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Ten Years After the REAL Classification: Open Issues

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haematologica

The origin and power of a name

Ancient Greek

αιμα [aima] = blood;
αιματος [aimatos] = of blood,
λογος [logos]= reasoning

Scientific Latin

haematologicus (adjective) = related to blood

Scientific Latin

haematologica (adjective, plural and neuter, used as a noun) = hematological subjects

Modern English

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Ten Years After the REAL Classification: Open Issues

Bologna, October 6-9, 2004

Guest Editor: Sante Tura

HODGKIN'S LYMPHOMA. WHY 30% OF THE PATIENTS IS STILL UNCURABLE?

Ten Years After the REAL Classification: Open Issues

6th October 2004

Hodgkin's disease coming into the 21st century

The successful use of cytotoxic drugs and/or radiation in the treatments of Hodgkin's disease anticipated understanding of the biological nature of the disease by almost 30 years. Increasing knowledge on the biology of this disease is opening new opportunities for targeted therapy in the near future.

The vast majority of patients presenting in stage III or IV are treated with combination chemotherapy. With the passage of time, the composition and schedules of the drug regimens have varied and some have been used with radiation therapy. The general standard in North America (and many centers in Europe) outside of clinical trials is 6-8 cycles of the ABVD regimen (adriamycin, bleomycin, vinblastine, dacarbazine). The Cancer and Leukemia Group B (CALGB) has conducted two trials in which ABVD was prospectively compared to MOPP (mustine, vincristine, procarbazine, prednisolone) alone, 12 months of MOPP alternating with ABVD, and a MOPP-ABV hybrid. A long-term follow-up of these trials showed that ABVD produced an equivalent or superior progression-free survival to that of the other regimens without the toxicity of alkylating agents - namely more profound myelosuppression, sterility and leukemogenesis. There was no difference in overall survival because second-line therapy could salvage relapsed patients. Combination chemotherapy in general, including ABVD, used for stage III/IV disease should achieve a clinical complete response rate of at least 70-80% with an approximately 20-30% relapse rate over time. The number of negative prognostic factors directly influences the outcome. The majority of patients present with 2-3 negative factors. When 5+ are present, there is an overall five-year survival that is somewhat less than 50%. These poor-risk patients represent only 7% of cases. In addition to clinical prognostic factors in advanced disease, there may be biological factors that correlate with response to chemotherapy. One such factor is positivity for Epstein-Barr viral latent antigen (LMP-1), which is associated with a favorable outcome. Tissue eosinophilia and elevated serum levels of interleukin-10 have been associated with an inferior failure-free survival. With the salvage rate of 30-50%, overall survival at five years could be in the range of 80%. The potential *cure* rate is in the range of 70%. With conventional dose chemotherapy regimens, the overall survival outcome is likely to be similar regardless of regimen. The major differences in the follow-up will be in the toxicity. Although the *standard* is ABVD, there is certainly a need for newer regimens that might offer the potential for an improved outcome. The German Hodgkin Lymphoma Group (GHSG) pioneered the development of BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisone) - a regimen based on a model intended to improve failure-free survival by 10-20% by modest dose intensification. The same group also tested an escalated version, given every 32 days, that increased the doxorubicin 140% (from 25-30 mg/m^2); cyclophosphamide 190% (650-1200 mg/m^2) and etoposide 200% (100-200 mg/m^2) and required growth factor support. This was compared to regular dose BEACOPP and COPP/ABVD as the standard in a prospective randomized trial. With a median follow-up of 56 months, the freedom-from-treatment failure rate was 87%, 76%, and 69%, respectively. The overall survival at five years was 91%, 88% and 83%. The escalated BEACOPP produced a significantly superior overall survival to that achieved by COPP/ABVD but not that by standard BEACOPP. The escalated BEACOPP regimen was, however, also associated with greater hematologic toxicity. The BEACOPP regimen (standard dose) is now being compared to ABVD in a prospective randomized trial.

Stanford V is a combined modality program that entails three months of weekly chemotherapy and steroids followed by irradiation (36-44Gy) to sites of initial bulky disease and those showing residual radiographic signs. An analysis of 142 patients demonstrated an 89% freedom-from-progression rate at a median of 5.4 years with an overall survival of 96%. These excellent results need confirmation, but it should be noted that 32% of patients had bulky (< 1/3 intrathoracic diameter) stage I or II disease. There were no treatment deaths or apparent sterilization. The vast majority (91%) in the series received radiation therapy.

The Milan NCI group conducted the pilot trial with VEBEP (etoposide, epirubicin, bleomycin, cyclophosphamide, and prednisolone) - a regimen given for 8 cycles, followed by complementary radiotherapy to sites of prior disease. The complete remission rate was high (94%) with 78% freedom-from-progression at six years. These excellent results were associated with gonadal damage, although this was

reversible in half of the men. This again illustrates the toxic risks associated with more intensified treatment. It is possible that a relatively small fraction of patients (10-20%) will be spared the necessity of salvage therapy when higher dose alkylating agents or etoposide-containing regimens are used compared to ABVD or ABVD-like regimens (with or without radiotherapy). This means that 60-70% of patients are exposed to a variety of unnecessary toxic risks. If salvage therapy is effective in that 10-20% group as opposed to in patients with *de novo* refractory or induction failures, then the group of patients treated with the less toxic regimen could show an equivalent survival with less overall toxicity. This emphasizes the importance of prognostic factors. However, it still remains to be seen whether more cytotoxic regimens will prove superior to ABVD in overall disease-related survival in patients with clinical factors predicting a poorer overall survival.

Many trials, including those of the BEACOPP and VEBEP regimens, do not permit an assessment of the impact of chemotherapy alone because they include radiation. A recent EORTC trial tested the value of complementary involved-field radiotherapy in stage III/IV patients in complete remission after completing 6-8 cycles of MOPP/ABV in a randomized trial. After six years of follow-up, the relapse-free and overall survival for the radiation group was 85% and 82%, respectively, versus 82% and 88% for the non-irradiated group. All patients with partial remission received irradiation, achieving outcomes similar to those of the patients with complete remission. Radionuclide scans to determine the status of residual mass in partial remission patients were not performed. It is conceivable that some of the patients in partial remission may have been in a pathologic complete remission as well.

The approach to localized Hodgkin's disease has changed. Limited radiation has been used with systemic therapy. Low-risk patients are now considered for chemotherapy alone. The demonstration that non-alkylating agent-containing regimens, such as ABVD, do not cause associated myelodysplasia or sterility has led to their widespread use in early low-risk disease. Prospective trials of chemotherapy alone vs. Radiotherpy or combined modality treatment have cast some doubt on the need for radiation. The clinical balance between efficacy and toxicity remains the challenge of clinical research.

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New Therapeutical Approaches

Chairmen: G. Pizzolo, G. Torelli

Bexxar. Tositumomab and Iodine-I^{131} Tositumomab

In June 2003, Bexxar was approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with CD20-positive, follicular, non-Hodgkin's lymphoma, with and without transformation, whose disease is refractory to rituximab and has relapsed following chemotherapy. Summarized here are the results of studies that led to the approval of Bexxar, as well as other studies that may further our understanding of the position this treatment should take in the overall management of B-cell lymphoma.

Rationale

The rationale for radioimmunotherapy of B-cell lymphoma is strong. Lymphomas are exquisitely sensitive to ionizing radiation. Relapses rarely occur in irradiated sites of disease. There is an abundance of well-characterized lymphoid surface antigens that can serve as targets for monoclonal antibodies labeled with various radioisotopes. Monoclonal antibodies, especially those specific for the CD20 antigen, have shown promising efficacy by themselves.

Radiolabeled antibodies can overcome some of the inherent limitations of unlabeled antibodies. Because ionizing particles emitted from the disintegrating atomic nucleus of a radioisotope can potentially travel through any number of cell diameters, depending on their energy, many tumor cells within a tumor can be killed by a single radiolabeled antibody bound to a tumor cell. In addition, when multiple radiolabeled antibodies are bound to cells within a tumor, a crossfire of ionizing particles is created (Figure 1) further intensifying the radiation dose to tumor cells. This effect is particularly important in bulkier tumors in which vascular access for antibodies to reach cells deep within a tumor may be limited. Furthermore, tumor cells either lacking the target antigen or expressing a small amount of antigen can be killed by crossfiring particles. In addition, immune mechanisms of cell killing by antibodies (eg, antibody-dependent cellular cytolysis and antibodydependent complement cytolysis) do not have to be competent in the patient for radioimmunotherapy to be effective. Also, direct mechanisms of antibody-mediated cell killing such as induction of apoptosis are not

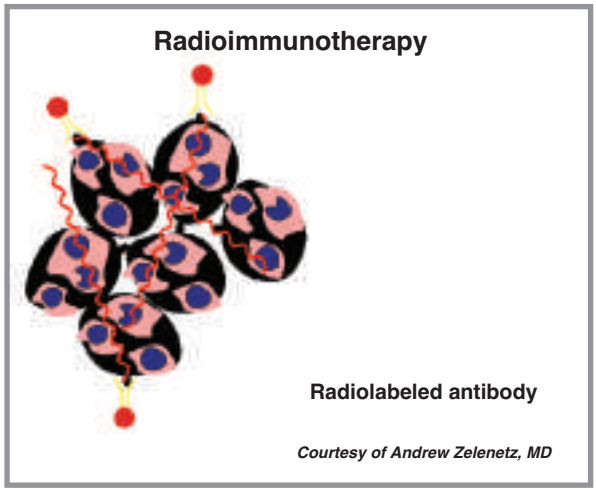

Figure 1. β particles emitted by a radiolabeled antibody bound to a target antigen can travel through multiple cells. A crossfire effect is created when more than one radiolabeled antibody binds to a tumor mass.

necessarily required. Finally, mechanisms of resistance to either immune or direct antibody cell killing can be overcome by radiation effects. It is possible, however, that both antibody and radiation-mediated anti-tumor effects can be retained, thus creating a multi-pronged attack on tumor cells.

Composition of Bexxar

Tositumomab is the monoclonal antibody component of Bexxar, while I-131 is the radionuclide used to label this antibody. Tositumomab was originally named anti-B1 and was developed by investigators at the Dana Farber Cancer Institute in the late 1970s. It is a murine IgG2a antibody that is specific for the CD20 antigen (formerly known as the B1 antigen) expressed by more than 95% of B-cell lymphomas. CD20 is known to play a role in cell proliferation and differentiation and can act as a calcium channel. It is not shed or internalized upon antibody binding, thus representing a stable target. Tositumomab is effective in mediating antibody-dependent cellular cytolysis (ADCC), complement-dependent cytolysis, cell-cycle arrest, and apoptosis *in vitro*; it also causes B-cell tumor regression *in vivo* in an animal model. I-131 is coupled to tositumomab by a simple covalent reaction. This radioisotope emits 0.6 Mev β particles with a pathlength of 0.8 mm. Thus, with this pathlength, these β particles can potentially deliver ionizing radiation over approximately 60 lymphoma cell diameters; this provides ample opportunity for crossfire effects within a tumor as described above. I-131 also emits low L.E.T. (linear energy transfer) γ rays that readily travel through tissue and can be detected externally with a γ detection device such as a γ camera or Geiger counter. While these γ rays are not believed to contribute significantly to anti-tumor effects and do impose certain modest radiation precautions to limit exposure of individuals in the vicinity, they are useful in monitoring the in vivo biodistribution and the rate of clearance of the radiolabeled antibody. This monitoring ability is important since biodistribution and clearance rates can be significantly influenced by tumor burden, splenic size, and amount of bone marrow involvement. Thus, by assessing these γ emissions over time after a trace-labeled dose of radiolabeled antibody is administered to a patient, a therapeutic dose can be later scaled to provide an individualized dose for each patient, potentially avoiding the over- and under-dosing that could occur if only a set dose were to be used.

Although the physical half-life of I-131 is 8 days, biological clearance is rapid and is primarily through the kidneys and gut. In the vast majority of cases, this treatment can be given in an outpatient setting in the United States.

Bexxar is supplied to a treatment site by overnight delivery. Tositumomab is supplied already labeled with I-131 after conjugation is performed at a central site (Nordion in Canada). A trace-labeled dose (also called a dosimetric dose) and a therapeutic dose are ordered separately. A separate vial of unlabeled tositumomab accompanies each shipment. This unlabeled antibody is given to the patient prior to the radiolabeled antibody to optimize the biodistribution of the radiolabeled antibody.

Phase I/II Trial (RIT-I-000)

The principal anticipated toxicity of radioimmunotherapy is myelosuppression. At the beginning of the clinical development of Bexxar, two main approaches to dosing were envisioned; one in which the dose would be non-myeloablative and thus not requiring hematopoietic stem cell support and the other using myeloablative doses with stem cell rescue. Both approaches have since been extensively studied, but the non-myeloablative dosing approach is that which the FDA evaluated for marketing approval. Hence, this discussion is confined to this latter dosing regimen. The dosing regimen, maximally tolerated dose, and preliminary efficacy data for the non-myeloablative treatment regimen were based on the results of a phase I/II single-center study conducted at the University of Michigan which began in April 1990.[1] A total of 59 patients with chemotherapy relapsed/refractory low-grade, transformed low-grade, or *de novo* intermediate-grade lymphoma were included. Patients were required to have bone marrow biopsies showing no more than 25% of the hematopoietic marrow space to be

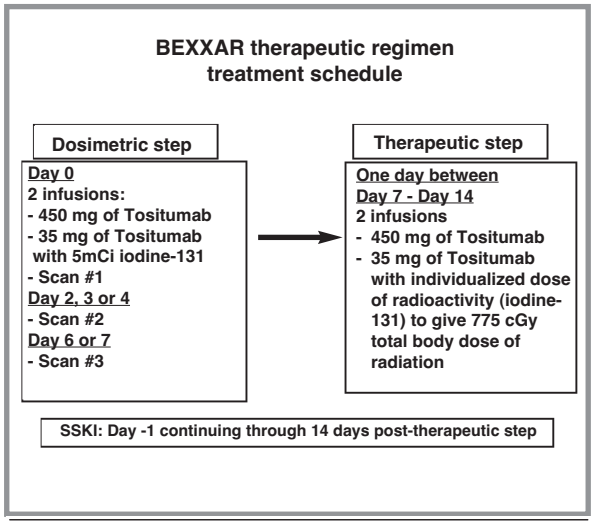

Figure 2. The Bexxar therapeutic regimen is given in two discrete steps - a dosimetric and therapeutic step. Information on the radiolabeled antibody's residence time after the dosimetric dose is used to calculate a therapeutic dose individualized for each patient.

Table 1. Bexxar studies in rituximab-naïve patients.

