LIFE AND DEATH IN GERMINAL CENTERS: A NEW PERSPECTIVE ON HUMAN B CELL LEUKEMIAS AND LYMPHOMAS

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I. INTRODUCTION

In 1988 Graham Smith presented a Medical Grand Rounds entitled, "Recent Developments in the Non-Hodgkins Lymphomas". He ended it by stating, "perhaps in more than any other lineage, rapid advances and understanding of regulation of growth and development of normal cells sets the stage for exciting new developments in the management of malignancies". (Smith, 1988) . Today's Medical Grand Rounds will be a further attempt on my part to relate normal B cell development to medically relevant topics. This is a theme I have returned to many times since my first Medical Grand Rounds here over twenty years ago. A few years ago I discussed immunodeficiencies in this framework (Capra, 1993). Today I would like to focus on malignancies of lymphocytes. Since B cell malignancies represent the bulk of adult leukemias and lymphomas and in order to focus the Grand Rounds, the subject will entirely be on B cell malignancies. Specifically I hope to illustrate for you how newer insights into our understanding of how B cells mature through germinal centers as they go through that remarkable process that leads to high affinity class switched antibodies has provided us with new tools to classify and treat human B cell malignancies. After a short review of normal B cell development we will review the classification of lymphoid neoplasms going over the stages of the various classification schemes from morphologic to immunophenotypic to genetic and to the status of the immune receptor genes. We will then discuss minimal residual disease detection. Finally, I hope we have time at the end to discuss the relationship of these processes in the overall scheme of a germinal center reaction (Burrows, et al., 1995).

Neoplasms of the immune system are a heterogeneous group of tumors whose cells of origin may be the lymphocyte, the histocyte, or other cell components of the immune system. Each neoplasm is thought to be a monoclonal expansion of malignant cells, although this has only been conclusively demonstrated for lymphocytic tumors. These neoplasms often retain many morphologic, functional, and migratory characteristics common to their normal cell counterparts. With increasing understanding of the normal immune system, it has become possible to classify many malignant immune disorders according to their cell of origin. Monoclonal antibodies to cell-surface antigens permit the identification of B or T lymphocyte proliferations. By immunophenotyping, malignant lymphocytic neoplasms can be related to stages of normal B or T lymphocyte development and maturation.

Establishing clonality of a B lymphocyte proliferation is usually accomplished by the demonstration of a single class of heavy-and/or light-chain cell-surface immunoglobulin. At the DNA level, clonality can be confirmed by the presence of a single immunoglobulin gene rearrangement. In precursor B lymphocyte neoplasms where surface immunoglobulin is not present, gene rearrangement studies are necessary to demonstrate clonality (Portlock, 1992a).

Non-Hodgkins lymphomas may occur at any age, although they are rarely diagnosed during the first year of life. They occur with increasing frequency throughout adulthood. The incidence is estimated to be approximately 35,000 cases per year in the United States, with males affected more often than females. Moreover, male predominance is most evident among young patients in association with the aggressive histologic subtypes of lymphoblastic and Burkitt's lymphomas.

Geographic clustering is characteristic of some non-Hodgkin's lymphomas: Burkitt's lymphoma in central Africa: adult T cell leukemia/lymphoma in southwestern Japan and the Caribbean; and small intestinal lymphoma with associated immunoglobulin disorders in the Middle East.

Preceding immune dysfunction has been associated with the development of aggressive non-Hodgkin's lymphomas. Congenital immunodeficiency states associated with lymphoma include severe combined immunodeficiency, ataxia-telangiectasia, Wiskott-Aldrich syndrome, X-linked lymphoproliferative syndrome, and common variable immunodeficiency. Transplant recipients, patients with autoimmune states, and patients with AIDS (acquired immunodeficiency syndrome) also have increased risk of developing lymphoma (Portlock, 1992b).

II. HUMAN ANTIBODIES, B CELL DEVELOPMENT AND GERMINAL CENTERS

A. Immunoglobulins

Immunoglobulin is the crux of the humoral immune response. As a cell surface receptor on B lymphocytes, immunoglobulin is responsible for instigating cellular processes as diverse as activation, differentiation, and even programmed cell death. As secreted antibody in plasma and other bodily fluids, immunoglobulin is able to bind foreign antigen, thereby either neutralizing it directly, or initiating steps necessary to arm and recruit effector systems such as complement or antibody-dependent cell cytolysis by monocytic phagocytes.

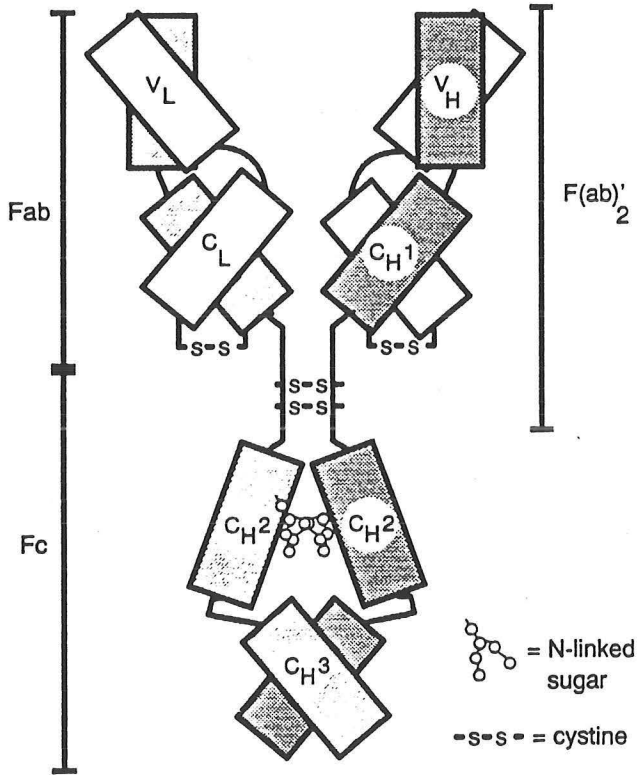
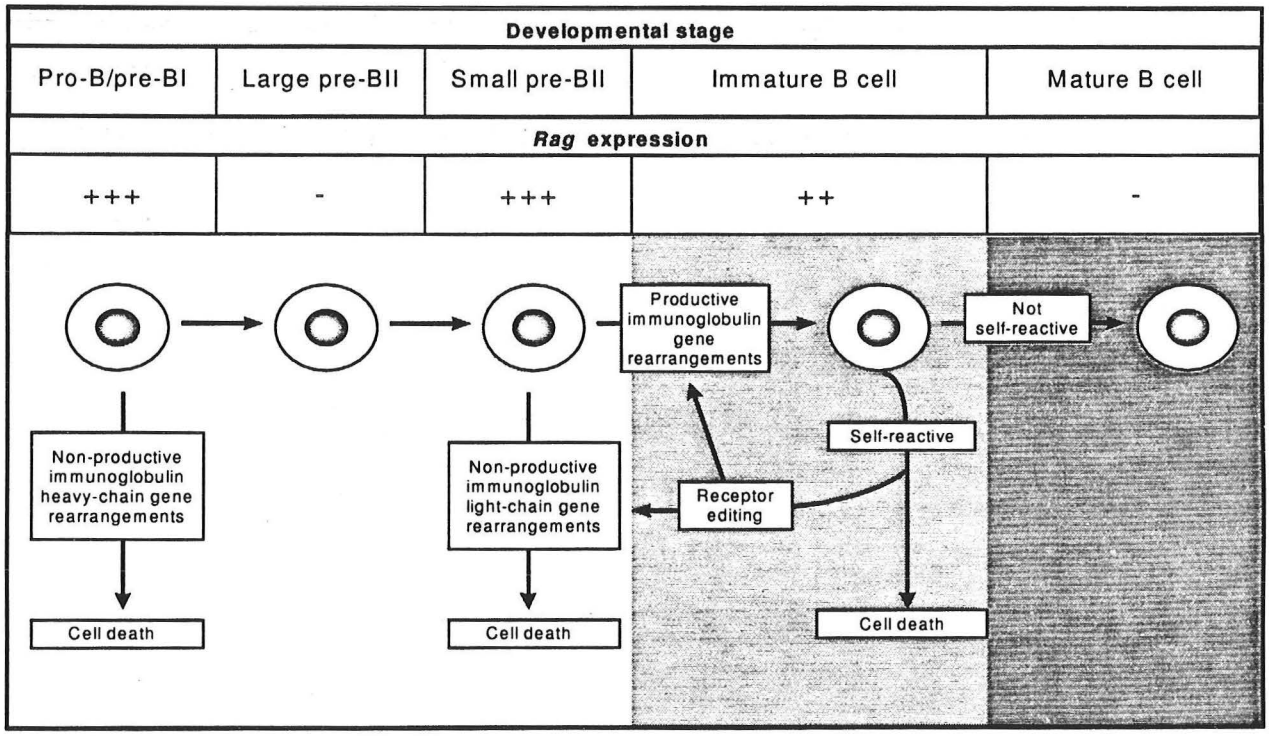


Diagram of a prototypic immunoglobulin monomer (IgG). Each *rectangle* represents an immunoglobulin domain of a heavy (*dark shading*) or light (*no shading*) chain. Labels on the *rectangles* follow standard domain nomenclature. Disulfide bonds crosslinking the two heavy chains at the hinge are denoted by *black bars* as are those crosslinking the heavy and light chains. The N-linked carbohydrate shown attached to C_H2 is found in all immunoglobulin classes although some classes are also glycosylated elsewhere. Major proteolytic fragments are denoted by *bars* flanking the molecule. Note that the boundary of Fab' extends below the hinge disulfides. Note also the dimerization of all domains except C_H2. (*From Carayannopoulos and Capra, 1991*).

The ability of immunoglobulin to perform such a wide array of duties can be attributed to evolution's clever usage of a structural paradigm, the immunoglobulin domain, and its duplication, diversification, and elaboration upon that design to endow it with an assortment of functional qualities (Frazer and Capra, 1998).

Despite the variety of purposes served by immunoglobulin molecules, one feature remains common to virtually all considerations of immunoglobulin structure and function: immunoglobulins have an amazing capacity to interact with other molecules. In one sense, immunoglobulins must be able to effectively bind a finite set of invariant partners, such as Fc receptors, signal-transducing molecules, and components of the complement cascade. In another sense, immunoglobulins, collectively, must meet the challenge of being able to recognize an essentially infinite array of antigenic determinants. More remarkable, perhaps, is the fact that immunoglobulin is frequently called upon to fulfill both of these binding responsibilities simultaneously, and in such a way as to mediate significant biological effects. As such, immunoglobulin molecules may be viewed as a marriage between the constraints engendered by biological continuity and the quest for diversity superimposed upon this evolutionary framework.



Developmental stages of human B cells.

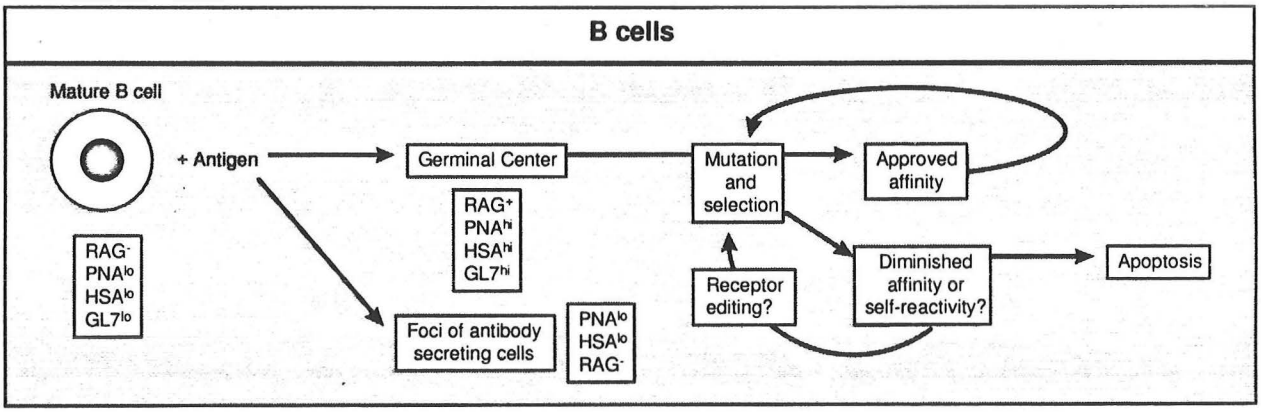
The lengths to which evolution has gone in order to bestow immunoglobulin with these conflicting capabilities has been the subject of intense scientific scrutiny, and has yielded innumerable fascinating insights into immunology, genetics, protein chemistry, and the discipline of biology as a whole. In trying to understand how antibody is able to recognize such a multitude of different specificities, science has benefited from the discovery of both VDJ recombination and somatic hypermutation. In an attempt to reconcile the incongruity entailed by the observation of highly divergent N-terminal regions coupled to constant C-terminal domains,

research has gained not only the once-heretical "two genes, one polypeptide" hypothesis, but also the concept of isotype switching. Thus, studies into immunoglobulin diversity have proven to be extremely profitable scientific endeavors. In addition, while diversity has been a hallmark of the study of immunoglobulin since it was first recognized to be a salient feature, several aspects which derive from immunoglobulins' underlying uniformity have been used to glean understanding into protein structure/function relationships in general (Frazer and Capra, 1998).

B. Normal B Cell Development

The function of B lymphocytes is to produce the antibodies described above (the humoral immune response). Naïve B lymphocytes are generated within the bone marrow and migrate into the secondary lymphoid organs where they either die or are recruited into the re-circulating B cell pool (Liu et al., 1996).

