

# Haematologica

## Journal of Hematology

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2. Burgess AW, Begley CG, Johnson GR, et al. Purification and properties of bacterially synthesized human granulocyte-macrophage colony stimulating factor. *Blood* 1987; 69:43-51.
3. The Royal Marsden Hospital Bone-Marrow Transplantation Team. Failure of syngeneic bone-marrow graft without pre-conditioning in post-hepatitis marrow aplasia. *Lancet* 1977; 2:242-4.
4. Anonymous. Red cell aplasia [editorial]. *Lancet* 1982; 1:546-7.
5. Karlsson S, Humphries RK, Gluzman Y, Nienhuis AW. Transfer of genes into hemopoietic cells using recombinant DNA viruses [abstract]. *Blood* 1984; 64(Suppl 1):58a.

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6. Ferrata A, Storti E. *Le malattie del sangue*. 2nd ed. Milano: Vallardi; 1958.
7. Hillman RS, Finch CA. *Red cell manual*. 5th ed. Philadelphia: FA Davis; 1985.
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9. DuPont B. Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. *Proceedings of the third annual meeting of the International Society for Experimental Hematology*. Houston: International Society for Experimental Hematology; 1974. p. 44-6.
10. Bieber MM, Kaplan HS. T-cell inhibitor in the sera of untreated patients with Hodgkin's disease [abstract]. Paper presented at the International Conference on Malignant Lymphoma Current Status and Prospects, Lugano, 1981:15.
11. Worwood M. Serum ferritin. In: Cook JD, ed. *Iron*. New York: Churchill Livingstone; 1980. p. 59-89. (Chanarin I, Beutler E, Brown EB, Jacobs A, eds. *Methods in hematology*; vol 1).
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**Indolent  
Lymphoma**  
Workshop

**Bologna, Italy**

**October 18-19, 1999**

***Guest Editors: Sante Tura, Pier Luigi Zinzani***

**BIANCA**

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## Foreword

The therapy of aggressive non-Hodgkin's lymphoma was somewhat standardized a few years ago and has allowed physicians to obtain encouraging results. Researchers are currently investigating the timing of some treatment options (e.g. ABMT used either as a front-line, a post-induction or a post-relapse choice) and the possible role of new drugs administered either alone or in combination regimens.

Indolent lymphoma has been dormant for a long time. Once upon a time wait-and-see used to be a legitimate choice and achieved excellent survival rates shared in by several institutions. Most researchers have felt for a long time that conventional chemotherapy cannot modify the survival of patients with advanced-stage indolent lymphoma.

Recently, however, we have reached a turning point. The progressive development of high-dose chemotherapy treatments followed by rescue with stem cells in young patients has led us to assess their therapeutic efficacy. We have felt ourselves compelled to switch from a cautious and somewhat passive approach to an aggressive one.

Molecular biology laboratories have been developing useful tools for identifying and monitoring minimal residual disease, allowing them to ultimately allowing clinicians to evaluate the real impact of complete remission quality on survival. The daily acquisition of new biological data influencing both clinical and therapeutic decision-making processes, the recent development of monoclonal antibodies targeting surface antigens expressed by most if not all indolent lymphomas, and the constant improvement of the results obtained with both conventional and high-dose chemotherapy, all justify the need for this international scientific workshop on indolent lymphoma.

It is a time of great fervor and expectations. It is a time of conspicuous increase of understanding, focused mainly on biology and therapy of this intriguing subset of lymphoma. All the more reason, therefore, for establishing what is certain and what is not, what can be immediately, or shortly, applicable to the daily routine of the hospital and what still needs for confirmation.

These are the reasons for which this workshop has been organized.

We feel indebted with all participants and particularly to all the contributors of this issue, to those who submitted the papers published herein, because we are certain that the careful reading of this supplement issue will be of great help to researchers and physicians – and of course their patients – by focusing our current knowledge of and future potential strategies for indolent lymphoma.

*Sante Tura  
Pier Luigi Zinzani*

**BIANCA**

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is a Latin adjective, neuter and plural,  
used in this context as a noun:  
it means “hematologic subjects”.

The appropriate English translation is therefore

**Journal of Hematology.**

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## Histopathology of indolent B-cell lymphomas

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Since the 1970s, several very different classifications of malignant lymphomas have been used around the world. The resulting lack of uniform diagnostic criteria for lymphoid tumors has given rise to considerable problems for both pathologists and clinicians, and has seriously hampered comparison of studies reported in the literature. In theory, as with all other tumor types, lymphomas should be classified on the basis of their supposed histogenesis in order to provide maximum information on their biology, natural history and response to therapy. In practice, however, since our knowledge of the immune system is still insufficient for this approach to be applied in all cases, a *biologically correct* lymphoma classification is not currently feasible. Despite this, many hematopathologists agree that by pooling morphologic, immunophenotypic and molecular findings it is possible to enumerate a large series of distinct entities that can be recognized and diagnosed in routine practice. In 1994, on the basis of the work of the *International Lymphoma Study Group*, a list of "real", clearly characterized anatomo-clinical entities that can be readily recognized with currently available techniques was published as a proposal for an up-to-date practical classification (*Revised European American Lymphoma Classification: REALC*) of malignant lymphomas (Table 1).<sup>17</sup>

### **Histologic grade and clinical aggressiveness**

Before the advent of immunophenotyping and the molecular biology techniques that have allowed the identification of many lymphoid neoplasms as separate entities, it was thought that non-Hodgkin's lymphoma constituted a single generic disease with various degrees of aggressiveness that could be revealed on the basis of morphology and clinical findings. This concept encouraged the conviction that it should be possible to devise a single grading system capable of predicting the clinical course of the disease. This was the principle behind the Working Formulation, in which lymphoid tumors were divided into three prognostic groups (indicated by grades of clinical malignancy) on the basis of the sur-

vival of the patients recruited in the original study. Nevertheless, each of these wide-ranging categories is actually known to contain a large number of conditions that differ greatly as regards their etiology, presentation, natural history, epidemiology and response to treatment. Moreover, each single variety of lymphoma displays its own spectrum of degrees of morphologic and clinical aggressiveness. As a result, it no longer appears possible to categorize lymphoid tumors on the basis of a generic grading system that would be tantamount to considering as a single entity different types of lung cancer, such as the carcinoid, squamous cell carcinoma, adenocarcinoma and small cell carcinoma. Nor can the degree of malignancy of a lymphoma be realistically determined on the basis of cell size, as was envisaged by the Updated Kiel Classification (UKC).<sup>11</sup> Indeed, this principle would lead to mantle cell lymphomas and anaplastic large cell lymphomas (ALCL) being interpreted, respectively, as low- and high-grade forms, in exact contrast to the findings of a validation study on REALC promoted by the U.S. National Cancer Institute in March 1994, which demonstrated that mantle cell lymphomas are associated with a 5-year survival rate of less than 30%, while that of ALCL is about 80%.<sup>22</sup>

### **Clinical categorization of non-Hodgkin's lymphomas**

The large series of different entities that can be distinguished on morphologic, immunophenotypic and biological grounds and that are generally included under the umbrella term *non-Hodgkin's lymphoma* can be ordered on the basis of various principles, including their supposed normal counterpart within the immune system, their morphologic appearance and their clinical characteristics. For the practising oncologist, the most rational criterion is their predictable behavior. Thus, patients with lymphoid tumors can be divided into different main groups on the basis of the characteristics of the process at the time of presentation and their life expectancy, as proposed by Dan Longo *et al.*<sup>13,21</sup>

### **Indolent lymphomas**

Indolent lymphomas are considered to be those associated with a survival measurable in years, independently of whether or not any therapy is applied.

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**Table 1. Revised European-American Lymphoma classification.****B-cell neoplasms**

- I. Precursor B-cell neoplasms: precursor B-lymphoblastic leukemia/lymphoma
  - II. Peripheral B-cell neoplasms
    1. B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma
    2. Lymphoplasmacytoid lymphoma/immunocytoma
    3. Mantle cell lymphoma
    4. Follicle center lymphoma, follicular  
Provisional cytologic grades: I (small cell), II (mixed small and large cells), III (large cells)  
Provisional subtype: diffuse, predominantly small cell type
    5. Marginal zone lymphoma  
Extranodal (MALT type +/- monocytoid B cells)  
Provisional subtype: nodal (+/- monocytoid B cells)
    6. Provisional entity: splenic marginal zone lymphoma (+/- villous lymphocytes)
    7. Hairy cell leukemia
    8. Plasmacytoma/plasma cell myeloma
    9. Diffuse large B-cell lymphoma\*
- Subtype: primary mediastinal (thymic) B-cell lymphoma
10. Burkitt's lymphoma
  11. Provisional entity: high-grade B-cell lymphoma, Burkitt-like\*

**T-cell and putative NK-cell neoplasms**

- I. Precursor T-cell neoplasm: precursor T-lymphoblastic leukemia/lymphoma
- II. Peripheral T-cell and NK-cell neoplasms
  1. T-cell chronic lymphocytic leukemia/prolymphocytic leukemia
  2. Large granular lymphocyte leukemia  
T-cell type  
NK-cell type
  3. Mycosis fungoides/Sézary syndrome
  4. Peripheral T-cell lymphomas, unspecified\*  
Provisional cytologic categories: medium-sized cell, mixed medium and large cell, large cell, lymphoepithelioid  
Provisional subtype: hepatosplenic  $\gamma\delta$  T-cell lymphoma  
Provisional subtype: subcutaneous panniculitis T-cell lymphoma
  5. Angioimmunoblastic T-cell lymphoma (AILD)
  6. Angiocentric lymphoma
  7. Intestinal T-cell lymphoma (+/- enteropathy associated)
  8. Adult T-cell lymphoma/leukemia (ATL/L)
  9. Anaplastic large cell lymphoma (ALCL), CD30 +, T- and Null-cell types
  10. Provisional entity: anaplastic large cell lymphoma, Hodgkin's-like

**Hodgkin's disease**

- I. Lymphocyte predominance
- II. Nodular sclerosis
- III. Mixed cellularity
- IV. Lymphocyte depletion
- V. Provisional entity: lymphocyte-rich classical HD

\*These categories are thought likely to include more than one disease entity.

These lymphoproliferative disorders have very variable clinical presentations. Some are constantly systemic diseases, often with leukemic manifestations. Others have an extranodal primary presentation and can remain localized for long periods, even in the absence of any therapy. Yet others correspond to tumors with nodal presentation, which can have widespread immune system involvement at the time of diagnosis. This leads to the basic distinction of three fundamental subtypes of indolent lymphoma: disseminated

**Table 2. List of indolent B-cell lymphomas.**

1. Disseminated lymphomas/leukemias  
B-cell chronic lymphocytic leukemia  
Lymphoplasmacytic lymphoma/immunocytoma  
Hairy cell leukemia  
Splenic marginal zone B-cell lymphoma  
Plasmacytoma/multiple myeloma
2. Extranodal lymphomas  
Marginal zone B-cell lymphoma of mucosa associated lymphoid tissue (MALT) type
3. Nodal lymphomas  
Small lymphocytic lymphoma  
Follicle center cell lymphoma  
Marginal zone B-cell lymphoma  
Lymphocyte predominance Hodgkin's disease

leukemias/lymphomas, extranodal forms and nodal ones. On clinical grounds, indolent lymphomas display *low-grade* histologic characteristics with a strong prevalence of small cells and a blastic ratio of less than 25% of the total population. Furthermore, the number of mitotic figures is low, according to the criteria of the UKC,<sup>11</sup> and constantly less than 5 mitoses per high-power field. A common feature of many different histologic types of indolent lymphoma is the tendency to undergo histologic transformation into a high grade form, with a corresponding acceleration of the clinical course. In the following the morphologic characteristics of B-cell indolent lymphomas will be described; the subclassification and immunophenotypic profiles of these lymphomas are reported in Tables 2 and 3, respectively.

**B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL)**

Morphologically, B-CLL/SLL is mainly constituted by small lymphoid elements, with the contemporary presence of some prolymphocytes and paraimmunoblasts, which in some areas can be numerous enough to give rise to pseudofollicles. The REALC underlines the diagnostic importance of the prolymphocyte and paraimmunoblast components that are usually arranged in pseudofollicles. Indeed, in the presence of inadequate fixation, when small lymphocytes can assume a cleaved nuclear profile, the presence of prolymphocytes and paraimmunoblasts can allow definite recognition of the process.

Furthermore, in the REALC, the B-CLL/SLL category includes the rarer forms of B prolymphocytic lymphoma (B-PLL): this decision is based on the observation that over time B-CLL tends to enrich itself in prolymphocytic forms (the so-called prolymphocytoid crisis), in such a way that – even when FAB criteria<sup>8</sup> are applied – the distinction between the two leukemic forms can become quite arbitrary.<sup>5,7</sup> B-CLL and B-PLL probably represent the two extremes of a single disease, provided with different degrees of aggressiveness. Finally, it should be noted that in the

Table 3. Phenotypic profile of indolent B-cell lymphomas.

Markers	CD79a	CD20	CD10	CD5°	CD23	CD68/KP-1	Slg	Clg	CD30	CD15	EMA	bcl-1	bcl-2	bcl-6
<i>Histotype</i>														
B-CLL	+	+	-	+	+	-	++	-/+	-	-	-	-	+	-
LP	+	+	-	-	-/+	e	+	+	e	-	-	-	+	-
FCL	+	+	+	-	-	-	+	-	e	-	-	-	+#	+
MZL	+	+	-	-	-	+/-	+	+/-	-	-	-	-	+W	-
HCL	+	+	-	-	-	+	+	-	-	-	-	-	+	-
MM	+/-	-	-	-	-	-	-	+	+/-	-	+/-	-	-/+W	-
HD-LP	+	+	+	-	-	-	+	-	e	-	+	-	+	+

Abbreviations. B-CLL: B-cell chronic lymphocytic leukemia. LP: Lymphoplasmacytic lymphoma/Immunocytoma. FCL: Follicle center cell lymphoma. MZL: Marginal zone lymphoma. HCL: Hairy cell leukemia. MM: multiple myeloma/plasmacytoma. HD-LP: Hodgkin's disease lymphocyte predominance. \*weak; °detectable by highly sensitive procedures on routine sections from briefly fixed samples; #strong.

REALC, the forms listed in the UKC as *lymphoplasmacytoid immunocytomas* are included in the B-CLL/SLL category owing to the absence of any significant clinical, prognostic morphologic or phenotypic differences that could justify a clear-cut distinction from B-CLL.<sup>10</sup>

#### **Lymphoplasmacytic lymphoma/immunocytoma**

This has practically become a category by way of exclusion. In fact, it comprises neoplasms that do not display characteristics that would allow their inclusion among the small B-cell, mantle cell, centrofollicular or marginal zone forms. The lymphoplasmacytic lymphoma of the REALC actually corresponds to the *lymphoplasmacytic immunocytoma* of the UKC, or in other words to the original description of Waldenström's disease:<sup>1</sup> it is made up of lymphoid elements that range from small lymphocytes (CD5<sup>-</sup>) to mature plasma cells by way of lymphoplasmacytoid forms.

#### **Hairy cell leukemia**

Hairy cell leukemia is morphologically made up of small-medium sized B-cells, with variably shaped, round, oval or cleaved nuclei with a fairly wide cytoplasmic rim provided with the characteristic villous projections that give the form its name. The population is generally confined to the peripheral blood, bone marrow and the red pulp of the spleen, while the lymph nodes are only very rarely involved. In addition to typical B-cell antigens, the cells express the receptor for interleukin 2 (CD25) and the CD103 integrin (a cell-adhesion molecule). In paraffin sections, as well as being positive for pan-B markers such as CD20 and CD79a, the neoplastic cells often express the molecule CD68/KP-1 and are labeled by antibody DBA.44.

Hairy cell leukemia is one of the tumors in which it is of fundamental importance to monitor minimal residual disease (MRD) following therapy: this goal can be easily achieved by the cheap immunohistochemical assay in paraffin sections. Indeed, patients treated with the more recent approaches such as interferons, 2-chloro-deoxyadenosine or deoxy-

cyformycin have been shown to retain isolated residual hairy cells trapped within hyperplastic or fibrotic marrow, the recognition of which is difficult or even impossible at pure morphologic evaluation.

#### **Plasmacytoma/plasma cell myeloma**

Plasma cells form the neoplastic elements of myeloma or plasmacytoma. The former term is used when the neoplasm is found in the bone marrow, causing skeletal destruction, while the latter is employed for the rarer tumors arising at extramedullary sites. Full histopathologic evaluation must take account of three important prognostic factors:

- grade* – evaluated on the basis of the cytologic characteristics of the plasma cells, this includes the small-cell and Marschalkò low-grade forms, the cleaved, asynchronous and polymorphous intermediate ones, and the high-grade blastic variety;
- growth pattern* – this can be interstitial, nodular or diffuse, the last having the worst prognosis;
- stage* – this is assessed on the basis of the ratio between the total bone-marrow cell population and the neoplastic component.

As well as confirming the neoplastic nature of the plasma cell population under study and allowing its precise quantification, immunohistochemical characterization of plasmacytomas (monotypic expression of  $\kappa$  or  $\lambda$  immunoglobulin light chains) is indispensable for the search for MRD in the bone marrow following therapy.

#### **Extranodal marginal zone lymphoma**

This is a new category, which has been introduced by the REALC. Histogenetically, the tumor can be traced to elements of the marginal zone surrounding the mantles of normal follicles, which is scarcely perceptible in the lymph nodes and clearly evident in Malpighian bodies in the spleen. B-cell lymphomas of the marginal zone can be divided into three categories: extranodal, nodal and splenic.

The extranodal forms correspond to the mucosa-associated lymphoid tissue (MALT) lymphomas described in the early eighties by Isaacson.<sup>3</sup> These are

primary B-cell lymphomas most frequently found in the intestine, stomach, skin, lung, respiratory airway, salivary gland and thyroid.<sup>3,9,18</sup> It is interesting to note that, with the exception of the intestine where MALT is normally present as Peyer's patches, in the other sites the lymphoid tissue appears in an acquired form following infective or more often autoimmune inflammatory processes.<sup>9,12,14,20</sup> The neoplastic elements display a peculiar cytologic profile, perifollicular aggregation, and a tendency to attack the epithelial component; they can colonize local nodes or – more rarely – migrate to other MALT sites. Their phenotypic features (such as frequent expression of the CD11c and CD68 molecules) and molecular characteristics (high number of somatic mutations) correspond to those of marginal zone elements. These lymphomas have the following relevant characteristics: 1) they can regress after disappearance of the infective agent and/or autoimmune condition which in conjunction with the T-cell system stimulus supported the B-cell clonal expansion; 2) they tend to remain localized for a long time in the primary site, where they can become multifocal; 3) left to themselves they evolve in the course of several years to a histologically highly aggressive large B-cell form; 4) in some cases they present characteristic chromosomal aberrations [+3, t(11;18)].<sup>9,12,14,20</sup> These factors are important for the therapeutic strategy, which at least initially can be fairly conservative.<sup>9,12,14,20</sup>

#### **Nodal marginal zone lymphoma**

Since the cytological, architectural and phenotypic features of the nodal variety do not differ from those of the extranodal form, the differential diagnosis must be made by the exclusion of an evident MALT lymphoma in any of its characteristic sites.<sup>15-17,19</sup>

#### **Splenic marginal zone lymphoma**

The splenic form<sup>15-17,19</sup> has rather different features from those of the two varieties of B-cell marginal zone lymphoma quoted above. In particular, it most often displays: a) dissemination, with intrasinusoidal bone marrow infiltration; b) presence of a leukemic component (with a "villous" appearance in at least 50% of cases); c) splenic involvement, both ring-like around Malpighian follicles and plurifocally in the red pulp; d) considerable variability in its cytologic details.<sup>5</sup> This tumor has been included in the REALC as a provisional entity, since further studies are needed to shed light on its histogenesis and in particular whether it derives from the marginal zone alone or tends to reproduce all the differential features of B-cells in the white pulp of the spleen.<sup>16</sup>

#### **Follicle center cell lymphoma**

This category comprises both the centroblastic/centrocytic and centroblastic follicular forms of the UKC.<sup>2,6,11</sup> Their inclusion as a single group is justified by their shared histogenesis (from centrofollicular cells), phenotype (CD10+, CD5-, CD23-,

bcl-2+, aggregates of follicular dendritic cells CD21+, CD23+, R4/23+) and chromosomal anomalies t(14;18). In view of the variable ratios of centroblastic/centrocytic elements a grading system has been proposed (I: CB<25%, II: CB=25-50%, III: CB> 50%).

#### **Lymphocyte predominance (LP) Hodgkin's disease/lymphoma (HD)**

The REALC includes HD in the light of the proven lymphoid nature of neoplastic cells: in particular, the classification basically follows the HD categorization that emerged from the Rye conference, but introduces a major conceptual distinction between the nodular LP form (the so-called paragranuloma) and all the remaining histologic varieties, referred to as *common type*. HD-LP is characterized by the peculiar morphology of the neoplastic elements (L&H or popcorn cells), the rarity or absence of diagnostic cells, and the B-cell nature of the small lymphocytes that constitute the *milieu* of the disease with the exception of the elements surrounding the popcorn cells that belong to the T-system and are CD57+. The neoplastic elements also express a peculiar molecular profile: in particular, they constantly carry B-associated antigens (CD19, CD20, CD22, CDw75, CD79a, J-chain), are CD45+, react occasionally and weakly with anti-CD30 antibodies, frequently appear EMA+ and are negative for both CD15 and EBV. Finally, the clinical behavior of HD-LP is quite different from that of common HD: it does not display a bimodal age distribution; bone marrow involvement can be detected even in patients with only a single affected lymph node; relapse tends to be late with possible evolution into a large B-cell diffuse form of lymphoma. All this contrasts with the typical features of the other histologic varieties of HD, in which: a) the disease tends to spread in an orderly and progressive manner, b) the accompanying *milieu* is composite with a strong T-cell component, c) the diagnostic elements and cellular variants are CD30+, CD15+, EBV+/-, CD45-, EMA- and more often do not express B-cell associated antigens, even though some studies have reported reactivity with the L26/CD20 antibody.

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## Cytogenetics of indolent lymphomas

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During the past decades a large amount of data on non-Hodgkin's lymphomas (NHL) has been produced by morphologic, immunophenotypic, cytogenetic and molecular genetic analyses. Clonal cytogenetic aberrations are present in 60 to 90% of patients in several series. The presence of a single abnormality is rare. Usually, the karyotype is hyperdiploid (47-50 chromosomes), with cytogenetically normal and abnormal cells in the same sample. Translocations, deletions and duplications are the most frequent structural aberrations, while inversions are rare.<sup>1</sup> The cytogenetic aberrations are useful not only as diagnostic markers, but also as a means to identify important oncogenes involving the pathogenesis of NHL. Lymphomas characterized by these genetic rearrangements have specific cytologic features and clinical behavior. Characteristic chromosomal translocations lead to the deregulation of proto-oncogenes, by juxtaposition with regulatory elements of genes normally expressed in the cells, most frequently immunoglobulin (Ig) genes. The t(8;14), with deregulation of the c-myc proto-oncogene, is associated with Burkitt's lymphoma (BL); t(14;18) and rearrangements of BCL-2 oncogene with follicular lymphoma (FL) and the t(11;14) involving BCL-1 (PRAD-1/CCDN1) oncogene with mantle cell lymphoma (ML); chromosome band 3q27 with BCL-6 rearrangements are involved in diffuse large cell lymphoma (DLCL). The t(2;5) has been reported in CD30<sup>+</sup> anaplastic large-cell (ALC) lymphoma. Recently, particular morphologic, immunophenotypic, cytogenetic and molecular characteristics have been related to different clinical forms of indolent lymphoma, modalities of progression and survivals.<sup>2,3</sup>

### Follicular lymphoma

The reciprocal t(14;18)(q32;q21) constitutes the most common chromosomal translocation in human malignant lymphoid malignancies. It is considered the genetic hallmark of follicular lymphoma (FL) and is present in 80-90% of cases. This translocation leads to the juxtaposition of the BCL-2 proto-oncogene located on band 18q21 to regulatory sequences of the Ig heavy chains (IgH) gene on band 14q32, resulting in enhanced BCL2 expression.<sup>4-7</sup> The consequence is high levels of BCL-2 protein in the cells.<sup>8</sup> The break-

points on chromosome 18 cluster in 2 regions: approximately 70% of breakpoints occur in the major breakpoint cluster region (M-BCR) and the remaining cases in the minor breakpoint cluster region (m-BCR).<sup>8</sup> BCL-2 encodes a membrane protein that has been localized to mitochondria, endoplasmic reticulum and perinuclear membrane.<sup>9</sup> BCL-2 overexpression inhibits apoptosis, conferring a survival advantage to the t(14;18)-positive cells. By itself it does not render the cells malignant; additional genetic alterations (mutations, microdeletions, etc.) may occur causing the malignant transformation.<sup>10</sup> Importantly, a small percentage of FL does not have rearrangements of BCL-2. Variant translocations involving the  $\kappa$  and  $\lambda$  immunoglobulin light chain genes: t(2;18)(p11;q21) and t(18;22)(q21;q21) are biological equivalents.

Conventional cytogenetics reveal the highest proportion of cases positive for t(14;18) (80-90%), while Southern blot and/or polymerase chain reaction (PCR)-based assays are positive in about 75% of the cases.<sup>11</sup> This discrepancy is probably due to breakpoints localized 3' to the classic breakpoint, outside the range revealed by the probes and primers usually used. However, PCR technology has the advantage that it is much more sensitive than karyotype analyses or Southern blot assays. Recent observations pointed out that single cells with t(14;18) may even be present in the peripheral blood of normal individuals. The finding of t(14;18)-positive cells in non-neoplastic tissue highlights the need to interpret positive PCR results, e.g. in studies of minimal residual disease, with caution.<sup>12</sup>

In the clinical progression to aggressive diffuse lymphoma, cytogenetic studies have shown secondary chromosomal abnormalities. Additional aberrations are trisomies 7 and 12 and breaks in 1p32-36, 1q21-q23, 6q21-q25 (20% of cases) and 17p alterations. These aberrations have been associated with a poor survival or a higher risk of transformation into DLCL.<sup>13</sup> They may be present at diagnosis, but also during progression of disease. Less frequent additional abnormalities are: +18, +20, +21, del(13)(q32) and +2.

A significant amount of high grade DLCL (30%) shows the t(14;18), often associated with p53 mutations and occasionally with del(6q) and/or c-myc rearrangements, usually described as histologic transformations of a previous FL.

The t(14;18) can be diagnostically useful and applied to evaluating the minimal residual disease

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by molecular studies. Rearrangements of 3q27 band, the locus of BCL-6 gene related to DLCL, have been described in approximately 5-10% of cases of FL.

#### **Marginal zone B-cell lymphoma**

Few cytogenetic studies of this lymphoma exist because fresh tumor tissue is difficult to obtain and poor *in vitro* growth hampers the observation of a significant number of metaphases. Nevertheless, trisomy 3 has been described as the most consistent chromosomal abnormality and strictly associated with this malignant disease.<sup>14</sup> In larger series whole or partial trisomy 3 has been reported in 56-78% of cytogenetically abnormal cases and in up to 60% of cases, when interphase FISH had been applied, of low-grade MALT lymphomas and 27% of splenic lymphomas. In a recent study cytogenetic and/or interphase FISH analysis revealed the trisomy in 22/36 (61%) of cases of MZBCLs, with similar frequencies in extranodal, nodal and splenic lymphomas.<sup>14-17</sup> Trisomy 3 has been described rather infrequently (6-24%, mean 12%, of cases with an abnormal karyotype) in other types of B-NHL.<sup>17,18</sup>

Trisomy 3 is often associated with structural changes of the long arm of the same chromosome (3q); considering the variety of breakpoints and partner chromosomes involved in the latter rearrangements, these structural aberrations are perhaps of secondary nature.<sup>17</sup> The molecular mechanisms of trisomy 3 are unknown. A gene dosage effect with over-expression of genes located on chromosome 3 has been suggested. B-cell surface antigen B7 was mapped on 3q13-q21,<sup>19</sup> the PBX2 homeobox gene on 3q22-q23<sup>20</sup> and a gene coding for a subunit of interleukin 12 gene on chromosome 3. Moreover, 3q27 was the locus of BCL-6 oncogene associated with DLCL, but it was not found to be rearranged in these cases.<sup>16</sup>

Trisomies 18, 7 and 12 have been described to occur non-randomly but less frequently than trisomy 3.<sup>14,15,21</sup> The t(11;18)(q21;q21) is the most common translocation in low-grade but not in high-grade MALT lymphomas<sup>22,23</sup> and structural aberrations of chromosome 1 recurrently involved the chromosomal regions 1p22, 1p34 and 1q21.<sup>14,15,21</sup>

#### **Lymphoplasmacytoid lymphoma**

The t(9;14)(p13;q32) is associated with approximately 50% of the subtype of NHL with plasmacytoid features and has been proposed as the specific cytogenetic marker of this disease.<sup>24</sup> The locus of the PAX-5 (paired homeobox-5) gene, has been mapped to band 9p13. PAX-5 gene encodes a B-cell transcription factor involved in the control of B-cell proliferation and differentiation. Several observations suggest that the t(9;14) causes deregulated expression of the gene by juxtaposition to the Ig regulatory elements. The result is a maturation arrest at the pro-B cell stage and its overexpression results in proliferation of B-cells.<sup>24</sup>

Other translocations involving 9p13 and various chromosome loci: 1q25, 3q27, 7q11, 12q13, 12q21, 19p13, 9q13 have been described.<sup>25</sup> These observations suggest that multiple chromosomal regions may contribute to the PAX-5 deregulation because of the juxtaposition of different regulatory elements. This is a similar mechanism to that observed in the chromosome translocations involving the BCL-6 in DLCL.<sup>26</sup>

No other genetic abnormality has been detected at significant frequencies in this type of lymphoma.

#### **Mantle cell lymphoma**

The cytogenetic hallmark of mantle cell lymphoma (MCL) is the translocation t(11;14)(q13;q32), that juxtaposes the BCL-1 locus on chromosome band 11q13 to the IgH gene on 14q32.<sup>27</sup> This translocation leads to overexpression of the cell cycle regulatory protein cyclin D1 (PRAD1 or CCND1).<sup>28</sup> The translocation can be detected cytogenetically in approximately 75% of cases. Cyclin D1 is overexpressed in >95% of MCL. It can also be revealed by double color FISH.<sup>29</sup> Since this translocation is detectable by PCR, molecular studies can be performed to evaluate minimal residual disease.

The most frequent additional abnormality is trisomy 12.

#### **Abnormalities of chromosome 1**

These aberrations are described in all histologic subtypes of NHL as a negative prognostic marker; a high incidence of breaks occurs in the regions 1p32-36 and 1q21-q23. Abnormalities of the short arm of chromosome 1 are found in many neoplastic diseases, ranging from neuroblastoma to hematologic neoplasms. Rearrangements of the distal segments of 1p have been observed in more than 10% of NHLs cases.<sup>1</sup> Additional or lost material often cannot be identified by banding analysis alone. However, the FISH technique offers a good opportunity to define the origin of these abnormalities. The pathogenetic impact could theoretically be due to any of the following mechanisms: increased dosage of genes on translocated material, loss of suppressor genes or recombination leading to fusion genes, or gene deregulation.<sup>30</sup>

#### **6q deletion**

Deletions of the long arm of chromosome 6 are among the most frequent chromosome aberrations in solid tumors (malignant melanoma, ovarian carcinoma, breast carcinoma) and hematopoietic malignancies. They occur in 4-13% of patients with ALL and in 20-30% of patients with NHL. Deletions at 6q have been reported either as an additional aberration or sole cytogenetic abnormality in lymphoid tumours, supporting the pathogenetic role of these aberrations. They are not specific to any subtype of NHL. The relatively limited power of the resolution of conventional cytogenetics has not helped the identification of the specific regions involved.<sup>31</sup> Molecular

studies have identified 2 regions of minimal deletion: RMD-1 at 6q25 to 6q27 and RMD-2 at 6q21 to 6q23 suggesting the presence of 2 or more tumor suppressor genes. In particular RMD-1 has been described to be associated with low and intermediate grade NHL, whereas RMD-2 is preferentially associated with high grade NHL. Del(6q) has been associated with an unfavorable prognosis.

### Trisomy 12

This is a more common numerical aberration, present in about 30% of FL and DLCL with bad prognosis.

### Additional abnormalities

Approximately 74% of cytogenetically abnormal NHLs displayed secondary aberrations: 66% in low grade, 85% in intermediate grade and 71% in high grade lymphomas. The mean number of additional abnormalities per lymphoma is: 4.6 in low grade, 6.7 in intermediate grade and 3.6 in high grade. The most frequent secondary numerical and structural abnormalities are +X, -Y, dup(1q), del(6q), +7 and +12.<sup>32</sup>

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## Molecular pathophysiology of indolent lymphomas

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Analogous to most human cancers, the genetic lesions involved in indolent lymphomas include the activation of proto-oncogenes and disruption of tumor suppressor genes.<sup>1</sup> In contrast to many types of epithelial cancers, the genome of indolent lymphoma cells tends to be relatively stable and is not subject to the generalized random instability which characterizes many types of solid cancers.<sup>2,3</sup> Historically, detection of recurrent, non-random chromosomal abnormalities by karyotypic analysis of indolent lymphoma metaphases has provided the major clue towards the identification and cloning of most of the genetic alterations of these diseases.<sup>2</sup>

Chromosomal translocation is the main mechanism of proto-oncogene activation in indolent lymphoma.<sup>1</sup> Like most types of hematopoietic neoplasms, chromosomal translocations of indolent lymphomas are constituted by reciprocal and balanced recombination events between two specific chromosomal sites. These translocations characteristically recur within a specific clinico-pathologic category of indolent lymphomas and are clonally represented in each tumor case. All chromosomal translocations of indolent lymphomas share a common feature, i.e. the presence of a proto-oncogene mapping to the vicinity of one of the two chromosomal recombination sites. As a consequence of the translocation, the proto-oncogene is juxtaposed to heterologous regulatory sequences which are derived from the partner chromosome and are invariably represented by antigen receptor loci. Because antigen receptor genes are expressed at sustained levels in normal cells corresponding to the differentiation stage of the lymphoma, the common consequence of the translocation is deregulated expression of the proto-oncogene.

Disruption of tumor suppressor genes in indolent lymphomas occurs through mechanisms similar to those associated with other human cancers and generally lead to biallelic inactivation of the gene, most frequently achieved through a combination of deletion, mutation and methylation. The tumor sup-

pressor genes most commonly involved in indolent lymphomas are p53 and p16.

The clinico-pathologic heterogeneity of indolent lymphomas is reflected by a high degree of heterogeneity in the molecular pathophysiology of these disorders. The following pages contain a summary of the molecular pathways associated with the main categories of indolent lymphoma recognized by the REAL classification.<sup>4</sup>

### **B-cell chronic lymphocytic leukemia**

The molecular pathogenesis of B-cell chronic lymphocytic leukemia is still largely unknown. Mutations of the p53 gene and loss of heterozygosity in 17p, the p53 site, are found in a small fraction (10 to 15%), of B-cell chronic lymphocytic leukemia cases.<sup>5,6</sup> A higher frequency of p53 alterations is observed after transformation of B-cell chronic lymphocytic leukemia into Richter's syndrome, a highly aggressive lymphoma with a poor clinical outcome,<sup>5</sup> suggesting that p53 may be involved in the genetic mechanisms underlying progression of B-cell chronic lymphocytic leukemia.

A number of dominant oncogenes, including c-MYC, BCL-1, and BCL-2 genes, have been widely investigated in B-cell chronic lymphocytic leukemia; none of these genes, however, has shown clear associations with the disease.<sup>6</sup> Since high levels of BCL-2 expression are consistently seen in B-cell chronic lymphocytic leukemia cells,<sup>7</sup> it is conceivable that they result from mechanisms other than chromosomal translocation.

Despite the paucity of information regarding the molecular lesions associated with B-cell chronic lymphocytic leukemia, cytogenetic studies have revealed several recurrent chromosomal abnormalities.<sup>2</sup> Trisomy 12 is found in approximately 35 % of B-cell chronic lymphocytic leukemia cases evaluated by interphase fluorescent *in situ* hybridization and correlates with a poor survival.<sup>8,9</sup> Based on karyotypic and deletion mapping studies, it is likely that the 13q14 chromosomal region harbors a novel tumor suppressor gene that is involved at high frequency in B-cell chronic lymphocytic leukemia.<sup>10</sup> In fact, deletions of 13q14 occur in approximately 60% of cases when analyzed by sensitive molecular tools, but the relevant gene has not been identified.<sup>10</sup>

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### Lymphoplasmacytoid lymphoma

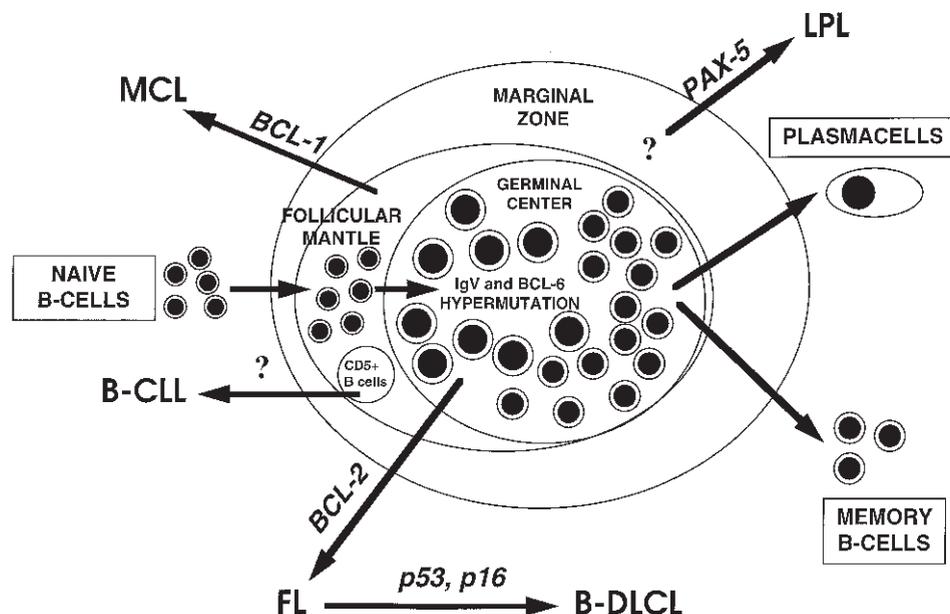
The t(9;14)(p13;q32) translocation associates with approximately 50% of lymphoplasmacytoid lymphomas (Figure 1).<sup>11</sup> The chromosomal breakpoints of t(9;14)(p13;q32) involve the IgH locus on chromosome 14q32, and, on chromosome 9p13, a genomic region containing the PAX-5 (Paired Homeobox-5) gene.<sup>11</sup> PAX-5 encodes a B-cell specific transcription factor involved in the control of B-cell proliferation and differentiation.<sup>11</sup> Presumably, the juxtaposition of PAX-5 to the IgH locus in lymphomas carrying t(9;14)(p13;q32) causes the deregulated expression of the gene, thus contributing to tumor development.