Study Description/Name	Overall response (%)	Median TTP (mos.) (range)	Complete response (%)	Median TTP (mos.) (range)
Chemotherapy refractory (RIT-II-004)	47%	13.2 (3.2-48.7)	20%	48.7 (10.5-48.7)
Chemotherapy-relapsed or refractory (RIT-II-002†)	59%	NR [b] (3.2-58.9+)	36%	NR+ (6.3-58.9+)
Chemotherapy-relapsed or refractory LG-NHL (RIT-II-001)	49%	14.4 (3.0+-62.1+)	26%	60.1 (11.6-62.1)+
Chemotherapy-relapsed or refractory (RIT-I-000††)	64%	15.2 (3.7-95.8+)	28%	2 9.1 (3.7-95.8+)

**excludes patients who only received unlabeled Tositumomab (Arm B); †NR: not reached; ††excludes 17 patients with intermediate- and high-grade lymphoma. The Bexxar therapeutic regimen in not indicated for chemotherapy -refractory, low-grade or follicular NHL.*

involved with lymphoma, platelet counts of at least 100,000/mm^3 and an absolute neutrophil count of at least 1,500/mm^3, adequate renal function, and an ability to comply with radiation safety instructions. These requirements were retained for all subsequent studies and continue to be important criteria for the clinical use of Bexxar.

The established dosing regimen is outlined in Figure 2. It consists of two discrete steps, a dosimetric step and a therapeutic step. In the dosimetric step, a 1-hour intravenous infusion of 450 mg unlabeled tositumomab is given followed by a small radiolabeled antibody dose (5 mCi). Pre-dosing with unlabeled antibody was found to improve the biodistribution of the radiolabeled antibody (increasing tumor dose, decreasing dose to the spleen, prolonging residence time). Gamma camera scans are then obtained at baseline and on two other occasions over the ensuing week to evaluate the biodistribution of the radiolabeled antibody and to determine the radioactivity in the patient at each time point to establish the rate of clearance. One week after the dosimetric dose, the therapeutic step is administered in which unlabeled tositumomab is again given but this time followed by a dose of radiolabeled antibody that is individualized for the patient based a simple calculation of total-body clearance rate over the preceding week. The dose is tailored to deliver a maximally tolerated total-body dose (75 cGy for patients with at least 150,000 platelets/mm^3, 65 cGy for those with between 100,000 to 149,000 platelets/mm^3, and 45 cGy for patients with a history of prior hematopoietic stem cell transplant). Unlike chemotherapy, the treatment is over at this juncture with no repeated cycles. Non-radioactive iodide such as a saturated solution of potassium iodide (SSKI), is given orally during and for a limited time after the therapeutic dose to help protect the thyroid gland from absorbing free I-131 released from the radiolabeled antibody.

Patients with low-grade or follicular lymphoma had a particularly high response rate (64% overall, 38% complete) and long durations of remissions. The complete remissions lasted a median of 29.1 months and 7 patients have had remissions of 7 to 11 years duration in the most recent update of the data. Patients with intermediate or high grade lymphomas fared less well (41% overall response rate, 0% complete). These data were the basis for focusing subsequent multi-center trials on low-grade or follicular lymphoma.

Multi-center studies on chemotherapy relapsed/refractory low-grade or transformed low-grade lymphoma

Table 1 summarizes the efficacy data from the subsequent multi-center studies conducted in patients with chemotherapy relapsed or refractory low-grade or transformed low-grade lymphoma considered by the FDA. The RIT-II-001 trial included 47 patients and was designed to validate the dosing methodology developed at Michigan.[2] The RIT-II-002 trial randomized 78 patients to receive either the radiolabeled antibody treatment or the antibody in its unlabeled state to determine the value added by the radionuclide component.[3] The radiolabeled antibody treatment was found to be superior to unlabeled antibody in overall response rate (OR = 55% vs. 19%) and complete response rate (CR = 33% vs. 8%). The RIT-II-004 study was designed to assess efficacy in 60 patients with chemotherapy-refractory disease (no response

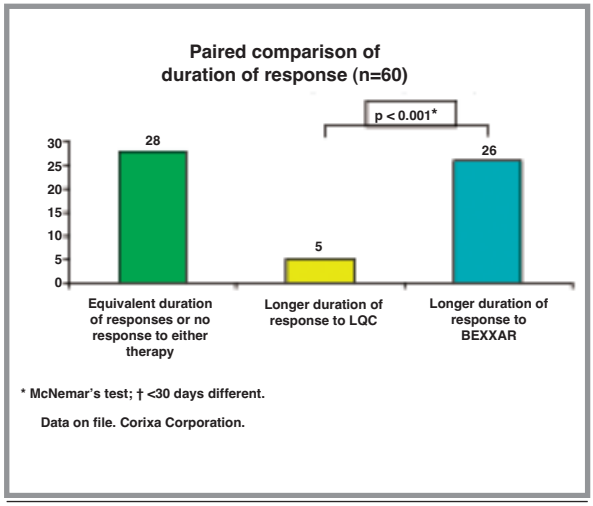

Figure 3. A comparison of duration of responses to Bexxar compared to the last chemotherapy regimen received prior to entering the RIT-II-004 trial for patients with chemotherapy-refractory low-grade or transformed low-grade lymphoma.

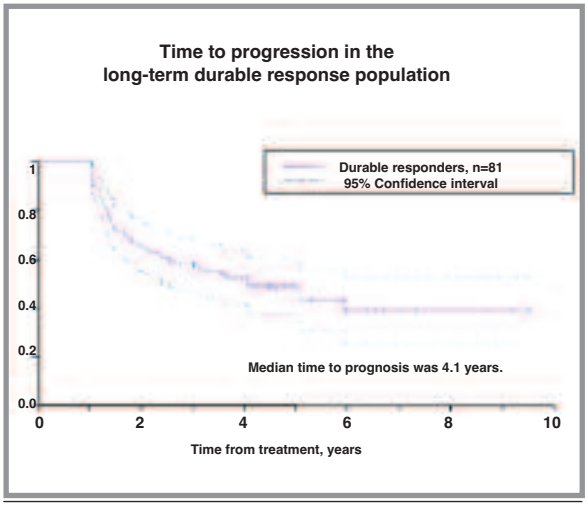

Figure 4. Time to progression in patients enrolled in 5 clinical studies of Bexxar who had at least one year of remission.

Table 2. Response data from RIT-II-004.

	Last qualifying chemotherapy (n=60)	BEXXAR (n=60)	p value*	p value†
Response	7/60 (12%)	28/60 (47%)	< 0.001	NA
Median (95% CI) duration of response for responders (months)	4.1 (3.0-5.4)	11.7 (6.9-47.2)	< 0.001	< 0.001
Complete response	1/60 (2%)	12/60 (20%)	0.002	NA
Median (95% CI) duration of response for complete responders (months)	4.8 NA	47.2 NA	0.003	0.001

**p values for response rates based on McNeman's test vs. 0.50; p-values for durations based on generalized McNeman's test: †paired Prentice-Wilcoxon test for censored data for comparison of duration; NA: not applicable.*

Table 3. Response to Bexxar in Rituximab-refractory patients.

	Response rate (%) (95% CI*)	Median duration of response (Mos.) Range
Overall response	63% (45%-79%)	25 (4+-35+)**
Complete response	29% (15%-46%)	NR*** (4-35%)
Median duration of follow-up	26 months	

**CI: confidence interval; **complete response rate: pathological and clinical responses; ***NR: not reached.*

or response lasting less than 6 months to the last chemotherapy received).[4] As shown in Figure 3, the number of patients who had a longer duration of response to Bexxar than to chemotherapy was about 5 times the number who had a longer duration to chemotherapy (p<0.001). The OR rate was higher to Bexxar than to chemotherapy (47% vs 12%) as was the CR rate (20% vs 2%, respectively) (Table 2). Taken together, these trials indicated high response rates in patients previously-treated with chemotherapy. Notably, the responses, especially complete responses, were durable, often lasting years. These data support the use of Bexxar as an effective salvage treatment for these types of patients.

Bexxar for Rituximab-refractory disease

A multi-center study was conducted in 35 patients who were refractory to rituximab and who had also had prior chemotherapy.[5] The overall and complete response rates were 63% and 29%, respectively, with the median duration of response being 25 months. The median duration of complete responses had not been reached with a median follow-up of 26 months (Table 3). These data supported the use of Bexxar as an effective salvage treatment for rituximab-refractory patients.

Durable responses

The response rates and safety data (discussed below) of the previously described studies in both rituximab-refractory disease and rituximab-naïve patients were pivotal in the FDA approval process. Also considered was the finding that 32% of patients had responses lasting more than one year. The median time to progression for these patients was 4.1 years with remissions continuing at 10 years (Figure

Table 4. Hematologic toxicity (n=230)

	Platelets	ANC	Hemoglobin
Grade 3/4*	53%	63%	29%
Median duration of Grade 3/4	32 days	31 days	23 days
Grade 4†	21%	25%	5%
Median duration of Grade 4	28 days	16 days	10 days
Median Nadir	43,00 mm^3	690 cells/ mm^3	10 gm/dL
Median time to Nadir	34 days	43 days	47 days

*Definition of grade 3/4: Platelets <50,000 mm^3 ANC <1,000 cells/mm^3 Hemoglobin <8 gm/dL
†Definition of grade 4: Platelets <25,000 mm^3 ANC <500 cells/mm^3 Hemoglobin <6.5 mg/dL

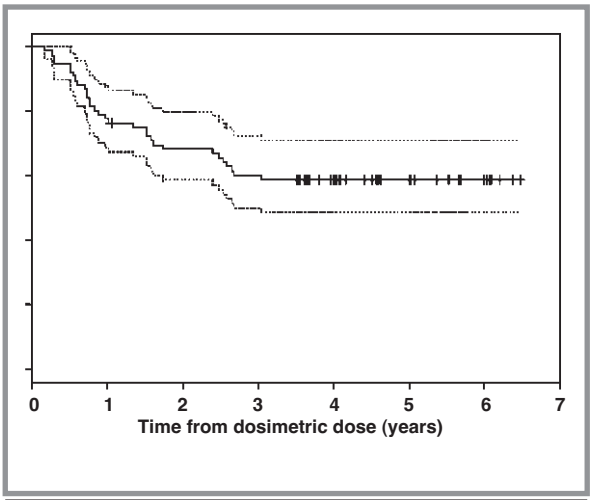

Figure 5. Progression-free survival in 76 patients with follicular lymphoma given Bexxar as front-line therapy.

4). This was considered remarkable for such heavily pretreated patients. Thus, a combination of safety data, response rates, and the durability of responses led to FDA approval.

Safety

Acute adverse events were assessed in the 230 patients from the above studies and an additional 765 patients from an expanded access program were added for analysis to assess for long-term and serious adverse events. Acute non-hematologic toxicities were typical of those seen with antibody infusions and were almost always grade 1 or 2. Infusions were remarkably well-tolerated, with only 7% requiring interruption or adjustment in rate because of side effects. Hematologic toxicity is summarized in Table 4. Nadirs in blood counts were later (at 4 to 7 weeks) than those typically seen with chemotherapy. Although grade 3 or 4 toxicity was common, only 8% of patients experienced a serious infection requiring hospitalization and hemorrhagic events were very rare. Only 27% of patients received one or more types of hematologic support measures such as growth factors or transfusions.

Human anti-mouse antibodies (HAMA) became detectable in only 10% of patients. Approximately 10% developed hypothyroidism, as determined by an elevated level of thyroid stimulating hormone. Myelodyplasia or acute leukemia was reported in 32 of 994 patients.[6] The annualized incidence was between 1 to 2%, not unlike that expected for chemotherapy by itself. Cytogenetic analyses consistently revealed abnormalities of chromosomes 5 and 7, typical of alkylating agent exposure. The relative contribution of radioimmunotherapy to the induction of these disease states still remains to be determined.

Retreatment with Bexxar and other treatments after Bexxar

A multi-center study has been performed including 32 patients who had previously responded to Bexxar and who had relapsed. The OR and CR rates were 56% and 22%, respectively, with the median duration of response being 10.7 months and the median duration of complete response not being reached. In addition, the hematologic toxicity of the second treatment was comparable to that of the first. Thus, it appears Bexxar can be given more than once and suggests other myelosuppressive therapies can be tolerated after Bexxar. Indeed, Dosik *et al.* have shown that a variety of chemotherapeutic regimens can be tolerated after Bexxar and that harvesting of hematopoietic stem cells is still possible.[7]

Earlier treatment, including frontline treatment

Given the promising results seen in heavily pretreated patients, the outcome of this treatment when employed earlier in the management of low-grade lymphoma is of considerable interest. Davies *et al.* recently reported a study that evaluated Bexxar as a treatment for either a first or second recurrence after chemotherapy.[8] The OR rate was 76% and CR rate was 49%. With a median follow-up of 3 years, the median duration of all responses was 1.3 years and was not reached for complete responders.

In a study conducted at the University of Michigan, 76 previously untreated patients with advanced-stage follicular lymphoma received Bexxar as a single agent for frontline treatment.[9] The overall and complete response rates were 95% and 75%, respectively. The 5-year progression-free survival was 60% for all patients and 77% for those who achieved a complete response (Figure 5). With a median follow-up of close to 5 years, no cases of myelodysplasia or acute leukemia have been observed.

Bexxar has also been given after initial chemotherapy induction (such as CHOP, fludarabine, and CVP) in the frontline setting.[10-12] The results from these trials are promising and show a high rate of conversion from partial to complete responses with the addition of Bexxar after the chemotherapy phase of treatment. Randomized trials that are further examining the role of Bexxar in the frontline setting are currently underway.

These data strongly suggest that radioimmunotherapy should be considered earlier in the course of treatment of this disease rather than waiting for the disease to become refractory to other treatments.

The future

Anti-CD20 radioimmunotherapy has gained a place in our therapeutic armamentarium. Future studies will further clarify its role in the management of not only low-grade or follicular lymphoma for which is has been approved, but of other types of B-cell lymphomas whose outcomes stand improvement.

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Clinical activity and safety profile of immunotherapy with epratuzumab (humanized anti-CD22) in B-cell non-Hodgkin's lymphoma

Therapy with rituximab (chimeric anti-CD20) is widely employed in the treatment of non-Hodgkin's lymphoma (NHL).[1,2] While this agent can enhance the efficacy of chemotherapy,[3,4] and can have substantial single agent activity, its effects are limited and additional agents and approaches are necessary. Antibodies directed against other antigens, and with potentially different mechanisms of action, could be useful in rituximab-resistant patients and could perhaps also synergize with rituximab and/or chemotherapy. A variety of other immunotherapy targets are currently under preclinical or clinical evaluation.

