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Chronic Lymphocytic Leukemia 2003

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Chronic Lymphocytic Leukemia 2003

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ENRICA MORRA AND MARCO MONTILLO

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Chronic Lymphocytic Leukemia - Update 2003

Foreword

During the last decade, there has been a resurgence of interest in research about chronic lymphocytic leukemia (CLL). An understanding of the molecular basis of this hematologic malignancy has led to the appreciation that several different B-cell diseases are represented under this name.

Several lines of data now suggest that B-cell chronic lymphocytic leukemia may actually be two diseases, reflecting the mutated and unmutated state of the immunoglobulin heavy-chain gene. The current use of fluorescent in situ hybridization permits a more accurate evaluation of the cytogenetics of the malignant cells, identifying distinct subsets of patients with strong correlations between the chromosome abnormality, clinical course, response to therapy and outcome. There have been as well important therapeutic advances in the last years. Several recently reported trials have helped to transform our paradigms for the treatment of CLL. A clear example of this is that fludarabine is now used as the preferred initial treatment for the disease. Nevertheless, the failure to cure patients has led to explore new strategies and for the availability of new drugs. An increasing number of new biological agents are being evaluated, including Campath-1H, recently approved for the treatment of fludarabine-resistant CLL. There has been a marked increase in the use of submyeloablative transplants, offering a more immunology-based therapy than standard bone marrow transplantation, potentially with less toxicity.

The reason we decided to organize this Meeting was to talk about the state of the art in CLL but also to stimulate a new generation of young investigators to study this fascinating disease.

We hope this Meeting will be interesting and stimulating and we take the opportunity to thank all Authors of this book for their contribute to make this possible.

Last but not least, our think goes to all patients who continue to look to us for hope; let us not disappoint them.

Enrica Morra, Marco Montillo

Morphologic and immunophenotypic characterization of chronic lymphocytic leukemia

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Introduction

Chronic lymphocytic leukemia (B-CLL), the most common leukemia in the Western world,¹ is a chronic lymphoproliferative disorder characterized by increased accumulation of long-lived B lymphocytes that are arrested in the G₀ phase of the cell cycle and seem to be refractory to programmed cell death.² However, B-CLL is a quite heterogeneous disease, considering that the cell morphology, membrane markers, cytogenetic and molecular abnormalities and histology of lymphopoietic tissues (bone marrow, lymph nodes and spleen) are not uniform in all cases. In particular the mutational status of immunoglobulin variable region (IgV_H) genes has allowed B-CLL to be categorized into two different entities: with mutated or unmutated IgV_H genes.³ According to these differences, the clinical features and patients' median survival are extremely various, ranging between 8 and 25 years.⁴

The risk of developing B-CLL increases progressively with age and is two times higher for men than for women. Environmental factors do not seem to play a major role in the pathogenesis of B-CLL. Family and population studies have shown that familial aggregation of B-CLL occurs more often than would expected by chance,⁵ with first degree relatives of B-CLL patients have a higher risk of being affected than members of the general population.⁶

Diagnostic criteria

Various arbitrary levels of absolute lymphocyte count have been suggested for the diagnosis of B-CLL, e.g. $\geq 10 \times 10^9/L$ according to the criteria of *International Workshop on Chronic Lymphocytic Leukemia (IWCLL)*;⁷ $\geq 5 \times 10^9/L$ according to the guidelines of *National Cancer Institute (NCI)*.⁸ The evaluation of cell morphological features and the demonstration of a monoclonal population of B lymphocytes with characteristic markers, allow the disease to be diagnosed and distinguished from other mature B-cell neoplasms within the WHO lymphoma classification.⁹

Morphology

B-CLL cells in peripheral blood smears, stained with standardized May-Grünwald-Giemsa, are typically

small (median volume 211.5 fL) with dense nuclear chromatin clumped in coarse blocks, nucleoli that are usually inconspicuous or not visible on light microscopy and scanty cytoplasm.

On the basis of cytomorphologic differences in cell size, nuclear and cytoplasmic outlines, characteristics of the nuclear chromatin, detectable nucleoli and the nucleo-cytoplasm (N/C) ratio, the French-American-British (FAB) group defined morphologic criteria¹⁰ to classify B-CLL into typical and atypical. The latter includes two subgroups categorized respectively as *mixed cell type CLL* with a spectrum of cells from small to large lymphocytes but with less than 10% of cells being prolymphocytes, and chronic lymphocytic leukemia/prolymphocytic leukemia (CLL/PL) with a variable proportion of prolymphocytes ranging between 10 and 55%.

Prolymphocytic leukemia (B-PLL) is generally characterized by a high white cell count with more than 55% of prolymphocytes in the peripheral blood (Table 1).

According to the presence and number of different lymphoid cells (small size lymphocytes, large lymphocytes, prolymphocytes, pleomorphic lymphocytes, cleaved lymphocytes, immunoblasts, granular lymphocytes, lymphoplasmacytoid cells), recognised by some authors in the morphologic features of B-CLL lymphocytic proliferation (Table 2), it has been possible to make a prognostic correlation between morphology and duration of survival.^{11,12} In particular, the B-CLL characterized by small or granular lymphocytes seems to be associated with a good prognosis;¹³ atypical morphology seems to be correlated with a worse prognosis.¹⁴

Immunophenotype

The immunophenotypic characterization of surface cell markers allows a monoclonal population of B lymphocytes to be identified at an early stage, when the lymphocyte count is below $5 \times 10^9/L$.¹⁵

B-CLL cells express surface membrane immunoglobulin (SmIg) weakly; the kind of light chain is κ in 60–80% and λ in 20–40% of cases. The heavy chain most often expressed is μ . The cells are positive with monoclonal antibodies (MoAb) which identify the B lineage such as CD19, CD20. In addition the great majority of cases are positive with CD5 (also reactive with T-lineage cells). The CD23 is almost constantly expressed on B-CLL cells (Figure 1); only the 5% of the B-CLL cases are reported to be CD23-negative. Reactivity with CD22, CD79b and FMC-7 (typically expressed in PLL) is

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Table 1. B-CLL: FAB classification (J Clin Pathol 1989; 567-84).

Typical CLL	
Atypical CLL	
a) "mixed cell type"	(prolymphocytes <10%)
b) CLL/PL	(prolymphocytes >10%<55%)
PLL	(prolymphocytes >55%)

CLL: chronic lymphocytic leukemia; PLL: prolymphocytic leukemia.

uncommon and generally associated with atypical morphology and a poor prognosis.

From a diagnostic point of view, adequate immunological phenotyping can be obtained through the evaluation of these 6 different markers: CD5, CD23, CD22, CD79b, FMC7 and intensity of expression of Smlg.

The combination of markers which characterizes B-CLL according to the *scoring system* proposed by Matutes and coll., represents an easy guideline to discriminate between B-CLL and other B-cell chronic lymphoproliferative disorders (B-CLD), particularly when the morphologic features are atypical (Table 3).^{16,17}

Finally, some authors have demonstrated a correlation between the expression of CD38 on B-CLL cells and germline status of IgVH genes and they have also demonstrated the association of both with clinical poor prognosis of B-CLL patients.¹⁸

Histopathology

B-CLL lymphocytes typically infiltrate the bone

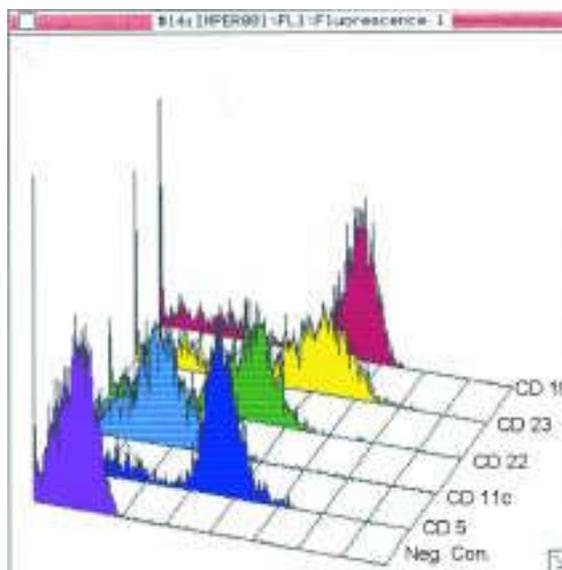


Figure 1. FACS profile in a B-CLL case with a typical immunophenotype (CD5⁺/CD19⁺/CD23⁺/CD22[±]/CD11c⁻).

marrow (> 30% of nucleated cells) and their proportion gradually increases in the advanced phases of disease. A bone marrow core biopsy is required to define the patterns of bone marrow infiltration. Four histologic patterns of infiltration have been described: interstitial, nodular, mixed (interstitial and nodular) and diffuse. The pattern of bone marrow infiltration correlates with clinical stage and has an independent prognostic value.¹⁹

Although the diagnosis of B-CLL is made on peripheral blood and bone marrow smears, the lymph node biopsy allows the evaluation of the

Table 2. CLL: leukemic B lymphoid cells.

	Size	Chromatin	Nucleolus	N/C ratio
Small lymphocytes ^{A,B,C}	< 2 RBC	clumped in coarse blocks	absent	high
Large lymphocytes ^{A,B,C}	> 2 RBC	clumped	inconspicuous or small	low
Prolymphocytes ^{A,B,C}	> 2 RBC	clumped	one, prominent	low
Pleomorphic lymphocytes ^{A,B,C}	>2 RBC	clumped	central and prominent	variable
Cleft lymphocytes ^{A,B,C}	1-2 RBC	homogeneously coarse	absent or inconspicuous	high
Immunoblasts ^{B,C}	> 3 RBC	finely dispersed	>1; prominents	low
Granular lymphocytes ^{B,C}	> 2 RBC	clumped	absent	low
Lymphoplasmocytes ^C	> 2 RBC	clumped	absent or small	low

Note: A: FAB 1989; B: Melo JV 1986; C: Orfao 1988.

Table 3. Scoring system for the differential diagnosis of chronic B-cell disorders based on the immunological profile (Am J Clin Pathol 1997;108: 378-82).

Marker	Score	
	1	0
CD5	positive	negative
CD23	positive	negative
FMC-7	negative	positive
SmIg	weak	moderate/bright
CD22/CD79b	weak/negative	moderate/bright

Chronic lymphocytic leukemia: 4-5; prolymphocytic leukemia: 0, 1, 2; hairy cell leukemia: 0, 1; follicular lymphoma (leukemic phase): 0, 1, 2; mantle cell lymphoma: 1-2; splenic lymphoma with villous lymphocytes: 0-1.

cytologic aggressiveness and growth pattern of the disease. Three growth patterns have been described: diffuse, *pseudofollicular* and tumor-forming.²⁰ The last subtype can evolve in two different ways, histologically defined as prolymphocytoid and paraimunoblastic.²¹

In some patients the evolution of B-CLL is characterized by the development of a unilateral, impressive lymphadenopathy and systemic symptoms (Richter's syndrome) with a histologic appearance of a clonally related large B-cell lymphoma.²²

Conclusions

The combination of morphologic and immunophenotypic analyses of B-CLL cells is particularly important in order to distinguish *morphologically atypical* and *immunophenotypically atypical* cases. In general, although atypical B-CLL cases do exist, these should not be accepted as such without a very careful evaluation of any morphologic and immunophenotypic characteristics that could identify a CLD with leukemic expression. In this regard, the combination of morphologic and immunologic parameters and analysis of cytogenetic and molecular biology features of leukemic cells could be of help not only to make a correct differential diagnosis, but also to identify distinct disease profiles in B-CLL.²³ In particular, the association between the trisomy 12, deletions at 6q, t(14;19) and B-CLL with mixed-cell type morphology, and the association between 17p deletion, t(11;14) and CLL/PLL, seem not to be random, but rather reflect some biological differences.^{24,25} The evaluation of these morphologic, immunophenotypic and molecular-cytogenetic differences seems to be very important in the diagnostic work-up of B-CLL and in the clinical management of B-CLL patients.

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Soluble molecules as prognostic factors in B-cell chronic lymphocytic leukemia

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The possible prognostic significance of a large variety of molecules detectable in the serum or plasma in patients with B-cell chronic lymphocytic leukemia (B-CLL) has been widely investigated during the last 15 years. These molecules, generally referred to as *soluble molecules*, include enzymes, membrane antigens, cytokines and their receptors, released by leukemic and/or bystander cells, involved in the functional mechanisms of cellular expansion.

The search for a more precise prognostic assessment in patients with B-CLL led to the development of a variety of prognostic models which recently began to take advantage from the availability of new biological features, such as "mutated" or "non mutated" immunoglobulin gene status, CD38 antigen and ZAP-70 expression. The purpose of this presentation is to review available data on soluble molecules in B-CLL in order to assess their role as prognostic markers.

Table 1 gives an overview of the publications on soluble molecules as prognostic factors in B-CLL, with the molecules' proven/suggested role. For some of them, which appear on the basis of available data more relevant as possible prognostic factors, further (although concise) details are provided below.

Soluble CD23 (sCD23)

CD23 is a functionally relevant molecule, expressed on the surface of B-CLL cells, which is detectable as a released cleaved form (sCD23) in the serum. The possible prognostic role of the level of sCD23 has been quite extensively investigated.¹⁻⁹ It has been clearly demonstrated that levels are higher in B-CLL patients than in normal control subjects. The values tend to correlate with stage and tumor mass and higher values have been found in patients with a worse survival. Hence the inclusion of sCD23 in some prognostic models. However, its independent prognostic significance is not that clear.

β 2-microglobulin (β 2-M)

Almost 20 years since the first report on β 2-M, this molecule still remains a promising prognostic marker in B-CLL. Several reports, often on small series of patients, demonstrated higher serum levels in patients than in controls, and that within patients higher values were associated with adverse prognostic features at presentation and with worse survival.^{7,8,10-15} When evaluated

in the context of other prognostic parameters in some reports, the level of β 2-M appears to maintain an independent prognostic value.

Serum thymidine kinase (s-TK)

Thymidine kinase, is a cellular enzyme known to be involved in a *salvage pathway* for DNA synthesis. s-TK levels in cancer patients seem to reflect the proliferative activity of the tumor. The serum TK activity in CLL patients is probably related to the number of dividing tumour cells as a result of tumor mass and rate of tumour cell proliferation, since s-TK levels correlate with the proliferative activity of CLL cells. Several reports showed a strict correlation between s-TK levels and adverse prognostic features in B-CLL and the prognostic significance appears independent from that of other parameters.¹⁵⁻²⁰ The ability of s-TK levels to identify a subgroup of patients with early, non-smoldering B-CLL seems particularly interesting.¹⁸ Recently, it has been shown that high s-TK levels correlate strictly with unmutated immunoglobulin gene status.²⁰

Tumor necrosis factor- α (TNF α)

TNF α is produced by leukemic lymphocytes in B-CLL and acts as an autocrine and paracrine growth factor in this disease. A recent report demonstrated that TNF α plasma levels correlated with disease characteristics at presentation, prognostic factors and survival.³⁷⁻⁴⁰ In particular, TNF α concentration appeared to be an independent predictor of survival.⁴⁰

Circulating CD20 (cCD20)

A recent study demonstrated that significant levels of circulating CD20 can be detected in the plasma of patients with B-CLL.⁴¹ Since cCD20 does not derive from shedding of the corresponding membrane-bound molecule, it has been hypothesized that cCD20 is part of a membrane complex or fragment, hence the use of the term *circulating* rather than *soluble* CD20. The variation in plasma CD20 level among patients with CLL likely reflects variations in tumor mass, rate of cell proliferation, and rate of cell turnover as well as the activity and ability of the reticuloendothelial system to remove products of breakdown of leukemic cells. cCD20 levels appear to correlate positively with adverse prognostic features and patients with higher values had significantly shorter survival than those with lower cCD20 concentrations. The prognostic value of cCD20 was independent of Rai staging or hemoglobin level.

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Table 1. Publications on soluble molecules as prognostic factors in B-CLL with their proved/suggested role.

<i>Molecule</i>	<i>Year of publication</i>	<i>Correlation with disease activity/prognosis</i>	<i>Independent prognostic value</i>	<i>References</i>
sCD23	1993-2002	+	±	1-9
β2-M	1980-1999	+	+	7,8,10-15
s-TK	1984-2003	+	+	15-20
sIL-2R/CD25	1987-1997	±	-	1,6,13,21-23
sCD8	1989-1994	±	-	1,22-24
sICAM-1	1994-1997	+	±	22,25,26
IL-6/sIL6-R	1995,2002	+	-	27,28
sVEGF	1999	+	±	29
sVCAM	1998	±	-	30
IL-8	1999,2003	±	-	31,32
sCD44	1997,2001	+	+	33,34
sCD27	1998	+	-	35
IL-10	2001	+	±	28
Fas	2001	+	-	36
TNF-α/TNF-R	1992-2002	+	+	37-40
cCD20	2003	+	+	41
others		+	±	1,42,43

Conclusions

After so many years since the first reports on the possible use of *soluble* molecules as prognostic markers in B-CLL and after so many reports regarding so many molecules, a final statement on their role is still lacking. Indeed this issue should be investigated in the context of prognostic models based on the combination of clinical findings and data emerging from the new biological features, such as immunoglobulin gene mutational status, cytogenetics, CD38 antigen and ZAP-70 expression.

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Cytogenetic and molecular cytogenetic features in chronic lymphocytic leukemia

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Cytogenetic analysis is an important tool for a refinement of the classification of hematopoietic neoplasms and the information derived represents a major prognostic factor in many forms of leukemia. Technical improvements in the field of molecular cytogenetic studies have allowed for a better understanding of the biology of neoplastic cells, pinpointing some genetic aberrations responsible for the transformation process (primary anomaly) and for tumor progression (secondary aberrations).

The study of chromosome aberrations in low-grade lymphoproliferative disorders, such as chronic lymphocytic leukemia (LLC) is hampered by the low mitotic index of the neoplastic cells and by sub-optimal quality of the metaphases. The use of mitogens, particularly phorbol esters and lipopolysaccharide from *E. coli*,¹ allowed for the identification of several recurrent chromosome aberrations, such as trisomy 12 and 13q deletion, which proved to be of prognostic significance.² An important advance in our knowledge on the cytogenetic profile of CLL was contributed by fluorescence *in situ* hybridization (FISH) studies, which allowed for the identification of specific chromosome anomalies in interphase cells.

The increasing awareness of the heterogeneity of CLL, resulting from morphologic and immunologic studies in the 1990s, prompted the FAB group to propose a reference scheme for the classification of CLL,³ identifying three related forms, namely typical CLL with <10% large lymphocytes (LL) or prolymphocytes (PL), atypical CLL with 10–55% LL or PL and prolymphocytic leukemia (PLL) with >55% PL.

More recently, surface marker analysis and genetic studies have added important information on the biological diversity underlying CLL, revealing new perspectives for an integrated classification of this disorder.

The contribution of molecular cytogenetic investigations to a complete diagnostic work-up in CLL are summarized here.