### Follicular lymphoma

Follicular lymphoma derives from germinal center B-cells (Figure 1). Chromosomal translocations that involve BCL-2 are the hallmark of follicular lymphoma, being detected in 80% to 90% of cases.<sup>1</sup> The BCL-2 gene was identified by molecular cloning of the t(14;18)(q32;q21) translocation, which is present in virtually all cases of follicular lymphomas as well as in a proportion of B-lineage diffuse large cell lymphomas.<sup>1</sup> The translocation joins the BCL-2 gene at its 3' untranslated region to IgH sequences, result-

ing in deregulation of BCL-2 expression because of the nearby presence of Ig transcriptional regulatory elements.<sup>12-14</sup> Approximately 70% of the breakpoints on chromosome 18 are clustered within a major breakpoint region (MBR), while the remaining cases usually break in the more distant minor cluster region (mcr).<sup>12-14</sup>

The BCL-2 gene encodes a 26-kDa integral membrane protein that has been localized to mitochondria, smooth endoplasmic reticulum and perinuclear membrane.<sup>14</sup> Whereas most proto-oncogenes of lymphoid neoplasia directly enhance cell growth, BCL-2 has no ability to promote cell cycle progression or cell proliferation but rather controls the cellular apoptotic threshold by preventing programmed cell death.<sup>14</sup> BCL-2 is only one member of a family of apoptotic regulators, which also includes BAX and BCL-X.<sup>14</sup> It is now clear that BCL-2 exists as part of a high molecular weight complex generated through heterodimerization with BAX.<sup>14</sup> The inherent ratio of BCL-2 to BAX determines the functional activity of BCL-2. When BAX is in excess, BAX homodimers dominate and cell death is accelerated; conversely, when BCL-2 is in excess, as in lymphomas carrying BCL-2 rearrangements, BCL-2/BAX heterodimers are the prevalent species and cell death is prevented.



**Figure 1.** Genetic pathways in indolent B-cell lymphomas. The major compartments of mature B-cells are schematically represented as a secondary follicle containing the germinal center and the mantle zone. The CD5+ B-cell compartment is also shown. The major categories of indolent B-cell lymphomas recognized by the REAL classification are indicated as B-CLL (for B-cell chronic lymphocytic leukemia), LPL (for lymphoplasmacytoid lymphoma), MCL (for mantle cell lymphoma), and FL (for follicular lymphoma). In a fraction of cases, FL may undergo histologic transformation to B-lineage diffuse large cell lymphoma (B-DLCL). The putative histogenetic derivation of each lymphoma category is indicated by an arrow originating from the relevant B-cell compartment. Arrows are flanked by the cancer related gene which is selectively involved in the pathogenesis of each specific category of indolent lymphoma, and, based on current knowledge, represents the genetic hallmark of the disease. In the case of B-CLL, the relevant cancer-related gene has not been identified.

The precise contribution of BCL-2 deregulation to follicular lymphoma development is complex. The pathogenicity of BCL-2 lesions in the context of follicular lymphoma is substantiated by the ability of BCL-2 specific antisense oligonucleotides to inhibit the growth of human B-cell lymphomas bearing BCL-2 translocations.<sup>15</sup> *In vivo*, however, the BCL-2 transgene leads to a pattern of polyclonal hyperplasia of mature, long lived B-cells resting in G<sub>0</sub>, which, despite morphologic similarities, contrasts with the consistent monoclonality of human follicular lymphoma.<sup>16</sup> Hence the view that BCL-2 activation is not sufficient for follicular lymphoma development, and that other genetic lesions or host factors are required. A strong candidate is chronic antigen stimulation and selection which would synergize with BCL-2 in driving follicular lymphoma expansion. Over time, and analogous to what happens in humans, some BCL-2 transgenic mice progress to develop aggressive, clonal diffuse large cell lymphomas which have acquired additional genetic lesions.<sup>17</sup>

Other oncogenes involved in lymphomagenesis, such as c-MYC and p53, do not appear to be involved in follicular lymphoma. Deletions of chromosome 6 are present in 20% of cases.<sup>18</sup> Over time, follicular lymphomas tend to convert into an aggressive lymphoma with a diffuse large cell architecture.<sup>4</sup> This histologic shift is generally accompanied by the accumulation of p53 mutations.<sup>19</sup> Rearrangements of c-MYC or inactivation of p16 may also occasionally accompany the histologic transformation of follicular lymphoma.<sup>20,21</sup>

### **Mantle cell lymphoma**

Mantle cell lymphoma is a relatively rare lymphoma of CD5<sup>+</sup> B cells originating from the mantle zone surrounding reactive follicular centers (Figure 1). The t(11;14)(q13;q32) translocation and BCL-1 rearrangement are the characteristic abnormalities of mantle cell lymphoma.<sup>1,22,23</sup> The BCL-1 locus was originally identified as a breakpoint site on chromosome 11 in B-cell malignancies carrying the t(11;14)(q13;q32) translocation.<sup>22,23</sup> Translocations involving BCL-1 are characteristically detected in 70% of mantle cell lymphomas.<sup>1,4</sup> BCL-1 translocations are selective for mantle cell lymphoma and, despite initial suggestions, are not found in B-cell chronic lymphocytic leukemia.<sup>1,4,6</sup>

The BCL-1 translocation results in the juxtaposition of the IgH locus on chromosome 14 to sequences from chromosome 11.<sup>22, 23</sup> The relevant oncogene, i.e. cyclin D1, lies 200 kb apart from the BCL-1 locus.<sup>24</sup> The cyclin D1 gene, also known as PRAD1 or CCND1, is a member of a family of proteins that regulate cell cycle progression.<sup>24</sup> As for other D-type cyclins, cyclin D1 is thought to act primarily as a growth factor sensor integrating extracellular signals with the cell cycle machinery. The pathogenetic role of BCL1 activation in human neoplasia

is suggested by the ability of cyclin D1 overexpression to transform cells *in vitro* and to contribute to B-cell lymphomagenesis in transgenic mice.<sup>25-27</sup> Since mantle cell lymphoma frequently simulates other low grade lymphoproliferative diseases, the detection of BCL-1 translocations is considered a relevant diagnostic marker for proper classification of this type of disorder.<sup>4</sup> A subset of mantle cell lymphomas also carry p53 or p16 mutations.<sup>21,28</sup> These genetic lesions denote a particularly poor prognosis for the patient.

### **Mucosa-associated lymphoid tissue (MALT) lymphomas**

Compared to other categories of indolent lymphomas, the understanding of the molecular pathogenesis of MALT-lymphomas is still in its early stages. In the case of gastric MALT-lymphomas, the majority of tumors are associated with *Helicobacter pylori* (*H. pylori*) infection.<sup>29</sup> It has been suggested that gastric MALT-lymphomas may be dependent upon antigen stimulation by *H. pylori* since malignant lymphoid cells respond to *H. pylori* antigens and since the lymphoma may regress, at least partially, upon eradication of infection.<sup>30</sup> The potential role of antigen in MALT-lymphoma pathogenesis is further supported by the observation that MALT-lymphoma cells harbor the genotypic clue of antigen-experienced B-cells, i.e. somatic hypermutation of Ig genes.<sup>1</sup> Whether the development of MALT-lymphoma arising in body sites other than the stomach is also dependent upon antigen stimulation and selection remains an open question. In this respect, it is remarkable that thyroid MALT-lymphoma is generally a sequela of Hashimoto's thyroiditis, an autoimmune process causing the exposure of B-cells to thyroid-derived autoantigens.

Among genetic alterations commonly involved in other lymphoma types, only BCL-6 rearrangements and p53 mutations have been detected in MALT-lymphomas, though at low frequency.<sup>31,32</sup> Cytogenetic studies, however, have pointed to several abnormalities recurrently involved in these tumors. The most frequent of these abnormalities are trisomy 3 and t(11;18).<sup>2, 33</sup> The genes implicated by these aberrations are not yet known.

### **Conclusions**

A number of general considerations can be drawn from the extensive body of studies on genetic lesions in indolent lymphomas summarized in these pages. First, the well recognized clinical and histologic heterogeneity of lymphoid malignancies reflects, and possibly is caused by the extreme degree of diversity among the molecular lesions associated with each type of indolent lymphoma (Figure 1). Second, it may be presumed that improved molecular diagnosis will soon translate into differential therapeutic protocols according to the tumor genotype, thus leading to therapeutic stratification and, it is to be hoped, improved patients' survival. Molecular evaluation of minimal residual disease in the patient's blood/bone marrow

as well as in grafts (bone marrow or peripheral blood stem cells) for autologous transplantation procedures is also likely to influence therapeutic decisions and the clinical follow-up. Third, lymphoid neoplasia results from the accumulation of multiple genetic lesions interplaying in the same clone. In this respect, follicular lymphoma is the best characterized example of multistep tumorigenesis among indolent lymphomas.

In addition to the current applications of genetic lesions in the management of indolent lymphomas, other uses may be developed in the future. Of particular appeal is the possibility that therapy might be directed at correcting the precise genetic lesion responsible for the development of each single type of indolent lymphoma. Such therapeutic strategy should, by definition, be largely specific to the lymphomatous cells and hence devoid of the major side effects presently encountered with standard therapeutic regimens. Initial results from *in vitro* studies indicate the potential feasibility of gene targeting in controlling indolent lymphoma growth and aggressiveness.<sup>15</sup> Yet, as with all human neoplasms, many issues must be resolved before the therapeutic use of gene targeting can be introduced into clinical practice.

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## Prognostic factors in follicular lymphoma: a clinical and methodologic challenge

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Follicular lymphoma (FL) is the second most frequently reported type of non-Hodgkin's lymphoma (NHL) in the *Non-Hodgkin's Lymphoma Classification Project*.<sup>1</sup> Its frequency, estimated from a study population of 1,403 NHL patients, was 22% (306 patients). These lymphomas were classified as follicular small-cleaved cell, follicular mixed cell and follicular large cell lymphoma in the Working Formulation and as follicular centroblastic/centrocytic or follicular centroblastic lymphoma in the Kiel classification. FL patients have a relatively indolent course, the 5 year overall survival rate is estimated at 70% and median survival duration from diagnosis ranges from 7 to 10 years. This tumor is not, however, curable with conventional treatment, demonstrating a constant annual death rate of 8%,<sup>2</sup> in contrast to the pattern of aggressive NHL in which late deaths are widely attributed to underlying mortality of the population at large. It is well known that FL may convert to diffuse large cell lymphoma during the course of the disease which is generally difficult to control with treatment. Moreover, some FL patients have poor progression-free and overall survival rates suggesting that this group is not homogeneous and that pre-treatment prognostic factors may be identified. Consequently, the characterization of high or low risk patients with FL would have important therapeutic implications. High-risk patients could be candidates for new therapeutic approaches such as high dose chemotherapy with autologous stem cell transplantation. In addition, the identification of different risk patterns would help in the design and interpretation of therapeutic trials.

This review summarizes the efforts to develop clinically based prognostic models and focuses on the main methodologic difficulties.

### **Identification of clinical prognostic factors**

The most reliable end-points are progression-free survival and overall survival. Achievement of complete remission is not very relevant in FL patients because of difficulties in the evaluation of bone-marrow involvement and the need to use a molecular marker. Many investigators have identified the clinical

pre-treatment variables associated with a poor survival in univariate analysis in their own series (Table 1). Interpretations must, however, be made cautiously because of generally small sample sizes, treatment heterogeneity and varying follow-up durations. As in aggressive NHL, the most reliable prognostic factors are age, B symptoms, performance status, extranodal sites of disease, marrow involvement, LDH level and  $\beta_2$  microglobulin.<sup>2-5</sup> Morphologic parameters such as percentage of large cells, architectural particularities or mitotic activity have a poorly reproducible predictive value and are not widely recognized.

### **Predictive model based on clinical pre-treatment prognostic factors**

Features that retained independent significance in multivariate analysis were used to develop prognostic factor models predictive for an individual patient's risk of death. All models (Table 2) incorporated parameters of bulk of the disease and the extent of tumor involvement but were not validated either by an internal procedure (data splitting or bootstrap) or in other large set of patients.<sup>4, 6-8</sup> More recently, the GELA identified 3 independent prognostic factors for poor survival: B-symptoms, age above 60 and at least 3 nodal sites > 3 cm.<sup>5</sup> Another approach was to demonstrate the applicability of the largely validated International Prognostic Index (IPI) for aggressive NHL to patients with FL. This index relies on the following five factors: age, performance status, stage, LDH and number of extranodal sites.<sup>9</sup> In recent studies, the IPI succeeded in separating FL patients into subgroups with different survival patterns.<sup>1,5,10</sup> However, its main weakness was that very few patients (< 10%) could be assigned to the adverse prognostic group. Moreover, the prognostic influence of the IPI factors has not been validated in the same way by univariate analysis in FL patients, and other factors, which were not significant for aggressive NHL, may have a greater predictive relevance in FL. In fact, the IPI discriminating power could not be considered as sufficient to allow a risk-based treatment allocation. A new predictive model based on large-scale (around 3,000 patients) multicenter trials, is currently being prepared by the International Prognostic Factor Project for FL. The primary outcome is overall survival. Statistical analysis is planned in order to overcome the weaknesses of the previously cited studies. It

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**Table 1. Univariate analysis of prognostic factors for follicular lymphoma.**

In the literature [2-4]	In the GELF86 trial [5]
Percentage of large cells/architecture	—
High mitotic activity	—
Cytogenetic abnormalities	—
Male gender	—
Age > 60 or 70 years	Age > 60 years
Ann Arbor stage III-IV	Ann Arbor stage IV
Systemic symptoms	Systemic symptoms
Performance status > 1	—
Number of extranodal sites > 1	Number of extranodal sites > 1
Tumor bulk > 7 or 10 cm	Tumor bulk > 7 cm
Hepato-splenic enlargement	Liver involvement
Diffuse marrow infiltration	Bone marrow involvement
Anemia	—
LDH > 1 N	LDH > 1 N
$\beta_2$ microglobulin > 1 N	—
—	At least 3 nodal sites > 3 cm
—	Serous effusions or local compressions

might provide a relevant index to guide initial therapeutic choice with individual patient's risk of death related to the number of adverse factors. However, this standard approach does not account for competing risks between successive relapses, histologic transformations, death in remission or in progression. Because treatment options for FL vary from abstinence to high dose chemotherapy, we think that an interesting approach could be to distinguish pre-treatment prognostic features from parameters that are more directly related to response to treatment.

#### **Prognostic factors associated with response to treatment**

Response to treatment demonstrates the chemosensitivity of an individual patient's disease and is highly correlated with survival. But what is the most important event in the course of FL in terms of death rate and outcome? The GELA recently showed that probability of histologic transformation was 22% at 5 years and 31% at 10 years.<sup>11</sup> Transformation was associated with poor outcome with a median survival duration of 7 months. The predictive factors were non-complete remission, low serum albumin level (< 35 g/L) and elevated  $\beta_2$  microglobulin (> 3 mg/L). On the other hand, Johnson *et al.* showed that following recurrence, the median duration of survival was 4.5 years.<sup>12</sup> Age, together with previous and continuing responsiveness to therapy were the principal determinants of survival.

In this particular lymphoma, several types of events appear to be of interest. Rather than considering a single (OS) or a composite (EFS) outcome, a more complex statistical methodology could be used. A multistate model, like the illness-death model, allows the investigation of the uniformity of prognostic fac-

**Table 2. Major prognostic index for follicular lymphoma.**

	Litam [6, 15]	Romaguera [4]	Leonard [8]	Cameron [7]	Decaudin [5]
Sex		×	×		
Age			×	×	×
Performance status			×	×	
Systemic symptoms				×	×
Ann Arbor Stage			×	×	
Number of extra-nodal sites	×				
Lymph node size	×				×
Degree of marrow infiltration	×				
Digestive tract involvement				×	
Hemoglobin level			×		
LDH level	×				
$\beta_2$ microglobulin level	×				

tors or treatment effect on different events such as relapses or death.<sup>13</sup> This approach may add important information on therapeutic strategy. For example: if younger patients have an increased risk of transformation or relapse and older patients an increased risk of death without recurrence, for the former, lymph node biopsy may have to be repeated in other sites looking for a discordant histology and, for the latter, treatment toxicity has to be carefully managed. The problem has recently been considered in relation to breast cancer with local or distant recurrences, but the same approaches can be applied to other tumor entities such as FL.<sup>14</sup>

In conclusion, the IPI created for aggressive NHL is not discriminant enough to guide a risk based therapeutic strategy for FL patients. Waiting for the achievement of a specific prognostic index,  $\beta_2$  microglobulin level may add information to standard prognostic factors. In further studies, a multistate approach could give more statistical nicety, particularly when intensive or expensive procedures are proposed.

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## Biological prognostic factors in indolent lymphoma

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The category of indolent lymphoma is a heterogeneous group of lymphoproliferative disorders with different presenting features, clinical courses and responses to treatment. This heterogeneity also applies to individual patients within each histologic category. For example, the survival of patients with follicular lymphoma, which is the commonest histologic subgroup of indolent lymphoma, may range from 2 to over 10 years from diagnosis. The possibility of identifying features at presentation with adverse prognostic impact has relevant clinical implications since new aggressive treatment approaches may nowadays be used with the aim of a possible cure for a proportion of patients. In this context, the choice of the candidates for these new approaches is crucial, hence the need for a reliable method for prognostic assessment.

The commonest and more easily applicable methods of stratifying patients with non-Hodgkin's lymphoma (NHL) into risk categories have so far been largely based on the evaluation of clinical features at presentation. The most popular and widely recognized proposal derived from this approach is the international prognostic index (IPI).<sup>1</sup> Originally applied to high-grade NHL, it was subsequently proved to be of some value also in the large majority of NHL categories. In recent years several studies have addressed the possible prognostic significance of a number of biological features characterizing NHL at presentation. Our aim is to review and discuss the available information on the relevance of biological prognostic factors in indolent lymphoma.

### Many methodologic approaches for many biological features

The biological events associated with and/or responsible for uncontrolled cell proliferation and spreading in NHL can be investigated by a variety of approaches, including traditional biochemistry and immuno-derived detection of serum/plasma molecules, flow cytometry and immunohistochemistry, cytogenetic and molecular technologies. The combined use of these approaches allows the detection of a number of features, from now on referred to as

biological markers, which can be grouped, for simplicity, into the following categories: serum/plasma markers, immunophenotypic markers, cytogenetic and molecular biology markers. Clearly, there is some overlapping between these groups, since a given biological event can be investigated by different markers included in each group.

### Serum/plasma markers

In this review, we do not consider the prototypic serum marker LDH, which has already been included in prognostic models.<sup>1</sup> LDH also appears to be a reliable prognostic marker in indolent lymphoma<sup>2</sup> and since it is available on a routine basis for virtually all patients it probably represents the gold-standard marker against which any other potentially useful prognostic markers should be validated.

Biological serum markers so far investigated as possible prognostic markers in indolent lymphoma include a variety of cell-derived soluble molecules, cytokines and cytokine receptors.

### $\beta_2$ -microglobulin

This molecule is released into the blood and body fluids from the surface membrane of most nucleated cells. Increased serum levels of  $\beta_2$ -microglobulin ( $\beta_2$ M) are found in a wide range of malignant and non-malignant conditions. In low-grade lymphomas high  $\beta_2$ M serum levels are associated with a lower complete remission (CR) rate and poorer survival.<sup>2,3</sup> In primary gastric lymphoma, serum levels of  $\beta_2$ M, together with LDH, were found to be of independent prognostic value in identifying groups at different risk, whereas IPI classification was devoid of prognostic significance.<sup>4</sup> Overall, available information appears still too preliminary to reach a conclusion on the role of  $\beta_2$ M serum determination for prognostic assessment in indolent lymphoma.

### Thymidine kinase

Over the last 20 years there have been several studies on the possible prognostic value of detecting thymidine kinase (TK) in NHL patients, including those with low-grade histology. Although some correlation was demonstrated between increased TK levels and outcome in low-grade NHL,<sup>5</sup> this parameter appears devoid of any independent prognostic significance.

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### **Soluble adhesion molecules**

Soluble forms of various adhesion molecules, such as sICAM-1, sVCAM-1 and sCD44, have been investigated as possible prognostic factors in NHL, including the low-grade histology form of the disease.<sup>6-8</sup> Although the circulating levels of each of these molecules were shown to correlate with advanced stage, presence of B symptoms and other features associated with an unfavorable outcome, they did not have independent prognostic value.<sup>6-8</sup>

### **TNF and TNF receptor family molecules**

Some evidence suggests a role for a number of the TNF/TNF-receptor (TNFR) family molecules in growth and differentiation activity of malignant lymphoid cells. Circulating levels of TNF- $\alpha$  and TNFR (p55- and p75-TNFR) have been shown to bear prognostic significance in NHL.<sup>9,10</sup> In a series of 40 patients with indolent lymphomas, increased circulating levels of TNF- $\alpha$  at presentation were significantly correlated with more advanced stage.<sup>9</sup> Although this parameter was not statistically significant in terms of prediction of response, when associated with high levels of soluble CD23 it contributed to identifying a selected group of non-responders. In another study, which included 54 patients with low-grade histology out of 124 patients with NHL, patients at low and high-risk for freedom from progression and overall survival could be identified on the basis of TNF, p55- and p75-TNFRs levels.<sup>10</sup> The low-risk group had TNF, p55 and p75 levels below median values, while the high-risk group had values for the three parameters above these limits. The addition of these parameters to the IPI yielded an improvement of the predictive value of the latter. Although these data appear of interest, their relevance for patients with indolent lymphoma is difficult to establish since the analysis was not performed separately for this group of patients.<sup>10</sup>

The serum concentration of the soluble form of CD95, another molecule of the TNF/TNFR family involved in the mechanisms of cell apoptotic death, has also been investigated in low-grade NHL, without any evidence of prognostic correlations.<sup>11</sup>

### **IL-6**

Several studies suggested a possible role for IL-6 in malignant lymphomas. Circulating IL-6 in NHL is derived from malignant cells<sup>12</sup> and its levels appear to be strictly correlated with C-reactive protein.<sup>13</sup> In a study addressed to investigate the prognostic significance of IL-6 serum concentration in indolent lymphoma at diagnosis, elevated levels were found only in a minority of patients. In these individuals, high IL-6 levels were associated with adverse disease features predictive of a shorter failure-free survival (FFS). Nevertheless, an independent statistical significance could not be demonstrated.<sup>14</sup>

### **IL-10**

At diagnosis patients with NHL have significantly

higher serum IL-10 levels than normal individuals and patients in CR.<sup>15</sup> The prognostic significance of these increased levels varies according to the assay used.<sup>15</sup> Overall, IL-10 serum concentrations do not seem to be of prognostic value in patients with low-grade histology.<sup>15,16</sup>

### **Other molecules**

The soluble IL-2 receptor (corresponding to the released p55  $\alpha$ -chain of the IL-2R complex) has been extensively investigated over the years in malignant lymphomas. Although some correlation could be found between sIL-2R serum concentration and a number of clinical features,<sup>17</sup> no convincing evidence has emerged on its prognostic role in indolent lymphoma.

Increased serum concentrations of CA125, a marker mainly utilized in the context of ovarian carcinoma, were found in about one third of patients with low-grade NHL.<sup>18</sup> The possible prognostic role of this marker remains to be investigated. High pre-treatment serum values of vascular endothelial growth factor (sVEGF) were found to be associated with poor overall survival in a small group of patients with low-grade NHL.<sup>19</sup> The association was not, however, of independent statistical significance.

### **Cytogenetic and molecular biology markers**

In recent years, several cytogenetic abnormalities (mainly aberrant rearrangements or deletions) have been identified in indolent lymphomas. Some of these abnormalities are quite aspecific or even secondarily acquired, while others have been shown to identify, or to be preferentially associated with, particular types of lymphomas. These aberrant rearrangements differ in nature from the monoclonal immunoglobulin rearrangement which was historically the first molecular biology marker to be associated with lymphoid neoplasia. Recently, the molecular biology of a number of such cytogenetic abnormalities and of their anomalous translation products has been partly clarified. As a consequence, understanding of ontogenetic and pathogenetic mechanisms thus far elusive has been enlightened and new tools for diagnostic and prognostic evaluation have been generated. The concept of minimal residual disease as a prognostic indicator has also been profoundly re-shaped by the availability of such novel molecular biology markers. Pruning down the vast array of cytogenetic/molecular abnormalities in indolent lymphomas to those demonstrated to be of any value in a clinical prognostic perspective, we may identify and classify them as follows.

#### **Lymphoma-specific rearrangements**

These rearrangements are usually translocation-based abnormalities detectable in the neoplastic population, but not in normal cells and preferentially associated with some types of lymphoma. The close association with specific lymphomas suggests

that such translocations/rearrangements are likely to be related to pathogenetic mechanisms relevant to the development and/or maintenance of the neoplasia, although some of these rearrangements have been reported in normal subjects.<sup>20</sup>

#### *t(14;18)(q32;q21) and t(11;14)(q13;q32)*

These are the two best characterized cytogenetic/molecular biology markers among indolent lymphomas. The *t(14;18)* is detectable in 60-80% of patients with follicular lymphoma (FCL) as well as in a proportion of those with diffuse large cell lymphomas. The *Bcl-2* gene is fused with the *IgH* locus and deregulated expression of the *Bcl-2* anti-apoptotic protein follows.<sup>21</sup> The *t(11;14)* occurs in the majority (70-100%) of patients with mantle cell lymphoma (MCL). The *Bcl-1* gene is fused to *IgH* locus and transcriptional deregulation of cyclin D1 follows.<sup>22,23</sup> The prognostic relevance of these translocation-based markers is primarily based on the fact that they identify particular nosologic entities requiring different approaches. Moreover, in a study which included 247 patients with indolent follicular lymphoma the *Bcl-2* rearrangement site was an independent prognostic factor for FFS, useful for identifying patients requiring different treatments, the absence of the translocation defining the patients with the poorest prognosis.<sup>24</sup>

#### *t(8;14)(q24;q32) and t(9;14)(p13;q32)*

These translocations have been reported to identify two subsets of low-grade lymphomas characterized by indolent presentation. The first translocation is distinct from that observed in high-grade lymphomas,<sup>25</sup> while the second one characterizes low-grade lymphomas with plasmacytoid differentiation.<sup>26</sup>

### **Lymphoma-specific rearrangements**

Though not specific for a given lymphoma, these rearrangements have some prognostic impact when present. Usually, they are deletions or mutations of genes relevant for regulation of proliferative or apoptotic events.

#### *p53*

This molecule plays a key role in the activation of apoptosis especially when DNA damage can not be repaired. Mutations related to inactivation of the *p53* tumor suppressor gene are among the best studied in cancer. *p53* mutations have been described in several subtypes of lymphoid neoplasia, including indolent lymphomas, with a frequency of mutations ranging from 5% to 40%. In studies including 53 and 23-well characterized MCL patients, *p53* mutations predicted a poor outcome.<sup>27,28</sup> *p53* mutations are associated with a poor prognosis in indolent lymphomas in terms of both more aggressive presentation and progression to high-grade lymphoma types. In a series of 25 cases the co-expression of *p53* mutation and *Bcl-*

2 rearrangement could account for variation in the clinical behavior seen in indolent follicular lymphomas, and lead to an accelerated accumulation of genetic abnormalities and to progression.<sup>29</sup>

#### *Retinoblastoma gene product*

In MCL, *Bcl-1* rearrangement leads to overexpression of cyclin D1, which participates in the control of cell cycle progression by interacting with the retinoblastoma gene product (pRb), a well known tumor suppressor gene. In a series of 23 patients with MCL, pRb was expressed in all cases but no gene rearrangements could be detected by Southern blot analysis.<sup>30</sup> Though the expression of pRb correlated significantly with the proliferative activity of the lymphoma and blastic cases were associated with increased expression of the phosphorylated protein, pRb appeared normally regulated in relation to the proliferative activity of the lymphoma.<sup>30</sup> This suggests that cyclin D1 overexpression in MCL may overcome the suppressive growth control of a normal pRb.<sup>30</sup>

#### *TNF-α polymorphisms*

In a series of 273 lymphoma patients, genetic polymorphism leading to increased TNF production was a prognostic factor in high grade lymphomas, but not in follicular lymphomas.<sup>31</sup> However, due to relevance of the TNF/TNFRs complex as specified above,<sup>9,10</sup> the possible prognostic role of TNF/TNFRs polymorphisms in indolent lymphomas is worth further evaluation.

#### *Other abnormalities*

Homozygous deletion at chromosome 9p21 as well as gain of chromosome 7 have been reported to mark the progression from indolent to aggressive follicular lymphoma.<sup>32,33</sup> These chromosomal changes are considered to represent important secondary genetic events characterizing histologic progression in follicular lymphomas. In contrast, in a series including 149 patients with indolent lymphoma, translocation breaks were observed at 14q32 (66%), 18q21 (56%), 1q21-23 (17%), 1p32-36 (14%), 6q21-25 (13%), trisomy 3 (13%), trisomy 18 (9%), 8q24 (8%), trisomy 12 (8%), trisomy 7 or 21 or 11 (6%), but there was no correlation with survival duration or type of treatment.<sup>34</sup> In a series which included 45 cases of MCL (32 typical and 13 blastoid variants) increased number of chromosomal imbalances and high-level DNA amplifications were associated with blastoid variants and, in particular, increased number of gains, gains of 3q, 12q and losses of 9p were associated with a shorter survival of the patients.<sup>35</sup>

### **Immunophenotypic markers**

Several immunophenotypic methods are currently employed to analyze the expression of proteins on isolated cells or tissue sections in lymphoma. Following the production of a large panel of reagents

and the introduction of highly sensitive retrieval techniques for unmasking antigens on fixed samples, immunohistochemistry provides a practical and highly informative tool for studying large retrospective series of stored tissue samples. Besides having a relevant role in defining the precise nature of neoplastic cells, many immunophenotypic markers, which can often detect peculiar abnormalities in subsets of cases within a given entity, appear of value in the prognostic assessment of NHL. The markers discussed in this section must be evaluated within a cell population using a "quantitative" rather than a "qualitative" approach, so that criteria for their evaluation must be strictly defined and all the technical steps need to be carefully standardized.<sup>36</sup>

### **Proliferation markers**

A number of studies are available concerning the proliferation index of lymphomas based on the demonstration of S-phase by flow cytometry, the bromodeoxyuridine labeling index, and/or the analysis of the percentage of cells expressing antigens associated to the cell cycle. These last are best determined immunohistochemically using monoclonal antibodies which recognize the Ki-67 antigen or recombinant parts of its structure on cryostat sections, or, more recently, on paraffin embedded material.<sup>37,38</sup> According to most of these studies, when low-grade NHL were considered as a whole, elevated S-phase rate, bromodeoxyuridine, and/or Ki-67 labeling indices appeared to represent adverse prognostic parameters, often with independent statistical significance.<sup>39-46</sup> Nevertheless, when specific subtypes of low grade NHL are considered separately this is not always confirmed. Contrasting data have been provided on the prognostic significance of the Ki-67 labeling index in FCL,<sup>47,48</sup> possibly because of the lack of reproducible criteria so far available on the grading of this subtype of low-grade NHL.

More informative data regarding the prognostic significance of cell proliferation markers will perhaps be gained in future studies if large series of specific subtypes of indolent NHL, precisely diagnosed on the basis of morphologic, immunophenotypic and molecular biological features of archive material are investigated.

### **Cell-cycle regulatory molecules**

Progression through the cell-cycle in eukaryotic cells is mediated by positive regulators of cell proliferation, such as cyclins and cyclin-dependent kinases (cdks), and negative regulators such as those encoded by the p53 and RB genes, as well as different cdk-inhibitors such as p21<sup>WAF1</sup>, p27<sup>KIP-1</sup>, p16<sup>MTS1</sup>, p15<sup>MTS2</sup> and others.<sup>49</sup> Because of their central role in regulation of cell proliferation these molecules are potentially involved in the pathogenesis of different types of lymphoma, and appear good candidates as prognostic markers.

### *Cyclin D1*

Overexpression of cyclin-D1 (also known as PRAD-1) is observed in the vast majority of MCL, as a consequence of the t(11;14).<sup>50,51</sup> The main prognostic relevance of the demonstration of nuclear overexpression of cyclin-D1 derives from its invaluable role in the diagnostic process of a specific subtype of NHL, i.e. MCL, which is characterized by a peculiarly poor prognosis. Immunohistology is currently effective and practical and is mandatory in the diagnosis of small/intermediate B-cell NHL that express the CD5 antigen. It must be taken into account that nuclear expression of cyclin-D1 can be observed, although at lower intensity, in hairy cell leukemia.<sup>52</sup>

### *p27<sup>KIP1</sup>*

p27<sup>KIP1</sup> is a cell cycle inhibitor that is able to engender cell cycle arrest in response to inhibitory stimuli, e.g. TGFβ or cAMP, lack of adhesion, cell contact inhibition. Moreover, its expression is required to coordinate cell cycle arrest during differentiation. In lymphoid tissues p27<sup>KIP1</sup> expression is inversely related to the proliferative index.<sup>53</sup>

Some data are already available on the possible prognostic relevance of p27<sup>KIP1</sup>. In a series of 105 NHL cases (high- and low-grade) low p27<sup>KIP1</sup> expression was significantly associated with a poor prognosis.<sup>54</sup> In fact, low-grade lymphomas, characterized by low proliferation indices, in fact strongly express p27<sup>KIP1</sup> in their nuclei. An interesting exception is hairy cell leukemia, an indolent B-cell lymphoproliferative disorder that is characterized by low proliferative activity, but absent or very low levels of p27<sup>KIP1</sup> expression.<sup>55</sup>

MCL is also characterized by a peculiar uncoupling of p27<sup>KIP1</sup> protein expression from the proliferative rate.<sup>56</sup> In fact, the expression of p27<sup>KIP1</sup> in MCL is variable, and its intensity is inversely related to prognosis.<sup>57</sup> A few data are also available on the prognostic significance of other cell-cycle regulators such as cyclin E<sup>54</sup> or the inhibitor p16INK4A, the expression of which seems to be associated with disease progression in mucosa-associated low-grade lymphomas and FCL.<sup>58</sup>

### *p53*

The possibility of detecting abnormal expression of p53 in human tumors by immunohistochemistry has been widely debated. In lymphomas, p53 overexpression (as defined by accumulation of immunoreactivity in a large proportion of nuclei) is observed in a relatively high number of high-grade B-cell NHL and MCL but only in a few cases of other NHL subtypes. In most studies p53 mutation and overexpression were significantly correlated with poor prognosis.<sup>59-66</sup> Interestingly, the combined immunohistochemical evaluation of p53 and p21<sup>WAF1</sup> (a cdk-inhibitor which is a mediator of p53 tumor suppression) is a valuable means of assessing the functional status of the p53

tumor suppressor gene product in NHL with potential application as a prognostic parameter in individual cases.<sup>67</sup>

### Conclusions

In spite of the large number of methodologic approaches currently used to investigate key biological features in NHL, available data on the identification of biological markers bearing independent prognostic significance in indolent NHL still appear immature. This is because, with a few exceptions, each individual marker has been mainly investigated in heterogeneous and often small series of patients, including high- and low-grade lymphomas and/or a variety of histologic subtypes. As a consequence, the possible prognostic power of individual markers in each subtype of indolent NHL might have been either missed or overestimated.

Confident identification of valuable prognostic markers in indolent NHL will derive from the accurate evaluation of properly selected and well standardized markers, investigated in large series of homogeneously treated patients with a given subtype of indolent lymphoma.

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## Alkylating agent therapy for follicular lymphoma

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Alkylating agents were first introduced into the therapy of malignant lymphoma almost half a century ago following the synthesis of chlorambucil at the Institute of Cancer Research. It rapidly became clear that chlorambucil was substantially more effective in those lymphomas with a longer natural history, namely, in the terminology of the time, follicular lymphoma and lymphosarcoma. Administered, orally, in low doses, continuously it resulted in regression of lymphadenopathy, gradually but usually incompletely in a substantial proportion of patients. It was observed early that such regressions were almost invariably apparent within six weeks. There is no evidence to suggest that cyclophosphamide, introduced shortly after chlorambucil yields different results.

There are many different schedules of dosage and duration of administration of alkylating agents given alone. Chlorambucil may be given in blocks of one week per month at a relatively high dose, in fortnightly pulses at somewhat lower doses, or continuously at lower doses still. The published overall response rate, using response criteria applicable in the 1970s and 1980s is approximately 80%, 25% being *complete* for patients receiving chlorambucil as the first therapy.

Evidence from Stanford indicates that continuing therapy to *maximum response* leads to a higher complete remission rate than if therapy is arbitrarily stopped after a given length of time. All the data, however, support the contention that the treatment is, for most people with follicular lymphoma excellent palliation, but rarely curative, the repeatedly *responsive* nature of the illness obscuring the early benefit of the advantage of *complete over good partial* remission. Hence it is very difficult to produce compelling evidence for one regimen being better than another. There is, however no evidence to support continuous, indefinite administration of alkylating agents. Although *maintenance* chlorambucil has been shown to improve the duration of first remission, it makes no impact on overall survival. Furthermore, excessive continuous administration of alkylating agents has been clearly demonstrated to predispose

to both myelodysplasia and second malignancy.

Not only is alkylating agent (alone) therapy effective at the initial presentation of follicular lymphoma: repeated responsiveness to the same therapy at least three times is the rule rather than the exception, provided transformation to large B cell lymphoma has not occurred, according to single center data derived from a relatively small number of closely observed patients.

Do these findings imply that chlorambucil or cyclophosphamide alone should be the treatment of first choice, at least at the first indication for therapy for all patients with follicular lymphoma? Not necessarily. On the one hand, they reflect the success of the use of a group of drugs which, used intelligently, may have increased the survival of patients from 5-10 years compared to the time before they were available. On the other, the lack of compelling evidence that any early intervention, with more drugs, higher doses of drugs or the use of biological therapy, in combination with moderately aggressive chemotherapy (which in meta-analysis has been reputed to confer some survival advantage) has led to cure or a major change in the shape of the survival curve is disappointing. It still raises the question as to whether, however boring it may be, chlorambucil alone should be the control arm of randomized trials.