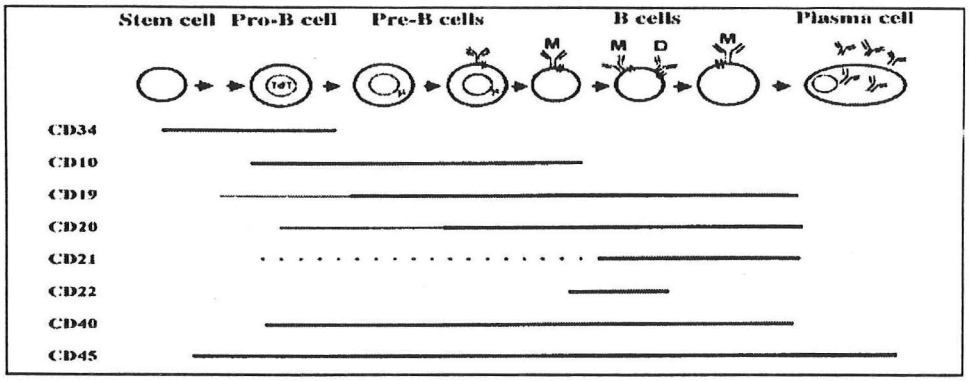
In the past I have presented to this group the notion that it is useful to divide the process of B cell development into an antigen-independent and an antigen-dependent one. The antigen-independent phase takes place almost exclusively in the bone marrow. During this time B cell precursors undergo a rearrangement of their V, D and J gene segments and under the influence of a myriad of enzymes including Terminal Deoxynucleotidal Transferase (TdT) as well as exonucleases, the initial antibody repertoire is developed. Naïve B cells leave the bone marrow and enter the peripheral blood as IgM^+/IgD^+ cells. The various stages of these cells from pre/pro to naïve B lymphocytes have been well studied. We will return later to the various B cell malignancies that occur at these stages of differentiation.



Abbreviated life history of human B cells.

From the peripheral blood B cells home to secondary lymphoid organs. Within secondary lymphoid organs such as the tonsil, lymphnodes, and spleen they encounter antigen (generally in the context of T cells and dendritic cells) in structures that are referred to as lymphoid follicles. Here two remarkable processes take place that leave permanent DNA changes on these B cells. The first is the process of somatic hypermutation which takes place in germinal centers. The second is the process of isotype switching, that is, the process whereby the IgM cell surface receptor for antigen becomes changed into an IgG, IgA or IgE secreting cell that eventually enters one of two pathways: the plasma cell pathway or the memory B cell pathway. In both instances these cells exit the follicle and re-enter the peripheral circulation.

Plasma cells home to the bone marrow where under the influence of various stromal factors within the bone marrow produce the vast bulk of circulating antibody. Memory B lymphocytes (which have stopped undergoing somatic mutation) await a new encounter with antigen where they re-circulate through secondary lymphoid follicles yet again and produce even higher affinity receptors (Nossal 1994a, 1994b).

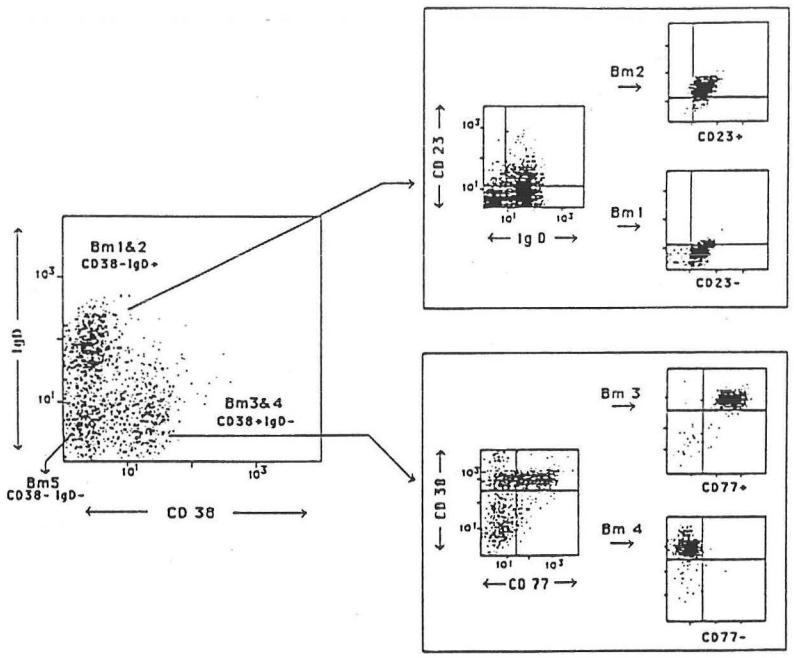


Development of human B lineage cells. TdT is found in the nucleus of pro-B cells, and the expression of cytoplasmic μ heavy chains marks the onset of the pre-B cell stage. The surface receptor illustrated on the late stage pre-B cell is composed of μ heavy chains plus the ΨLC complex and is non-covalently associated with the Igα/β heterodimer. The receptors on B cells are composed of either μ (M) or δ (D) heavy chains disulfide bonded to either κ or λ light chains; both sIgM and sIgD also associate with Igα/β. Expression of differentiation antigens is illustrated by horizontal lines, the thickness of which is proportional to expression levels. (*Adapted from Burrows, et al., 1995)*

C. Human B Cell Subsets

A decade ago we had a clear understanding of the stages of antigen independent differentiation of both mouse and human B cells. These were easily studied with molecular rearrangements, surface phenotypes and the like. Human mature B cells, however, have eluded further classification until recently. The detailed analysis of the sites of antigen driven B cell activation in animal models has demonstrated that naïve B cells undergo successive differentiation stages within various compartments of peripheral lymphoid organs to become high affinity memory B lymphocytes or plasma cells. Several laboratories including those of our collaborators, Yong-Jun Liu and Jacques Banchereau have pioneered studies of human tonsillar lymphocytes (Pascual, et al., 1994; Liu, et al., 1996 and Galibert, et al., 1996). Their analysis has resulted in a now well accepted differentiation scheme which has allowed various investigators to separate such mature B lymphocytes into various subsets.

The basis of the separation involves two surface markers, IgD and CD38. Banchereau and co-workers first divided human tonsillar B lymphocytes into four groups based on double sorting with these two markers. Subsequently, additional populations were further subdivided although that is less crucial for our analysis today.

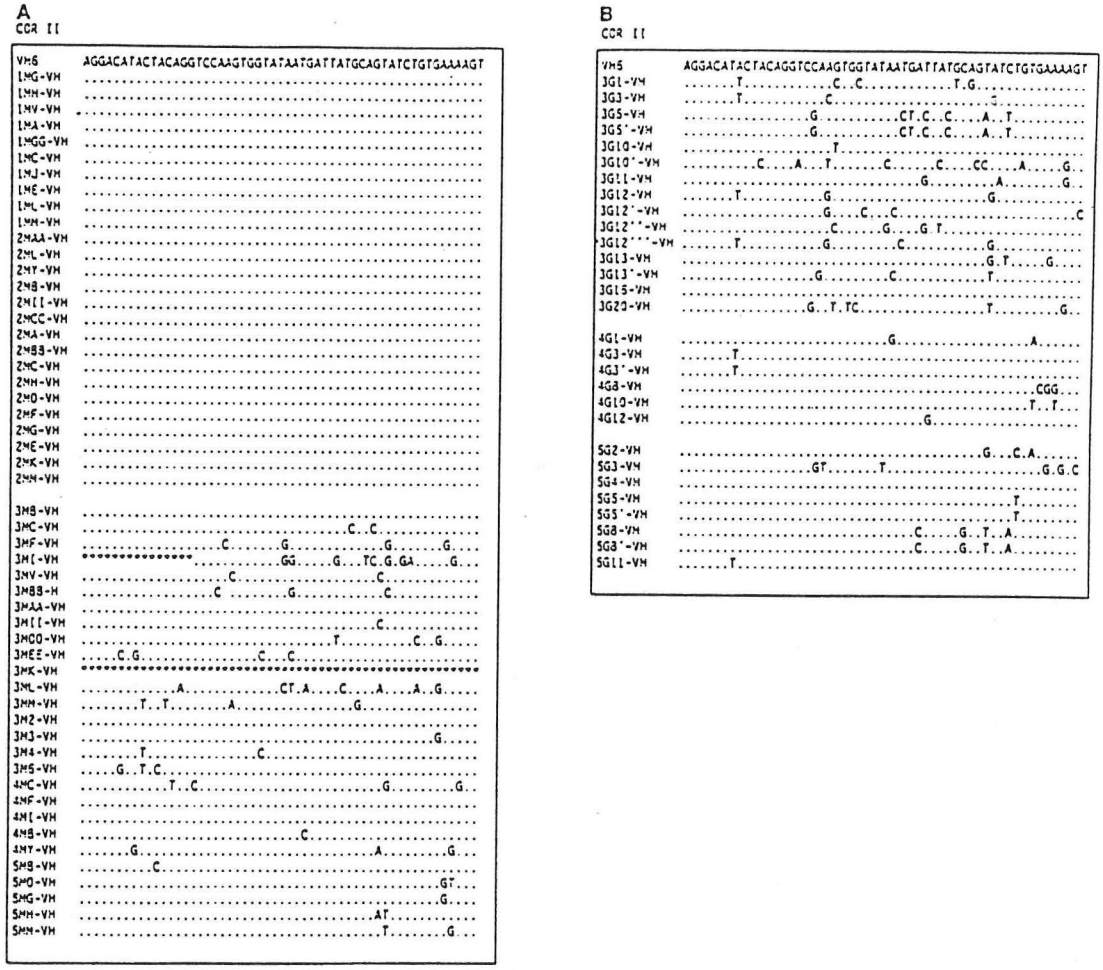


Immunofluorescence FACS analysis of tonsil B cells to identify IgD$^+$ CD38$^-$ FM B cells, IgD$^-$ CD38$^+$ GC B cells, and IgD$^-$ CD38$^-$ memory B cells. IgD$^+$ B cells were further sorted into CD23$^-$ (Bm1) and CD23$^+$ Bm2 cells. CD38$^+$ B cells were sorted into CD77$^+$ (Bm3) and CD77$^-$ (Bm4) cells *(From Pascual et al., 1994).*

The first and perhaps most important observation was that the naïve follicular mantle IgD$^+$/CD38$^-$ B cell consisted of two subsets, CD23$^-$ and CD23$^+$. An extensive analysis of this group of cells that we refer to as Bm1 and Bm2 (follicular mantle B cells) is shown in Table I, but, **The most important thing for our discussion today is that an analysis of the immunoglobulin transcripts from Bm1 and Bm2 cells revealed no cases of somatic mutation and only the IgM and the IgD isotypes.** That is, there had been no switching activity and the antibody genes are unaltered. While it would be getting ahead of ourselves it is well known that chronic lymphocytic leukemia, the most common adult leukemia, is of the IgM isotype (with rare exceptions) and no somatic mutation has occurred in the immunoglobulin genes. Therefore, we can imagine that peripheral leukemias that had these characteristics might arise from Bm1 and Bm2 cells. We will return to this later. These follicular mantle B cells that we first isolated from human tonsils have subsequently been isolated from virtually all human lymphoid organs. They represent naïve B cells that have not encountered antigen. Most importantly, they have not been through the process of somatic mutation.

The second most important observation of Banchereau and his colleagues concerns the IgD$^+$/IgM$^+$/CD38$^+$ B cells which are the germinal center founder cells. These cells represent about 10% of tonsillar B cells and contain most of the phenotypic markers that in other species have been identified with germinal center B cells including CD10, CD71 and CD77. They lack most of the markers expressed on follicular mantle B cells such as CD5, CD23 and CD44. They express Fas but not Bcl2 and are sensitive to rapid spontaneous apoptosis during *in vitro* culture. Most of them are proliferating B cells as indicated by the expression of the proliferation related nuclear antigen Ki67 or contain high DNA content as analyzed by Hoerst 33342 staining (see Table I). These cells can be further subdivided with the phenotypic marker CD77 into centroblasts (Bm3 cells) and centrocytes (Bm4 cells). These cells have been analyzed extensively in several laboratories and the critical observation in relationship to human

malignancies that relates to the normal physiology of these B cells is that **while both centroblast and centrocytes are undergoing somatic mutation only centrocytes are isotyped switched!** Again, getting again ahead of our story we can envision that any human B cell malignancy that is of the IgG, A or E isotype must have passed through the centrocytic stage. While IgM cells that are well somatically mutated are likely of centroblastic origin. Below, the degree of somatic mutation and isotype switching that is evident in this group of cells is shown.



Nucleotide sequence comparison of the CDR2 of V_H6 containing transcripts from each of the subsets. ***(A)*** **IgM transcripts.** ***(B)*** **IgG transcripts.** ***(Asterisk),*** **base pair deletion** ***(From Pascual et al., 1994).***

Finally, those B cells that are IgD$^-$ and CD38$^-$ have all the characteristics of memory B lymphocytes. That is, their immunoglobulins genes are somatically mutated and the cell surface markers (see Table I) are characteristic of memory cells in other species. Finally, plasma cells emerge from this reaction as isotype switched highly mutated secreting cells that as mentioned above home to the bone marrow where they produce large amounts of antibody (Hodgkin, et al., 1996).