Cytogenetic profile

Clonal chromosome anomalies can be detected by G-banding analysis in 50% of cases.⁴ Some authors⁵

claimed that chromosome anomalies are more frequently encountered in advanced stages of the disease; however more recent studies did not revealed any difference in terms of frequency of clonal aberrations in early vs advanced stages, either in typical CLL⁶ or in atypical CLL.⁷ It is reasonable to assume that the detection of chromosome anomalies may be more difficult in the presence of limited disease, when cell proliferation is slow and that improved culture conditions may facilitate cytogenetic investigations at any disease stage. Recently, a novel culture method, using a CD40-ligand expressing cell line, was set up. This proved to be able to promote divisions in virtually all cases, with a 90% incidence of clonal chromosome anomalies.⁸

Data from the largest series, collected by Juliusson *et al.* in a multicenter study, showed that: a) 604/662 cases were analyzed successfully; b) the most frequent anomaly was trisomy 12, present in 19% of all assessable cases, followed by structural anomalies of 13q, present in 10% of the cases; c) other recurrent chromosome changes were 14q aberrations and 11q anomalies in 8% of the cases, 6q anomalies in 6% of the cases; and d) <5% of the cases carried abnormalities of 17p or of chromosomes 1, 8, 7, 18. Subsequent studies showed that the incidence of clonal aberrations may be higher in atypical CLL.^{9–12}

Molecular cytogenetics

FISH techniques have been used successfully in CLL, this leukemia being characterized by a low mitotic index and by the overgrowth or residual normal lymphocytes under mitogen stimulation, which may lead to false negative results when using conventional cytogenetic analysis.¹³ Early in the 1990s FISH studies showed that 10–15% of CLL with a normal karyotype or without analyzable metaphase cells carried +12 in interphase cells. The percentage of cells with +12 was rather low in some cases, suggesting that this chromosome anomaly may sometimes be superimposed on an already transformed clone. Sequential chromosome studies showed that the size of the trisomic clone did not vary substantially during the course of the disease and that preferential homing of trisomic cells to involved lymph nodes with respect to peripheral blood may occur.¹⁴ It is worth noting that a relative increase of the percentage of trisomic cells was observed following cytoreduction by alkylating agents, suggesting that cells harboring this chromosome imbalance may be less chemosensitive than cytogenetically normal cells.¹⁵

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Later on, the employment of cosmid, BAC and YAC clones allowed for the study of a number of chromosome lesions in interphase cells, such as deletions of 13q14, 11q22-23, 17p13, 6q21.

Thus, a number of studies showed that 13q14 deletions may be found in at least 40% of cases of CLL.^{16,17} These deletions invariably involve a DNA segment distal to the Rb gene. At least 9 genes were cloned from the commonly deleted segment but no oncosuppressor gene has so far been identified. Interestingly, a novel class of genes, *microRNA* genes were recently characterized; it is thought that these may potentially play a role in the transformation process.¹⁸

Following the demonstration that the p53 gene may be involved in the progression of lymphoid neoplasms,^{19,20} attention was devoted to the 17p13 deletion/p53 deletion in CLL. Convincing evidence was provided that up to 5% of CLL cases carried 17p deletions involving the p53 gene, that the morphology of the cells in these patients was consistent with the CLL/PL variant of the FAB classification and that the disease usually runs an aggressive course refractory to purine analogs.²¹ These findings are not surprising when considering that this cytogenetic lesion is found in a number of conditions, especially in the presence of disease progression, prolymphocytoid transformation and Richter's syndrome.^{22,23}

An 11q22-23 deletion was shown to occur in up to 20% of CLL. Morphology in these patients was typical and prognosis was unfavorable, especially in the young-age group.²⁴ Deletion mapping studies using several YAC probes covering a large 11q region showed the minimal region of loss to be centred around the 11q22-23 bands, where the *ataxia teleangiectasia-mutated* (ATM) gene is located. The involvement of the ATM gene through a loss-of-function mechanism is plausible, since some cases were shown to carry inactivating mutations affecting the remaining allele.^{25,26}

Deletion of chromosome 6q centred around the 6q21 band occurs in 2-5% of the cases and is associated with atypical morphology, CD38 positivity and an intermediate prognosis.²⁷

Other recurrent aberrations occurring in <1% of the cases are the t(14;19)(q32;q13), which is almost invariably associated with an aggressive disease, trisomy 8q and 3q and t(14;18)(q32;q21).

There is consensus that t(11;14)(q13;q32) is not found in typical CLL, this translocation being the marker of mantle cell lymphoma (MCL), where it is found in virtually all cases when using sensitive molecular cytogenetic techniques.^{28,29} However, a number of studies described the occurrence of t(11;14) involving the BCL1 locus in a form of chronic lymphoproliferative disorder possibly corresponding to the CLL/PL.^{9,30-33} These chronic leukemias usually, though not invariably, displayed

a mantle-cell phenotype, (CD5⁺, CD19⁺, FMC7⁺, CD23⁻, bright expression of slg) and showed a peculiar clinical picture, with primary bone marrow (BM) and peripheral blood (PB) involvement, splenomegaly and absence of nodal disease. Transformation into prolymphocytic leukemia and Richter's syndrome was reported in some cases.^{22,34} Cell morphology, the clinical picture and the pattern of evolution of this chronic lymphoproliferative disorder are distinctive from those of leukemic MCL and some authors proposed naming this cytogenetic entity of chronic lymphoproliferative disorder as *mantle cell leukemia*.^{35,36} Interestingly an elegant study of a large series of MCL with t(11,14) identified a subset of patients with non-nodal disease, primary BM and PB involvement, and a *mutated* pattern of the variable portion of the Ig gene.³⁷ Whereas a fraction of CLL patients may carry *mutated* Ig genes, this feature is unusual in MCL, a disease showing an *unmutated* configuration of the Ig gene in the majority of cases. Thus this recent finding adds to the heterogeneous clinicobiological picture of CLD with the t(11,14).³⁷ As a matter of fact, besides being found in MCL and multiple myeloma, the t(11,14) can also be found in a spectrum of lymphoid disorders, including prolymphocytic leukemia, splenic lymphoma with villous lymphocytes and CLL/PL. It is reasonable to assume that the transformation of CD5⁺ B-lymphocytes in the follicle mantle may give rise to a spectrum of lymphoid neoplasias, ranging from the classical form of MCL, to a *de novo* leukemic condition with primary BM and PB involvement and morphological features suggestive of atypical CLL. This scenario is reminiscent of the existing relationship between small lymphocytic lymphoma and classical CLL, which represent different clinicobiological manifestations deriving possibly from the same transformed cell.⁹

The salient correlations between chromosome lesions and phenotype of CLL are summarized in Table 1.

Chromosome aberrations and prognosis

Some cytogenetic features have an established role in risk assessment at diagnosis.

Cytogenetic features

Studies using banding techniques revealed a number of correlations between cytogenetic findings and hematological features, resulting in the compilation of an international database co-ordinated by Juliusson and coworkers.^{2,12} The salient prognostic information deriving from the analysis of these data are summarized below:

a) there is a statistically significant correlation between the percentage of abnormal metaphases and survival: those patients with only normal metaphases (NN karyotype) at diagnosis and dur-

Table 1. Salient correlations between some chromosome lesions and phenotype in CLL.

<i>Cytogenetic aberration</i>	<i>Frequency</i>	<i>Morphology</i>	<i>IgVH gene</i>	<i>Clinical picture</i>
del 13q14	40-50%	typical	>80% mutated	Indolent clinical course if single anomaly
+12	10-15%	atypical	50% mutated	Worse prognosis with respect to 13q- single (cytogenetic analysis)
6q-	2-5%	atypical	50% mutated	High WBC count, intermediate prognosis
11q-	10-20%	typical	>80% unmutated	Adenopathies, unfavorable course, especially in the young
17p-	2-5%	atypical	>80% unmutated	Unfavorable outcome, refractory to purine analogs
t(11;14)(q13;q32)	<1%	atypical (*)	50% mutated	Variable clinical course, usually aggressive
t(14;19)(q32;q13)	<1%	atypical	?	Aggressive clinical course

(*) CLL/PL sharing several features with MCL (see text for discussion).

ing the course of the disease have a median survival in excess of 11 years; those with normal and abnormal metaphases (AN karyotype) have a median survival of 8 years, whereas those patients with only abnormal metaphases (AA karyotype) have a median survival shorter than 5 years. These observations seemingly reflect a correlation between *in vitro* and *in vivo* growth of the abnormal clone: those patients with an abnormal clone overgrowing the residual normal cells have a more aggressive disease.

b) There is a gradient in survival according to the number of chromosome anomalies, the best outcome being associated with a normal karyotype, the worst with 3 or more aberrations in the same clone; patients carrying 1-2 aberrations tend to have an intermediate survival. The correlation between karyotype complexity and survival is highly reproducible in a number of hematopoietic neoplasms, reflecting a strong relationship between genetic instability and aggressive disease.

c) Some specific chromosome aberrations have an independent prognostic predictivity: the 13q deletion is a favorable factor if present as the sole anomaly; trisomy 12 and 11q- have an adverse impact, as do 14q32 translocations. It is worth noting that many patients included in previous studies with 14q32 breaks did in fact carry the t(11,14). Many of these patients would nowadays be classified as having leukemic MCL; however, 1% of CLL patients enrolled in a German multicenter trial carried t(11;14), identified by FISH analysis (Dohner H, personal communication, 3rd International GIMEMA Conference, Lecce 2003).

Specific chromosome abnormalities

FISH has two advantages over conventional cytogenetic analysis: 1) it allows for the detection of specific chromosome lesions in non-dividing cells which would be missed by metaphase analysis and, 2) it is able to detect loss of chromosome material in the order of magnitude of one hundred-kb; deletions of this size are far beyond the resolution power of banding analysis.

Thus, the percentage of patients with a demonstrable chromosome lesions rises from 50% with conventional cytogenetic analysis to 70-75% by FISH using a 4-probe panel for the detection of trisomy 12q13 and of deletions 13q14, 17p13 and 11q22-23. An additional 10% of patients can be shown to carry a 6q21 deletion, 14q32 translocations and partial trisomy 3q or 8q. A cytogenetic classification was defined to allocate every patient into a single category based on the following hierarchy: 17p- > 11q- > +12 > 13q- > other abnormalities. The frequency of each cytogenetic lesion in a large study was as follows: 13q- 36%; trisomy 12q 14%; 11q- 17%; 17p- 7%, other abnormalities 8%, no demonstrable lesion 18%.³⁸

The median survival was different in these groups: those patients with 13q- survived 133 months, those with trisomy 12q 114 months, those with no detectable aberration 111 months; those with 11q- 79 months and those with 17p- 32 months. Median survival in patients with trisomy 12q (detected by FISH) was not different from that in patients with no cytogenetic lesion, a finding at great variance from data obtained by karyotype analysis. The reason for this discrepancy is not clear. It is worth noting that the heterogeneity of series of patients in different studies and the inclusion of

patients with a minority of trisomic cells in the trisomy 12q group may be confounding factors.

Specific chromosome lesions also have an impact on other clinical variables: for instance those patients with 17p- show the shortest treatment-free interval, whereas those with 13q- tend to have stable disease not requiring therapy for many years. The 6q- anomaly is associated with high white cell counts, with a relatively short treatment-free interval and with CD38 positivity.²⁷

Conclusions and perspectives

Cytogenetic and molecular cytogenetic analysis can identify distinct disease subsets in CLL. A FISH approach to the cytogenetic classification of CLL has the merit of being relatively easy, quick and reproducible, allowing for the correct classification of the majority of patients and for the identification of two specific chromosome lesions (i.e. 17p- and 11q-) of clinical importance. However, it fails to identify other cytogenetic patterns with prognostic significance (i.e. karyotypic patterns and karyotype complexity) and it does not allow for the separation of trisomy 12 as a distinct entity from the very large group including CLL patients with 13q-, normal karyotype, other abnormalities.

While approximately 80% of CLL patients can be correctly included in a specific cytogenetic category, some cases have miscellaneous aberrations. The definition of the hematologic profile in those patients carrying novel recurrent chromosome abnormalities, such as 4q21 anomalies, 6p24-25 translocation, 1p36 breaks, t(2,14) is an area of intensive research.^{39,40} Specific cytogenetic lesions, genetic markers, such as the configuration of the immunoglobulin gene, and immunophenotype^{41,42} represent important tools for a modern diagnostic work-up of CLL.

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Immunoglobulin genes: characteristics and prognostic prediction in early stage chronic lymphocytic leukemia

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B cells are designed to manufacture immunoglobulin (Ig). Ig resides in the surface membrane of the B cell where it has the function of recognizing and responding to exogenous antigens. Recognition is via the variable (V) regions, which differ in sequence from one B cells to another, and provide a complete repertoire of potential antigen combining sites. Cutting and pasting the component gene segments produces the extreme diversity of the B cell receptor.^{1,2} This recombinatorial process, which occurs in the bone marrow, is mediated by proteins encoded by the recombination-activating genes, RAG1 and RAG2.³

For the heavy chain of Ig, selection takes place from the potentially functional germline genes with 51 V_H genes divided into seven families (V_H1 to V_H7), 27 D genes and 6 J_H genes. The joining of V_H to D and D to J_H is not precise, with non-templated nucleotides being inserted by terminal deoxytransferase (TdT) or templated nucleotides being deleted by exonucleases, both in a random manner.⁴ This introduces a further huge diversity into the shape of the Ig molecule, especially as the D segment can thus be read in any of the three frames.⁵ The consequence is that the third complementarity-determining region (CDR3) of any given lymphocyte is unique, providing a clonal signature for any tumor deriving from it.

Rearrangement of the light chain variable region genes occurs in a similar manner, involving single-step recombinations of V_K/J_K or V_λ/J_λ gene segments but with no D segments. In pre-B cells the B-cell receptor combines Ig heavy chains with the surrogate light chain encoded by the V_{preB} and $\lambda 5/14.1$ genes.⁶ Successful rearrangement of the light chain genes down-regulates expression of the pre-B cell complex and suppresses further rearrangement. If the rearrangements produce a non-functional Ig or an autoreactive antibody, apoptosis is induced. The cells can escape from this by rearranging the other allele, most commonly of the light chain. This process is known as receptor editing. Occasionally B-cells express both κ and λ light chains, indicative of failure of allelic exclusion.

On completion of these processes the B-cell leaves the bone marrow for the periphery where it may meet its appropriate antigen. This encounter induces affini-

ty maturation, usually in the germinal centers of the peripheral lymphoid organs. Here, somatic mutation occurs under the influence of CD40+ve T cells, cytokines and antigen-bearing follicular dendritic cells.⁷ The rate of introduction of base pair changes is of the order of 10^{-4} – 10^{-3} per generation. Probably because of structural reasons the mutations tend to cluster in the CDRs.

A further genetic arrangement, again usually occurring in the germinal center, induces Ig class switching from IgM plus IgD to IgG, IgA or IgE. Which isotype is chosen is determined by cytokine secretion.⁸ The constant region genes at the heavy chain locus each has a 5' switch region composed of tandem repeats. Isotype switching occurs between two switch regions, looping out the intervening constant region genes, although an alternative mechanism involving RNA splicing leading to multiple isotypes being generated may also occur.⁹

Both somatic mutation and class switching require the influence of activation-induced cytidine deaminase (AID). This enzyme was discovered by Honjo *et al.*¹⁰ by cDNA subtraction analysis of a switch-stimulated cell line, which revealed a cDNA with sequence homology to the cytidine deaminase acting on the mRNA of apolipoprotein B. Knockout mice for this gene were unable to class switch or somatically mutate Ig genes.¹¹ At the same time linkage analysis of microsatellite markers in the autosomal recessive version of the hyper-IgM syndrome, mapped the defective gene to 12p13.¹² Since human AID had also been mapped to 12p13, it was a simple step to discover crippling mutations of this gene in the autosomal recessive version of the hyper-IgM syndrome.¹³ How the gene acts is still controversial. It may be an RNA editing enzyme like its homologue, but there is also evidence that it directly deaminates dC residues in DNA.¹⁴ Recently studies on AID in CLL have emerged but no clear picture is apparent.

Cells leaving the germinal center become either memory cells or plasma cells, according to the cytokine environment. Plasma cells primarily migrate to bone marrow, but also to spleen, lymph nodes and the mucosal associated lymphoid tissue. Memory cells form part of the circulating pool, but are also found in peripheral lymphoid organs and in the marginal zone of the spleen.¹⁵

Sixty per cent of peripheral blood B-cells from normal individuals are naïve cells with unmutated IgVH genes; 40% are memory cells carrying somatically mutated IgVH genes and expressing surface CD27.¹⁶ Only a small proportion of naïve cells express CD5,¹⁷

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although this proportion is higher in early life. The majority of circulating memory cells express surface IgM (some also express IgD). Only a minority show evidence of class switching.

Because CLL cells express CD5 it was generally accepted that CLL cells were derived from the minor population of CD5+ve naïve B cells, and this was confirmed by early sequences of the IgVH genes in CLL, which were in germline configuration.¹⁸⁻²⁰ However, reports began to appear in the literature detailing evidence of somatic mutation, and this was summarized in 1994 with a review of the literature by Schroeder and Dighiero²¹ which found that 36/75 reported cases had IgVH genes with less than 98% sequence homology to the appropriate germline gene. The figure of 98% was chosen because the range of polymorphisms of IgVH genes was unknown and it was thought that they could account for that degree of disparity.²² However, some of the cases reported by Schroeder and Dighiero were atypical; some were CD5 negative and there was a disproportionate number with class switched IgVH genes. These were not all cases of classical CLL.

Following this, a multicenter study of 64 patients with surface IgM⁺, CD5⁺ CLL also found two groups of roughly equal numbers with respectively mutated and unmutated V_H genes.²³ Although no clinical detail was available otherwise to distinguish the two subsets, the authors were able to confirm that some cases of CLL had somatic mutations, and also that the presence or absence of somatic mutations was biased towards the use of particular V_H genes.

In 1997 our group examined the IgV_H genes of 22 patients with classical B cell CLL segregated according to karyotype. Tumors with trisomy 12 had unmutated V_H genes but those with 13q14 abnormalities detected by conventional cytogenetics had evidence of somatic mutations.²⁴ Since it has been previously shown that CLL patients with trisomy 12 have a poorer survival than those with abnormalities at 13q14²⁵ this pointed to an association between clinical status and degree of somatic mutation and we extended the study to 84 patients looking particularly at overall survival. The striking finding to emerge was that patients with unmutated IgV_H genes had a median survival of 9.7 years, while those with mutated IgV_H genes had a median survival of 24.4 years.²⁶ At the same time Damle *et al.*²⁷ in a series of 64 patients published back-to-back with ours, found a median survival 9 years for the unmutated group and 17 years for the mutated group. Others have since confirmed these findings in both CLL and small lymphocytic lymphoma²⁸⁻³⁰ and our own series, now extended to 274 patients, gives the same highly significant difference in survival (Figure 1).