Be that as it may, the opportunity for developing more refined therapeutic strategies, whether curative or palliative, with possibly more sensitive and relevant end points is greater now than since the alkylating agents were introduced.

From the chemotherapy standpoint, the purine analogs alone or in combination offer an alternative to alkylating agents. *Megatherapy* with hemopoietic stem cell rescue induces long remissions but carries a risk of myelodysplasia.

Interferon may become established as a component of *conventional* therapy. It is possible to induce regression of disease with comparatively non-toxic antibody therapy which has now been licensed: antibodies may be used to deliver irradiation with exciting results. The technology is now available for constructing DNA vaccines, clinical trials of which are in progress.

Will these advances supplement or complement the alkylating agents? Time will tell.

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## Conventional treatment of indolent lymphomas: role of CHOP or CHOP-like chemotherapy

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The Working Formulation<sup>1</sup> divided all non-Hodgkin's lymphomas into three prognostic grades, low, intermediate, or high, based on survival after therapy. However, many clinical investigators subsequently found it more convenient to divide the non-Hodgkin's lymphomas into two categories, indolent and aggressive. In this frame of reference, the indolent non-Hodgkin's lymphomas frequently included Working Formulation categories A, B, C, D, and E. The new REAL Classification<sup>2</sup> attempts to define actual disease entities. The indolent behaving group of diseases would include B-CLL/SLL, lymphoplasmacytoid, mantle cell, follicle center, and marginal zone B-cell lymphomas. These indolent lymphomas were generally characterized as being malignancies of small B lymphocytes, which had low proliferation rates with a high proportion of resting cells. Frequently there was nodal and bone marrow involvement, initial sensitivity to chemotherapy and radiotherapy, but increasing resistance to treatment with each relapse and finally a tendency to transform to a more aggressive large cell lymphoma.

In the early 1990s, the clinical presentation and natural history of these newly recognized pathologic entities had not been studied in large series of uniformly treated patients to determine whether these entities differ clinically from the other relatively indolent lymphomas with which they have been previously classified. The *Lymphoma Committee of the Southwest Oncology Group (SWOG)*, therefore, reviewed the pathology and clinical course of 376 previously untreated patients with advanced stage disease of Working Formulation categories A, B, C, D, or E, who received CHOP in SWOG studies 7204, 7426, or 7713.<sup>3</sup> This analysis benefited from the fact that the median follow-up was 16.5 years and that all patients received treatment with the best available current therapy for aggressive lymphomas.<sup>4</sup>

All of the 376 cases were reviewed by three lymphoma pathologists (Drs. Bharat Nathwani, Peter Banks, and Thomas Grogan) and a consensus diagnosis was reached. The diagnosis of mantle cell lymphoma was made in 36 patients (10%) and the diagnosis of marginal zone lymphoma was made in 43 patients (11%). After forty-nine other cases had been excluded, 248 cases remained in WF category A

through E. WF A included 54 patients, WF B 127 patients, WF C 29 patients, WF D 24 patients, and WF E 14 patients.

### Clinical results

#### Mantle cell lymphoma

The clinical characteristics of the patients with mantle cell lymphoma were compared to those of the remaining 248 patients in WF categories A through E. Median age of both groups was 55 years with a range from 18-81 years. There was a male predominance in patients with mantle cell lymphoma (81%) compared to those with WF A-E (54%) ( $p=0.009$ ). The failure-free survival for the 36 patients with mantle cell lymphoma was significantly shorter than that of the 248 remaining patients with WF A-E. The 10-year failure-free survival estimate was only 6% compared to 25% for WF A-E ( $p=0.0002$ ). The overall 10-year estimated survival was also significantly lower for the mantle cell lymphoma patients (8%) than that of the patients with WF A-E (35%) ( $p=0.0001$ ). In fact, the failure-free survival and overall survival estimates for the patients with mantle cell lymphoma were lower than those for WF A, WF B, WF C, WF D, or WF E when examined as separate groups.

#### Marginal zone B-cell lymphoma

The characteristics of the marginal zone lymphoma patients were very similar to those of the remaining patients in WF A-E with a median age of 51 years (range: 23-76) and 51% being male. However, the percentage of patients with gastrointestinal involvement was higher in the marginal zone group (23%) than in the remaining WF A-E patients (4%) ( $p<0.001$ ). In addition to the fact that the MALT lymphomas were mucosa-based and all extranodal, while the monocytoid B-cell lymphomas were node-based, the subclassification of the marginal zone lymphomas into MALT lymphomas and monocytoid B-cell lymphomas failed to demonstrate any significant differences in clinical presentation except that the MALT lymphoma group did have more patients with gastrointestinal involvement than the monocytoid B-cell group (8/19, 42% vs. 2/21, 10%;  $p=0.03$ ). The extranodal sites of involvement for the MALT lymphomas included: 8 gastrointestinal tract, 4 skin, 2 parotid, 2 lung, 2 breast, and 1 nasopharynx. Nodal involvement was found in 15/19 (79%) of

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MALT lymphomas.

The failure-free survival of the 43 patients with marginal zone lymphoma was similar to that of the 248 remaining patients with WF A-E. The 10-year failure-free survival estimate was 36% compared to the 25% for WF A-E ( $p=0.26$ ). The overall 10-year estimated survival was also not significantly reduced for the marginal zone lymphoma patients (39%) compared to that of the patients with WF A-E ( $p=0.83$ ). Furthermore, if one prefers to compare the failure-free survival and overall survival for the 43 MZL patients to that of the 210 patients with classically defined low-grade lymphomas (WF A, B, and C), the results were also similar ( $p = .22$  and  $.89$ , respectively).

The subclassification of the marginal zone lymphomas into MALT lymphoma and monocytoid B-cell categories did allow recognition of significant differences in failure-free survival and survival. The 10-year estimated failure-free survival for patients with MALT lymphoma was 21% compared to 46% for monocytoid B-cell lymphoma ( $p=0.009$ ). The overall 10-year estimated survival for patients with MALT lymphoma (21%) was significantly lower than that of patients with monocytoid B-cell lymphoma (53%) ( $p=0.007$ ). Although the failure-free survival of MALT lymphoma patients was not significantly reduced compared to that of patients with WF A-E ( $p = 0.12$ ), overall survival was reduced ( $p = 0.02$ ).

#### **Small lymphocytic lymphoma and follicular lymphoma**

If one wishes to subdivide the remaining indolent lymphomas further, it becomes evident that WF A or small lymphocytic lymphoma has a shorter time to treatment failure and overall survival than the follicular lymphomas WF B, C, or D. The 54 WF A patients had a 13% 10-year failure-free survival (FFS) and a 19% 10-year overall survival (OS) compared to 28%, 35%, and 39% FFS and 42%, 38%, and 44% OS for WF B, C, or D, respectively. Although there may be early differences in the failure-free or overall survival of WF B, C, or D, these differences disappear with prolonged follow-up.<sup>5</sup> Furthermore, studies have demonstrated that it is extremely difficult for even expert hematopathologists to reproducibly subdivide the follicular lymphomas into WF B, C, or D6.

#### **Treatment recommendations**

Treatment recommendations for the small lymphocytic lymphomas remain controversial to say the least. Patients with advanced stage indolent non-Hodgkin's lymphoma may be treated with various approaches ranging from deferred initial therapy (watch and wait), single agent alkylating agents, radiation therapy, to combination chemotherapy. Each can be effective when used by experienced clinicians. None has yet produced curative therapy. In general, CHOP or CHOP-like regimens are more toxic and therefore are not indicated for the initial treatment of indolent lymphomas unless patients need rapid

tumor debulking. Unfortunately the prognosis for these patients has not changed in over twenty years. Median survivals of only 5-10 years cannot be considered good. Clearly innovative treatment strategies are needed. The use of cytokines such as interferon; monoclonal antibodies with or without radioisotopes or toxins, purine analogs, and even high dose therapy with stem cell rescue are all under investigation.

The facts that patients with advanced stage, mantle cell lymphoma have a high proliferative rate resulting from overexpression of cyclin D1, the shortest median survival of all these patients, and that all have died demonstrates conclusively that patient with mantle cell lymphoma should be categorized as having an aggressive lymphoma. Given that the patients in these studies were treated with CHOP, it is also clear that standard therapy for aggressive lymphomas does not produce durable long-term remissions and cure in mantle cell lymphoma. These patients are, therefore, candidates for innovative treatment approaches. Several such approaches being studied include dose intensification with colony-stimulating factor support, and high dose therapy with stem cell rescue. The results are eagerly awaited.

In contrast, patients with advanced stage, marginal zone B-cell lymphoma have a clinical course similar to that of other patients in WF A-E although patients with advanced stage MALT lymphomas may have a more aggressive course than previously recognized. This latter conclusion awaits data from larger numbers of patients with advanced stage MALT. Today, patients with advanced stage marginal zone lymphoma can be managed as patients with any other indolent lymphoma.

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## Purine analogs in the management of indolent lymphoproliferative disorders

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The two major low grade lymphoproliferative disorders seen in clinical practice are low grade lymphoma [including follicular lymphoma (FL) and small lymphocytic lymphoma (SLL), and chronic lymphocytic leukemia (CLL)]. CLL is the systemic counterpart of SLL. For many years, conventional treatment comprised alkylating agent-based regimens using chlorambucil or cyclophosphamide as single agents or combined with corticosteroids, vincristine or doxorubicin. Now a new group of drugs has become available for clinical research, namely the purine analogs. The first of these to be studied was deoxycoformycin (DCF), then more recently 2-chlorodeoxyadenosine (2-CDA), and fludarabine. The activity of these agents in lymphoid malignancies became apparent in phase I clinical trials<sup>1-3</sup> and subsequently was confirmed in extended phase II clinical trials.<sup>4-6</sup> DCF differs from 2-CDA and fludarabine in that it is a potent inhibitor of adenosine deaminase (ADA). ADA is a key enzyme in the purine salvage pathway as it catalyzes the irreversible deamination of adenosine to inosine through an intermediate transition state. On the other hand, 2-CDA and fludarabine are poor substrates for ADA and these drugs work through different mechanisms of action<sup>7</sup> (illustrated in the article by Dr. Plunkett in this symposium).

While active in a broad range of lymphoid malignancies, DCF has predominantly been studied in hairy cell leukemia in which it was demonstrated to be more potent than interferon in achieving complete remissions (CR) and to be active in interferon-refractory patients.<sup>8</sup> 2-CDA also entered the arena of clinical research predominantly as an agent in hairy cell leukemia producing a high rate of CR with a single infusion of therapy.<sup>9</sup> Fludarabine, on the other hand, was registered for use as an agent for previously treated patients with refractory and relapsed CLL.<sup>10</sup> The spectrum of activity of these agents has now been broadened and all are known to be active in both low grade lymphoma and CLL.

### Chronic lymphocytic leukemia

Before fludarabine was demonstrated to be a major new agent in CLL, most patients received initial therapy with chlorambucil either as a single agent

or combined with prednisone.<sup>11</sup> Some studies utilized cyclophosphamide as the alkylating agent usually combined with vincristine and prednisone (CVP).<sup>12,13</sup> Subsequently investigators evaluated the role of doxorubicin in combination with cyclophosphamide, vincristine, and prednisone in CAP, CHOP and mini-CHOP regimens.<sup>14-16</sup> French investigators demonstrated that, compared to the CVP regimen, mini-CHOP was able to improve the survival of Binet stage C patients and this latter regimen has continued to be explored.<sup>16</sup> However, once patients became refractory to alkylating agent regimens, there was no effective therapy available. Fludarabine was first agent demonstrated to be active in low grade lymphoma and CLL.<sup>3</sup> Twenty-two CLL patients were treated (median age of 63 years), eight patients having Rai stage III-IV disease. Myelosuppression was the most frequent toxicity. One patient achieved a complete remission and three a partial response.

The next major study used fludarabine as a single agent in previously treated and refractory patients with CLL.<sup>10</sup> Sixty-eight patients received fludarabine as a single agent at 25-30 mg/m<sup>2</sup>/day for five days. Using older criteria for response, 10 patients obtained a CR and 30 a partial remission (PR) for an overall response rate of 59%. The response rate in Rai stage III disease was 58% and Rai stage IV disease 50%. Applying the NCI Working Group criteria to these data showed a difference in response rate with the CR rate being 14%, nodular PR, using NCIWG criteria, 24% and 28% a PR. Major conclusions from that study was that fludarabine was an important new agent in the management of previously treated patients with CLL with the major toxicities being myelosuppression, immunosuppression and the development of infection. The major infections of concern were episodes of pneumonia and fevers of unknown origin. Septicemia was uncommon. This study was followed at MDACC by a series of other confirmatory studies; five days of fludarabine combined with prednisone gave an overall response rate of 52% in 169 patients, once a week fludarabine obtained a response rate of 24% in 46 patients and three-day fludarabine a 46% response rate in 80 patients.<sup>17-19</sup> In none of these studies was it possible to demonstrate that there was a survival advantage of fludarabine as a single agent compared to earlier anthracycline-containing combinations. Other studies have confirmed the response rate in previously

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treated patients with CLL with responses varying from 31–67%.<sup>20</sup>

A randomized, comparative trials of fludarabine with the CAP regimen (cyclophosphamide, doxorubicin, and prednisone) was conducted.<sup>21</sup> The study was multi-national. Patients with previously untreated Binet stage B or C disease or relapsed B-CLL previously treated with chlorambucil or non-anthracycline regimens were included. Patients received either fludarabine 25 mg/m<sup>2</sup>/day, days 1–5 or CAP (cyclophosphamide 750 mg/m<sup>2</sup>/day on day 1, doxorubicin 50 mg/m<sup>2</sup>/day on day 1, and prednisone 40 mg/m<sup>2</sup>/day on days 1–5). Both treatments were given for a total of six courses. One hundred previously untreated patients were entered into the study together with 96 previously treated patients. The overall response for fludarabine was 60% versus 44% for CAP ( $p=.02$ ). The response rate for fludarabine was higher in both untreated and previously treated patients but the result was not significantly superior in the untreated patients. However, 48% of patients previously treated responded to fludarabine versus only 27% for CAP ( $p=.04$ ). There was no survival advantage in the overall study or in the various treatment subcategories although significantly longer event-free survival was noted for fludarabine versus CAP ( $p=.01$ ). Subsequent randomized clinical trials have been reported in abstract form. In the NCI Intergroup Study with large numbers of patients, the overall response rate was 70% versus 43% for fludarabine versus chlorambucil at a dose of 40 mg/m<sup>2</sup> as a single dose every four weeks.<sup>22</sup> The CR rate using the revised NCIWG criteria<sup>23</sup> was 27% for fludarabine versus 3% for chlorambucil.

Fludarabine has also been used as initial therapy in CLL for patients with progressive stage I-II disease or advanced stage disease. Seventy-one patients received fludarabine alone and in 103 patients, fludarabine was combined with prednisone.<sup>17</sup> The CR rate was 29% with another 32% obtaining a nodular partial response. The overall response rate was 79%. The  $\beta_2$ -microglobulin level, age, and immunoglobulin levels were associated with probability of achieving complete remission. No survival advantage was demonstrated between the CR patients and nodular PR patients although the CR patients had a longer time-to-progression. No significant survival advantage compared to that obtained with two preceding historical treatments at the M. D. Anderson Cancer Center, namely CAP and POACH, has been demonstrated.<sup>24</sup>

### **2-chlorodeoxyadenosine (2-CDA)**

2-CDA was reported as an effective new agent for the treatment of CLL in 1988.<sup>25</sup> Subsequently the series was expanded and 90 patients received 2-CDA as a 0.1 mg/kg/day continuous infusion for seven days (0.14 mg/kg/day) or as a bolus infusion for five days. Most patients had Binet stage C disease. These patients had also been extensively previously treated

and six patients had previously failed to gain any benefit from fludarabine. The response rate was 44% with only 4% obtaining a CR.<sup>26</sup> Myelosuppression was the most prominent toxicity with opportunistic infections such as *Listeria*, *cryptococcus*, and herpes viruses being associated with significant infections. Subsequently other authors confirmed the activity of 2-CDA in previously treated patients with response rates varying from 31–67%.<sup>20</sup> 2-CDA has also been used either intravenously or orally as initial therapy in small subsets of patients. The overall response rate is in the range of 70–85%.<sup>27,28</sup>

DCF has been studied in small subsets of patients as a single agent predominantly in previously treated patients. The response rate has varied between 15–35%.<sup>20</sup> In a group of 13 patients who received DCF as initial therapy, six obtained a partial response. Thus, it is obvious that all three agents have substantial activity. Fludarabine has been most extensively investigated as a single agent and combined with corticosteroids in CLL; pentostatin and 2-CDA have been less studied, at least in the United States. All three agents share problems of immunosuppression.

### **Combination studies**

As purine analogs do not appear to be curative in CLL, a number of combination approaches have been evaluated. Mitoxantrone or cyclophosphamide has been added to combinations of corticosteroids and fludarabine. The rationale behind this is that fludarabine inhibits the repair of DNA damage associated with the use of mitoxantrone or alkylating agent regimens.<sup>7</sup> The combination of fludarabine, mitoxantrone, and dexamethasone (FND), discussed subsequently, is highly active in low grade lymphoma. A regimen was developed for the treatment of CLL consisting of fludarabine 30 mg/m<sup>2</sup>/day for three days and mitoxantrone 10 mg/m<sup>2</sup> on day 1. No corticosteroids were used in the CLL studies as there is no evidence that corticosteroids provide additional benefit to fludarabine in CLL. Indeed the rate of opportunistic infections by *Pneumocystis carinii* and *Listeria* is increased by the addition of corticosteroids.

The response rate to fludarabine and mitoxantrone (FM) was not significantly different to that seen with fludarabine alone or combined with prednisone. The combination of fludarabine with cyclophosphamide (FC) utilizes fludarabine 30 mg/m<sup>2</sup>/day for three days and cyclophosphamide 300 mg/m<sup>2</sup>/day for three days.<sup>29</sup> The response rate to FC in all the categories in which it has been evaluated is superior to that for single agent regimens. The combination is not associated with any hair loss and the major morbidity is episodes of infection which are more prominent in patients with extensive prior therapy. The response rate compared to that of fludarabine is illustrated in Table 1.

The rate of cyto-reduction with the FC combination is more rapid than that with the single agents. The incidence of infections is similar to that caused by the

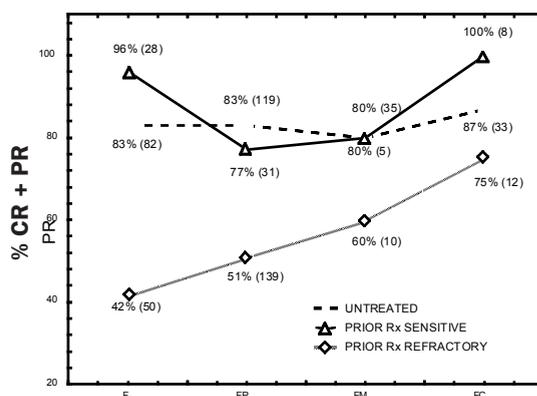
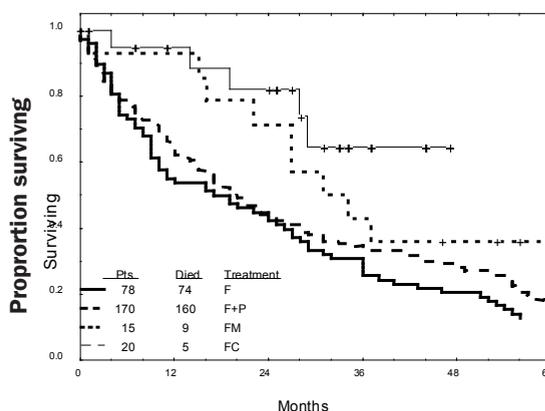
**Table 1. Comparison of fludarabine versus fludarabine+CTX.**

Group	% Overall response		p value
	Fludarabine	Fludarabine + CTX	
Untreated	79	88	NS
Alkylators	56	85	.007
Fludarabine-sensitive	30	72	<.001

single agents. Additional morbidities include nausea which occurs in 30–40% of patients with vomiting in 5–10%. Rashes occur in 7% of patients. Additional studies have utilized GMCSF to try and prevent infections and enhance the activity of antigen-presenting cells. More recently FC has been combined with amifostine to decrease toxicity to normal marrow cells. The results of these studies are still pending. No significant difference in complete remission rate or overall response rate has been found in patients receiving FC as initial therapy compared to fludarabine as a single agent (Figure 1). There is a suggestion of an improved time-to-treatment failure but the study is still in an early stage. On the other hand, in previously treated patients there is evidence that FM and FC both enhance survival compared with F + FP (Figure 2). Patients received these regimens as initial salvage therapy after having been treated with alkylating agents but not having previously received any purine analogs. The survival advantage is more marked in patients who are alkylating agent refractory than in patients who have merely received alkylating agents and are possibly still sensitive. Subsequent studies will explore combination approaches including monoclonal antibodies such as rituximab.

### Low grade lymphoma

Low grade lymphomas traditionally included follicular center-cell lymphomas such as follicular small cleaved cell (FSC) and follicular mixed cell lymphomas. The other category is small lymphocytic leukemia. The REAL classification and the *to be published* World Health Organization classification will allocate these and other lymphomas to the low grade lymphoma or indolent lymphoma category. These are accumulative diseases rather than proliferative diseases. The discoveries that bcl2 protein causes resistance to apoptosis or programmed cell death and that the 14;18 translocation juxtaposes the bcl2 oncogene with the immunoglobulin heavy chain resulting in dysregulation of bcl2 have been pivotal.<sup>30</sup> Rearrangement occurs in 80–85% of follicular lymphomas. The abnormal bcl2 gene provides a marker which can be used to follow the disease by use of the polymerase chain reaction (PCR). This enables the detection of one rearranged cell among 10<sup>5</sup> cells. Most patients, even those in complete clinical remission, can still be found to have cells with the bcl2

**Figure 1. Response to fludarabine regimen: untreated, prior Rx sensitive, and prior Rx resistant.****Figure 2. Survival CLL prior Rx by treatment.**

rearrangement.<sup>31</sup> This has led to interest in the use of bcl2 PCR studies as surrogate endpoints for clinical response. The staging system that is commonly used is the Ann Arbor Staging System with patients in stage I and II being candidates for curative therapy. Most patients are, however, in stage IV, involvement of bone marrow being common. Application of the International Prognostic Index for lymphomas has not been very useful as stage and performance status are not very applicable to most patients with low grade lymphoma. Other important prognostic factors are serum  $\beta_2$ -microglobulin level and tumor bulk.

Small lymphocytic lymphoma (SLL) is a difficult disease to treat. SLL is not associated with the t(14;18) or rearrangement bcl2. Bcl2 is, however, overexpressed in the majority of cases. The majority of patients with SLL present with stage IV disease.

### Conventional therapy

Most patients with stage I and II disease are treated with involved field radiation therapy. These patients have real prospects of cure;<sup>32</sup> however, very late relaps-

es do occur. Total lymphoid irradiation and combined chemoradiation have been studied in stage I and II low grade lymphomas but have not been established as superior at this point.<sup>33</sup> Complete remissions can be obtained in stage III LGL by total lymphoid irradiation and combined chemotherapy<sup>34</sup> and long term relapse-free survival rates are approximately 60%. Within the follicular lymphomas, it appears that follicular mixed cell lymphoma is more likely to benefit from combination chemotherapy than single agent therapy.<sup>35</sup> The classification of the disease into follicular mixed cell (FMC) or follicular large cell (FLC) lymphoma is not always clear.

### Newer therapies

Purine analogs have been extensively used in low grade lymphoma. Both fludarabine and 2-CDA have marked activity in previously treated patients with relapsed or refractory LGL. Salvage regimens with fludarabine have given response rates varying from 30–75% and with 2-CDA 43–88%.<sup>36</sup> Pentostatin is also active with response rates <30%.<sup>36</sup> Marked single agent activity has led to combinations being developed. Studies are underway of 2-CDA combined with mitoxantrone and other agents.<sup>37</sup> At the M.D. Anderson Cancer Center, a phase I clinical trial of fludarabine, mitoxantrone, and dexamethasone (FND) resulted in an overall response rate of 71% with 43% complete remissions. In the phase II trial on 51 patients, 94% responded with 47% obtaining a complete remission.<sup>38,39</sup> Subsequently this regimen has been applied in a front line randomized comparison with the alternating triple therapy (ATT) regimen, an aggressive, rotating multi-drug combination.<sup>40</sup> These regimens are being compared in a randomized fashion in patients with bcl2 rearrangement. The overall response rate in both arms of the study is above 95% with more than 80% obtaining clinical complete remissions. It appears that two-thirds of the patients also become PCR negative for the bcl2 rearrangement.<sup>41</sup> Prophylaxis with cotrimoxizole should be given to lymphoma patients receiving purine analogs and corticosteroids. A combination of fludarabine and cyclophosphamide has been used in 27 patients: all patients responded and 89% obtained CR.<sup>42</sup>

### Molecular remission

Most patients treated with regimens varying from chlorambucil up to CHOP do not become PCR negative.<sup>43</sup> PCR negativity has been obtained using standard dose chemotherapy followed by high dose therapy and autologous bone marrow transplantation at the Dana Farber Cancer Institute. Patients becoming PCR negative after the transplant have a significantly longer failure-free survival.<sup>44</sup> Sixty-eight percent of patients became PCR negative in the peripheral blood after treatment with the aggressive ATT regimen.<sup>40</sup> The advent of targeted therapy with monoclonal antibodies such as rituximab<sup>45</sup> has led to great interest in combining these agents with chemotherapy. The present

MDACC study is examining the use of FMP followed by rituximab versus FMP with simultaneous rituximab. Early reports of this study show no difference in tolerance or toxicity. Campath-1H antibodies are effective in irradiating peripheral blood and bone marrow disease but early studies in low grade lymphoma were associated with toxicity.<sup>46</sup> Other studies on the use of radioimmunotherapy with antibodies directed predominantly against CD20 are also underway. Thus the purine analogs have assumed a significant place in clinical trials. In addition to the studies reported with mitoxantrone and cyclophosphamide other investigators are evaluating the use of purine analogs combined with idarubicin.

### Conclusions

Purine analogs, despite our lack of knowledge about their mechanism of action have obvious marked activity in low grade lymphoproliferative diseases. Within a decade they have moved from being salvage therapy to being studied as frontline treatment. Fludarabine has been established as the most active single agent in CLL and combination programs are being used as front line therapy at institutions such as the MDACC. Further insights into the mechanism of action of the purine analogs promise to lead to further advances in this group of diseases.

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## The role of radiation therapy

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In the Revised European American Lymphoma Classification the non-Hodgkin's lymphomas (NHL) shown in Table 1 are recognized as indolent lymphomas.<sup>1,2</sup>

Follicular small cleaved cell NHL (FSCC NHL, follicular grade I) is the most common of the indolent lymphomas, but extranodal marginal zone B-cell lymphomas (MALT) are also frequently seen in clinical practice; both subtypes will be discussed in this review.

### Follicular small cleaved cell NHL, stage I-II

Many published studies have demonstrated the efficacy of radiation therapy in the treatment of clinically staged patients with localized follicular small cleaved cell, non-Hodgkin's lymphoma. Patients with early stage disease are curable with local-regional irradiation. The updated series from Stanford University details the results of radiation therapy in 177 patients treated over a 30+ year period.<sup>3</sup> Out of the 177 patients, 73 had stage I disease and 104 had stage II indolent NHL. Staging laparotomy was performed in 25% of patients and 20% of patients had extranodal presentations, Total nodal irradiation/subtotal nodal irradiation (TLI/STLI) was given to 41 patients and involved field (IF) or extended field (EF) irradiation was delivered to 133 patients. Staging laparotomy and total nodal irradiation were used in the early years of the study. Histology was follicular grade 1 in 57% of cases and follicular grade 2-3 in 43% of patients. The median follow-up was 7.7 years. The 5-year, 10-year, 15-year and 20-year survivals were 82%, 64%, 44%, and 35%, respectively. The 5-year, 10-year, 15-year and 20-year disease-free survivals were 55%, 44%, 40%, and 37%, respectively. Only 5/47 patients followed for 10-years or longer have relapsed. This study demonstrates that a substantial percentage of patients with early stage FSCC NHL never have their disease permanently controlled by local-regional irradiation.

The techniques of radiation therapy (field arrangement and size, and dose) selected for the treatment of non-Hodgkin's lymphoma are guided by the histologic subtype, the stage of disease, and the patterns of failure. Early-stage indolent lymphomas treated with RT alone have been shown to relapse in

extra-nodal sites or in nodal sites distant from the radiation fields when IF or EF radiation therapy has been used. This observation has led some researchers to recommend TLI for patients with FSCC NHL. Several factors, however, argue against the use of such extensive radiation therapy for patients with early stage indolent lymphomas. On multi-variant analysis, for patients with stage I-II disease, there is no evidence that the use of extended-field or TNI provides for a survival advantage compared to involved-field or regional irradiation. There are concerns about increased toxicity and the possibility that subsequent treatment, if needed, will be compromised as more than 50% of stage I-II patients eventually will develop a recurrence and require more aggressive treatment.<sup>3</sup> In addition, there is a high conversion rate of FSCC NHL to a more aggressive histology over time, requiring treatment with chemotherapy.<sup>4,5</sup> Prior treatment with TLI may compromise marrow reserve and limit subsequent multi-agent chemotherapy given either for recurrent indolent NHL or for NHL transformed to a more aggressive histology. Also, there is an increased risk of late complications including second cancers with large field irradiation.<sup>3</sup> Finally, there is an increasing role for high dose chemotherapy and total body irradiation (with autologous hematopoietic stem cell rescue) in the management of recurrent disease. Such treatment may be difficult to deliver after TLI. All these considerations make TLI unattractive as an initial therapy for early stage indolent lymphoma.

For patients with stage I-II FSCC NHL, our current recommendations include the use of regional radiation therapy fields. This strategy consists of irradiating the involved nodal region plus one additional

**Table 1. Indolent lymphomas: REAL classification.**

#### Indolent nodal lymphomas

- Follicular grade 1 (small cell)
- Follicular grade 2 (mixed)
- Follicular grade 3 (large cell)

#### Indolent extranodal lymphomas

- Extranodal marginal B-cell lymphomas (MALT)

#### Indolent disseminated leukemia/lymphoma (CLL)

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uninvolved region on each side of the involved nodes. For example, the treatment field for lymphoma of the inguinal nodes would include the ipsilateral femoral, inguinal, and external iliac nodes. The treatment of a stage I lymphoma of the right supraclavicular nodes would at least include the ipsilateral axillary, supraclavicular and cervical nodes. The cervical, supraclavicular, oropharyngeal, and nasopharyngeal nodes would be irradiated in patients with involvement of Waldeyer's ring. The recommended dose for patients with follicular small cleaved cell, non-Hodgkin's lymphoma is 3,000-3,600 cGy with a boost to areas of initial involvement to 3,600-4,000 cGy. When there is a possibility of significant morbidity from treatment, such as long-term xerostomia from irradiation of the majority of the salivary glands, lower doses to the uninvolved nodal areas are recommended (i.e. 2,500-3,000 cGy).

The role of combination chemotherapy in the management of early-stage follicular lymphoma is unclear. At least three randomized studies conducted in the 1970s failed to demonstrate that non-adriamycin containing combination chemotherapy regimens plus radiation therapy were superior to radiation therapy alone.<sup>6-8</sup> Two more recent studies report on the use of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy in addition to radiation therapy for stage I and II low grade lymphomas.<sup>9,10</sup> In one study there was an improved freedom from relapse favoring the initial combined modality therapy; in the other study no significant differences were seen. Neither study demonstrated differences in survival. In part, the choice of therapy may lie in the careful assessment of prognostic factors. Most patients with Ann Arbor clinical stage I or II follicular small cleaved cell and follicular mixed lymphomas should have a good prognosis following local-regional radiation therapy alone. For patients whose prognosis is less certain, such as patients with stage II disease with multiple sites of involvement or bulky nodes, or patients with follicular large cell histology, chemotherapy followed by involved field irradiation may provide more durable remissions.

#### **Follicular small cleaved cell NHL, stage III-IV**

The optimal treatment strategy for patients with advanced-stage low-grade lymphoma is controversial.<sup>11</sup> Treatment has followed one of two divergent approaches; an aggressive approach that may include extensive radiation therapy, combination chemotherapy, or both, and a conservative approach that consists of no initial treatment followed by a palliative single-agent chemotherapy or involved field radiotherapy when treatment is needed. Although many years of clinical investigation have failed to prove that immediate aggressive therapy improves survival compared to conservative therapy,<sup>11</sup> the median survival of only 7 to 8 years from diagnosis in patients with stage III-IV disease has prompted the continuation of

aggressive treatment approaches.<sup>12-14</sup> Advanced-stage indolent lymphomas are responsive to single and multiple-agent chemotherapy, radiation therapy, and combined modality treatment approaches.<sup>11</sup> Unfortunately, the responses are not durable, lasting a median of only 2 years. In many studies, less than 10% of patients with low-grade lymphomas remain in remission for 5 years or more. Despite the indolent nature of follicular lymphomas, most patients ultimately die of their disease. Of 147 previously untreated patients enrolled at St. Bartholomew's Hospital in various protocols, ranging from no initial therapy to conservative treatment with single alkylating agents, only 53 of 147 patients remain alive; 94 patients have died and only 18 patients died from causes unrelated to lymphoma.<sup>13</sup>

Radiation therapy in the treatment of patients with stage III-IV FSCC NHL serves two roles, as palliative treatment and as part of a potentially curative approach in clinical trials. Radiation therapy has much to offer as a palliative approach. For patients with isolated disease that has returned after remission or has not responded as well as other sites to chemotherapy, involved field irradiation can allow patients to go significant periods of time without the need for additional combination therapy. Furthermore, indolent NHL can produce troublesome symptoms through bone or spine involvement, through pressure on peripheral nerves, by disfiguration via large neck or axillary nodes, by intraorbital involvement, and through many other mechanisms. Indolent NHL is very sensitive to radiation therapy and often nodes resistant to chemotherapy will begin to shrink after 4-5 radiation treatments. Sometimes even a large volume of lymph nodes can be encompassed because the low doses of radiation needed result in minimal toxicity to normal tissues.

As a curative approach, total nodal irradiation combined with chemotherapy has been used for the treatment of stage III patients in past studies. McLaughlin and colleagues reported on the use of CHOP chemotherapy and TLI in 74 patients with follicular lymphomas. The relapse-free survival at 5 years was 52%. This study did not provide sufficient evidence that patients were cured by this approach.<sup>15</sup> The NCI initiated a prospective randomized study comparing conservative treatment (no initial therapy) with aggressive combined modality therapy with ProMACE/MOPP CT followed by low-dose (24 Gy) total lymphoid radiation therapy.<sup>16</sup> Eighty-nine patients were randomized. The disease-free survival was significantly higher in the combined modality therapy group at 4 years (51% vs 12%), however, no differences in overall survival were seen. As these studies have failed to show a survival advantage for an initial aggressive approach in the management of patients with advanced stage indolent lymphomas, new approaches are being developed that for the most part do not include the use of TLI.

High-dose cyclophosphamide and total body irradiation followed by autologous bone marrow transplantation (ABMT) has been tested by a number of groups in patients with low-grade lymphomas either in high risk patients as up-front treatment or for patients who relapse.<sup>17-20</sup> The latter approach is more common. Trials have used donor bone marrow that has been *purged* of malignant cells by multiple cycles of *ex vivo* treatment with specific antibody and complement in combination with this approach.<sup>17,21,22</sup> There is some data to suggest that this may be the preferred approach over the use of non-purged marrow. Now that long-term data following autologous bone marrow transplantation are becoming available there are data to suggest that the median survival from diagnosis may be higher in patients who undergo ABMT than in patients treated with conventional management (unpublished data, from Dr. Arnold Freedman, Boston, MA, USA).

Finally, advances in monoclonal antibody based therapies, with or without radioisotopes, provide hope for patients with advanced stage indolent NHL. Please see the excellent review by Multani and Grossbard.<sup>23</sup>

#### **Molecular biology in detecting occult disease**

Techniques that detect the presence of the t(14;18) and other translocations may serve as very sensitive indicators of minimal residual disease in the bone marrow or peripheral blood<sup>24</sup> and as a measure of the efficacy of *in vitro* purging of bone marrow before autologous transplantation.<sup>22</sup>

However, despite the technical development of these sensitive techniques, there are insufficient data to support their use in formulating treatment recommendations. Whereas 10-15% of patients with stage I or II disease have circulating lymphoma cells, it is not clear that local radiotherapy should be withheld solely on that basis. Nor does the presence of these cells necessarily predict clinical relapse. Of 63 patients with low-grade lymphoma in clinical remission only six had rearrangements in one study. Nineteen patients relapsed, but only one relapsed patient had a rearrangement in the peripheral blood prior to relapse. The other patients with circulating abnormalities had been followed a median of two years without recurrence of NHL.<sup>25</sup>

Two other studies using the t(14;18) in the blood of patients with low-grade NHL failed to show a role for molecular detection in the management of patients with low grade NHL. Price<sup>24</sup> studied 15 patients in long-term remission; PCR was positive in 6 of 8 patients presenting with stage III-IV disease; but positive in none of 7 patients with stage I-II disease. Finke<sup>26</sup> studied 21 stage I-II patients; PCR was negative in 13 patients and positive in 8 patients; only one patient relapsed in each group. In contrast, data suggest that PCR analysis of transplanted purged

autologous marrow can predict for the risk of recurrence following high dose chemotherapy/radiation therapy and stem cell transplantation.

#### **Extranodal marginal zone B-cell NHL (MALT)**

The indolent extranodal lymphomas associated with mucosal associated lymphoid tissues (MALT) involve the GI tract, salivary glands, breast, thyroid, orbit, conjunctiva, and lung. As these diseases tend to remain localized for long periods of time, local treatment (surgery or local/regional irradiation) is very effective. In particular, low doses of radiation therapy (30 Gy) will almost always control sites of disease. The use of chemotherapy for MALT lymphomas has received limited attention as this indolent HNL does not routinely require the use of systemic treatment in patients with early stage disease. In one study of 24 patients with low-grade MALT treated with daily cyclophosphamide or chlorambucil for 12-24 months the complete response rate at 1 year was 75% and approximately 50% continued to be in remission at the time of the study.<sup>27</sup> Although these results are favorable, control of limited disease is superior with radiation therapy and the use of chemotherapy should be limited to patients with advanced stage disease.