All of these processes are under the direction of a series of newly discovered interacting ligand receptor complexes including CD40, CD40 ligand, and deficiencies in this regard lead to some of the immune deficiency syndromes that I discussed with you three years ago in these Grand Rounds (Capra, 1993).

Table 1.

B cell subsets	FM		sIgD+ GC		GC		Memory
	IgD+ CD38-		IgD+ CD38+		IgD- CD38+		IgD- CD38-
	Bm1	Bm2	Bm2	Bm3δ+4δ	Bm3	Bm4	Bm5
CD23	-	+	-	-	-	-	-
CD39	+	+	?	?	-	-	+
CD44	+	+	±	±	-	±	+
CD10	-	-	+	+	+	+	-
CD71	-	-	+	+	+	+	-
CD77	-	-	±	±	+	-	-
IgM	+	+	+	-	±	±	±
IgG	-	-	-	?	+	+	+
Fas	-	-	+	+	+	+	-
Ki67	-	-	+	+	+	+	-
Somatic mutation	-	-	+	+	+	+	+
Switching activity	-	-	-	-	+	+	-

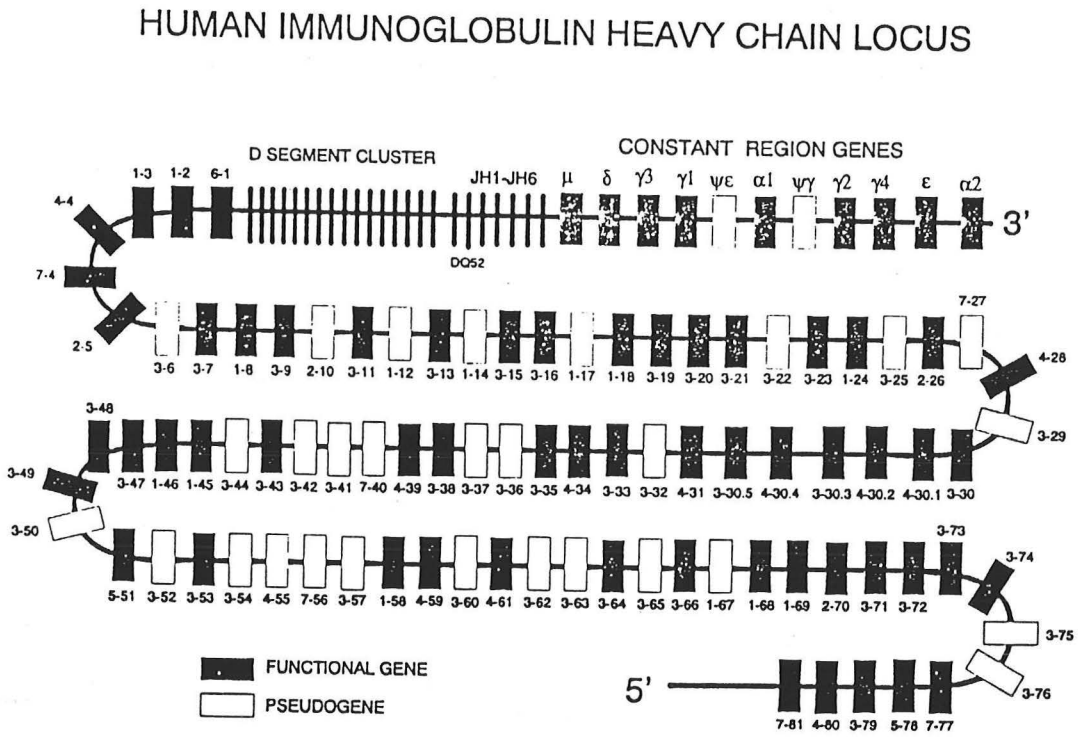
(+)= positive; (±)= low levels; (-)= negative.

Recent studies from Max Cooper's laboratory suggest that memory B lymphocytes migrate to the bone marrow in humans as do plasma cells. Analysis of their homing receptors, duration of bone marrow residence and ultimate fate is needed for a complete understanding of clinical disorders such as multiple myeloma in which aberrant B lineage cells accumulate in the bone marrow (Liu and Banchereau 1996b; Paramithiotis and Cooper, 1997).

D. Over-utilization of Specific Human Immunoglobulin Gene Segments

The human V_H and Vκ loci have been completely mapped and the coding regions have been completely sequenced from several individuals. Thus, we have a fairly complete understanding of the genomic structure of two of the three crucial variable region receptor families of B cells (human lambda has received less attention to date). During the past several years many investigators have studied Ig gene expression in normal B cells at various stages of development and in various B cell disorders. The human V_H families have been divided into six or seven groups and the kappa families into four to six groups and the utilization of these various families has been studied extensively (Stewart and Schwartz, 1994; Davidkova, et al., 1997; Brezinschek et al., 1997). Several lines of evidence suggest that certain individual V_H and Vκ genes are overexpressed at early stages of ontogeny (and as we shall see later also in B cell malignancies) and in autoantibodies (not a subject we will return to). Some of these genes, for example, are used by as much as ten or fifteen percent of specific repertoires rather than the expected one or two percent based on the frequency of these various genes. Kraj, et al., have presented data that nearly ten percent of the V_H genes of all cDNA libraries from PBLs are the V_H4-34 gene (Kraj, et al., 1995). Since this represents one of 100 human V_H segments this would indicate a dramatic overexpression of this gene. This is an area of intense investigation in our own and other laboratories because this particular gene segment is used exclusively in human cold agglutinins one of the CLL variant lymphomas.

This will not be further detailed other than introduce the notion that the representation of each V_H and Vκ gene is not proportional to its frequency in the germline. Thus, when we encounter B cell malignancies we might anticipate that some or all of these malignancies might reflect this preferential utilization of V gene segments.



Organization of the Human V_H Locus. Schematic diagram of the human heavy chain gene complex. The variable region genes are located at the 5' end of the locus and the constant region genes are found at the 3' end. Members of different V_H families are interspersed throughout the V_H portion of the locus. D segments are represented by black lines, J genes are represented by blue lines and constant regions are shown in red. Functional gene segments are represented by solid boxes and pseudogenes are shown as open boxes. This map is not drawn to scale.

E. Molecules That Are Critical For The Development of Germinal Centers

When immunoglobulin genes were sequenced at the germline level elements were noted (particularly 5' of the coding regions) that were referred to as octamers. They seem to be preserved across species and across various families of B cell receptor variable regions. Soon an octamer binding factor was found that was shown to be a B cell specific transcription factor. This has grown to a large family of molecules that are thought to be involved in the transcription of immunoglobulin genes. These factors bind other factors required for immune responses and recently many of these factors have been deleted from the mouse genome by homologous recombination. In general, when this happens, germinal center formation is severely impeded if not eliminated (Liu and Banchereau, 1996a). For example, the OBF/1 co-activator has now been shown to be essential for the formation of germinal centers (Kim, et al., 1996). Previous studies have shown that mice that are deficient for the key molecules involved in T cell/B cell interaction such as CD40, CD40 ligand, and MHC Class II or transgenic mice that produce large amounts of soluble CTLA-4 also lack the ability to form germinal centers (Kelsoe, 1996; Shubart, et al., 1996). Mice lacking lymphotoxin α, lymphotoxin β or TNF-α and their receptors are also impeded in germinal center formation. While one must be cautious that the impairment of germinal center formation may not be a direct consequence of knocking out TNF-α, lymphotoxin α, or lymphotoxin α,β and their receptor genes it does provide us with clues as to

how primary follicles and follicular dendritic cells play an important role in the survival and recruitment of newly generated non-self reactive B cells into the re-circulating B cell pool (Schubart, et al., 1996).

F. The Germinal Center And Tolerance

There are two stages at which self reactive B cells need to be eliminated in mammals. The first occurs during the process of antigen independent gene rearrangement that takes place in the bone marrow. One can envision that during this process occasionally receptor molecules are generated that are self reactive. These cells die in the bone marrow never to reach the peripheral lymphoid tissues. This phenomenon is known as primary B cell tolerance. Additionally, as somatic hypermutation is occurring in germinal centers and the process is generally viewed as random, it is possible, indeed likely that self reactive clones emerge in the germinal centers and therefore, need to be eliminated. This is generally referred to as secondary B cell tolerance. We will return to these points later as we approach human lymphoid malignancies that produce self reactive antibodies (Kelsoe, 1996; Pulendran et al., 1997)).

III. STATUS OF THE IMMUNE RECEPTOR GENES

Based on a modern understanding of immunoglobulin gene rearrangement, somatic mutation and the germinal center reaction, there are a few relatively simple maneuvers that can be applied to the structure of the immune receptor genes that provide us with an enormous amount of new information concerning the origin of each of these tumors. For example, since all of the human V_H, V kappa and J gene segments have been sequenced, a simple DNA sequence of the rearranged gene in a B cell tumor allows us to draw the conclusion as to whether that B cell has even been in an environment to engage the somatic hypermutation machinery. Those tumors that are pre-follicle will have no mutations whereas those that arise in the follicle or after the follicle will be peppered with somatic mutation.

MUTATIONS IN Ig GENES

	Mutations	
	Present	Ongoing
Pre-follicle (Bone Marrow → Naïve (Bm 1, Bm 2)	NO	NO
Follicle (Germinal Center) (Bm 3, Bm 4)	YES	YES
Post-follicle (Plasma cell, Memory Cell) (Bm 5)	YES	NO

A division of lymphocytes based on the status of mutations in the immunoglobulin genes.

Additionally, one can assess a phenomenon termed 'intraclonal variation' by sequencing several different immunoglobulin genes from the same tumor. Thus, during the initial biopsy of a solid tumor or analysis of the peripheral blood of the leukemia, rather than sequencing a single

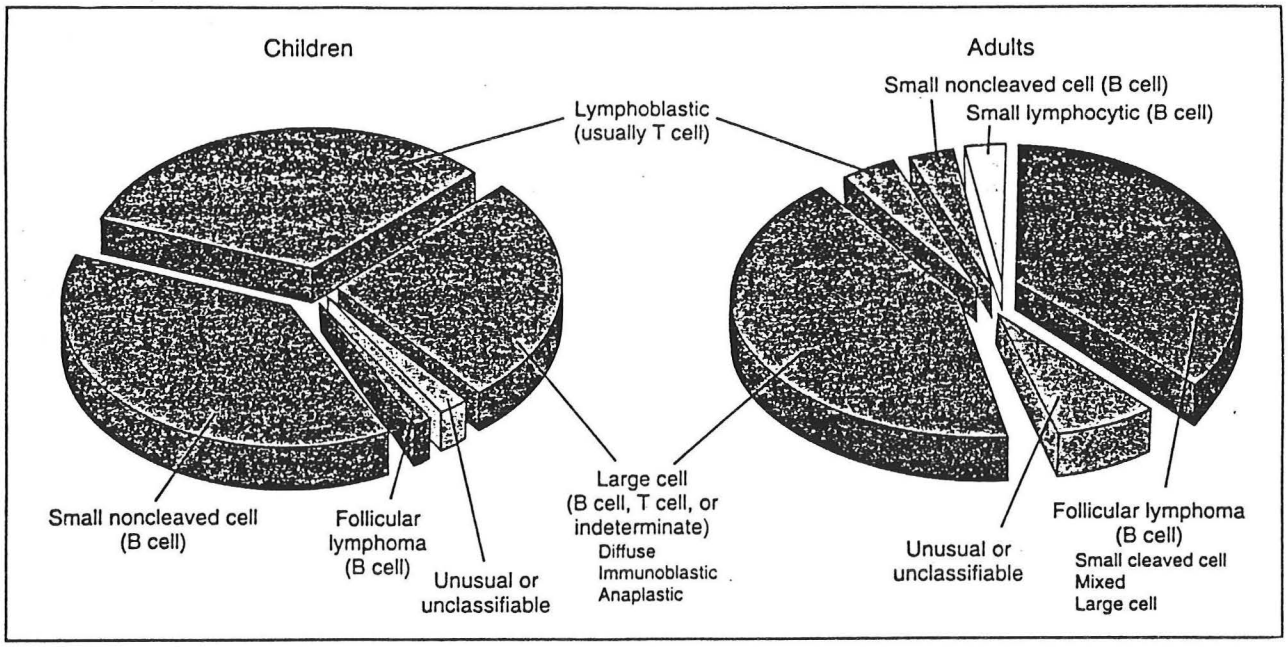
rearrangement, if a dozen or so are sequenced they either all have the same sequence or there are slight variations. These slight variations are termed 'intraclonal variations' and indicate that the tumor arose in a cellular population in which the somatic hypermutation machinery has not yet been turned off. Along the way the isotype of the tumor can be easily determined and again, since we know that isotypic switching does not occur until the Bm4 subset, one can easily eliminate a series of possible clonal origins for the tumor by this approach. Examples of these are illustrated where some of the tumors that we will discuss have been classified as to whether they contain mutations and/or whether there is intraclonal variation.

INTRACLONAL VARIATION

• B-ALL		NO
• B-CLL	$CD5^+$ (95%)	NO
	$CD5^-$ (5%)	YES
• BURKITTS LYMPHOMA		YES
• HAIRY CELL LEUKEMIA		YES
• MYELOMA		NO

Adapted from Bakkus et al. 1992, Kluin et al. 1997, Pritsch et al., 1997.

These relatively simple maneuvers have provided hematologists and oncologists studying human B cell tumors with considerable information. B-ALL and the vast majority of B-CLL's (essentially all those that are $CD5^+$ which represents over 95% of B-CLL's) show no intraclonal variation, whereas $CD5^-$ CLL's, Burkitt's lymphomas and hairy cell leukemia demonstrate intraclonal variation. Myelomas, on the other hand, representing end stage plasma cells are remarkably stable and show no intraclonal variation.