The CD22 antigen is commonly expressed on normal and malignant B cells, with a comparable distribution to that of CD20.[5] Epratuzumab (hLL2) is a humanized IgG1 monoclonal antibody directed against the CD22 antigen, and is under evaluation as a lymphoma therapy both in unlabeled and radiolabeled forms.[6-10] Preclinical studies both *in vitro* and in murine models have demonstrated clear anti-lymphoma activity, which appears to be related at least in part to antibody-dependent cellular cytotoxicity (ADCC).[11] At the Center for Lymphoma and Myeloma at the Weill Medical College of Cornell University, we conducted phase I/II trials with this agent, evaluating doses of 120-1000 mg/m^2/weekly for 4 treatments.[12] Most toxicities were grade 1 and were primarily infusion reactions such as fevers, rigors, and hypotension, which were uncommon despite the short administration time of only 30-60 minutes. No-dose limiting toxicity was observed. While temporary depletion of B cells was observed in some subjects, no effects on hematologic parameters, serum immunoglobulins, or blood chemistry were noted. Serum antibody levels persisted for 3-4 months following therapy and human anti-human antibody (HAHA) formation was rare. Objective responses were demonstrated in follicular NHL and in diffuse large B-cell NHL, and some were durable over several years. In follicular NHL, 24% of patients responded overall, with 43% of patients in the 360-mg/m^2 dose group and 27% in the 480 mg/m^2 group responding. Three patients with follicular lymphoma (6%) had a complete response (CR), 2 of whom were receiving 360 mg/m^2 and 1 receiving 480 mg/m^2, and 6 patients (12%) had a partial response (PR), 4 receiving a dose of 360 mg/m^2 and 2 receiving 480 mg/m^2. Seven follicular NHL patients had sustained responses of greater than 8 months and 3 of these responses were ongoing up to 25 months after treatment. No responses were observed in patients with other indolent histologies (primarily chronic lymphocytic leukemia/small lymphocytic leukemia). In diffuse large B cell lymphoma, across all dose levels and histologies, objective responses were observed in 5 patients, including 3 complete responses. In patients with diffuse large B-cell lymphoma, 15% had objective responses.[13] Overall, 11 (20%) DLBCL patients experienced some tumor mass reduction. The median duration of objective response in DLBCL was 26.3 weeks and the median time to progression for responders is 35 weeks. Several patients in this study received a second course of epratuzumab at the time of disease progression. In two patients who had demonstrated an objective response to their first course, a second round of administration again resulted in tumor regression.[14]

As the efficacy of most single agent treatments of cancer is limited, combinations offer the possibility of improved outcomes relating to additive or synergistic effects. As with other active agents, it is therefore important to assess epratuzumab in combination with other antibodies and/or chemotherapy for lymphoma. Preclinical studies have demonstrated that rituximab therapy may upregulate the expression of CD22,[15] and murine experiments showed that epratuzumab + rituximab treatment may have greater effectiveness than monotherapy.[16] We have preliminarily assessed a regimen of combination immunotherapy with epratuzumab plus rituximab.[17] Patients were treated with epratuzumab 360 mg/m^2/week over 60 minutes followed by rituximab 375 mg/m^2/week in the standard fashion, both over 4 weeks. Preliminary analysis demonstrates that the combination regimen results in manageable drug-related toxicity which is comparable in degree and nature to that observed with rituximab monotherapy, and generally NCI grade 1 or 2. As far as concerns the efficacy analysis, the majority of subjects achieved an objective clinical response, with nearly all responders having a complete response or complete response - unconfirmed. Follow-up is ongoing to establish time to progression parameters. A larger multicenter study has gone on to evaluate this combination regimen and the results suggest that there may be benefits in some subsets of patients.[18] In a non-comparative study with a limited number of patients, response rates in small lymphocytic lymphoma (SLL) (50%) and rituximab-relapsed (75%) patients appear greater than those typically seen with rituximab alone. Additionally, a pilot study of CHOP (cyclophosphosphamide, doxorubicin, vincristine, prednisone) with rituximab and epratuzumab as initial therapy of diffuse large B-cell lymphoma is currently underway.[19] Preliminary results suggest that this regimen is well tolerated (with toxicity comparable to CHOP-R alone). Complete response rates are high, and larger studies are planned.

In an era in which virtually all patients with B-cell lymphoma receive rituximab at one time or another, assessment of novel antibody approaches is exceedingly complex. Given the potentially overlapping mechanisms of action of antibodies, and resistance patterns based on both tumor and host characteristics (such as Fc receptor polymorphisms), it is unclear whether new antibodies or combinations may be most useful in overcoming rituximab resistance (in resistant patients) or in enhancing sensitivity (in sensitive patients). Given the heterogeneous nature of patients treated recently in most trials of new agents (generally sensitive to prior treatment with rituximab +/- chemotherapy), interpretation of efficacy results is difficult, particularly those involving comparisons to results expected with rituximab alone. One might expect that benefits of any combination regimen may be confined to specific patient subsets defined by histology and sensitivity (or lack of sensitivity) to prior therapy. To help clarify the picture, in the Cancer and Leukemia Group B (CALGB) as part of the evaluation of new agents in follicular lymphoma, there will be a phase II study which will assess the activity of rituximab in patients previously treated with a chemotherapy + rituximab combination. These results will provide a benchmark for comparison with studies of novel combinations such as epratuzumab + rituximab in order to provide a better sense of the benefits of the combination. At this point, the single agent activity of epratuzumab in the two most common NHL subtypes and favorable toxicity profile suggest that epratuzumab is a promising agent in lymphoma therapy. Several current and planned trials will establish the role and optimal setting for epratuzumab in the treatment of patients with B-cell malignancies.

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Treatment of anemia with epoetin α in patients with hematologic malignancies

Cancer- or chemotherapy-related anemia can produce debilitating symptoms, such as fatigue and weakness, which negatively affect patients' ability to maintain daily life activities and cope with cancer. Anemia is common in patients with hematologic malignancies, occurring in up to 70% of patients, and its severity depends on the specific malignancy diagnosed, extent of disease, and type and duration of chemotherapy received. Epoetin-α significantly increases hemoglobin (Hb) and reduces transfusion requirements in anemic patients with cancer receiving chemotherapy (including patients with hematologic malignancies), and growing evidence supports the role of epoetin-α in helping to alleviate anemia-related symptoms and improve quality of life (QOL). Despite the adverse consequences of anemia and the clinical benefits of erythropoietic therapy, nearly half of patients with hematologic malignancies do not receive any anemia treatment. Clinical practice guidelines recommend erythropoietic therapy in patients with cancer-related anemia and Hb d10 g/dL, with treatment optional for patients with Hb >10 g/dL to <12 g/dL. However, several studies in patients with solid tumors or hematologic malignancies and mild-to-moderate anemia (Hb e8 g/dL to d12 g/dL) receiving chemotherapy have demonstrated a positive effect of earlier epoetin-α therapy on hematologic and QOL parameters. Additional investigation of earlier treatment with epoetin-α and the target Hb level to optimize clinical benefit for patients with hematologic malignancies has recently been completed and will further characterize the value of anemia treatment in this population.

Introduction

Anemia in hematologic malignancies can arise from neoplastic infiltration of the bone marrow, hemolysis, nutritional deficiencies, and myelosuppressive chemotherapy.[1] The reported incidence of anemia in patients with hematologic malignancies ranges from 40% to 70%, and its severity varies depending on the specific malignancy diagnosed, extent of disease, and type and duration of chemotherapy received.[2-4] In the European Cancer Anemia Survey (ECAS), 73% of 2316 patients with hematologic malignancies were anemic (hemoglobin [Hb] <12 g/dL) at some point during the 6-month survey (71% of newly diagnosed cases, 84% of those with recurrent or persistent disease, and 55% of patients in remission).[5] However, despite the known adverse effects of anemia on patient's well-being and quality of life (QOL), fewer than half (46%) of these patients received treatment for their anemia.[5] In patients who did receive anemia treatment, it was initiated at lower Hb levels (mean, 8.9 g/dL) than currently recommended by evidence-based guidelines for the treatment of cancer- and chemotherapy-related anemia.[6,7] Guidelines issued by the American Society of Hematology (ASH) and the American Society of Clinical Oncology (ASCO) recommend epoetin-α as a treatment option for patients with Hb d10 g/dL, with treatment for Hb >10 g/dL to <12 g/dL determined by clinical circumstances,[6] and guidelines issued by the National Comprehensive Cancer Network (NCCN) recommend erythropoietic therapy for patients with Hb d11 g/dL.[7] Thus, the continuing issues for clinicians are at what Hb level to initiate erythropoietic therapy, and to what level should Hb be increased in patients with hematologic malignancies and chemotherapy-related anemia to optimize clinical benefit. The objective of this review is to provide an overview of clinical studies of epoetin-α for the treatment of anemia in patients with hematologic malignancies.

Effect of anemia on quality of life

Cancer- or chemotherapy-related anemia is associated with debilitating symptoms, such as fatigue and weakness, that negatively affect patients' QOL, including their ability to continue to work and cope with their disease.[8-10] Fatigue in these patients has also been linked with depression, lack of motivation, and disturbances in mood and cognition.[9] A recent report highlighted the clinical significance of QOL improvements in patients with cancer and anemia.[11] In this study, QOL data from a randomized, double-blind trial evaluating epoetin α treatment (150 IU/kg to 300 IU/kg administered subcutaneously [SC] 3 times weekly [TIW]) in patients with cancer and anemia receiving chemotherapy were compared with population-normative QOL data, using the Functional Assessment of Cancer Therapy-Anemia (FACT-An) questionnaire.[11] Using this tool, QOL of the patients with cancer and anemia was found to be significantly lower than that of the normal population, and treatment of these anemic patients with epoetin-α was associated with clinically meaningful improvements in QOL.[11] A clinically meaningful QOL change has been interpreted as the minimally important difference in QOL scores between patients with cancer and anemia receiving epoetin α vs placebo.[12] For the FACT-General (FACT-G) and FACT-An scales, a 2.5-point and 4.2-point increase from baseline, respectively, is considered clinically meaningful.12 For the 100-mm Linear Analog Scale Assessment (LASA), clinically meaningful increases in QOL scores are 9.6 mm for Energy, 8.7 mm for Activity, and 9.8 mm for Overall QOL.[12]

Defining a target hemoglobin level

In retrospective analyses of data from 3 community-based trials of epoetin α administered 3 times weekly or once weekly to patients receiving chemotherapy with or without radiation for solid tumors or hematologic malignancies, the maximal incremental gain in QOL associated with a 1-g/dL increase in Hb occurred when Hb increased from 11 g/dL to 12 g/dL.[13] These results were confirmed by a subsequent incremental analysis in patients with solid tumors or nonmyeloid hematologic malignancies receiving radiation therapy with concomitant or sequential chemotherapy.[14] These incremental analyses suggest that even in patients with mild anemia (Hb e10 g/dL to d12 g/dL), historically left untreated, epoetin α can elicit increases in Hb that result in clinically important improvements in QOL. To maximize QOL outcomes in these patients, treating to a target Hb level of 12 g/dL should be considered a target Hb that is recommended by NCCN guidelines.[7] The current prescribing information for epoetin α in the US (PROCRIT®; Ortho Biotech Products, L.P.; Bridgewater, NJ, USA) suggests treating to a target Hb range of 10 g/dL to 12 g/dL in patients with nonmyeloid malignancies and chemotherapy-induced anemia, and withholding treatment if Hb exceeds 13 g/dL.[15]

Clinical trials of epoetin α in patients with hematologic malignancies and anemia

Epoetin α administered once weekly or three times a week has been shown to significantly increase Hb, reduce transfusion requirements, and improve QOL in 4 large, community-based, open-label trials of more than 7500 anemic patients with solid tumors and hematologic malignancies receiving chemotherapy with or without radiation.[16-19] Similar results have been reported from 3 randomized, double-blind, placebo-controlled trials in patients with multiple myeloma or other hematologic malignancies receiving either epoetin α[20,21] or epoetin β[22] (an epoetin analog not approved for use in the US) for the treatment of chemotherapy-related anemia. In addition, several small studies in patients with hematologic malignancies support the efficacy of epoetin α in correcting anemia associated with the cancer and its treatment.[23-29]

The efficacy of epoetin α in patients with hematologic malignancies and mild-to-moderate anemia has also been demonstrated in a randomized, double-blind, placebo-controlled trial.[30] Littlewood and colleagues evaluated the effect of up to 28 weeks of treatment with subcutaneous epoetin α 150 to 300 U/kg three times a week compared with placebo on hematologic and QOL outcomes in patients with solid tumors or hematologic malignancies receiving non-platinum chemotherapy in a randomized, double-blind, placebo-controlled, multicenter trial.[31] Patients eligible for this study were required to have Hb ≤10.5 g/dL. Patients with Hb >10.5 g/dL to ≤12.0 g/dL were also eligible if their Hb had decreased at least 1.5 g/dL per cycle or month since initiation of chemotherapy. Randomization was stratified by tumor type (solid or hematologic) and baseline Hb (≤10.5 g/dL vs >10.5 g/dL to ≤12.0 g/dL).[31] Of the 375 patients included in the intent-to-treat population, 251 received epoetin-α and 124 received placebo.[31] A total of 115/251 (46%) epoetin α patients and 58/124 (47%) placebo patients had hematologic malignancies (non-Hodgkin's lymphoma, multiple myeloma, chronic lymphocytic leukemia, and Hodgkin's disease), and a subgroup analysis was subsequently performed on the 173 patients with hematologic malignancies.[30] In addition, 16% of the epoetin α patients and 12% of the placebo patients had a baseline Hb >10.5 g/dL.[31]

The mean change in Hb from baseline, the percentage of responders (with response defined as an Hb increase ≥2 g/dL at any point during the study unrelated to transfusion) and transfusion rates for each treatment arm stratified by the overall intent-to-treat population, patients with hematologic malignancies, and patients with baseline Hb >10.5 g/dL are presented in Table 1. In patients with hematologic malignancies, treatment groups were similar with respect to mean baseline Hb (epoetin α, 9.9 g/dL; placebo, 9.7 g/dL) and distribution of hematologic malignan-

Table 1. Results From a randomized, double-blind, placebo-controlled trial of epoetin α in anemic patients with cancer receiving chemotherapy.[30,31]

	All patients	*Patients with hematologic malignancies**	*Patients with baseline Hb >10.5 g/dL*
Treatment group, n (%)			
Epoetin α	251 (100) [36/33/14/17]	115 (46)	42 (17)
Placebo	124 (100) [37/44/9/11]	58 (47)	15 (12)
Transfusion rate,%			
Epoetin α (n = 251)	25†	25	7
Placebo (n = 124)	40	43	20
Mean Hb increase from baseline, g/dL			
Epoetin α (n = 244)	2.2‡	2.2	2.3
Placebo (n = 115)	0.5	0.3	-0.4
% Responders, (Hb increase e2 g/dL)			
Epoetin α (n = 244)	71‡	75	81
Placebo (n = 115)	19	17	0

