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Impact of Molecular Biology on Our Understanding of Non-Hodgkin Lymphoma

M. Brada

INTRODUCTION

NON-HODGKIN lymphoma (NHL) is a clonal expansion of B or T lymphocytes during various stages of differentiation. A number of non-random chromosomal rearrangements and translocations have long been recognised, the most frequent of which are t(8;14) and t(14;18). Molecular analyses of chromosomal rearrangements have identified genes adjacent to the breakpoint sites which are deregulated and are considered to play an important role in oncogenesis. The majority of translocations identified also involve immunoglobulin genes in B-cell and T-cell receptor genes in T-cell neoplasms. Intensive research effort is directed at molecular studies of these and other less frequent chromosomal alterations with the aim of defining mechanisms and detecting new genes involved in oncogenesis. For the clinician the increased understanding of the individual steps of oncogenesis and their regulation provides a potential target for therapeutic intervention. The maturation of lymphoid cells is accompanied by rearrangement of immunoglobulin (Ig) and T-cell receptor (TCR) genes as part of the normal mechanism of generating Ig and TCR diversity. Specific rearrangements and translocations can be exploited as clonal markers in diagnosis and monitoring of disease.

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MOLECULAR EVENTS

Immunoglobulin and TCR genes

The somatic rearrangement of Ig and TCR is a mechanism for generating antibody and TCR diversity [1]. Rearrangement of Ig gene occurs early in lymphoid maturation and follows a defined sequence of recombination steps of initial joining of Diversity (D) and Joining (JH) genes followed by VHDJH joining (VH = variable region gene). Heavy chain rearrangement is followed by κ and λ light chain recombination (for review see Alt *et al.* [2, 3]). The rearrangement of heavy chain and light chain genes is specific for each B-cell and its clone. However,

mature B-cells can undergo further somatic recombination and mutation [4–6]. The rearrangement of TCR genes follows a similar sequence [7, 8] and can also be used as a clonal marker. During the maturation of T lymphocytes, TCR γ chain rearrangement and δ chain recombination (or deletion) precede β and α chain rearrangement [9, 10]. These normal clonal rearrangements have no known role in oncogenesis.

myc gene translocation

Endemic and most cases of sporadic Burkitt's lymphoma (BL) are associated with t(8;14) where the c-*myc* gene is translocated from its site on the long arm of chromosome 8 (band q24) to the derivative chromosome 14 adjacent to the Ig heavy chain gene [11–13]. In the less frequent translocations of t(2;8) and t(8;22) the c-*myc* gene is brought to proximity of κ or λ Ig light chain genes on chromosomes 2 and 22 [14]. The t(8;14) translocation has been most intensively studied and results in constitutive expression of c-*myc*. Although the individual steps are not fully defined and may involve the control mechanisms of transcription as well as increased mRNA stability, the final result is an increase of *myc* protein which may stimulate proliferation (for review see McKeithan [13]).

The product of the c-*myc* gene is a nuclear binding protein which plays a role in the regulation of cell proliferation. C-*myc* mRNA is increased when resting (G_o) cells enter cycling phase [15] and the protein is involved in the stimulation of DNA synthesis [16]. The oncogenic potential of c-*myc* has been demonstrated in animal studies. Transfection of activated c-*myc* gene into human Epstein–Barr virus (EBV) infected lymphoblasts causes tumour when cells are injected into mice [17]. Transgenic mice with *myc* subjected to an enhancer control develop active lymphoid proliferation and B-cell tumours [18, 19].

t(14;18) *and bcl-2 gene*

The translocation t(14;18) is present in the majority of low grade nodular lymphomas and in 20–30% of high grade lymphomas. It involves Ig heavy chain genes on chromosome 14 and in the majority of cases the *bcl-2* gene on chromosome 18. The translocations cluster in two short breakpoint regions on chromosome 18. A major breakpoint region (mbr) lies within the untranslated region of *bcl-2* exon. A small proportion of translocations occur within the minor cluster region (mcr) which lies 3′ of the *bcl-2* gene. On chromosome 14 the translocation is within the J region of Ig heavy chain [20]. The translocation is thought to be mediated by an Ig recombinase which recognises heptamer-spacer-nonamer sequences involved in recombinase action of Ig and TCR gene rearrangement [21]. Further studies of the detailed control mechanisms of recombinase activity are eagerly awaited to explain the inappropriate action of this enzyme.