The two subsets are clearly different. Those with unmutated V_H genes have a more malignant dis-

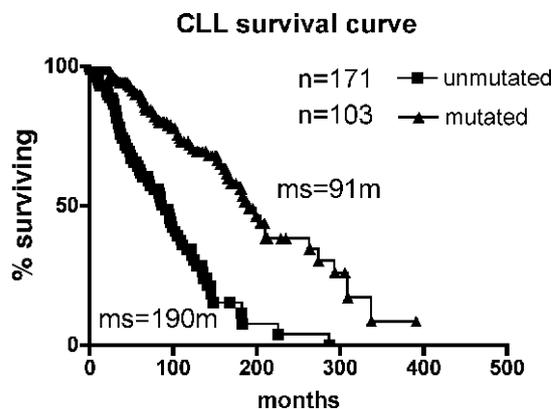


Figure 1. Survival curve mutated vs. unmutated cases of CLL (n = 274).

ease than those with somatic mutations. They are significantly more likely to have advanced stage disease, progressive disease, atypical morphology and trisomy 12 as an isolated karyotypic abnormality, whereas those with somatic mutations were more likely to have stable stage A disease with typical morphology and chromosomal deletions or translocations at 13q14.²⁶ Unmutated cases have a male:female ratio of 3:1, whereas those with mutations are equally common in both genders.

There is a biased use of IgV_H genes. The use of the 51 IgV_H genes in normals is not random. Brezinschek *et al.* analyzed the gene usage by individual normal B cells in the blood of three (youngish) individuals.³¹ Overusage of the V_H3 family was seen. The V₃₋₂₃ gene was most commonly used, followed by V_{3-30.3}, V₃₋₃₀, and V₃₋₀₇. One possible reason for this predominance is the duplication of these segments in some haplotypes. In CLL the most notable bias is the use of V₁₋₆₉ by the unmutated subset^{23,26} often with the D₃₋₃ gene segment and the J_H6 gene. The biased use of the V₁₋₆₉ gene, particularly the 51p1 allele, in CLL was first reported by Kipps *et al.*¹⁸ and has since been confirmed by most workers in this field. In comparison to the normal IgV gene usage, V₄₋₃₄ is also overused, especially in the mutated subset.^{23,26} A surprising observation was that the V₃₋₂₁ gene had a poor prognosis whether or not there were somatic mutations.³² These patients tended to use the same J_H gene and to have a very short and similar CDR3 with virtually no remaining D segment gene.³³ Such an arrangement strongly suggests a single type of stimulating event. A similar argument has been adduced for the strange finding of several cases of CLL from around the planet with class-switched Ig using V₄₋₃₉ linked to D₆₋₁₃ and J_H5.³⁴

The meaning of these biases is unclear. The control information derives from an examination of lymphocytes from three individuals, all under the age of 50,³¹ whereas most patients with CLL are much older. Inbred strains of mice show a biased usage of V_H gene usage with age³⁵ and a similar bias has been suggested in elderly humans.³⁶ However, using the G6 antibody which reacts only with the 51p1 epitope, no biased usage of this gene was found in elderly normals.³⁷ Another possible explanation is that superantigen engages with framework regions of the B cell receptor, stimulating the lymphocyte outside the germinal center.²⁶ Recently, the Liverpool group has suggested that patients using the mutated V₃₋₂₁ gene have a higher than expected incidence of p53 abnormalities.³⁸

Gene expression profiles of the two subsets demonstrate remarkable similarity, but they can be distinguished by the expression of a few hundred genes from a pool of 12,000 genes tested.^{39,40} The most important of these distinguishing genes is *ZAP-70*,³⁹ a gene important to signaling via the T cell receptor and not previously thought to play a part in B cell signaling. Several authors have now demonstrated that expression of *ZAP-70* protein, detected by flow cytometry correlates well with unmutated IgV_H genes and survival, and may be used as a surrogate marker for the technically difficult IgV gene sequence.^{41,42}

From the clinician's perspective the importance of this new knowledge is how it affects treatment. For many years it has been established that early treatment of early stage disease carries no benefit compared to watching and waiting for progression.⁴³ However, this opinion is based on series treated ineffectually with chlorambucil, which were not stratified according to modern prognostic markers. It is now possible to produce molecular remissions even in advanced disease using combinations of purine analogues and monoclonal antibodies. Clinical trials are surely now warranted of effective treatment of early stage disease stratified by more informative prognostic markers.

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Therapy of B-cell chronic lymphocytic leukemia: traditional approach or new strategies?

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After the introduction of purine analogs, the interest in B-cell chronic lymphocytic leukemia (CLL) and, namely, the issue of CLL therapy has incredibly increased. Beside the common approach, many new therapeutic options are now available, from the use of purine analogs alone or in combination chemotherapy regimens, immunotherapy with monoclonal antibodies, to intensification programs with high dose therapy and autologous stem cell rescue, to allogeneic transplantation after myeloablative or non-myeloablative conditioning regimens.

However, one of the most important advances in the last few years are the biological studies on CLL, which are going to enable clinicians to distinguish different subsets of patients with different prognoses.

Following the advances in the knowledge of CLL biology and prognosis, the concept of a *risk-adapted* therapy program in this disease is now well accepted, with therapeutic choices ranging from traditional approaches to alternative strategies.

In fact, before planning CLL therapy it is of paramount importance to correctly categorize the disease in order to discriminate between CLL and other indolent B-cell lymphoproliferative disorders. The evaluation of clinical and biological prognostic risk should then be assessed according to common and more recent parameters such as cytogenetics and molecular profile, at least in young patients who could be suitable for therapy intensification.

The appropriate choice among the different therapeutic options must be made considering not only the first line therapy, but also further lines of treatment, because of the well known natural history of the disease; in other words it would be advisable to try to design a long-term treatment strategy. Last but not least, the feasibility and the cost of the program should be considered, taking into account the elevated incidence of the disease, in order to avoid the unjustified use of expensive treatments on a large scale.

Another important aspect in CLL therapy is the definition of response. In the past, therapy was aimed at simple control of the disease and the response to therapy was evaluated on clinical and hematologic ground.

More recently, it has been demonstrated that, after intensive approaches, it is possible to reach a molecular remission, as expressed by a germ-line configuration of Ig genes. Molecular remission is associated with a longer duration of response. However, since CLL is an indolent disease, often presenting in elderly age, it is very important to verify the advantage of new treatments also in terms of overall survival (OS).

Traditional approaches

The oldest and most employed traditional CLL therapy is chlorambucil (CLB), commonly given as single agent with or without prednisone, at a low dose, intermittently, for a relatively short period.

As reported in Figure 1, regimens with a wide range of dose intensities of chlorambucil have been reported in the literature.

However, our own experience in two consecutive randomized cooperative trials^{1,2} clearly demonstrated the superiority of continuous high-dose CLB (HD-CLB) over both standard-dose intermittent CLB (SD-CLB) and CHOP combination chemotherapy in terms of response rate and OS. Moreover, when compared with fludarabine as a single agent, the HD-CLB regimen produced a similar response with comparable OS according to our data from a subsequent randomized trial.³ Figure 2 illustrates the response rates obtained by HD-CLB therapy in three randomized trials, with a clinical response rate ranging between 86% and 92%. In other words, around 10–12% of patients with advanced CLL have primary resistance to HD-CLB.

Unfortunately, so far, CLB is commonly used at low dose in cyclic schedules, more appropriate for the treatment of elderly patients in whom therapy is aimed at a clinical control of the disease with the minimal possible toxicity. Even in important prospective trials the adopted CLB dose has been chosen at a very low level thus hampering the possibility of evaluating the efficacy of this drug.⁴

Another personal experience deals with the problem of therapy duration. From the survival analysis of patients achieving partial response in the two initial HD-CLB trials^{1,2} we found that the use of maintenance treatment with low-dose CLB was associated with a longer OS (Figure 3).

Following this observation we retrospectively analyzed the impact of the duration of maintenance therapy on the outcome of a series of 114 CLL cases consecutively observed and we found that the longer the

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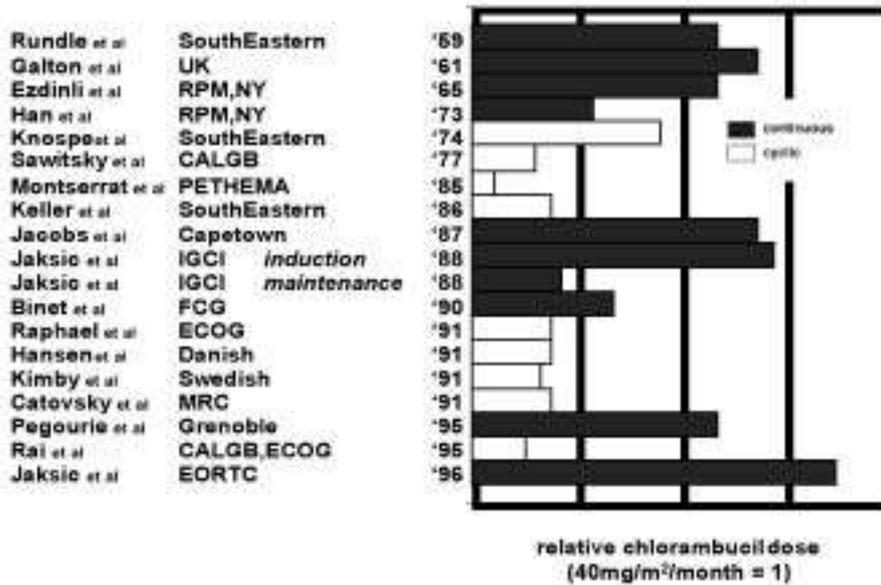


Figure 1. Historical review of CLB dose-intensity.

treatment the better the outcome (Figure 4). Therefore, from the overall results of our own experience we can conclude that HD-CLB followed by low-dose CLB maintenance represents a very good option for traditional treatment of CLL.

Alternative strategies

In recent years it has been demonstrated that the achievement of a molecular remission in CLL is possible; thus, the problem whether to aim treatment to the cure of the disease has been raised.

It has been reported, mainly by the MD Anderson group, that fludarabine in combination with cyclo-

phosphamide can induce a high response rate (around 90%), with an elevated number of complete remissions and with a possible advantage in terms of OS.⁵

Following the introduction of immunotherapy with anti-CD20 monoclonal antibody (rituximab), a more eradicating approach, consisting in the combination of fludarabine, cyclophosphamide and rituximab, was proposed by the MD Anderson group. The first results are apparently very exciting, even in the unfavorable subset of relapsed and resistant patients. Unfortunately, so far, none of these regimens have been tested in comparative

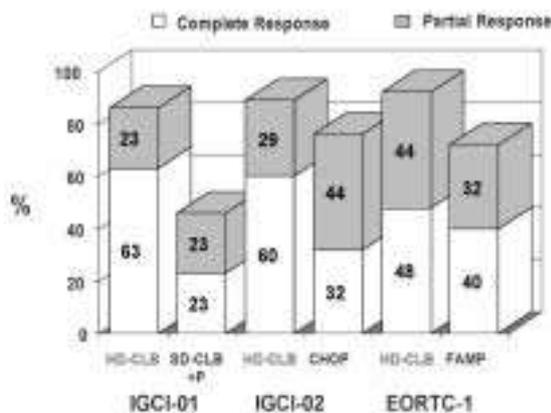


Figure 2. Response rate according to type of therapy in three HD-CLB trials.

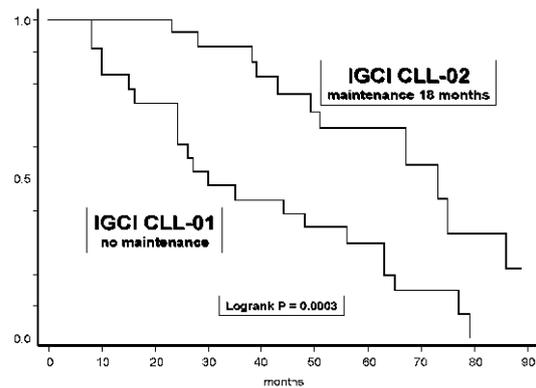


Figure 3. Survival by maintenance of patients in partial remission from IGCI CLL-01 and CLL-02 trials. In CLL-01 PR case did not receive maintenance; in CLL-02 PR cases received maintenance treatment with low-dose CLB for 18 months.

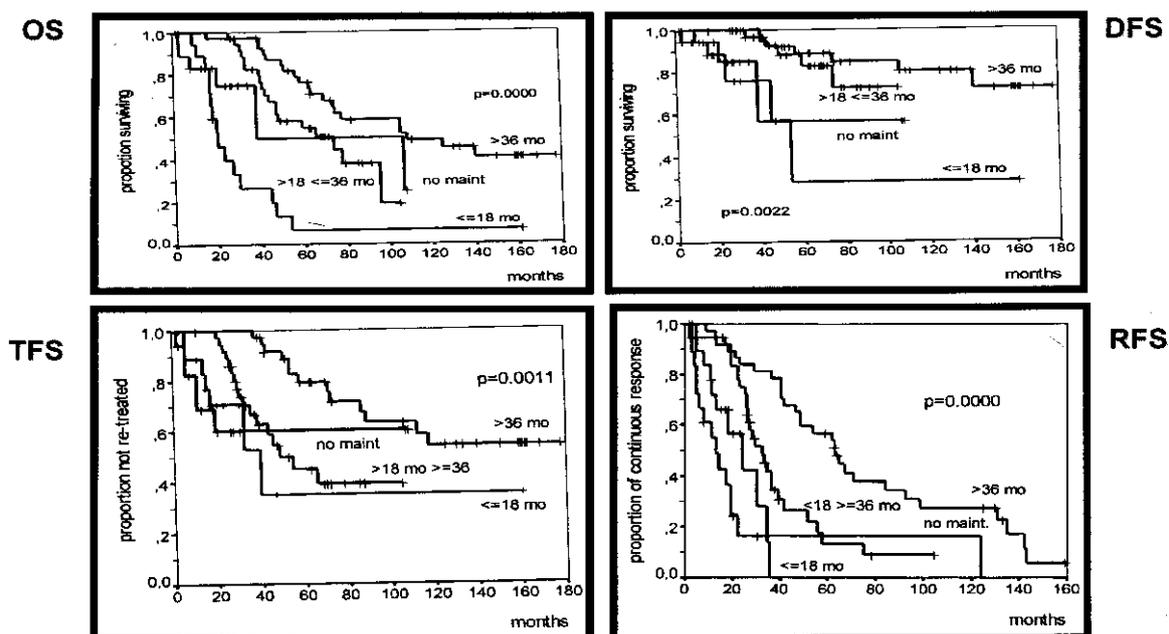


Figure 4. OS by low-dose CLB maintenance duration in 114 cases with a median follow-up of 71 months. Patients are divided according to maintenance duration: >36 months vs. >18-≤36 vs. ≤18 vs. no maintenance.

randomized trials, so that the real advantage in terms of survival still needs to be confirmed.

In addition it should be mentioned that these treatments have relevant toxicity, mainly consisting in infectious and autoimmune complications.

Another approach aimed to eradicate the disease is autologous or allogeneic stem cell transplantation. With the prolongation of the follow-up of transplanted patients it is becoming evident that autologous transplantation is not able to eradicate the disease, although it can induce molecular remissions and very long response duration with a good quality of life.

As far as allogeneic transplantation is concerned, the experience in CLL is still rather limited and apparently characterized by an important transplant-related mortality; however, it seems that this approach can cure the disease. Thus, much hope is now directed towards alternative transplant approaches such as autografting with the addition of anti-CD52 and/or anti-CD20 monoclonal antibodies or allogeneic transplantation with reduced intensity conditioning regimens. At the moment larger experience on these approaches is still needed.

Conclusions

According to the information we already have in our hands, we can answer the question of whether to use traditional or alternative strategies in CLL treatment by concluding that different sets of patients should be addressed to different strategies.

For elderly patients a traditional approach with dose modulation according to the general clinical condition should be employed. For younger cases with therapy requirement but without unfavorable biological prognostic factors, traditional effective first-line treatment could be proposed, and then innovative strategies in the case of resistance, progression or frequent relapses.

In case of young patients with unfavorable prognostic features, immediate treatment with therapeutic approaches aimed to eradicate the disease is advisable.

In all cases, it would be very useful to try to include these experiences in prospective clinical studies. In fact, only the results of ongoing and future randomized trials will give us the possibility to improve the planning of the overall treatment program for B-CLL patients.

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Evolving strategies in the treatment of chronic lymphocytic leukemia with purine analogs

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In patients with chronic lymphocytic leukemia (CLL) the initial response to alkylating agents, alone or combined with corticosteroids, is good. However, this approach is characterized by palliative rather than curative intent, and disease progression ultimately occurs as a result of resistance.¹ With the aim to improve the efficacy of treatment, combination chemotherapy, including low dose anthracyclines² and high dose daily chlorambucil regimen³ was adopted, with better overall results.

Since their introduction in the early 1980s purine analogs, such as fludarabine, cladribine and the adenosine deaminase inhibitor 2-deoxycoformycin, have produced higher remission rate than alkylator based regimens.

Fludarabine (9-β-D-arabinofuranosil-2-fluoro-adenine monophosphate) is a fluorinated analog of aradenine that is relatively resistant to deamination by adenosine deaminase. It has proven to be effective in the treatment of lymphoproliferative disorders, including CLL, low-grade lymphoma, Waldenström's macroglobulinemia and prolymphocytic leukemia.

Cladribine is a purine analog that closely resembles fludarabine. The major differences between the two drugs are the presence in cladribine of both a chlorine atom on the 2 carbon of the purine ring (fludarabine has a fluorine atom) and deoxyribose (fludarabine has an arabinose). These differences lead to the variation of dosing schedules: 20–30 mg/m² for fludarabine versus 4–5 mg/m² for cladribine. Cladribine is highly effective against hairy cell leukemia and produces clinical results similar to those of fludarabine in the treatment of patients with advanced CLL.⁴

As regards the mechanism of action of these drugs in B-CLL, the growth arrested clonal B lymphocytes undergo apoptosis if exposed to fludarabine. It has recently been demonstrated that chemotherapy-induced apoptosis includes as a key step an early cleavage of p27kip1 by caspases.⁵

Single agent studies with fludarabine have demonstrated a higher complete response rate and overall response rate compared with historical experience with

alkylator based regimens.⁶ These results have subsequently been confirmed by comparative clinical trials of fludarabine versus CAP, a CHOP-like regimen without vincristine,⁷ and chlorambucil,⁸ showing both studies also a significant prolongation of time to progression.

Furthermore, a randomized comparison of fludarabine, CAP, and CHOP in 938 previously untreated stage B and C CLL patients was conducted by the French Cooperative Group on CLL.⁹ Although the long term results have shown that fludarabine treatment is not associated with a survival benefit, when compared to CHOP or CAP, the better tolerance, the quality and the high number of remissions and the longer time-to-relapse make the use of this drug, alone or in combination, interesting for an alternative non-palliative management of CLL.

Fludarabine as a single agent

At present fludarabine appears to be the most active agent to have been evaluated in CLL. Single agent therapy usually consists of 25 mg/m²/day for five days, every 4 weeks for 6 cycles.