*CLL, chronic lymphocytic leukemia; Hb, hemoglobin; HD, Hodgkin's disease; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma. *Numbers in brackets indicate the proportion of patients with NHL, MM, CLL, and HD, respectively. †p =.0057; ‡p<.001.*

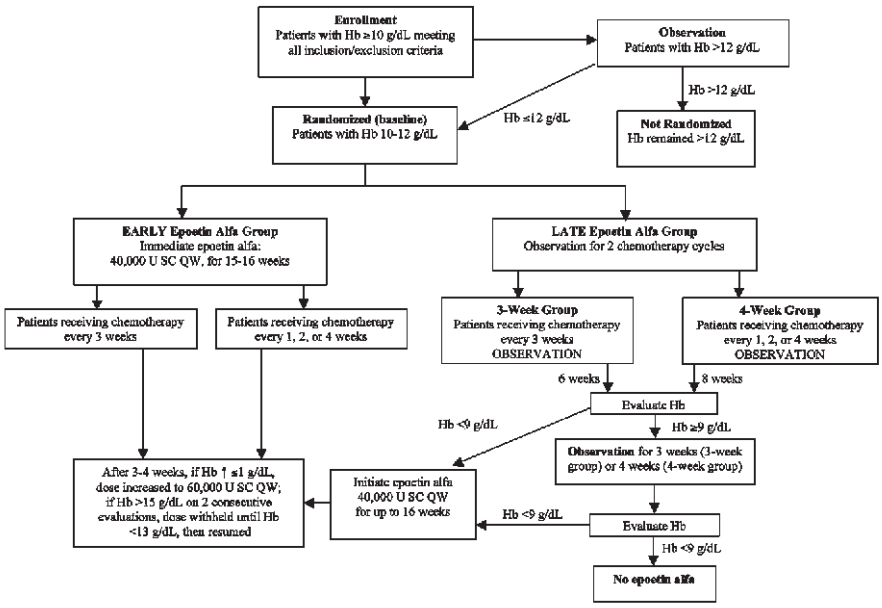

Figure 1. Treatment schema of a prospective, open-label, randomized trial of epoetin α treatment in mildly anemic patients with hematologic malignancies. Hb, hemoglobin; SC, subcutaneously; QW, once weekly; ↑, increase.

cies.[30] Patients with hematologic malignancies treated with epoetin-α had substantially better outcomes than placebo patients for Hb change (mean increase from baseline to final measurement, 2.2 g/dL vs 0.3 g/dL, respectively); hematologic response (75% vs 17%); transfusion utilization (mean rate after day 28, 25% vs 43%); and QOL improvements as measured by FACT-G, FACT-An, and LASA.[30,31] With respect to QOL improvements, patients with hematologic malignancies treated with epoetin alfa had clinically meaningful[12] improvements in FACT-G Total (5.8 points), FACT-An Fatigue (4.3 points), and LASA Energy (10.1 mm) and Activity (11.6 mm).[30]

In comparison, the overall study population (patients with solid tumors and hematologic malignancies) who received epoetin-α therapy had a mean increase in Hb of 2.2 g/dL (mean baseline Hb, 9.9 g/dL) whereas theplacebo recipients had a mean increase of 0.5 g/dL (mean baseline Hb, 9.7 g/dL); teh response rates were 71% and 19%, respectively ($p<0.001$ for each parameter).[31] In the overall study population, significantly fewer epoetin α-treated patients than placebo-treated patients were transfused after day 28 (25% vs 40%; $p = 0.0057$). In addition, patients treated with epoetin α had significantly greater improvements in QOL than did patients treated with placebo for FACT-G (+2.5 vs -3.6 points; $p = 0.004$); FACT-An Fatigue (+3.0 vs -2.2 points; $p = 0.004$); FACT-An Anemia (+4.0 vs -2.6 points; $p = 0.0007$); and LASA Energy (+8.1 vs -5.8 mm; $p = 0.0007$), Activity (+7.5 vs -6.0 mm; $p = 0.0018$), and Overall QOL (+4.8 vs -6.0; $p = 0.0048$). A subsequent multivariate regression analysis of these QOL data demonstrated a significant positive correlation between Hb increases and QOL improvements.[32]

In the subset of patients from the full cohort with a baseline Hb >10.5 g/dL, epoetin α patients had a mean increase in Hb of 2.3 g/dL from baseline compared with a mean decrease of 0.4 g/dL among the placebo-receiving patients. The response rate for patients with Hb >10.5 g/dL was 81% for those receiving epoetin α compared with 0% for those receiving placebo.[31]

Thus, the results of this study support the use of epoetin α in patients with solid tumors and hematologic malignancies with mild-to-moderate anemia receiving chemotherapy, and suggest that significant improvements in QOL can be attained with the correction of milder anemia.

Use of epoetin α in patients with hematologic malignancies and mild anemia

The results of the studies described here using epoetin-α therapy in patients with hematologic malignancies and anemia, the findings from the incremental analyses,[13,14] and data from other studies suggesting that earlier treatment with epoetin-α in patients with a variety of tumor types may be beneficial,[33] raise the question of whether patients with hematologic malignancies and mild anemia (Hb 10 g/dL to 12 g/dL) undergoing chemotherapy should receive epoetin-α therapy. In an effort to address this question, a recently completed prospective, open-label, randomized study evaluated hematologic response, transfusion requirements, QOL, and

health care resource utilization following treatment with epoetin α once weekly in mildly anemic patients with chronic lymphocytic leukemia, multiple myeloma, Hodgkin's disease, and non-Hodgkin's lymphoma scheduled to undergo chemotherapy for at least 4 months.[34] The objectives of the study were to evaluate the correlation between changes in Hb and QOL as measured by LASA and FACT-An, to compare the effects of immediate treatment of anemia with epoetin-α versus delaying treatment until anemia became more severe, and to assess whether improvements in QOL affected health care resource utilization and work/productivity. The study schema for this trial is presented in Figure 1. Final results from this study will provide further insight into whether treating patients with hematologic malignancies and mild anemia with epoetin-α at the start of chemotherapy is safe and has beneficial effects on Hb levels, QOL parameters, health resource utilization, and work/productivity outcomes.

Conclusions

Despite the high prevalence of anemia in patients with hematologic malignancies, recent survey results in Europe highlight the undertreatment of anemia in this population.[5] However, there is growing evidence of the value of anemia correction in patients with hematologic malignancies, both on Hb levels and QOL parameters. In both community-based and well-controlled randomized studies of anemic patients with hematologic malignancies, treatment with epoetin-α significantly increases Hb, decreases transfusion utilization, and improves QOL. Several studies have also suggested that earlier treatment with epoetin-α in patients with mild-to-moderate anemia can improve Hb and QOL.[31,33] Current evidence and guidelines suggest treating these anemic patients to a target Hb in the range of 10 g/dL to 12 g/dL. However, epoetin α administered at the start of chemotherapy in patients with hematologic malignancies and mild anemia (Hb e10 g/dL to d12 g/dL) may have favorable effects on QOL, health care resource utilization, and work/productivity in these patients.

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Pixantrone - a novel chemotherapy agent active in lymphoma

Pixantrone is a novel aza-anthracenedione with activity in experimental tumors. In animal models, this agent appears to have a reduced potential for causing delayed cardiotoxicity. Pixantrone has structural similarities with mitoxantrone as well as with anthracyclines. In preclinical studies it showed an antitumor activity superior to thato of mitoxantrone and doxorubicin, and exhibited excellent synergy with platinum.

Encouraging results were observed in two early studies. In a phase I study of pixantrone there were three long-lasting responses (two complete responses and one partial response): one of the complete responses lasted 24 months.

In a phase II study, 33 patients with aggressive relapsed non Hodgkin's lymphoma were treated with pixantrone as a single agent. There were nine (27%) confirmed responses (5 complete and 4 (12%) partial and four transient (unconfirmed) partial responses. Some of these responses were long-lasting and contnued for several months.

No clear drug-related or clinically relevant cardiac events were observed in the phase I studies. However, in these patients the cumulative exposure to pixantrone was low. In the phase II trial, in which more patients received higher therapeutic doses, four cardiac events occurred during the study period. All of these patients had been previously treated with full doses of doxorubicin, and had additional cardiac risk factors. These preliminary data suggest that pixantrone should not be used in patients with abnormal cardiac conditions. This novel agent is being further explored in indolent lymphomas as well as in aggressive lymphomas in combination with cytarabine. Pixantrone has also be used to replace doxorubicin in the CHOP regimen of combined chemotherapy.

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What drugs are behind the corner in the treatment of lymphomas?

We are in an exciting time in the management of patients with lymphomas. Therapies are moving away from the non-specific cytotoxic agents and towards more targeted approaches. A number of chemotherapy agents with unique mechanisms of action are currently available, including bendamustine, gallium nitrate, bortezomib, histone deacetylase inhibitors, and rapamycin analog (CCI-779). In addition, an ever growing number of biological agents is available for clinical trials, including monoclonal antibodies directed at a variety of target antigens (e.g. CD20, CD22, CD23, CD80, CD30), and antisense molecules to enhance the activity of these agents. The future lies in combining the chemotherapeutic and biological therapies in a rational manner that will optimize their activity, and to identify those patients most likely to respond to these strategies. Through these new technologies and with these unique agents we will be able to improve the cure rate of patients with lymphomas.

Introduction

Despite advances in the treatment of the lymphomas over the past few decades, results are clearly not satisfactory. Combinations of chemotherapy regimens such as CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) with the monoclonal antibody rituximab have extended the survival of patients with aggressive non-Hodgkin's lymphoma (NHL),[1] resulting in an increase in the number of patients being cured from about 35-40% with CHOP to about 60% with the combined therapy. Nevertheless, that means that almost 40% of patients will still die from this disease. Despite high response rates to a variety of therapeutic approaches, indolent NHL and mantle cell lymphoma (MCL) remain incurable disorders. Few effective options are currently available for refractory Hodgkin's lymphoma. Therefore, novel therapeutic strategies are needed to improve the prognosis of these patients.

Renewed interest in older agents

Gallium nitrate. In studies from the 1980s gallium was reported to produce a response rate in excess of 40% in both indolent and aggressive NHL.[2] Toxicities included hypocalcemia, nausea, vomiting, renal, and anemia without significant neutropenia or thrombocytopenia. Optic neuritis was a problem and hindered further development of this agent. Lower doses are prescribed when this drug is used to treat hypercalcemia, and optic neuritis has been rare. A recently completed multicenter phase II trial using current diagnostic and response criteria is undergoing analysis and will hopefully reproduce the earlier response rates without significant untoward effects.

Bendamustine. Bendamustine combines the properties of a nitrogen mustard with a purine analog. As monotherapy or as part of combination chemotherapy protocols for first-line or subsequent treatment, it has produced impressive response rates in patients with chronic lymphocytic leukemia (CLL) and NHL.[3] At least additive activity with rituximab has been demonstrated.[4] A study by Rummel *et al.*[4] accrued 60 patients, of whom 49 were evaluable, including 17 patients with follicular lymphoma (FL), 14 with MCL, 14 with immunocytoma and 4 with marginal zone NHL. Most had failed to benefit from either fludarabine or cladribine. The overall response rate was 92% including 65% complete remissions. Confirmatory studies are ongoing in the United States.

New agents

Bortezomib. The proteasome is a large, multicentric protease complex that is present in all eukaryotic cells. It degrades proteins adorned by ubiquitin, including those involved in cell cycle control and tumor growth. The proteasome is also required for activation of NFκB which maintains cell viability through the transcription of inhibitors of apoptosis and elaboration of adhesion molecules and growth factors. NFκB also induces drug resistance.

Bortezomib (PS-341, Velcade), the first proteasome inhibitor in clinical trials is a specific and selective inhibitor of the 26S proteasome,[5] and plays a regulatory role in multiple cellular pathways involving cell cycle, transcription factor activation, cell trafficking, and apoptosis. Bortezomib has recently been approved by the FDA for the treatment of relapsed and refractory multiple myeloma, following results of a trial in which more than 30% of relapsed and refractory patients responded.[6] A recently reported, randomized trial showed that this drug is superior to dexamethasone in relapsed and refractory patients.[7] Recent studies have demonstrated impressive activity with this agent in several histologic types of NHL as well.[8,9] In the experience of O'Connor *et al.*,[8] there was a complete remission (CR) and 5 partial remissions (PR) among 8 evaluable patients with FL, 5 PR in 10 patients with MCL, PR in both patients with marginal zone lymphoma, but no responses in those with small lymphocytic lymphoma. Goy *et al.*[9] reported 6 CR and 5 PR among 23 evaluable patients with MCL, and a CR and 2 PR among 19 evaluable patients with other B-cell lymphomas. The drug is reasonably well tolerated with adverse effects including sensory and motor neuropathy and thrombocytopenia.[10] Since Hodgkin's lymphoma has a high level of NFκB expression, this agent is currently in clinical trials in this malignancy within the CALGB as a single agent and, if activity is noted, will be entered into multiagent combinations.

Histone deacetylase inhibitors

Depsipeptide inhibits tumor cells through histone acetylation and inhibits signal transduction through MAP kinase and causes p53-independent G1 arrest. Either alone or in combination with hypomethylating agents, depsipeptide induces cellular proteins that may have critical effects on apoptosis, proliferation and susceptibility to immunologic manipulation. In phase I and II trials, 73% of evaluable patients with peripheral T-cell NHL or mycosis fungoides, showed objective responses, including two complete responses.[11] Toxicities included anemia, leukopenia, neutropenia, thrombocytopenia, fatigue, anorexia, nausea, vomiting, elevated AST/ALT, increased creatine phosphokinase, hypocalcemia, asymptomatic electrocardiographic changes and supraventricular arrhythmias. Studies are ongoing in CLL as well as in B-NHL and peripheral T-cell lymphomas.

Suberoylanilide hydroxamic acid (SAHA) is a small molecule that induces differentiation and/or apoptosis in transformed cells *in vitro* and inhibits tumor growth in animal leukemia and lymphoma models. SAHA inhibits the activity of class I and II HDACs and is selective in altering gene expression. SAHA exhibits synergistic anticancer activity with radiation, kinase inhibitors, cytotoxic agents and differentiating agents. It also has excellent bioavailability and is administered by the oral route. Phase I trials have been conducted with both intravenous and oral preparations. In the first trial in patients with lymphoma including 29 heavily pretreated patients, 25 of whom had NHL or Hodgkin's lymphoma, activity was observed in 6 patients including 1 CR and 1 PR 12. A phase II trial in aggressive NHL is in development. Another active HDAC inhibitor in clinical trials is MS-275.