The result of t(14;18) is an increase in the expression of a *bcl-2* protein, which is a mitochondrial membrane associated protein [22, 23]. Insertion of *bcl-2* protein into the pre-B cell line has been demonstrated to prolong survival of these cells independent of growth factors [24]. In transgenic mice the presence of *bcl-2* with Ig enhancer is associated with follicular hyperplasia. Occasional tumours may arise which contain c-*myc* rearrangement [25]. It therefore appears that the presence of increased amounts of *bcl-2* protein confers survival advantage as well as prolonging the survival of B-cells. It requires activation of other oncogenes such as c-*myc* for the development of tumour.

Other translocations

In t(11;14) translocation described in small cell lymphocytic lymphoma and chronic lymphatic leukaemia (CLL), the *bcl-1* gene on chromosome 11 is translocated next to the Ig heavy chain gene [26–28]. In T-cell lymphomas two major translocations have been described — t(8;14) and t(7;14) which involve the variable region of the TCR α chain on chromosome 14 and the Ig heavy chain. Other translocations involving chromosome 14 have also been reported [29]. The cloning of these translocation sites may identify further genes involved in oncogenesis. The main interest in NHL has concentrated on the gain of function of oncogenes. As in other malignancies, loss of suppressor gene activity may also play an important role and abnormalities of the p53 gene on chromosome 17 have been demonstrated in NHL.

Viruses and lymphomas

The majority of endemic Burkitt's lymphoma (BL) cases carry copies of EBV genome in the lymphoma cells. EBV DNA and EBV proteins (especially EBV nuclear protein 2) are also present in lymphoma cells of AIDS associated lymphoma [30, 31] and in the lymphoid proliferation of other immune deficiency states [32]. Akin to the immortalising properties of EBV infected cell lines, the virus is thought to induce proliferation of B-lymphocytes as one of the steps in the development of lymphoma, particularly in immune deficiency states. In states of impaired immune surveillance the loss of B and T lymphocyte interaction may result in uncontrolled proliferation, described as B-cell lymphoproliferative disorder [33].

The EBV genome in host cells becomes circular during latency and this is accomplished by the joining of a number of 500 bp tandem repeat DNA sequences at both ends of the linear molecule. Circularisation produces a specific fused terminal restriction fragment. Analysis of the structure of the termini and the number of terminal repeats can be used to determine the clonality of a tumour [34].

Human T-lymphotropic virus 1 (HTLV-1) virus is associated with adult T-cell leukaemia/lymphoma. The virus is incorporated into T-cells and molecular studies have determined some of the steps in the activation of T-cell proliferation through trans-acting genes without involvement of known oncogenes.

CLINICAL APPLICATION OF MOLECULAR BIOLOGY TECHNIQUES

Diagnosis

Clonality of non-Hodgkin lymphoma has been demonstrated by specific rearrangement of Ig and TCR genes in the DNA of tumour tissue from B-cell and T-cell lymphoma respectively [35–39]. Ig or TCR gene rearrangement is therefore used in the diagnosis of NHL [40]. False negative results may occur and are most likely due to sampling error where the portion of the biopsy specimen analysed does not contain tumour tissue. Where immunocytochemistry with conventional B-cell and T-cell specific antibodies is unable to define cell lineage, DNA hybridisation studies help provide the answer. However, cases containing both Ig and TCR rearrangement have been described [41] which may represent an early "progenitor" genotype or lineage infidelity. Tumours of previously unclear lineage such as Ki-1 lymphoma and some cases of "histiocytic" lymphoma have been ascribed to B or T cell lineages (for review see [42, 29]).

