since receptor expression is only moderately diminished, even though the percentage of cells able to provide IL4 is decreased by half. This anti-apoptotic effect could also play a role in the relative insensitivity of the leukemic cells to cytotoxic therapy[57 99].

There is also an interesting parallel with histologic findings[99]. The morphology of B-CLL in bone marrow, peripheral blood, and other tissues includes a predominant infiltrate of small, compact lymphocytes, and few large cells called "prolymphocytes". In tissue, these prolymphocytes accumulate in what are known as proliferation centers. Cell cycle markers such as Ki-67 (a nuclear

protein expressed exclusively during active phases of the cell cycle) are negative in the population of small cells, but positive in the larger "prolymphocytes"[100]. See figure 13 (page 60).

One could speculate that these prolymphocytes are predominantly TNFα secretors and/or TNFα receptor-positive, while the rest of the tumor cells are long lived IL4 producers and acceptors. It appears reasonable that the proliferating group may downregulate IL4 receptors, and the resting majority would express low levels of receptors for TNFα. This scenario would be inverted in the aggressive type of leukemia.

Non-neoplastic lymphocytes or monocytes accompanying the leukemic cells may provide B-CLL cells with cytokines[52-54], but these populations vary in total numbers and proportion, from case to case and during the course of the disease[52 102] . Most of the B-CLL cases analyzed contained populations of T lymphocytes, and in all of the low grade cases the CD4:CD8 ratio was within normal limits (approximately 2:1, data not shown). In many patients, numbers of residual non-leukemic B and T lymphocytes are drastically reduced, particularly in the aggressive stages. This was the case in the high risk patients investigated here. In addition, nearly all of these high risk patients showed a CD8 subset predominance, with inversion of the CD4:CD8 ratio.

Therefore, it would seem that both, the B-CLL cells, and their T cell entourage may generate a TH2 type, IL4-providing environment in the majority of indolent B-CLL populations, in which the tumor cells would be signaled by the cytokine, perhaps bypassing cognate interaction, into a pseudo-post activation, "refractory" and apoptotic-resistant state. Paradoxically, the effects seem limited to cell life span, and are not reflected in proliferative activity.

These conditions would be reinforced by the presence of other TH2 associated cytokines in the patients serum, such as IL10 [83] and IL6[73]. IL6 and IL10 have

been detected in the B-CLL serum, with a neutralizing effect on TNFα proliferative stimuli.[73 74 84]

There is an additional or alternative aspect in which this TH2 type of cytokine milieu could be involved in the leukemic process, and that is by promoting the peculiar refractory-like expression of the BCR complex in B-CLL cells[38 39]. The message to skew the splicing of 79a gene towards the truncated 79a variant could be provided by an IL4-mediated signal, rather than a physiologic, antigen or T-cell provided interaction[37].

The aggressive cases would develop in an IL4 poor environment, in which TH2 type signaling would be diminished and the abnormal B lymphocytes would proliferate in response to TNFα. It is also well known how brighter expression of lineage markers and surface immunoglobulin typically accompanies disease progression[30 31 101]. These fully expressed molecules would revert the "anergic" state , enabling the B-CLL cell for activation and response to proliferative stimuli. The effects of IL4, but also of IL10 and IL6 would be minimal at that time, given the reported decrease in their serum levels in the high risk patients. Therefore they would not exert the proposed TNFα inhibitory effect noted during the low risk phase[73 74 84 87].

Whether CD8+ T –IL4 producing cells [52 53] play a role in the switch-off of the TH2 environment, upregulation of TNFα secretion and receptor expression, or in the maintenance of a low level IL4 production, remains to be investigated. To date, the CD8+ subset in B-CLL has been related to facilitating the autoimmune processes that often accompany the disease[54].

What mechanisms bring about these developments in B-CLL? Is there a natural progression from the indolent to the severe stages as longevity favors the chances for genetic aberrations, or are these changes a deficient response to otherwise normal environmental interactions?