Thalidomide derivatives

Initial interest in the use of thalidomide in the treatment of lymphomas was stimulated by its activity in multiple myeloma and Waldenström's macroglobulinemia.[13] Unfortunately, the activity has been minimal in indolent NHL based on a recently closed study conducted by the CALGB (unpublished data). A recent abstract did suggest important activity for the combination of thalidomide and rituximab in patients with relapsed and refractory mantle cell lymphoma.[14] However, the use of thalidomide is complicated by its impressive toxicity including somnolence, constipation and neuropathy. Newer generation immunomodulatory derivatives (IMiD) are currently in development. IMiD markedly stimulate T-cell proliferation, as well as interleukin-2 and interferon-γ (IFN-γ) production. CC-5013 (REVLIMID) is 50 to 2000 times more potent than thalidomide in stimulating T-cell proliferation and 50 to 100 times more potent than thalidomide in augmenting IL-2 and IFN-γ production.[15] In a phase I trial in 27 patients with refractory multiple myeloma there was no dose limiting toxicity up to 28 days; however, grade III myelosuppression was encountered after that point at the 50 mg/day dose making 25 mg/day the maximum tolerated dose.[16] Activity was noted in 71% of patients. Of particular note was the absence of the somnolence, constipation and neuropathy seen with thalidomide. The Cancer and Leukemia Group B (CALGB) will soon activate a randomized phase II trial in relapsed/refractory follicular NHL of rituximab versus rituximab plus REVLIMID.

Pixantrone is an anthracenedione with activity in relapsed and refractory NHL.[17,18] A potential benefit of this agent is that it causes less cardiotoxicity than do anthracyclines. Additional studies are ongoing to characterize its efficacy better.

Liposomal vincristine has a longer half-life than standard vincristine and reaches higher concentrations in tumors than nerves. Thus, it appears to be more active and better tolerated than the standard formulation and is being tested as a single agent and in combinations.[19]

Targeted and biological agents

Antisense oligonucleotides are chemically modified single-strand DNA molecules with a nucleotide sequence that is complementary to the target mRNA. Therefore, such agents are capable of inhibiting expression of the target gene. The Bcl-2 gene is a potentially important target because it is overexpressed in most follicular NHL and chronic lymphocytic leukemias, and in about a third of large B-cell NHL. Bcl-2 upregulation is thought to be responsible for maintaining the viability of tumor cells as well as inducing a form of multi-drug resistance. Elevated Bcl-2 also correlates with poor response to therapy in NHL. These observations, and others, have stimulated interest in exploring an antisense against Bcl-2 and other genes important to tumor survival.

In order to inhibit the target mRNA, antisense oligonucleotides must first be incorporated into cells by endocytosis. The oligonucleotide then inhibits gene expression by hybridization with the mRNA, followed by cleavage of the mRNA by recruitment of RNAse-H and other endonucleases.

G3139 (oblimersen sodium; Genasense (Genta Incorporated, Berkeley Heights, NJ, USA) is the first antisense molecule to be widely tested in the clinic for the treatment of human tumors. G3139 is a phosphorothioate oligonucleotide consisting of 18 modified DNA bases (i.e., an 18-mer) that targets the first 6 codons of Bcl-2 mRNA to form a DNA/RNA duplex.

In the first phase I study of G3139 in 21 patients with NHL,[20] a complete remission was attained in one patient with low-grade lymphoma who had progressive disease in nodes and bone marrow after 2 prior regimens; this response has continued for longer than

3 years. Subjective improvement was also noted in the majority of patients who entered the study with tumor-related symptoms. This agent is also active in relapsed/refractory patients with MCL.[21] Side effects primarily include neutropenia, thrombocytopenia, and fatigue. Although the response rate to oblimerson as a single agent is modest, it has potential to augment the activity of other agents, such as rituximab, fludarabine and cyclophosphamide, and, therefore, this drug will have its greatest impact in combination strategies. Such multiagent regimens are currently under clinical investigation including CHOP for lymphoma and fludarabine and rituximab for CLL.

New monoclonal antibodies

Epratuzumab (anti-CD22). Epratuzumab is an anti-CD22 monoclonal antibody with activity in both low grade and aggressive NHL.[22,23] A combination with rituximab has been evaluated; however, the results were not clearly superior to either agent alone suggesting that the optimal dose and schedule were possibly not used in that study.[24]

Galiximab (anti-CD80). CD80 is an immune costimulatory molecule present on the surface of NHL cells. Galiximab is a macaque-human chimeric anti-CD80 antibody with *in vivo* anti-lymphoma properties that is actively being studied in patients with refractory NHL. The antibody is well tolerated except for mild fatigue, nausea, and headaches, and has single agent activity of about 19% at the higher dose levels in phase I studies.[25] Based on preclinical data suggesting synergy, a phase I/II study of the combination of galiximab and rituximab has recently been completed and is undergoing analysis 26. A phase II study will soon be activated within the CALGB of the combination of rituximab and galiximab in previously untreated follicular NHL.

Humanized anti-CD20. Several humanized anti-CD20 monoclonal antibodies are also in early stages of clinical development. These are intended to reduce infusion-related events, thus shortening administration time, while potentially being more effective. One of these, *IMMU-106* has also been combined *in vitro* with the anti-CD22 monoclonal antibody, epratuzumab, and clinical trials will soon be underway.[27]

Lumiliximab (anti-CD23). For patients with CLL/SLL, a new primatized, anti-CD23 monoclonal antibody, is under evaluation. Lumiliximab (IDEC-152) has shown *in vitro* synergy with rituximab.[28] Phase I testing has been completed and demonstrated the safety and activity, of this agent, which is now in phase II trials.

Bevacizumab (Avastin). Angiogenesis factors such as basic fibroblast growth factor (bFGF) upregulate bcl-2, delaying programmed cell death.[29] Bevacizumab is a recombinant, humanized monoclonal antibody to vascular endothelial growth factor. This antibody has been approved in the US for the treatment of advanced colorectal cancer. However, data suggesting a potential role for angiogenesis in lymphoid malignancies have stimulated interest in the study of this agent in NHL as well. The Southwest Oncology Group has completed a phase II trial in relapsed aggressive NHL and the results are being analyzed.

Anti-CD30 monoclonal antibodies

The CD30 antigen expressed on both Reed-Sternberg cells in HL and the malignant cells of anaplastic large cell NHL provides an excellent target for antibody therapy. Several anti-CD30 antibodies are currently being studied in clinical trials. They have been well tolerated, but the dose and schedule need to be optimized for greater activity.[30,31]

Conclusions

We are in an exciting time in the management of patients with lymphomas. Therapies are moving away from the non-specific cytotoxic agents and towards more targeted approaches. Technologies such as genomics and proteomics provide the opportunity to develop disease-specific and even patient-specific therapies. There is an ever growing list of new biological and targeted agents. The future lies in combining biological therapies in a manner that will optimize their activity, with reduced dependence on the more toxic and non-specific cytotoxic drugs, in identifying those patients most likely to respond to these therapies, and in monitoring disease status and prevent recurrence. Applying our knowledge of the immunology and biology of these diseases to a rational combination of agents will finally lead to the realization of the goal of curing patients with indolent lymphoid malignancies.

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State of the Art

Chairmen: S. Amadori, F. Mandelli

Immunophenotyping: its past and present role in studies of human lymphoma

Prior to the 1980s, hematopathologists had to rely largely on classical morphologic features when diagnosing lymphoma. The establishment in the 1970s of techniques for making monoclonal antibodies therefore represented an important new advance. However, the first monoclonal antibodies were raised in immunology laboratories, where they were selected on the basis that they reacted with external epitopes on living white cells. As a consequence, although these antibodies could often be used on cryostat sections of human tissue, very few of them were reactive with paraffin-embedded tissue, and their value to the diagnostic pathologist was limited.

Paraffin-reactive monoclonal antibodies began to emerge in the 1980s but many of them were reactive with molecules that were not lineage-specific (e.g. CD15, CD43, CD75). It became evident that many of these molecules were carbohydrate epitopes or heavily glycosylated proteins, and it was not clear that it would ever be possible to produce antibodies reactive with classical lineage markers in routine biopsy samples. However, an antibody reported in 1984 was to prove of considerable significance in this regard.[1] This reagent, L26, was raised in a pathology laboratory in Hokkaido and selected, by screening on tissue sections, because of its high specificity for B cells. It reacted with paraffin-embedded tissue, but it failed to work by flow cytometry and it was not until 1990 that it was shown to react with an intracellular epitope on the CD20 molecule.[2] This observation provided evidence that a classical lineage-specific marker could be detected if the antibody was directed against the right epitope, and it complemented studies in the late 1980s in which it was shown that T cells in paraffin-embedded tissue could be detected using antibodies against intracellular epitopes on the epsilon chain of CD3.[3,4] These studies together suggested that antibodies will often react with leukocyte-associated molecules in paraffin sections if the target epitope is intracellular rather than on the cell surface, the reason being that intracellular protein sequences often lack the easily denatured three-dimensional structure characteristic of external epitopes.

Subsequently, *paraffin-reactive* antibodies against a substantial number of classical leukocyte surface molecules have been raised, and it is now possible to phenotype lymphomas in tissue sections almost as extensively as can be achieved using cell suspensions. Some of these markers represent an extension of work from immunological laboratories, where *CD* leukocyte molecules were defined. However, the list of markers detectable in biopsy tissues has been supplemented by a growing number of intracellular molecules identified not by immunologists but by biochemists or molecular biochemists, and the scope of phenotyping has expanded as a result. Early examples of clinically relevant markers of this sort included TdT, lysozyme and the anti-apoptotic protein BCL2.[5] More recent categories of intracellular markers detectable in routine sections comprise transcription factors (e.g. PAX-5, BCL6, MUM-1)[5] and molecules involved in cell signalling.[6] Molecules in the latter category show a degree of lineage restriction very similar to that of classical surface *CD* markers. They have the advantage that they are not confined to the surface membrane and therefore may show very extensive cytoplasmic labeling, and in some instances they are preferable to classical markers (e.g. in chronic lymphocytic leukemia, where expression of CD20, the classical *pan-B* marker, is often weak).

Future applications of immunohistological labeling

Antibodies suitable for use on paraffin-embedded tissue have proved crucial to the categorization of lymphoma entities (both in the sense of establishing entities, such as those of the REAL classification, and also in terms of the everyday process of practical diagnosis). What is the likely scope of immunophenotyping in the context of human lymphoma in the future, now that many issues of classification and diagnosis have been resolved?

Improving lymphoma diagnosis

The success of current classification schemes should not distract attention from the fact that some lymphomas are still difficult to diagnose with certainty. Some markers do not work well on routine biopsy material; and some entities are *diagnoses of exclusion*, defined by the absence of specific immunohistochemical markers. Mantle cell lymphoma is an obvious example of the former category (or at least was until recently), since the only specific marker (cyclin D1) was notoriously unreliable, and in practice diagnosis was often based on equivocal cyclin D1 staining and the absence of CD23. This problem has recently improved, however, with the availability of a better antibody against cyclin D1. However, in the context of lymphoplasmacytoid lymphoma or marginal zone lymphoma, diagnosis still relies heavily on morphology and the absence of markers of other entities. There is a clear need for

new markers that are selective for these disorders.

All lymphoma clinical services regularly encounter patients whose tumor is suggestive of Burkitt's lymphoma but not diagnostic (*Burkitt-like* lymphoma). In the REAL scheme such cases were thought frequently to represent diffuse large cell lymphomas that mimic Burkitt's lymphoma morphologically. However, the WHO classification argues that such cases (so-called *atypical Burkitt/Burkitt-like* lymphomas) are often a form of true Burkitt's lymphoma. It was also suggested that the title *atypical Burkitt/Burkitt-like* lymphoma should be used only *for cases with proven or with strong presumptive evidence of MYC translocation*, but in practice cytogenetic/fuorescent *in situ* hybridization (FISH) data are rarely available for routinely biopsied cases. Attempts to find a morphologic/phenotypic profile that correlates sufficiently closely with MYC translocation to be used as a surrogate have been unsuccessful, and new markers are needed to resolve such dilemmas.

Subdividing lymphoma categories

It appears unlikely that major new types of lymphoma will be defined in the future, at least in developed countries. However, several traditional categories have been subdivided in recent years into clinically distinct subgroups: for example, extranodal marginal zone lymphoma, chronic lymphocytic leukemia and diffuse large cell lymphoma are all now thought to be divisible into at least two subgroups. However, these subgroups were detected using molecular biological/genetic techniques that are unsuitable for routine use, and there is therefore a need for immunohistological markers for these sub-entities. Anaplastic large cell lymphoma represents a good example of how immunohistological techniques can replace molecular biological methods. The (2;5)(p23;q35) translocation and its variants are present in only a proportion of cases in this diagnostic category. The production of an anti-ALK monoclonal antibody provided a reliable and simple means of detecting this anomaly and made FISH and genetic methods largely redundant. Similarly, positive labeling of germinal centers for BCL2 is a good surrogate for the presence of the t(14;18) translocation. Several studies have now shown, in the case of chronic lymphocytic leukemia, that immunocytochemical labeling for the ZAP-70 kinase can be used as a marker for the Ig unmutated clinically less favorable form.[7,8] However, the correlation is far from perfect, and other markers should continue to be sought. The distinction between translocation-positive and -negative MALT lymphomas can also at present only be made by polymerase chain reaction (PCR) or FISH procedures, and an antibody-based procedure is also needed in this context. There are reports that the *germinal center* subtype of diffuse large B-cell lymphoma can be detected by immunostaining (for CD10 and BCL6), but other markers would also be of value. This natural progression from the initial detection of a genetic abnormality by molecular biological analysis to the development of robust immunohistological techniques of diagnostic value is likely to continue in the future as new lymphoma subgroups are defined.

Defining molecular functional status

The traditional aim of immunohistological studies is to establish whether a cellular marker is absent or present (and, if present, to make a semi-quantitative assessment of the intensity and cell-to-cell variation in expression). However, this throws no light on the functional status of the molecule, which may be highly relevant to understanding mechanisms of lymphomagenesis and defining subgroups. One approach to the immunohistological detection of the functional status of a molecule is to explore alterations in its subcellular localization. Molecules that relocate from the cytoplasm to the nucleus include the related molecules c-REL and BCL3 in mediastinal large B-cell lymphoma and anaplastic large cell lymphoma respectively,[9-11] suggesting activation of the NF-κ B pathway, and BCL10 in MALT and nasal lymphoma.[12-15] Furthermore, we have recently demonstrated nuclear relocalization of NFATc1, suggesting activation of the calcineurin pathway, in a subset of diffuse large B-cell lymphomas and also in Burkitt's lymphoma. It is therefore likely that a systematic search will reveal nuclear relocalization of other molecules indicative of signal pathway activation in other human lymphomas. Intracellular signaling is dependent on a dynamic and balanced process of phosphorylation and de-phosphorylation of a large number of different proteins. In the past we have shown that extensive phosphorylation of intracellular proteins by the hybrid ALK kinases in anaplastic large cell lymphomas results in strong immunohistological positivity for phosphotyrosine in routine tissue sections.[16] Other lymphomas do not show comparable degrees of tyrosine phosphorylation on immunohistological analysis,[17] but activation of signaling pathways (either physiological in origin or because of an acquired genetic event) may nevertheless occur and several laboratories have started to explore this possibility using antibodies specific for *phospho-epitopes*, i.e. epitopes created on a molecule when tyrosine, serine or threonine is phosphorylated. Preliminary results suggest that a minority of antibodies to phospho-epitopes are suitable for immunohistological use on paraffin sections.