Half or more of patients with gastric NHL have the indolent MALT type. The optimal treatment of gastric MALT lymphoma remains to be determined. Gastric MALT lymphoma is frequently associated with chronic gastritis and *Helicobacter pylori* infection. Based on clinical observations, it has been postulated that *H. pylori* infection leads to the accumulation of MALT in the stomach and that gastric MALT lymphomas arise within this *acquired* MALT tissue. This has prompted speculation that eradication of the *H. pylori* infection might lead to tumor regression. Promising early results have been seen with the use of antibiotics for gastric MALT NHL. In one study from the German MALT Lymphoma Group, 33 patients with low grade MALT were treated with antibiotics. At one year median follow-up more than 70% of patients remained in complete remission.<sup>28</sup> However, in a follow-up study, 22 of 31 patients in continuous complete remission (median follow-up 16 months), had a monoclonal B-cell population on PCR analysis leaving open the question of the durability of the complete response after antibiotics.<sup>29</sup>

Patients who are *H. pylori* negative may be less likely to respond to antibiotics than *H. pylori* positive patients; initial treatment of *H. pylori* negative patients with antibiotics remains under study. For patients who have persistent disease after antibiotics, local regional irradiation therapy is the treatment of choice. Good results have been obtained with total or partial gastrectomy, however, this approach has been associated with long-term morbidity. Local/ regional radiation through a two or three field approach (to avoid

the left kidney) will provide local control and relief of symptoms in greater than 90% of patients. Although more information is needed to determine the optimum radiation therapy dose, 30 Gy appears sufficient to control disease in most patients.<sup>30</sup>

Over 80% of patients with pulmonary NHL have indolent histology; nearly 80% of these are MALT.<sup>31</sup> The 5-year survival of patients with pulmonary MALT is above 90%. Often surgery is the initial treatment for patients with pulmonary MALT; however, development of recurrent disease is common and moderately low-dose radiation therapy has the potential to provide durable remissions. Gastrointestinal MALT is less common than gastric MALT. Approximately 25% of patients with gastrointestinal NHL have MALT. Very few data exist on the success of treatment of these patients. The most common sites are the jejunum and the ileo-cecum.

Lymphomatous involvement occurs in the orbit and in the conjunctiva with about equal frequency, accounting for between 5 and 14% of all extra-nodal presentations. These locations should be considered individually as they are often histologically distinct and have different natural histories. Conjunctival lymphoma tends to be localized, but may be associated with advanced disease. Due to its infiltrative nature, conjunctival lymphoma recurs with a high frequency following surgical excision alone. Surgery is used for diagnosis, but local radiation therapy to the entire conjunctiva is the definitive treatment of choice. Treatment of conjunctival lymphoma can be accomplished with either electrons or with photons. We generally use electrons as the lens can be protected with daily placement of a tungsten eye shield. Dunbar *et al.* from the Massachusetts General Hospital have recommended doses of 2,400-3,000 cGy for conjunctival lymphoma without local recurrence in a series of 12 patients treated with electron beam radiation therapy.<sup>32</sup>

CT scanning is useful for the evaluation of the extent of disease and important for radiation treatment planning when the orbit is involved. Various treatment field arrangements can be employed depending on the location of the disease (i.e. disease limited to the anterior portion of the orbit, involving both eyes, or involving the lachrymal glands). In treatment of orbital HNL in contrast to conjunctival NHL, it is not possible to block the lens and cataract development is more common. When disease is unilateral, a single anterior photon field can be used to avoid dose to the contralateral eye, however, this field produces a higher anterior dose and more dose than optimal to the lachrymal gland resulting in a greater risk of some eye dryness. Alternatively an anterior wedged pair can allow for better dose distribution within the tumor and less dose to normal tissues. Doses restricted to 30-36 Gy will control nearly 100% of indolent NHL of the orbit while maintaining a low risk of toxicity to the lachrymal glands and retina.

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## Interferons in the management of indolent non-Hodgkin's lymphomas

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During the years 1980-1985, it was shown that interferon- $\alpha$  (IFN- $\alpha$ ) was a drug active in some chronic lymphoproliferative disorders, especially hairy-cell leukemia. Following on these first results, several phase II trials of IFN- $\alpha$  in low-grade (indolent) non-Hodgkin's lymphomas (NHL) were undertaken; the response rate in the main trials was around 50% even in patients with chemoresistant disease.<sup>1</sup>

The mechanisms of action of IFN- $\alpha$  have since been extensively studied but remain largely unknown. IFN- $\alpha$  produces direct antiproliferative effects on tumor cells (inhibition of thymidine utilization, induction of thymidine phosphorylase activity, induction of double-stranded RNA-activated protein kinase activity), increases host-mediated immune defenses and inhibits angiogenesis.<sup>2,3</sup>

Interactions between interferon- $\alpha$  and cytotoxic drugs have been tested using either inhibition of *in vitro* growth of lymphoid cell lines or inhibition of tumor growth in nude mice. These studies showed additive or synergistic effects between IFN- $\alpha$  and some cytotoxic drugs which are active in NHLs such as cyclophosphamide, doxorubicin, prednisone or vinca-alkaloids.<sup>4</sup>

These results led to numerous phase III trials of IFN- $\alpha$  in low-grade or follicular only NHLs. Although these trials were quite different in their design, 3 groups of trial can be considered according to the place of IFN- $\alpha$  in the treatment program:

- IFN- $\alpha$  as a maintenance treatment after maximum response to conventional chemotherapy. IFN- $\alpha$  treatment was compared to abstention.<sup>5-8</sup>
- IFN- $\alpha$  combined with chemotherapy, either a single alkylating agent (chlorambucil or cyclophosphamide), or multiagent chemotherapy containing or not doxorubicin. Patients were treated either with chemotherapy alone or with chemotherapy concomitantly associated with IFN- $\alpha$ .<sup>9-12</sup>
- IFN- $\alpha$  tested first in association with an induction chemotherapy regimen, then as a maintenance treatment (4-arm randomized trial).<sup>13-15</sup>

Most of these trials have been subjected to a meta-analysis with inclusion of individual data in the mod-

el.<sup>16</sup> The results are summarized in Table 1. Despite the fact that these trials are heterogenous in their design (type of chemotherapy, type of IFN- $\alpha$ , dose and treatment duration, type of low-grade NHLs, prognosis factors, etc) several conclusions can be drawn:

1. most trials show that IFN- $\alpha$ , either as a maintenance treatment or in association with chemotherapy, increases median progression-free survival (PFS) (see Table 1). Some trials show that this improvement in PFS is translated into an increase in median overall survival (OS);
2. the beneficial effects of IFN- $\alpha$  are most significant in patients with adverse prognosis factors at initial staging. For instance, the *German Lymphoma Study Group* found the best effects occurred in patients with B symptoms.<sup>7</sup> In the GELF study, the improvement in PFS and OS was significant in patients with a high-tumor burden. It would be most useful to reach a consensus on prognostic factors in follicular lymphoma (FL) in order to select patients who would gain maximum beneficial effects from IFN- $\alpha$  treatment;
3. the meta-analysis conducted by Rohatiner<sup>16</sup> showed that the beneficial effects of IFN- $\alpha$  on PFS and OS were most significant in patients who had been treated previously or concomitantly with an intensive chemotherapy regimen, especially one containing an anthracycline or an anthracenedione such as the COPA regimen in the ECOG study,<sup>9,10</sup> the CHVP regimen in the GELF study,<sup>11,12</sup> the prednimustine-mitoxantrone association in the German study, or CHOP-bleomycin in the Mexican trial.<sup>6</sup> The beneficial effects of IFN- $\alpha$  are far less clear in patients treated with a single alkylating agent or CVP;<sup>5,13-15</sup>
4. no difference in efficacy and tolerance between IFN- $\alpha$  2a and IFN- $\alpha$  2b seems to exist;
5. the meta-analysis results suggest that there is a threshold dose of IFN- $\alpha$  required to produce beneficial effects ( $36 \times 10^6$  IU/month).<sup>16</sup> The German study group observed very similar results with a threshold dose of 9 MU/week.<sup>7</sup> It should be emphasized that this conclusion relies on a retrospective analysis. No prospective study has tested the role of dose of IFN- $\alpha$  when associated with chemotherapy;
6. some studies suggest that a long duration of IFN- $\alpha$  administration may improve the results. In the

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Table I. Results of treatment with interferon alpha (IFN $\alpha$ ) of low-grade non-Hodgkin's lymphomas.

Author, date (Ref.)	IFN $\alpha$ dose	IFN $\alpha$ duration (/wks.)	Chemo- therapy (months)	No. of pts	Median TTP (months)			Median survival (months)		
					without IFN $\alpha$	with IFN $\alpha$	p	without IFN $\alpha$	with IFN $\alpha$	p
<b>IFN<math>\alpha</math> as maintenance<sup>1</sup></b>										
Hagenbeek <i>et al.</i> , 1998 <sup>5</sup>	9MU/m <sup>2</sup>	12	CVP	242	22	33	0.054	No difference <sup>5</sup>		
Avilès <i>et al.</i> , 1996 <sup>6</sup>	15MU	12	CEOP-CVP	98	46	>108	<0.01	74	>108	<0.001
Unterhalt <i>et al.</i> , 1995 <sup>7</sup>	15MU	until prog.	CVP	170	15	21	<0.05	NR	NR	NR
			PmM	180	27	43	<0.05	NR	NR	NR
Dana <i>et al.</i> , 1998 <sup>8</sup>	6MU/m <sup>2</sup>	12	ProMACE- MOPP	279	40	58	0.18	77% <sup>9</sup>	80% <sup>9</sup>	NS
<b>IFN<math>\alpha</math> in association<sup>2</sup></b>										
Smalley <i>et al.</i> 1992, 1998 <sup>9,10</sup>	30MU/m <sup>2</sup> *	6-9	COPA	249	23	35	0.008	68	94	0.04
Solal-Céligny <i>et al.</i> , 1993, 1998 <sup>11,12</sup>	15MU	18	CHVP	242	18	34	<0.005	66	83	<0.02
<b>IFN<math>\alpha</math> in association + maintenance<sup>3</sup></b>										
Peterson <i>et al.</i> , 1997 <sup>13</sup>	6MU/m <sup>2</sup>	24	CPM	581 2,045	31% <sup>9</sup>	32% <sup>9</sup>	0.99 0.40	67% <sup>9</sup>	69% <sup>9</sup>	0.99
					42%	46%				
Arranz <i>et al.</i> , 1998 <sup>14</sup>	9MU/m <sup>2</sup>	12	CVP	144	22	>36	0.0003	82% <sup>#</sup>	70% <sup>#</sup>	0.3
Rohatiner <i>et al.</i> , 1997 <sup>15</sup>	9MU	4.5	CBC	108	No difference in progression-free and overall survivals <sup>5</sup>					

Abbreviations: CVP = cyclophosphamide-vincristine-prednisone; CEOP = CVP + epirubicin; PmM = prednimustine-mitoxantrone; ProMACE-MOPP = alternate treatment with procarbazine-adriamycin-cyclophosphamide-etoposide then mechlorethamine-vincristine-procarbazine-prednisone; COPA or CHVP = CVP + adriamycin; CPM = cyclophosphamide; CBC = chlorambucil; PFS = progression-free survival; NR = not reported.

<sup>1</sup>IFN $\alpha$  given in patients with complete or partial response after induction chemotherapy; <sup>2</sup>IFN $\alpha$  given as a concomitant (GELF) or sequential (ECOG) association with chemotherapy; <sup>3</sup>patients with low-grade disease are selected for adverse prognosis factors; <sup>4</sup>four-arm trials with a first randomization of induction treatment with or without interferon and a second randomization of responders for a maintenance treatment with or without interferon; <sup>5</sup>results not given in the report; <sup>6</sup>results for patients randomized for maintenance treatment. \*Given during 1 week every month just before each chemotherapy cycle; <sup>#</sup>6-year survival; <sup>9</sup>5-year survival.

GELF study, 18 months of treatment was associated with a significant improvement in PFS and OS. In the German study, IFN- $\alpha$  treatment is maintained until progression or toxicity and there is a significant improvement in PFS (data on OS not mentioned). Both of these trials showed the most significant beneficial effects of IFN- $\alpha$ ;

- IFN- $\alpha$  produces significant adverse reactions, e.g. flu-like disease, especially at the onset of treatment, and asthenia, depression, decrease in libido during the chronic phase of treatment. Tolerance to the treatment may be improved in many patients by concomitant use of antipyretics and antidepressant drugs. No prospective analysis of quality-of-life (QOL) has been performed during trials up to date. In a retrospective analysis using the Q-TwIST method, we have shown that, almost whatever decrease in QOL reported by the patient during treatment with IFN- $\alpha$ , the beneficial effects on quality-adjusted survival remained significant.<sup>17</sup>

More than 1,500 patients have been included in randomized trials of IFN- $\alpha$  in low-grade NHLs. Few trials of the treatment of neoplastic hematologic diseases have included such a large number of patients. The results have allowed us to conclude that IFN- $\alpha$

has an important role in the treatment of FL, especially for patients with adverse prognostic factors and in combination with an anthracycline-containing regimen. Nevertheless, these trials are heterogenous (dose and duration of treatment with IFN- $\alpha$ , histologic type and tumor burden of the NHL, type of chemotherapy). So, no standard administration scheme of IFN- $\alpha$  can yet be proposed.

Several new indications for IFN- $\alpha$  in NHLs are under testing:

- one randomized study showed an improvement in median PFS and median OS in patients with large-cell NHL treated with IFN- $\alpha$  after maximum response to chemotherapy. This trial registered only a small number of patients and the results need to be confirmed;<sup>18</sup>
- after intensive therapy with autologous stem cell transplantation for NHL, IFN- $\alpha$  may have two roles; it may: a) decrease the relapse rate by acting on the residual disease; b) hasten the recovery of the immune deficiency, and strengthen the immune reactions against tumor cells (expression of ligands by tumor cells, stimulation of NK and cytotoxic cells). Some phase II studies have tested the feasibility of IFN- $\alpha$  treatment given after autol-

ogous stem cell transplantation either in association with interleukin 2<sup>19</sup> or with cyclosporin A.<sup>20</sup> A European randomized trial is presently examining the role of IFN- $\alpha$  given alone after autologous stem cell transplantation for relapse of lymphoma;

3. as IFN- $\alpha$  stimulates cell-surface antigen-expression, increases localization of antibodies into tumors, and may augment antibody-dependent effector cell functions, there is a rationale for associating IFN- $\alpha$  with other immunotherapies. A first trial of an association of rituximab and IFN- $\alpha$  has been reported.<sup>21</sup> No additive toxicities were observed and a 45% response rate was observed in 38 patients with previously treated low-grade NHL.

Almost 20 years after the first trials of IFN- $\alpha$  in non-Hodgkin's lymphomas, it can be shown that there is undoubtedly a role for IFN- $\alpha$  in the management of NHLs. Although numerous treatment modalities can be proposed for patients with low-grade NHL, there is a need for continuing clinical trials with IFN- $\alpha$  in follicular NHLs.

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## Long-term follow-up of autologous bone marrow transplantation in patients with relapsed follicular lymphoma

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Patients with low grade follicular non-Hodgkin's lymphomas (NHL) are not cured by conventional treatment.<sup>1</sup> Although the median survival for these patients is 8 to 10 years, the disease-free survival for previously untreated patients given conventional therapy is generally between 18-36 months.<sup>1-4</sup> In contrast to lymphomas of more aggressive histology most of which relapse within 2 years of the patient completing initial treatment, a significant number of follicular lymphomas do not recur until beyond 3 years. Therefore, long follow-up is critical to assess the impact of treatment on remission duration and survival in these diseases.

The rationale for the use of high dose ablative therapy in follicular lymphoma is based upon the fact that relapsed patients can continue to respond to further conventional treatment and salvage regimens. This approach is being applied more frequently in patients with relapsed low grade NHL, in spite of uncertain efficacy.<sup>5-13</sup> One problem with these studies is that the length of follow-up has been relatively short, considering the long natural history of these diseases. An alternative to clinical assessment of remission status may be molecular studies of minimal residual disease. Following autologous bone marrow transplantation (ABMT) in patients with follicular lymphoma, the detection of minimal residual disease by polymerase chain reaction (PCR) has been shown to be a useful surrogate marker for relapse and may help obviate the need for extended follow-up in a disease in which late relapses are common.<sup>9,14-17</sup>

In this study, we report the results of 153 patients with relapsed follicular NHL who underwent high dose chemoradiotherapy and anti-B cell monoclonal antibody purged ABMT between 1985 and 1995. These results suggest that a significant proportion of these patients with follicular NHL experience prolonged disease-free and overall survival.

### Design and Methods

#### Selection of patients and treatment protocol

Patients were eligible for this study if they were less than 65 years of age and had relapsed follicular small cleaved cell or follicular mixed NHL. For all patients, a minimal disease status had to be attained through chemotherapy, radiotherapy, or both, prior to entry as previously described.<sup>6,18</sup>

Preparative therapy consisted of cyclophosphamide 60 mg/kg of body weight, infused on each of two consecutive days before radiotherapy. TBI was administered in fractionated doses (200 cGy) twice daily on three consecutive days (total of 1200 cGy) in 144 patients, 9 patients received 1,400 cGy. Supportive care was provided as previously described.<sup>9</sup>

#### Collection, processing, and infusion of marrow

Bone marrow was obtained, then treated *in vitro* with anti-B cell monoclonal antibodies and rabbit complement as previously described. After treatment, the cells were cryopreserved as described elsewhere.<sup>9</sup>

#### PCR analysis

Nested PCR amplifications at the major breakpoint region (MBR) and minor cluster region (mcr) of the bcl-2/IgH rearrangement of t(14;18) were performed as previously described.<sup>19</sup>

### Results

#### Patient characteristics

One hundred and fifty-three consecutive patients (median age 43 yrs) with follicular NHL in sensitive relapse or incomplete first remission underwent ABMT between 3/85 and 5/95. Prior to ABMT, 120 of the patients had follicular small cleaved cell (FSC), and 33 had follicular mixed (FM) histology. At diagnosis, 102 patients (67%) had stage IV disease, largely because of bone marrow involvement. At some time prior to consideration for ABMT, extranodal sites of involvement, exclusive of the marrow, were present in 35% of patients.

#### Prior therapy

All patients had previously received combination

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chemotherapy. The median number of regimens with which patients were treated was 3 (range 2-7). At some time in their history, 27% of patients had received involved field radiotherapy and one patient had been previously treated with TBI (150 cGy). A complete response (CR) at anytime during their disease course, including the time of bone marrow harvest was documented in only 78 (51%) of the patients. At bone marrow harvest, only 46 (30%) of the patients were in CR. Of the 107 patients in partial response (PR) at harvest, 72 had residual BM involvement.

### **Treatment outcome**

Of the 153 patients treated, only one early treatment-related death was seen. Another patient died of chronic liver disease without evidence of lymphoma at 46 months. Of the remaining 151 patients, 63 have had relapses (as of September 1998). Seventy-nine patients remain alive and in CR with a median follow-up of 61 months (range 24-156 months). The Kaplan-Meier estimate of the percentage of patients alive and disease-free following ABMT is 42% at 8 years. The estimate of the overall survival at 8 years is 66%. The survival from diagnosis for the entire group of patients is 69% at 12 years.

### **Second malignancies**

Following ABMT, second malignancies have developed in 18 patients. These include solid tumors in 5 patients, while one patient developed acute lymphoblastic leukemia 28 months post-ABMT. Twelve patients have developed myelodysplasia with one evolving into acute myelogenous leukemia, from 9 to 64 months post-ABMT. Ten of these patients have died, six patients had no evidence of recurrent lymphoma. Four of the 10 patients died following allogeneic BMT (alloBMT). The remaining 2 patients who are alive after the development of myelodysplasia, both have relapsed NHL as well.

### **PCR analysis following ABMT**

Bone marrow samples were available from the BM harvest and post-immunologic purging in 113 of the patients in whom a bcl-2/IgH rearrangement could be PCR amplified from diagnostic tissue. PCR detected residual disease at the time of BM harvest in all informative patients. Following immunologic purging, PCR analysis revealed no detectable disease in 48 of these patients (42%), whereas in the remaining 65 patients (58%), PCR detectable disease persisted after purging. The effect of marrow purging was examined in these 113 patients. Among the 48 patients who were PCR negative after purging, there have been 6 relapses. In contrast there have been 49 relapses among the 65 patients who were PCR positive after purging. The 8-year FFR for the PCR negative patients is 83%, while the FFR for the patients who were PCR positive post-lysis is 19% ( $p=0.0001$ ).

Following ABMT, BM samples were obtained at six monthly intervals for two years and yearly thereafter.

Continued PCR negativity in follow-up BM samples was strongly predictive of continued CR.

### **Discussion**

In this report, we present the results of high-dose chemoradiotherapy and ABMT in 153 consecutive patients with relapsed indolent follicular NHL treated between 1985 and 1995. Considering that the median disease-free survival (DFS) of second remission with conventional therapy for patients with this disease is 13 months, our study shows a significant prolongation of DFS with high dose therapy.<sup>4</sup> Moreover the median survival after first relapse from conventional therapy for advanced stage patients is 5 years.<sup>2</sup> The overall survival following ABMT in this series was 66% at 8 years, suggesting that we may be observing a prolonged survival with high dose therapy.

We previously reported in patients undergoing ABMT for follicular NHL in second or greater remission as well as first remission,<sup>9,15</sup> that the presence of minimal residual disease in the reinfused marrow was the most significant prognostic factor for relapse. However, these studies suggested that only a subset of patients presently benefit from purging. Considering that 89% of the patients who relapsed were reinfused with a PCR+ BM, further improvements in techniques to yield a more tumor-free stem cell product are likely to have an impact on DFS.

The observation that minimal residual disease present at the time of ABMT and during follow-up is predictive for relapse has stimulated novel approaches toward eradicating minimal residual disease. Potential approaches include: amplification of effector cells with cytokines; tumor cell vaccination; vaccination with idiotypic peptides; and monoclonal antibodies. These immunomodulatory and targeted therapies may be particularly well suited to patients who are at high risk of relapse following high dose therapy, specifically those receiving a PCR+ stem cell product and those who are persistently PCR+ after transplant.

It is clear that other strategies are needed to improve the results of autologous transplantation. Radio-immunoconjugates have been used in both myeloablative and non-myeloablative doses with high response rates.<sup>20-24</sup> AlloBMT has been employed in a limited fashion in patients with relapsed follicular NHL.<sup>10,25-27</sup> The overall survival rates for patients undergoing conventional alloBMT are quite similar to those undergoing autologous BMT, because despite the significantly higher treatment-related mortality, there is a lower relapse rate. This lower relapse rate with alloBMT as well as anecdotal responses to donor lymphocyte infusions are evidence for a graft-versus-lymphoma effect.<sup>28,29</sup> One strategy to reduce the high morbidity and mortality of alloBMT, while generating a graft-versus-lymphoma effect, would be to combine selective T cell depleted allogeneic BMT<sup>30,31</sup> with donor lymphocyte infusions. Ongoing and future studies in

these areas as well as in the development of more effective ablative regimens, tumor cell purging and treatment of minimal residual disease may provide more effective treatment than current treatments which currently benefit only a minority of patients.

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## High-dose chemotherapy with PBPC autograft in low grade non-Hodgkin's lymphoma

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The availability of hemopoietic growth factors and peripheral blood progenitor cells (PBPC) has significantly reduced the toxicity of high dose chemotherapy and autografting. This approach is now commonly employed with a toxicity similar to second and third generation chemotherapy regimens.<sup>1-3</sup> The high dose strategy is therefore now considered not only as a rescue option, but also as first line in the treatment of high risk lymphoma patients.<sup>4</sup>

The high dose sequential (HDS) chemotherapy scheme proposed several years ago by Gianni's group at the Milan Cancer Institute has represented the keystone for all subsequent high dose programs including PBPC support.<sup>5</sup> Such an approach has been extensively employed by the Milan group and at our Institution for the treatment of patients with relapsed or high grade lymphoma patients.<sup>6-8</sup> In histologically indolent subtypes the frequent involvement of bone marrow by neoplastic cells is often a major obstacle to the employment of the original HDS. In order to render this approach suitable also for indolent forms, a few modifications have been made, including intensification of the debulking phase and later harvest postponed to the end of the high dose phase.<sup>9</sup> The goal was to achieve extensive tumor mass reduction prior to harvesting and to make the risk of harvesting residual contaminating cells as low as possible.

Preliminary results with the modified and intensified HDS (i-HDS) have been very encouraging. We were able to obtain good clinical responses with no evidence of minimal residual disease in the PBPC harvests, as assessed by PCR analysis, in patients with advanced stage follicular lymphoma.<sup>10</sup> Results were also good in terms of both tolerability of the treatment and amount of harvested PBPC.<sup>11</sup> This article is an update of data reported in the pilot study with i-HDS in follicular lymphoma; in addition, we will show data collected in patients with low grade non-follicular lymphoma receiving the same treatment; finally we will outline possible future direc-

tions of the study, including the use of the anti-CD20 monoclonal antibody rituximab for *in vivo* purging purposes prior to PBPC harvests, and a randomized study to assess the efficacy of i-HDS compared to conventional treatments.

### Patient characteristics and treatment plan

In our pilot study, 46 patients with low-grade lymphoma were treated with i-HDS and were evaluated for toxicity and clinical response. Patient selection criteria included age below 60, biopsy-proven diagnosis of low-grade lymphoma, advanced-stage disease with requirement of undelayed treatment and no previous exposure to chemotherapy or extended radiotherapy. Seventeen patients had lymphocytic lymphoma and 29 follicular lymphoma. The main clinical features of the two groups of patients are summarized in Table 1. In the lymphocytic subgroup, histologic and immunophenotypic analyses showed 10 patients with lymphocytic lymphoma or chronic lymphocytic leukemia, 6 with monocytoid/marginal zone lymphoma and one with lymphoplasmacytoid lymphoma. Of 29 patients with follicular lymphoma, 10 had either transformed or discordant histology. As shown in Table 1, virtually all patients had advanced stage disease most often with disease-related symptoms and bone marrow involvement.

The treatment schedule comprised 2 APO courses, including 4 total doxorubicin administrations at 75 mg/sqm, followed by 2 DHAP courses. The subsequent high-dose phase included sequentially: hd-etoposide (VP16) (2 g/m<sup>2</sup>), hd-methotrexate (8 g/m<sup>2</sup>), a 30-day cytotoxic drug-free interval, hd-cyclophosphamide (CY) (7 g/m<sup>2</sup>) followed by PBPC harvest; hd-mitoxantrone (60 mg/m<sup>2</sup>) + L-PAM (180 mg/sqm) with PBPC autograft concluding the program. G-CSF was given following hd-VP16 and CY as well as following PBPC autograft.

### Response to treatment and long-term follow-up

Overall the scheme proved to be feasible, with acceptable toxicity, although two toxic deaths occurred. One patient receiving warfarin for deep vein thrombosis at presentation had a fatal cerebral hemorrhage in spite of normal platelet counts prior to the hd-phase. A second patient died of cerebral hem-

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**Table 1. Main clinical features.**

Total patients	46
Age (yrs), median (range)	47 (36-62)
Sex M/F	23/23
Histology:	
<i>lymphocytic lymphoma</i>	17
<i>follicular lymphoma</i>	19
<i>transformed lymphoma</i>	10
Stage III-IV	44
BM involvement	37
Disease-related symptoms	30

**Table 2. Main treatment toxicity.**

	VP16	CY	Autograft
Days to:			
WBC > 1,000/mmc	11 (8-17)	12 (9-18)	11 (9-25)
PLTs > 50,000/mmc	11 (0-23)	14 (0-22)	9 (0-365)
Patients with fever	5	14	28
Severe infections	2	0	1
Grade III-IV oral mucositis	0	0	9

VP16 = etoposide 2 mg/m<sup>2</sup>; CY = cyclophosphamide 7g/m<sup>2</sup>; autograft was performed with PBPC ( $\geq 8 \times 10^6$ /kg CD34<sup>+</sup> cells) preceded by mitoxantrone 60 mg/m<sup>2</sup> and L-PAM 180 mg/m<sup>2</sup> as conditioning regimen.

\*Two pneumonia and 1 CMV infection.

**Table 3. Clinical response to i-HDS in indolent lymphoma.**

	<i>Lymphocytic lymphoma</i>		<i>Follicular lymphoma</i>	
	n	(%)	n	(%)
CR	11	(65)	24	(83)
long-term CR	6	(35)	20	(69)
range (mos)	21-101		14-108	

orrhage while in persistent thrombocytopenia following autograft performed with bone marrow (BM) cells; this was the only patient displaying poor mobilization in whom we could not employ PBPC for autograft. No other severe toxicities were recorded, with the exception of fever, a few documented infectious episodes and grade 3-4 oral mucositis. Main hematologic and extrahematologic toxicities are summarized in Table 2. One patient in complete remission (CR) was diagnosed as having myelodysplasia which progressed to acute leukemia over 1 year: she is now in CR of her second tumor at 16 months. This was the only second tumor recorded in this series of heavily treated patients with indolent lymphoma.

Marked tumor regression was observed in the great

majority of patients and 34 (74%) reached CR. As shown in Table 3, CR was achieved by 11 patients (65%) of the lymphocytic subgroup, and 23 (79%) of the follicular subgroup; 5 more follicular patients (17%) had a short-lasting response followed by tumor progression and death within a few months; four of these patients had transformed histology.

At a median follow-up of 4 yrs 39 patients are alive. There was no statistically significant difference in the survival curves between lymphocytic and follicular subgroups. In contrast, a significant difference was observed in CR duration, as summarized in Table 3. Indeed, in the lymphocytic subgroup 4 out of 11 patients in CR had disease recurrence and overall 6 patients are presently alive in continuous complete remission (CCR); in the follicular subgroup, 3 of the 23 patients in CR relapsed and 20 patients are long-term survivors with no signs of disease progression.

The good tumor response of patients with follicular disease was further documented by PCR analysis of minimal residual disease (MRD). Nineteen patients were evaluated for MRD; 13 (68%) of them were PCR negative on either PB or BM harvests. This suggests that intensive chemotherapy delivered prior to progenitor cell harvest has an *in vivo* purging effect. A similar rate of PCR negativity was reported in follicular lymphoma by the Dana Farber group using an *ex vivo* purging approach.<sup>12</sup> In the experience of the Boston group, the achievement of PCR negativity was highly predictive of prolonged disease-free survival.<sup>13</sup> In our series too, all but one patient with at least one PCR negative harvest remained in prolonged clinical and molecular remission after autograft. MRD was also evaluated in 6 lymphocytic patients: neither pre-transplant harvests nor post-graft follow-up samples showed evidence of negative PCR. Thus, among indolent lymphomas, PCR negativity seems easier to achieve, either by *in vivo* chemotherapy purging or by *ex vivo* manipulation, in follicular lymphoma patients.<sup>14</sup>

### Perspectives

In the last few years, in the field of high-dose therapy for indolent lymphoma we have focused our interest on two main goals: first, to improve the procedure to harvest uncontaminated PBPC in the highest fraction of patients; second, to evaluate whether the promising results obtained in pilot studies at our Institution can be reproduced at a multicenter level, with large series of patients.

To increase the chances of harvesting PCR negative PBPC we developed, in collaboration with Gianni's group, a novel HDS scheme which includes the administration of anti-CD20 rituximab. The humanized monoclonal antibody is scheduled following high-dose cytotoxic drug administration in order to exploit the maximal anti-B cell effect immediately prior to PBPC harvest. Preliminary results from a pilot study in 15 patients with follicular or mantle cell lym-

phoma were encouraging. Marked tumor regression was observed in all patients. In addition, PBPC devoid of molecularly detectable tumor contaminating cells could be collected from all but one patient. Thus, rituximab addition to the HDS program proved to be effective and enhanced the *in vivo* purging effect of high-dose chemotherapy.

The efficacy of the high-dose approach is also under evaluation in a multicenter study. Several Italian hematology centers are participating in an ongoing study which enrolls consecutive patients with previously untreated, high-risk follicular lymphoma. Early results seem satisfactory, at least in terms of feasibility, tolerability and antitumor efficacy of the high-dose sequential program. We are now planning a randomized, controlled trial comparing i-HDS possibly potentiated with rituximab and conventional therapy.

In conclusion, our results along with data reported by several other groups demonstrate the efficacy of high-dose chemotherapy approaches in indolent lymphoma, particularly in the follicular subtype. An unexpectedly high proportion of patients may reach durable clinical remission often in the absence of molecularly detectable residual disease. The unanswered question is whether high-dose chemotherapy should be chosen as front-line therapy in high-risk patients or whether it should be reserved to rescue patients progressing after conventional treatment. The availability of anti-CD20 rituximab suitable for both conventional and high-dose strategies should be considered when planning studies which address this issue.

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## Allogeneic transplantation for non-Hodgkin's lymphoma

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### Allogeneic transplantation - graft vs. lymphoma effects

As the limitations of autologous transplantation for lymphoid disorders have become more apparent, renewed interest in allogeneic transplantation and clinical application of potential graft vs lymphoma (GVL) effects has been generated. The clinical evidence for GVL effects remains anecdotal. We and others have reported a number of cases of lymphoma regression in allogeneic recipients after withdrawal of immunosuppression or after infusion of donor lymphocytes.<sup>1-3</sup> Among nine patients who could be evaluated for the effects of donor lymphocyte infusion (DLI) or withdrawal of graft-versus-host disease (GvHD)-prophylaxis, responses occurred in six.<sup>2</sup> These effects have since been demonstrated in several additional patients, and attainment of molecular remission in a patient with follicular lymphoma has also been demonstrated.<sup>4</sup>

A second way in which GVL effects can be demonstrated is by the analysis of the relationship between graft-versus-host disease (GvHD) and disease recurrence after allogeneic transplantation. In one retrospective study by the EBMT, a lower recurrence rate was found for patients with chronic GvHD vs those without cGvHD (0% vs 35%,  $p=0.02$ ).<sup>5</sup> In two other retrospective analyses, one focusing on low grade lymphoma<sup>6</sup> and the other on intermediate grade lymphoma,<sup>7</sup> no relation between the incidence of GvHD and disease recurrence could be demonstrated. This may be due to the relatively small number of patients analyzed and the low overall incidence of disease recurrence in the latter studies.

Besides the presence of GVL effects, the lack of involvement by lymphoma cells in the graft, may also contribute to a decreased recurrence rate after allogeneic transplantation. There is considerable evidence that, in autologous transplantation, the infusion of occult lymphoma cells contributes in many cases to disease recurrence. To quantify the contribution of occult lymphoma cells to recurrence, data on twin transplants in lymphoid malignancies are required. Only a few such studies are available.<sup>8-11</sup> They indicate relatively low recurrence rates, but the numbers of patients treated in these studies is too small to make an accurate assessment of recurrence rates.

Whether due to lack of tumor contamination or to a GVL effects, a large number of studies indicate a lower recurrence rate after allogeneic transplantation when compared with autologous transplantation. This seems to be true for most types of lymphoid malignancies with the possible exception of Hodgkin's disease. The only prospective study comparing allogeneic with autologous BMT was reported by Ratanatharathorn *et al.*, and indicates a significantly decreased recurrence rate after allogeneic BMT, and a trend toward improved disease-free survival.<sup>12</sup>

Recommendations regarding the use of bone marrow transplantation need to consider disease characteristics and results of therapeutic alternatives. As the natural history and treatment options for patients with different subsets of lymphoid malignancies differ widely, we will subsequently discuss allogeneic transplantation for follicular lymphoma and another subset, mantle cell NHL, separately.

### Follicular lymphoma

#### Conventional treatment for follicular lymphoma

The median survival of patients affected by follicular lymphoma is 7 to 9 years. Follicular lymphoma usually responds well to initial chemotherapy, but patients almost invariably relapse. At the time of first recurrence, the median life expectancy decreases to approximately 4 years and further decreases with subsequent recurrences.<sup>13,14</sup>

#### Allogeneic transplantation for recurrent low grade lymphoma

Allogeneic bone marrow transplantation has generally been reserved for far advanced patients with low grade lymphoma. We reported encouraging results with extended disease free survival in 12 of 15 heavily pretreated patients.<sup>15,16</sup> Other groups have also reported a high fraction of long term remissions.<sup>17-20</sup> Relapse rates after allogeneic transplants have been substantially lower than after transplantation of purged autologous transplants, most likely due to the graft-vs-lymphoma effect.<sup>17,18</sup>

Verdonck *et al.* published a study of 28 patients comparing results with allogeneic vs. autologous transplants in patients with advanced low grade lymphoma.<sup>18</sup> The patients had received 2 to 5 prior conventional chemotherapy regimens. Eighteen patients, all with chemotherapy-sensitive disease, received autologous BMT and 10 patients (all with overt mar-

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row involvement and 7 with chemotherapy-resistant disease), underwent allogeneic transplantation. A common conditioning regimen, cyclophosphamide plus total body irradiation was used for all patients. All allogeneic BMT patients achieved complete remission, 3 patients had a treatment-related death, and 7 patients were alive in remission at a median follow-up of 41 months. Of the autologous transplant recipients, none died of treatment-related complications. Complete remission was achieved in 67%, but only 3 of 18 patients are alive and disease-free. The probability of disease-progression among allogeneic transplants was 0% vs. 83% for autologous recipients ( $p = 0.002$ ) and progression-free survival at 2 years was 68% and 22% respectively ( $p = 0.049$ ).

Attal *et al.* also performed a retrospective case-control analysis of 216 patients reported to the French Bone Marrow Transplant Group Registry from 1986 to 1996.<sup>17</sup> Seventy-two allogeneic transplants were matched with 144 autologous grafts on the basis of age, disease status and conditioning regimen. Patient characteristics were comparable for sex, age (median 40 years), stage, interval from diagnosis to transplant (33 months), number of prior chemotherapy regimens (2). Fifty-three percent were refractory to chemotherapy; 75% received a TBI containing conditioning regimen. Median follow-up was 34 months. A comparable initial complete response rate occurred after transplant (allo = 86%, auto = 78%). The allogeneic transplants had a significantly lower relapse rate of 12% at 60 months with a plateau after 15 months in contrast to 55% with autologous transplantation without an apparent plateau ( $p < 0.001$ ). Transplant-related mortality was higher after allogeneic BMT (30% versus 4% with autotransplantation),  $p < 0.001$ ). The 4-year event free survival was not significantly different, 53% for allogeneic BMT and 45% for autologous transplantation. The previously mentioned analysis by the EBMT registry similarly indicated a lower recurrence rate after allogeneic transplantation, but a worse overall survival.<sup>15</sup>

The *International Bone Marrow Transplant Registry* recently analyzed results of allogeneic BMT from HLA identical sibling donors in 113 patients transplanted by 50 teams.<sup>15-20</sup> Median age was 38 (range 15-61) years. The median interval from diagnosis to transplant was 24 months and the median number of prior chemotherapy regimens was 2. Eighteen percent of the patients had small lymphocytic lymphoma, 46% had follicular small cleaved cell lymphoma and 36% had follicular mixed cell lymphoma. Eighty-one percent of the patients had stage IV disease at diagnosis, most commonly due to the presence of bone marrow involvement. At transplant, 14% of the patients were in complete remission and 71% continued to have stage IV disease. Thirty-seven percent were considered chemotherapy resistant. Twenty-nine percent of the patients had a performance status  $\leq 80\%$ . The conditioning regimen contained TBI in 82% of the

patients. Median follow-up for surviving patients was 22 months after transplantation. Three year probability of disease-free survival was 49% (95% CI: 39-59) and the probability of disease recurrence was 16% (95% CI 9-27). The probability of treatment-related mortality was 28% (95% CI 19-39). In multivariate analysis a decreased Karnofsky score, presence of chemotherapy resistant disease and the use of a non-TBI regimen were independent predictors of survival.