In the majority of cases of Hodgkin's disease Ig and TCR genes can only be detected in their germ line form. DNA from biopsy material containing a large proportion of Reed-Sternberg

(RS) cells and from cell suspensions enriched for RS cells may show Ig gene rearrangement [43–50]. TCR gene rearrangement has also been detected and is not related to the proportion of RS cells [51, 52]. Although these findings may complicate the diagnostic application of Ig and TCR gene rearrangement studies such cases do not pose any diagnostic difficulty on conventional histology. *bcl-2* rearrangement has also been detected by the polymerase chain reaction in Hodgkin's disease tissue [53] and it is speculated that follicular lymphoma and Hodgkin's disease may coexist or they may share common pathogenesis [54].

The application of Ig and TCR gene rearrangement studies to lymphoid proliferations previously described as "pseudo-lymphoma" defined a number of conditions as clonal lymphoid disorders. These include lymphoid aggregates in the orbit, salivary gland pseudo-lymphoma and lymphoid proliferation in salivary glands in association with Sjögren's syndrome. Clonal nature of T-cell disorders has been confirmed in cases of Sezary syndrome, mycosis fungoides, T-γ lymphocytosis and in a proportion of cases of angioimmunoblastic lymphadenopathy [55, 56] as well as in cases of lymphoid proliferation associated with coeliac disease [57]. The finding of clonality of a lymphoid lesion is however not diagnostic of malignancy [58]. Diagnostic application has been reviewed by Griesser *et al.* [29].

Southern blot hybridisation to detect gene rearrangement for diagnostic purposes is at present a labour intensive and time consuming technique. Polymerase chain reaction (PCR) [59] which amplifies specific DNA sequences is considerably faster and requires smaller amounts of DNA for analysis. It has been applied to amplify sequences across the Ig gene rearrangement sites by using conserved V region primers and J sequence primers. The limited number of Vδ genes of TCRδ also allow for TCR amplification [60]. Ig gene PCR can be used as an initial screening test to detect lymphoma in histological material for diagnostic purposes [61]. Sequencing of the variable region of Ig or TCR gene from lymphoma tissue can generate tumour specific probes and primers which can also be used for PCR or *in situ* hybridisation [62].

Molecular analysis of *bcl-2* rearrangement can be used in the diagnosis of low grade lymphoma to distinguish it from benign proliferation. The relatively high frequency of detection in high grade tumours exclude it as the only marker for histological grade. *bcl-2* antibodies have been used for diagnostic purposes to detect low grade lymphoma [63]. However, the *bcl-2* protein is also present in proliferating normal B-cells and the diagnostic value of this finding is at present not clear.

Oligoclonality

Immunosuppressed patients, such as recipients of organ transplants may develop lymphoproliferative lesions akin to lymphoma. Their clonal nature has been demonstrated by Ig gene rearrangement, although several sites from the same patient exhibit different rearrangements, demonstrating oligoclonality [64]. The clonal nature of individual lymphoproliferations in organ transplantation has also been confirmed by demonstrating clonality of inserted episomal EBV genome [65]. Oligoclonal lymphoproliferations have also been found in other immune deficiency states and on occasions were associated with severe EBV infection [66].

Apparent biclonality of lymphoma has been demonstrated in approximately 10% of lymphomas by κ/λ staining and by gene rearrangement criteria. However, cells of phenotypic diversity observed at different tumour sites and progression of nodular to diffuse histology may involve the same clone. Using gene rearrangement criteria alone in the assessment of clonality of lymphoid tissue is complicated by the finding of further recombination events and frequent mutations within the hypervariable region of V genes in mature B-cells [4–6]. To overcome this, studies of clonality should include the more stable translocation markers. The apparent biclonality probably reflects the development of subclone rather than the presence of two independent clones.