Correlating genetic and immunohistological analysis

Recent years have seen the definition of many genetic abnormalities in human lymphoma, either via cytogenetic and gene cloning/sequencing studies or from microarray studies. It is clearly of potential value to see how these data correlate with immunohistological labeling, both to validate the data and to develop surrogate tests for underlying anomalies. The introduction of ZAP-70 as a marker of unmutated CLL (as referred to above) was prompted by gene expression data. Furthermore, we have recently shown that the broad defect in the expression of B-cell-associated genes identified by profiling Reed-Sternberg cell lines[18] is matched by abnormalities detectable by immunohistological labelling of routine biopsies.[19] However, it cannot be assumed that gene expression abnormalities always correlate with differences in protein detection identified by immunohistological labeling, and there is an ongoing need to compare gene expression profiles with immunohistological patterns.

Identifying new genetic abnormalities by immunohistological screening

One aim of cytogenetic analysis and gene expression profiling is to provide clues to underlying acquired chromosomal and genetic abnormalities, and this can indicate candidate molecules against which to produce new antibodies for immunohistological use. However, an alternative approach, which has yet to be widely explored, is to start by screening for immunohistological abnormalities and then to perform FISH analysis for genetic alterations or supervised cluster analysis of gene expression data. For example, it would be of interest to investigate whether the minority of cases of diffuse large B-cell lymphoma in which there is loss of signaling molecules show a distinctive pattern of RNA expression. Furthermore, the striking nuclear re-localization of NFATc1 in some diffuse large B-cell lymphomas and in Burkitt's lymphoma (*see above*), may point to a group of cases with abnormalities in gene expression that may be detectable by microarray profiling.

Identifying the normal counterpart of neoplastic lymphoid cells

The idea that hematopoietic tumors derive from a normal counterpart is widely held (although it can be difficult in some instances to prove), and in consequence the study of reactive lymphoid tissue is relevant to the study of lymphoma. For example, we recently identified rare mononuclear cells with a distinctive pattern of kinase expression (Syk- and Hck-positive) at the periphery of the thymus that may represent the cell of origin of some T lymphoblastic leukemias.[6] We have also identified a population of large B cells in T cell-rich (interfollicular) areas of lymphoid tissue. These *T-cell-associated large B cells*, which may show a dendritic morphology, have an Ig V region mutation profile characteristic of post-germinal center cells, and may be the peripheral equivalent of *asteroid* B cells in the thymic medulla.[20] It was suggested that they might represent the cell of origin of Hodgkin's disease, and there is recent support for this idea from two gene profiling studies of mediastinal B-cell lymphoma (a neoplasm believed to arise from B cells in the thymic medulla) which reported a similarity between this tumor and Hodgkin's disease.[21,11] This suggests that the asteroid B-cell type in the thymus can give rise, presumably through the acquisition of different genetic abnormalities, to these two different diseases. This model would also predict that a peripheral equivalent of mediastinal B-cell lymphoma may exist arising from interfollicular *T cell-associated large B cells*.

The immunophenotypic characterization of rare lymphoid cell types that may give rise to lymphomas (and that often escape the attention of immunologists) may therefore be of continuing relevance in the context of hematologic malignancies.

Identifying prognostic markers

One of the most widely pursued aims of immunohistological studies of lymphomas over many years has been to identify patterns of protein expression that are predictive of clinical behavior. However, there are several reasons why this approach, despite its popularity, should be treated with some caution.

Firstly, putative markers should be assessed within a homogeneous entity, to avoid confusion between proteins that are directly associated with an underlying genetic abnormality and prognostic markers within a disease subgroup. For example, cyclin D1 expression in small cell lymphomas is now viewed as prognostically unfavorable because it identifies a specific disease (mantle cell lymphoma) and not as an independent risk factor. By the same token, ALK protein has been cited as a favorable prognostic marker in *anaplastic large cell lymphoma*: however, many hematopathologists would argue that this is because immunostaining for this protein defines a distinct disease which is different from *ALK-negative ALCL*.

Secondly, any prognostic markers within a homogeneous entity are identified by statistical analysis of disease progression in large groups of patients. However, for the individual patient such findings translate into probability rather than absolute prediction, and they may be considered irrelevant to clinical management, particularly when there is a limited choice of alternative therapeutic strategies. For example, it is still rare for clinicians (or patients) to ask if a case of diffuse large B-cell lymphoma belongs to the good-prognosis *germinal center* category, even though the validity of this observation is accepted and immunohistological techniques are said to detect many of these cases.[22-25]

By the same token, BCL2 protein has been shown in several studies to be of prognostic significance in diffuse large B cell lymphoma, but this information rarely informs clinical decisions.

A third problem lies in differences between immunohistological results obtained in different laboratories. Many examples can be cited, including the fact that the prognostic significance of BCL2 in diffuse large B-cell lymphoma has been found in some studies but not others. Furthermore, prognostic associations based on the intensity or the number of positive cells are inevitably subject to observer bias. Immunohistological markers of prognostic significance are therefore not readily found, and one suspects that if new markers emerge in the future they will often prove to be secondary to underlying genetic alterations. However, with these caveats in mind, there is clearly an argument for continuing to search for new prognostic markers by screening clinical material (using tissue array techniques) with large panels of antibodies.

Conclusions

Immunophenotypic studies of human lymphoma have proved invaluable in the past for the establishment of lymphoma categories and for practical diagnosis. However, the list of antibodies suitable for use on routine biopsies continues to grow, with the consequence that the value of immunohistology in the context of lymphoma has still not been fully exploited.

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T-cell and NK-cell lymphomas: current status and new perspectives

Mature T-cell neoplasms are derived from mature or post-thymic T cells. Because NK-cells are closely related, and share some immunophenotypic and functional properties with T cells, these two classes of neoplasms are usually considered together.[1]

Mature T-cell and NK-cell neoplasms are relatively uncommon. In a large international study which evaluated lymphoma cases from the United States, Europe, Asia, and South Africa, T-cell and NK-cell neoplasms accounted for only 12% of all non-Hodgkin's lymphomas.[2] The most common subtypes of mature T-cell lymphomas are peripheral T-cell lymphoma, unspecified (3.7%) and anaplastic large cell lymphoma, T/null (2.4%).

Epidemiology

T-cell and NK-cell lymphomas show significance variations in incidence in different geographical regions and racial populations. In general, T-cell lymphomas are more common in Asia.[3] These differences result from both a true increased incidence, as well as a relative decrease in the frequency of many B-cell lymphomas, such as follicular lymphoma, seen commonly in North America and Europe. One of the main risk factors for T-cell lymphoma in Japan is the virus, HTLV-1. In endemic regions of southwestern Japan, the seroprevalence of HTLV-1 is 8-10%. The cumulative life-time risk for the development of adult T-cell leukemia/lymphoma (ATLL) is 6.9% for a seropositive male and 2.9% for a seropositive female.[4] Other regions with a relatively high seroprevalence for HTLV-1 include the Caribbean basin, where Blacks are primarily affected over other racial groups.[5] Differences in viral strain may also affect the incidence of the disease.[6,7]

Another major factor affecting the incidence of T-cell and NK-cell lymphomas is racial predisposition. Nasal and nasal-type NK-cell lymphomas, and aggressive NK-cell leukemia are much more common in Asians than they are in other races.[8] In Hong Kong, nasal NK-cell lymphoma is one of the more common subtypes, accounting for 8% of cases. By contrast, in Europe and North America, it accounts for less than 1% of all lymphomas. Other people at increased risk for this disease are individuals of Native American descent in Central and South America, and Mexico.[9,10] These populations are genetically linked to Asians, and are believed to have emigrated to the American continent from Asia, either over the Aleutician land bridge or a water route.[11] Overall, the incidence of T-cell and NK-cell malignancies does not appear to be changing, although long term epidemiological data are not available, as it is only recently with modern immunophenotypic and molecular tools that these neoplasms have been reliably distinguished from B-cell lymphomas.

Survival

T-cell and NK-cell lymphomas as a group are clinically aggressive. Patients have a much poorer response to therapy and shorter survival than those with either B-cell lymphomas or Hodgkin's lymphoma.[2] One of the few T-cell lymphomas with a good response to therapy is anaplastic large cell lymphoma, systemic. The poor response to therapy may be due to an intrinsic drug resistance for many of these neoplasms.[12] In addition, many T-cell and NK-cell neoplasms present with advanced stage disease, also conferring a poor prognosis.[13] T-cell lymphomas have been most commonly treated with standard chemotherapeutic protocols largely developed for B-cell lymphomas, and then empirically applied to T-cell diseases. Few clinical trials have been restricted to the T-cell lymphomas, in part due to the relative rarity of these neoplasms. Recently, more targeted therapies have been developed for some diseases, such as adult T-cell leukemia/ lymphoma. For example, antiviral therapy with zidovudine and interferon α has produced complete remissions in some untreated patients.[14] Some protocols have used immunotherapy, coupled with toxins or radionuclides, to target highly expressed antigens on the surface of the neoplastic cells.[15-18] On a theoretical basis it should be possible to target the upregulated ALK tyrosine kinase in anaplastic large cell lymphoma, analogous to the molecularly targeted therapy of chronic myelogenous leukemia.[19]

Pathophysiology

T-cell lymphomas manifest the immunophenotypic features of post-thymic T lymphocytes. There are two major classes of T cells: $\alpha\beta$ T-cells and $\gamma\delta$ T cells.[20] This distinction is based on the structure of the T-cell receptor. The $\alpha\beta$ and $\delta\gamma$ chains are each composed of an external variable (V) and constant (C) portion. They are both associated with CD3, which is identical in both T-cell subsets. CD3 contains γ,δ, and ε chains. While NK-cells do not have a complete T-cell receptor complex, they do usually express the ε chain of CD3 in the cytoplasm, which can be recognized by polyclonal antibodies to CD3.

$\gamma\delta$ T cells are $CD8^+$, or $CD4^-/CD8^-$, and also CD5. Along with NK cells, they are cytotoxic effector cells. Both are components of the innate immune system, and do not require antigen sensitization to be active. $\gamma\delta$ T cells comprise less than 5% of all normal T cells, and show a restricted distribution, being found mainly in the splenic red pulp, intestinal epithelium, and other epithelial sites. It is notable that these sites are

more commonly affected by γδ T cell lymphomas, which are relatively rare.[21-23] γδ T cells are not MHC restricted in their function, and represent a first line of defense against bacterial peptides, such as heat shock proteins.[20] They are often involved in responses to mycobacterial infections, and mucosal immunity.

More recently, the pattern of cytolytic molecules has been investigated and correlated with both cellular origin and function. For example, to date five granzymes have been demonstrated in human cells.[24] These enzymes are similar in structure, but differ in their substrate specificity and chromosomal locations. Granzyme M (Gr M), a novel member of this family, has unusual enzyme specificity, preferring cleavage after methionine, leucine, or norleucine.[25,26] It has been suggested that this enzyme may play role in the effector phase of innate immune responses.[27] Its expression is restricted to NK cells, $CD3^+$ $CD56^+$ T-cells, and γδ T cells, but it is absent in other cytotoxic T-cell subsets. Therefore, it may serve as a marker of the innate immune system, as distinguished from cytotoxic T cells belonging to the adaptive immune system.

In a recent study investigating the expression of Gr M in T-cell and NK-cell lymphomas, Gr M was preferentially expressed in nasal NK/T-cell lymphomas (100%), γδ T cell lymphomas (100%), and ETTCLs (85%), but was largely negative in other cytotoxic T-cell malignancies including ALCL (6%), and subcutaneous panniculitis-like T-cell lymphoma (SPTCL) (11%).[28] As expected, it was also negative in mycosis fungoides/Sézary's syndrome (3%), and angioimmunoblastic T-cell lymphoma (0%), neither of which has cytotoxic features. These results suggest that the Gr M-positive lymphomas are derived from innate immune cells.

The innate immune system is distinguished from the adaptive or antigen-driven immune system, which is dependent on antigenic sensitization. Most T cells in peripheral blood and peripheral lymphoid organs belong to this system. This T-cell compartment is heterogeneous and functionally complex, and includes naïve, effector (regulatory and cytotoxic), and memory T-cells. $CD4^+$ T cells are primarily regulatory, acting via cytokine production, while $CD8^+$ (and double negative) T cells are primarily cytotoxic. Most nodal peripheral T cell lymphomas are derived from these more functionally mature T cells.

αβ T cells are divided into two major subtypes, $CD4^+$ and $CD8^+$. In normal lymphoid tissues, $CD4^+$ cells exceed $CD8^+$ cells, and a similar ratio is seen among malignant diseases. CD4 T cells or *helper T cells* are mainly cytokine secreting cells, whereas CD8 T cells are mainly involved in cytotoxic immune reactions. CD4 cells are divided into two major types, based on their cytokine secretion profile. Th1 cells secrete interleukin (IL)-2 and interferon γ, but not IL-4, 5, or 6. By contrast, Th2 cells secrete IL-4, 5, 6, and 10.[20] Th1 cells provide help mainly to other T cells and macrophages, whereas Th2 cells provide help mainly to B cells, in their production of antibodies.[29]

Many of the clinical manifestations of T-cell lymphomas can be related to cytokine expression by the neoplastic cells. For example, the hypercalcemia associated with ATLL has been linked to secretion of factors with osteoclast-activating activity.[30-32] The hemophagocytic syndrome seen in many T-cell and NK-cell malignancies has been associated with secretion of both cytokines and chemokines.[33,34]

NK cells share some functions and markers with cytotoxic T cells. They can express CD2, CD7, CD8, CD56, and CD57, all of which can be seen in some T-cell subsets. As noted above, they also are often positive for the ε chain of CD3. However, they are usually positive for CD16, which is less often positive on T cells. Both NK cells and cytotoxic T cells express cytotoxic proteins, including as perforin, granzyme B, and T-cell intracellular antigen (TIA)-1.[35] These antigens are also seen on cytotoxic T-cell and NK-cell malignancies.[36]

Subtypes of T-cell and NK-cell lymphomas

The classification of T-cell and NK-cell neoplasms proposed by the WHO emphasizes a multiparameter approach, integrating morphologic, immunophenotypic, genetic, and clinical features (Table 1). Clinical features are of particular importance in the subclassification of these tumors, in part due to the lack of specificity of other parameters. T-cell lymphomas show great morphologic diversity, and a spectrum of histological appearances can be seen within individual disease entities. The cellular composition can range from small cells with minimal atypia to large cells with anaplastic features. Such a spectrum is seen in anaplastic large cell lymphoma, adult T-cell lymphoma/leukemia, and nasal T/NK-cell lymphoma, as selected examples. Moreover, there is morphologic overlap between disease entities. Many of the extranodal cytotoxic T-cell and NK-cell lymphomas share similar appearances, including prominent apoptosis, necrosis, and angioinvasion.[37]

In contrast to B-cell lymphomas, specific immunophenotypic profiles are not associated with most T-cell lymphoma subtypes. While certain antigens are commonly associated with specific disease entities, these associations are not entirely disease-specific. For example, CD30 is a universal feature of anaplastic large cell lymphoma, but can be expressed, usually to a lesser extent, in other T-cell and B-cell lymphomas. Similarly, while CD56 is a characteristic feature of nasal NK/T-cell lymphoma, it can be seen on other T-cell lymphomas, and even multiple myeloma.[38-40] Additionally, within a given disease entity, variation in the immunophenotypic features can be

Table 1. Mature T-cell and NK-cell neoplasms.