These data indicate that high dose chemoradiotherapy and allogeneic bone marrow transplantation is potentially curative for patients with advanced low grade lymphoma. Unlike the case with autologous transplants, there have been very few relapses after 2 years from transplantation. The potential efficacy of allogeneic transplantation must however be balanced against its risks and the long natural history of indolent lymphoma. Transplants have generally been performed after failure of one to several chemotherapy regimens. On the other hand, results are likely to be better relatively early in the course of the disease than after high level drug resistance develops or the patient becomes debilitated. Given the long natural history of low grade lymphomas and the low relapse rates observed with transplants even with advanced disease, we reserve the use of allogeneic transplantation until after failure of initial chemotherapy.

#### **Allogeneic vs autologous transplantation in recurrent low grade lymphoma**

The available data do not provide clear guidelines regarding the relative role of allogeneic vs. autologous bone marrow transplantation. All studies report a lower relapse rate with allotransplantation, presumably due to graft-vs-lymphoma effects, but this benefit is outweighed to a variable degree by a higher rate of treatment-related mortality. Certainly, a number of patients who are ineligible for autologous transplantation due to extensive marrow involvement, may achieve durable remissions after allogeneic transplantation. Given the durability of complete remissions achieved, it is generally our policy to recommend allogeneic transplants for patients with matched sibling donors rather than autologous transplantation. Treatment decisions must however be individualized. Patients with long first remissions and low risk features may obtain a long second remission with salvage chemotherapy alone, and the relative risks and benefits of all treatment alternatives should be presented to potential transplant recipients.

### **Mantle cell lymphoma**

#### **Natural history and conventional management of mantle cell NHL**

Mantle cell lymphoma is a B-cell lymphoma originating from a normal counterpart in the mantle of the lymphoid follicle.<sup>21</sup> The disease is associated with

t(11;14)(q13;q32) and rearrangement of bcl-1 in a large percentage of cases. In the past, the disease was often confused with either CLL or diffuse large cell lymphoma. In the past five years, it has been recognized more reliably and the clinical features have been better described. Patients often present with widespread disease; bone marrow and peripheral blood involvement are common as is involvement of the gastrointestinal tract.<sup>21-27</sup> Despite morphologic and phenotypic similarities to CLL, the prognosis of mantle cell lymphoma is worse than that of CLL or of follicular lymphoma. In general the responses to chemotherapy are also less durable than those achieved in other types of diffuse lymphoma.<sup>22-26</sup> Estimated 10 year survival in a recent series was only 8%.<sup>24</sup>

### **Intensive chemotherapy and autologous or allogeneic transplantation for mantle cell lymphoma**

Because of the poor long-term survival in mantle cell lymphoma, many centers have recommended autologous or allogeneic transplantation as consolidation of remission.<sup>21,28,29</sup> Encouraging early results have been reported after autologous transplantation.<sup>30</sup>

Blay *et al.*,<sup>31</sup> reported on 18 patients with diffuse centrocytic lymphoma, including nine who had a confirmed diagnosis of mantle cell lymphoma, who underwent autologous transplantation in partial or complete remission. The conditioning regimen contained TBI in 11 cases and was BCNU-based in six. One patient received a combination of etoposide, carboplatin and ara-C. Seven patients received bone marrow and 12 received G-CSF stimulated peripheral blood progenitor cells. With a median follow-up of 30 months, progression-free survival was 75%.

Dreger *et al.* reported on 12 patients with stage III/IV mantle cell lymphoma who received two cycles of induction with Dexamethasone-BEAM, followed by consolidation with autologous transplantation.<sup>32</sup> Six patients received TBI containing regimens and two received high dose chemotherapy regimens. With a median follow-up of 12 months post-transplant, all patients were alive and in remission.

Khouri *et al.*, reported on 25 patients with aggressive mantle cell lymphoma who received an intensive induction regimen to be followed by autologous or allogeneic transplantation in first remission.<sup>28</sup> Conditioning for transplantation consisted of cyclophosphamide and TBI. Four patients received allogeneic transplantation, the rest underwent autologous transplantation. With a median follow-up of 25 months (range 7-44 months), 21 patients remained free of disease. Actuarial event free survival at three years was estimated at 72% (95%CI 45-98). This was significantly improved compared with a historical control group.

Finally, Stewart *et al.* reported on nine patients with mantle cell lymphoma in relapse who underwent

autologous transplantation and achieved a two year disease-free survival of 34%.<sup>29</sup> Haas *et al.* reported on 13 patients. With a median follow-up of 18 months, disease-free survival was 74%.

Follow-up in all of these studies is rather limited. In contrast, the Dana-Farber group, in a retrospective analysis of 26 patients with recurrent or refractory mantle cell lymphoma, found a very high recurrence rate after autologous transplantation.<sup>33</sup> By two years after transplantation, more than half of the patients had relapsed. The high recurrence rate may be due to the high percentage of occult bone marrow involvement in these patients and the inability to achieve adequate purging of the marrow. In four mantle cell lymphoma patients in whom no residual lymphoma was infused (including 2 allogeneic and one syngeneic recipient), only one patient relapsed. This series casts serious doubts on the long-term curative potential of autologous transplantation in mantle cell lymphoma, at least with currently available methodology. On the other hand, after allogeneic transplantation, very few if any recurrences have been observed, and we currently recommend this for patients who have a matched sibling donor.<sup>28</sup>

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## A review of the international experience with allogeneic bone marrow transplantation in follicular lymphoma

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### Historical background

Follicular lymphoma (FL) usually has an *indolent* clinical behavior and is frequently associated with overexpression of the bcl-2 gene secondary to the t(14;18) chromosomal translocation. Until the nineties, no treatment has been able to modify the tendency of the disease to respond and relapse until transformation towards a more aggressive chemo-resistant disease. The more recently adopted high-dose approach significantly prolonged the clinical responses, but sensitive and specific molecular studies suggest that most of these patients still have minimal residual disease in their bone marrow or peripheral blood prior to overt clinical relapse. However, the achievement of a molecular remission in the peripheral blood has been demonstrated in a significant proportion of cases treated with intensive chemotherapy and the risk of relapse is lower in patients receiving an intensification with high-dose chemo-radiotherapy followed by molecularly negative autologous peripheral blood stem cell (PBSC) infusion compared to those rescued with molecularly positive cells. So far, prospective comparative studies have failed to recognize a significant survival advantage of any treatment over the other.

### Allogeneic bone marrow transplantation

Since the early nineties, durable complete remissions have been described in patients with FL treated with an allogeneic bone marrow transplantation (BMT) and more recently it has been shown that some of these patients appear to remain in molecular remission at last follow-up.<sup>1-6</sup> Moreover, it is relevant that sustained responses have also been achieved with an allogeneic transplant performed at a very late stage of the disease and finally the existence of a graft-versus-lymphoma (GVL) effect has been suggested by the disease regressions following the donor leukocyte infusion in patients relapsing after an allogeneic transplant.<sup>7-10</sup>

Patients receiving an allogeneic transplant for a FL also have a disappointingly high transplant-related mortality (TRM) and consequently their low relapse risk does not translate into a better survival in com-

parison with cases treated with an autologous transplant.<sup>11-14</sup> However, we must note that allogeneic transplant has been frequently performed in poor candidates to an autologous transplant because of chemo-resistant disease or extensive bone marrow involvement. Therefore, these risk-matched retrospective comparative studies may have failed to recognize any difference between autologous and allogeneic transplants which could exist if allogeneic transplant had been performed in patients with more favorable features. Another important aspect limiting these comparative retrospective studies is that the histologic features, which influenced the outcome of allogeneic transplant in low-grade lymphomas,<sup>15</sup> could not be reviewed.

More recently, an advantage of allogeneic over autologous transplant in FL has been suggested both by the achievement of a durable response with an allogeneic transplant in patients relapsing after an autologous transplant<sup>16,17</sup> and by the observation that unmanipulated allogeneic BMT produced a better event-free survival than autologous transplant in patients with a low tumor burden, but not in those with more advanced disease stage at transplant.<sup>18</sup> However, another recent study is in favor of allogeneic BMT also in patients with advanced poor-risk disease since it leads to prolonged disease-free survival which rarely occurs after autologous stem cell transplant.<sup>19</sup> It is noteworthy that T-cell depletion was applied only in the latter study. Therefore, the conflicting results of these two studies in transplants performed in patients with poor prognostic features may also suggest that T-cell depletion is an effective measure to reduce the TRM of allogeneic BMT without ablating its strong GVL effect.<sup>18,19</sup> In fact, a reduced risk of TRM has been described with T-cell depleted transplants<sup>20-23</sup> and with transplants after non-myeloablative conditioning regimens in poor candidates for standard allogeneic transplant.<sup>24-26</sup> These techniques would make allogeneic transplant safer and thereby applicable in a larger proportion of patients with FL at an earlier stage of their disease. However, it is not clear whether T cell depletion or non-myeloablative conditioning regimens will be associated with a higher risk of relapse which might still be curable with donor leukocyte infusion. Very sensitive and specific monitoring of the GVL effect through cell therapy may be realized in patients with

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FL carrying the t(14;18) chromosomal translocation with real-time quantitative polymerase chain reaction (PCR) analysis<sup>27,28</sup> rather than with the less sensitive and specific qualitative nested and single round PCR methods.<sup>29</sup>

Faster engraftment has been described with allogeneic PBSC transplantation compared to allogeneic BMT.<sup>30</sup> Moreover, T-cell depleted allogeneic PBSC transplant was associated with a lower relapse rate than T-cell depleted BMT.<sup>30</sup> However, this study included patients with various diseases, therefore the potential benefit of T-cell depleted allogeneic PBSC transplant over allogeneic BMT in patients with FL remains to be explored.

The growing interest in allogeneic transplant in patients with low-grade lymphoma is also indicated by the number of transplants performed since 1990 from a matched unrelated donor (MUD). The *National Marrow Donor Program* (NMDP) reported a 34% progression-free survival at 2 years in 40 patients with data indicating that, similarly to allogeneic transplants from an HLA-identical siblings, the main drawback of MUD transplant in patients with non-Hodgkin's lymphoma (NHL) is the very high TRM.<sup>31</sup> Antilymphoma responses were observed in adults achieving a mixed-chimerism with unmanipulated BMT from an HLA-mismatched family donor without myeloablative conditioning.<sup>32</sup> Future studies of allogeneic transplants from MUD or from HLA-mismatched related donors are warranted.

#### Reports from the international registries

The first report of the *European Group for Blood and Marrow Transplantation* (EBMT) indicated that allogeneic BMT may be successfully performed in patients with intermediate-grade or high-grade NHL.<sup>33</sup> In particular, the overall results were strongly related to disease status at transplant: patients with minimal residual disease having lower relapse rate and lower TRM than those transplanted with active disease.<sup>33</sup> The prognostic value of disease status at transplant was also confirmed in a second EBMT study: no survivors beyond 8 months post-transplantation in 10 patients transplanted (5 allogeneic and 5 autologous) with disease refractory to chemotherapy, patients transplanted in partial remission doing worse than those transplanted in complete remission among those with chemosensitive disease.<sup>34</sup> However, the prognostic value of disease status at time of allogeneic transplant could not be evaluated in patients with FL since, at that time, very few of such patients had received allogeneic transplant at the participating EBMT centers.

In the nineties more allogeneic transplants in patients with low grade NHL were performed in Europe, with 113 cases being reported in 1997 by the EBMT in a large comparative study of allogeneic transplant with autologous transplant for malignant lymphoma.<sup>13</sup> In particular, this third EBMT study has

shown that allogeneic BMT is highly effective for low grade lymphoma with a 42% progression-free survival and a 50% survival probability at 4 years.<sup>13</sup> The GVL effect was suggested in all histologic categories of NHL by the significantly lower relapse rate after allogeneic transplant than after autologous transplant.<sup>13</sup> Interestingly, the disease status at transplant did not influence overall survival in 113 patients with low grade lymphoma while it did so in high-intermediate grade NHL.<sup>13</sup> This observation supports a stronger GVL effect in low grade lymphoma compared to lymphomas with intermediate-high grade histologies. Despite the reduced relapse rate following allogeneic BMT, this study showed a survival disadvantage for these patients due to a higher TRM with allogeneic compared with autologous transplant.<sup>13</sup> Similar overall results of allogeneic transplant in low-grade lymphoma were subsequently confirmed by an observational study of 113 patients conducted at 50 centers participating in the *International Bone Marrow Transplant Registry* (IBMTR).<sup>35</sup> In the IBMTR study, most patients came from non-EBMT centers and therefore were not included in the previous EBMT comparative analysis.<sup>13</sup> Rare recurrences and high TRM were observed in the two largest international registry studies of allogeneic transplants in patients with low-grade lymphoma.<sup>13,35</sup> A prognostic analysis was conducted in the IBMTR study demonstrating that higher survival was associated with pre-transplant Karnofsky performance score greater or equal to 90%, chemotherapy-sensitive disease, use of a TBI-containing conditioning regimen, and age less than 40 years.<sup>35</sup> Three out of these prognostic features are not related to the transplant procedure itself, but may be useful to select patients having a better chance of survival after an allogeneic transplant.

#### Conclusions

The interest of transplant centers in allogeneic transplant in younger patients with FL has grown worldwide in the nineties mainly as a consequence of the disappointingly high relapse rates registered after autologous transplants and the promising results with allogeneic transplants derived from the large EBMT and IBMTR registry data.<sup>13,35</sup> Therefore, further studies should be directed to optimal exploitation of the powerful GVL effect after allogeneic BMT in patients with poor prognosis FL.

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## Rituximab anti-CD20 antibody therapy of B cell non-Hodgkin's lymphomas

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The approval of the chimeric anti-CD20 monoclonal antibody (mAb) Rituximab (IDEC-C2B8, Rituxan, Mabthera®) by the US FDA for the treatment of patients with relapsed low-grade or follicular lymphoma represented a milestone in the development of new agents for the treatment of cancer that have greater tumor specificity and less host toxicity. In the 18 months since drug approval, a large number of clinical trials have been initiated evaluating this new agent alone and in combination with conventional therapy. This paper will highlight the development of the mAb and the progress that has been made in the treatment of patients with B cell malignancies.

### **Rituximab (IDEC-C2B8, Rituxan, Mabthera®) chimeric anti-CD20 mAb**

Rituximab is a chimeric mAb containing the murine variable binding regions from IgG1 anti-CD20 mAb IDEC-2B8. The chimeric mAb has human IgG 1  $\kappa$  constant regions. Because of the human Fc constant regions, the mAb has much greater interaction with human immune effector mechanisms. *In vitro* and *in vivo*, the mAb binds to the CD20 antigen in a restricted fashion. *In vitro* tests using human complement and human antibody dependent cellular cytotoxicity (ADCC) effector cells demonstrate that the chimeric mAb is more active in killing B cell lymphoma lines than the murine parent mAb.<sup>1</sup> In addition, antibodies binding to CD20 may induce direct effects including the inhibition of proliferation and the induction of apoptosis in some B cell lymphoma lines.<sup>2-4</sup> Additional crosslinking may augment these effects.<sup>5</sup> In some of these studies the mAb appears to sensitize resistant cells to the effects of cytotoxic chemotherapy. However, in patients, the exact mechanism of tumor cell killing remains undefined. Rapid tumor lysis has been observed in some patients with high levels of circulating tumor cells in the blood, especially those tumor types that express high levels of the CD20 antigen such as prolymphocytic leukemia, some mantle cell and CLL-like histologies.<sup>6,7</sup> In some of these patients, symptoms are suggestive of complement activation. In most patients, the tumor response is much more gradual, occurring over several weeks to several months, suggesting that either ADCC or direct effects may be involved.

### **Single agent trials with rituximab**

The first studies evaluated single doses ranging from 10 to 500 mg/m<sup>2</sup>,<sup>8</sup> followed by weekly doses x 4 of 125-375 mg/m<sup>2</sup>. Mild infusional side effects were usually noted. These side effects included fever, chills and rigors along with mild upper respiratory symptoms and rash. They were most often grade I or II. More serious events including hypotension and bronchospasm were observed in less than 10% of patients. Adverse events were more likely to occur during the first mAb infusion and were rare with the second to fourth treatments. The cause of the infusional toxicity is not known, but may be due to the lysis of circulating B cells (normal and tumoral) and to the release of cytokines such as TNF- $\alpha$ , IL6 or IL-1. These symptoms were observed in earlier clinical trials of other murine mAbs in patients with circulating tumor cells. The lack of symptoms during the subsequent infusions may be explained by the persistence of mAb from the initial infusion and the depletion of B cells in the peripheral blood from the initial infusion. As mentioned above, more severe infusional related symptoms occurred in some patients. Although the results have not yet been extensively analyzed, many of these patients had high levels of circulating tumor cells with bright expression of CD20 in the peripheral blood. An alternate dosing schema involving treatment with a small dose of the mAb to first clear out the circulating tumor B cells and treatment with hydration and allopurinol has been proposed by some investigators for this population.<sup>7</sup> However, it is not clear that the more serious events can be anticipated and patients should receive adequate monitoring for infusional related symptoms during their initial mAb infusion.

There are several reports of single agents being active in the treatment of patients with relapsed low-grade non-Hodgkin's lymphoma (NHL).<sup>9,10</sup> The response rate is approximately 60% for patients with follicular histology, but only 12-15% for patients with small lymphocytic NHL. The median time to progression for responding patients is 11-13 months in these trials. Responses were observed in patients with bulky disease, patients relapsing following bone marrow transplantation and in patients resistant to chemotherapy, suggesting different mechanisms of action. Coiffier also reported single agent activity using 8 doses of the mAb in mantle cell NHL and in diffuse large cell NHL.<sup>11</sup> In this trial the response rate

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was approximately 30%, including responses in some elderly patients with aggressive NHL treated with mAb as initial therapy prior to conventional chemotherapy. Other responses have been reported in abstract form in Waldenström's macroglobulinemia (immunocytoma), post-transplant lymphoproliferative disorders, and CLL. In some patients with working formulation category A histology (small lymphocytic), altered pharmacokinetics of the mAb have been observed and treatment with larger doses of the mAb are being explored. For the majority of patients, however, the dose of 375 mg/m<sup>2</sup> x 4 weeks allows mAb to accumulate in the serum and persist for 3-6 months.<sup>12</sup> It is not clear whether larger doses of the mAb or more prolonged treatment will improve the response rate or duration of remission. Studies evaluating these approaches and using maintenance mAb every 6 months are underway.

### ***In vivo purging***

From the very first clinical trial with single agent rituximab, the specificity with which the mAb is capable of eliminating circulating B cells from the peripheral blood was evident.<sup>8</sup> Following a single infusion of 50-100 mg/m<sup>2</sup> rituximab, B cells detectable using flow cytometry will have been eliminated from the majority of patients with follicular NHL. Following treatment with 4 infusions of the mAb the majority of patients will become PCR negative for the t(14:18) translocation. Interestingly, the peripheral blood may be cleared even in patients who do not achieve an anti-tumor response in lymph nodes. In contrast, patients with small lymphocytic lymphoma or mantle cell lymphoma with circulating tumor cells seem to be more resistant to peripheral blood clearance. In most studies, normal peripheral blood B cells return in 6-9 months. This effect allows strategies designed to use pre-treatment with rituximab prior to stem cell collection to purge tumor cells *in vivo* from the PBSC collection. A number of trials are underway to evaluate this approach by combining rituximab with stem cell mobilization regimens with G-CSF alone or in combination with chemotherapy.

### ***Combinations with chemotherapy or cytokines***

The single agent activity and minimal toxicity associated with rituximab along with some evidence of synergy with conventional chemotherapeutic agents provide the rationale for combination studies. Although a large number of such trials are ongoing, only a few have been reported. It remains unclear whether the best strategy is to combine the mAb with the chemotherapy or to use them in sequence. The South Western Oncology Group in the USA has completed enrollment of more than 100 patients with newly diagnosed advanced stage follicular NHL treated with 6 cycles of the CHOP regimen followed by 4 infusions of rituximab. Patients are being followed for toxicity, response rate, disease free and overall survival and being monitored using PCR assays for

effect on molecular markers of the disease.

Czuczman recently reported combining 6 doses of rituximab with 6 cycles of CHOP chemotherapy for patients with low-grade or follicular NHL.<sup>13</sup> Two doses of the mAb were given prior to the first cycle and following the 6th cycle of CHOP. An additional dose was administered prior to the 3<sup>rd</sup> and 5<sup>th</sup> cycles. Nearly all patients responded to the treatment and 75% of patients remain progression-free after 2 years of follow-up. Toxicity was that expected from the mAb and from the chemotherapy. Link *et al.* recently reported on the combination of 6 cycles of CHOP with 6 doses of rituximab (a dose given 3 days prior to each chemotherapy cycle) for patients with aggressive large B cell NHL.<sup>14</sup> Again, a high response rate without unexpected toxicity was observed. Longer follow-up from all three of these trials and results from subsequent randomized clinical trials are required to prove the efficacy of these combinations.

Because of the long half-life of the mAb, it is probably not critical exactly when the mAb is given with the chemotherapy, as it will be continuously present following the first few doses in most patients. However, the question of whether the mAb would be more effective in combination (due to synergy) compared to that in sequence (treatment of minimal residual disease) remains unanswered and will await the results of clinical trials.

### ***Rituximab re-treatment***

Rituximab may be used as a retreatment option for patients with a prior response to the mAb. In a recent series approximately 40% of patients had a second response,<sup>15</sup> and interestingly those patients often had longer remissions than they had gained from the earlier course. Some patients have now received up to 4 different courses of the mAb and have had 4 responses over a period of 5-6 years. The reason why all the patients did not respond is not known. In a very few cases, selection of a CD20 negative clone has been documented. However, in most cases the tumor continues to express the CD20 antigen, implying that either the effector mechanisms are less active or that the tumor has developed resistance to the anti-tumor effects of the mAb (direct or immune mediated). This is not a surprise because earlier studies with anti-idiotypic mAbs demonstrated that when highly active selective pressure is applied to a tumor, escape mutants can arise. Strategies focused on augmenting effector mechanisms or increasing CD20 antigen expression by treatment with cytokines such as G-CSF, GM-CSF, IL-2 or interferon- $\alpha$  in order to enhance anti-tumor activity are being evaluated.

### ***Conclusions***

The development and approval of rituximab represent significant advances in the treatment of patients with B-cell NHL. It is clear that the agent induces tumor responses that are of benefit to our patients with relapsed low-grade histology. Exploration of the

use of mAb alone and combined with current chemotherapy regimens needs to be completed in order to determine the effect on the natural history of relapsing follicular NHL. Studies should be performed on combination therapy, both as front line treatment and during relapses, for patients with aggressive NHL. Randomized clinical trials will be required to determine the ultimate place of this new therapy in the treatment of patients with CD20 expressing B-cell NHL.

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## Rituximab sequentially administered to a first-line treatment with CHOP chemotherapy in follicular non-Hodgkin's lymphoma patients

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Follicular non-Hodgkin's lymphomas (FL-NHL) represent about 30 percent of newly diagnosed NHLs and occur most commonly in middle-aged patients, with the median age at diagnosis being 50 years. The neoplastic clone of the great majority (up to 80%) of FL-NHL patients bears the t(14;18) translocation in which the Bcl-2 proto-oncogene on chromosome 18 is translocated to the Ig heavy chain (IgH) region on chromosome 14 creating a hybrid Bcl-2/IgH gene. Molecular cloning of this translocation allowed a polymerase chain reaction (PCR)-based assay to be set up for the diagnosis and monitoring of minimal residual disease.<sup>1</sup> Despite the prolonged median survival time of these patients,<sup>2-4</sup> both conventional and experimental therapeutic approaches have failed to modify overall survival, although high dose therapy might provide some advantage at least in terms of freedom from disease progression.<sup>5</sup> The clinical results highlight the need to improve the management of minimal residual disease in an attempt to avoid therapeutic protocols associated with major toxicities. Vaccine and genetic therapies, as well as immunotherapy with monoclonal antibodies, might provide powerful new strategies to reach this goal in the near future.<sup>6-8</sup> Of the different monoclonal antibodies tested in clinical trials, the chimeric mouse/human anti-CD20 antibody rituximab (IDEC Pharmaceuticals, San Diego, CA and Genentech, Inc, San Francisco, CA, USA) has so far been the most extensively studied.<sup>9-15</sup> This molecule is an IgG1  $\kappa$  antibody containing murine light and heavy chain variable regions, and human  $\gamma$  1 heavy chain and  $\kappa$  light chain constant regions. The antibody reacts specifically with the CD20 antigen which appears during the pre-B cell development stage of B-cell differentiation, is expressed on the surface of malignant and normal B-cells and is not present on stem cells and plasma-

cells. In addition, the CD20 antigen is not shed and does not appear to undergo modulation in response to antibody binding, thus representing an ideal target for monoclonal antibody based immunotherapy. Moreover, preliminary results indicate that rituximab can revert the bone marrow and peripheral blood lymphocytes of patients with PCR detectable t(14;18) positive cells to a negative status.<sup>13</sup> With the aim of identifying an effective and non-myeloablative strategy for the molecular eradication of minimal residual disease, the present multicenter study was designed to evaluate the capacity of rituximab to induce negativization of the Bcl-2/IgH chimeric gene in patients persistently positive after CHOP induction therapy.

### Design and Methods

#### Patients

Ten Italian centers were selected for the study. To be eligible, each patient was required to have a histology proven diagnosis of CD20 positive follicular NHL as defined by the REAL classification and a positive molecular analysis for Bcl-2/IgH t(14;18) translocation in the bone marrow and peripheral blood. Patients were required to be more than 18 and less than 65 years of age, with clinical stage II-IV disease (according to the Ann Harbor staging system) and an ECOG performance status of 0.1. After first time treatment with six cycles of CHOP chemotherapy, patients had to be in partial or complete clinical response to be eligible for rituximab treatment. To be included patients needed to be molecularly positive on two consecutive determinations four and six weeks after the completion of CHOP chemotherapy (baseline). If one of the two tests proved negative, a third molecular assessment was performed within 2 weeks; this third evaluation conclusively established positivity or negativity for the molecular marker. Patients were not included if they had other NHL subtypes, had progressive or stable disease after CHOP, had received prior chemotherapy for the lymphoma, had clinically significant

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abnormal cardiac, liver or renal function unrelated to the lymphoma, history of other cancers, major surgery within the last 4 weeks, opportunistic infections, HIV positive serology, unacceptable hematologic status or CNS involvement. The study was conducted according to rules of good clinical and laboratory practice, and the principles of the Declaration of Helsinki and had been approved by the local Ethics Review Committees.

### Study design and monitoring of minimal residual disease

The study was an open label, multicenter phase II study to assess whether the neoplastic clone in patients with follicular NHL carrying the t(14;18) translocation can be reduced and potentially eliminated by the administration of rituximab. Patients with a molecularly proven t(14;18) were assigned to receive 6 cycles of CHOP chemotherapy. Four weeks after the last molecular determination confirming the presence of t(14;18) positive cells in the bone marrow and/or peripheral blood, patients were planned to receive four weekly intravenous infusions of rituximab (375 mg/m<sup>2</sup>). Every week patients were examined at the investigating center to check efficacy and safety parameters. The molecular follow-up always had to be performed on both bone marrow and peripheral blood samples, and was planned as follows: after the third cycle of CHOP, and at 4 and 6 weeks after the sixth cycle of CHOP (baseline). Thereafter, the molecular monitoring was performed 12, 28 and 44 weeks after baseline.

### Endpoints

The primary endpoint of the study was to assess the capacity of rituximab to induce negativization of the Bcl-2-IgH chimeric gene in the bone marrow and peripheral blood of patients with a partial or complete clinical response after CHOP chemotherapy and with molecularly detectable residual disease. Another primary objective of the study was to evaluate the safety of rituximab when administered sequentially to CHOP chemotherapy. A secondary endpoint was to evaluate the clinical activity on residual disease after CHOP induction chemotherapy.

### Molecular evaluation of Bcl-2/IgH rearrangement

The molecular evaluation of follicular lymphoma patients bearing the t(14;18) chromosome abnormality was carried out by PCR analysis of the Bcl2-IgH rearrangement according to published methods with only minor modifications.<sup>16</sup> Briefly, the initial amplification was carried out in a 50 µL final volume using 1 µg of DNA with the following oligonucleotides: 5' CAGCCTTGAAACATTGATGG 3' for the MBR or 5' CGTGCTGGTACCACTCCTG 3' for the mcr and 5' ACCTGAGGAGACGGTGACC 3' for the J<sub>H</sub> consensus region. Samples were amplified with an initial denaturation at 95°C for 10 min, followed by either 27

**Table 1. Molecular analysis of Bcl-2/IgH chimeric gene in follicular non-Hodgkin's lymphoma patients referred for the study.**

Overall entry	121 cases
PCR positive	89/121 (74%)
PCR negative	32/121 (26%)
MBR+	81/89 (91%)
mcr+	8/89 (9%)

MBR+= major breakpoint region; mcr+= minor cluster region.

cycles of denaturation for the MBR or 30 cycles for mcr. Each cycle was performed with 1 min of denaturation at 94°C, 1 min of annealing at 55°C for the MBR amplification or 1 min at 58°C for the mcr, and 1 min of extension at 72°C. The final extension period was prolonged to 10 min. A nested PCR reaction (30 cycles) was performed using 1 µL of a 1:10 dilution of the first-round amplification product using oligonucleotide primers internal to the original primers: 5' TATGGTGGTTTGACCTTTAG 3' for the MBR or 5' GGACCTTCCTTGGTGTGTTG 3' for the mcr and 5' ACCAGGGTCCCTTGGCCCCA 3' for the J<sub>H</sub> consensus region, with 1 min of denaturation at 94°C, 1 min of annealing at 58°C and 1 min of extension at 72°C. The final extension period was again prolonged to 10 min. A 25-µL aliquot of PCR product was analyzed on a 2% agarose gel containing ethidium bromide in Tris-borate electrophoresis buffer and visualized under UV light.

### Results

One hundred and twenty-one previously untreated patients with a morphologic diagnosis of follicular NHL were referred to our laboratories for molecular analysis of t(14;18). By means of the PCR assay we searched for the presence of Bcl-2/IgH positive neoplastic cells within the bone marrow and peripheral blood mononuclear cells; 89 (74%) patients had a detectable rearrangement while 32 (26%) proved negative. Among the 89 Bcl-2/IgH positive patients, 81 (91%) had a rearrangement within the major breakpoint region (MBR) and 8 (9%) within the minor cluster region (mcr) (Table 1). Patients positive for t(14;18) were assigned to receive CHOP and the molecular evaluation repeated after 3 and 6 cycles of chemotherapy confirmed that this anthracyclin-containing regimen can induce clearance of t(14;18) positive cells from the bone marrow and peripheral blood in a significant proportion of previously untreated follicular patients.<sup>17</sup> Indeed, as shown in Table 2, after 3 cycles of CHOP 18 of 66 (27%) evaluable patients proved to be PCR negative both in the bone marrow and peripheral blood lymphocytes; similarly, after 6 cycles of CHOP 19 of 52 (36%) evaluable patients were molecularly negative. At this time, 33 patients who had reached a com-

**Table 2. Molecular analysis of Bcl-2/IgH chimeric gene in previously untreated follicular non-Hodgkin's lymphoma patients.**

CHOP chemotherapy	PCR negative
Before therapy	0/66 (0%)
After 3 cycles	18/66 (27%)
After 6 cycles*	19/52 (36%)

\*The molecular analysis was conducted on bone marrow and peripheral blood mononuclear cells on 2 consecutive determinations.

**Table 3. Molecular follow-up analysis after rituximab administration in follicular NHL patients treated with sequential CHOP and rituximab.**

PCR status	12 weeks (n=25)	28 weeks (n=17)	44 weeks (n=9)
BM-/PB-	13	12	5
BM-/PB+	1	0	0
BM+/PB-	9	4	1
BM+/PB+	2	1	3

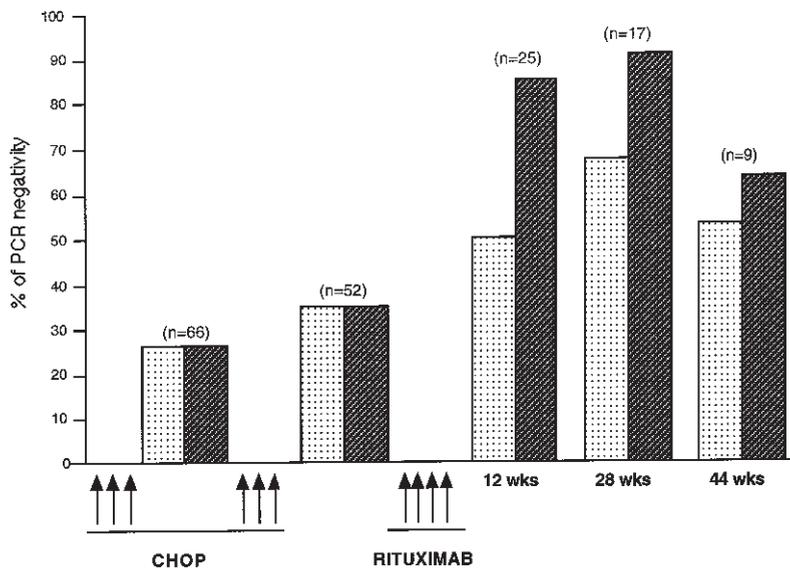
Pb, peripheral blood; BM, bone marrow.

plete or partial clinical remission after CHOP chemotherapy, but were still PCR positive, started rituximab therapy. Of these patients, 25 have completed rituximab treatment and entered the minimal residual disease follow-up program which was performed again on bone marrow and peripheral blood samples collected, as planned, 12, 28 and 44 weeks after enrollment into the study. At the first determinations after the four weekly infusions of Rituximab, half of the

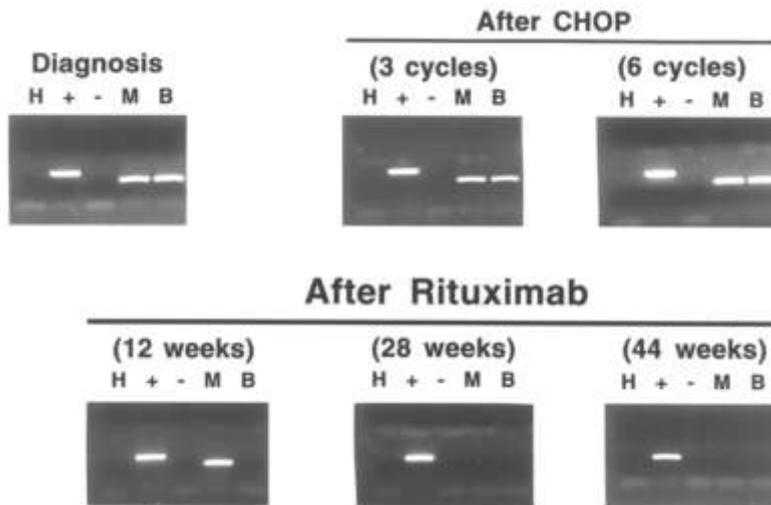
patients had reached a molecular complete remission defined as the clearance of the Bcl-2/IgH chimeric gene from both the bone marrow and peripheral blood. Notably, the disease appears to be more persistent in the bone marrow than in the peripheral blood, since after the first and second follow-ups, more than 85% of patients were found to be molecularly negative as assessed using peripheral blood while a lower proportion of patients reached the same result when bone marrow was used for the assessment (Table 3 and Figure 1). Nonetheless, several PCR positive cases in the bone marrow at the first follow-up had become negative by the second molecular analysis, thus indicating an apparent progressive clearance of the neoplastic clone (Figure 2). Nine of the 19 patients molecularly negative at the end of CHOP chemotherapy and who did not receive rituximab were also molecularly evaluated 12 weeks after baseline; 3 of them had become bone marrow PCR positive.