Distribution of histologic subtypes of non-Hodgkin's lymphoma in children and adults.

IV. LYMPHOMA CLASSIFICATION

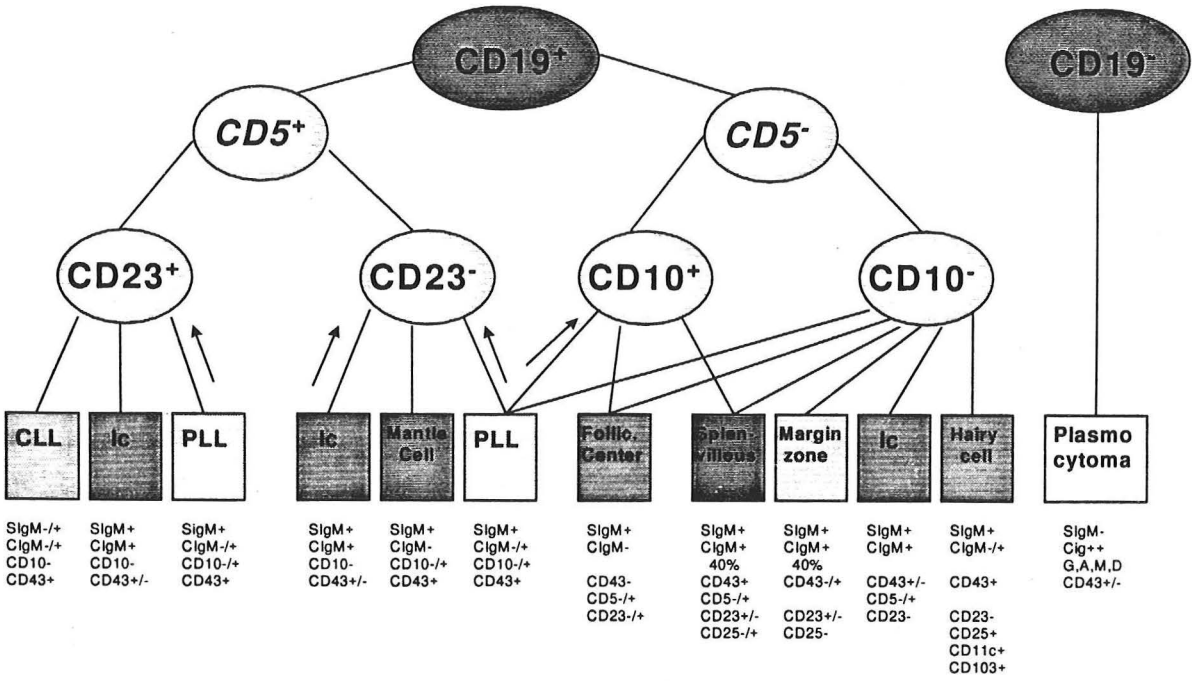
The classification of lymphomas has been contentious and frustrating for clinicians and pathologists for over 50 years. The source of frustration primarily derives from the enormous pleomorphism of lymphomas and the difficulty in assigning normal cellular counterparts to these malignant B cell tumors. Over the course of the last four decades there have been four distinct formulations that have worked their way to the status of being internationally approved. The most recent revision was devised about two years ago. Since different classification schemes depend upon entirely different properties, it is extremely difficult to draw parallels between them. In retrospect, many of the categorizations that were done three and four decades ago were enormously prescient of contemporary thinking while others were hopelessly flawed particularly in their simplicity.

LYMPHOMA CLASSIFICATION

- **RAPPAPORT (1966)**
  Based on histologic and cytologic characteristics of lymphoma cells (mainly descriptive)
- **KIEL AND LUKES AND COLLINS (1974)**
  Based on relationship between malignant lymphomas and their normal counterparts within the lymphatic system
- **WORKING FORMULATION (1982)**
  Based on clinical parameters and H & E morphology, grouping according to their natural history, response to therapy, and overall survival
- **"REAL" CLASSIFICATION (Revised European American Lymphoma Classification) (1994)**

Until recently, lymphomas were characterized on the basis of two basic systems. One was the histologic characterization particularly with hematoxylin and eosin stains. These formulations came out of the mid-sixties and are referred to by among other terms as the Kiel classifications. About 15 years ago a new system was devised that took into account the prognosis of patients with these diseases and a separate classification of lymphomas developed around the notion of high grade, medium grade and low grade malignancies. Other classifications describe cells as large and small in attempts to devise classification schemes that combine the three approaches have been frustrating.

About 15 years ago immunophenotyping became common and the utilization of polyclonal and monoclonal antibodies revolutionized the classification of lymphomas. B and T cell malignancies were easily distinguished and even subsets of B cells were classified based on the presence or absence of distinct cell surface markers. Later, genetic phenotyping came into common use and translocations and the overexpression of certain oncogenes was added to the classification scheme. Along the way attempts at relating lymphomas to their normal cellular counterparts were made but were often abandoned in the face of the definitiveness of immunophenotyping and genetic analysis.



A simple algorithm to immunophenotype human lymphomas ***(Adapted from Marjanovic and Ihan, 1997).***

A couple of examples of how lymphoma classifications are complicated would be to consider the distinction between diffuse and nodular lymphomas. In general, diffuse lymphomas have a prognosis of six to twelve months and with treatment, about half of the patients can be "cured" with combination chemotherapy and radiotherapy. While nodular lymphomas have eight to sixteen year survival periods, treatment is probably irrelevant except for the relief of symptoms and these patients are never cured.

In 1994 a group of hematopathologists met and proposed the Revised European American Lymphoma classification or 'REAL' system. It was formulated and thought to have the following advantages. 1) It incorporated all nodal and extranodal lymphoid tumors including Hodgkin's disease. 2) It was histologically updated by recognizing new categories such as mantle cell lymphoma. 3) In tumors that need further validation to be considered as separate entities (such as diffuse large B cell lymphomas) it avoided morphologic rating. The clinical course of lymphomas being influenced by factors other than histology (tumor burden, cell kinetics, apoptotic rate, and so forth); and 4) It included all molecular data (phenotype, karyotypic analysis, genotype, etc.) which could assist in the diagnosis. This formulation was originally published in *Blood* in 1994 by Harris, et al., (Harris et al., 1994) and updated by (Pileri, 1995) and most recently by (Heildemann, et al., 1996).

One of the major new insights that has been incorporated into the REAL classification concerns the status of the immune receptor genes and it is through this process that the major take home message of these Grand Rounds is directed. As we have learned by our study of immunoglobulin gene rearrangement, prior to exiting the bone marrow, the immunoglobulin genes are rearranged into V and J for light chain and V, D and J for heavy chain. These are in germline configuration, that is, there is no somatic mutation and the process is fairly stable.

Thus, any neoplasm that involves a pre-B cell has its immunoglobulin genes in an unrearranged form whereas once that stage has been reached we can type it rather easily in the laboratory as a rearranged immunoglobulin gene. Additionally, we have discussed two new processes. That is, since somatic mutation takes place in germinal centers it should now be obvious that any tumor that arises prior to the passage of that B cell through the germinal center will continue to have its immunoglobulin genes in germline configuration, that is, unmutated. It also follows any tumor that arises from the B cell lineage after the passage through the germinal center will have immunoglobulin genes that show evidence of somatic mutation. Finally, as indicated above, the process of somatic mutation ends by the time a B cell reaches the memory compartment. Therefore, if the tumor is sampled and there is variation in the immunoglobulin gene sequence between different parts of the tumor, the likelihood is that the tumor arose from a germinal center B cell (for example, a centroblast or centrocyte) while those that arise from memory and plasma cells will have stable sequences over the long course of the tumor and when it is sampled in different compartments. Finally, since we have learned that the isotype switch does not occur until late in the course of the germinal center reaction, tumors that are IgG or A or E are by definition Bm4 cells or beyond.

B cell markers		*Immature lymphoid marker*		*Non-lineage-specific markers*	
CD9	B cell antigen (p24)	TdT:	terminal deoxynucleotidyl transferase (expression on the nuclear membrane)	CD11b	complement (C3bi) receptor type 3 (CR3)
CD10	common ALL antigen(p100)			CD11c (p150,95)	adhesion molecule
CD19	pan B cell antigen (p95)			CD25	IL-2 receptor
CD20	B cell antigen (p35)			CD34	precursor cell antigen
CD21	B cell antigen (p140); complement (C3d) receptor type 2 (CR2)			CD38	T10 antigen
				HLA-DR	MHC class-II antigen
CD22	B cell antigen (p135)				
CD23	B cell antigen (low affinity Fc epsilon receptor) (p45)				

Markers used to immunophenotype B cells

Incorporating these new insights into lymphoma classification has provided powerful new insights into diagnosis. However, the proof that these are useful to clinicians can only be if they pass the test of utility in therapy and prognosis. And in that case, while most of the evidence suggests that this is a major new advance in our understanding, all of the evidence is not yet in. Let us now turn and review these in turn.

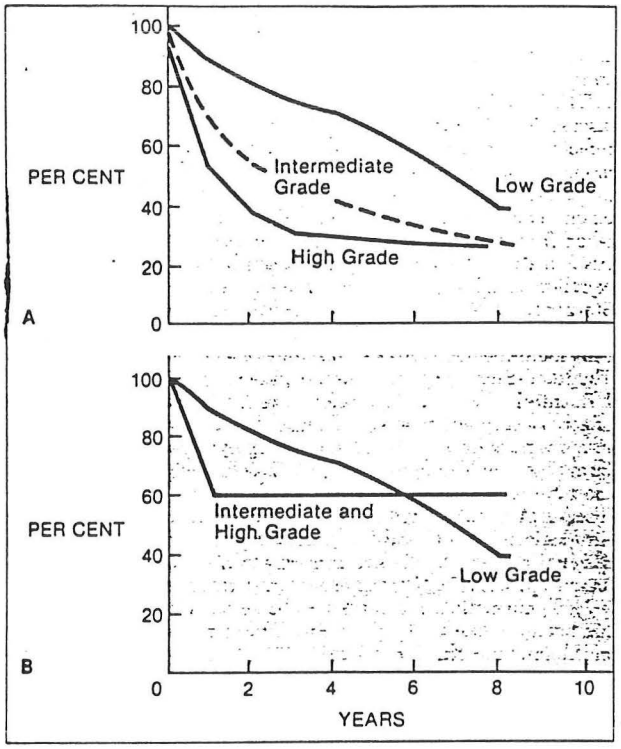
SOME EXAMPLES OF THE CLASSIFICATION OF B CELL LYMPHOMAS

Kiel Classification	Working Formulation	REAL
Low Grade	Low Grade	
B-cell CLL	Small lymphocytic lymphoma	B cell CLL
Plasmacytic leukemia	Plasmacytoma	Plasma-cytoma/ myeloma
High Grade	Intermediate-Grade	
Centroblastic lymphoma	Diffuse, large cell lymphoma	Diffuse large B cell L.
Burkitt's Lymphoma	High Grade	
	Small non-cleaved Burkitt's Lymphoma	Burkitt's Lymphoma

Summary of Classification Systems: Non-Hodgkin's Lymphomas of B-Cell Lineage

Kiel Classification	Working Formulation	REAL Classification
Low-Grade Lymphomas	Low-Grade Lymphomas	
B-cell chronic lymphocytic leukemia B-cell prolymphocytic leukemia	Small lymphocytic L consistent with CLL	B-cell chronic lymphocytic leukemia
Lymphoplasmactytoid immunocytoma	Lymphoplasmacytoid L/immunocytoma	Small lymphocytic L B-cell prolymphocytic leukemia
Lymphoplasmactyic immunocytoma	Small lymphocytic, plasmacytoid L.	Lymphosplasmacytoid L.
	Small lymphocytic L.	Extranodal marginal zone B-cell L.
Monocytoid L., including marginal zone	Small lymphocytic L.	Nodal marginal zone B-cell L.
	Small lymphocytic L.	Splenic marginal zone B-cell L.
Hairy cell leukemia		Hairy cell leukemia
Plasmacytic L.	Plasmacytoma	Plasmacytoma/Myeloma
Centroblastic/centrocytic L., follicular	Follicular L. predominantly small cleaved cell	Follicle center L., follicular, grade I
Centroblastic/centrocytic L., follicular	Follicular L. mixed small and large cell	Follicle center L., follicular, grade II
	Intermediate-Grade Lymphomas	
Centroblastic L., follicular	Follicular L. predominantly large cell	Follicle center L., follicular, grade I
Centroblastic/centrocytic L., diffuse	Diffuse L. small cleaved cell	Follicle center L., diffuse, small cell
	Diffuse, mixed small and large cell L.	
Centrocytic L., diffuse	Diffuse L. small cleaved cell	Mantle cell Lymphoma
High-Grade Lymphomas		
Centroblastic L.	Diffuse, large cell Lymphoma	Diffuse large B cell Lymphoma
	High-Grade Lymphomas	
B immunoblastic L.	Diffuse large cell immunoblastic L.	Diffuse large B cell L.
B-large cell anaplastic L. ($Ki\text{-}1^+$	Diffuse large cell immunoblastic L.	Diffuse large B cell L.
Burkitt's Lymphoma	Small non-cleaved, Burkitt's L.	Burkitt's Lymphoma
	Small non-cleaved, non-Burkitt's L.	High grade B-cell L., Burkitt-like
Lymphoblastic L.	Lymphoblastic L.	B-precursor lymphoblastic L.