Leukemic or disseminated
T-cell prolymphocytic leukemia
T-cell granular lymphocytic leukemia
Aggressive NK-cell leukemia
Adult T-cell lymphoma/leukemia (HTLV1+)
Extranodal
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-type T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Cutaneous
Mycosis fungoides/Sezary's syndrome
Anaplastic large cell lymphoma, primary cutaneous type
Nodal
Peripheral T-cell lymphoma, not otherwise characterized
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma, systemic type

seen. For example, hepatosplenic T-cell lymphomas are usually of γδ T-cell phenotype, but a minority of cases is of αβ derivation.

Finally, in contrast to B-cell lymphomas, there are no convenient immunophenotypic markers of monoclonality, although the presence of an aberrant immunophenotype may point towards a diagnosis of malignancy[41] Therefore, molecular studies, most commonly polymerase chain reaction (PCR) studies for rearrangement of the T-cell receptor genes, are generally required in order to evaluate the clonality of a T-cell proliferative process.[42,43] Presently, specific genetic features have not been identified for many of the T-cell and NK-cell neoplasms. One of the few exceptions is anaplastic large cell lymphoma, which is strongly associated with the t(2;5) and other variant translocations.[44] However, the molecular pathogenesis of most T-cell and NK-cell neoplasms remains to be defined.

For the above reasons, clinical features at present play a major role in the subclassification of T-cell and NK-cell neoplasms. Several broad clinical groups are delineated: (i) leukemic or disseminated; (ii) nodal; (iii) extranodal; and (iv) cutaneous. The diseases within these broad clinical groupings often share clinicopathologic and immunophenotypic features.[37]

New perspectives

Most aspects of the WHO classification have been validated since its publication, but new insights have been obtained for some entities. Blastic NK-cell lymphoma is a disease that was postulated to be of precursor NK-cell derivation, based on the expression of CD56.[45] However, more recent studies suggest that the cell of origin is a precursor to plasmacytoid monocytes, a specialized type of dendritic cell.[46-48] This cell is closely related to the myeloid/macrophage system, and the disease has a high incidence of bone marrow involvement. It is not know n why the skin is so frequently involved,[49] but there is also a relatively high incidence of cutaneous involvement in more mature plasmacytoid monocyte proliferations.[50]

Further insights have been obtained regarding neoplasms derived from γδ T-cells. Hepatosplenic T-cell lymphoma was the only tumor type regularly recognized as being of γδ T-cell origin. However, mucocutaneous γδ T-cell lymphoma appears to be emerging as a distinct entity.[21] These tumors are aggressive, and tend to involve skin and mucosal sites with high frequency. Although they may resemble other entities, such as subcutaneous panniculitis-like T-cell lymphoma (SPTCL), they are clinically more aggressive.[51] In addition, histological differences are seen. For example, in cutaneous γδ T-cell lymphomas, the infiltrates often extend from the epidermis to the subcutaneous tissue, whereas typical SPTCL is usually restricted to the subcutis. Functionally γδ T-cell lymphomas are part of the innate immune system, representing a primitive type of immune response.[28]

The WHO classification includes both ALK$^+$ and ALK$^-$ tumors under the heading of anaplastic large cell lymphoma (ALCL), systemic, but recommends that the presence or absence of expression of ALK be stated in each case. The exact nature of ALK$^-$ ALCL remains controversial.[52,53] Whether these cases constitute a distinct entity, or are part of the spectrum of peripheral T-cell lymphoma, unspecified, remains to be determined. The spectrum of translocations involving gene partners other than *NPM* in ALCL has been expanding.[54] Moreover, somewhat unexpectedly, the classical *NPM/ALK* translocation has been identified in cases of ALK$^+$ large B-cell lymphoma.[55] Further insights into the molecular pathogenesis of T-cell lymphomas in general should help to resolve many issues regarding their classification, and, potentially, their treatment.

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Vaccine therapy: mobilizing immune attack against lymphoma

When planning an attack, it is first useful to understand the enemy. In terms of lymphoma, the ten years following the REAL classification have been highly productive in adding to the knowledge of lymphoma at the molecular level. Gene expression profiling, in particular, has emerged as a technology which can provide insight into the subsets which exist within disease categories, especially in diffuse large B-cell lymphoma (DLBCL).[1,2] This approach might also reveal new targets for immune attack. In parallel, genetic analysis of the B-cell receptor (BCR), a critical molecule for survival of normal peripheral B cells, continues to reveal new features of B-cell malignancies.[3] This has been clearly illustrated for chronic lymphocytic leukemia, in which the presence or absence of somatic mutations in the immunoglobulin (Ig) variable (V) region genes is now a major prognostic indicator.[4,5] For follicular lymphoma (FL), the presence of somatic mutations in the *IgV* genes, and the continuing accumulation of intraclonal mutations, reflects the influence of the germinal center (GC) site and points to stimulation of tumor cells via the BCR. The question now is how this stimulation is maintained and whether it influences tumor growth and survival. Once this is understood, rational approaches to immune attack will be facilitated.

Surface Ig as a critical molecule for B-cell tumors of the germinal center

Tumors commonly rely on interaction with environmental elements to maintain growth and survival, and lymphomas are no exception. There are many strategies to achieve this, but in the case of B-cell tumors of the GC, we have recently implicated surface Ig (sIg) in this interaction. For FL, the fact that tumor cells retain expression of sIg, even when one allele is disrupted by the t(14;18) translocation, points to a potential role in maintenance or growth. By analyzing the *IgV*-gene sequences, we found that FL cases have acquired sequence motifs appropriate for addition of oligosaccharide chains. Similar sites are infrequent in normal B cells, but are detectable not ony in FL, but also in a subset of DLBCL, and in certain other tumors located in the germinal center.[6,7] Sites are introduced by somatic mutation and, since they appear to be positively selected, could be involved in tumor pathogenesis. The heterogeneity of expression in DLBCL is interesting, and we are currently investigating the incidence of sites in the subsets defined by expression of critical genes which appear to have different clinical behavior.[8]

We have now identified the added sugars as oligomannose-type glycans. Importantly, the sugar pattern differs from that in the sIg Fc region, which contains

the expected range of complex type glycans, indicating that the normal glycan processing pathway is intact. The *unfinished* oligomannose glycans, uncommon on cell surface glycoproteins, are apparently exposed, since they can bind specifically to mannose-binding lectin (*manuscript in preparation*). There appears, therefore, to be a contribution to tumor pathogenesis that involves molecules of the innate immune response and that is independent of classical antigen recognition. Interruption of binding of oligomannose glycans to lectin-like receptors could provide a novel specific targeted therapy. These findings might also have relevance for the success of anti-idiotype antibody in the treatment of FL, since antibody could interfere with this binding.

Idiotypic Ig as a target for immune attack

Idiotypic (Id) determinants expressed by sIg of lymphoma cells provide ideal targets for immune attack.[9] Antibody is a key mediator of attack,[9] and the efficacy of anti-Id antibody therapy is intriguing.[10] Clearly lymphoma cells are susceptible to antibody attack, as is evident from the success of anti-CD20,[11] and it is possible that anti-Id cold be even more effective due to direct action via the BCR.[12,13] However, technical difficulties in making individual Id-specific antibodies make this approach untenable for wide clinical application. It is slightly easier to make Id protein vaccines, and clinical trials of Id protein coupled to KLH are producing promising outcomes,[14,15] with a multicenter randomized trial now in progress. In addition to antibody, T cells recognizing peptides derived from somatically mutated sequences in the V-region complementarity determining regions (CDR) have been detected in vaccinated patients and could also have a role in clinical effectiveness.[16] Even Id protein vaccines are technically demanding, and, to reduce this, we opted to develop DNA vaccines containing the Id-encoding variable region genes, VH and VL, linked together as a single chain Fv (scFv).[17,18] The scFv appears able to fold and express Id determinants of the parental Ig.[19]

DNA fusion vaccines

DNA vaccines offer an opportunity to deliver specific tumor antigens in a form to induce selected effector pathways. The backbone CpG motifs in the plasmid activate innate immunity, releasing cytokines which drive a Th1-dominant response against encoded antigen.[20] While injected muscle or skin cells provide a depot of antigen, transfer to antigen-presenting cells must occur, and design modifications can target expression to the endoplasmic reticulum or to intracellular sites. In the case of scFv, we chose to direct protein to the endoscopic reticulum to ensure optimal folding. However, in spite of activation of innate immunity, scFv alone was poorly immunogenic.[18] We therefore fused a gene encoding a non-toxic fragment (Fragment C (FrC)) of tetanus toxin to the scFv in order to activate T-cell help. This dramatically improved performance in pre-clinical models, leading to anti-Id protection against lymphoma.[18,21] The immunoenhancing action of the FrC is likely to be due to an amplification of T-cell help. By harnessing $CD4^+$ Th cells from the large, non-tolerized anti-microbial repertoire, it may be possible to bypass or reactivate the anergized anti-tumor T cells. This strategy of linked T-cell help activated via fusion genes can promote both antibody and effector T cells.[22] In terms of induction of antibody, these fusion gene vaccines can also be effective for other antigens[21,23] and offer a simple strategy to raise monoclonal antibodies against any antigen without having to isolate the protein. Other groups have used different immunoenhancing molecules delivered via DNA. One trial used mouse Ig constant region sequences to amplify immunity against Id determinants, but only weak responses were induced, possibly because the foreign sequences were not sufficiently immunogenic in human subjects.[24] Many molecules are also being tested in DNA vaccines with the aim of increasing co-stimulation, innate immunity and cytokine levels.[25] The possibilities of manipulating immunity are almost infinite.

Clinical trial

The striking pre-clinical results with DNA scFv-FrC have allowed us to move to a pilot clinical trial of DNA scFv-FrC fusion genes in patients with follicular lymphoma. DNA is injected at an intramuscular site at weeks 0,1,2,4,8 and 12 with a dose escalation from 500-2,500 μg per injection. Individual vaccines are required, and each scFv protein has to be expressed as a recombinant molecule for measuring immune responses. This slows progress and the trial is still proceeding, but we have made preliminary assessments of responses against both the FrC and Id components of the vaccine in 10 patients. Encouragingly, 8/10 have responded to FrC by increasing antibody levels or by an increased T-cell response. Although these are memory responses due to previous exposure to tetanus toxoid, they do at least show that the vaccines are presenting antigen to the immune system. The two patients who failed to respond had initial splenic involvement with tumor and may have had residual disease. This confirms the initial observation that vaccination will operate best when tumor burden is very low or absent. T-cell responses to Id were seen in 5/7 of those evaluated among the 8 responders to toxoid. All 5 patients remain in complete remission or with very low volume of local disease at 1-2 years from the start of vaccination.

Modified DNA fusion vaccines to activate $CD8^+$ cytotoxic T cells

For most tumors, attack by cytotoxic T cells (CTL) is likely to be the most effective strategy. Not only are

CTL able to kill target cells, but the range of intracellular tumor antigens expressed only as peptides associated with MHC class I is large. We have therefore re-engineered our initial fusion gene design to optimize induction of CTL.[26] Our new vaccines contain a minimized FrC sequence to avoid potentially competitive MHC class I-binding epitopes. The phenomenon of immunodominance noted in natural CTL responses induced by viral infection tells us that the response tends to focus on a limited number of epitopes, and we need to ensure that these are tumor-derived.[27] A second design modification was to place an encoded tumor-derived peptide sequence at the C-terminus of the minimized FrC sequence.[26,27] This leads to efficient liberation of the target peptide by aminopeptidases in the endoscopic reticulum, followed by binding to the MHC class I in that site. In several models, we have shown a highly efficient induction of peptide-specific CTL able to kill target tumor cells.[28] So far, we have been able to induce immunity against cancer testis antigens, overexpressed transcription factors and viral epitopes. We have demonstrated that tolerance can be broken in models by inducing CTL against H-Y male-specific epitopes in males. Combinations of vaccines are being explored for dual attack on separate antigens to ensure that tumor cells cannot escape by antigen loss. In terms of clinical testing, we are currently using the design to induce CTL in transplant donors against an epitope from cytomegalovirus. The concept here is to protect the recipient against dangerous reactivation of virus during the vulnerable period of immunosuppression.

Problems in translating of DNA vaccines into the clinic

The common problem of translating results in pre-clinical models to human subjects is particularly acute for DNA vaccines. One reason for this is that certain critical limitations, such as dose and volume of DNA injected, are difficult to overcome. For example, it is known that if the optimal volume of 50 μL used for intramuscular injection in mice is reduced to 5 μL, all response is lost.[29] It is clearly impossible to increase the 50 μL appropriately on a weight basis in human subjects, and it is likely that our volume of 1 mL is not ideal. There are two simple physical strategies which could overcome this limitation, electroporation and injection of DNA attached to microparticles. Both have been described in detail, and it is clear that they amplify response to naked DNA dramatically.[29,30] The mechanism is unclear, but in both cases is likely to include higher transfection rates, together with increased inflammatory responses, possibly involving attraction of antigen-presenting cells to the injection site.[29,30] Both electroporation and microparticle injection are in current clinical use for different applications, making their addition to DNA vaccination feasible. We have focused initially on electroporation and have found that it amplifies both antibody and cellular responses dramatically in pre-clinical models. To test the approach in patients, we are currently planning a clinical trial of electroporation using a DNA fusion vaccine against prostate cancer.