## Discussion

The use of PCR analysis to detect the Bcl-2/IgH chimeric gene in NHL lymphoma patients bearing the t(14;18) chromosome aberration is a powerful tool to investigate minimal residual disease which has been proposed as a crucial prognostic factor to determine the clinical response to different therapeutic modalities. A positive correlation between molecular eradication of the Bcl-2/IgH chimeric gene and a good clinical outcome has been reported in patients with a previously untreated follicular lymphoma who received conventional anthracyclin-containing chemotherapeutic regimens.<sup>17</sup> A similar positive correlation has also been found in patients undergoing autologous transplantation in second or further remission either



**Figure 1. Molecular eradication of Bcl-2/IgH bearing cells in follicular NHL patients after 3 and 6 cycles of CHOP and four infusions of rituximab. White columns represent patients achieving a complete molecular response (PCR negativity in both the bone marrow and peripheral blood) whereas black columns indicate patients PCR negative only in the peripheral blood.**



**Figure 2.** Representative experiment showing the progressive clearance of Bcl-2/IgH positive cells after rituximab administration. PCR analysis was performed, at the indicated time points, on patients' bone marrow (M) and peripheral blood (B) mononuclear cells. As negative controls, a sample containing all PCR reagents without DNA was amplified (H) in parallel with DNA extracted from peripheral blood mononuclear cells obtained from a normal donor (-). As positive control, DNA was extracted from the Bcl-2/IgH positive DoHH2 cell line (+).

with *in vitro* purged bone marrow derived stem cells<sup>18-21</sup> or with circulating peripheral blood progenitor cells mobilized and purged *in vivo* with high dose sequential chemotherapy.<sup>22</sup> Our study was designed to investigate whether the sequential administration of 6 cycles of CHOP chemotherapy and four intravenous weekly infusions of the chimeric anti-CD20 monoclonal antibody rituximab could induce a comparable or greater eradication of the neoplastic clone as monitored by PCR analysis of bone marrow and peripheral blood samples in a group of previously untreated follicular NHL patients. Our results confirm that CHOP chemotherapy is capable of inducing molecular negativization in at least one third of the patients. Among patients achieving a molecular remission after CHOP, the clearance of the lymphomatous clone seems to be rapid since this result is already reached after 3 cycles of chemotherapy in the majority of patients. Whether this reflects a peculiar sensitivity to chemotherapy or a lower tumor burden in the bone marrow and peripheral blood still remains to be determined. Not surprisingly, however, in most cases the neoplastic clone was not cleared by conventional chemotherapy either from the bone marrow or from the peripheral blood and these patients were enrolled in the rituximab program. Many of these patients became negative following four injections of rituximab and some of them remained in long-lasting molecular remission. We noticed that the clearance of the Bcl-2/IgH chimeric gene was progressive after rituximab, since several cases became negative at the second molecular follow-up. It is tempting to speculate that this late and progressive disappearance of the neoplastic clone can be explained on the basis of the available phar-

macokinetic data indicating that measurable amounts of the chimeric anti CD20 antibody can be found up to six months after the end of treatment.<sup>13</sup> Another intriguing observation is the apparent resistance of the neoplastic clone within the bone marrow. This result is in keeping with other studies which also suggested that molecular analysis of minimal residual disease performed on bone marrow samples has more reliable prognostic implications.<sup>18</sup> In another recently published study it was confirmed that the marrow aspirate more frequently remains PCR positive even though peripheral blood PCR was reported to be as informative as bone marrow for the detection of minimal residual disease and for its prognostic value. On the basis of these results, it has been proposed that patients who achieve and sustain a molecular response have a better failure-free survival than those who either revert back to PCR positivity or who never achieve a molecular response.<sup>17</sup> Although the short follow-up of our patients does not allow us to draw firm conclusions, the high proportion of complete (bone marrow and peripheral blood) and partial (only peripheral blood) molecular responses so far observed seems to be promising. Despite the high proportion of PCR negativizations, some cases had subsequent molecular and clinical relapses which highlights the importance of prolonged follow-up of this cohort of patients.

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## Radioimmunotherapy of non-Hodgkin's lymphomas

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The clinical effectiveness of antibody serotherapy of B-cell lymphomas has now been clearly established by a series of trials from several institutions employing chimeric anti-CD20 antibodies (e.g., rituximab [Rituxan®]) and radiolabeled anti-CD20 antibodies.<sup>1-12</sup> Non-radioactive chimeric anti-CD20 antibodies have been shown to produce objective remissions in 50-60% of patients with follicular lymphomas and in a third of patients with aggressive lymphomas.<sup>2,3</sup> Furthermore, the toxicity of the chimeric rituximab antibody has been generally less than that of standard chemotherapy and anti-chimeric antibody immune responses occur in <1% of patients. Nevertheless, unlabeled anti-CD20 antibodies have significant limitations. Half the patients with indolent non-Hodgkin's lymphomas (NHL) and two-thirds of patients with large cell lymphomas and mantle cell lymphomas fail to respond to rituximab and only 5-10% of the observed responses are complete.<sup>2,3</sup> For these reasons, we and others have investigated the potential for enhancing the efficacy of anti-B cell antibodies by conjugating them to radionuclides.<sup>5</sup> This approach is attractive because radiolabeled antibodies kill tumor cells by emitting  $\beta$  particles which are effective even in patients with defective host immune effector functions. The exquisite radiosensitivity of hematologic malignancies makes radioimmunotherapy particularly attractive for targeting B-cell lymphomas.

### The CD20 antigen

The CD20 antigen appears to be an ideal target for radiolabeled antibody therapy. It is a 35,000 molecular weight non-glycosylated phosphoprotein which is present in high density on the surface of > 95% of B-cell lymphomas. Although its precise function is controversial, it appears to play an important role in B-cell cycle progression and may also function as a calcium channel.<sup>13</sup> In contrast to many other B-cell surface antigens, CD20 does not circulate freely in the bloodstream nor does it undergo endocytosis after antibody binding. These features are markedly advantageous, since circulating antigen

could act as a *blocking factor* preventing access of anti-CD20 antibodies to tumor cells. Furthermore, internalization of iodine-131-labeled anti-CD20 could lead to degradation and "dehalogenation" of iodine-131-radioconjugates,<sup>14,15</sup> thereby attenuating their efficacy.

### Choice of radionuclide

Both iodine-131 and yttrium-90 have been used effectively for radioimmunotherapy of B cell lymphomas, and experts do not yet agree on which radioisotope is better. I-131 has been used in the majority of clinical trials and is convenient for several reasons: it is readily accessible; it can be attached to antibodies using simple radiochemical reactions (Chloramine T or iodoGen reactions); it is inexpensive; and it can be used for both imaging and therapy. Most importantly, I-131 has demonstrated impressive success in treating both thyroid carcinoma and non-Hodgkin's lymphomas.<sup>5-12</sup> Disadvantages of I-131 include its tendency to be released rapidly from tumor target cells after endocytosis,<sup>14-16</sup> and its emission of energetic gamma rays which present a potential radiation risk to family members and to health care personnel.

Yttrium-90 emits  $\beta$  particles which are five times as energetic as those from I-131. It has a half-life of 2.5 days, is easily utilized on an outpatient basis, is stably retained by tumor cells even after endocytosis,<sup>16</sup> and emits very few gamma particles. On the other hand its absence of gamma emissions means that surrogate isotopes such as indium-111 must be used for imaging purposes. In addition, Y-90 is more expensive and less accessible than I-131. Furthermore Y-90 has a tendency to accumulate non-specifically in the liver and bones, though this problem is mitigated by utilization of newer, stabler chelation methods.

### Impact of tumor burden and splenomegaly

Many investigators have published studies showing that the accumulation of radiolabeled antibodies in tumor sites decreases as tumor size increases.<sup>17-19</sup> The adverse effect of increasing tumor burden on accumulation of radioimmunoconjugates is believed to result from diffusion and convection barriers which impede penetration of large immunoglobulin molecules into large tumors.<sup>20,21</sup> Human clinical trials have usually supported results of animal

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experiments in demonstrating an adverse impact of tumor size on antibody penetration, though the implications of this finding for treatment remain controversial. In a phase I study of I-131-labeled anti-B cell antibodies, we found that patients with tumor burdens less than 500 cc in volume were more likely to achieve a *favorable biodistribution* than patients with tumor burdens greater than 500 cc ( $p < .0001$ )<sup>8</sup> (a *favorable biodistribution* was rigorously defined in this study to mean every assessable tumor site received more radiation than any of the critical normal organs).

Splenomegaly seems to exert a similar adverse effect on antibody biodistributions. In our phase I biodistribution study, we found that only 2 of 16 patients with significant splenomegaly achieved a *favorable biodistribution* compared to 17 of 22 with normal spleens and 5 of 5 splenectomized patients.<sup>8</sup> We have assumed that the deleterious effect of splenomegaly on antibody biodistributions is due to the entrapment of anti-B cell antibodies by large numbers of resident CD20-expressing B-cells in the spleen. The trapping of I-131-anti-CD20 antibodies in the spleen limits the availability of I-131-anti-CD20 antibody to other tumor sites, for example lymph nodes. Other clinical investigators have also reported splenic trapping of therapeutic antibodies, although this phenomenon can be partially circumvented by pre-infusion of a large dose of unlabeled *cold* antibody prior to the radiolabeled antibody.<sup>6,7,22</sup>

Despite the unfavorable influence of large tumor burdens and splenomegaly on antibody penetration into tumor sites, it has been shown that even patients with these unfavorable characteristics can achieve sufficient concentrations of radioimmunoconjugates in tumor sites to attain meaningful clinical benefit, though the chances of achieving a remission, particularly a complete remission, are diminished compared to those of patients with smaller tumor burdens and normal spleen sizes.<sup>12</sup> We have shown that patients with bulky disease can achieve better antibody biodistributions if they are treated with a combined approach which includes initial cytoreduction with chemotherapy or splenectomy to debulk the tumor prior to I-131-anti-CD20 antibody therapy.<sup>9</sup>

#### **Selected clinical trials of radioimmunotherapy**

Several groups have now reported the efficacy of radiolabeled antibody therapy for patients with B-cell lymphomas. Although excellent responses have been reported using radiolabeled antibodies targeting several different B-cell surface antigens including idiotype immunoglobulin, class II histocompatibility antigens, and CD22, the highest response rates, highest complete response rates and longest remission durations have been reported in trials using radiolabeled anti-CD20 antibodies.<sup>6-12</sup> One hundred and sixteen patients were treated in multi-center trials using non-myeloablative doses of I-131-anti-B1 (anti-CD20)

antibody (Bexxar®, Coulter Pharmaceutical Inc., South San Francisco, CA, USA) according to a dosing regimen originally developed by Mark Kaminski.<sup>6,7</sup> In this approach, patients are first given a dosimetric infusion of 450 mg/m<sup>2</sup> of unlabeled anti-B1 antibody followed by 30 mg of trace-labeled I-131-anti-B1. Serial whole body gamma imaging is then performed three times over the subsequent week to determine the rate of radioimmunoconjugate metabolism. An individualized therapeutic infusion of I-131-anti-B1 is then administered 7-14 days after the trace-labeled infusion using the same doses of antibody but with an augmented dose of I-131 calculated to deliver 75 cGy of whole body irradiation (usually 100-150 mCi). A response rate of 78% including 46% complete responses was observed in the 116 patients.<sup>29</sup> Myelosuppression was dose-limiting, with grade 4 neutropenia seen in 15-20% of patients 4-6 weeks after treatment. Non-hematologic toxicities included mild fevers, chills, fatigue, and nausea. This same regimen was used by Dr. Kaminski to treat 34 newly diagnosed, previously untreated patients with low grade lymphomas at the University of Michigan. All of the previously untreated patients achieved objective remissions, including 56% complete remissions, with I-131-anti-B1 antibody.<sup>23</sup> It is noteworthy that the complete responses often developed very slowly over months as documented by serial CT scanning.

Susan Knox *et al.* administered yttrium-90 (Y-90)-labeled anti-CD20 antibodies to 18 patients with relapsed B cell lymphomas (4 treated with the anti-B1® antibody and 14 with the IDEC Y2B8® antibody) using single doses of 13.5 to 50 mCi of Y-90.<sup>11</sup> Six complete remissions and seven partial responses were observed (overall response rate, 72%), with a median response duration of six months. Doses of Y-90 greater than 50 mCi produced severe hematologic suppression, requiring stem cell reinfusion, but no other serious toxicities were observed. Witzig recently also investigated the efficacy and toxicity of Y-90-labeled anti-CD20 antibodies in a phase I/II trial of IDEC-Y2B8® antibody.<sup>12</sup> Patients were treated with either 100 or 250 mg/m<sup>2</sup> of unlabeled rituximab followed by Y-90 labeled Y2B8 (0.2, 0.3, or 0.4 mCi/kg) with 250 mg/m<sup>2</sup> being determined as the optimal dose of unlabeled rituximab. This trial also established the maximally tolerated dose of Y-90 without stem cell support to be 0.4 mCi/kg. The overall response rate was 67%, including 26% CRs and 41% PRs. Eighty-two percent of patients with low grade lymphomas responded (27% CRs, 56% PRs), compared to 43% of patients with intermediate grade lymphomas. As in other trials of radioimmunotherapy, hematologic toxicity was dose-limiting. Only one patient in this trial developed an anti-globulin (HAMA/HACA) immune response.

Other investigators have reported radioimmunotherapy trials targeting B lymphocyte surface antigens other than CD20. DeNardo *et al.* pioneered treatment

of lymphoma patients with radiolabeled antibodies using an I-131-labeled-anti-DR antibody (Lym-1).<sup>24</sup> DeNardo treated 30 patients with relapsed NHL and CLL with fractionated doses (30-60 mCi) of an I-131-Lym-1 given at 2-6 week intervals and observed three CRs (10%) and 14 PRs (47%) (overall response rate, 57%). As in many other trials, myelosuppression (especially thrombocytopenia) was dose-limiting. The same investigators later investigated the efficacy of increasing doses of I-131-Lym-1 (40-100 mCi/m<sup>2</sup>), and reported an overall response rate of 52% (33% CRs and 19% PRs).<sup>25</sup> David Goldenberg and his coinvestigators infused I-131-labeled anti-CD22 antibody LL2 into 21 patients with relapsed B-cell lymphomas, achieving objective remissions in 4 (24%), including one complete response.<sup>26,27</sup> Finally, yttrium-90-labeled anti-idiotypic antibodies were administered to 9 NHL patients by White *et al.*<sup>28</sup> which achieved two complete responses and one PR.

### **High dose radioimmunotherapy with autologous stem cell transplantation**

Our group in Seattle has focused on studying the potential of myeloablative doses of I-131-labeled anti-B cell antibodies given in conjunction with autologous stem cell support to circumvent the dose-limiting hematologic toxicity.<sup>8-10</sup> In a phase I dose-escalation trial, we evaluated the biodistribution of I-131-labeled anti-CD20 (anti-B1<sup>®</sup>) and anti-CD37 (MB-1) antibodies in 43 patients with relapsed B-cell lymphomas. A protein dose of 1.7\*<sup>i</sup> mg/kg achieved optimal biodistribution with the anti-CD20 (anti-B1<sup>®</sup>) antibody, whereas 10 mg/kg was required to achieve an optimal biodistribution with the anti-CD37 antibody. Nineteen patients received therapeutic infusions of I-131-anti-CD37 or anti-CD20 antibodies calculated to deliver a specified maximal dose of radiation ranging from 10 to 31 Gy (234-777 mCi) in an escalating manner to critical normal organs. Fifteen patients required autologous bone marrow reinfusion. Non-hematologic toxicities included nausea, fever, thyroid dysfunction, and transient, mild elevations of liver function tests, but these were generally mild if doses were limited to less than 23 Gy. At higher doses which delivered 27 Gy or more to the lungs, significant but reversible cardiopulmonary toxicity was observed. Eighteen of 19 patients treated with therapeutic infusions on this protocol obtained objective remissions (95%), including 16 complete responses (84% of treated patients).

In a subsequent phase II trial, we administered 1.7 mg/kg\* of I-131-anti-B1 antibody to 21 patients using an individualized dose of I-131 (345 to 785 mCi) which was estimated to deliver approximately 27 Gy to the dose-limiting normal organ (generally the lungs) with higher doses of radiation being delivered to tumor sites (27-92 Gy).<sup>9</sup> Previously stored autologous bone marrow (19 patients) or peripheral blood stem cells (2 patients) were reinfused into patients

after the body activity fell to 2 mR/hr or lower at 1 meter. Seventeen of the 21 patients (81%) eventually achieved complete remission and one achieved a partial remission for a total response rate of 86% (one patient converted from a partial response to a complete remission after the original paper was published). Twenty-nine of the patients treated with I-131-anti-B cell antibodies in these phase I & II trials at our institution were treated with the anti-B1 (anti-CD20) antibody. We have recently reviewed and published long-term follow-up results of these 29 cases treated with I-131-anti-B1 with stem cell transplantation.<sup>10</sup> After a median follow-up of 42 months, we found that remission durations ranged from 3 to 87+ months after radioimmunotherapy. The median remission duration has not yet been reached but will be in excess of 38+ months for patients with indolent lymphomas.<sup>10</sup> Kaplan-Meier estimates of overall survival and progression-free survival are 68% and 42%, respectively, after a median follow-up of 42 months. The projected overall survival at six years for patients having indolent lymphomas (n=19) was 78%, compared with 43% for patients with aggressive histologies ( $p=0.07$ ). Progression-free survival at six years was estimated to be 51% for patients with indolent lymphomas compared to 20% for aggressive histologies ( $p=0.04$ ).

Our current protocol attempts to improve upon single agent I-131-anti-CD20 radioimmunotherapy by combining myeloablative doses of I-131-anti-B1 with high dose VP-16 (60 mg/kg), cyclophosphamide (100 mg/kg), and autologous stem cell transplantation. Although the final results of this ongoing clinical trial are not yet available, it appears that these agents can be safely combined with acceptable toxicity. The maximally tolerated dose of I-131-anti-CD20 which can be safely given in this combination appears to be a dose delivering no more than 25 Gy of irradiation to the heart and lungs. Currently, the overall survival is 84% and the progression-free survival is 74% for 47 patients entered in this phase I/II study (median follow-up 18 months [unpublished data]).

### **Summary**

Radiolabeled anti-B cell antibodies are safe and effective agents which have shown encouraging efficacy and acceptable toxicity for treatment of patients with relapsed lymphomas. Ongoing clinical trials should define more accurately the nuances of treatment regimens which will achieve optimal clinical efficacy.

\*Recent recalibration of the anti-B1 antibody protein concentrations used in the phase I and II trials by Coulter Pharmaceuticals (Palo Alto, CA, USA) with a revised antibody extinction coefficient suggests that the doses of anti-B1 antibody administered were actually 0.35, 1.7, and 7.0 mg/kg rather than 0.5, 2.5 and 10 mg/kg, respectively, as originally reported.<sup>8,9</sup>

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## Adoptive immunotherapy for B-cell lymphoma

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Although patients with advanced stage non-Hodgkin's lymphoma (NHL) often achieve clinical complete remission (CR), the majority of these patients ultimately relapse. The source of such relapse in NHL, as in other malignancies, is most likely the residual lymphoma cells that are below the limit of detection using standard diagnostic techniques. Therefore considerable effort has been made over the past decade to develop new techniques that have greatly increased the sensitivity of detection of neoplastic cells. In particular, the identification of specific gene rearrangements and chromosomal translocations in neoplastic cells has permitted the development of sensitive molecular techniques that are capable of detecting minimal residual malignant cells. With the development of these more sensitive techniques, especially by the application of polymerase chain reaction (PCR) technology, the presence of residual neoplastic cells in patients in complete clinical remission, commonly called *minimal residual disease* (MRD), has been demonstrated clearly. It would seem obvious that if such residual lymphoma cells can be detected in a patient additional therapy would be necessary for cure. However, this has never been conclusively established for the minimal residual numbers of neoplastic cells that can now be detected in patients following achievement of a clinical CR. The critical issue now is to determine whether such sensitive detection of residual detectable lymphoma cells by PCR can identify those patients who will relapse. If this proves to be the case, then molecular biology techniques will become a routine part of staging and follow-up of patients and redefine our concept of CR, such that our goal should be to aim for a *molecular CR*.

Tumor specific DNA sequences occur at the sites of non-random chromosomal translocations and are candidates for detection by PCR amplification if the sequence of the breakpoints are known. Most follicular lymphomas exhibit t(14;18), most mantle cell lymphomas express t(11;14), and small subsets of patients have characteristic chromosomal transloca-

tions. However, the majority of patients with lymphoid malignancies do not demonstrate non-random chromosomal translocations. In these cases an alternative strategy must be developed to detect MRD. During normal lymphoid maturation, B-cells and T-cells undergo rearrangement of their antigen receptors, the Ig and TCR genes respectively. Lymphoid neoplasms usually rearrange TCR or Ig genes or both, and their clonal progeny have this identical antigen receptor rearrangement. B-cell neoplasms including NHL, acute lymphoblastic leukemia (ALL), myeloma and chronic lymphocytic leukemia (CLL) undergo somatic rearrangement of the IgH locus providing a useful marker of clonality and stage of differentiation in these tumors. Previous studies from our own and from other laboratories have supported the notion that eradication of PCR detectable disease is indeed associated with improved outcome.<sup>1-5</sup> In our own studies PCR detection of disease identifies two groups of patients at high risk of relapse. Persistence of PCR detectable disease in the stem cell collection after immunologic purging is associated with greatly increased likelihood of relapse.<sup>6</sup> Similarly, persistence, reappearance or quantitative increase in PCR detectable disease in the patient after high dose chemotherapy is also associated with high likelihood of relapse.<sup>1-5</sup> Improved outcome might therefore be achieved by increasing the efficacy of purging or by approaches to eradicate minimal residual disease in the patient. An ideal approach to treat such minimal residual disease is by immunotherapy. Such therapy would be most likely to be effective when the patient has minimal disease. Moreover, the use of PCR assessment allows this therapy to be targeted to those patients at sufficiently high risk of relapse to merit such an approach.

The potential role of immunotherapy has been demonstrated in patients with B-cell malignancies. The use of CD20 mAbs has clearly demonstrated immunotherapy can be an effective treatment for patients with B-cell lymphoma or leukemia.<sup>7,8</sup> Recent advances in basic immunology have allowed us to recognize the important pathways involved in the initiation and expansion of a T-cell mediated response against tumors. Since the major co-stimulatory molecules are expressed on professional antigen presenting cells (APCs) it has become clear why tumor cells function poorly as APCs since they fail to express these

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molecules. A number of investigators have focused on the use of dendritic cells (DCs) as the major APCs pulsed with tumor associated antigens to trigger anti-tumor immunity.<sup>9,10</sup> An obvious target for such an approach in the B-cell malignancies is the tumor clone-specific idiotype (Id) of B-cell lymphoma and leukemia. This antigen has been extensively studied and has been targeted using a number of immunotherapeutic strategies.<sup>11-14</sup> Very convincing data have been reported regarding CD4<sup>+</sup> T-cell and antibody responses against Id and there is also clear evidence for clinical efficacy of Id-directed immunotherapy. This strongly suggests that B-cell leukemia and lymphoma can be recognized by the patients' immune system. However, one major disadvantage of Id-directed immunotherapy is that the Id is specific for each patient's clonal B-cell population and therefore every patient has to be treated with an individual drug or reagent that is still very difficult to produce. This treatment might therefore not be widely applicable, but limited to a few specialized centers. One of the disadvantages of targeting a single antigen or even a single peptide is the chance of immunoselecting for antigen-loss variants of the tumor by inducing an efficient immune response against the antigen targeted. An alternative approach is to convert the tumor cells that could be turned into efficient APCs that might be capable of presenting a whole array of tumor antigens so that immunoselection might be prevented. We and others have been able to demonstrate that leukemia and lymphoma B-cells can be turned into very efficient *tumor-APCs*.<sup>15,16</sup> Crosslinking CD40 on normal and malignant B-cells induces important adhesion and costimulatory molecules and significantly upregulates MHC class I and II expression as well as antigen processing in these cells. Moreover, important cytokines including IL-12 are induced in normal and malignant B-cells after CD40 stimulation. These cells become very efficient APCs for allogeneic and autologous T-cells.

Patients who achieve CR but have persistence of MRD would be ideal candidates for immunotherapy. Unfortunately, a large body of data demonstrates that the immune system is defective with a restricted T-cell repertoire after chemotherapy and/or stem cell transplantation. These defects would most likely hamper the success of vaccination strategies since vaccination depends on the induction of T-cell responses *in vivo*. The adoptive transfer of *ex vivo* generated autologous lymphoma-specific T-cells might overcome this limitation. Studies of donor lymphocyte infusion after allogeneic BMT for patients with CML strongly support the claim that T-cell therapy after chemotherapy can be effective.<sup>17</sup> In addition adoptive T-cell transfer of EBV- respectively CMV-specific T-cells after allo-BMT is effective.<sup>18</sup>

We are currently conducting clinical trials of CD40 activated lymphoma cells as vaccines to induce tumor specific immunity. We are also conducting

clinical trials of adoptive T-cell specific immunotherapy performing expansion of tumor specific autologous T-cells *ex vivo*. Pre-clinical studies are underway attempting also to induce tumor specific allogeneic T-cells so that donor lymphocyte infusions might maximally target tumor antigens, potentially decreasing the likelihood of graft versus host disease.

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## Translational development of patient-specific vaccination for follicular lymphoma

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It is known that the idiotypic determinants (Id) of the immunoglobulin synthesized by follicular lymphoma (FL) cells are unique and can therefore serve as a tumor-specific antigen.<sup>1,2</sup> In a pilot study in humans, we pioneered the use of purified autologous Ig protein as a vaccine for patients with FL.<sup>3</sup> This study demonstrated that FL Ig could be made immunogenic, primarily by the induction of antibody responses. However, because these patients were already in clinical remission, standard tumor shrinkage criteria could not be used to assess anti-tumor responses.

In about 85-90% of cases FL is characteristically associated with a chromosome translocation that brings the *bcl-2* gene, located on chromosome 18, under the transcriptional influence of the immunoglobulin heavy chain gene on chromosome 14. The precise codons involved in the translocation are patient-specific, but the rearrangements cluster in particular regions: the major breakpoint region (MBR: 65% of the breakpoints) and the minor cluster region (MCR: 25% of the breakpoints). The remaining 10% occur between the MBR and the MCR. It has been shown that the *bcl-2*/IgH translocation involving the MBR can be reliably utilized as a molecular marker for minimal residual disease (MRD) using a very sensitive PCR technique.<sup>4,5</sup> Previous studies have demonstrated that most FL patients in clinical CR following standard chemotherapy still harbor *t*(14;18)-bearing tumor cells detectable by PCR.<sup>6,7</sup> Furthermore, patients with persistent tumor cells in bone marrow and/or peripheral blood appear to be at increased risk of relapse. Based on promising preclinical studies,<sup>8</sup> we began testing a novel Id vaccine formulation for its ability to induce tumor-specific T-cell immunity, as measured by the ability of patient T-cells to specifically lyse their own tumor cells *in vitro*, and to exert anti-tumor effects, measured by elimination of *t*(14;18)-bearing cells from the peripheral blood of uniformly treated FL patients in first CR.

### Clinical protocol

The protocol opened under IRB approval in 1994. Of the 35 patients who signed informed consents, 23 (66%) achieved a clinical complete response to chemotherapy. However, 3 of these 23 patients had to be excluded shortly afterwards because of either early relapse or vaccine production difficulties, leaving a homogeneous group of 20 patients with FL in first CR who actually received vaccine treatment. Specifically, previously untreated patients with a diagnosis of stage III or IV follicular small cleaved cell or follicular mixed lymphoma underwent excision of a peripheral lymph node as starting material for vaccine production and were then uniformly treated with a minimum of 6 monthly cycles of prednisone, doxorubicin, cyclophosphamide, and etoposide (ProMACE,<sup>9</sup> modified) to CR. Restaging and documentation of CR at the end of chemotherapy, pre-vaccination, and post-vaccination required absence of disease as detected through physical examination, and common staging procedures, including bilateral bone marrow biopsies, chest and abdomen CT scans and lymphangiograms.

Ig was purified from each patient's tumor by a heterohybridoma fusion technique and conjugated to keyhole limpet hemocyanin (KLH) as previously described.<sup>3</sup> Identity of fusion products and the patient's FL was verified by comparing their Ig VH CDR3 sequences.<sup>10</sup> After 6-15 months of immune recovery following the completion of chemotherapy, each of the 20 patients remaining in CR received 4 monthly vaccinations s.c. with autologous lymphoma-derived Ig (0.5 mg)-KLH mixed with free GM-CSF as an immunologic adjuvant. For each course, 3 additional doses of GM-CSF were administered daily at the same vaccination site. In addition, an identical booster vaccination was given 2 months later. Thus, each patient received a total of 5 vaccinations.

### Molecular evaluation

*Bcl-2*/IgH gene translocations were amplified by a nested PCR previously described,<sup>6</sup> using peripheral blood mononuclear cell (PBMC)- and/or bone marrow (BM) cell-derived genomic DNA. In our hands, this PCR consistently revealed a sensitivity of detection of 1 malignant cell in a total of 100,000 cells.

For each timepoint, 10 replicates were tested in parallel in the same experiment, along with a positive con-

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trol consisting of genomic DNA obtained from the RL cell line, and negative controls consisting of genomic DNA obtained from the DHL-16 cell line and buffer alone. A single positive PCR reaction out of 10 replicates was considered sufficient to score the timepoint as positive.

As an internal control, the  $\beta$ -actin pseudogene was amplified from all DNA samples. Furthermore, the molecular evaluation was performed in 2 independent laboratories at 2 separate sites by individuals blinded to clinical information. Almost complete concurrence of results was observed between replicates of individual samples and between the 2 sites (96% of individual PCR reactions yielded identical results).

Finally, we determined the optimal cellular source of the genomic DNA template, by comparing pre-vaccination PBMC and BM samples head to head. Initially, both BM and PBMC samples were analyzed in parallel for 7 patients. All 7 PBMC samples yielded unique bcl-2 gene rearrangements, while these rearrangements were successfully amplified from only 3 BM samples obtained at the same pre-vaccination timepoint. On the basis of these results, we completed our molecular analysis on serial PBMC samples.

Of the 20 patients who achieved CR after ProMACE chemotherapy and completed Id vaccination, 11 had tumors which demonstrated amplifiable MBR bcl-2/IgH gene rearrangements, and were thus suitable for molecular analysis of their MRD. Bcl-2/IgH rearrangements for all 11 patients were formally sequenced and confirmed to contain unique combinations of sequences derived from the bcl-2 intron, JH complex, and N region nucleotides.

All 11 patients were PCR positive in PBMC at study entry (pre-chemotherapy), as well as pre-vaccination, despite remaining in clinical CR. However, 8 of these patients converted to PCR negativity immediately after completion of vaccination. All 8 patients have maintained PCR negativity in follow-up samples for a median of 15+ months after completion of vaccination (range: 8+ to 32+ months). Furthermore, bcl-2/IgH breakpoints amplified from pre-vaccine PBMC genomic DNA were formally sequenced and found to be identical to those amplified from their corresponding primary tumor lymph nodes. The remaining 3 patients have remained continuously PCR positive. Thus, at least 8 of 11 (73%) patients cleared residual t(14;18)-bearing cells from the peripheral blood as a consequence of Id vaccination.

### Clinical course

Eighteen of 20 patients remain in continuous, first complete remission (median: 39+ months from completion of chemotherapy, range: 25+ to 50+). Two patients relapsed at 15 and 7 months after completion of vaccine therapy. One had never cleared the t(14;18)-bearing cells from the peripheral blood; the other patient did not have the MBR rearrangement and so molecular CR status could not be established.

### Conclusions

The availability of a sensitive molecular marker for residual FL cells provided the opportunity to assess, for the first time in a systematic manner, the question of anti-tumor effects in response to vaccination. Our PCR assay provided a level of sensitivity of detection that optimally distinguished between pre-vaccination PBMC samples, which were uniformly positive, and samples obtained post-vaccination, which have been continuously negative in 8 of the 11 patients with MBR-positive tumors. Although the long-term clinical significance of molecular remissions in patients with FL remains to be determined,<sup>7,11</sup> vaccination either further reduced the tumor burden beyond that already achieved by chemotherapy in these patients or led to the redistribution of residual tumor to sites other than the peripheral blood.

GM-CSF may be a critical component of this vaccine formulation, both for generation of CD8+ T-cell immunity and for potency of anti-tumor effects.<sup>8</sup> In fact, our earlier clinical protocol using the same immunogen administered without GM-CSF revealed primarily humoral, without convincing CD8+ T-cell, responses.<sup>3</sup>

The results of the current study thus provide the rationale for a definitive, prospective randomized trial comparing combination chemotherapy alone to the same chemotherapy followed by KLH-conjugated Id protein vaccination given with GM-CSF in a study that uses remission duration as an endpoint.

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## Mechanism-based rationales for combination therapies of lymphoid malignancies

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Nucleotide analogs now comprise the major class of therapeutic agents used in the treatment of hematologic malignancies. For instance, pentostatin and more recently cladribine have each had a major impact on the natural history of hairy cell leukemia. Cytosine arabinoside and 6-thioguanine are standard components of most therapeutic regimens for acute leukemias, but the cure rate is in need of improvement, particularly in adult patients. Finally, fludarabine is clearly active in indolent B-cell malignancies, both as initial treatment and for disease that has become refractory to treatment with alkylating agents. Nevertheless, it is apparent that fludarabine alone is unlikely to generate a significant population of long-term survivors. Thus rationales are needed for the design and evaluation of nucleoside analog-based therapies that feature combinations with other effective anti-leukemia agents. New knowledge of the varied mechanisms of action of nucleoside analogs, as well as an appreciation of the biology of tumor cells, provide an opportunity to develop mechanism-based strategies in the design of combination therapies.

The mechanisms of action of such well known analogs as thiopurines, cytosine arabinoside, fludarabine, and cladribine, all appear to require the incorporation of the drug into DNA before therapeutically useful activities are seen. Once incorporated, some nucleotide analogs then serve as relatively poor substrates for the addition of subsequent nucleotides by DNA polymerases and the process of DNA replication thereby becomes inhibited. The current hypothesis stipulates that analog-induced delays in DNA replication are sensed by cells, and that these sensing mechanisms signal for additional processes responsible for removal of the analog. If the cell is unable to remove the analog by proof-reading DNA repair mechanisms that would permit resumption of DNA replication, the stalled replication fork will eventually generate a signal initiating cell death by apoptosis. Thus, incorporation into

DNA is essential for nucleotide analog-induced cell death. A major limitation to the potential effectiveness of nucleoside analogs is the fact that only a small fraction of most tumors are actively in cycle, and yet fewer are in S phase when the drugs are administered. Therefore, therapeutic strategies are needed to expand the fraction of the tumor population that is synthesizing DNA.

Our approach to increasing the number of cells engaged in DNA synthesis has focused on conditions that induce DNA repair. Because these repair processes share many of the same DNA polymerizing and metabolizing enzymes that are required for DNA replication, it has been hypothesized that incorporation of a nucleotide analog into a patch of repairing DNA by these enzymes would prevent the repair of the damaged DNA. In addition, it is possible that the incorporated analog would be sensed by mechanisms similar to those which monitor replication, an action that might also activate the apoptosis signal. Thus, combinations of nucleosides with alkylating agents or platinum derivatives, which are known to initiate DNA repair processes, might act to sensitize cells that are otherwise kinetically resistant to nucleoside analogs.

### Fludarabine

Fludarabine exhibits several novel mechanisms of DNA synthesis inhibition which are different from ara-C, suggesting that fludarabine may be more effective in combination with drugs that induce a DNA repair response.<sup>1</sup> The triphosphate, F-ara-ATP, is the major intracellular metabolite of fludarabine. The concentration of F-ara-ATP in CLL lymphocytes reaches 30-60  $\mu\text{M}$ ,<sup>2,3</sup> a range in excess of concentrations required to compete with normal nucleotides to inhibit specific enzymatic actions effectively in model systems. The retention of F-ara-ATP by CLL lymphocytes is variable among individuals, but is generally characterized by a median half-life of 12-15 hr.<sup>3</sup> Thus, it is a relatively long-lived active metabolite, which probably accounts for the observed efficacy of daily dosing schedules.

F-ara-ATP inhibits the synthesis of deoxynucleotides by ribonucleotide reductase,<sup>4</sup> depleting the cell of dNTPs required for DNA replication and repair. This probably facilitates the incorporation of

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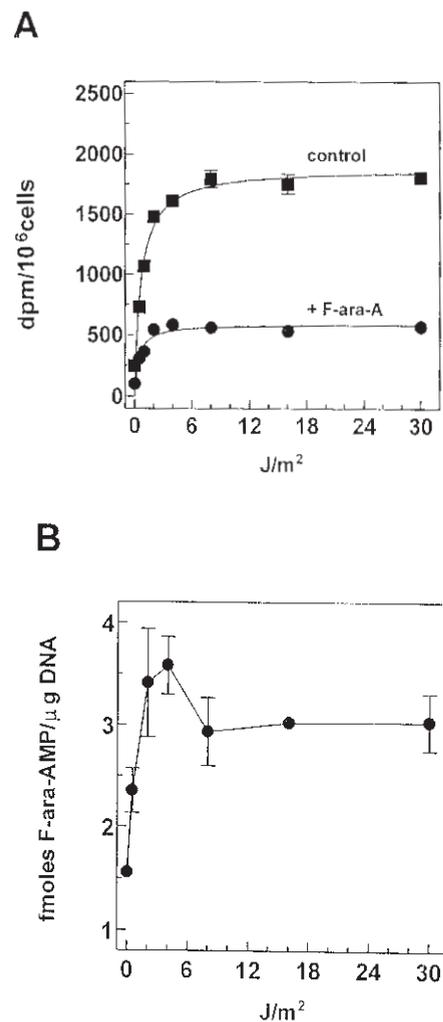
more F-ara-ATP into DNA because the ratio of the cellular concentrations of dATP to F-ara-ATP is lowered. The incorporation of fludarabine nucleotides into DNA is strongly correlated with loss of cell viability;<sup>5</sup> this is manifested by several distinct mechanisms. First, initiation of DNA synthesis on the lagging strand with primer RNA by DNA primase is inhibited by F-ara-ATP.<sup>6</sup> Second, F-ara-ATP competes with dATP for incorporation into DNA; once in the DNA, it is a poor substrate for the addition of a subsequent deoxynucleotide by any of several DNA polymerases, and thus frequently terminates DNA synthesis at the site of analog incorporation.<sup>5</sup> Third, the 3'→5' exonuclease proof-reading activities associated with pol  $\epsilon$ , which is thought to enhance the fidelity of DNA replication by removing mispaired nucleotides from the elongating DNA chain, is only poorly capable of removing 3'-terminal F-ara-AMP<sup>5,7</sup> relative to normal nucleotides and ara-CMP.<sup>8</sup> Fourth, when DNA ligase encounters a piece of DNA that is terminated by a fludarabine nucleotide, it is difficult to join that DNA to an adjacent DNA.<sup>9</sup> Furthermore, the free nucleotide, F-ara-ATP, is directly inhibitory to human DNA ligase. When fludarabine nucleotide incorporation in DNA reaches a critical level, the process of apoptosis is activated, or released, and the cell dies.<sup>10-12</sup> Finally, F-ara-ATP is a substrate for RNA polymerase II, thus transcription is prematurely terminated and protein synthesis affected.<sup>13</sup>

These multiple mechanisms of action distinguish fludarabine from other inhibitors of DNA synthesis and repair such as ara-C and hydroxyurea. The inhibition by F-ara-ATP of components of DNA synthesis that are required for DNA repair suggests that combining fludarabine with agents which initiate DNA repair, will create new targets for F-ara-ATP action. To this end, we have demonstrated synergistic activity of fludarabine and ionizing radiation in tumor-bearing mice,<sup>14,15</sup> and in combinations with chemotherapeutic agents which produce cross-links or adducts that signal DNA repair are a second approach to generating targets for the action of fludarabine nucleotides. An example of this tactic is the combination of fludarabine with cisplatin. Together, these drugs produce synergistic cytotoxicity in growing cells in culture; the mechanism of this toxicity involves inhibition of the repair of cisplatin-induced cross-links in the DNA.<sup>16</sup> A cisplatin-resistant cell line exhibits more rapid removal of cross-links than parental cells, indicative of a greater capacity for DNA repair. Treatment of the resistant cells with fludarabine slows the rate of cross-link removal to that of control cells, suggesting that fludarabine may be useful in reversing resistance to cisplatin.<sup>16</sup>

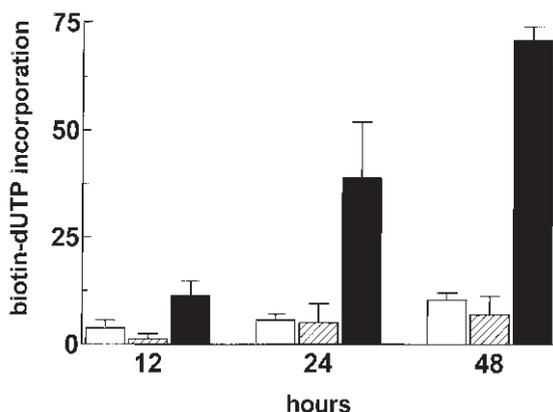
#### UV radiation and fludarabine

We conducted a proof-of-principle study of this hypothesis using quiescent human lymphocytes activated into DNA repair with UV light.<sup>17</sup> In keeping with

earlier studies, UV irradiation stimulated unscheduled DNA synthesis, and this was saturable with radiation dose (Figure 1A). Pre-treatment with fludarabine nucleoside (F-ara-A) almost completely inhibited this unscheduled DNA repair response. Using [<sup>3</sup>H]F-ara-A, it was possible to determine that this was accompanied by the incorporation of the analog into the repairing DNA of these lymphocytes (Figure 1B). Flow cytometry studies demonstrated that throughout this treatment the lymphocytes remained quiescent with G<sub>0</sub> DNA content. These results are consistent with the conclusion that UV irradiation initiated nucleotide excision repair, and that the flu-



**Figure 1. Induction of DNA repair by UV irradiation and inhibition by F-ara-A.** A. Lymphocytes from healthy donors were incubated with 3 mM F-ara-A for 2 hr (closed symbols) or not (open symbols) followed by the indicated doses of UV irradiation. Immediately thereafter, [<sup>3</sup>H]thymidine was added and after 2 hr, radioactivity associated with DNA was quantified as a measure of DNA repair. B. Lymphocytes were incubated with 0.85  $\mu$ M [<sup>3</sup>H]F-ara-A for 2 hr, and irradiated with the indicated doses of UV. After 2 hr, radioactivity associated with DNA was quantified as a measure of F-ara-A incorporation.<sup>17</sup>



**Figure 2. Apoptosis induction in quiescent lymphocytes after treatment with fludarabine, UV, or their combination.** Lymphocytes from healthy human donors were irradiated with  $2.0 \text{ J/m}^2$  of UV (open bars), incubated with  $3 \mu\text{M}$  F-ara-A for 2 hr. (stripped bar), or with  $3.0 \text{ mM}$  F-ara-A for 2 hr before irradiation with  $2.0 \text{ J/m}^2$  (solid bars). Apoptosis was measured by the TUNEL assay using flow cytometry.<sup>17</sup>

darabine nucleotide was incorporated into the repairing DNA of the cells. Although neither fludarabine nor UV alone caused significant apoptosis as judged by morphology, DNA fragmentation, or TUNEL assay by flow cytometry, combining these treatments resulted in much greater than additive killing, approaching 80% of the population by 48 hours (Figure 2). These proof-of-concept studies provided a theoretical basis for the design of combination therapy trials in the clinic.