Adapted from Hiddemann, et al., *Blood* **88**:4085-4089, 1996.



A: Actuarial survival according to histologic grade, based on 1975 data, as reported in the Working Formulation (1982).
B: Hypothetical actuarial survival according to histologic grade, based on 1985 data.
(Adapted from Portlock, 1992b).

V. THE LYMPHOMAS

(1) Precursor B cell Neoplasm: Precursor B Lymphoblastic Leukemia/Lymphoma (B-LBL)

This leukemia was previously called lymphoblastic (diffuse poorly differentiated lymphocytic) B cell type leukemia.

There are about 2,000 cases of acute lymphoblastic leukemia in the United States every year of which approximately half represent precursor B cell neoplasms. There are two general types that are distinguishable by the presence or absence of cytoplasmic immunoglobulin. Otherwise these pre-B cell leukemias have the common phenotype of being $CD22^+$, $CD79a^+$, $CD19^+$ and being surface immunoglobulin negative. These tumors generally are TdT^+ and are RAG positive.

The genetic features of this disease are that the immunoglobulin heavy chains are usually rearranged. The light chain genes may or may not be rearranged. Otherwise, there are no consistent genetic abnormalities in B-LBL.

Children are more commonly effected than adults. This disease accounts for about 80% of acute lymphoblastic leukemia and about 20% of lymphoblastic lymphoma. Although the vast majority of precursor B cell neoplasms present as acute leukemias with bone marrow and peripheral blood involvement, there are a small percentage of these cases that present as solid tumors in the skin, bone or lymphnodes without any bone marrow involvement. The disease is highly aggressive but frequently curable with available therapy. The postulated normal counterpart is the bone marrow derived precursor B cell. In the categorizations that I have described previously, it would be a cell prior to a Bm1 cell.

PRECURSOR B-CELL NEOPLASM: PRECURSOR B-LYMPHOBLASTIC LEUKEMIA/LYMPHOMA (B-LBL)

- **IMMUNOPHENOTYPE:** **TdT^+ $CD19^+$ $CD79a^+$ $CD10^+$ sIg^- cMu^-**
- **GENETIC FEATURES:** **Ig heavy chains rearranged**
- **CLINICAL FEATURES:** **Disease of children (80% of ALL) present as acute leukemias poor prognosis**
- **POSTULATED NORMAL COUNTERPART:** **Bone marrow derived precursor B cell**

(2) Chronic Lymphocytic Leukemia

All the rest of the B cell neoplasms are peripheral B cell neoplasms. Chronic lymphocytic leukemia (CLL) is a hematologic neoplasm characterized by the accumulation of mature-appearing lymphocytes in the peripheral blood associated with infiltration of the bone marrow, spleen, and lymph nodes. The disease is uncommon before the 4th decade of life and is usually seen in patients of over 50 years of age. **It is the most common form of chronic leukemia in the United States.** Enlarged lymphnodes in patients with B-CLL show a characteristic infiltrate. The predominant cell is a small lymphocyte. There's ocasionally a small nucleolus. Larger lymphoid cells are always present. The immunophenotype of these cells is that they have faintly positive surface IgM, they have B cell associated antigens such as CD19, CD20 and CD79a and they are invariably CD5$^+$ and CD23$^+$. CD23 is useful in distinguishing B-CLL from mantle cell lymphoma (see the next section).

The genetics of the immunoglobulin genes are that the heavy and light chain genes are rearranged, trisomy 12 has been reported in about one third of the cases and abnormalities of 13q14 are seen in about 25% of cases.

B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

• IMMUNOPHENOTYPE:	sIg$^+$ (both IgM and IgD), B cell associated antigen positive (CD19, CD20, CD79a), CD5$^+$, CD23$^+$
• GENETIC FEATURES:	Ig chains are rearranged; trisomy 12 in one third
• CLINICAL FEATURES:	90% of CLL in U.S. older adults Involves bone marrow and PB

The postulated normal counterpart is the recirculating CD5$^+$, CD23$^+$ peripheral B cell which in the classification scheme that I have described is typically a Bm2 cell.

For the purposed of today's discussion there are 3 key issues in a consideration of CLL that impinge on these Grand Rounds. 1) Is the disease heterogeneous? 2) What is the normal cellular counterpart of B-CLL? 3) What is the relationship of B-CLL in autoimmune disease? These issues are all heavily inter-related but will be discussed separately.

Is B-CLL a heterogeneous disease?

Since B-CLL is the most common form of chronic leukemia in the United States, most series report over 500 cases so the numbers here are rather significant and staggering. About 95% of patients with B-CLL have IgM bearing cells that are CD5$^+$. About 5% bear IgG and are

$CD23^-$. The importance of this distinction should be evident in terms of the cellular origin and the relationship of the tumor to the germinal center reaction that I have discussed to this point. Specifically, since the vast majority of cases of B-CLL are surface IgM, surface IgD^+ (albeit weak in both instances) and $CD5^+$, we assume that these cells derive from Bm2 cells (see below) while the IgG/$CD23^-$ types would derive from later stages of B cell development (perhaps Bm4 or Bm5). These distinctions are only important if they impinge on either treatment or prognosis and the evidence in this regard is simply not in. Many studies suggest that the IgG phenotype of B-CLL has a poorer prognosis, is less responsive to therapy and has a poorer survival than the IgM B-CLL. But, not all studies confirm these suggestions.

Whether the $CD5^+$ CLL cells result from malignant transformation of cells committed to a particular lineage (B-1 cells), or cells at a particular stage of normal B cell differentiation (mature, recirculating B cells), is a matter of current debate. Overall, most CLL cells bear sIgM and sIgD, and express unmutated VH and VL region genes, thus resembling the Bm2 cell subset that populates the FM of normal peripheral lymphoid organs (Kupper et al., 1991; Rassenti and Kipps, 1993). Up to 35% of the cases, however, express a single isotype, and molecular analyses of IgG CLL cells show the unequivocal accumulation of somatic mutation within the corresponding variable region genes (Cai et al., 1992; Hashimoto et al., 1995), suggesting a post-germinal center origin (Bm5) for these cells. Therefore, it is conceivable that more than one stage of B cell differentiation be the potential host for the malignant transformation that results in this disease. Indeed, different subgroups of CLLs express non-FM markers, like CD11b and the GC marker CD38, and they are heterogeneous for the expression of molecules involved in the adhesion with other cells or with extracellular matrix proteins.

Recent studies suggest that CD38 expression distinguishes at least two groups of CLL with different responses to anti-IgM antibodies and propensity to apoptosis (Zupo et al., 1996). In fact, while anti-CD38 antibodies block lymphopoiesis and prevent the spontaneous apoptosis of normal human GC B cells, $CD38^+$ CLL cells have been reported to undergo apoptosis upon treatment with these antibodies. Additionally, apoptosis of $CD38^+$ CLL cells can be prevented by rIL4, but not by exposure to CD40L, a molecule that rescues normal GC B cells from programmed cell death.

In the future, a more systematic approach to the classification of CLLs based on the expression of markers associated with different developmental stages of peripheral B cells in combination with Ig sequencing analysis should help establishing a more accurate connection between normal B cells and their pathological counterparts, as well as perhaps offer a better understanding of the prognostic implications for different disease subgroups.

V gene usage in B-CLL

B-CLL has been extensively studied for Ig V_H and V_L gene segment utilization because (1) there are many cases in virtually every tumor registry and (2) because there is no somatic mutation in the V genes it is relatively easy to assign the germline counterpart in each case. Almost all studies have come to the conclusion that there is an 'over-representation' of specific V genes, although surprisingly, different studies have come to different conclusions as to which V genes are over-represented. The difficulty in most of the studies that have been published

relate to the method of analysis which often is a southern blot or perhaps a PCR of a specific V_H or V_L gene family rather an exact sequence and specific assignment. Thus, some studies have come to the conclusion that specific V_H families were over-represented but when individual genes were sequenced there did not seem to be a specific over-representation of a particular gene. However, it is fair to say that almost all studies have come to the conclusion that the specific V genes utilized in CLL are skewed and more likely represent the skewing that is seen in the fetal repertoire. This (see below) may be a reflection of the cellular counterpart in the $CD5^+$ fetal B lymphocyte.

Status of the immune receptor genes.

As indicated above, 95% of B-CLL's of the IgM isotype and of these essentially all represent immunoglobulin genes in so called germline configuration, that is, without somatic mutation. All of these results suggest that B-CLL represents the malignant counterpart of a relatively immature B cell – specifically a B cell that has yet to enter the central regions of a germinal center but clearly has exited the bone marrow. We typically consider this to be the Bm2 cell which, as discussed earlier, are never somatically mutated.

The IgG CLL's on the other hand are, in general, heavily somatically mutated and a number studies have suggested that the somatic mutations in the IgG CLL's has a specific pattern to it that have suggested to some authors the workings of an antigen driven immune response. This has led to the speculation that at least some forms of CLL may represent chronic antigen stimulation with a specific pathogenic virus.

Relationship to autoimmunity.

Depending upon the study from 10% - 80% of people with CLL have manifestations of autoimmune disease. Certainly, the number of autoantibodies detected in the circulation of patients with B-CLL is far in excess of that seen in the normal population. There are some who interpret this in the light of the fact that there are 100,000 B lymphocytes per *cc* in the circulation rather than 5,000 and argue that this represents nothing more than one would anticipate with this extraordinary number of circulating B lymphocytes circulating. Others suggest that this is unrelated as the B cells in the circulation are clonally related and the number of <u>different</u> autoantibodies is quite dramatic. The subject needs to be discussed in two separate ways. First, the polyclonality of the autoantibodies that are found in B-CLL and, secondly, the monoclonal nature of a number of the B-CLL tumors themselves.

For example, autoimmune hemolytic anemia occurs in about 25% of B-CLL patients at some time during the course of the disease. Although the pathogenic autoantibodies occasionally may be produced by the malignant B cell clone, in most cases the autoantibodies appear to be may by remnant normal B lymphocytes. This view is supported by the observation that the antibodies eluted from the red blood cells are typically polyclonal IgG antibodies. On the other hand, the antibodies produced by the CLL cells are usually IgM and are always monoclonal. However, there are indeed dramatic cases of CLL in which the autoantibody produced has specific reactivity and is the cause of an autoimmune disease such as autoimmue hemolytic anemia, cold agglutinin disease and the like.

The enigma of the CD5$^+$ B cell.

As mentioned above, 95% of CLL patients are CD5$^+$, CD5 is a marker of fetal B lymphocytes in mice and is present in early B cell differentiation in humans. In mice there has been a dramatic association between CD5$^+$ B cells and autoimmune disease, specifically autoantibodies to a large variety of important autoantigens are produced by CD5$^+$ B cells. In both mice and humans, B cells that arise early in ontogeny are enriched for CD5 expression. Fetal and CLL CD5$^+$ B lymphocytes are unresponsive to lectins and are unable to cap surface Ig. Furthermore, CD5$^+$ B cells from tonsilar tissue of mature individuals express the surface antigen phenotype of mantle zone B cells. Thus, is has been suggested (but not proven) that CD5$^+$ B cells of the mantle zone and the CLL B cells are both the progeny of cells that arise early in human ontogeny. Since the fetal heavy chain Ig repertoire exhibits a restricted variable region utilization, the V domains of CLL B cells have been used to test this hypothesis. Most investigators have concluded that there is an association between the CD5$^+$ B cell and the CLL B cell based on this non-random utilization.

The difficulty in human immunology is that the CD5 marker is not as stable as it apparently is in the mouse and in humans the CD5 marker tends to be more a differentiation marker rather than a lineage specific marker. Thus, the exact position of the CD5 antigen during B cell ontogeny in the human is still unclear.

(3) Mantle Cell Lymphoma

By the mid 1970's the non-Hodgkin's lymphomas had been classified as either well differentiated (small lymphocytic) or poorly differentiated (small cleaved cell). About this time, the term "lymphocytic lymphoma of intermediate differentiation" was used to describe those non-Hodgkin's lymphomas that were difficult to classify (Berard *et al.* 1974, Berard, 1975). The term centrocytic lymphoma also became popular as many believed some lymphomas to be of germinal center origin (Lennert *et al.*, 1975). Others thought these tumors corresponded to cells of the primary lymphoid follicle and the mantle zones of secondary lymphoid follicles. In the 1980's two groups described distinctive types of follicular lymphomas that were characterized by the proliferation of atypical small lymphoid cells in wide mantles around benign germinal centers, and the term mantle zone lymphoma came into use (Weisenburger, *et al.*, 1982, Palutke *et al.*, 1982). This neoplasm was thought to represent the follicular counterpart of diffuse intermediate lymphocytic lymphomas. In the early 1990's it became clear that these were all variants of the same disease. That is, lymphocytes of the primary lymphoid follicles and the mantle zones of the secondary lymphoid follicles are the next differentiation stage or the next location for the same lymphocyte. Currently, the accepted term for this classification is mantle cell lymphoma and it corresponds to approximately 5% of all non-Hodgkin's lymphomas (Weisenburger & Armitage, 1996).

The immunologic features of mantle cell lymphomas are; surface IgM+, IgD+, cells 60% of whom are lambda. They are positive for pan B cell antigens such as CD19, 20, 22, 24 and HLA DR. They are usually, but not always, CD5+, they are negative for CD23 (Argatoff *et al.*, 1997). A major breakthrough in the classification of these tumors occurred when it was

discovered that cytogenetically they shared a t(11;14) (q13; q32) translocation (Rimokh *et al.*, 1993). The molecular counterpart of the translocation is a *bcl-1* rearrangement that leads to cyclin D1 (*bcl-1*, PRAD1) proto-oncogene over-expression.