Detection of minimal disease

DNA hybridisation with Ig and TCR gene probes detects clones of neoplastic cells with 1–5% sensitivity [35, 67, 68]. With this technique lymphoma cells have been demonstrated in peripheral blood and bone marrow when not detectable by conventional means. A third of patients with active NHL may have circulating lymphoma cells, and the peripheral blood involvement appears to be related to the extent of disease, although lymphoma cells can be detected in a proportion of patients with clinical stage I and II disease and in patients with normal bone marrow [67, 69, 70]. Circulating clonal T cells have also been detected in patients with mycosis fungoides/Sezary syndrome [71]. These findings may have important clinical implications but at present their prognostic significance is not clear. Detecting clones of lymphoma cells in peripheral blood of patients clinically free of disease does not appear to predict for subsequent recurrence [70]. A sensitive method of detecting lymphoma cells in bone marrow and peripheral blood may also help in assessing remission status and may have an important role in bone marrow transplantation, particularly if autologous marrow is used.

The sensitivity of detection of minimal disease can be significantly increased by the use of polymerase chain reaction (PCR). This has been particularly applied to the study of nodular lymphoma which carries the t(14;18) translocation [72, 73]. With primers specific for *bcl-2* gene at the mbr or mcr loci and primers for the JH region of Ig it is possible to differentially amplify t(14;18) translocation sequence and detect up to $1:10^5$ cells bearing chromosomal translocation. This has been applied to the detection of tumour DNA from lymph nodes, bone marrow and peripheral blood of patients who are apparently disease free [74].

PCR has become a very powerful tool in clinical practice [75] but the interpretation of results with detection of such small numbers of lymphoma cells is not clear. The technique is also prone to error [76] and thus finding clones of abnormal cells in patients otherwise in remission should not at present be taken as an indication for further therapy. This is particularly so in the face of low grade lymphoma where cells carrying t(14;18) have been found in patients in long term remission [77, 78]. Nevertheless, the studies of minimal disease may provide an insight into the course of disease and may help as a prognostic indicator particularly if aggressive treatments such as autologous bone marrow transplantation and bone marrow purging are used.

Prognosis

Although the presence of t(14;18) is of no clear prognostic significance in nodular lymphomas this translocation is also detected in a proportion of patients with high grade NHL. The presence of such rearrangement may predict a relapsing course of disease, although so far it has no influence on the overall survival of patients with high grade NHL [79]. The study of other chromosomal translocations and other changes, such as

deletions and chromosomal losses, may provide further biological information and predict the clinical course of disease [80–82].

Current molecular technology also includes flow cytometric techniques which measure cellular DNA content and proliferative activity and this can be carried out on paraffin embedded material. Both DNA content and proliferative activity of lymphoma tissue have been correlated with prognosis [83, 84].

Therapy

Although the present understanding of molecular events in lymphoma has not yet been therapeutically exploited the potential exists that manipulation of the *myc* and *bcl-2* systems may alter the proliferation and differentiation in established malignancy.

There has been a major drive towards development of specific antibodies for therapy in NHL. Anti-idiotype antibodies appear to be useful in a proportion of patients with lymphoma [85], but the benefit is usually short-lived. Molecular analysis of the Ig gene of lymphoma tissue from patients treated with anti-idiotype antibodies has suggested that the failure of response is due to somatic mutations within the Ig gene which occur in mature B-cells [86]. The increased understanding of the molecular structure of antibodies has also led to the production of genetically engineered chimaeric antibodies with human sequences [87–89].

CONCLUSION

Molecular biology techniques have enormously advanced our understanding of the biology of NHL and related tumours. This information together with advances in molecular techniques has already been exploited in clinical practice for diagnosis, detection of minimal disease and as prognostic indicators. The oncogenic steps and subsequent control mechanisms of growth and differentiation are complex and likely to become more so. Clinicians live in expectation of finding critical steps which will be susceptible to selective external manipulation. Let's all hope . . . .