The response rate to fludarabine and survival varied according to the number of previous treatments, the stage of the disease, and whether or not patients were refractory to alkylating agents. Other factors associated with survival were age and albumin levels. Age greater than 70 years was also adverse prognostic factor for response. Complete remission (CR) ranged from 74% in previously untreated patients to 57% in previously treated non-refractory and 28% in previously treated refractory, respectively.¹⁰

The median time to progression of untreated patients responding to fludarabine was 33 months and was 21 months for previously treated patients. With at least 3-year follow-up, the median survival of previously untreated patients has not been reached and for previously treated (not refractory) median survival was 29 months and for the previously treated (refractory) patients was 9 months. Time to progression is significantly affected also by response to treatment: patients of any age achieving a CR after fludarabine (as a single agent or combined with prednisone) is 42 months, with 20% of patients expected to still be in remission at eight to ten years.¹¹ Patients who have more than 10% CD5 and CD19 co-expressing B-cells or have κ or λ excess in the marrow have a shorter remission duration than patients without evidence of persistent CLL clones.¹¹

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Survival after progression of the disease was also affected by the type and degree of previous treatments. No successful salvage regimen after initial fludarabine therapy was shown for patients refractory to alkylating agents, while fludarabine achieved further remissions in patients who had received fludarabine as initial treatment or were not refractory to alkylating agents.⁹

The response to salvage therapy with fludarabine in patients who had failed or relapsed after receiving fludarabine correlated with their initial response (Table 1).¹²

Activity of oral Fludarabine phosphate

Seventy-eight (96.3%) of 81 patients with previously treated B- CLL received 10 mg tablets of fludarabine phosphate to a dose of 40 mg/m²/d for 5 days, repeated every 4 weeks, for a total of 6 to 8 cycles.¹³ Overall remission rate was 46.2% (CR, 20.5%; PR, 25.6%) according to IWCLL criteria. In terms of clinical efficacy the oral formulation is similar to the i.v. formulation.

Toxicity

Very few organ side effects are observed at conventional doses. Treatment related side-effects are mainly infectious complications, especially in pre-treated patients, and autoimmune phenomena. This is due to the worsening of the immune impairment of CLL patients caused by purine analogs. Infectious toxicity is acceptable, with no severe episodes among untreated patients. The vast majority of patients usually receive prophylaxis against infection with trimethoprim sulfamethoxazole. Autoimmune phenomena are observed in untreated patients.¹⁴

The safety profile of fludarabine oral formulation¹³ is comparable to that of i.v. formulation with the exception of more frequent, but predominantly mild, gastrointestinal adverse events (nausea and vomiting or diarrhoea).

Combination regimens of purine analogs and alkylating agents

Chlorambucil and Cyclophosphamide (CTX) were the mainstay of treatment of CLL before the discovery of Fludarabine.

Combinations of Fludarabine and Chlorambucil were abandoned because of increased toxicity from overlapping myelosuppression and immune suppression. Furthermore, an increased risk of therapy related myeloid leukemias has been observed in patients with CLL after treatment with Fludarabine and Chlorambucil.¹⁵

Fludarabine + Cyclophosphamide

CTX was chosen for combination with Fludarabine because preclinical studies suggested additive or synergistic activity of these two agents.^{16,17} Flu-

Table 1. Response to fludarabine retreatment in patients failing or relapsing from fludarabine regimens (Keating et al.,¹²)

Initial response	Fludarabine retreatment			
	CR	PR	Total pts.	%
Overall response	13	28	63	67
Complete remission	10	8	25	72
Nodular partial remission	3	13	23	64
Partial remission	0	7	11	70
Failure	0	4	-	2

Table 2. Comparison of response rates to Fludarabine (F), Fludarabine+Cyclophosphamide (FC) and to FC+Rituximab (FCR) in untreated CLL patients (Wierda et al.,¹⁹).

Therapy	Fludarabine	FC	FCR
No. patients	82	53	135
CR rate (%)	35	43	63
OR rate (%)	85	91	95

darabine in fact inhibits repair of DNA damage caused by agents such as cyclophosphamide and mitoxantrone. Fludarabine 30 mg/m² is administered intravenously over 30 minutes daily for 3 days and cyclophosphamide 300 mg/m² over 1 hour daily for 3 days. Courses were repeated six times, every 4 to 6 weeks, depending on recovery of blood counts.¹⁸

Overall, this regimen proved to be substantially more active than Fludarabine as a single agent in all phases of CLL management. Fludarabine and cyclophosphamide produced $\geq 80\%$ response rates in all patients not refractory to Fludarabine at the start of therapy as well as a 38% response rate in patients who were refractory to Fludarabine. Although the CR rate was not increased in previously untreated patients, residual disease assessed by flow cytometry was rare and median time to progression was not reached after a median follow-up of 41 months.¹⁸ Myelosuppression and infection were the most significant complications of this treatment. Pneumonia or sepsis occurred in 25% of patients, and were significantly more frequent in patients who were refractory to Fludarabine at the start of combination chemotherapy.

The comparison of response rates to Fludarabine and to the combined regimen Fludarabine + Cyclophosphamide (FC) is shown in Table 2.

Pentostatin + Cyclophosphamide

Pentostatin 4 mg/m² with Cyclophosphamide 600 mg/m² were administered on day 1 of each cycle, with cycles repeated every 3 weeks for six treatments to 23 patients with previously treated CLL. There were 17 responses (74%), including four CR. The combination proved safe and effective, so that the authors are currently studying a regimen including Pentostatin, CTX and Rituximab.²⁰

Cladribine + Cyclophosphamide

Patients with refractory or recurrent CLL received Cladribine 4 mg/m²/day and Cyclophosphamide 350 mg/m²/day both administered intravenously for 3 days every 4 weeks. Nine of 20 (45%) had a response, whose median duration was 12 months. Although inferior to the combination Fludarabine + Cyclophosphamide, this regimen showed interesting activity in patients with advanced CLL. Myelosuppression was the major dose-limiting toxicity.²¹

Combination of fludarabine with monoclonal antibodies

Targeted monoclonal antibodies (MoAbs) offer a promising alternative for the treatment of refractory CLL. The target antigens used therapeutically in CLL include CD20, which is expressed on normal and malignant B-cells (but at low density on B-CLL), and CD52, which is highly expressed on normal and malignant B- and T-lymphocytes, including CLL, but not on haematopoietic stem cells.

The anti-CD20 MoAb Rituximab may be effective in CLL when used at high doses²² and when combined with chemotherapy²³ (Keating *et al.*, 2000). The response rate correlates with sensitivity or refractoriness of the patients to Fludarabine.

Fludarabine + Rituximab have been used in concurrent or sequential combination,²⁴ with higher ORR and CR rate in the concurrent (90% and 47%, respectively) versus the sequential arm (77% and 28%, respectively). In this study the response rates increased after consolidation therapy with Rituximab.

The combination of Fludarabine + Cyclophosphamide + Rituximab (FCR) has further improved the complete response rate already obtained with FC (Table 2). Molecular responses have been achieved in 56% of 55 CR patients.¹⁹ In previously treated patients the response rate to FCR is highest in patients who are still considered sensitive to fludarabine,²⁵ but remissions occurring after salvage therapy are significantly shorter than initial remissions.

Fludarabine + Campath-1H

Another major new agent investigated at the present time is the anti-CD52 monoclonal antibody Alemtuzumab (Campath-1H). Campath-1H

demonstrated activity in previously-treated patients, including those that were Fludarabine refractory, with a 33% of response rate.²⁶ Campath is more active at clearing the disease from blood, bone marrow, and spleen and less active in bulky lymph nodes. Therefore, it has demonstrated good efficacy in the setting of patients with near complete remission, or partial remission, with some patients becoming polymerase chain reaction (PCR) negative.

The sequence of combination chemotherapy programs followed by monoclonal antibodies in the minimal residual disease is the new paradigm for the next decade. Alemtuzumab proved to be able to eradicate minimal residual disease (MRD) in previously treated patients.²⁷ More recently, treatment with fludarabine for debulking and Campath-1H to purge residual disease was capable of eradicating CLL to levels that are undetectable by flow cytometry or PCR.²⁸ Furthermore, of sixteen patients treated with this sequential therapy including fludarabine and Campath-1H, the collection of peripheral blood stem cells was feasible, with excellent yields in 92.8% of patients primed with the combination of intermediate dose Ara-C and G-CSF.²⁹

Clinical studies have demonstrated that the purine analogues are the most effective chemotherapeutic agents in CLL. Combined with alkylating agents, response rate and time-to-treatment-failure have been significantly improved. When combined with Rituximab, the results are strikingly improved, especially in previously untreated patients. The nucleoside analog Fludarabine and the two monoclonal antibodies Campath-1H and Rituximab have emerged as the three most promising agents for first-line treatment in CLL.

The aim of the studies currently in progress is to find the most effective way of combining these agents to increase the incidence of CR rate, which is the first step to obtain a long term control of the disease. The further integration of this frontline approach with procedures targeted to eradicate minimal residual disease (MRD) and to maintain a MRD-negative status (e.g. autologous and allogeneic bone marrow transplantation) may be an appropriate strategy to switch the goal of therapy from palliation to cure.

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Risk adapted management of chronic lymphocytic leukemia: update on the cooperative trials of the German Chronic Lymphocytic Leukemia study group

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Summary

The diagnosis and treatment of chronic lymphatic leukemia (CLL) are currently undergoing great change. With numerous new therapeutic procedures being available (purine analogs, high-dose treatment and monoclonal antibodies) the options for the treatment of CLL are considerably more diverse now than a few years ago, and currently include procedures that take into account comorbidity and risk. At the same time, it should be emphasized that many important questions regarding the treatment of CLL remain unresolved. Therefore, it is important to treat CLL patients in multicenter studies such as those proposed by the German CLL study group (GCLLSG).

In the past, the standard treatment for CLL patients in advanced stages was oral monotherapy with chlorambucil.¹ Chlorambucil is an alkylating drug that is usually well tolerated. Remission rates of up to 40% can be achieved with chlorambucil, but complete remissions are achieved only rarely, and partial remissions are of short duration. There is *no* advantage from combining chlorambucil with corticosteroids, except in autoimmune complications. There are several, largely equivalent, methods of administration.¹

The treatment of CLL has changed greatly since the introduction of the purine analogs fludarabine and 2-chlorodeoxyadenosine (cladribine). Purine analogs are the first substances that achieve a relatively high rate of complete remission, even used alone as monotherapy.² Fludarabine has been studied more thoroughly than cladribine. Fludarabine is administered intravenously at a dose of 25 mg/m² from days 1–5 of cycles given at intervals of 4 weeks. Up to six cycles are given.

As primary treatment, fludarabine results in response rates of approximately 80%, and about a third of all patients achieve complete remission.^{3,4} In patients who have previously been treated with alkylating drugs, the response rates are between 12 and 55%, and in patients who are resistant to alkylating drugs, between 20 and 40% 5–7. In the single phase III study to date, which compared fludarabine directly with chlorambucil in pre-

viously untreated patients, fludarabine achieved higher response rates (70% vs. 43%), more complete remissions (27% vs. 3%) and a longer period of survival free of progression (33 months vs. 17 months), but with no clear extension of total survival.⁸ In comparison with more intensive multiple chemotherapy regimens, such as cyclophosphamide, doxorubicin and prednisone (CAP) or cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP), fludarabine is at least as effective with regard to response rates and survival free of progression.⁹ In relapses after previous treatment with fludarabine, a second line of chemotherapy with fludarabine results in further, high response rates of between 67 and 83%.¹⁰

The main adverse effects of purine analogs are myelosuppression and lymphocytopenia, with low counts of CD4⁺-positive T lymphocytes, although this does not lead to a higher rate of infection, than that associated with similarly myelosuppressive treatments, such as CAP. However, the spectrum of pathogens differs from that occurring after myelosuppressive treatment with alkylating agents.¹¹ In about 1–5% of cases, autoimmune cytopenia occurs during treatment.¹² Tumor lysis syndrome can occur rarely during treatment with fludarabine.¹³ The efficacy of cladribine is similar to that of fludarabine.^{14–16}

A new modality in CLL treatment: purine analog in combination therapy

In order to improve treatment with purine analogs further, combinations of fludarabine with cyclophosphamide and/or mitoxantrone have been studied, because these drugs act synergistically *in vitro*.¹⁷ The combination of fludarabine and cyclophosphamide is by far the best studied (Table 1). It produces response rates of more than 80% in pre-treated patients.^{18–22} The combination of fludarabine and epirubicin in a study of previously treated patients also produced response rates of over 80%; among these patients, about 30% achieved complete remission.²³ In another study, the triple combination of fludarabine, cyclophosphamide and mitoxantrone induced complete remissions in 50% of relapsed patients.²⁴ The most common side effects of these fludarabine combinations are severe infections.

Monoclonal antibodies: rituximab and alemtuzumab

Monoclonal antibodies bind to CLL cells via defined surface antigens and kill leukemic cells by various

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Table 1. Response rates of fludarabine and cyclophosphamide combinations (some selected references).

Author, year ^{Ref}	Combination	N	CR	PR	OR
O'Brien, 2001 ²⁰	F 30 mg/m ² & CY 500/350/300 mg/m ² d1-3	93 CLL	16%	72%	88%
Hallek, 2001 ¹⁹	F 25 mg/m ² & CY 250 mg/m ² d1-3	36 CLL	16%	75%	91%
Frewin, 1999 ²¹	F 25 mg/m ² & CY 250 mg/m ² d1-3	10 NHL	0%	50%	50%
		7 CLL	28%	43%	71%
Flinn, 2000 ²²	F 20 mg/m ² d1-5 & CY 600 mg/m ² d1, G-CSF 5 µg/kg d8+	43 NHL	60%	32%	92%
		17 CLL	47%	53%	90%
Bosch, 2001 ²⁴	F 25 mg/m ² d1-3 & CY 300 mg/m ² d1-3, Mitoxantrone 6 mg/m ² d1	60	50%, 18 mol. CR	28%	78%

F = fludarabine; CY = cyclophosphamide; G-CSF = granulocyte colony stimulating factor. CR = complete remission, OR = overall response rate, PR = partial remission.

mechanisms (apoptosis, complement activation, antibody-mediated cellular cytotoxicity). Monoclonal antibodies provide an opportunity to treat CLL and maintain remissions. The efficacy of monoclonal antibodies against CD20 (rituximab)²⁵ or against CD52 (alemtuzumab, formerly called campath-1H)²⁶ is currently under examination in clinical studies. Most experience with monoclonal antibodies has been gained in the treatment of relapses of CLL. In studies to date, rituximab alone led to partial remissions of short duration in 20–41% of patients.^{27,28} When dose intensified rituximab regimens are applied, the response rates may increase up to 75%.^{29,30} In patients with high leukemia cell counts in the peripheral blood, there is a danger of severe adverse effects, caused not only by the release of cytokines from the leukemic cells, but also by the agglutination of leukemic cells in small blood vessels.^{31,32}

Alemtuzumab is directed at CD52, which is expressed by normal B and T lymphocytes and almost all CLL cells. Undesirable effects of alemtuzumab are myelosuppression and T-cell depletion, which can lead to infectious complications.³³ Alemtuzumab shows its effect particularly in the peripheral blood and bone marrow. To date, alemtuzumab has been used primarily in relapses, and results in a response in about 42% of cases, with a relapse-free interval of > 12 months.³⁴ This monoclonal antibody was, therefore, approved in 2001 for the treatment of CLL relapses after failure of fludarabine treatment. A response rate of 89% was achieved in primary treatment.³⁵

The use of alemtuzumab as a post-remission consolidation therapy has been studied by the GCLLSG and by an Italian pilot study to test the hypothesis that leukemic cells (minimal residual disease) can be permanently eliminated in this way. Montillo and colleagues showed that subcutaneous administration of alemtuzumab three times a week for 6 weeks in escalating doses up to 10 mg may

be an efficient and safe way to achieve molecular remissions in a significant number of CLL patients.³⁶ Our own experience shows that conventional dosing with 30 mg alemtuzumab three times weekly following standard fludarabine or fludarabine-cyclophosphamide chemotherapy might be even more efficient, but provokes infectious complications in the majority of patients (*Wendtner et al., unpublished data*). Taken together, these results illustrate that post-remission consolidation therapy with alemtuzumab induces complete molecular remissions. However, the optimal timing and dosing schedule for alemtuzumab as consolidation therapy remain to be determined.

Chemoimmunotherapy – combinations of fludarabine with rituximab and alemtuzumab

The combination of fludarabine with monoclonal antibodies has been added recently to armamentarium of treatment options for CLL and has yielded very promising results (Table 2). The results obtained so far suggest that there is a synergistic anti-leukemic effect of fludarabine and rituximab. The overall response rates are between 77 and 95%. Most importantly, the rate of complete remissions obtained with this combination is between 20 and 66% except in fludarabine-resistant patients (7%). In particular the combination of fludarabine, cyclophosphamide and rituximab induced complete remissions of 66% of untreated patients, a remission rate never previously obtained in the primary treatment of CLL.³⁷ In another study, 104 patients received fludarabine plus rituximab either simultaneously (n=51; on day 1 of each course) or sequentially (n=53; 6 courses of fludarabine followed by rituximab for 4 weeks).³⁸ The response was significantly higher if rituximab was given simultaneously with fludarabine. The overall response rate was 90% versus 77%, with 47% versus 28% complete remissions (Table 2). These results suggest a synergistic mode of action of both drugs. Again, the major

Table 2. Response rates to combination therapies consisting of fludarabine or fludarabine/cyclophosphamide with rituximab (some selected results).

Author, year ^{Ref}	Therapy	N	CR	nPR	PR	OR
Manero 2001 ³⁹	FC+R Alkylator-resistant	15	20%	13%	33%	80%
	FC+R F-sensitive	60	30%	15%	33%	
	FC+R F-resistant	27	7%	11%	41%	
Byrd, 2001 ³⁸	FC+R	51	47%		43%	90%
	FC+R	53	28%		49%	77%
Wierda, 2001 ³⁷	FC+R primary therapy	79	66%	14%	15%	95%
Schulz, 2002 ⁴⁰	F+R	29	34%		52%	90%

FC = Fludarabine and cyclophosphamide; R = Rituximab; NPR = nodular partial remission; further abbreviations see Table 1.

side effect of this combination therapy was severe, sometimes opportunistic, infections.

A combination of alemtuzumab and fludarabine was tested by Kennedy *et al.* in 6 patients who were refractory to fludarabine.⁴¹ Five patients responded to this therapy, and one had a complete molecular remission. One patient had pneumonia caused by *Pseudomonas*. There was no CMV reactivation. Taken together, these results suggest that the combination of fludarabine with monoclonal antibodies (rituximab and alemtuzumab) has a strong potential to achieve complete molecular remissions.

High-dose therapy followed by autologous or allogeneic stem cell transplantation

Myeloablative high-dose (chemo)therapy, with subsequent autologous or allogeneic stem cell transplantation (SCT), is the most intensive form of chemo-immunotherapy in CLL, and often combines all available elements of anti-leukemic treatment such as cytotoxic agents, monoclonal antibodies, total body irradiation and – in the case of allogeneic transplantation – cellular immunotherapy. It remains an experimental treatment for CLL which should be restricted to the framework of studies.^{42–44} A detailed description of these treatment modalities is beyond the scope of this manuscript. However, it should be pointed out that the treatment-related mortality of autologous SCT is currently 5–10%.⁴³ Because of the lack of randomized studies, it is unknown whether this therapeutic approach results in a better long-term prognosis than conventional chemotherapy. The results of a European phase III trial are urgently awaited.