MANTLE CELL LYMPHOMA	
• IMMUNOPHENOTYPE:	sIgM$^+$ sIgD$^+$ 60% lambda Pan B cell antigen positive (CD19, 20, 22, 24) and HLA-DR Usually CD5$^+$ Negative for CD 23
• GENETIC FEATURES:	t(11;14) (q13; q32) bcl-1
• NORMAL COUNTERPART:	Bm1

The REAL classification is the first major classification officially incorporating mantle cell lymphoma and providing objective diagnostic criteria. As discussed above, it also represents a paradigm shift from previous pathologic classifications of proliferative disease which were based solely on light microscopic morphologic features (Davis & Szczarkowski, 1996). The REAL classification defines disease entities based on incorporation of current available immunologic and genetic characterization of the lympho-proliferative clones.

Mantle cell lymphoma is particularly difficult to characterize morphologically and phenotypically. However, the over-expression of cyclin D1 is a common finding as well as the cytogenetic translocation noted above (Molot *et al.*, 1994). The immunoglobulin heavy chain locus on chromosome 14 is translocated to the *bcl-1* region on chromosome 11. This is one of the mechanisms causing the upregulation of expression of the PRAD1 gene located about 120kb from the *bcl-1* majortranslocation cluster (Ott *et al.*, 1997). The PRAD1 gene encodes one of the key cyclins involved in the control of the cell cycle.

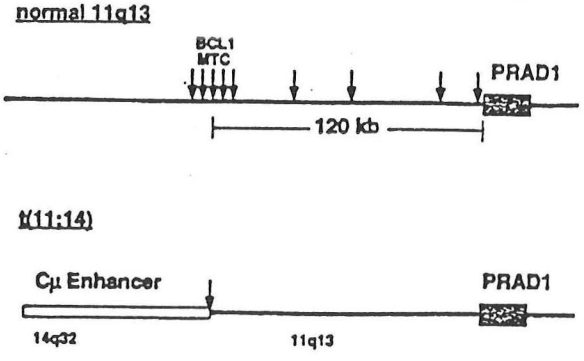
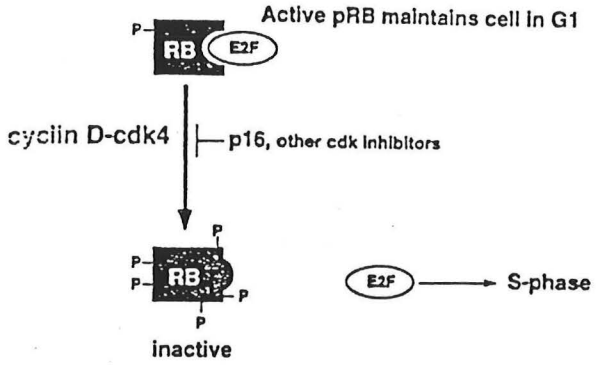


Diagram of the t(11;14) in MCL. An 11q13 breakpoint at the *bcl-1* major translocaiton cluster (MTC) is shown; other described breakpoints (arrows in upper panel) exist as close as 1 kb from the first exon of the PRAD1 gene.



Schematic diagram showing the functional interrelationships of cyclin D1.

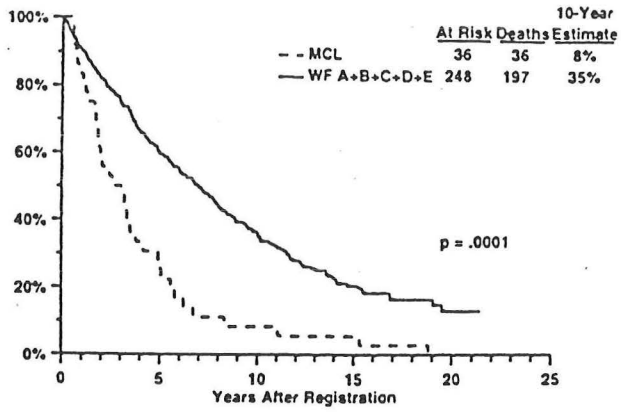
Over-expression of PRAD1 prevents the cell from leaving the cell cycle and causes continuous cell proliferation. While upregulation of cyclin D1 has been described in various non-lymphoid malignancies, **MCL is so far the only lymphoma characterized by this finding.** Figures above illustrate the translocation as well as the schematic showing the functional interrelationships of cyclin B1.

Clinically patients with mantle cell lymphoma represent a sub-population of patients with lymphoma. There is a male to female ratio of 4:1 and their prognosis is generally poorer than that for most other lymphomas.

Table 2. Clinical Features of MCL at Initial Presentation

Median age	60 yr
Male-to-female ratio	4:1
Generalized lymphadenopathy	90%
Splenomegaly	60%
Hepatomegaly	30%
PB lymphocytosis	30%
BM infiltration	80%
GI involvement	20%
Waldeyer's ring involvement	10%
Ann Arbor Stage III/IV	90%
B symptoms	40%
Bulky disease	30%
Poor performance status	20%
Elevated lactate dehydrogenase	40%
Elevated β-$_2$-microglobulin	55%

The neoplastic cells of MCL appear to correspond to the naïve B cells that normally home to and reside in primary lymphoid follicles and the mantle zones of secondary follicles. As such, they correspond to a subset of normal follicular B cells that are thought to transform into germinal center cells in response to antigen. The relationship between the nodular (mantle zone) and the diffuse lymphocytic forms of MCL is biologically analogous to the germinal center cell lymphomas of follicular and diffuse types respectively, whereas the blastic forms of MCL are analogous to the transformed lymphomas arising in other low grade lymphomas.



Overall survival of patients with MCL compared with those having Working Formulation (WF) types A through E.

There is practical clinical value to knowing the level of cyclin B1 expression in mantle cell lymphomas (Uchimaru, *et al.*, 1997). Uchimaru, *et al.* measured cyclic D1 in 45 hematopoietic cell lines in 40 clinical specimens of patients with mantle cell lymphoma. In myeloid cell lines the level of cyclin D1 expression varied, but among lymphoid tumors only those of mantle cell lymphoma are increased (Link & Zutter, 1995). Small aliquots of clinical specimens could be used with a PCR assay using RT-PCR. This assay has proven to be superior to *bcl-1* analysis as *bcl-1* rearrangements are detectable easily in only 50-70% of MCL's probably because of the large target region for possible rearrangement breakpoints. However, essentially every patient with MCL has over-expression of cyclin D1.

In a recent clinical study from Vancouver, (Argatoff *et al.*, 1997) 80 cases of MCL were studies. They found increased mytotic activity blastic morphology and peripheral blood involvement at diagnosis were prognostically important factors. They utilized many of the assays described above for cyclin to group their patients and related them to patient prognosis.

Several studies have demonstrated that the V_H genes in this order are not somatically mutated as would be predicted for naïve B cells (Hummel *et al.*, 1994).

(4) Burkitt's Lymphoma

Burkitt's lymphoma is an aggressive B cell malignancy that primarily affects children. There are two major sub-groups of Burkitt's lymphoma which differ in geographic distribution. The 'high incidence' or endemic form is restricted to certain parts of equatorial Africa and the 'low incidence' or sporadic form is seen world wide. The endemic form is highly associated with Epstein Barr virus because the tumor cells consistently carry the EBV genome whereas the sporadic form has a more uneven association with EBV ranging from 10%-85% genome positivity in different areas. Recently, the incidence of sporadic Burkitt's lymphoma has shown a dramatic increase in human immunodeficiency virus infected patients and the data from Europe and North America indicate that over 50% of these tumors are EBV linked. It appears that the pre-disposing factor here is the ability of HIV to chronically stimulate the B cell system rather than acting through T cell suppression.

Burkitt's lymphoma has been called 'undifferentiated lymphoma' in the past but has been called Burkitt's lymphoma for many, many years. Burkitt's tumor cells are monomorphic medium sized cells with round nuclei and relatively abundant basophillic cytoplasm which may give the cells a "cohesive" appearance. Cytoplasmic lipid vacules are usually evident on imprints or smears. The tumor has an extremely high rate of proliferation as well as a high rate of spontaneous cell death. A "starry sky" pattern is usually present and imparted by numerous benign macrophages that have ingested apoptotic tumor cells. Burkitt's cells are virtually all surface IgM^+, $CD19^+$, 20^+, 22^+ and $79a^+$, the so called B cell associated antigens. Burkitt's lymphomas are $CD5^-$ and $CD23^-$.

BURKITT'S LYMPHOMA

- **IMMUNOLPHENOTYPE:**
  - $sIgM^+$
  - B cell associated antigens = ($CD19^+$, 20^+, 22^+, $79a^+$)
  - $CD5^-$
  - $CD23^-$
- **GENETIC FEATURES:**
  - c-myc translocations 14 t (8;14), 2 t (2;8) or 22 t (8;22)
- **CLINICAL FEATURES:**
  - One third of non-African pediatric lymphomas
  - Highly aggressive
- **NORMAL COUNTERPART:**
  - (Bm3)

Even though the International Lymphoma Study Group defined in 1994 the postulated normal counterpart of this tumor type as a "B cell of unknown differentiation stage", the correlation between Burkitt's cells and germinal center small, non-cleaved cells has been widely accepted since first proposed by Lukes and Collins. However, it is important to emphasize that this tumor preferentially grows in non-GC environments, like the jaw or the ovary.

Phenotypically, Burkitt's cells express B cell markers (CD19, CD20, CD22, CD79a), germinal center markers (CD10and CD38), and centroblast-associated antigens like CD77, resembling the normal population of Bm3 cells (Gregory et al., 1987). Additionally, they lack FM markers like CD5 and CD23. These tumors indeed behave as centroblasts by displaying an extremely high rate of proliferation and spontaneous cell death.

Molecular analyses of the Ig variable region genes reveal an extensive pattern of somatic mutation and lack of *in vivo* intraclonal expansion within Burkitt's lymphoma cells in the majority of cases studied, suggesting that the malignant transformation giving rise to this tumor occurred after and/or switched off the process of somatic mutation (Chapman et al., 1996). However, when cultured in the presence of anti-IgM antibodies and anti-CD3 activated T cells, tumor cells can be induced to undergo *in vitro* somatic mutation (Denepoux et al., 1997).

(5) Follicular Lymphoma

Follicular (nodular) lymphomas constitute the largest subgroup of non-Hodgkin lymphomas. Follicular lymphomas originate from mature B lymphocytes (centroblast/centrocyte) of germinal centers (Gerdes and Stein 1982; Freedman et al., 1992). The majority of human follicular lymphomas start as B cell tumors of low malignancy, but have a tendency to become aggressive during the later stages of the disease, usually several years after the onset of the lymphoma.

Approximately 85% of FLs are associated with the t(14;18) translocation that places the bcl-2 oncogene into juxtaposition with the joining (J_H) gene segment cluster of the Ig heavy-chain (H) gene locus. In most cases, the breakpoints on chromosome 18 are clustered either in the 3' end of the last bcl-2 exon or within the 3'-untranslated region of the gene, leaving the open reading frame (ORF) intact. The t(14;18) translocation upregulates expresion of the bcl-2 gene product that induces prolonged cell survival by blocking programmed cell death (apoptosis). In general FL cells that undergo transformation to a higher-grade NHL retain the t(14;18) translocation and acquire secondary genetic abnormalities including non-random chromosomal changes, c-myc gene rearrangement, or p53 tumor suppressor gene mutations suggesting that heterogeneous genetic lesions and different molecular mechanisms underlie this neoplastic evolution.

Intensive structural analysis of the hybrid bcl-2/IgH gene sequence in FLs showed that the translocated bcl-2 gene may undergo further alterations during the natural course of the disease. Somatic mutations of the ORF changing the structure of the p26-bcl-2α protein, major and minor deletions in the hybrid bcl-2/IgH gene sequences, or multiple different bcl-2 gene rearrangements have been reported in FL cells. To gain further insight into the secondary alterations of the translocated bcl-2 gene, the nucleotide sequence of the breakpoint and ORF regions of the bcl-2 oncogene has been analyzed in sequential biopsy specimens from patients with FL that showed no histologic alterations in subsequent biopsy specimens or underwent morphologic transformation to diffuse large-cell lymphoma (DLL). Results indicate that DLL cells may represent transformants of the preexisting FL clone, or may emerge from an unrelated B-cell clonotype through a novel and discrete bcl-2 translocation event. Most of the data also suggest that somatic mutations of the ORF of the bcl-2 gene are associated with higher-grade NHL cells.

To expand a bit on the above, the common feature of the follicular lymphomas is a 14/18 chromosome translocation, associated with about 85% of cases (Tsujimoto et al., 1985, 1986, 1987; Kneba et al., 1991). The break point in chromosome 14 is a JH immunoglobulin gene element. In the chromosome 18 there are two different stretches of DNA approximately 150-500bp in length, where the translocations are usually found. These translocation areas are 3' to the putative oncogene *bcl-2* (Tsujimoto et al., 1985, 1986). The translocation takes place at the pre-B cell stage when the D-J joining of the immunoglobulin heavy chain gene is attempted. The expression of the *bcl-2* gene is increased in these lymphoma cells, likely because the promoter of the *bcl-2* gene is under the positive effect of immunoglobulin enhancers. Bcl-2 protein is believed to play a role in the longevity of cells, presumably by preventing programmed cell death (apoptosis) (Liu et al., 1991; Hockenbery et al., 1990; Vaux et al., 1992; Merino et al., 1994).