### Cyclophosphamide

Cyclophosphamide is an alkylating agent which shows activity against a wide variety of hematopoietic neoplasms, some solid tumors, and in the context of bone marrow transplantation. As a prodrug, it requires metabolism, primarily by mixed function oxidases of the liver, to produce 4-hydroxycyclophosphamide. This metabolite and its acyclic tautomer aldophosphamide are transported to the blood, and eventually these prodrugs enter other tissues, including the target tumor cells, and give rise to phosphoramidate mustard, the proximal alkylating species.<sup>18,19</sup> Peak alkylating levels in plasma are found 2 to 3 hours after cyclophosphamide administration, and seem to be maintained for several hours.<sup>20</sup> The cytotoxic lesion is thought to result from the formation of interstrand DNA cross-links, which are found after incubation with the activated prodrug 4-hydroperoxy-cyclophosphamide *in vitro*<sup>21</sup> and following *in vivo* cyclophosphamide administration.<sup>22</sup> Interstrand cross-links involve predominantly N7 of guanine, although reaction with the phosphate backbone<sup>23</sup> and O<sup>6</sup> position of guanine<sup>24</sup> occur to a lesser extent. DNA-protein

cross-links are also formed; the kinetics of single-strand and double-strand DNA breaks, which have been found long after the initial alkylations, suggest that these are the result of DNA cross-linking and are not caused directly by the drug.<sup>25</sup>

Few studies on the cellular pharmacodynamics of cyclophosphamide in clinical samples have been reported. DNA single-strand breaks and interstrand cross-linking were studied in mononuclear cells from the peripheral blood of women receiving simultaneous combination treatment with cyclophosphamide and carboplatin for ovarian cancer.<sup>26</sup> Although both parameters were increased after treatment, it was not possible to establish correlations with response. Such determinations in normal tissues may serve as a dosimeter for comparisons among patients, although the usefulness of such measurements as predictors of response comes into question, particularly when applied to relapsed and possibly resistant disease. DNA interstrand cross-linking was measured in the leukemic lymphocytes of a patient with CLL.<sup>27</sup> Maximum cross-linking was observed 12 hours after *i.v.* cyclophosphamide injection; there was a significantly lesser amount at 24 hours, suggesting a repair process. Although the precise mechanism of repair by removal of DNA interstrand cross-links by bifunctional alkylating agents such as phosphoramidate mustard, melphalan, busulfan, or cisplatin has not been elucidated, *in vitro* studies with human CLL lymphocytes indicate a kinetic process which varies between individuals.<sup>28-31</sup> Strategies to inhibit the repair of interstrand cross-links using DNA synthesis inhibitors such as ara-C or hydroxyurea have shown some success in preclinical systems,<sup>16,32</sup> but with the possible exception of ara-C and cisplatin combinations in low grade lymphoma,<sup>33</sup> there has not been a notable increase in response rates when these approaches have been translated into the clinic.

### Synergistic mechanisms of action

As an extension of this study, we sought to evaluate the DNA repair hypothesis in quiescent human lymphocytes using 4-hydroperoxycyclophosphamide, a precursor of cyclophosphamide, a DNA repair-inducing agent that is widely used in the clinic. As an assay of DNA repair, we applied single cell gel electrophoresis (comet) analysis.<sup>34,35</sup> This assay has an established application in human lymphocytes incubated *in vitro*<sup>36</sup> and during therapy with alkylating agents.<sup>37,38</sup> Although alkylation by cyclophosphamide will activate base excision repair, nucleotide excision repair, and the removal of interstrand DNA cross-links, the results obtained with 4-HC are qualitatively similar to those obtained by UV light which was chosen originally because it activates nucleotide excision repair almost exclusively.

As seen after UV irradiation, treatment with 4-HC for 15 min also elicited induction of unscheduled DNA synthesis. Most of this was due to single strand

**Table 1.** Action of fludarabine and 4-hydroperoxycyclophosphamide on the normal human lymphocytes. Lymphocytes from healthy volunteers were incubated with 10  $\mu$ M F-ara-A alone for 2 hr, 40  $\mu$ M 4-hydroperoxycyclophosphamide alone for 15 min, or with the combination, first with 10  $\mu$ M F-ara-A for 105 min and then addition of 40  $\mu$ M 4-hydroperoxycyclophosphamide for 15 min. Cells were washed and the tail moment was analyzed by comet assay at the indicated times.

Repair time, hr	Relative tail moment		
	F-ara-A*	4-HC <sup>o</sup>	F-ara-A & 4-HC <sup>o</sup>
0	0.0 $\pm$ 0	8.3 $\pm$ 1.5	15.3 $\pm$ 2.4
2	0.5 $\pm$ 0.7	1.0 $\pm$ 1.2	11.0 $\pm$ 3.6

\*n = 2; <sup>o</sup>n=4.

breaks revealed under the alkaline conditions, whereas double strand breaks (neutral pH conditions) were a minor component (not shown). We interpret these results to indicate that 4-HC treatment initiated a DNA repair response that included removal of alkylated DNA nucleotides, and that the single strands are diagnostic of these processes. There was also a dose-dependent response when fludarabine triphosphate was loaded into cells by incubation with 0.1 to 30  $\mu$ M F-ara-A followed by a 15 min exposure to 20  $\mu$ M 4-HC (not shown). As was the case after UV induction of nucleotide excision repair (Figure 1B), this was accompanied by inhibition of unscheduled DNA synthesis and incorporation of [<sup>3</sup>H]F-ara-A into the DNA of the lymphocytes, which remained in G<sub>0</sub>. When the induction of DNA repair by 4-HC was evaluated by the single cell gel electrophoresis (comet) assay, it was clear that F-ara-A alone had no effect on comet formation (Table 1). In lymphocytes treated with 4-HC alone, the significant increase in tail moment that was observed initially (zero hour) diminished rapidly after washing into fresh medium, suggesting rapid completion of the processes requiring DNA incision/ligation. However, when cells were first loaded with fludarabine triphosphate and then treated with 4-HC and washed into fresh medium, the original comet signal was greater than after 4-HC alone, and failed to recover to control values (Table 1). The difference between the 4-HC and 4-HC + F-ara-A values was statistically significant ( $p = <0.001$ , two-way ANOVA test). Both MTT assays of cell viability and pulsed-field gel electrophoresis of high molecular weight DNA fragmentation which accompanies apoptosis indicated that the effect of the combination was greater than the sum of the effect of each drug alone (not shown).

### Summary

Our results support the hypothesis that initiation of DNA repair in quiescent cells will permit the incorporation of a nucleotide analog into the DNA of these cells. Combining what are essentially non-tox-

ic levels of agents (cisplatin, cyclophosphamide) or modalities (UV or ionizing radiation) that initiate DNA repair with nucleoside analogs such as fludarabine resulted in substantially greater killing than the sum of either agent alone and that suggests that the combination is acting by a mechanism different from that of either agent alone. This is consistent with the working hypothesis, which postulates that agents that induce DNA repair in quiescent cells will enable the incorporation of nucleotide analogs into the DNA of quiescent cells, thereby initiating signals for cell death. Recent clinical trials designed to implement this rationale have proved its effectiveness in the treatment of indolent hematologic diseases.<sup>39</sup>

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## Gastric MALT lymphoma

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**M**arginal zone B-cell MALT lymphomas comprised 7.6% of more than 1,400 non-Hodgkin's lymphomas in a recent international evaluation of the clinical significance of the REAL classification. In the past most cases would have been misinterpreted as pseudolymphomas, although many features contribute to their allocation as malignant conditions: monoclonality is usually demonstrable and histologic transformation has been described, as well as the possibility of dissemination to the regional lymph nodes and sometimes to the bone marrow. Moreover, non-random chromosomal aberrations can be detected, the most common being the t(11;18)(q21;q21) present in at least one third of cases. The recent identification of the genes at the breakpoints (the apoptosis inhibitor gene API2 and a novel 18q gene, MLT) suggests that this translocation may result in a survival advantage for MALT lymphoma B-cell clones. A second non-random translocation, much more rarely detected, the t(1;14)(p22;q32), might confer an increased capacity of autonomous growth to the tumor by means of inactivating mutations and overexpression of the BCL10 gene.

Paradoxically, MALT lymphoma usually arises as a consequence of a pre-existing disorder in mucosal sites where lymphocytes are not normally present. The onset of MALT lymphomas in the stomach is preceded by the acquisition of MALT as a result of *Helicobacter pylori* infection. The micro-organism can be found in the gastric mucosa in nearly all instances and there is a body of epidemiologic, biological, molecular and clinical data suggesting a link between *H. pylori*-chronic gastritis and MALT lymphoma of the stomach. This link supports the hypothesis that *H. pylori* provides the antigenic drive to the lymphoma growth in the stomach.

A tentative explanation for the pathogenesis of gastric MALT lymphomas may be that B- and T-lymphocytes are recruited in the gastric mucosa as part of the immune response; B-cell proliferation is secondary to specific activation of reactive T-cells by *H. pylori* and cytokines. It is not clear whether the B-cell activation requires the continuous presence of the

micro-organism as an antigenic source or whether it is related to an indirect autoimmune mechanism. In fact, the tumor B-cells most often show antibody specificity for auto-antigens and, to proliferate, need contact-dependent help from intratumoral T-cells which is apparently mediated by CD40 and CD40 ligand (CD154) interactions. This immunologic stimulation mediated by mucosal T-cells may explain the tendency of low-grade MALT lymphoma to remain localized and to regress after *H. pylori* eradication.

Genetic alterations can, however, continue to occur until a point is reached at which autonomous (i.e. *H. pylori*-independent) growth is possible, and additional alterations can result in high-grade transformation. The exact mechanism of this transition from *H. pylori* infection to MALT lymphoma is still unclear. Most patients with *H. pylori* gastritis do not develop lymphoma. It is, therefore, widely accepted that additional environmental or genetic factors must play a pivotal role in gastric lymphomagenesis.

The histologic features of low-grade B-cell lymphomas of MALT type are similar regardless of the site of origin. The pivotal feature of low-grade MALT lymphoma is the presence of a variable number of lymphoepithelial lesions that can be defined as unequivocal invasion and partial destruction of gastric glands or crypts by aggregates of tumor cells. Lymphoepithelial lesions are of striking relevance for the diagnosis of low-grade gastric MALT lymphoma; however they can sometimes be seen in the context of non-neoplastic mucosa-associated lymphoid tissue.

Although diagnosis of low-grade B-cell MALT lymphomas from gastric biopsies is usually easy, early or borderline cases can be confused with *H. pylori*-related follicular gastritis and in a few cases the distinction on purely morphologic grounds can be difficult. Since lymphoma is a clonal outgrowth of cells that have acquired certain genetic alterations, possible diagnostic support can come from the finding of a monoclonal B-cell population; molecular demonstration of monoclonality must, however, be interpreted in the context of the histologic pattern and should not have a diagnostic value *per se*.

MALT lymphoma usually remains localized to the initial site, but sometimes can present with involvement of multiple mucosal sites. Some cases have been identified with simultaneous gastric and intestinal involvement; thyroid and salivary gland MALT lymphomas may also disseminate to the gastroin-

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testinal tract. It has been postulated that this may be due to particular homing properties similar to those of the normal B-cells of the MALT. Within the stomach low-grade MALT lymphoma is often multifocal. Microscopic lymphomatous foci can be present at sites distant from the main tumor and may explain the reports of frequent relapses in the gastric stump after surgical excision.

Some diagnostic problems can arise in the presence of an increased number of large cells, a finding that may suggest histologic progression to a high-grade lymphoma. In fact, only the *low-grade* MALT lymphomas have been included as extranodal MZB-CL in the REAL classification, while *high-grade* MALT lymphomas, with or without a low-grade component, are still included in the diffuse large-cell lymphoma (DLCL) group as a distinct disease with aggressive clinicopathologic features.

Histologic grading of MALT lymphomas in gastric biopsies is often problematic; a small component of low-grade MALT lymphoma can be identified in a significant proportion of high-grade lymphomas, and conversely, foci of high-grade (large-cell) lymphoma can be seen in low-grade MALT lymphoma, suggesting transition from one to the other, as occurs in other low-grade lymphomas. The prevalence and time interval of histologic transformation of MALT lymphomas are unknown. Isaacson and Chan have proposed that the presence of compact confluent clusters, or sheets of large cells may indicate the emergence of new clones and can be used as the criterion for diagnosing transformation into a high-grade MALT lymphoma. However, general agreement has never been achieved. More recently de Jong *et al.* proposed the distinction of the following four groups which may have prognostic relevance: a) *pure low-grade MALT lymphoma*, defined by clusters of less than 5 blast cells and with no evidence of a diffuse blastic component; b) *low-grade MALT lymphoma with high-grade component* in which clusters of blasts of 5 to 20 cells (and occasionally single large clusters) can be found and with a diffuse blastic component below 10% of the tumor cells; c) *high-grade MALT lymphoma with low grade component* showing large clusters of more than 20 cells and/or a diffuse blastic component of more than 10%, sporadic lymphoepithelial lesions may still be present; d) *high-grade lymphoma without low grade component* with only a diffuse blastic (large cell) component.

Whether this last group should be categorized as a MALT lymphoma remains controversial; at least some of these lymphomas are derived from the MALT, as strongly supported by the epidemiologic findings of Parsonnet *et al.*, who found that *H. pylori* infection precedes the growth of both low-grade MALT lymphoma and DLCL gastric lymphomas.

There is increasing evidence that eradication of *H. pylori* with antibiotics can be effectively employed as the sole initial treatment.

We recently reviewed a multicenter series of 93

patients with low-grade gastric MALT lymphomas of the stomach from northern Italy (Brescia and Varese) and southern Switzerland (Bellinzona). The disease was most often localized in the antrum or multifocal. No statistically significant difference was apparent in either overall survival or event-free survival between patients who received different initial treatments (chemotherapy alone, surgery alone, surgery with additional chemotherapy or radiation therapy, or antibiotics against *H. pylori*). The actuarial 5-year overall survival was 82% in the series as a whole. In this series, 49 patients (all with stage-I disease) were given only antibiotics as initial treatment; eradication of *H. pylori* was achieved in 97% of patients and histologic regression of the MALT lymphoma was documented in 67% of patients (95% CI 51% to 80%) after *H. pylori* eradication. The median time required to achieve histologic regression was 5 months (range 3-18 months). A first update of the German multicenter trial has also been recently published and confirms, with a median follow-up of 2 years, the efficacy of antibiotics in inducing apparently durable lymphoma remission.

The use of antibiotics as first-line therapy may avert or at least postpone the necessity for more aggressive treatment in most patients and in our opinion, the indolent nature of the disease in most cases makes a conservative approach advisable, with antibiotic therapy as the sole initial treatment. Any of the highly effective antibiotic regimens recently proposed can be used, *provided that strict oncologic and endoscopic follow-up is carried out* with multiple repeated biopsies taken two-three months after treatment to document *H. pylori* eradication, and, subsequently, twice yearly for two years, then at least once yearly to monitor the histologic lymphoma regression.

Whether treatment for *H. pylori* will definitely cure the lymphoma and prevent its relapse is still unknown; indeed, the effect of antibiotics on the lymphoma clones appears to be suppressive rather than ablative. Some cases have been reported in which tumor recurrence was documented following *H. pylori* re-infection, suggesting that some residual dormant tumor cells can still be present despite clinical and histologic remission. In addition, relapses have been documented in the absence of *H. pylori* re-infection, indicating the presence of B-cell lymphoma clones that have escaped the antigenic drive.

The efficacy of antibiotic therapy may be reduced in cases with locally advanced disease, with bulky masses or deep infiltration of the gastric wall, and in cases associated with increased numbers of large blasts, but in our experience eradication of *H. pylori* is also worthwhile in these cases.

Chemotherapy with single-agent chlorambucil and local radiotherapy have been shown to be capable of inducing complete remissions in most cases and can be used in patients who do not respond to antibiotics. The need for surgical resection should be re-

defined. Surgery has been widely used in the past with excellent results for localized disease. However, follow-up endoscopy may reveal, in the remaining gastric mucosa, the re-appearance of lymphoepithelial lesions that can be responsible for local recurrence. Indeed, the fact that MALT lymphoma is often a multifocal disease suggests that clear excision margins are not necessarily a guarantee of radical resection.

Low-grade MALT lymphomas with focal high-grade areas might behave like other low-grade MALT lymphomas when treated with surgery or chemotherapy or both but their susceptibility to being cured with antibiotics has still to be adequately studied.

The best treatment for high-grade gastric lymphoma is still very controversial, particularly with respect to the role of surgery. In our opinion gastric lymphoma with aggressive histologic features must be aggressively treated. However, eradication of *H. pylori* should be attempted (in addition to chemotherapy) in the setting of high-grade MALT lymphoma, since this approach may eliminate an antigen-dependent component that upon stimulation might be responsible for tumor recurrence.

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## Non-gastrointestinal MALT lymphoma

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**M**ucosa-associated lymphoid tissue (MALT) lymphomas were first described by Isaacson and Wright in 1983<sup>1</sup> in a small series of patients with low-grade B-cell gastrointestinal lymphomas. Although MALT lymphomas occur most frequently in the stomach, they have also been described in various non-gastrointestinal sites, such as salivary gland, conjunctiva, thyroid, orbit, lung, breast, kidney, skin, liver and prostate.<sup>2-18</sup> While this particular entity was not included in the *Working Formulation for Clinical Usage*, in the updated Kiel classification it was listed as a monocytoid cell lymphoma; in the R.E.A.L. classification of 1994, the MALT lymphomas were definitively classified among the marginal zone B-cell lymphomas. MALT lymphomas are characterized by neoplastic marginal cells which display variable combinations of colonization of reactive germinal centers, plasmacytic differentiation, and destructive epithelial infiltration, forming lymphoepithelial lesions. The risk of a diagnostic dilemma is reduced by the favorable prognosis of this low-grade lymphoma and its tendency to remain localized for long time to the primary site. Paradoxically, MALT lymphomas only occasionally arise from sites where MALT is normally present, such as the tonsil and Peyer's patches. The reason for this seems to be that MALT lymphomas generally arise in lymphoid tissue that has been acquired as a result of some pre-existing disorder: for example, *Helicobacter pylori* colonization in the stomach,<sup>19,20</sup> follicular bronchiectasis in the lung,<sup>21</sup> autoimmune diseases in the salivary (Sjögren's disease) and thyroid (Hashimoto's thyroiditis) glands,<sup>22,23</sup> and reactive or inflammatory lesions of the orbit.<sup>24</sup> The literature reports a correlation between hepatitis C virus (HCV) infection and extranodal MALT lymphomas.<sup>25-27</sup> On therapeutic grounds, in contrast to nodal lymphomas, low-grade MALT lymphomas respond favorably to local treatments such as surgery and/or local radiation therapy. The outcome and prognosis of low-grade MALT lymphomas are more favorable than those for other extranodal lymphomas.

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### Design and Methods

From January 1988 to October 1997, 75 patients with previously untreated non-gastrointestinal MALT lymphoma were admitted to nine Italian institutions. Up to 1994, the histologic and cytologic diagnosis of MALT lymphoma was made according to the updated Kiel classification, after which time the R.E.A.L. classification was used. For the purposes of this study, the exact diagnoses of all the cases were reviewed according to the R.E.A.L. classification. The diagnosis was made solely on the basis of incisional biopsy, transbronchial lung biopsy/bronchoalveolar lavage (BAL) or surgical excision, and was determined on hematoxylin-eosin and Giemsa-stained preparations, supported by immunohistochemical analysis. The Ann Arbor system was used for staging; all patients were HIV-negative.

Staging evaluation included initial hematologic and chemical surveys and physical examination, in addition to chest roentgenograms and computerized tomography of the chest and abdomen, abdominal ultrasonography and gastro-duodenoscopy. Bone marrow biopsy and ENT examination were performed in all patients. The patients with conjunctival disease underwent complete ophthalmic examination, including double eversion of the upper eyelids to examine the upper fornix.

### Therapeutic approaches

Patients were treated according to disease stage and disease location. Patients with localized lymphoma were generally treated with surgery or local radiation therapy, while other patients received adjuvant chemotherapy. Patients with advanced stage disease were treated with chemotherapy: either in single-agent (chlorambucil, cyclophosphamide, or fludarabine) or polychemotherapy regimens (cyclophosphamide, doxorubicin, vincristine, and prednisone [CHOP], CHOP-like regimens, or vepesid, mitoxantrone, cyclophosphamide, vincristine, prednisone, and bleomycin [VNCOP-B]).<sup>28</sup> In addition, in four patients with conjunctival disease and one patient with a lachrymal involvement, therapy was restricted to local intralésional administration of  $\alpha$ -interferon ( $\alpha$ -IFN) (3 MU three times a week) for 4 to 6 weeks.

### Response criteria

Complete response (CR) was defined as the complete absence of all clinical evidence of the lymphoma

for at least 6 weeks; reduction of at least 50% of known disease for at least 6 weeks was rated as a partial response (PR). Patients with stable or progressive disease were considered as having no response. Survival and relapse-free survival curves were calculated according to the method of Kaplan-Meier. The survival curve was measured from diagnosis until death; the time to treatment failure interval was calculated from the end of induction therapy to the first evidence of relapse (for CRs) or to the first evidence of progression (for PRs).

## Results

### Patient characteristics

Thirty-five patients were males and 40 were females. Their ages ranged from 27 to 91 years (median 58 years). The lymphomas were localized in the lung (19 patients), orbital soft tissue (16 patients), skin (7 patients), thyroid (7 patients), lachrymal gland (6 patients), conjunctiva (6 patients), salivary gland (6 patients), breast (3 patients), eyelid (2 patients), larynx (1 patient), bone marrow (1 patient), and trachea (1 patient). With respect to stage at diagnosis, 47 (63%) patients had stage IE or IIE and 28 (37%) had advanced stage disease. Thirteen patients had bone marrow involvement. Eight (11%) patients were HCV-positive. Most had localized disease: lung (2 patients), orbital soft tissue (1 patient), skin (1 patient), salivary glands (1 patient), lachrymal gland (1 patient), and larynx (1 patient). One case manifested in the eyelid but was accompanied by bone marrow involvement. As regards the correlation with autoimmune diseases, 1 (14%) patient of the 7 with thyroid localization had previously diagnosed Hashimoto's thyroiditis, and 1 (17%) patient of the 6 with lachrymal gland lymphoma had previously diagnosed Sjögren's disease.

Of the 75 patients, 59 (79%) obtained a CR and the remaining 16 (21%) showed a PR. Among stage IE and IIE patients, 41/47 (87%) patients obtained a CR and 6 (13%) a PR while, among the 28 patients with advanced stage disease, 18 (64%) obtained a CR and 10 (36%) a PR. At present, 52/59 (88%) are still in CR at a median follow-up of 42 months (range 8 to 126 months). Three of them are in a second CR after relapses at 12, 41, and 42 months. All but 2 of the patients are still alive with a median follow-up of 47 months (range 8 to 126 months). One patient died of colon carcinoma (orbit soft tissue localization in CR) after 43 months, and one of gastric hemorrhage (a lachrymal gland localization in PR) after 35 months. The estimated time to treatment failure of all 75 patients is 30% at 5 years and the estimated overall survival of all 75 patients is 95% at 5 years, while the 5-year time to treatment failure is 29% for early stages (IE-IIE) and 32% for advanced stage disease.

### Specific sites: treatment and response

**Pulmonary lymphoma.** Of the 19 patients with pulmonary lymphoma, 14 received chemotherapy alone,

2 surgery plus chemotherapy, 2 surgery alone, and the remaining patient had chemotherapy followed by radiation therapy. Fifteen (79%) patients obtained a CR and 4 (21%) patients a PR.

**Orbital soft tissue lymphoma.** Of these 16 patients, 11 received chemotherapy alone, 3 local radiotherapy, and 2 had chemotherapy plus radiation therapy. Twelve (75%) patients obtained a CR and 4 (25%) patients a PR.

**Skin lymphoma.** Initial surgery was performed in all 7 patients, after which 6 patients received chemotherapy and 1 had local radiation therapy. Five (72%) patients obtained a CR and 2 (28%) a PR.

**Thyroid lymphoma.** All 7 patients had local tumor excision followed by chemotherapy, either alone (5 patients) or in association with local radiotherapy (2 patients). All patients obtained a CR (100%).

**Lachrymal gland lymphoma.** Of these 6 patients, 2 received surgery alone, 2 had surgery followed by chemotherapy, 1 patient received chemotherapy alone, and the remaining patient had local  $\alpha$ -IFN alone. Four patients (67%), obtained a CR and 2 (33%) a PR.

**Conjunctival lymphoma.** These 6 patients received either local administration of  $\alpha$ -IFN alone (4 patients) or surgery alone (2 patients). All 4 patients treated with  $\alpha$ -IFN obtained a CR (67%), while the 2 patients who received surgery alone had a PR (33%).

**Salivary gland lymphoma.** All 6 patients received chemotherapy, and 2 of them also had local radiation therapy. CR was achieved by 4/6 (67%), and 2 (33%) patients reached a PR.

**Other sites.** All patients who had other sites involved obtained a CR: breast (3 patients; local excision plus chemotherapy), eyelid (2 patients, 1 of whom had local radiation therapy and 1 surgical treatment), larynx (1 patient with local radiation therapy), bone marrow (1 patient with chemotherapy), and trachea (1 patient with local radiotherapy).

**Bone marrow involvement.** Twelve (16%) patients (3 with pulmonary, 3 with salivary gland, 2 with lachrymal gland, 2 with orbital soft tissue, and 2 with conjunctival lymphoma) also had contemporary involvement of the bone marrow. Four (34%) of these patients achieved a CR and have continued to maintain this response, while the other patients obtained a PR with minimal residual disease in the bone marrow.

**Localized vs advanced disease.** Among the 47 patients with I-II stage disease the CR rate was 87%, while in the 28 with advanced disease (stage IV) it was 64%. Therapy, which was selected independently of stage, was based on the specific localization of MALT and on the tumor burden.

### Relapse patterns: the localizations

Of the 10 patients who relapsed, there were 3 with pulmonary lymphoma, 2 with orbital soft tissue involvement, 2 with skin lymphoma, 1 with conjunc-

tival involvement, 1 with salivary gland lymphoma, and 1 with breast lymphoma. Seven of the relapses were local recurrences (in the vicinity of the removed tumor), and three were in other sites; all were histologically documented as low-grade MALT lymphoma. The 3 recurrences distant from the primary site were: breast and contralateral breast; salivary gland and further lymph nodes of the neck; and orbital soft tissue and further skin relapse. Three of the 10 patients obtained a second CR: in detail, 2 with recurrence in another site (breast-contralateral breast and orbit soft tissue-skin) and 1 with local relapse (eyelid); these three patients are still alive and disease-free at 36, 16 and 13 months after their second treatment responses. The remaining patients obtained PR after the retreatment; all are currently alive with disease.

No statistically significant difference was observed between the treatment failure curves with respect to the five main locations because of the low number of patients. No relapses/progressions were observed in the thyroid subgroup.

## Discussion

Although as many as 60% of all MALT lymphomas occur in the gastrointestinal tract (especially the stomach) where mucosa-associated lymphoid tissue normally occurs, the tumor often involves non-mucosal epithelia (e.g. salivary gland, thyroid, conjunctiva, breast) or mucosal sites without a significant amount of normal lymphoid tissue (e.g. lung), or occasionally non-epithelial tissues (e.g. orbital soft tissue). The non-gastrointestinal sites most frequently involved by MALT lymphomas are the lung and orbital soft tissue.

Regarding the clinical characteristics of the disease, our data confirm the specific features of non-gastrointestinal MALT lymphoma outlined in previous reports by Thieblemont *et al.*<sup>29</sup> and Zinzani *et al.*<sup>30</sup> In particular, there was a predominance of female patients with a median age of 55-60 years in the patients of the present series, and the lung and orbital soft tissue were the most frequent locations. Among our MALT lymphomas, the correlation with autoimmune disease seems to have been less strong than in other types of lymphoma. In the current study, there was a CR rate of 79% and an overall response rate of 100%. With respect to the specific locations, all 7 (100%) thyroid lymphoma patients obtained a CR, while for the other sites which affected at least 6 patients (lung, orbital soft tissue, skin, lachrymal gland, conjunctiva, and salivary gland) the CR rates ranged from 67% to 79%. A particularly poor response was encountered among those patients with concomitant mucosal and bone marrow involvement,<sup>31</sup> who had a CR rate of only 34%. Of the 59 CRs achieved, only 10 (17%) relapsed. Overall, the time to treatment failure rate was 30% at 5 years; the thyroid subset had the worst time to treatment failure rate.

After a median follow-up of 47 months (range 8 to 126 months) overall survival is 95%. Relapses occurred as local recurrences, dissemination to other organs, or in different locations of the same extranodal site. One third of the relapsed patients obtained a second CR, and all the relapses have shown at least a PR with a good outcome.

The therapeutic approach was evaluated in relation to the different organs involved. In particular, chemotherapy was privileged for lung disease, while surgical excision and radiotherapy or local  $\alpha$ -IFN were preferred for the orbital soft tissue and conjunctiva, respectively. It should be emphasized that the local intralesional administration of  $\alpha$ -IFN for conjunctival and lachrymal gland locations proved effective in 5 patients, producing a CR without relapse with this treatment alone.

To the best of our knowledge, this is the largest reported series to date of non-gastrointestinal MALT lymphomas. Our data confirm that the prognosis of this particular entity is more favorable than that of other extranodal lymphomas. However, several aspects await clarification such as the issues of homing receptors and the antigenic dependency of the lymphoma. With regard to the specific therapeutic approaches, our data underline the effectiveness of combination therapy (chemotherapy plus radiation therapy) in the majority of sites, the real effectiveness of local treatment with  $\alpha$ -IFN in particular locations where radiation therapy could cause unpleasant and dangerous sequelae, and the role of chemotherapy (using CHOP or CHOP-like regimens) in the lung. Nevertheless, no firm conclusions can be drawn regarding the most appropriate treatment with respect to the stage of the lymphoma. In fact, for MALT lymphoma patients the therapeutic approaches need to be tailored according to the specific site and this makes it extremely difficult to stratify the different early and advanced stage treatments. In terms of response duration, patients with disease in the thyroid and lachrymal glands seem to have the best prognosis, while those with skin disease have a poorer outcome. The demonstrated possibility of achieving good results and long-lasting survival in all patients by means of therapeutic approaches differentiated according to the particular extranodal location underlines the importance of correctly identifying non-gastrointestinal MALT lymphomas.

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## Cutaneous lymphomas

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**P**rimarily cutaneous lymphomas (PCL) are an extremely polymorphic group of malignant B- and T-cell clonal neoplasias originating in the skin with different clinical, histologic and immunophenotypic findings.

For many years these diseases have been diagnosed exclusively using morphologic criteria according to histologic classifications employed for nodal lymphomas. In the last decade progresses in immunohistochemistry and acquisition of new clinico-pathologic data have demonstrated that previous classifications are inadequate for categorizing cutaneous lymphomas. In fact, the correlation between histotype and prognosis used for nodal lymphomas cannot be applied to a lymphoma with the same histomorphology arising in the skin. PCL should only be classified by using a combination of clinical, histologic and immunophenotypic parameters. A proposal for a separate classification of the group of primary cutaneous lymphomas, taking into account all the above mentioned data, was recently published by Willemze *et al.*<sup>1</sup> A modified *consensus classification* was adopted from the EORTC group on cutaneous lymphomas (Table 1).<sup>2</sup> Unlike in the existing classification schemes, the terms indolent, intermediate and aggressive are used to underline clinical behavior and prognosis. The groups labeled as provisional contain possibly new clinico-pathologic entities.

### Primary cutaneous T-cell lymphomas

#### *Mycosis fungoides*

Mycosis fungoides (MF) is an epidermotropic cutaneous lymphoma characterized by small to medium-sized cerebriform T-cell lymphocyte infiltration of the skin.<sup>3</sup> Its etiology is unknown, but it typically occurs in adult life and has an indolent course with slow progression. At onset the disease affects the skin and only in later stages may it involve lymph nodes and internal organs.

*Clinical findings.* The classical form of MF, known as Alibert-Bazin MF, presents as patches evolving into plaques and nodules. In the patch stage skin lesions are characterized by single, or rarely, multiple erythematous-scaling or eczematous patches, generally

localized on the trunk and legs. Subsequently sharply defined, slightly elevated, dark red plaques arise from pre-existing patches. Comedo-like inclusions and sebaceous cyst hyperplasia are frequently observed, and hairs are reduced. After several years, reddish-brown or purplish indurated red nodules or tumors, occasionally with softly mushroom appearance, develop. These may occur on any part of the surface of the body but there is a predilection for the face. A dermopathic lymphadenopathy becomes evident.

In the erythrodermic form, described by Hallopeau and Besnier in 1891, the skin becomes abruptly and diffusely bright red, thickened and hardened, with a superficial lymphadenopathy. The number of Sézary cells in the peripheral blood is not significant although the condition can assume features similar to those of Sézary syndrome and is nowadays considered a related entity.

*Mycosis fungoides d'emblée*, described by Vidal and Brocq in 1885, is a cutaneous lymphoma manifested by tumors. Most cases are now considered different types of cutaneous lymphomas (pleomorphic small/medium or large cell lymphoma) rather than true MF.

*Histology.* In early stages a superficial, patchy, non-specific infiltrate containing scattered atypical lymphoid cells, with focal exocytosis, can be observed. Fully developed lesions show band-like infiltrates of varied thickness and density, composed of neoplastic cells and variable numbers of inflammatory cells (mainly plasmacytes and histiocytes), which occupy the papillary dermis and invade the epidermis. Small and medium sized lymphocytes with indented or cerebriform nuclei (Lutzner cells), are found either in single units or in nests within the epidermis (Pautrier's microabscesses). In a more advanced tumor stage the infiltrate occupies the whole dermis, reaching the subcutaneous fat. Progression to a large T cell lymphoma may be observed; this is often associated with an aggressive clinical course.

*Immunohistochemistry.* MF cells in a well-developed lesion characteristically express the bcl-2 anti apoptotic marker and an  $\alpha\beta$  helper "primed" T-cells phenotype:

$\beta F-1^+$ ,  $CD2^+$ ,  $CD3^+$ ,  $CD4^+$ ,  $CD5^{+/-}$ ,  $CD6^+$ ,  $CD7^{+/-}$ ,  $CD8^-$ ,  $CD25^-$ ,  $CD29^+$ ,  $CD30^-$ ,  $CD45RO^+$ . In early stages the infiltrate is formed by a mixture of neoplastic and reactive T-lymphocytes, the number of  $CD4^+$  and  $CD8^+$  cells being similar in most cases.