The treatment-related mortality of allogeneic SCT is between 25 and 50%⁴⁴ preventing the more frequent use of this modality in CLL. Non-myeloablative conditioning protocols could improve the

results.⁴⁵ The graft-versus-leukemia effect is exploited by donor lymphocyte transfusions, which are efficient in CLL.^{46,47}

Current and future investigations by the German CLL Study Group

The GCLLSG has developed a concept of comorbidity- and risk-adapted treatment of CLL in the framework of clinical studies. The long-term aim of these studies is continuous optimization of treatment in order to achieve higher response rates, longer periods free of disease, a better quality of life and, perhaps in the future, a cure for the disease. In the CLL1 protocol for patients in Binet stage A, the risk of progression is first determined. Patients with a high risk of progression (non-nodular bone-marrow infiltration or lymphocyte doubling time of <12 months and elevation of serum thymidine kinase or of serum β 2-microglobulin) are either observed or treated with fludarabine after randomization. Patients with a low risk of progression are observed. The CLL1 protocol will be replaced during the next months by the CLL7 protocol, which will be conducted as a joint protocol by the French and German CLL study groups, investigating the benefit of early treatment with a triple combination of fludarabine, cyclophosphamide and rituximab in patients at high risk (as defined by molecular cytogenetics, elevated serum thymidine kinase, short lymphocyte doubling time, and unmutated immunoglobulin status) versus the same triple combination as deferred treatment.

When a clear indication to treat is presented (Binet stage C or stage B with symptoms), treatment will be initiated. The CLL4 protocol for patients aged up to 65 years compared fludarabine with the combination of fludarabine and cyclophosphamide. The study was closed in July 2003. So far, the results of the CLL4 protocol have shown

that fludarabine plus cyclophosphamide causes more myelosuppression, but induces twice as many complete remissions as fludarabine alone. Moreover, the combination seems to prolong the event-free and progression-free survival of the patients. The CLL4 protocol was replaced in July 2003 by the CLL8 protocol comparing fludarabine plus cyclophosphamide versus these two drugs plus rituximab in the primary treatment of CLL.

The CLL5 protocol, for patients aged 66 years and above, compared chlorambucil with fludarabine. We have learnt from this trial that fludarabine can be given to elderly patients without a significant increase in toxicity compared to that in younger patients. This CLL5 protocol will be replaced in the next few months by the CLL9 protocol testing the benefit of darbepoietin- α as supportive treatment for patients with relevant comorbidity being treated with dose-reduced fludarabine monotherapy.

The protocols are constantly being extended by innovative procedures. New substances or combinations are tested in phase II protocols (CLL2G and CLL2I with alemtuzumab; CLL2H with CHOP plus rituximab). High-dose chemotherapy with autologous stem cell replacement (CLL3C protocol) or allogeneic stem cell transplantation (CLL3X protocol), are also being tested by the GCLLSG.

We hope that the clinical trials of the GCLLSG will contribute to an improved outcome of patients with CLL.

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Combinations of chemotherapy and immunotherapy in chronic lymphocytic leukemia

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Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia diagnosed in the western hemisphere, accounting for 25% of all adult leukemias. CLL is usually considered a disease of the elderly. However, about 10% to 15% of patients reported in different clinical trials are under 50 years of age. Patients with low-risk, early stage disease are generally just followed closely, as there is no evidence of a survival benefit associated with early intervention. Previously, with the onset of disease symptoms or progressive cytopenias, patients were treated with oral alkylator agents with or without prednisone. While alkylator therapy is both easy to administer and effective in providing palliation for patients with CLL, complete remission with this approach is quite rare. The initial identification of the efficacy^{1,2} of fludarabine (FAMP) treatment for alkylator-refractory CLL has altered the therapeutic approach taken for most patients. Three prospective phase III trials were performed in untreated CLL: these trials showed both a superior response rate^{3,4} and progression free-survival³⁻⁵ with FAMP therapy versus alkylator-based therapy but not a survival advantage. Indeed, although fludarabine has become an acceptable first-line treatment for most patients with symptomatic CLL, most patients unfortunately do not attain complete remission with FAMP and all eventually relapse following this therapy. In order for the therapeutic outcome of patients with CLL to progress, therapies must be identified that effectively eliminate CLL refractory to FAMP and that are synergistic with this or other active agents which have a side effect profile that allows effective combination approaches. Significant advances in the development of monoclonal antibodies have improved targeting of leukemic cells with acceptable toxicities. Lymphomas and leukemias are particularly well suited for monoclonal antibody therapies given the identification of multiple tumor cell-specific antigens that are not shared by other tissues. Monoclonal antibodies are targeted; do not produce extramedullary toxicity and they may greatly increase the antitumor effect of chemotherapy. To date, targeted monoclonal antibodies (MoAb) offer a promising alternative to chemotherapy in refractory CLL. Among such antibodies, two have emerged with

some promise: rituximab, a chimeric human-mouse MoAb that recognizes and binds to the CD20 antigen, and alemtuzumab (Campath-1H), a humanized MoAb with specificity toward the CD52 surface antigen.

Early experience with both antibodies demonstrated that they are easy to administer, have good tolerability and single-agent activity in both relapsed non-Hodgkin's lymphomas (NHL) and CLL.^{6,7} As encouraging as these results may be, MoAbs are not curative by themselves. Therefore, the next logical step is to explore combinations of them with other agents in order to improve the clinical response rate and the long-term outcome of patients with these disorders. As experience is being accumulated with MoAb combinations in CLL, new insights are being gained into response patterns and effects on minimal residual disease which have not been reached with chemotherapy alone and which might form the basis for new therapeutic paradigms in a disease that is still considered incurable.

Rationale for monoclonal antibody combinations in chronic lymphocytic leukemia

The major mechanism of action of MoAbs is thought to depend on the activation of human effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated lysis. The consequence of the cytotoxic activity of these components, which are not usually recruited by more traditional chemotherapy agents, is clearance of malignant cells.⁸ However, there is now increasing *in vitro* evidence that MoAbs are also capable of inducing apoptosis.^{9,10} It has been demonstrated that treatment of lymphoma cell lines with rituximab sensitizes the cells to the cytotoxic and apoptotic effect of other chemotherapy drugs, including FAMP. One mechanism by which rituximab sensitizes cells to the cytotoxic effects of chemotherapy *in vivo* is through the downregulation of anti-apoptotic pathways.^{11,12} Synergistic activity of MoAb combinations might therefore overcome some of the problems and limitations of MoAbs when they are used as single agents. The expression of CD20 antigen on the surface of CLL cells is weak when compared to that on follicular lymphoma or other NHL cells.¹³ This, in part, is reflected by the lower response rates to rituximab as a single-agent in patients with CLL than in patients with other low NHLs. Furthermore, the expression of surface antigens might vary substantially among CLL patients, thus rendering antigen-antibody binding and subsequent biological and clinical effects rather unpredictable. It is

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apparent from experience with single-agent MoAbs that these drugs clear some anatomic compartments more efficiently than others. Although alemtuzumab achieves almost complete clearance of the peripheral blood, response rates in patients with organomegaly or bulky lymphadenopathy are rather modest.⁷ Finally the use of single-agent MoAb therapy might not be sufficient to capture chemotherapy-refractory cells. This observation is based on experience on rituximab dose-intensity studies in which the response rate to single-agent rituximab at increasing dose levels was higher if patients retained sensitivity to purine analogs such as FAMP.¹⁴

The CD20 antigen

CD20 is a B-cell-specific surface protein.¹⁵ It is expressed in the pre-B stage and later stages of development, but on lymphoid stem cells. The expression of CD20 continues through B-cell maturation until the plasmacytoid immunoblast phase; CD20 is only weakly expressed on plasma cells.

CD20 has four cell membrane-spanning domains; it probably functions as a calcium channel. Binding of CD20 by anti-CD20 antibody can affect cell cycle progression.¹⁶ The aminoacid sequence of CD20 is similar to that of the β -subunit of the high affinity IgE receptor. These proteins are members of a family of proteins that play a role in signal transduction. CD20 appears to participate in signaling through cross-linking, and CD40 and/or the MHC class II molecule may play a role in this cross-linking process.¹⁷ In some cell lines, anti-CD20 antibodies can induce apoptosis.¹⁸ CD20 is expressed on most malignant B-cells, with nearly 90% of B-cell lymphomas expressing the CD20 antigen. Virtually 100% of B-CLL patients expressed CD20, although the expression can be dim.¹⁹

Rituximab

Rituximab is a chimeric antibody directed against CD20. Preclinical studies utilizing rituximab demonstrated that this chimeric antibody is effective in lysing cells via both human complement-dependent cytotoxicity and ADCC.²⁰ Binding of CD20 by antibody does not appear to induce antigen modulation or internalization, thus making the CD20 antigen an excellent therapeutic target.

Following US Food and Drug Administration approval a certain number of trials with rituximab in CLL were initiated but significant concern existed among clinical and laboratory investigators regarding the eventual role of rituximab in the treatment of this disease. Indeed, the target for rituximab, CD20, is much more dimly expressed on CLL cells than on follicular lymphoma cells,²¹ and the immune defect relative to ADCC²² and its complement function was well demonstrated.²³ Pharmacokinetic studies, from a limited number of cases enrolled in the pivotal trials, demonstrated a strong correlation between mean plasma antibody concentration and response. Pharmacokinetic studies of patients with small lymphocytic leukemia (SLL) showed a lower pre-treatment plasma trough concentration of rituximab before the second and fourth infusions, and at 1 week, 1 month and 3 months post treatment, as compared with levels in patients with other low-grade histologies.²⁴ The reasons for the rapid clearance of rituximab in patients with CLL/SLL are not clear but might be diminished target binding, higher tumor load, or rapid antibody metabolism. Indeed, the aforementioned pharmacokinetic findings may provide insight into why hypogammaglobulinemia is common in advanced CLL; a previously presumed mechanism of diminished production may be superseded by one of accelerated destruction. In addition, recent prelim-

Table 1. Results of trials using rituximab in combination with chemotherapy.

Investigator	Regimen	n	Patient Population	Response Rates
Byrd <i>et al.</i> , ²⁷	Sequential fludarabine/rituximab	53	Chemotherapy naive	15 (28%) CR 26 (49%) PR
	Concurrent fludarabine/rituximab	51	Chemotherapy naive	24 (47%) CR 22 (43%) PR
Keating <i>et al.</i> , ³⁰	Rituximab/fludarabine/cyclophosphamide	135	Chemotherapy naive	90 (67%) CR 20 (15%) nodular PR 18 (13%) PR
Polliack <i>et al.</i> , ²⁹	Rituximab/fludarabine/cyclophosphamide/mitoxantrone	14	Chemotherapy naive	10 (71%) CR 3 (21%) nodular PR
Garcia-Manero <i>et al.</i> , ³¹	Rituximab/fludarabine/cyclophosphamide	136	Previously treated	28 (21%) CR 18 (13%) nodular PR 50 (37%) PR

inary work has suggested that CLL patients may differ from both healthy volunteers and other lymphoma patients in having a significant amount of free soluble plasma CD20, which increases as the disease progresses.²⁵

It was not long after the approval of the use of rituximab for relapsed and refractory low-grade NHL, that combination studies of the antibody with chemotherapy agents were pioneered in patients with NHL.²⁶ Likewise, combination chemotherapy with anti-CD20 MoAb to exploit a potential synergy in the treatment of CLL has been proposed by several groups. Table 1 provides an overview of current studies of rituximab combined with chemotherapy in CLL.

Rituximab/fludarabine

The cancer and Leukemia Group B (CALGB) conducted⁹ a phase II trial of 104 previously untreated CLL patients who were randomized to receive either fludarabine for 6 cycles followed by rituximab or fludarabine concurrently with rituximab on days 1 and 4 of cycle 1 and on day 1 of cycles 2-6.²⁷ According to the National Cancer Institute (NCI) Working Group criteria,²⁸ the data suggest that concurrent chemotherapy and rituximab yields higher overall response rates (ORRs) and complete response (CR) rate than does the sequential combination. Forty-six of 51 patients (90%) in the concurrent treatment arm responded while in the sequential treatment arm 41 of 53 patients (77%) responded and 15 of these 53 patients (28%) achieved a CR, (Figure 1). No significant difference was observed with respect to toxicities and rate of infections. While 9 of the first 44 patients (20%) in the concurrent treatment arm developed significant infusion-related toxicities, no infusion-related toxicities were observed when a stepped-up dosing approach was used for rituximab during the first induction course.

Rituximab/fludarabine/cyclophosphamide/mitoxantrone followed by stem cell transplantation

Dr. Polliack and colleagues used a combination of fludarabine/cyclophosphamide/mitoxantrone for 4-6 cycles as primary therapy, followed by 4 weekly infusion of rituximab as secondary therapy and purging prior to stem cell harvesting.²⁹ Fourteen patients with *de novo* CLL were treated. All of the patients responded, with 10 patients (71%) achieving a CR and 3 patients (21%) achieving a nodular partial response (PR). Interestingly, 4 out of 10 complete responders (29% of patients treated) achieved a molecular remission, indicating eradication of minimal residual disease.

Rituximab/fludarabine/cyclophosphamide

Building on their successful fludarabine/cyclo-

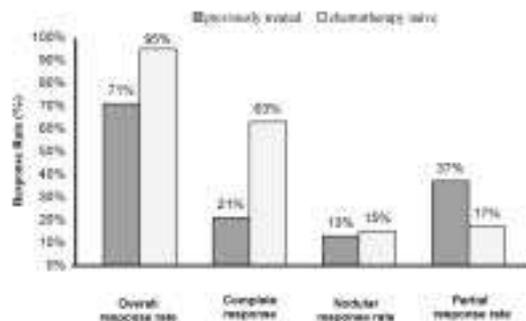


Figure 1: Response to Rituximab+Fludarabine(±) cyclophosphamide in previously untreated chemotherapy naive CLL patients (Byrd et al. Blood 2003)

phosphamide combination as a backbone, the group at the M. D. Anderson Cancer Center added rituximab concurrently with each course of chemotherapy.³⁰ During the first cycle rituximab was given at a dose of 375 mg/m² on day 1, followed by fludarabine/cyclophosphamide on days 2-4; in all subsequent cycles, rituximab was given at a dose of 500 mg/m² on the same day as the first doses of the chemotherapy agents. One hundred and thirty-five previously untreated patients have been studied. A complete response, as defined by NCI Working Group criteria, was documented in 90 patients (67%). Twenty patients (15%) and 18 patients (13%) achieved a nodular PR and a PR, respectively. Grade 3/4 neutropenia was seen in 60% of the cycles administered, and grade 3/4 thrombocytopenia was demonstrated in 7% of the cycles. Overall, the regimen was well tolerated, and approximately 70% of the patients were able to complete all 6 courses. In addition to producing the highest CR rate reported with any CLL induction regimen, this study documented molecular remission, as demonstrated by polymerase chain reaction (PCR) negativity for immunoglobulin (Ig)H in 57% of the complete responders. These data support the suggestion that the antibody/chemotherapy combination is effective and able to eradicate minimal residual disease in a substantial proportion of patients. Using the same regimen in 136 patients with relapsed CLL, a study by Dr. Garcia-Manero and colleagues showed an ORR of 71%, with a CR rate of 21%.³¹ Of 14 complete responders in whom IgH gene rearrangements were studied by PCR, 5 (36%) became negative, indicating that achieving molecular response is possible even at the more advanced stages of CLL.

The CD52 antigen

CD52 is a heavily glycosylated antigen expressed at high levels (approximately 5×10⁵ molecules/cell) on most normal and malignant mature lymphocytes but not on hematopoietic stem cells.^{32,33} It is the shortest cell surface protein characterized to date, and consists of only 12 amino acids attached indirectly to the cell membrane through a glycoprotein

I (GPI) anchor. CD52 is not modulated either *in vitro* or *in vivo* in the presence of bivalent MoAb. The appearance of CD52- negative tumor cells following alemtuzumab treatment is extremely rare and it has been possible to treat some patients successfully with multiple courses of MoAb.³⁴

Alemtuzumab

Campath 1-H contains the six complementary determining regions (CDRs) of a murine anti-CD52 MoAb grafted onto human IgG1 and κ genes.³⁵ Although the effector functions of alemtuzumab are not fully understood, cross-linking of the CD52 antigen by the MoAb may induce apoptosis *in vitro*.³⁶ Both antibody-dependant cellular cytotoxicity and complement binding are necessary for cell lysis *in vitro*.³⁷ This mechanism of action does not overlap with the known mechanisms of action of any existing chemotherapies, thereby limiting possibilities of cross resistance.³⁸

Combination regimens in patients with relapsed/refractory CLL

There are only few reports on alemtuzumab in combination regimens for the use in previously treated patients with disease progression.

The first reported experience with the combination of Campath-1H and FAMP in CLL was used in patients showing refractoriness to each agent used singly.³⁹ Six patients who had received a median of 8 courses of FAMP (range 4-10 courses) and 16 weeks of Campath-1H (range 8-32 weeks) were treated. Five patients responded, one had a complete response according to NCI WG criteria. The responses observed were better in each patient than responses after each agent used singly. Complete morphologic bone marrow responses were seen in 3 patients, including eradication of disease measured by sensitive flow cytometry in two.

The preliminary results of a trial conducted in this setting by the German CLL study Group have recently been reported.⁴⁰ This phase-II trial investigated the safety and efficacy of a combined modality treatment with FAMP and Campath-1H in patients with relapsed CLL. The primary objective of the study was to assess the feasibility of and overall response rate to the combination. Secondary objectives included assessment of the response duration and rate of molecular responses. The schedule consisted of an escalation phase (phase A) of Campath-1H up to 30 mg within 3 to 14 days, followed by the combination therapy with fludarabine + Campath-1H (phase B). FAMP was administered at the standard dosage of 30 mg/m²/d (day 1-3) immediately before the antibody-infusion (30 mg absolute) and repeated on day 28 for a total of 4 cycles. A total of 18 patients were included in this phase-II study: 14 have completed the therapy and are evaluable for response. The baseline characteristics of these 14

patients were: median age 59.7 years (range 38-80); median number of prior regimens 2 (range 1-4). The responses in these 14 evaluable patients were CR 9, PR 3, SD 1, and PD 1. Side effects consisting of fever, chills and exanthema of the skin were mild and mainly related to the first campath-1H-infusions. Transient grade III-IV hematologic toxicity was noted in patients with pre-existing high-grade bone marrow infiltration. One patient died due to fever of unknown origin: this patient was heavily pretreated with large tumor masses.

Campath-1H as treatment of minimal residual disease

A brief report published in 1997 suggested that alemtuzumab may be effective for *purging* residual disease in patients who achieve a maximal response with purine analogs.⁴¹ In this small study, alemtuzumab induced CR in 5/6 patients who had residual disease following induction therapy with either FAMP or deoxycoformycin.

These favorable results prompted us to test alemtuzumab systematically as post-remission treatment in patients with CLL.⁴² The primary objective of our study was to define the role of alemtuzumab as treatment of residual disease after FAMP. We also wanted to demonstrate the feasibility of collecting peripheral blood stem cells (PBSC) and the quality of harvest after alemtuzumab therapy. Intravenous administration of alemtuzumab is often associated with infusion reactions, which consist of fever, rigors and nausea, caused by the release of cytokines such as tumor necrosis factor α and interleukin-6. These reactions, which may be grade 3-4 severity, occur most often after the initial infusions. An additional objective of this study was to assess the safety profile of alemtuzumab administered subcutaneously.