FOLLICLE CENTER LYMPHOMA

- IMMUNOPHENOTYPE: sIg^+ $CD5^-$
- GENETIC FEATURES: t(14;18) involving rearrangement of the *bcl-2* gene in 85-95% of cases (suggests that failure to switch off BCL-2 may contribute to the development of a lymphoma
- CLINICAL FEATURES: Adult disease, widespread at diagnosis, indolent course
- POSTULATED NORMAL COUNTERPART: Germinal center B cells, both centrocytes and centroblasts (Bm3 and Bm4)

Thus, follicular lymphomas are malignancies of germinal center B cells. The process of somatic hypermutation of immunoglobulin genes, which occurs during the generation of memory B cells in germinal centers appears to be a general feature of FLs and can result in considerable intraclonal heterogeneity. Tumor cells without surface immunoglobulin are presumably generated in most FLs by mutations that result in stop codons and/or dysfunctional residues. It is therefore significant that the vast majority of FLs are surface immunoglobulin positive even after treatment with tumor-specific anti-idiotypic antibodies. Long-term retention of an idiotypic determinant has also been demonstrated in one FL that mutated its immunoglobulin genes. These observations suggest there may be selection for surface immunoglobulin expression in FLs and are consistent with the notion that antigen stimulation may play a role in the growth of these tumors.

The role of antigen selection during the clonal evolution of a FL can be studied by analyzing immunoglobulin gene mutations. Mutations appear to mostly localize to the immunoglobulin variable regions and occur randomly in the framework regions (FWRs) and complementarity-determining regions (CDRs). Antigen contact is thought to occur primarily through amino acid residues in the CDRs. As a result, these mutations can be positively selected and variable genes from antigen-selected clones often have large accumulations of replacement mutations in their CDRs. Moreover, clustering of replacement mutations in CDRs can be used as an indicator of antigenic selection. Past studies of immunoglobulin genes in FLs were potentially limited with regard to mutation analyses because the corresponding germ-line variable genes were never identified.

Heterogeneity of Ig genes in the follicular lymphoma population

Recently there has been a report where the nucleotide sequences for the heavy chain variable region (V_H) genes of a FL had been sampled on multiple occasions over a 2-year time period. The V_H segment expressed by this case was found to be a mutated version of an already reported germ-line gene termed V_H4-21 (V_H4-34). Analysis of the VH gene mutations suggested that an antigen may have been acting to stimulate the growth of the tumor and to select cells for growth that retained their immunoglobulin receptor but mutated the binding site.

Levy and colleagues found that DNA purified from some follicular lymphomas displays sequence heterogeneity in the V(D)J-regions of the immunoglobulin genes (Levy et al., 1987; Zelenetz et al., 1991, 1992). They have suggested that the heterogeneity of the immunoglobulin V regions found in follicular lymphomas is caused by the somatic hypermutation mechanism operating in the malignant B cells (Cleary et al., 1986; Levy et al., 1987; Zelenetz, et al., 1991, 1992). In a study of an autoreactive human B cell lymphoma, the heterogeneity of rearranged VD region of the IgH gene were also observed from longitudinal clinical specimens. The specimens included splenic lymphoma at diagnosis and lymph node relapses 3 and 4 years later (Friedman et al., 1991).

(6) Diffuse Large Cell Lymphoma

Diffuse large cell lymphoma is a common and aggressive subtype of B cell non-Hodgkin's lymphoma that frequently harbors genetic alterations in the BCL-6 gene: About 50% of these lymphomas contain BCL-6 translocations and 75% have mutations in a putative 5' regulationary region of the gene. Because these genetic changes invariably spare the BCL-6 coding region, the contribution of BCL-6 to lymphomagenesis is likely to be a subversion of its role in nontransformed cells. Consistent with this possibility, BCL-6 protein is expressed at the highest levels in germinal center B lymphocytes, which are the cells from which diffuse large cell lymphomas may arise. BCL-6 is a potent transcriptional repressor, but its natural target genes have not been identified.

DIFFUSE LARGE CELL LYMPHOMA

- **IMMUNOPHENOTYPE:** B cell associated antigens + ($CD19^+$, $CD20^+$, $CD22^+$, $CD79a^+$)
- **GENETIC FEATURES:** *bcl*-2 gene rearranged in ~50%
- **CLINICAL FEATURES:** 30% -40% of adult NHLs
  Usually a single node
  Agressive, but potentially curable
- **POSTULATED NORMAL COUNTERPART:** Germinal center B cells, Bm 3 or Bm 4

Large B-cell lymphomas constitute 30% to 40% of adult NHLs; the median age is in the sixth decade, but the range is broad, and these tumors may be seen in children. Patients typically present with a rapidly enlarging, often symptomatic mass at a single nodal or extranodal site; up to 40% are extranodal. Large cell lymphomas are aggressive but potentially curable with aggressive therapy. Although several studies have reported a slightly worse prognosis for immunoblastic than large follicular center cell types, other studies have failed to confirm this. Cases of multilobated B-cell type are often extranodal.

The malignant cell that proliferates in cases of DLCL resembles morphologically a centroblast (Bm3) or an immunoblast, and most of the time the tumor sample contains a mixture of these two cell types. Additionally, normal non-B cells (T lymphocytes and histiocytes) may accumulate within the tumor. Malignant cells express sIg, mainly of the IgM isotype, B cell markers like CD19, CD20, CD22, CD79a, and various degrees of CD45, CD5 and CD10. Available studies of the surface phenotype of these tumors does not allow, therefore, their unequivocal assignment to a normal B cell counterpart.

The main genetic alteration associated with DLCL is the translocation and/or mutation within the promoter arear of the gene encoding the transcriptional repressor BCL-6. Under normal circumstances, the BCL-6 gene product is exclusively expressed within germinal center B cells (both centroblasts and centrocytes), and mice with a disrupted BCL-6 gene fail to develop germinal centers, suggesting that the BCL-6 protein plays an important role at this stage of B cell differentiation. Although there is no formal evidence to support a direct role of the genetic alteration of the BCL-6 repression activity could participate in the malignant transformation of cells in which this gene is usually functional, the germinal center centroblast and centrocyte.

Additional evidence to support the notion of a CG origin of DLCL derives from the analysis of Ig variable region genes transcripts within these tumors, which reveals the unequivocal presence of somatic mutation without intraclonal variation (Hsu and Levy, 1995). Where in the process of differentiation of the DLCL normal counterpart sits compared to the two previously described GC-derived lymphomas (Burkitt's and Follicle Center Lymphomas), deserves further study.

(7) Multiple Myeloma

Multiple myeloma represents a malignant proliferation of plasma cells. The terms multiple myeloma and myeloma are used interchangeably. The disease results from the uncontrolled proliferation of plasma cells derived from a single clone. The tumor, its products and the host response to it result in a number of organ dysfunctions and symptoms of bone pain or fracture, renal failure, susceptibility to infection, anemia and often manifestations of hyperviscosity. There are approximately 1,000 new cases of myeloma in the United States every year. It is primarily a disease of the elderly and its incidence increases with age. The median age at diagnosis is about 65.

Some basic observations of plasma cell development.

First, let us recall that plasma cells originate from antigen specific B cells after a number of developmental steps within different microenvironments. We have reviewed this process and appreciate that all plasma cells have effectively passed through a germinal center reaction and participated in T cell dependent secondary responses within the germinal centers where follicular dendritic cells and T cells are present. There is considerable evidence that normal plasma cells are heterogeneous and the relevance of that to an understanding of the origin multiple myeloma is crucial. Plasma cells generated in macrophage rich areas after T cell

independent antigen selection, secrete IgM and are short lived. A primary T cell dependent response in the extra-follicular areas also gives rise to short lived plasma cells.

The properties of multiple myeloma plasma cells and of the monoclonal immunoglobulins they produce are somewhat different. Multiple myeloma paraproteins may be directed against a wide variety of infectious agents suggesting that the development of myeloma may be caused by antigen stimulation. Next the isotype of multiple myeloma plasma cell is generally IgG or IgA demonstrating that the predominant phenotype of multiple myeloma tumor cells is that of a post-switch B cell. Also, the clonal proliferation involves a cell population that has already passed through the stage of somatic hypermutation of immunoglobulin genes. As the process of Ig gene somatic hypermutation occurs in the germinal centers of secondary follicles following antigen stimulation and is coupled to the isotype switch, this means that peripheral lymphoid organ germinal centers have a central role in the development of myeloma. Thus, the observation that myeloma is a neoplasm of plasma cells that have a post-switch phenotype, shows somatic mutation, and may produce monoclonal immunoglobulin with targeted antibody activity might suggest that the evolution of myeloma is in the antigen triggered process. However, the specific causal antigen is unknown.

Contrasting with the distribution of normal plasma cells, multiple myeloma plasma cells localize uniquely in the bone marrow. Even if the lamina propria of the intestine contains more immunoglobulin producing cells than all other tissues in the body, it is never a site where multiple myeloma develops – not even IgA producing myeloma. Likewise, the involvement of the spleen and lymph nodes which is typical of Waldenström's macroglobulinemia is very unusual in multiple myeloma.

The information available on the B cell population that feeds the downstream compartment of plasma cells and disseminates the disease indicates that this population has been generated in peripheral lymphoid organs during secondary T cell dependent antibody responses. These plasma cells are programmed to home to the bone marrow and are committed to differentiate in close association with the bone marrow microenvironment. Thus, the existing data suggest that the most likely candidate for the physiological B lymphocyte equivalent of the multiple myeloma plasma cell precursor is either an activated B memory cell or a plasmablast.

The most important take home message of our newer information concerning these cells is that they do not arise in the bone marrow but they home to the bone marrow.

PLASMA CELL MYELOMA

- **IMMUNOPHENOTYPE:** sIg$^-$ cIg$^+$ CD19$^-$ CD20$^-$ CD22$^-$ CD38$^+$
- **GENETIC FEATURES:** IgH and L chains rearranged or deleted
- **POSTULATED NORMAL COUNTERPART:** Plasma Cell

VI. MINIMAL RESIDUAL DISEASE: MOLECULAR APPROACHES

It is well known that at diagnosis patients with acute leukemia may have a total of 10^{12} malignant cells. Historically, a "complete" remission has long been based on a morphologic assessment of a bone marrow smear that demonstrates less than 5% neoplastic cells. However, these patients may still have as many as 10^{10} neoplastic cells in their body. Over the years, there have developed a number of techniques have been developed to detect tumor cells.

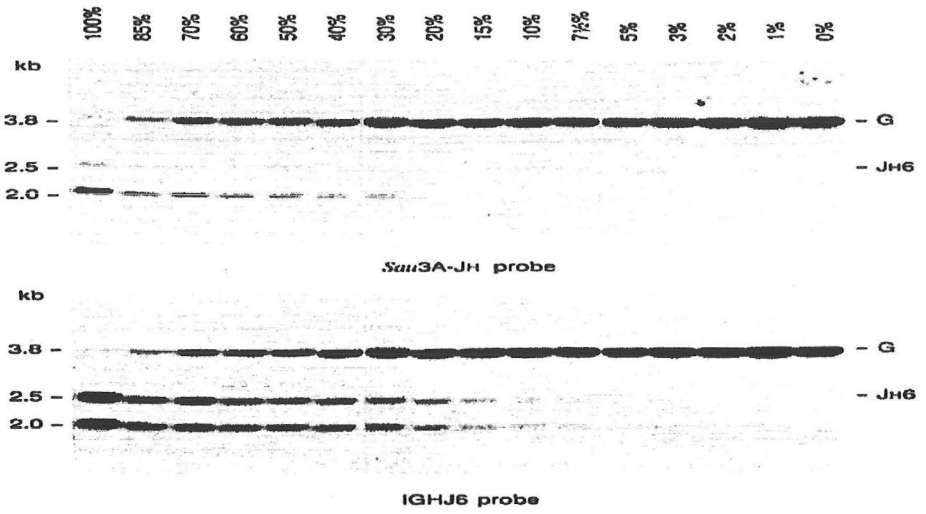
Tumor cell culture assays were introduced some years ago. Although several clinical studies have been reported, the specificity and sensitivity of *in vitro* culture techniques is still not established and positive results have not shown a strong correlation with relapse (Sievers and Lohen, 1995). Cytogenetics has a high false/negative rate which limits this assay for a minimal residual disease analysis.

Molecular biological approaches have been used with considerable success in this arena. Standard southern blot analysis for rearranged T and B cell receptor genes and for bcl-1 and bcl-2 translocations and for bcr/abl translocations typically demonstrates sensitivities on the order of 5%. This is especially good in follicular lymphomas (85% are positive for bcl-1 or 2) mantle cell lymphoma (50% are positive for bcl-1) and b-all (over 90% of which are positive for IgH rearrangements). There are problems with this technique in that it is still fairly low sensitivity with high numbers of false negatives. It is also a time consuming technique requiring 10 to 14 day turn around and it is fairly expensive.