What could prompt B-CLL cells to secrete these cytokines in such a particular profile? Further investigation will have to answer how to accommodate these findings in the larger context of B-CLL biology.

For starters, a larger series would be desirable to confirm the results of this study, and, ideally, a more "physiologic" method of signal amplification for the cytokine secretion experiments. Simultaneous detection of both cytokines and receptors, to investigate coexpression in the same cell, are now possible with new instrumentation.

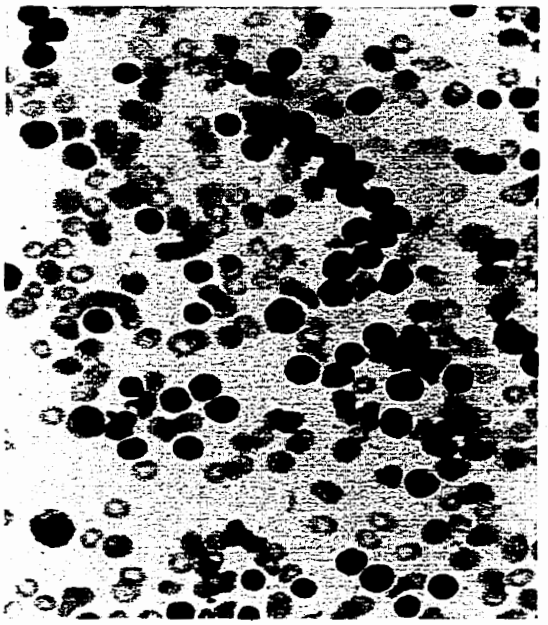
Investigation of sufficient numbers of cases segregated by stage is needed to gain insight into the variation within populations at a comparable stage of clonal evolution. Longitudinal testing of patients during the course of their disease, and in the appropriate instances, during disease progression may confirm the chain of events proposed earlier.

Correlation of these experiments with other, until now independent findings, such as the various karyotypic defects, expression of various activation, memory, or costimulatory molecules will offer a more comprehensive integration of knowledge for this leukemia.

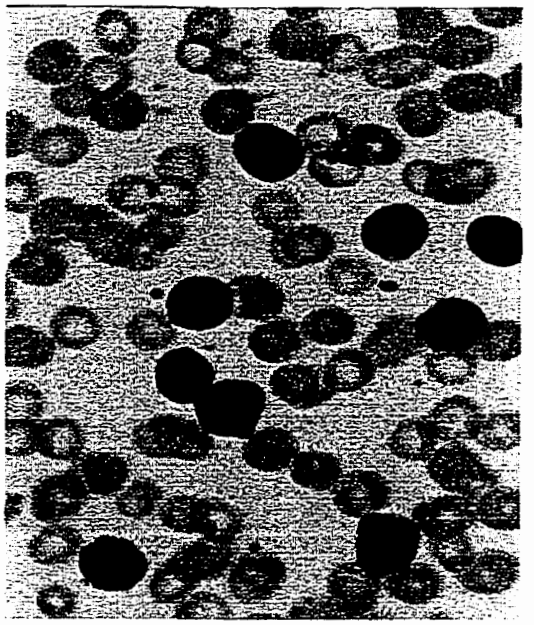
Finally, determination of cytokine production profiles in the emerging subtypes of B-CLL[16-18], ie: innate B-1 type (CD38+) versus hypermutated/memory type (CD38-) could be key to understanding associated disease behavior and the immunologic mechanisms at play.

This could translate into multiple potential therapeutic strategies, better adapted to different stages of the disease, and even to discriminate among distinct populations within a clone, enough to specifically target these with the appropriate agents. However, immunologic manipulation of the B-CLL microenvironment could prove the most fruitful and least toxic therapeutic modality.