Trial design

Alemtuzumab was given at least 8 weeks after the discontinuation of FAMP. It was administered subcutaneously, three times a week for 6 weeks, in escalating doses up to 10 mg, with premedication of 1 g paracetamol and 10 mg of chlorpheniramine. Patients received acyclovir and cotrimoxazole as prophylaxis from the start of treatment and until 3 months after the end of alemtuzumab treatment. Granulocyte colony-stimulating factor (G-CSF) at a dose of 5-10 μ g/Kg/day was started 8 weeks after completion of alemtuzumab in order to mobilize PBSCs. The PBSCs were collected when the number of CD34⁺ cells reached $\geq 10/\mu$ L. In those patients in whom mobilization of PBSC with G-CSF failed, the protocol allowed a second attempt of harvesting with intermediate-dose Ara-C (ID-Ara-C), 800 mg/m²/q12 h for 6 doses.

Restaging procedures, performed after completion of treatment with the purine analog, and imme-

Table 2. Characteristics of the 17 patients before Campath-1 treatment.

Patient	Age	Sex	Pre-treatment stage		Previous treatment	Status before Campath-1H	IgH rearrangement
			RAI	Binet			
1	44	M	IV	C	Chlorambucil+PDN; FAMP ×6	PRN	Monoclonal
2	57	F	I	A	FAMP × 8 (oral)	PR	Monoclonal
3	44	M	IV	C	FAMP × 6	PR	Monoclonal
4	55	M	IV	C	Chlorambucil+PDN; FAMP ×5	PRN	Monoclonal
5	58	M	II	A	FAMP × 8 (oral)	PRN	Monoclonal
6	62	M	IV	C	FAMP × 6	PRN	Monoclonal
7	61	M	II	A	FAMP × 6	CR	Monoclonal
8	57	M	I	B	FAMP × 6	PRN	Monoclonal
9	57	F	II	A	FAMP × 6; FAMP+cyclophosphamide ×3	PR	Monoclonal
10	62	F	II	A	FAMP × 6	PR	Monoclonal
11	49	M	II	B	FAMP × 4	CR	Monoclonal
12	60	M	I	B	FAMP × 6	CR	Monoclonal
13	55	M	I	A	FAMP × 8	RPN	Monoclonal
14	53	F	II	B	FAMP × 6	RP	Monoclonal
15	38	M	I	A	FAMP × 6	CR	Monoclonal
16	47	M	II	B	FAMP × 4	CR	Monoclonal
17	45	M	IV	C	FAMP+cyclophosphamide ×6	CR	Monoclonal

diately before and after alemtuzumab, included: bone marrow aspiration, trephine biopsy, immunophenotyping, and molecular study for IgH rearrangement. Ultrasound of the abdomen and standard radiography of the thorax were done in all patients. Response criteria were those defined by the NCI Working Group.

Patients' characteristics

The results of the treatment have recently been updated.⁴³ The characteristics of the seventeen patients treated with alemtuzumab are summarized in Table 2. Thirteen were males and 4 females, with a median age of 55 years (range 38–62). All patients received chemotherapy including FAMP before alemtuzumab: as first line treatment in 14 cases and as salvage therapy in 2 patients who were resistant to chlorambucil plus prednisone. The last patient was treated with the association of FAMP + cyclophosphamide (CTX). In one patient FAMP was administered, together with CTX⁴⁴ at disease progression after a previous response to single-agent FAMP. The median time from FAMP discontinuation to alemtuzumab initiation was 5 months (range 2–11).

Results

Before starting alemtuzumab, 6 patients were in complete morphologic and immunophenotypic remission, 5 were in partial remission (PR) and 6 were in nodular partial remission (PRN). All the patients had persistent residual disease as detected by PCR amplification of monoclonal IgH rearrange-

ments. As shown in Table 3, four patients who were in PR after FAMP treatment achieved CR after alemtuzumab, two of them showing a persistent monoclonal rearrangement of IgH gene. Four patients who were in PRN after FAMP improved to CR after alemtuzumab with, in one case, conversion to a polyclonal rearrangement of the IgH gene. Five of the 6 patients in CR after chemotherapy converted to a polyclonal rearrangement of the IgH gene after immunotherapy.

All but one patient proceeded to PBSC mobilization, which was successful in 2 cases after the initial attempt with G-CSF. In 14 patients, ID-Ara-C had to be administered after an inadequate PBSC harvest produced by growth factor alone. All but one patient had successful mobilization of PBSC ($CD34 \geq 2.5 \times 10^6/kg$). Fifty per cent of the cases yielded PBSC with polyclonal IgH gene rearrangements. Six patients have been transplanted so far. In three cases the patients were conditioned with cyclophosphamide plus total body irradiation while melphalan was administered to the remaining three patients. All cases showed rapid hematopoietic engraftment and none experienced major complications.

Other studies

The CALGB recently updated the results of a phase II study using sequential administration of FAMP followed by Campath-1H.⁴⁵ Fifty-seven patients with previously untreated CLL entered the CALGB 19901 study. After assessment of disease extent, patients with active disease received FAMP 25

Table 3. Results in the 17 patients after campath-1H treatment.

Patient	Disease Status		IgH Rearrangement	PBSC CD34+ x10 ⁶ /kg	PBSC IgH Rearrangement
	Before Campath-1H	After Campath-1H			
1	PRN	CR	Polyclonal	3.4 (G-CSF)	Polyclonal
2	PR	CR	Monoclonal	22.9 (ID-Ara-C)	Polyclonal
3	PR	CR	Monoclonal	3.2 (G-CSF)	Monoclonal
4	PRN	PRN	Monoclonal	27.0 (ID-Ara-C)	Polyclonal
5	PRN	CR	Monoclonal	37.1 (ID-Ara-C)	Monoclonal
6	PRN	CR	Monoclonal	18.7 (ID-Ara-C)	Monoclonal
7	CR	CR	Polyclonal	11.3 (ID-Ara-C)	Polyclonal
8	PRN	CR	Monoclonal	11.8 (ID-Ara-C)	Polyclonal
9	PR	CR	Polyclonal	10.0 (ID-Ara-C)	Monoclonal
10	PR	CR	Polyclonal	18.1 (ID-Ara-C)	Polyclonal
11	CR	CR	Monoclonal	62.3 (ID-Ara-C)	Monoclonal
12	CR	CR	Polyclonal	17.9 (ID-Ara-C)	Polyclonal
13	PRN	CR	Monoclonal	-	-
14	PR	PRN	Polyclonal	No Mob. (ID-Ara-C)	-
15	CR	CR	Polyclonal	19.2 (ID-Ara-C)	Monoclonal
16	CR	CR	Polyclonal	24.2 (ID-Ara-C)	Monoclonal
17	CR	CR	Polyclonal	40.5 (ID-Ara-C)	Polyclonal

mg/m²/day × 5 days intravenously, for a total of 4 monthly courses. Patients achieving stable disease (SD) or a better response were treated with a 6-week course of Campath-1H. The monoclonal antibody was given intravenously at a dose of 30 mg three times a week after stepping up the dose during the first week (3 mg for the first dose, increasing, as tolerated, to 10 mg and then to 30 mg).

After the FAMP phase of therapy, three patients (5%) achieved a CR and 28 (50%) a partial response. Thus, data on 39 patients who received alemtuzumab are available. The influence of Campath-1H on patients in PR after FAMP was evaluable in 24 patients: 9 of them improved to CR while 15 remained stable in PR. Out of 12 patients with SD after FAMP, 2 achieved a CR and 7 had a PR following alemtuzumab. Among 39 patients who entered the alemtuzumab phase of therapy, there were 14 CR (36%) and 22 PR (56%) with an overall response rate of 92% (NCI-WG 1996 criteria). Considering all 56 patients (intention to treat population) the incidence of CR and PR was 25% and 40%, respectively.

The preliminary results of a randomized trial conducted by the German CLL study Group have recently been reported.⁴⁶ The primary objective of the study was to compare the treatment effect of alemtuzumab consolidation with that of no further treatment on progression-free survival. Secondary endpoints were to evaluate response according to NCI criteria, presence of minimal residual disease and safety. Twenty-three evaluable patients were recruited into this study.²¹ Patients in remission fol-

lowing first line treatment with FAMP or FAMP+CTX were randomized to treatment with alemtuzumab or no further treatment. Eleven patients were randomized to receive alemtuzumab: the dose was escalated from 3 mg to 10 mg, to the target dose of 30 mg iv over the first 3 days followed by 30 mg, three times weekly, for 12 weeks.

Six months after randomization there was a trend toward improved response rates (CR and PR) in patients who received alemtuzumab therapy. At a median follow-up there was a trend toward longer progression-free survival in the alemtuzumab arm (no progression versus a mean of 21.6 months; $p=0.069$). Significantly more patients converted to molecular remission in the alemtuzumab consolidation arm ($p=0.048$). Unfortunately this study was stopped prematurely because 7/11 patients who received alemtuzumab suffered grade 3 or 4 infections. The authors are currently defining the optimal dose for alemtuzumab consolidation in patients with CLL in remission after FAMP-based chemotherapy.

Conclusions

After showing remarkable activity as single agents, the MoAbs rituximab and alemtuzumab are being increasingly used in combination with other active agents. The combinations have proved to be safe and active. Issues that remain to be resolved include the optimal sequence of combinations (concurrent or sequential) and the question of whether or not antibodies are better suited for use in secondary therapy during remission rather than during

induction. Sound knowledge of MoAb pharmacokinetics should help to generate a more rational design of combination studies with respect to optimal dose and route of administration. Indeed an important issue emerging from our study is the choice of the route of administration of alemtuzumab. The subcutaneous route of administration may be preferable one because adverse reactions are very rare and mild. A major objective in the design of future combination trials must, however, be to analyze the impact of these treatments on clearing minimal residual disease in the hope of eventually formulating a treatment strategy with curative intent for a disease that still remains incurable.

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Clinical pharmacology of the monoclonal antibodies rituximab and campath-1H

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Antibodies or immunoglobulins are glycosylated proteins produced by plasma cells, and specifically by activated B cells which complete the differentiation process.

Most patients treated with murine antibodies develop human anti-murine antibodies (HAMA). HAMA not only neutralize the murine antibody and its therapeutic properties, but may also cause cytotoxic reactions, such as serum sickness, nephrotoxicity and anaphylaxis, as a result of the antibody-protein interaction.^{1,2} In order to obviate these problems, monoclonal antibodies have been developed by genetic engineering.

The resulting chimeric monoclonal antibody differs from the normal antibody in that it possesses the human Fc domain but retains the murine Fab domain. The Fab region may also be attacked by specific antibodies called human anti-chimeric antibodies (HACA) but this kind of immune response is less common.

Thus chimeric antibodies are as specific as murine monoclonal antibodies while the presence of the human Fc fragment decreases incompatibility with the patient's body and favors interactions with cell functions. Indeed the Fc domain acts in synergy with the human mechanisms effecting the immune response.

Rituximab

Rituximab is a genetically engineered chimeric IgG1 kappa monoclonal antibody with human constant and murine variable regions. It specifically recognizes the CD20 antigen,³ a hydrophobic phosphoprotein expressed on mature B cells and most B-cell lymphomas but not on stem cells, pre-B cells or mature normal plasma cells. The CD20 antigen is tightly bound to the cell membrane, with only a small proportion exposed on the cell surface. It is not shed or internalized when bound by rituximab and its cell-surface expression is highly restricted. Rituximab kills CD20⁺ cells via different mechanisms which include complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).⁴ Moreover, it has been demonstrated that rituximab can induce apoptosis by CD20 cross-linking⁵ and increase the sensitivity of B-lymphoma cells to the cytotoxic effects of chemotherapeutic agents and toxins.^{6,7}

In the clinical setting, rituximab was initially investigated in dose ranging studies performed in patients with

relapsed low-grade non-Hodgkin lymphoma (NHL) in whom the dose was escalated from 10 to 500 mg/m².⁸ All patients received the planned dose and no dose-limiting toxicity was identified. Assessment of the dosing regimen used in these studies led to the selection of a dose of 375 mg/m² rituximab administered once weekly for four doses as the standard administration schedule, since it appeared biologically active and well tolerated.⁹

Nevertheless the spectrum of efficacy of rituximab is wide, the diseases treated are biologically heterogeneous, and the schedules and dosages may be quite varied. The optimal use of rituximab should be driven by a biological and pharmacokinetic rationale.

Drug assay

An enzyme-linked immunoassay (ELISA) is used to quantify the serum levels of rituximab.¹⁰

Diluted serum samples are allowed to react with purified polyclonal anti-rituximab antibody coated on a microtiter plate and with anti-human IgG labeled with horseradish peroxidase. After incubation and washings, substrate solution is added and absorbance is read at 492 nm. Rituximab concentrations in samples are determined by interpolation from a standard curve prepared diluting known amounts of rituximab in normal human serum. During analysis of clinical samples, a standard curve plus at least one set of quality control samples is assayed in each run. This method is highly sensitive (2 µg/mL), rapid, accurate and precise.

Clinical pharmacokinetics

The efficacy and safety of rituximab in patients with relapsed or refractory indolent NHL have been clearly documented but some issues remain controversial, such as the most effective dosage schedule, the role of combination therapy, and the possibility of using rituximab application in other malignancies or as a consolidation agent after chemotherapy.

Pharmacokinetic evaluation remains an important tool for the clinician when designing new therapeutic schemes and combinations and for selecting patients who can benefit from more tailored and individualized schedules of administration (i.e. higher dosages or repeated drug administrations).

Previous studies suggested that the pharmacokinetics of rituximab were non-linear at clinically relevant doses due to saturation of elimination mechanisms and to interaction between rituximab and tumor cells. Ritux-

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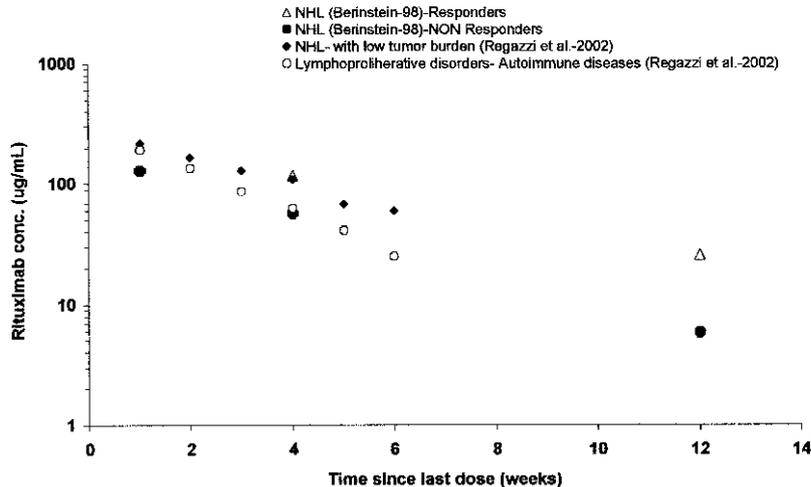


Figure 1. Terminal decay of Rituximab in different patient populations.

imab binding was found on lymphoid cells in the thymus, the white pulp of the spleen, and a majority of B-lymphocytes in peripheral blood and the lymph nodes. Following intravenous administration, rituximab is reproducibly detected in the cerebral spinal fluid at concentrations which are at most 0.1% that of matched serum.¹²

The clinical pharmacokinetic profile of rituximab was extensively studied by Berinstein *et al.*⁸ in a large number (166) of patients with recurrent low grade lymphoma: patients with higher peak and trough levels of circulating free rituximab exhibited higher response rates; the antibody serum concentrations were influenced by histological subtype of lymphoma, by the intensity of CD20 expression on the tumor cells and by tumor bulk. Several authors suggested that upon an initial infusion of the drug, antibody-coated B cells were rapidly depleted from the peripheral blood, lymph nodes and bone marrow, leaving a decreased number of tumor cells available for drug uptake upon subsequent infusions.¹¹

Since tumor cells function as a route of rituximab elimination, the drug clearance during repeated infusions appeared decreased and the half-life significantly prolonged, resulting in drug accumulation.

These suggestions were in agreement with previous observations that serum antibody levels were inversely correlated with baseline lymphocyte counts, as well as with two measures of baseline tumor bulk. Patients with malignancies related to higher tumor burden or high CD20 expression had lower serum rituximab concentrations as well as lower response rates to treatment.

In our experience with patients with a low tumor burden, when the treatment and the post-treatment periods were considered as one course the pharmacokinetic model adopted allowed adequate characterization of rituximab disposition both during and

after therapy, even when the drug administration schedule was different.

The accumulation and its extent are a result of administration frequency (half-life of the drug relative to the dosing interval) and indeed rituximab accumulation was observed during weekly dosing regimens in all reported studies. Because rituximab distribution and elimination appear very long, the extent to which the drug could accumulate in the body after multiple doses is difficult to estimate.

The very long distribution and elimination phases observed suggest that a long time is required for drug distribution into both central and peripheral compartments and for drug elimination (Figure 1). Several factors may be involved.

The form itself of rituximab influences the agent's catabolic rate and biodistribution, since intact monoclonal antibodies have a longer half-life.

Since stem cells supply the pool of depleted B cells, detected rituximab concentrations could also result from a balance between depletion and repletion of these cells. This phenomenon, together with the release of rituximab from coated but not lysed lymph node or B cells may substantially contribute to the disposition profile observed.

Campath-1H

Campath-1H is a humanized immunoglobulin G1 (IgG1) anti-CD52 found on the surface of human lymphocytes.

The CD52 antigen is a lipid-anchored glycoprotein abundantly expressed on lymphocytes. The antigenic epitope recognized by Campath-1H comprises the C-terminal amino acids together with part of the anchor.¹³ Campath-1H has been shown to rapidly deplete peripheral blood B cells and T cells, without affecting stem cells. It binds to the cell membrane of more than 95% normal human blood lymphocytes, as well as of B and T lymphomas, causing lysis

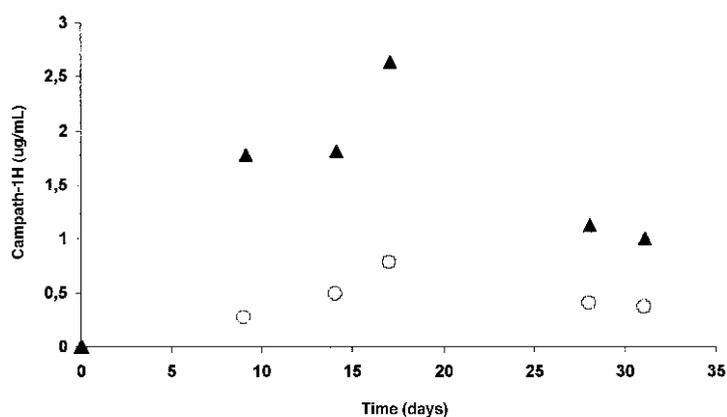


Figure 2. CAMPATH-1H serum levels in a typical patient with CLL, during the first month of treatment with 30 mg (2h-infusion) three times a week. ▲ serum levels just after drug infusion; ○ serum levels just before drug infusion.

by complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).^{14,15}

Campath-1H is being developed for the treatment of chronic lymphocytic leukemia (CLL) and low-grade non-Hodgkin's lymphoma, as an immunosuppressive agent in transplantation and for treatment of autoimmune diseases. It has also been used in stem-cell transplants for the prevention of graft-versus-host disease and graft rejection by depletion of T lymphocytes from both donor and recipient; nevertheless donor lymphocytes might contribute an anti-leukemia effect and lymphocyte depletion may exacerbate problems with immune reconstitution.