TABLE VI.
Genotypic Abnormalities That Can Be Used as Targets for Molecular Detection of MRD

		Frequency (%)*	
Chromosomal Abnormality	Molecular Target	Adults	Children
t(9;22)(q34;q11)	BCR-ABL (RNA)	25-40	4-6
t(1;19)(q23;p13.3)	E2A-PBX1 (RNA)	2-3	5-6
t(4;11)(q21;q23)	MLL-AF4 (RNA)	5	2
t(5;14)(q31;q32)	IL3-IGH (DNA)	<1	<1
t(11;19)(q23;p13)	MLL-ENL (RNA)	<1	<1
t(9;11)(p21-22;q23)	MLL-AF9 (RNA)	<1	<1
t(1;19)(q22;p13)	E2A-HLF (RNA)	<1	<1
t(8;14)(q24;q32.3)	MYC-IgH (DNA)	4-5	1-2

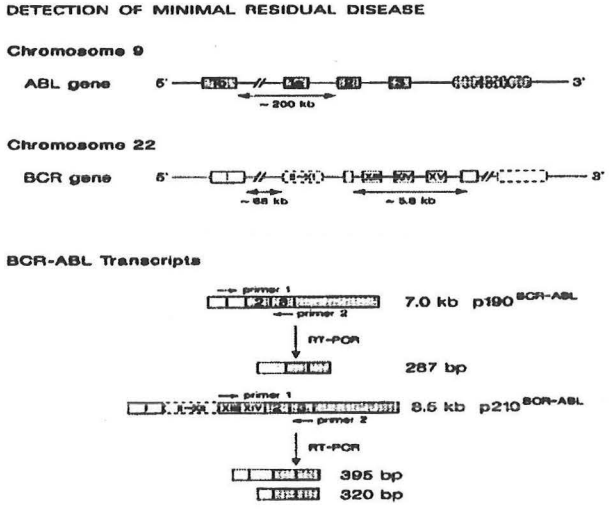
*The frequency within B-lineage tumors
Adapted from Campana and Pui, et al., ***Blood*** **85**:1416-1434, 1995.



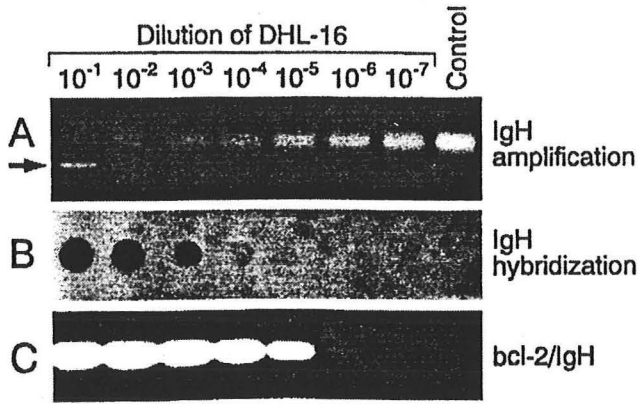
Dilution experiment in which B-CLL cells with two rearranged IgH alleles were diluted in PB-MNC from a healthy individual. The *Bgl*II filter was successively hybridized with the *Sau*3A-J_H probe (upper panel) and IGHJ6 probe (lower panel). In this dilution experiment the two IgH gene rearrangements of the B-CLL were still detectable in the 5% dilution mixture upon hybridization with the IGHJ6 probe. Upon hybridization with the *Sau3*A-JH probe one rearranged IgH gene band was visible down to the 10% dilution mixture, but the other rearranged band (representing a J_{H5} gene rearrangement) was only visible down to the 40-50% dilution mixture (*Adapted from Beishuizen et al., 1993*).

PCR

Currently PCR is the most sensitive technique for detecting one abnormal cell and 10^3-10^6 normal cells. An example of how this works is shown below:



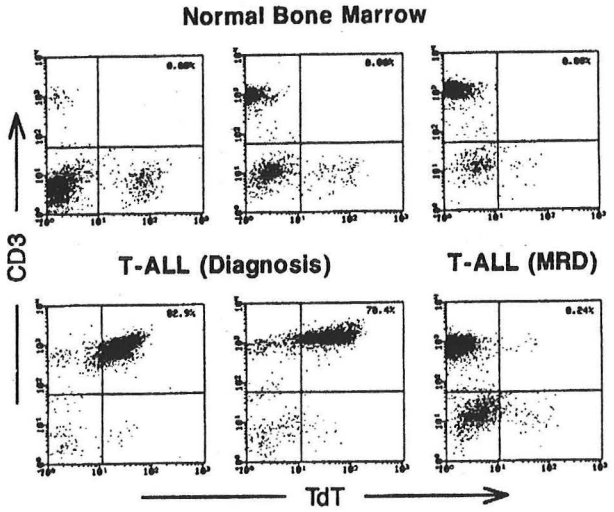
BCR/ABL recombination in Ph^+ leukemias. The breakpoints regions are indicated by double pointed arrows. The breakpoints in the majority of ALL cases map to the minor breakpoint cluster region located within the BCR first intron of approximately 68 kb. This produces a transcript of 7.0 kb encoding a protein of 190 kD. In most cases of CML, the breakpoints map to the major breakpoint cluster region of 5.8 kb, located downstream from minor breakpoint cluster region. This rearrangement produces 8.5-kb transcripts encoding a protein of 210 kD. Single-pointed horizontal arrows indicate the position of the primers used for RT-PCR (*Adapted from Campana and Pui, 1995*).



PCR amplification of serial dilutions of the DHL-16 cell line in normal PB mononuclear cells. (A) Ethidium bromide stained agarose gel electrophoresis of PCR product obtained using the FR3 consensus primers. (B) Dot blot hybridization of PCR product probed with the lymphoma specific oligonucleotide probe. (C) Ethidium bromide stained agarose gel electrophoresis of PCR product obtained using nested PCR amplification of the bcl-2/IgH mcr rearrangement. *Adapted from Zwicky et al., 1996.*

PCR is based on the knowledge that specific DNA sequences only exist in tumor circumstances (translocations, for example, or are clonally related to specific antibody variable region genes. These provide critical information that can be used to assess minimal residual disease in a highly sensitive way and over and over it has been demonstrated that malignant cells while are present long after the sensation of standard chemotherapy.

One critical issue relates to whether such analyses can be used to "push" chemotherapy even harder in order to demonstrate a complete loss of all malignant cells and here the evidence is not absolutely solid. The bulk of the data would suggest that patients with fewer malignant cells have fewer relapses and that when cells are modest in number additional chemotherapy is warranted. What is surprising is that even patients well into relapse still have levels or residual leukemia cell DNA. And in one study (Roberts, et al., 1997) despite continued remission in 17 patients with acute lymphoblastic leukemia evidence of residual leukemia was detected by PCR in 15 of the 17. This suggest that irradiation of all leukemia cells may not be a prerequisite for cure.



Detection of MRD by flow cytometry. BM mononucleated cells were permeabilied and labeled with antibodies to membrane plus cytoplasmic CD3 (y-axis) and nuclear TdT (x-axis). Immunophenotype was studied selectively on cells with lymphoid morphology. In 3 normal BM samples (top panels) less than 1:10,000 cells co-express TdT and CD3. By contrast, in 2 cases of T-ALL at diagnosis (bottom left and middle panels), most cells are $TdT^+/CD3^+$. In 1 patient with T-ALL in morphologic remission, 0.24% of lymphoid cells express TdT and CD3 simultaneously (bottom right panel), indicating residual disease. *From Campana and Pui, 1995.*

It has been expected that the advent of clonotypic polymerase-chain-reaction (PCR) methods for tracking small numbers of leukemic cells would make it possible to monitor the efficacy of therapy, identify residual or reemerging disease, and hence anticipate clinical relapses as a guide to management. Several studies have demonstrated persistence of the leukemic clone for 12 to 18 months after diagnosis, thereby providing a rationale for maintenance therapy. Others have shown an impressive correlation between the rapidity or extent to the reduction in the number of leukemic cells after induction therapy and the subsequent outcome or between persistently positive results on PCR testing or the presence of minimal residual disease and subsequent relapse. The addition of quantification of the PCR test with clonotype-specific primers derived from rearranged immunoglobulin or T-cell receptor genes or leukemia-related fusion genes promises to improve the accuracy and value of molecular monitoring. Current large-scale studies in Europe and the United States are attempting to determine how to optimize, simplify, and standardize PCR-based predictive screening.

Potential Sources of Error in Detection of MRD

	Type of Error	
Method	**False Negative Result**	**False Positive Result**
All	Limited number of cells available for study; heterogeneous distribution of leukemia	
In situ hybridization	Poor hybridization signals	Natural occurrence of aneuploidy in normal cells; artifactual colocalization of probes
PCR performed on antigen-receptor gene rearrangements	Degraded DNA; clonal change in rearrangement; oligoclonality	Cross-hybridization with similar sequences derived from normal lymphoid cells
Colony assays	Phenotypic switch	Antibody cross-reactivity; normal cells expressing leukemia-associated phenotypes

Adapted from Campana and Pui, et al., *Blood* 85;1416-1434, 1995.

Table VIII. Relationship of MRD Detection by PCR Amplification of IgH or TCR Gene Rearrangements to Clinical Outcome of Therapy

Authors	MRD Detected	→ Relapse*	No MRD	→ Relapse*
Yamada et al	3	0	2	1
Yokota et al	5	0	3	0
Biondi et al	3	3	13	3
Nizet et al	15	6	2	0
Neale et al	2	2	4	0
Potter et al	4	4	5	0
Deane et al	5	3	3	1
Ito et al	2	1	22	8
Kitchingman	5	5	6	2
Cave et al	5	3	15	3
Total	49	27 (55.1%)	75	18 (24.0%)

Samples included are from patients in morphologic remission studied more than 6 months from diagnosis. All studies had more than 6 months follow-up (if no relapse occurred). *Either hematologic or extramedullary relapse. Adapted from Campana and Pui *Blood* 85:1416-1434, 1995.

VII. A REVISED 'REAL' CLASSIFICATION

About A year ago, Hiddemann, et al., presented a revision of the 'REAL' classification that we have gone through today (Hiddemann et al., 1996). Recall that one of the hallmarks of the REAL classification was that the clinical elements of the tumors were not considered at all. After two years of use, there has been some rethinking of this issue and particularly those closer to the bedside have argued strongly that the clinically useful aspects of some of the previous classifications schemes had been lost, particularly those that allow one to estimate prognostic relevance of the diagnostic system. As such, there is some criticism that the REAL proposal lacks the translation into clinical groupings and hence deserves a further interpretation to facilitate its acceptance and clinical applicability. In this way, the tumors have been categorized some what differently and this shown in the last table of these Grand Rounds.

The important element that is added to this classification is the clinical grouping schema. That is, (1) indolent (low risk lymphomas), (2) aggressive lymphomas (intermediate risk) and, (3) very aggressive lymphomas (high risk). Hodgkin's disease is listed independently and is now grouped as a B cell malignancy. I had hoped to have time to review this but shall not.

Some common features are shared by a variety of different lymphomas that allowed them to be grouped into the designated categories. As indolent lymphomas are mostly diagnosed at advanced stages 3 and 4 are characterized by a slowly progressive clinical course during which spontaneous regression may be observed, sensitivity to chemotherapy is good and final disease eradication is rarely achievable by our current available methods. Aggressive lymphomas, on the other hand, have a more rapid clinical course. A moderate to good response to chemotherapy and a considerable proportion of patients experience long term disease free and overall survival after treatment. The very aggressive lymphomas show even more rapidly expanding growth characteristics and usually constitute a life threatening situation to the patient. In most of these circumstances effective therapy is however available and can be applied with a significant chance for cure.

All classifications of lymphomas are obviously subject to improvement. What today's Grand Rounds have attempted to do is bring the most recent information on normal B lymphocyte development to bear on this classification and to show how new molecular technologies are available to help in the diagnosis. In most instances these diagnostic tools provide important information to the clinician, either in determining prognosis, or most importantly, in providing clinical approaches to the management of these devastating and, unfortunately, rather common human neoplasms.

A REVISED REAL CLASSIFICATION OF B CELL NEOPLASMS

I. Indolent lymphomas (low risk)

- Chronic lymphocytic leukemia
- Lymphoplasmacytic lymphoma/immunocytoma/Waldenstrom's macroglobulinemia
- Hairy cell leukemia
- Splenic marginal zone lymphoma
- Marginal zone B-cell lymphoma
  - Extranodal (MALT-B-cell lymphoma)
  - Nodal (monocytoid)
- Follicle center lymphoma/follicular, (small cell)-grade I
- Follicle center lymphoma/follicular, (mixed small and large cell)-grade II

II. Aggressive lymphomas (intermediate risk)

- Prolymphocytic leukemia
- Plasmacytoma/multiple myeloma
- Mantle cell lymphoma
- Follicle center lymphoma/follicular, (large cell)-grade III
- Diffuse large B-cell lymphoma

III. Very aggressive lymphomas (high risk)

- Precursor B-lymphoblastic lymphoma/leukemia
- Burkitt's lymphoma/B-cell acute leukemia
- Plasma cell leukemia

Proposed clinical schema for malignancies of the B lymphoid system.

PHENOTYPIC MARKERS LINKING NORMAL B CELL SUBSETS AND B CELL NHLs

	Function	Mature B cell expression	Lymphoid tissue expression	B cell NHL expression
CD5	(-) regulation of antigen receptor-induced growth signals	Subset of normal B cells	Follicular mantle	CLL MCL
CD23	CD21 ligand Low affinity IgE receptor	Bm2	Follicular mantle	CLL
CD38	Signal transduction Cell adhesion	Centroblast (Bm3) Centrocyte (Bm4) Plasma cells	Germinal center Plasma cell rich areas	Burkitt's FCL DLCL?
CD77	Globotriaosylceramide Unknown function	Centroblast (Bm3)	Dark zone of germinal center	Burkitt's FCL? DLCL?

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