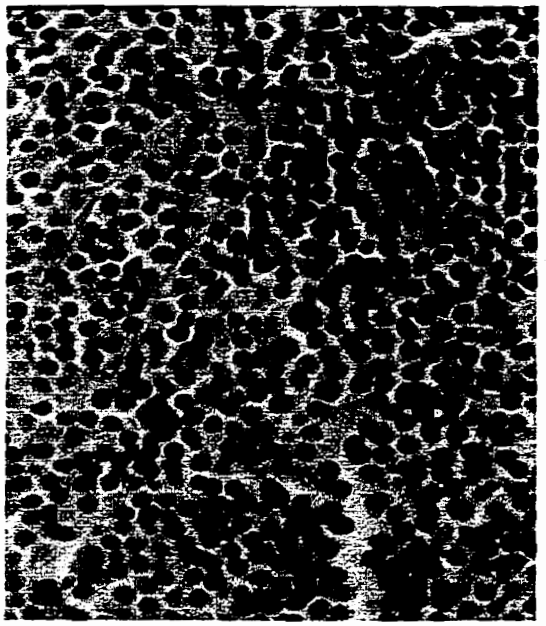
Figure 13



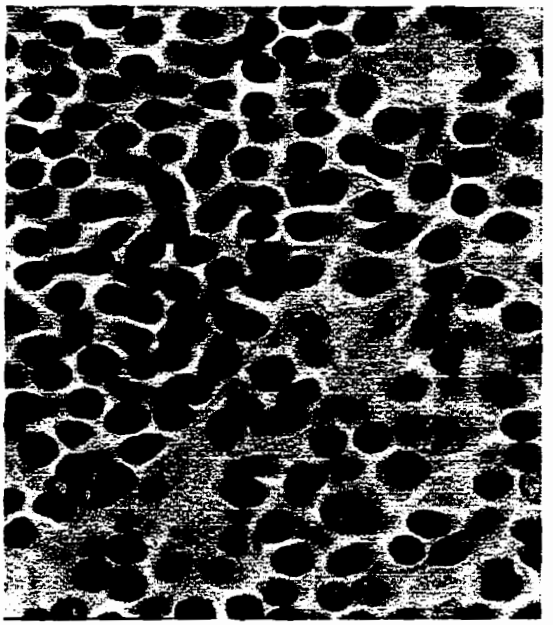
Peripheral Blood, W-G stain. Small compact B-CLL cells are seen throughout the field. Large pro-lymphocytes can be seen in the center and bottom left corner.



Peripheral Blood, W-G stain. High power view of B-CLL cells: Small, non dividing cell majority.



Lymph node section, H+ E stain. A Large Pro-lymphocyte is seen in the center surrounded by a monotonous massive population of small B-CLL cells.



Lymph node section, H+ E stain. A proliferation center with several pro-lymphocytes can be seen on the bottom right corner. Non dividing B-CLL cells occupy the rest of the field.

SUMMARY

The results suggest that the cells of B-CLL are subject to regulatory mechanisms mediated by cytokines. These mechanisms could be autocrine as well as paracrine. In general: a) intracellular IL4 and cell surface IL4 receptor expression were detected in the majority of the cells and in the majority of the patients; b) intracellular TNFa and cell surface TNFa receptor expression were detected in a minority of the cells in most of the patients.

When the patients were clinically categorized according to the Rai Classification, ie: low vs. high risk, the majority of the cells in the low risk cases produced IL4 and expressed IL4 receptor, while TNFa and TNFa receptor were detected in greater numbers of cells in the high risk cases.

The data collected provide a platform for proposing the following hypothesis: a minor population of cells in B-CLL is under the proliferative regulation of TNFa and its receptor, and these cells may constitute the clonogenic compartment. A second major population form as a result of the non-dividing progeny of the former cells, due to interference with their apoptotic pathway mediated by IL4 and its receptor.

I believe that this hypothesis will form the basis for new studies towards the clarification of our understanding of the underlying mechanisms of this important B cell malignancy.

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