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Role of High-Dose Chemotherapy and Autologous Bone Marrow Transplantation in the Treatment of Lymphoma

Thierry Philip and Pierre Biron

INTRODUCTION

EVEN THOUGH malignant lymphomas can be considered among the most sensitive of malignancies as regards response to chemotherapy and radiation therapy, there still exists a significant fraction of adult patients for whom intensive therapy and bone marrow transplantation (BMT) can be discussed. Advances in the area of bone marrow transplantation have been associated with numerous reports of very promising pilot studies [1]. However, no clear indication based on a randomised study is found in the world literature. The purpose of this review is to define the optimal timing for autologous bone marrow transplantation (ABMT) in intermediate and high grade non-Hodgkin lymphomas (NHL).

50 cases of diffuse NHL are observed every year for a population of 1 million adults [2]. 20 of them are observed in patients over 60 years old at diagnosis and 10 are localised at time of first symptoms. Thus only 20 diffuse NHL/year/1 million adults can be considered for bone marrow transplantation. Even with restricted indications, as many as 7/year/1 million can be considered for BMT in first complete remission, 2/year/1 million for BMT in first partial remission, 4/year/1 million for BMT in sensitive relapses, 2/year/1 million for BMT in non-explosive resistant relapses and possibly also an additional 2/year/1 million adults for primary refractory patients. A total of 17 BMT/year/1 million adults will mean between 300 and 500 indications in France and between 1200 and 2000 indications in the USA [1, 2]. As a comparison, only 700 BMT for lymphomas were recorded in the world betwen 1981 and 1985 and only 500 autologous bone marrow transplantations were performed in France in 1987 (the highest number of ABMTs in one single country in Europe) [2]. The necessity to clearly define indications for BMT in this disease is therefore clear and could be considered as a priority for public health in developed countries.

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TIMING FOR ABMT

What is optimum timing for ABMT in CR1 for intermediate-grade and high-grade lymphomas? The optimum timing for ABMT in Burkitt's lymphomas has been extensively reviewed [3,4]. For lymphoblastic lymphomas it is in fact comparable to lymphoblastic leukaemia. However, intermediate grade lymphomas, according to the International classification, are the most common NHL in adults [5]. The majority of reported regimens are able to produce 60% long term survival in this group [6]. It is important to consider therefore that 10% of the patients will never reach first CR and will progress on induction therapy, that 8% will only be in partial response after induction and that approximately 8% will die early of toxicity [6]. If these patients and patients over 60 years old at diagnosis are excluded, survival curves of 70–75% are common and indications for BMT very unsecure.

The selection of bad prognosis groups is thus mandatory if BMT is considered in first CR. It is now widely accepted that candidates for prospective studies can be defined as patients less than 55 years old at diagnosis, with at least 2 extranodal localisations or a tumour of at least 10 cm at diagnosis, with a bad Karnofsky score (< 70%) or with bone marrow or CNS disease at initial presentation [6]. This group is reported to have an expected survival with conventional regimen of 55% at 3 years (B. Coiffier, Centre Hospitalier Lyon). Only prospective and randomised studies are acceptable in this field. They should avoid protocols with high toxic death rates and they should include at least 150 patients in each arm. The European NHL group is currently studying this group of patients in a randomised multi-institutional European trial (C. Gisselbretch, chairman).

ABMT IN PRIMARY REFRACTORY PATIENTS

The term refractory lymphoma has frequently been utilised in an ambiguous context and thus needs to be better defined. In describing the results of salvage studies, the frequently used statement "patients who have failed front line regimens" is not appropriate. The setting in which these patients "failed" is much more important than the fact they failed. Those patients who fail to achieve a major response to front line chemotherapy regimens are without doubt the best example of refractory disease.