Since there is a fine balance between the risks of graft-versus-host disease and host-versus-graft reaction, relapse and infection, Campath-1H serum levels must be monitored during and after treatment.

Drug assay

Measurement of a humanized monoclonal antibody in human serum is challenging because the antibody, typically present at a concentration <20 µg/mL, is so similar to normal human immunoglobulin, whose concentration is approximately 10,000 µg/mL. The assay should measure intact antibody and not breakdown fragments.

Measuring serum levels of Campath-1H is important for optimizing the treatment dosing schedule. Available assays are complicated and difficult to adapt for throughput testing, with most assays being based on capture of Campath-1H using the CD52 antigen.

Campath-1H concentrations are presently measured by an indirect immunofluorescence assay and antibody concentration is calculated by comparison with a standard curve. After serum samples have been incubated with target cells HUT-78 (human T-cell lymphoma) Campath-1H levels are detected by a fluorescent-labeled anti-human IgG using flow

cytometry.

Rebello reported 1:2 serum sample dilution¹⁵ with wash buffer but we adopted 1:8 as being more accurate. Standard solutions are added to the diluted serum. They are then mixed with 50 µL of cell target suspension in a 96-well microtiter plate. Next the Campath-1H–HUT-78 complex is labeled with polyclonal anti-human IgG FITC specific for the Fc domain. The cells are analyzed by flow cytometry on a FACScan instrument. The median fluorescence intensity (MFI) of all cells in this region is plotted versus antibody concentration for standard samples. The quantification limit for Campath-1H concentration is 0.3 µg/mL.

Recently Manshoury *et al.*¹⁶ validated a simple sandwich enzyme linked immunosorbent assay (ELISA) to measure Campath-1H antibody levels. This assay has a 0.1 µg/mL limit of quantification and a CV of ±12%.

Nevertheless the flow cytometry assay has been most frequently used to analyze Campath-1H levels in clinical samples from various trials including the treatment of CLL, bone marrow and kidney transplantation.

Clinical pharmacokinetics

The pharmacokinetic and pharmacodynamic profile of Campath-1H was evaluated in a multicenter trial in NHL and CLL patients. Peak and trough Campath-1H levels in CLL patients increased over the first weeks of therapy and reached the steady-state within week 6.

The increase in serum concentrations corresponded to a marked decrease in the malignant lymphocytosis.

The patients with baseline peripheral lymphocyte counts >30,000/µL had significantly lower peak and trough Campath-1H levels than those with counts <30,000/µL. This suggests that malignant lymphocytosis is a blood compartment in which Campath-1H accumulates: when malignant lymphocytosis

decreases this compartment is eliminated and peak and trough Campath-1H levels increase. Therefore the interindividual variability of Campath-1H pharmacokinetics is related to the tumor burden and its distribution.^{15,16}

Usually Campath-1H is administered as a 2-hour intravenous infusion. The terminal half-life of Campath-1H was approximately 2–3 weeks.^{15,16}

The median plasma levels in CLL patients with residual disease, treated with 10 mg Campath-1H three times a week for 1 month were 0.42 µg/mL (range: 0–1.76 µg/mL) in those with complete remission (CR), while patients not responding to treatment had no detectable plasma Campath-1H. The authors of this study suggested that CLL patients with higher levels of residual disease may require higher doses of Campath-1H in order to eradicate their disease and that detectable plasma levels of Campath-1H may be necessary to achieve CR.¹⁷

Some authors have investigated, as more convenient for long-term treatment, the use of subcutaneous (sc) Campath-1H (three times weekly for 6 weeks, at escalating doses up to 10 mg) for the eradication of residual disease in the bone marrow of patients in clinical remission after fludarabine phosphate treatment. All patients improved to complete remission or partial remission and Campath-1H was able to eliminate residual disease in 47% of patients.¹⁸ Preliminary results have shown that peak serum levels, obtained 1 hour after sc administration, were virtually the same as the Campath-1H levels obtained at the end of the 2-hour infusion; trough serum levels were $\approx 30\%$ the corresponding values observed after intravenous administration (data normalized to the same dosage).

EC Morris *et al.*¹⁹ studied the pharmacokinetics of Campath-1H used for *in vivo* and *in vitro* T-cell depletion in allogeneic transplantation, since the relatively slow clearance of the immunoglobulin could impair immune reconstitution, affect rates of viral reactivation and limit efficacy of the donor T-cell mediated graft-versus-leukemia effect. In the group of patients treated with 100 mg Campath-1H *in vivo* over five days the median peak level was 13.7 µg/mL, occurring 15 min after the final dose. At 28 days, the median level was 1.0 µg/mL. The half-life from 4 to 32 days after the last infusion was 8 days. The Campath-1H concentrations remained at lympholytic concentrations (>0.1 µg/mL) for approximately 56 days post-transplant, 26 days longer than in the patients treated with 20 mg Campath-1H (lower dose) added *in vitro* to the stem cells prior to their re-infusion.

In conclusion, measuring Campath-1H to correlate its serum levels with clinical response and side effects, appears to be the most promising methodology to optimize dosing and scheduling of drug therapy.

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Risk of infections when using new therapeutic approaches for chronic lymphocytic leukemia

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Infectious complications have been known to be a major cause of morbidity and mortality in chronic lymphocytic leukemia (CLL) patients for many years.¹ Indeed, they are the leading cause of death in most series.² Infectious mortality ranges between 30–50%.³

Patients with CLL are predisposed to infections because of both the humoral immunodepression inherent to the hematologic disease, which is related to stage and duration of CLL, and further immunosuppression related to therapy with steroids and cytotoxic drugs.

The majority of infections in CLL are of bacterial origin and involve the respiratory system, pneumonia being the most frequent severe complication. Encapsulated bacteria (*Streptococcus pneumoniae*, *Haemophilus influenzae*) are predominant, but in the neutropenic post-chemotherapy phase *Staphylococcus aureus* can also be responsible for bacteremia and septicemia, especially in patients with hypogammaglobulinemia. *Herpes simplex* and *zoster* are the usual viruses found, particularly in advanced disease.⁴

The immunodeficiency and natural infectious history of alkylator-resistant, corticosteroid-treated patients appears to have changed with the introduction of fludarabine.⁵ Fludarabine has emerged as a salvage therapy for chlorambucil-resistant CLL, but the encouraging results have been complicated by severe and unusual infections due to sustained reduction of CD4-positive T lymphocytes.⁶ Furthermore a synergistic immunosuppressive role between concomitant fludarabine and corticosteroids has also been hypothesized.⁷ The introduction of monoclonal antibody therapies, which are usually given for refractory and relapsed disease, often in patients previously treated with fludarabine, can further increase the immunodepression; fungal and also atypical viral infections have been reported in these patients.

Pathogenesis of infection

The pathogenesis of infection in CLL is multifactorial, with both alterations due to primary disease process and immunosuppression by subsequent treatments; however, hypogammaglobulinemia, in particular, is predictive of an increased frequency of infection.

Hypogammaglobulinemia

The prevalence of hypogammaglobulinemia in CLL patients varies from 10 to 100% and is related to the duration and stage of the disease.⁸ Usually hypogammaglobulinemia is not reversible even if complete remission is obtained.⁹ A few cases of normalization have been described in patients who are complete responders to fludarabine treatment.¹⁰

Low levels of immunoglobulins have been associated with the frequency of infections in these patients, suggesting that the control of CLL also reduces the severity of infections. Different studies have shown that low immunoglobulins levels are related to infections.¹¹

CLL patients with hypogammaglobulinemia usually show bacterial infections, which are often recurrent, just as in patients with primary hypogammaglobulinemia. The most frequently isolated bacteria are *Streptococcus pneumoniae* and *Haemophilus influenzae*. Despite numerous reports correlating hypogammaglobulinemia and infection in CLL, the relationship between the level of a specific immunoglobulin class and the risk of infection is not well-established. Rozman *et al.*,¹² studying the natural history of a cohort of 247 patients with CLL, revealed that the survival in patients with immunoglobulins levels < 700 mg/dL at diagnosis was shorter than that of patients with levels > 700/dL; specifically, low levels of serum IgG and IgA, but not IgM, influenced survival. Rozman hypothesized that a predominant IgA deficiency is a prognostic factor for increased frequency of respiratory tract infections as it occurs in carriers of selective IgA deficiency. It is must not, however, be forgotten that patients with hypogammaglobulinemia may not have infections and that, contrarily, patients with CLL and normal Ig levels can be subject to recurrent infections; in fact, as well as, low Ig levels, it is important that B lymphocytes are able to form a specific response¹³ which is always defective in these patients.¹⁴ Recurrent bacterial infections from *Streptococcus* and *Haemophilus* are particularly associated with low levels of IgG.¹⁵

Cell-mediated immunity

Cell-mediated immunity is altered in CLL patients, but from the literature is difficult to understand whether this is a primary defect, strictly related to the hematologic disease, or whether it is always chemotherapy-induced.¹⁶ Although the absolute T-lymphocyte count is usually normal, reduced T-cell colony forming capacity, an increase in the percentage of T suppressor lymphocytes

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with a reduction of T-helpers and a decrease of CD4/CD8 ratio are seen. Defects in natural killer (NK) cell activity have also been reported.¹⁷ In addition, CLL patients have been found to have a poor delayed hypersensitivity response to a variety of skin tests antigens such as *Candida albicans*, mumps, and diphtheria toxoid.¹³

Neutropenia, neutrophil function and complement

The absolute neutrophil count and neutrophil function are usually normal in untreated CLL patients; neutropenia becomes pathogenetically more important in advanced disease and with more intensive chemotherapy utilization, predisposing patients to bacterial and fungal infections.

Enzyme deficiency (β -glucuronidase, lysozyme, myeloperoxidase) was found in neutrophils of some, but not all, patients with CLL.¹⁸ These defects may resolve with hematologic remission.

Heath *et al.*¹⁹ found reduced serum complement levels in CLL patients. Complement plays a crucial role in the control of some bacterial infections; opsonization with complement is necessary for subsequent interactions with neutrophils. A lower level of C3b fraction of complement was found in the sera of patients with a history of bacterial infections than in the sera of patients who had no prior infections. On the other hand, no relationship was demonstrated between complement levels and previous infection in another series of patients.²⁰

Other risk factors

Duration of disease

The risk of infection usually increases with the duration of disease, in relation to the natural history of the disease itself and to previous therapies undertaken; both these factors can reduce immunoglobulins level. The risk of a severe infection is 26% at 5 years, but increases to 57% in patients with profound hypogammaglobulinemia, and to 68% in patients with profound hypogammaglobulinemia and Binet stage C of disease.³

Advanced stage of disease

There is a significant correlation between the stage of disease and infections; infectious episodes are not only more frequent,²¹ but also more severe in patients in stage C (82%) rather than stage A (33%).⁸

Patients with stage C disease are a selected group predisposed to infections; the severe defect of immune response plays a critical role in infectious morbidity and mortality.

Infectious complications during conventional chemotherapy

Infections are the major cause of death in CLL patients. In a large study at the M.D. Anderson Can-

cer Center, 30% of patients died because of infection;²² these data were confirmed by other authors.²³ These retrospective studies were performed to verify different drugs combinations and not to investigate the origin of infections and the cause of death; the reported data often do not allow correct evaluation of the infectious episodes or any correlation with hypogammaglobulinemia, neutropenia, previous treatments and stage of CLL; moreover, the data are derived from selected populations with advanced or refractory disease, typical of third level centers, to which more complicated cases are admitted. Despite these limitations, it is evident that the incidence of infections in CLL patients is higher than in the general population, and it develops parallel to the progression of the haematologic disease.

With regard to the main sites of infections, pneumonia is more severe and frequent, particularly in advanced disease; bacteriemia and septicemia are common in neutropenic patients. In a prospective surveillance study the rate of nosocomial bacteriemia in CLL patients was 9.4% and more than 50% of cases of bacteriemia were present in patients with neutrophils $< 0.1 \times 10^9/L$.²⁴

Other common sites of infection include all the respiratory tract, the urinary tract, skin and soft tissue. Encapsulated bacteria (*Streptococcus pneumoniae*, *Haemophilus influenzae*) are the predominant pathogens in patients with CLL, but *Staphylococcus aureus* and various Gram-negative enteric pathogens, such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* are also frequently seen in patients with hypogammaglobulinemia.^{21,25}

Mycoses, in particular cryptococcal meningitis (2.4%) and disseminated histoplasmosis have been reported in CLL patients.^{26,27} Other reports describe sporadic cases of *Candida* and *Aspergillus* infections, which may be related to therapies resulting in more prolonged periods of neutropenia, in patients with advanced disease.

Viral infections are much less common than bacterial infections in CLL patients treated with conventional drugs, and usually affect patients with advanced stages who are receiving chemotherapy. These viral infections do not seem to be a significant cause of death, even if related morbidity can be high.²⁸ Finally, mycobacterial infections (*Mycobacterium tuberculosis* or atypical mycobacteria) have been reported rarely, especially in patients with a previous history of mycobacterial infection; the use of steroids scheduled in chemotherapy cycles or prescribed for autoimmune episodes (immune hemolytic anemia and/or thrombocytopenia), is an important risk factor of this infection. The prevalence of mycobacterial infection in CLL patients was 88 cases/10,000 patients.²⁹ Very sporadic cases of *Pneumocystis carinii* infections have been reported.³⁰

Table 1. Infection prophylaxis in CLL patients treated with innovative therapies.

<p><i>RISK FACTORS</i></p> <p>Binet stage B and C Previous chemotherapy Neutropenia Renal dysfunction Minor or no response to fludarabine CD4 count <200 cells/mL Age > 65 Ig titer < 400 mg /dL</p> <p style="text-align: center;">↓</p> <p><i>PROPHYLAXIS</i></p> <p><i>Pneumocystis carinii</i>: Trimethoprim- sulfamethoxazole (particularly in steroid therapy) One tablet 3 times a week</p> <p><i>Fungi</i>: Fluconazole or itraconazole 400 mg daily (if colonized) <i>Herpes</i>: acyclovir 400 mg twice daily (800 mg twice daily if previous severe infection)</p> <p><i>Ig replacement</i>: only patients with recurrent and severe bacterial infections caused by <i>Staphylococcus</i>, <i>Streptococcus pneumoniae</i> and <i>Haemophilus influenzae</i> 250 mg/kg every four weeks</p>

Infectious complications during purine analogs treatments

Until the 1980s the appearance of resistance to alkylators heralded evolution of the disease because of the lack of efficient salvage regimens. In the 1990s the introduction of purine analogs produced promising results, but was accompanied by a different spectrum of infections because of selective T-cell abnormalities which these agents cause.

Fludarabine is very active against indolent lymphoid neoplasms; its efficacy is due to its ability to reduce the number of lymphoid cells rapidly, even giving rise to tumor lysis syndrome in some cases.

Profound and prolonged suppression of the CD4 count occurs, with median CD4 counts decreasing <200/mL in 2-3 months of therapy. Although the CD4 count improves in the first 3 months after the completion of treatment, quantitative abnormalities may persist for 1-2 years. This immunosuppression and neutropenia secondary to therapy caused an increased number of infections, particularly by opportunistic pathogens, also in the absence of neutropenia or steroid therapy. Despite the reduced CD4 count the hematologic and clinical response to purine analogs increases macrophage cell activity and often also hypogammaglobulinemia; for this

reason infections are more frequent at the beginning of the disease and decrease as the CLL improves.

In a review of the literature³¹ related to fludarabine-associated opportunistic infections, in which 2,269 patients with low-grade malignancies who received fludarabine therapy were evaluated, the most notable infectious complications were respiratory tract infections and unexplained fever; 3.2% of these patients developed opportunistic infections during or after fludarabine treatment; 97% of these infections occurred in patients who had been previously treated with alkylating agents or corticosteroids. Opportunistic infections were due to *Pneumocystis carinii* (33%), mycoses (30%), *Listeria monocytogenes* (14%), also after many months from completion of therapy, mycobacteria (9%), CMV (7%), and herpes (6%); a very high incidence of localized *Varicella zoster* was also noted in several studies, particularly in patients with a CD4 count less than 50 cells/mL.³² The majority of these infections did not appear to be related to neutropenia or low levels of immunoglobulins. The high incidence of opportunistic infections (7-28%) in patients previously treated with alkylating agents or steroids reported by some authors^{31,33-36} suggests that the

utilization of steroids before and after fludarabine treatment increases immunosuppression, also increasing opportunistic infections. In fact, opportunistic infections seem be less frequent in other salvage chemotherapy cycles containing steroids, in which only 1.5% of patients had opportunistic infections.³⁷⁻³⁹

Finally, the risk of infections during fludarabine therapy increases with the use of higher than recommended doses, or when different purine analogs are given in association or sequentially.^{40,41}

Infectious episodes related to the use of monoclonal antibodies

Monoclonal antibodies are a desirable therapy because, unlike chemotherapeutic agents, they are specific for a tumor-associated target. The characteristic phenotype of CLL cells distinguishes them from leukemic forms of other lymphoid malignancies and provides excellent targets for the use of monoclonal antibody therapy.

Alemtuzumab (campath-1H)

The profound and long-lasting lymphocytolytic activity of campath-1H is responsible for severe and prolonged immunodepression which produces a major predisposition to infectious complications. The importance of correct prophylaxis of infective events appeared early in studies of campath-1H and this is evident if the results of the first studies are compared with those of the following studies, which also included prophylaxis for *Pneumocystis carinii* and *Herpes*.

In 1997 Bowden⁴² published a report of 7 cases of resistant/relapsed CLL in patients treated with subcutaneous campath-1H, in which the most important side-effect was reactivation of CMV infection seen in 3 patients during the period of lymphopenia. Two of these patients with febrile illness required i.v. ganciclovir; the third had subclinical reactivation.

Likewise, in the phase II study by Osterborg *et al.*⁴³ of 29 previously treated patients with advanced CLL the main side-effects of Campath-1H treatment were infections, which were related to long-lasting lymphocytopenia. No prophylaxis was given. Localized *herpes simplex* virus reactivation was noted in 11 patients and oral candidiasis in five. *Pneumocystis carinii* pneumonia was diagnosed in two patients. Four patients had bacterial pneumonia and septicemia was diagnosed in 3 cases and suspected in one. All patients recovered.

The pivotal trial included 93 patients treated at 21 centers in USA and Europe⁴⁴ who had experienced treatment failure from previous therapy with fludarabine and who had also received alkylating agents. Infections occurred in 51 (55%) patients during the study. These infections were mild to moderate in 26 cases and grade III to IV in 25 cases.

Septicemia occurred in 14 patients (15%), and two cases led to death. Herpes simplex was present in 6 cases. *Cytomegalovirus* reactivation in 7 patients caused concern. A total of 11 patients, all of whom with advanced disease, developed opportunistic infections during treatment and a further 7 did so in the follow-up period (1 *Pneumocystis carinii* pneumonia, 3 aspergillosis, 1 mucormycosis, 1 cryptococcal pneumonia, 1 *Listeria meningitis*, 4 *Herpes zoster* and 7 CMV reactivation). Six of 9 deaths were due to infections. Responders seemed to experience fewer infections than non-responders.