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**Table 1. EORTC classification of cutaneous lymphomas.**

Primary CTCL	Primary CBCL
<i>Indolent</i>	<i>Indolent</i>
Mycosis fungoides	Follicle center cell lymphoma
MF + follicular mucinosis	
Pagetoid reticulosis	
Lymphomatoid papulosis	Immunocytoma
Large cell CTCL, CD30+	
<i>Intermediate</i>	
Large B-cell lymphoma of the leg	
<i>Aggressive</i>	
Sézary's Syndrome	
Large cell CTCL, CD30-	
<i>Provisional</i>	<i>Provisional</i>
Granulomatous slack skin	Intravascular CBCL
CTCL, Pleomorphic S/M cells	Marginal zone B-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma	Plasmacytoma

Several macrophages and dendritic cells are usually present. Less frequently, MF cells show positive staining for CD7 or for CD45RA mAbs and not for CD45RO.

*Comment.* MF cells show TCR- $\beta$  and TCR- $\gamma$  rearrangement; identical histologic and immunohistochemical findings can be observed in cases of T-cell pseudolymphoma.

### Variants of mycosis fungoides

#### Large plaque parapsoriasis

In this condition there are scaly, erythematous, atrophic patches, with or without poikiloderma, generally located on the trunk and buttocks. In 10% of patients the condition progresses to overt MF. Immunophenotypically large plaque parapsoriasis is identical to MF and may show TCR- $\gamma$  rearrangement. Some investigators believe that large plaque parapsoriasis is a pseudo T-cell lymphoma.

#### Follicular mycosis fungoides and follicular mucinosis

Follicular mucinosis is considered to be a distinct form of MF, characterized clinically by hyperkeratotic follicular papules forming patches and plaques involving the head and the neck or generalized patches, often associated with alopecia. Histologic features include a primarily perivascular and periadnexal infiltrate with sparing of the epidermis (folliculotropism) and mucinous degeneration of the follicle. Follicular MF is a similar entity, but without follicular mucinosis. Immunologically the proliferating cells express the same phenotype as MF, cited above; activated CD25<sup>+</sup> and CD30<sup>+</sup> cells can be detected in the involved follicles.

#### Pagetoid Reticulosis (Woringer-Kolopp syndrome)

This is a strongly epidermotropic T-cell lymphoma.

*Clinical findings.* This syndrome is characterized by a circumscribed and sharply defined reddish-brown plaque, usually localized to the distal part of the extremities. Further lesions can develop and become confluent. The prognosis is usually very good.

*Histology.* The intra-epithelial neoplastic T lymphocytes show large, sometimes cerebriform nuclei and abundant clear cytoplasm. Pautrier's microabscesses are not formed.

*Immunohistochemistry.* Immunohistochemical data show cases expressing phenotype identical to MF cells; but in a significant number of cases (40% to 50%) different phenotypes have been reported (CD8<sup>+</sup>; CD4<sup>-</sup> and CD8<sup>-</sup>; TCR- $\delta 1^+$ ). It is matter of discussion, at to whether it is correct to define these cases as a variant of MF.

*Comment.* The rare disseminated type of reticulosis occurs in older males and is manifested by an eruption of inflamed and scaling patches, papulo-nodular lesions and tumors. This presentation is commonly observed in aggressive CD8<sup>+</sup> or  $\gamma\delta$  T-cells lymphomas.

#### Granulomatous slack skin (GSS)

*Clinical findings.* GSS is a rare variant of MF, characterized clinically by slow development of asymptomatic circumscribed pendulous lax lesions involving folds or the trunk.

*Histology.* Skin biopsy shows a dense granulomatous infiltration disposed in a palisade pattern around areas of dermal necrosis with destruction of elastic tissue. Higher magnification demonstrates neoplastic T-lymphocytes with cerebriform nuclei, macrophages and often multinucleated giant cells.

*Immunohistochemistry.* The neoplastic cells in these cases show the same phenotype as MF cells.

*Comment.* Cases of MF with other types of granulomatous reactions (i.e. sarcoid-like or granuloma annular-like) have also been reported.

#### Sézary syndrome

Sézary syndrome is considered a leukemoid form of MF. It is characterized by a triad of erythroderma, generalized lymphadenopathy and atypical clonal lymphoid cells (Sézary cells, >1,000 x mm) in the peripheral blood and occasionally in the bone marrow. The CD4/CD8 ratio is >10.

*Clinical findings.* Most patients are elderly and may develop the syndrome either *de novo* or following MF. In the initial stage the disease may mimic non-neoplastic dermatoses, but over time it progresses, producing erythroderma with pronounced itching, palmo-plantar hyperkeratosis, severe alopecia, onychodystrophy, and generalized lymphadenopathy. The median survival is shorter in patients with this syndrome than in those with MF.

*Histology.* Skin biopsy shows features similar to those found in MF, but the infiltration is often more monotonous.

onous and the epidermotropism less evident or even absent.

**Immunohistochemistry.** The immunophenotype of circulating and skin infiltrating SS cells is similar to that of MF cells.

### **Primary cutaneous CD30-positive lymphoproliferative disorders**

This group is composed of primary cutaneous CD30 positive large T cell lymphoma, lymphomatoid papulosis (LyP) and borderline cases in which there is a discrepancy between the clinical aspect, behavior and histologic appearance. Although all the authors agree that these three entities are parts of a wide spectrum of a disease, differentiation between them is important from clinical and therapeutic point of view.<sup>4</sup>

#### *Lymphomatoid papulosis*

This is a chronic, recurrent, self-healing, papulonodular eruption that clinically can resemble Mucha-Aberman disease, but histologically shows features suggestive (even if not diagnostic) of a CTCL.

**Clinical findings.** The papulo-nodular lesions usually disappear within 3-6 weeks, very often leaving a scar. In 10-20% of the cases the disease is concurrent with, followed or preceded by another lymphoma (MF, CD30<sup>+</sup> large T cell lymphoma or Hodgkin's disease).

**Histology.** The histologic features of LyP lesions can be distinguished into three types:

*Type-A:* a V-shape angiotropic infiltrate composed of scattered or small clusters of large atypical CD30<sup>+</sup> T-cells, mixed with other inflammatory cells (polymorphonuclear, lymphocytes, histiocytes).

*Type-B:* a band-like and perivascular epidermotropic infiltrate with a prevalence of small to medium sized atypical cells with cerebriform nuclei (like classical MF cells). Some cases of LyP may show both type A and B histologic pictures.

*Type-C:* a borderline histology with CD30<sup>+</sup> large T cell lymphoma.

**Immunohistochemistry.** The immunophenotype of atypical CD30<sup>+</sup> T-cells in the type A and type C variants is CD2<sup>±</sup>, CD3<sup>±</sup>, CD4<sup>+</sup>, CD7<sup>±</sup>, CD8<sup>-</sup>, CD25<sup>+</sup>, while in the type-B lesions the large cerebriform MF-like cells usually do not express the CD30 antigen. However, cases expressing the CD8<sup>+</sup> or double negative CD4<sup>-</sup>, CD8<sup>-</sup> phenotype are not rare. Recent immunohistochemical analysis using new markers of cytotoxic granules (TIA-1, Granzyme B) expressed from cytotoxic cells (CD8<sup>+</sup> and  $\gamma\delta$  T-cells, natural killer cells, CD4<sup>+</sup> clones in GVH disease), shows the strong positivity of the typical blasts of LyP (R. Willemze *et al.*, EORTC meeting, Munich, December 1996). This observation may, in part, explain the necrotic evolution of the lesions in several types of lymphomas.

### **Primary CD30<sup>+</sup> large cell T-cell lymphoma (C-ALCL)**

This is a CTCL composed of cohesive sheets of large tumor cells expressing CD30 antigen. There is

no prior history of LyP, MF or other CTCL.<sup>4</sup>

**Clinical findings.** It affects patients of a wide range of ages with a predilection for adults. It manifests as localized or disseminated nodules or tumors that may ulcerate. Complete or partial spontaneous regression may be observed in up to 25% of the patients. Prognosis is generally good. Involvement of regional lymph nodes occurs in approximately 25% of patients; this is not necessarily associated with an unfavorable prognosis.

**Histology.** Skin biopsy shows a diffuse infiltrate, composed of large CD30<sup>+</sup> (>70%) neoplastic cells, with characteristic morphology of anaplastic cells (clear nuclei, prominent nucleoli, abundant cytoplasm) or showing pleomorphic or immunoblastic features. In borderline forms (as in the *Regressing Atypical Histiocytosis*) an inflammatory mixed infiltrate with relatively few CD30<sup>+</sup> cells and prominent epidermal hyperplasia may be observed (LyP-like).

**Immunohistochemistry.** The neoplastic cells express a CD4<sup>+</sup>, CD30<sup>+</sup> T-cell phenotype with variable loss of T cell antigens (CD2, CD3, CD7) or a *null* phenotype. Co-expression of CD25 and CD30 activation antigen is observed in cells with the large anaplastic cytomorphology, while in cases of pleomorphic cytomorphology CD25-IL-2R is frequently negative. In 50% to 70% of cases the large cells express the TIA-1 and Granzyme-B markers of cytotoxic cells. The Ki-67 proliferation antigen is strongly positive. In contrast to nodal ALCL, in most cases with pleomorphic cytomorphology EMA and BNH-9 mAbs are negative and CD45-pan leukocyte marker is positive.

### **Primary cutaneous CD30<sup>-</sup> large T-cell lymphoma**

This is a rare variant of CTCL.<sup>3</sup> These T-cell proliferations are characterized clinically by solitary or generalized plaques or large tumors and, in contrast to CD30<sup>+</sup> large T-cell lymphomas, have an unfavorable prognosis. These neoplasms show a nodular or diffuse infiltrate composed of a varied number, but not less than 30%, of CD30<sup>-</sup> large pleomorphic T-cells, mixed with immunoblasts, with or without epidermotropism. Some of these cases are TIA-1 positive (personal observation).

### **Pleomorphic small/medium sized T-cell lymphoma**

**Clinical findings.** This pleomorphic lymphoma is a controversial provisional entity of CTCL, presenting as single or several deep-purplish nodules or tumors without patches typical of mycosis fungoides.<sup>5</sup>

**Histology.** A dense, diffuse and nodular infiltration in the dermis, often reaching the subcutaneous fat, is observed. Higher magnification demonstrates small-medium pleomorphic lymphocytes with indented and hyperchromatic nuclei. Epidermotropism may be absent.

**Immunohistochemistry.** The classical immunophenotype of pleomorphic cells is CD2<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, CD5<sup>+</sup>, CD7<sup>±</sup>, CD25<sup>±</sup>, CD30<sup>-</sup>, CD45RO<sup>+</sup>.

## Rare and aggressive CTCL

### **CD8<sup>+</sup> epidermotropic cytotoxic T lymphoma**

This is a variant of CTCL characterized by proliferation of pleomorphic small/medium or medium/large strongly epidermotropic T-lymphocytes.<sup>6</sup> However, CD8<sup>+</sup> marker can be expressed in cases showing the same course as CD4<sup>+</sup> MF.

*Clinical findings.* Clinical findings may be eruptive disseminated inflammatory patches, plaques, nodules and ulcerated tumors. Cases presenting with disseminated lesions have a very bad prognosis.

*Histology.* These aggressive cases show a lichenoid strongly epidermotropic band-like infiltrate into the acanthotic epidermis. In early lesions, intraepidermal lymphocytes with spongiosis and blistering or keratinocyte necrosis, as in GVH disease, can be seen.

*Immunohistochemistry.* Immunohistochemical studies of aggressive form disease show a CD3<sup>+</sup>, CD8<sup>+</sup>, TIA-1<sup>+</sup>, CD2<sup>-/+</sup>, CD7<sup>-/+</sup>, CD45RA<sup>+</sup>, Ki-67<sup>+</sup> T-cell infiltrate in the superficial dermis and in the epidermis. Other MF-like cases expressing CD8<sup>+</sup> are usually CD45RO<sup>+</sup> and CD45RA<sup>-</sup>.

A similar clinico-pathologic presentation can be observed in cases of primary  $\gamma\delta$  T-cell lymphoma.<sup>7</sup> In these cases the immunophenotype is TCR- $\delta$ 1<sup>+</sup>, CD3<sup>+</sup>, TIA-1<sup>+</sup>, CD7<sup>-/+</sup>, CD4<sup>+</sup>, CD8<sup>-/+</sup>, CD45RA<sup>+</sup>.<sup>7</sup>

### **Angiocentric lymphomas, CD56<sup>+</sup> NK, nasal and nasal-type**

These lymphomas<sup>3</sup> are characterized by angiocentric and angioinvasive infiltrates formed by a variable number of small, medium size pleomorphic or blastic tumor cells. Tumor cells express a highly defective immunophenotype (T-cell and CD56 or CD16 NK markers can be co-expressed).

### **Subcutaneous panniculitis-like T-cell lymphomas**

Subcutaneous lymphomas were recently proposed as a new CTCL type, and included in the REAL classification.<sup>8</sup>

*Clinical findings.* The prototype of this form should be considered malignant cytophagic panniculitis, a clinical entity showing deep subcutaneous nodules, with a panniculitic-like inflammatory evolution and worse prognosis.

*Histology.* The classical histologic finding is infiltration of adipose tissue by small/medium size lymphocytes, forming a "rim" around and into the fat lobules, and the presence of many macrophages with leuko-erythrophagocytosis in the adipose tissue.

*Immunohistochemistry.* Neoplastic T-lymphocytes are characterized by the expression of CD2<sup>+</sup>, CD3<sup>+</sup>, CD8<sup>+/-</sup> and TIA-1<sup>+</sup> markers. In some cases the infiltrate is formed of  $\gamma\delta$  T-lymphocytes.

### **Pseudolymphomas, T-cell type**

These pseudolymphomas form a heterogeneous group of disorders. Several cases were observed during the administration of anticonvulsant drugs

(phenytoin and carbamazepine), however, in most cases causative agents have not been identified.

*Clinical findings.* The cutaneous lesions varies from a localized papule, nodule or plaque to a more widespread generalized eruption. Spontaneous regression of the lesions after several months is usually observed. Intralesional or topical steroids are usually employed in localized eruptions.

*Histology.* Two possible patterns can be observed: the band-like pattern and the nodular pattern. In the former atypical cerebriform cells and isolated blasts are present, the epidermotropism is less pronounced than in MF and Pautrier's abscesses are absent. In the nodular pattern a perivascular nodular and diffuse infiltrate occupying the whole dermis and in some cases also the subcutaneous fat is observed. Atypical cells can be detected in the dermis and in the epidermis, the histiocytic component being substantiated in several cases, showing a granulomatous reaction.

*Immunohistochemistry.* CD2<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, CD5<sup>+</sup>, CD7<sup>-</sup> T-lymphocytes are predominant, but groups of CD22<sup>+</sup> B-cells can be present. Frequently, T-lymphocytes express DR or CD25 activation antigens, but CD30 is negative.

The cutaneous lesions of actinic reticuloid are erythematous-squamous infiltrative plaques localized to sun-exposed areas. Patients with this condition show cutaneous sensitivity to several contact photo-allergens.<sup>3</sup> The infiltrate simulates MF, although a less prominent epidermotropism is present with a hyperplastic spongiotic epithelium suggesting chronic dermatitis. Infiltrating cells have a T-cell immunophenotype with predominance of CD8<sup>+</sup> T-lymphocytes.

*Comment.* The differential diagnosis between cutaneous lymphomas and pseudolymphomas must be made combining clinical findings, histology, immunohistochemistry and molecular studies. However the most important criteria for diagnosis are clinical data (history, presentation, regression).

## **Therapy of CTCL CD4<sup>+</sup> MF and variants, indolent types**

### *Stage IA (T1-N0-M0)*

*Mycosis Fungoides (MF):* differential diagnosis from inflammatory reactions may be difficult. Local steroids and ultraviolet (UV) light may be used for early lesions; in cases with follicular involvement, PUVA (psoralen and UVA) and retinoids must be used to obtain a complete remission.

*Localized Pagetoid reticulosis:* localized lesions may be treated with radiotherapy, which can produce stable remission.

### *Stage IB (T2-N0-M0)*

Ultraviolet or PUVA therapy and retinoids, also in combination, or topical caryolisine are usually employed.

*Stage IIA (T1,2-N1-M0)*

The reactive lymphadenopathies are sensitive to systemic steroid therapy.

*Stage IIB (T3-N0,1-M0)*

When tumors are present, different therapy is needed. Local radiotherapy and systemic interferon-alpha therapy may be used, in association with retinoids or PUVA. In case of relapses total skin electron beam therapy (TSEBT) or mono/polychemotherapy can be used.

*Stage III (T4-N0, 1-M0-B0, 1)*

MF: the presence of erythroderma with or without adenopathy is an indication for apheresis/photopheresis treatment, in association with systemic corticosteroids, retinoids or interferon alpha. In case of relapse and progression mono and polychemotherapy may be used (pentostatin, fludarabine, methotrexate, cyclophosphamide, chlorambucil, CHOP, ProMACE-CytaBOM) or TSEBT.

*Sézary syndrome.* We observe the presence of several atypical cells in the peripheral blood (more than 1,000 mm<sup>3</sup>) and a red-brown erythroderma with specific adenopathies. The same therapies as those for erythrodermic MF may be used.

*Stage IVA (T1, 2, 3, 4-N2, 3-M0) and Stage IVB (T1, 2, 3, 4-N0, 1, 2, 3-M1)*

Mono or polychemotherapy as cited above may be used; experimental therapy such as autologous or heterologous bone marrow transplantation can be considered in some cases.

**Therapy of CD30+ lymphomas***Lymphomatoid papulosis*

In cases with benign evolution (rapid spontaneous regression without scars), no therapy is required. However, in some cases PUVA or systemic steroids can be used. There are some reports of methotrexate treatment.

*Primary cutaneous CD30+ large cell lymphoma*

In case of localized lesions, surgery and/or radiotherapy may be used. Only in the case of systemic involvement should polychemotherapy such as MOPP,

CHOP, ProMACE-CytaBOM, MACOP-B be used.

**Therapy of rare and aggressive CTCL**

*CD30- large cell lymphomas, subcutaneous panniculitis-like T-cell lymphoma, CD8+ epidermotropic cytotoxic lymphomas,  $\gamma/\delta$  lymphomas, NK-nasal type CD56+ lymphomas:* mono or polychemotherapy must be considered the first choice treatment, because of the frequent systemic involvement, progression and poor prognosis.

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## Splenic lymphoma with villous lymphocytes

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Splenic lymphoma with villous lymphocytes (SLVL) is a low-grade B-cell lymphoproliferative disorder which was first characterized by Melo *et al.* in 1987,<sup>1</sup> although cases with similar features had previously been described in the literature.<sup>2</sup> It has subsequently been included in the *French-American-British (FAB) Cooperative Group* classification of B lymphoid leukemias.<sup>3</sup> Although still a matter of some debate, it is likely that SLVL represents the leukemic counterpart of splenic marginal zone lymphoma (SMZL) and SLVL has since been classified according to the most recent REAL lymphoma classification as a provisional entity overlapping with SMZL4.

### Clinical features

SLVL is a relatively rare disease accounting for approximately 5% of all chronic B-cell disorders.<sup>5</sup> The etiology is unknown and it mainly affects a middle-aged to elderly population with a male predominance.<sup>6</sup> Patients may be asymptomatic, or may complain of discomfort related to an enlarging spleen or symptoms resulting from cytopenias. 'B' symptoms are unusual in uncomplicated or progressive SLVL. Patients usually present with moderate to massive splenomegaly; occasionally the spleen is not clinically palpable, but may be shown to be enlarged by imaging techniques. Lymphocytosis is almost invariably present, ranging in most cases from 10 to 40x10<sup>9</sup>/L.<sup>1</sup> Anemia and thrombocytopenia due to hypersplenism or, more rarely, bone marrow infiltration are seen in one third of patients. Observations of autoimmune anemia and thrombocytopenia are rare,<sup>5</sup> and even these few include one case of autoimmune hemolytic anemia thought to be induced by treatment with the purine analog, fludarabine (unpublished observation). Hepatomegaly is detected in around half of the patients, and one third have a monoclonal band, usually IgM but occasionally IgG. Free light chains may be detected in the urine. Lymphadenopathy is rare at diagnosis and, if present, is usually of small volume. Bulky lymphadenopathy should alert the clinician to the possibility of transformation to high-grade lymphoma, reported to occur in approximately 5% of cases of SLVL.<sup>5</sup>

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### Morphology

Examination of the peripheral blood film reveals a varying proportion of small to medium-sized cells with a high nucleo-cytoplasmic ratio. The nucleus is round or oval with mature and often clumped chromatin; in some cases the cells have a small nucleolus raising the problem of differential diagnosis with hairy cell leukemia-variant (HCL-V). The cytoplasm is moderately basophilic with fine villous projections, which may show polarity; a few cells with lymphoplasmacytic differentiation may be identified.<sup>1</sup> Leukopenic cases are rarely seen, although small numbers of abnormal lymphocytes are usually detected in the peripheral blood film. Morphology in SLVL may be confused with that of other B-cell diseases such as chronic lymphocytic leukemia (CLL), B-cell prolymphocytic leukemia (B-PLL), hairy cell leukemia (HCL), and HCL-V. However, careful examination of morphologic features in conjunction with additional diagnostic tools, such as cell markers and histology, should allow discrimination of SLVL from these other disorders in most cases.

### Immunophenotyping

The distinct immunophenotype of SLVL was fully characterized in a group of 100 patients by Matutes *et al.*<sup>7</sup> Cells from most or all patients express CD19, CD37, CD24, FMC7, HLA-Dr, moderate to strong surface membrane immunoglobulin staining (SmIg) with light-chain restriction and strongly express membrane CD22 (mCD22). CD10, CD23 and CD38 are positive in one third, CD25 in one quarter, and CD11c in half of the cases. A minority of cases express HC2, B-ly-7 and CD5. HC2 and B-ly-7 were found to be the most useful markers in distinguishing between SLVL and HCL. Therefore, the most usual phenotype in SLVL is CD5 (-), CD23 (-), CD79b (+), mCD22 (+), moderate to strong SmIg, FMC7 (+) and CD24 (+). Patients with SLVL have a low score (0-2) using the combination of 5 markers devised to aid the diagnosis of CLL<sup>8</sup> and similarly, the majority score only 1-2 (of 4) using the HCL panel of antibodies.<sup>9</sup>

### Histology

The bone marrow usually shows nodular/paratrabecular involvement, although interstitial infiltration has also been described. More recently, a unique pattern of bone marrow infiltration termed intrasinusoidal has been reported in both SLVL and SMZL, although its specificity has yet to be determined.<sup>10,11</sup>

A clear zone surrounding abnormal lymphoid cells, characteristic of HCL, is not seen in SLVL. Occasionally the bone marrow aspirate may not show an increase in lymphocytes (>30%) and may be interpreted as uninvolved. More usually, however, there is an increase in lymphocytes often with the typical morphology as seen in the peripheral blood. Spleen histology, when available, is characterized by nodular infiltration of the white pulp, with cells resembling marginal zone B-cells.<sup>12</sup> Varying degrees of red pulp infiltration are also observed, with sinusoidal invasion. Spleen histology is most useful in distinguishing SLVL from HCL and HCL-V in which infiltration of predominantly the red pulp is observed. Lymph nodes, when involved by low-grade disease, generally show diffuse replacement of follicles with preservation of sinuses.<sup>12</sup>

### Cytogenetics

Oscier *et al.*<sup>13</sup> described results of cytogenetic analysis in 31 patients with SLVL, in whom they found a high overall incidence of clonal abnormalities (87%), frequently complex. The most commonly detected abnormalities were deletions of the long arms of chromosome 7 (7q22/7q35), seen in 20% of cases. Breakpoints involving the short arm of chromosome 2 (2p11) and isochromosome 17q were also frequent, being found in 18% of cases. The translocation t(11;14)(q13;q32), which is characteristically associated with mantle cell lymphoma (MCL), was also detected in 18% of patients in this study. Further molecular analysis has suggested a degree of variation in the breakpoints at 11q13/14.<sup>15</sup> However, detection of this translocation in SLVL warrants careful review to exclude MCL, preferably by histology. Interestingly, trisomy 12 and deletion of the long arm of chromosome 13 (13q14) were infrequently seen although by fluorescence *in situ* hybridization (FISH) 13q14 deletions, most frequently involving the retinoblastoma gene locus, have been reported in 50% of cases.<sup>16</sup> A recent study of SLVL by our group looking specifically for trisomy of chromosome 3 by FISH, in an attempt to clarify the relationship between SLVL and SMZL, has shown an incidence of trisomy 3 in SLVL of 17%.<sup>17</sup> This is, in fact, comparable with some of the studies of SMZL in which trisomy 3 was detected by FISH,<sup>18</sup> and supports the hypothesis that SLVL and SMZL may represent different manifestations of the same disease. Another study of trisomy 3 in SMZL by cytogenetics and FISH has, however, reported a significantly higher detection rate.<sup>19</sup> To date, trisomy 3 has been reported to have been detected by conventional cytogenetic analysis in only one patient with SLVL.<sup>15</sup>

### Treatment and prognosis

The natural history of SLVL is that of a chronic clinical course, with up to one third of patients never requiring therapy.<sup>6,15</sup> At five years, >70% of patients are alive and a proportion of patients die of causes

unrelated to SLVL. Transformation into a high-grade lymphoma (comparable with Richter's transformation in CLL) is rare but documented in around 5% of patients.<sup>5</sup> An *observation only* policy is, therefore, acceptable for asymptomatic, non-progressive patients. Treatment is indicated for symptomatic disease, as in other low-grade B-cell disorders, usually manifested by an enlarging spleen with related cytopenias and/or a progressively rising white cell count (WCC). Splenectomy remains the first-line treatment of choice for most patients, following which more than 75% of patients will achieve significant clinical benefit with normalization of hemoglobin and platelet levels and, often, an improvement or normalization of WCC.<sup>6,15</sup> Splenic irradiation (10Gy given over two weeks) is an alternative for those patients in whom splenectomy is contraindicated, with good responses reported in between 40 and 100% of cases.<sup>6,15</sup>

Chemotherapy has its place in the treatment of SLVL, although when used first-line has been shown to worsen prognosis.<sup>15</sup> However, no patient in this study received purine analogs. Chemotherapy is generally required on relapse of the disease post-splenectomy, most usually manifested by a rising WCC. Results of the use of alkylating agents are, in general, disappointing with only a third responding overall.<sup>6</sup> However, some useful control of the WCC may be achieved. Cyclophosphamide may be of additional benefit in the treatment of associated autoimmune phenomena which, although rare, are recognized complications in SLVL.  $\alpha$ -interferon does not appear to have a role in the treatment of SLVL.

There are few data on the use of purine analogs in SLVL. The largest series reported by Bolam *et al.* described the achievement of 100% complete remissions in four previously treated patients, two of which proved to be durable.<sup>20</sup> We have earlier reported a durable partial response in one of two previously treated patients subsequently given therapy with fludarabine.<sup>5</sup> To date, there are no reports of fludarabine as first-line therapy in SLVL.

Similarly, data on the use of the related agents 2-deoxycoformycin (DCF) and 2-chlorodeoxyadenosine (2-CdA) are limited. We have reported one durable and one short-lived partial response to DCF in three patients with SLVL, again previously treated.<sup>5</sup> Two groups have described the use of 2-CdA in a total of four patients, with three partial responders but no complete remissions.<sup>21,22</sup>

In summary, patients without symptoms or signs of progression should be observed. When treatment is required, splenectomy is the treatment of choice reserving splenic irradiation for those unable to tolerate surgery. On relapse post-splenectomy, fludarabine should be considered, and 2-CdA or DCF used in non-responders to fludarabine or on relapse post-fludarabine. Combination chemotherapy is of use for patients with evidence of transformation, and may also benefit the underlying low-grade disease. Whether newer,

experimental agents may play a role in the treatment of this disease remains to be answered.

The prognosis for patients with SLVL is generally good, with an identical 5-year survival of 78% reported by two groups.<sup>6,15</sup> The median survival had not been reached on completion of either study, but these data should become available in forthcoming studies of SLVL with longer periods of follow-up.

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## Mantle cell lymphomas

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**N**ovel diagnostic techniques such as immunophenotyping and molecular analysis have provided new insights into the pathophysiology of malignant lymphomas and are increasingly used to complement the morphologic discrimination of lymphoma subtypes. These techniques allowed the more precise characterization of distinct lymphomas and provided the means to recognize new entities such as mantle cell lymphomas (MCL), marginal zone lymphomas or lymphomas that originate from mucosa-associated tissues (MALT lymphomas).<sup>1</sup>

Despite being described as a centrocytic lymphoma in the Kiel-classification,<sup>2,3</sup> MCL was not generally accepted as a separate entity until the detection of the translocation t(11;14) and the rearrangement of the bcl-1 gene that appear to be highly associated with this disease. The discovery of these abnormalities allowed the recognition that centrocytic lymphomas and lymphocytic lymphomas of intermediate differentiation described by Berard and Dorfmann<sup>4</sup> and Nanba *et al.*<sup>5</sup> as well as the mantle zone lymphomas first identified by Weisenburger *et al.*<sup>6</sup> all relate to the same disorder, for which the new term mantle cell lymphoma was proposed.<sup>7-9</sup> On this basis, the epidemiology, the morphologic, immunophenotypic and molecular characteristics as well as the clinical course and treatment results of MCL could be described more precisely.

### Epidemiology

Mantle cell lymphomas occur predominantly in elderly patients with a median age of 65-70 years and affect predominantly the male sex with a male to female ratio of 2.7-3:1.<sup>10-13</sup>

Mantle cell lymphomas account for approximately 5-8 % of all lymphomas and appear to be evenly distributed in different countries.<sup>14</sup>

### Pathogenesis

The detection of the t(11;14) translocation and the rearrangement of the bcl-1 gene not only facilitated the recognition of this lymphoma subtype but also provided an approach to unravel the biology and pathogenesis of MCL in greater detail. Hence, it

was shown that through the t(11;14) translocation the cyclin D1-gene is juxtaposed to the gene for the immunoglobulin heavy chain, which leads to an overexpression of cyclin D1 and a deregulation of the cell cycle.<sup>15-21</sup> This process impairs consecutive mechanisms of cell cycle regulation involving the retinoblastoma-protein, cyclin-dependent kinases (CDK) and CDK-inhibitory proteins such as p16, p15, p18 and p21.<sup>22,23</sup> In a considerable proportion of MCL mutations of p53 are found which are usually associated with a poor clinical outcome and a blastoid phenotype.

### Histopathology

Mantle cell lymphomas can present with various histologic growth patterns. According to a proposal by the *European Lymphoma Task Force* four subtypes can be discriminated:<sup>24</sup>

- nodular with residual germinal centers (mantle zone pattern);
- nodular without residual germinal centers and a loosely structured follicular dendritic cell (FDC) meshwork (primary follicle pattern);
- nodular without residual germinal centers and tight FDC-clusters;
- diffuse with/without residual germinal centers.

Cytologically, mantle cell lymphomas are characterized by a predominant population of atypical small to medium-sized lymphoid cells with irregular and indented nuclei. The chromatin is moderately coarse and the cytoplasm is scant. Atypical cells vary from those with cerebriform nuclei to others somewhat larger with finely dispersed nuclear chromatin. The latter cases have been referred to as the blastic variant. The mitotic rates may vary considerably among the different subtypes and may exceed 50-100 mitotic figures per 10 high-power fields particularly in the blastic variant form (Table 1). For clinical purposes the two main subtypes of typical and atypical MCL can be discriminated. The latter subgroup is characterized by a high mitotic rate and Ki67 expression and frequently involves p53 mutations.<sup>25-29</sup>

### Immunophenotype

Mantle cell lymphomas regularly have a high surface expression of IgM and IgD. The malignant lymphocytes express pan-B-lymphoid markers such as

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Table 1.

		<b>MCL VARIANTS</b>
<b>Cytology</b>		<b>Growth pattern</b>
<ul style="list-style-type: none"> <li>• Small- to medium-sized cells</li> <li>• Irregular nuclei (or mixture of irregular and round);</li> <li>• Fine condensed, not clumped chromatin</li> <li>• Small inconspicuous cytoplasm</li> <li>• Centroblasts or immunoblasts absent/rare</li> <li>• Variable mitotic rate, usually low</li> </ul>		<ul style="list-style-type: none"> <li>• Nodular with residual germinal centers (GC)(--mantle zone pattern)</li> <li>• Nodular without residual GC and loosely structured FDC-meshwork (--Pfe pattern);</li> <li>• Nodular without residual GC and tight FDC clusters (--GC colonization or induction of tight FDC clusters);</li> <li>• Diffuse with/without residual GC</li> </ul>
<b>Immunophenotype</b>		<b>Molecular biology and cytogenetics</b>
CD5+	CD10-/(+?)	CCND1 (PRAD1/bcl-1) overexpression and/or t(11;14)(q13;q32) translocation useful for confirmation but lack of detection does not exclude the diagnosis of MCL.
CD23/(+?)	CD11c-	
CD20 <sup>++</sup>	CD25-	
BCL-2 <sup>+</sup>	CD43 <sup>±</sup>	
		<b>TYPICAL MCL</b>
<b>Cytology</b>		<b>Growth pattern</b>
<ul style="list-style-type: none"> <li>• Predominance of small- to medium-sized round cells</li> <li>• Mixture of small- to medium-sized cells and blastoid cells</li> <li>• Predominance of blastoid cells</li> </ul>		<ul style="list-style-type: none"> <li>• Same features as in typical MCL</li> </ul>
<b>Immunophenotype</b>		<b>Molecular biology and cytogenetics</b>
CD5±	CD10-/(+?)	Same features and significance as in typical MCL
CD23/(+?)	CD11c-	
CD20 <sup>++</sup>	CD25-	
BCL-2+	CD43±	
FDC meshwork as in typical MCL		

CD19, CD20 and CD22 and are also positive for CD5. They usually lack the expression of CD23, CD10, CD11c and CD25 (Table 1 a,b).

### Features of clinical presentation

Mantle cell lymphomas occur predominantly at higher age and affect predominantly the male sex. Most patients are diagnosed at the advanced stage IV and have frequent involvement of the bone marrow. In 20-30% of cases leukemic dissemination is found. Approximately 40% of patients present with B-symptoms and bulky disease.<sup>10,11,30</sup> As compared to follicle center lymphomas, extranodal involvement is significantly more frequent and often involves the gastrointestinal tract (Table 2).

### Therapy

The increasing insight into the biology and pathogenesis of mantle cell lymphomas is in sharp contrast to the stagnation in clinical management. In spite of numerous efforts to improve on the prognosis of patients with mantle cell lymphomas by more intensive therapy, no substantial prolongation of disease free or overall survival has been achieved and the long term prospectives are dismal. As indicated by Table

2, a high proportion of cases actually responds to initial cytoreductive chemotherapy by a partial or complete remission. These are, however, of short duration and characterized by the rapid development of drug resistance. Hence, the median survival is in the range of only 3-4 years (Table 2). Within this context, the role of anthracyclines has been addressed more specifically. The only prospective randomized comparison testing these drugs was carried out by Meusers *et al.* This study showed no benefit from the addition of anthracyclines to the combination of cyclophosphamide, vincristine, prednisone.<sup>35</sup> In contrast, Zucca *et al.* reported a more favorable effect of this treatment on the basis of a non-randomized comparison with a historic control.<sup>36</sup> A better outcome was also suggested by data from the *European Organization for the Research and Treatment of Cancer (EORTC) Lymphoma Study Group* after intensified treatment with Pro-MAZE MOPP.<sup>37</sup> Still, long term survivors were rarely observed in these series. The evaluation of newer antineoplastic agents, in particular the purine analogs, also revealed relatively disappointing results.<sup>38,39</sup>

New treatment modalities are therefore urgently

Table 2. Features of presentation and clinical outcome.

Variable	Bosch et al. <sup>25</sup>	Velders et al. <sup>11</sup>	Zucca et al. <sup>36</sup>	Norton et al. <sup>31</sup>	Fisher et al. <sup>32</sup>	Teodorovic et al. <sup>37</sup>	Pittaluga et al. <sup>33</sup>	Berger et al. <sup>34</sup>	Majlis et al. <sup>25</sup>	Hiddemann et al. <sup>10</sup>
No. patients	59	41	65	66	36	64	55	52	46	45
Median age (yr)	63	68	64	62	55	58	68	58% > 60 yrs	54	64
Male:female ratio	3 : 1	1.6 : 1	2 : 1	3.7 : 1	4 : 1	2.7 : 1	6.8 : 1	NR	1.7 : 1	3.5 : 1
Molecular studies	Yes	Yes	No	No	No	No	Yes	No	Yes	No
Histologic distribution	N: 5% B: 10%	All diffuse	NR	N: 36% B: 15%	N: 39% B: 33%	N: 3%	N: 11%	N: 22%	N: 13% Mantle zone 26%	NR
Poor PS	50%	20%	20%	NR	NR	6%	NR	23%	NR	NR
Splenomegalie	44%	NR	35%	48%	NR	33%	NR	59%	NR	42%
Bone marrow involvement	75%	80%	58%	80%	53%	60%	66%	82%	69%	67%
Leukemic expression	58%	NR	20%	NR	NR	NR	NR	49%	NR	NR
GI tract involvement	17%	NR	15%	12%	19%	NR	NR	20%	24%	22%
Stage IV	86%	78%	72%	82%	NR	NR	62%	89%	82% (III & IV)	NR
Increased LDH level	40%	55%	30%	NR	NR	NR	NR	45%	NR	NR
CR	19%	32%	51%	9%	NR	52%	NR	31%	(**)	17%
CR + PR	65%	NR	86%	71%	86%	83%	NR	56%	NR	69%
Duration of CR (months)	17	25	44	10	6% (*)	21	NR	14	(**)	NR
Median survival (months)	49	32	42	36	8% (*)	45	32	52%	(**)	28

(\*) at 10 years; (\*\*) Variable according to histological subtype. Abbreviations: N: nodular; B: blastic; NR: not reported; PS: performance status; GI: gastrointestinal; CR: complete response; PR: partial response.

needed. Preliminary data from the *German Low Grade Lymphoma Study Group* and the *EORTC-Lymphoma Study Group* suggest that maintenance therapy with interferon- $\alpha$  may prolong remission duration.<sup>11,39</sup> This question is currently being addressed in a prospective randomized comparison by a European Intergroup trial. Current attempts also concentrate on evaluating the relevance of myeloablative radio-chemotherapy followed by peripheral blood stem cell transplantation for which preliminary conflicting results have been reported. The available data suggest applying this approach in the early phases of therapy after initial cytoreduction by conventional regimes rather than withholding is to later stages of treatment. In addition, myeloablation including total body irradiation appears to be beneficial.<sup>41-48</sup> New perspectives may also arise from antibody targeted therapy or antilymphoma immunotherapy.<sup>49,50</sup> These modalities are currently under development and may change the currently dismal prospective for patients suffering from mantle cell lymphomas.

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