In a compassionate-use protocol⁴⁵ which included 152 heavily pretreated fludarabine-refractory patients, CMV infection occurred in 4 patients (1.8%) and was fatal in one patient. Five patients died from infection (4 from pneumonia, 1 from gas gangrene).

Other studies in refractory /relapsed CLL patients have shown similar results with an increment of CMV reactivation and opportunistic infections.^{46,47}

Recently,⁴⁸ the incidence of CMV viremia during alemtuzumab therapy was evaluated in patients with relapsed/refractory disease receiving famciclovir prophylaxis (250 mg *PO bid* or equivalent); despite this prophylaxis, 15% of cases had CMV viremia at the median of 28 days after the first dose of campath-1H, but there was no clinical evidence of CMV organ disease. Ganciclovir treatment was produced prompt resolution of fever. By univariate regression analysis the following were not risk factors for CMV viremia: age, number of prior regimens, prior rituximab therapy, prior splenectomy, modified Rai stage, and number of neutrophils and lymphocytes, although there was a trend towards significance for prior rituximab therapy ($p=0.07$).

The frequency of CMV pneumonia following alemtuzumab treatment was determined by retrospectively evaluating pools of safety data to clarify the overall incidence of CMV complications following alemtuzumab therapy in 1538 patients with lymphoid malignancies.⁴⁹ The incidence of symptomatic CMV infection and/or reactivation was 3.6%. Nine (0.6%) patients had CMV pneumonia with 3 deaths. In these studies specific prophylactic therapy was not given.

Recently, Lundin *et al.*⁵⁰ reported on their experience of 41 patients with previously untreated CLL with advanced disease requiring treatment, who received alemtuzumab by the subcutaneous route. Infections were rare, but 10% of the patients developed CMV reactivation. These patients rapidly responded to i.v. ganciclovir. One patient, allergic to cotrimoxazole prophylaxis, developed *Pneumocystis carinii* pneumonia.

Montillo *et al.* found that the percentage of CMV reactivation was increased when alemtuzumab was given after fludarabine⁵¹ as sequential treatment in first-line therapy. All patients were monitored for

CMV reactivation weekly, during treatment and for six weeks after drug discontinuation. Three patients demonstrated CMV reactivation (20%) at the end of treatment without developing CMV disease; oral gancyclovir was administered in 2/3 cases, in one because of the presence of symptoms (fever, nausea) and in the other because of the elevated number of positive cells.

In conclusion, CMV infection should be suspected during febrile episodes in patients receiving cam-path-1H. Prompt therapy of confirmed or suspected CMV infections is crucially important to cure these patients.

Rituximab

Rituximab has been rapidly adopted by physicians for a wide range of B-cell malignancies because of its activity, its moderate side-effects and because of the possibility of using it in combination with chemotherapy. Alternative doses and schedules of administration have been explored in an attempt to improve the response rate in CLL patients. Rituximab as first-line treatment circumvents complications associated with myelosuppression and immunosuppression;⁵² of forty-four evaluable CLL patients who received single-agent rituximab for induction and maintenance treatment, only 3 experienced grade 3 infections (*Staphylococcus aureus* pneumonia, one patient; localized *Herpes zoster*, one patient; gastroenteritis, probably viral, one patient) and seven other patients had minor (grade 2) infections; no patients developed opportunistic infections while responding to rituximab.

A different therapeutic strategy was explored by the CALGB,⁵³ which conducted a randomized phase II trial of concurrent versus sequential fludarabine and rituximab with 51 and 53 patients per arm, respectively. Infections, predominantly mucocutaneous, occurred commonly in both regimens throughout therapy, with a similar overall frequency. Opportunistic infections were diagnosed in 8 patients (2 *Herpes zoster*, 3 *herpes simplex*, 1 case each of influenza A and Echo virus, 1 *Pneumocystis carinii* pneumonia) in the concurrent treatment arm and in 14 patients (2 *Herpes zoster*, 7 *Herpes simplex*, and 1 case each of Influenza A, CMV pneumonia, and *Pneumocystis carinii* pneumonia) in the sequential treatment arm. The majority of these opportunistic infections were viral in origin and often localized. On the basis of the infectious data derived from this trial the authors advise only preventive strategies against *Herpes virus* infections.

In a phase II trial⁵⁴ to evaluate the efficacy and safety of rituximab in pretreated CLL patients, infections occurred in 7 (24%) of 29 patients; five were grade 3-4 infections and consisted of sinusitis, pneumonia, generalized *Herpes zoster*, subcutaneous suppurative infection of the arm, septicemia resistant to antibiotic treatment and, later, a fatal

pulmonary aspergillosis.

In a phase I trial⁵⁵ in which rituximab dose was escalated up to 2,250 mg/m² myelosuppression was uncommon, and severe neutropenia (<0.5×10⁹/L) was seen in 11% of patients. Sepsis or fever of unknown origin (FUO) occurred in 10% of cases (*Escherichia coli* urinary tract, one patient; FUO, three neutropenic patients; FUO, one patient). Minor infections were seen in 10% of patients and included bronchitis, gastroenteritis, and urinary tract infections.

In conclusion, rituximab is associated with markedly reduced hematologic events such as severe neutropenia, as well as fewer associated infections. The incidence of infections was higher in patients who had received previous treatments; opportunistic infections were above all of viral origin, while other opportunistic infections were diagnosed only sporadically.

Rituximab + alemtuzumab

The combination of the two antibodies, rituximab plus alemtuzumab, has been studied by several groups.^{56,57} In the M.D. Anderson series,⁵⁷ of the 47 evaluable patients, all receiving antineoplastic prophylaxis with cotrimoxazole and valacyclovir, 25 (52%) experienced at least 1 infectious episode; fever of unknown origin was seen in 6 patients (13%), pneumonia in 5 cases (10%), and CMV reactivation without disease was seen in 13 cases (27%).

Prophylactic strategies

The prevention of infections is very important in CLL patients who are treated with innovative therapies.

- The data in the literature do not support a preventive use of high dose intravenous immunoglobulins, which should be given only in cases of severe, recurrent bacterial infections due to encapsulated bacteria;⁵⁸ in a randomized study, low doses of Ig (250 mg/kg every four weeks) were equivalent to higher doses (500 mg/kg) in protecting from severe bacterial infections.⁵⁹
- Prophylaxis of *Pneumocystis carinii* pneumonia in pretreated patients receiving fludarabine and/or alemtuzumab is current policy, even though there are no placebo-controlled studies that support this policy in this population of patients. As in HIV patients with a CD4 count less than 200 cells/mL sulfamethoxazole-trimethoprim (one double-strength tablet three times a week) became the prophylaxis of choice in these patients. The prophylaxis is given until at least 2 months after discontinuation of fludarabine treatment⁴ and for at least 6 months after completion of alemtuzumab treatment. Sulfamethoxazole-trimethoprim is also effective against listeriosis and other bacterial infections;

- it is inexpensive, relatively well tolerated and suitable for long-term use.³²
- Acyclovir is active against Herpes viruses and is usually performed as prophylaxis in patients treated with fludarabine and /or monoclonal antibodies (400 mg twice daily), particularly in patients who have had previous herpetic infections (800 mg twice daily).
 - Antifungal prophylaxis should be considered in this setting only if mucositis and fungal colonization are present, or during prolonged neutropenic episodes. *Aspergillus spp.* have been reported in patients treated with both fludarabine and alemtuzumab.⁶⁰ With the exception of nursing patients in HEPA rooms, there is no established prophylaxis against aspergillosis. Itraconazole (400 mg daily) may be the drug of choice if colonization is present because of its efficacy against both *Candida* and *Aspergillus*.
 - Reactivation of CMV should be suspected and carefully monitored during and after alemtuzumab treatment; only symptomatic infections should be treated.
 - Considering that only small series or sporadic cases of mycobacterial infections have been described and the historical frequency of cases is 8.8 per 1000 CLL patients,²⁹ prophylaxis against mycobacterial organisms should be considered only if previous history of this infection is present.
 - The suboptimal response to vaccination noted in CLL patients may be related to impaired antibody production. Anti-pneumococcal vaccination should be recommended, even in the presence of a reduced response.⁶¹ The response of CLL patients to influenza vaccine has also been studied. In a small series of CLL patients⁶² mean antibody titers after influenza vaccination were low, but sufficient especially in the early stages of disease. Reimmunization with influenza vaccine at 1 month could be necessary because of a decrease in antibody titers.

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Graft-versus-leukemia effect after reduced-intensity conditioning and allogeneic stem cell transplantation in patients with chronic lymphocytic leukemia

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Chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia in western countries where it accounts for 20–40% of all leukemias. The median age at diagnosis is close to 70 years. The course of the disease is heterogeneous: some patients have a survival which is not affected by the disease, whereas others die shortly after diagnosis. In order to understand these different behaviors better, new prognostic factors were investigated in the hope that these would identify a proportion of young patients with poor-risk disease who could benefit from intensive treatments. Thus, in association with traditionally accepted factors such as advanced disease stage and short lymphocyte doubling time, new risk factors have assumed clinical relevance: unfavorable cytogenetics,¹ mutational status of the variable region of the immunoglobulin heavy-chain genes (IgH),² and expression of CD38 or protein ZAP-70 on the leukemic cells.^{3,4} Despite a median survival of about 10 years, CLL remains incurable with standard treatments and most patients die of their disease; however about one third of the patients are under the age of 60 and 10–15% are younger than 50 years. The aim of treatment cannot be palliative in these patients, so young patients with advanced stage disease and one or more unfavorable prognostic factors and patients who have relapsed after conventional therapy (with purine analogs) can be considered eligible for autologous or allogeneic stem cell transplantation programs.

The studies performed on high dose chemotherapy and autologous stem cell transplantation demonstrated a low *transplant-related mortality* (TRM), but survival curves did not reach a plateau in the long term and patients relapsed even after 5 years of remission.^{5–8} Evaluation of minimal residual disease (MRD) by polymerase chain reaction (PCR) analysis of the rearrangement of the immunoglobulin heavy-chain gene is a powerful indicator of the relapse risk: persistence of MRD after autologous transplantation or the switch from a negative to a positive MRD status during the follow-up after autologous bone marrow transplantation are highly predictive for clinical relapse.^{9,10} Allogeneic stem cell transplantation (allo-SCT) has proven to be more effective than autografting; in fact different studies reported a low relapse rate, and the curves

of disease free-survival reached a plateau after 3 years.^{11–14} The superior tumor control provided by allografting suggests that CLL cells are susceptible to a graft-versus-leukemia effect (GVL). Several lines of evidence for the presence of GVL activity come from the response to withdrawal of cyclosporine, to donor lymphocyte infusions or to the onset of chronic graft-versus-host disease (GVHD).^{15,16} MRD positivity after allogeneic transplantation has a different significance than positivity after autologous transplant: in fact while persistence of MRD after autografting usually heralds disease recurrence, the detection of MRD after allogeneic transplantation does not necessarily predict clinical relapse. Some authors have reported delayed responses or persistently low level of MRD even 1 year after allografting, supporting the notion that the slow clearance of MRD is a demonstration of the immunologic control of the disease.^{17,18}

In spite of this, the overall survival (OS) after allogeneic transplantation is reported to be only 45–60% at 3–4 years post-transplant because of the substantial treatment-related toxicity when standard myeloablative regimens are used. In fact a recent update of the *European Group for Blood and Marrow Transplantation* (EBMT) database including 209 allografted patients showed a 3-year TRM of 40%.¹⁹ The patient's age, extensive pretreatment and CLL-associated incompetence of the immune system may be factors responsible for the high TRM. Thus, since the graft-versus-CLL effect seems to be crucial for the eradication of the disease, the intensity of the conditioning regimen may not be as important as in other diseases, particularly in patients with a chemosensitive disease. Allogeneic transplants using reduced intensity (non-myeloablative) conditioning regimens have been investigated in CLL. In this approach, the preparative regimen (including, in most cases, fludarabine along with melphalan, or low dose total body irradiation (TBI), or cyclophosphamide or campath-1H) is not aimed at eradicating the disease, but at providing sufficient immunosuppression to allow engraftment of allogeneic stem cells and the development of a graft-versus-tumor effect. The aim is to decrease TRM and to eliminate the leukemic cells through the graft-versus-tumor effect.²⁰

Several groups have exploited allogeneic transplantation after reduced intensity conditioning (RIC) in CLL. Khouri *et al.* demonstrated that successful allogeneic transplantation can be done in patients with lymphoid malignancies without prior myeloablation.²¹ He report-

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ed data on 8 CLL patients included in a group of 15 patients affected by lymphoproliferative disorders; the median age was 55 years (range 45-71). The reduced intensity conditioning regimen consisted of fludarabine and cyclophosphamide or fludarabine, cisplatin and cytarabine for patients affected by Richter's syndrome or intermediate grade lymphoma. Engraftment took place in 11 patients, of whom 8 achieved complete remission; TRM at 100 days was 6%. This study demonstrated the feasibility of RIC transplantation for lymphoproliferative disorders. The main problem was the high incidence of GVHD because the first patients did not receive GVHD prophylaxis in order to increase the GVL effect.^{22,23} The MD Anderson group also compared RIC with fludarabine/cyclophosphamide and myeloablative allo-SCT with Cy/TBI in CLL patients with similar prognostic factors (including resistance to fludarabine) but of different ages (57 vs 43 years). Toxicity was reduced with RIC but there was no difference in overall survival at 3 years (53% vs 48%). Patients transplanted earlier during the course of their disease, while chemosensitive and with no sign of transformation, appeared to have a better outcome (disease-free survival 64% vs 22% for chemosensitive and chemorefractory disease, respectively).²⁴

More evidence of the role of a graft-versus-tumor effect in CLL came from the studies of the Seattle group demonstrating the possibility of obtaining responses in patients allografted with minimal conditioning.²⁵ In fact, on the basis of results from canine models, McSweeney *et al.* studied the minimal dose of total body irradiation, that in association with mycophenolate mofetil and cyclosporine, allowed the engraftment while reducing the toxicity of conditioning regimen. Forty-five patients (median age 56 years) with HLA identical sibling donors, not eligible for myeloablative allo-SCT were treated. Among these 45 patients, 8 were affected by CLL; most of these were chemorefractory and had been previously treated with fludarabine. Three of these patients achieved complete remission, and two became PCR negative between 5 and 12 months post-transplant. Molecular remissions occurred while patients were still receiving the immunosuppressive treatment for GVHD, indicating that powerful GVT responses were generated.

Based upon the hypothesis that the slow kinetics of tumor cell growth in CLL would allow the graft to exert an antileukemic effect, Scheteling *et al.* treated 30 patients with a RIC regimen containing fludarabine, busulfan and antithymocyte globulin. Engraftment was successful in all patients; acute GVHD grade II to IV was observed in 17 patients and chronic GVHD was observed in 21 patients. Twelve patients achieved a complete remission; MRD was monitored in 8 patients who

reached complete remission and all of them achieved the molecular remission. The observation of late complete and molecular remission confirmed the evidence of a graft-versus-leukemia effect in advanced CLL and the feasibility of RIC was confirmed by the low non relapse mortality (15% at 2 years).²⁶

Finally a recent report from the EBMT described the outcome of 77 patients treated with RIC. Moderate conditioning regimens (low dose TBI or fludarabine-cyclophosphamide combinations) were administered to 56% of the patients, whereas 44% received more intense conditioning consisting of fludarabine-busulfan or high dose melphalan combinations. In 40% of the patients, *in vivo* T-cell depletion was performed with antithymocyte globulin or alemtuzumab. The incidence of GVHD grade II-IV was 34%; the estimated risk of chronic GVHD was 58%; it is worthy of note that all patients developing chronic GVHD achieved complete remission. The use of DLI produced durable remissions, with a median follow-up of 8 months. The estimated risk of TRM at one year was 18%. Event-free and overall survival rates at 24 months were 56% and 72%, respectively.²⁰

In our preliminary experience on 45 patients affected by advanced hematologic malignancies, we demonstrated the feasibility of RIC allo-SCT. The median age of patients was 49 years; 18 patients were chemorefractory and 26 had previously failed to benefit from an autologous transplantation. The conditioning regimen consisted of thiopeta 10 mg/kg, fludarabine 60 mg/m² and cyclophosphamide 60 mg/kg; GVHD prophylaxis was based on cyclosporine and short course methotrexate. Engraftment was successful in all patients; the TRM was 13% at a median follow-up of 10 months. Twenty-three patients achieved complete remission and 10 patients obtained molecular remission.²⁷ So far, we have transplanted 15 CLL patients with this RIC regimen. The median age was 56 years; 5 patients were chemorefractory and 8 in partial remission before the transplant; the median number of previous treatments was two, and 3 patients had previously failed to benefit from an autologous transplant. The median numbers of infused CD34⁺ cells/kg and CD3⁺ cells/kg were 5.9×10⁶ and 3.1×10⁸, respectively. All patients engrafted: the median time to 0.5×10⁹/L neutrophils was 12 days; the median time to 20×10⁹/L platelets was 13.5 days. At a median follow-up of 450 days, there were 8 complete remission and 5 deaths, of which 3 were transplant-related (GVHD and infections). Acute GVHD was observed in 8 patients; 2 of 13 evaluable patients developed chronic GVHD, one of which after DLI. Nine patients had a molecular marker and 6 of them had molecular follow-up: at a median follow up of 450 days 4 of 6 patients are in durable molecular remission.

In conclusion, RIC allo-SCT may be considered a promising treatment option for patients with high-risk CLL. The TRM is lower than that associated with myeloablative allografting. Studies are ongoing to evaluate the use of partial *in vivo* T-cell depletion with alemtuzumab in order to decrease the incidence of GVHD.^{28,29}

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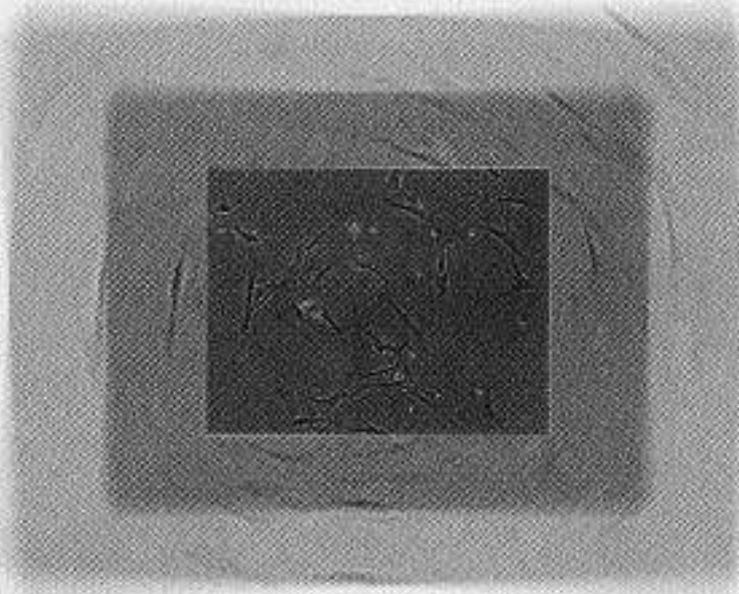


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