A popular approach, especially in the field of infectious diseases, is the so-called *prime/boost* vaccination strategy.[31] It appears that priming with naked DNA, followed by boosting with the same antigen delivered via a viral vector, is particularly effective in amplifying immune responses. Non-replicating pox viruses or adenoviruses are preferred vectors, and modified vaccinia Ankara (MVA) is particularly attractive as a vehicle since a number of host range and virulence genes have been deleted by extensive passaging *in vitro*. The principle has been tested in pre-clinical models and in human subjects using vaccines against human immunodeficiency virus[32] and malaria.[33] While exciting results are emerging, there is the concern that it could be difficult to use live viral delivery systems in immunosuppressed patients. There is also the problem that the ensuing immunity against components of the viral vector will suppress the further boosts likely to be needed to control residual cancer cells. In our recent experience, *prime/boost* can be carried out by using different DNA fusion vaccine designs, or by changing the physical delivery method, and this might have the same effect while avoiding the problems of viral vectors.

Concluding remarks

The REAL classification provided the foundation for understanding the nature of lymphoid malignancies at the molecular level. It has also added to our knowledge of the normal cellular counterparts of the tumor cells, thereby expanding our immunological expertise. We should now know how to use the immune system to attack lymphomas, and the first successes are already evident from antibody therapy. If we draw an analogy with infectious diseases, antibody might hold disease in check, but it is unlikely alone to effect a cure. Vaccination can both prevent infection and be used to treat infection *in situ* as was shown first by Pasteur with rabies. The tools of genomics are available both for identifying new targets, and for designing novel delivery systems. Once we can learn to use those tools effectively we should be able to offer patients a simple and non-toxic strategy to control tumor growth. However, patience is required in translational research, and regulatory restraints slow progress. The other question is whether drug treatment and clinical management can bring patients into remission with an intact immune capacity. This is a challenge, but there is little point in asking a defeated immune system to respond to an inadequate vaccine. The disappointing results from some trials in this setting should not be taken as a negative comment on

the reality of vaccination. It may be that refined immunologic responses will be the first surrogate endpoint, and that we have to wait to assess impact on disease.

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Marginal zone lymphoma: one or more entities

Chairman: F. Cavalli

The molecular pathology of mucosa-associated lymphoid tissue lymphoma

In the pathogenesis of mucosa-associated lymphoid tissue (MALT) lymphoma, *H. pylori* infection generates immunological responses that stimulate the growth of malignant B cells. This bacterial infection also triggers inflammatory responses by attracting and activating neutrophils. Activated neutrophils release reactive oxygen species that can cause a wide range of genetic damage and may thus play a role in the acquisition of genetic abnormalities in gastric MALT lymphoma. Three chromosomal translocations are associated with MALT lymphoma.

t(11;18)(q21;q21). In most cases positive for this translocation, t(11;18)(q21;q21) is the sole chromosomal aberration. In contrast, MALT lymphomas that are negative for this translocation commonly show a wide range of chromosomal aberrations, including trisomy of chromosomes 3, 12 and 18.[1,2]

In 1999, Dierlamm *et al.*, and subsequently Akagi *et al.* and Morgan *et al.* cloned the breakpoint of t(11;18)(q21;q21) and the genes involved were identified.[3] The translocation caused reciprocal fusion of the API2 and MALT1 genes. API2 inhibits the biological activity of caspases 3, 7 and 9 and is believed to be an apoptosis inhibitor. *MALT1* is involved in antigen receptor mediated NF-κB activation. As only the *API2-MALT1* but not the *MALT1-AP12* fusion transcript is consistently expressed in MALT lymphoma, the *API2-MALT1* fusion is likely to be oncogenic.[3]

The translocation occurs at variable frequencies within MALT lymphomas depending on the site of origin of the tumor, the frequency ranging from 0% in thyroid and salivary gland MALT lymphomas to 30% in gastric and 40% in pulmonary MALT lymphomas.[4-7] The translocation is more frequently seen in cases showed dissemination to regional lymph nodes or distal sites than in those confined to the stomach, and is associated with those not responding to *H. pylori* eradication.[6,8,9] Despite the strong association of t(11;18)(q21;q21) with adverse clinical features, the translocation is only rarely seen in transformed MALT lymphoma.[10]

The biological importance of the *API2-MALT1* fusion is indicated by the analysis of breakpoints at both the genomic and transcript levels. The *API2-MALT1* fusion transcripts are always in frame.[11] The API2 gene contains three N-terminal bacoluvirus IAP repeats (BIR), a middle caspase recruitment domain (CARD) and a C-terminal zinc binding RING finger domain. The MALT1 gene comprises an N-terminal death domain, followed by two Ig-like domains and a caspase-like domain. Within the API2 gene, all breakpoints occur downstream of the third BIR domain but upstream of the C-terminal RING domain, with 91% just before the CARD. In the MALT1 gene, the breakpoints occur in four different introns upstream of the caspase-like domain.[4-7] Thus, the resulting API2-MALT fusion transcripts always comprise the N-terminal region of API2 with three intact BIR domains and the C-terminal MALT1 region containing an intact caspase-like domain. The specific selection of these domains of the API2 and MALT1 gene to form a fusion product strongly suggests that they are required for the oncogenic activities of the fusion product.

t(1;14)(p22;q32). Like translocation-negative MALT lymphomas, t(1;14)(p22;q21)-positive cases often show trisomy of chromosomes 3, 12 and 18.[12] This translocation occurs in approximately 5% of MALT lymphomas and the translocation-positive gastric cases are typically those at advanced stage, which are unlikely to respond to *H. pylori* eradication.[7] Cloning of the breakpoint showed that t(1;14)(p22;q32) brings the *BCL10* gene under the control of the immunoglobulin gene heavy chain enhancer and deregulates its expression.[13] BCL10 contains a CARD domain in its amino terminal region. *BCL10* knockout mice studies showed that *BCL10* is essential for both the development and function of mature B and T cells and plays an additional role during neural tube closure.[14,15] In lymphocytes, *BCL10* specifically links antigen receptor signaling to the NF-κB pathway.[14,15] NF-κB activation in lymphocytes leads primarily to cellular activation, proliferation, survival and induction of effector function. In line with these observations, transgenic mice in which *BCL10* was linked to an Ig enhancer-containing construct showed a dramatic and specific expansion of splenic marginal zone B-cells, reminiscent of human marginal zone lymphoma.[16]

t(14;18)(q32;q21). This translocation brings the *MALT1* gene under the control of the immunoglobulin gene heavy chain enhancer and causes its overexpression.[17] It appears to occur more frequently in non-gastrointestinal MALT lymphomas, particularly those of the liver, lung and ocular adnexa.[18,19] However, its actual incidence in MALT lymphomas of various sites and its clinical relevance remain to be investigated.

Molecular connection of the three translocations

Mounting evidence indicates that the oncogenic activity of the three MALT lymphoma associated chromosomal translocations is linked by the physiological roles of *BCL10* and *MALT1* in lymphocytes. *BCL10* forms a complex with *MALT1* and and induces conformational changes that permit *MALT1* oligomerization[20] leading to activation of NF-κB. Using the knockout mice approach, it has been shown that *BCL10* specifically transduces the antigen receptor signaling to the NF-κB pathway.[14,15] Studies of *MALT1* knockout mice re-enforce the above findings and further show that *MALT1* operates downstream of *BCL10*.[21,22] In line with the physiological role of *BCL10* and *MALT1*, the expression of both proteins is primarily restricted to lymphoid tissues and predominantly in the cytoplasm of activated germinal center B-cells.[23,24]

In MALT lymphoma with t(1;14)(p22;q32), *BCL10* is believed to form oligomers via its CARD domain and thus can trigger MALT1 oligomerization and subsequently NF-κB activation. MALT1 is synergistic with *BCL10* in NF-κB activation.[25] In MALT lymphoma with t(14;18)(q32;q21), the oligomerization and activation of *MALT1* is thought to be dependent on *BCL10*. In line with this, both *MALT1* and BCL10 were found to be highly expressed in the cytoplasm of MALT lymphoma cells with t(14;18)(q32.q21).

API2-MALT1 has been shown to activate NF-∫B although neither wild type API2 nor wild type MALT1 alone has this activity.[20,25] BIR domains are capable of mediating oligomerization of proteins that contain these domains and it has been proposed that the *API2-MALT1* fusion protein is oligomerized through such an interaction to constitutively activate NF-κB.[25] Interestingly, the API2 gene promoter is known to be NF-κB responsive, so expression of the *API2-MALT1* transcript is likely to be stimulated by NF-κB. This could set up a positive feedback loop, resulting in continual NF-κB induction.

Apart from the oncogenic activity of the above chromosomal translocations directly associated with the NF-κB pathway, these genetic abnormalities may confer additional oncogenic activities as suggested by aberrant *BCL10* expression in MALT lymphoma. In normal B cells including those of the marginal zone of B-cell follicles, *BCL10* is expressed primarily in the cytoplasm23. However, in MALT lymphoma with t(1;14)(p22;q32), *BCL10* is strongly expressed in the nuclei. Moderate levels of nuclear BCL10 expression are also seen in up to 50% of t(1;14)(p22;q32)-negative MALT lymphomas, including almost all t(11;18)(q21;pq21)-positive cases. Furthermore, *BCL10* has been found to be expressed highly and selectively in the nuclei of the splenic marginal zone B cells in transgenic mice in which *BCL10* gene expression is driven by Ig enhancers. These observations suggest that aberrant nuclear *BCL10* expression may play a role in MALT lymphoma development but the biological activity of nuclear *BCL10* remains to be investigated.

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Extranodal presentation of non-Hodgkin's lymphomas

The incidence of Non-Hodgkin's lymphoma in western countries has increased substantially in the last 30 years with age-adjusted incidence rates rising by approximately 80% between 1973 and 2000 in the USA.[1,2] Extranodal presentations of lymphoma increased more rapidly than nodal disease, with race- and sex-specific increases of 3.0% to 6.9% per year for extranodal cases compared with 1.7 to 2.5% for nodal disease.[3] Primary extranodal disease accounts for between 24% and 48% of new lymphoma cases and often present as localized disease even at sites with no native lymphoid tissue.4-6 The exact definition of primary extranodal lymphoma, particularly in the presence of both nodal and extranodal disease, remains a controversial issue.

Criteria for diagnosing of primary extranodal lymphoma were proposed by Dawson for gastrointestinal lymphomas, and further refined by Lewin and Herrmann.[7-9] The original Dawson criteria stipulated that for a designation of primary extranodal lymphoma, patients had to present with their main disease manifestation in an extranodal site, have regional lymph node involvement only, with no peripheral lymph node involvement, and no liver or spleen involvement. Later these criteria were relaxed to allow for contiguous involvement of other organs (e.g. liver, spleen), and for distant nodal disease providing that the extranodal lesion was the presenting site and constituted the predominant disease bulk. The above mentioned criteria were initially made for the gastrointestinal lymphomas[7-9] and later extrapolated to extranodal localizations in general.[10] The designation of stage III and IV lymphomas as primary extranodal lymphomas is also controversial. Variable reporting criteria make it difficult to establish the true incidence of primary or localized extranodal lymphomas. Extranodal involvement occurring in the presence of predominantly nodal or disseminated disease may represent secondary extranodal disease spread. Some authors deal with this by discussing stage I and II presentation only as primary extranodal lymphomas, while others include as extranodal lymphomas those presentations with a dominant extranodal component with only minor nodal component.[4] Another area of controversy is designation of extranodal versus extralymphatic site. The taxonomy in extranodal lymphomas deserves comment in so far as the Ann Arbor classification recognizes Waldeyer's ring, thymus, spleen, appendix, and Peyer's patches of the small intestine as lymphatic tissues, and does not consider them as extranodal lesions. However, many clinicians separate nodal from extra-

Table 1. Frequency and sites of extranodal lymphomas in different countries.

	USA 1972	NL 1989	DK 1991	Canada 1992	Hong Kong 1984	CH 1997
Stomach	24	23	19	–	39	36
Small intestine	8	5	9	–	24	11
Colon and rectum	5	7	2	–	–	4
Head and neck	21	23	8c	34	22	19
Orbit	2	3	1	4	1	5
Central nervous system	2	6	7	10	–	1
Lung, pleura	4	5	5	1	–	1
Bone	5	3	9	4	4	3
Soft tissue	9	2	3	5	3	1
Breast	2	2	1	2		3
Skin (except mycosis fungoides)	8	2	11	4	3	6
Genitourinary tract	3	4	6	5	8	5

nodal presenting sites rather than lymphatic and extralymphatic sites and for this reason lymphoma arising in the spleen, tonsils and Waldeyer's ring are most often been included among the extranodal types. Extranodal lymphomas can arise in almost every organ (Table 1). In recent years, the greatest increases have been observed for the lymphomas of the central nervous system (CNS), followed by lymphomas of the gastrointestinal tract and the skin.[3]

In addition to the AIDS epidemic, other predisposing factors, such as both viral and bacterial infections, immunosuppressive treatments, exposure to pesticides and other environmental agents, may have contributed to the increased incidence.[6] While considerable progress has been made in the understanding of gastric lymphoma and its relationship to *Helicobacter pylori*,[11] the precise cause of most lymphomas remains to be elucidated. Recent data have provided some evidence supporting the hypothesis that, analogous to *Helicobacter pylori* in the stomach, other bacterial infections may be associated with the growth of mucosa associated lymphoid tissue (MALT) lymphoma at other sites.[12-14] *Borrelia burgdorferi*, the spirochete responsible for Lyme disease, may be implicated in the pathogenesis of at least a subset of cutaneous marginal zone B-cell lymphomas. We and others have shown that the micro-organism can be present in biopsy specimens of the cutaneous lymphoma, and a lymphoma remission can be achieved with antibiotic therapy aimed to the spirochete.[12] Ferreri *et al.* demonstrated the presence of *Chlamydia psittaci* in up to 80% of ocular adnexa lymphomas of either MALT type or non-MALT type histology and the possible activity of antibiotic in this setting too.[13] These data strongly associate the origin of extranodal marginal zone lymphomas from chronic inflammations, often associated with infectious conditions and/or autoimmune states.

The outcomes of extranodal lymphomas are difficult to ascertain. Since these tumours, numerous when considered together, are widely distributed throughout the body, it is difficult to find adequate series of any given site. Most reported data are limited to single institution retrospective series assembled largely by selective referral; still only a minority of the published data have been collected prospectively from population-based registries.[10]

Moreover, the literature on extranodal lymphomas lacks uniformity in histopathological classification. Many historical series were published before the recognition of MALT as the origin of many extranodal lymphomas and in general, classification of primary extranodal lymphomas was similar to that of nodal lymphomas, without consideration that their origin could be different. The first attempt to eliminate this problem was made only very recently with the general adoption of the REAL/WHO classification.[15-16] In prospective trials, extranodal lymphomas are usually included together with nodal lymphomas. Recently, the International Extranodal Lymphoma Study Group has originated a number of retrospective and prospective trials to clarify the management issues distinct to extranodal presentations (*http://www.ielsg.org*).

The histologic spectrum of extranodal lymphoma differs from that of nodal lymphoma with a predominance of frequently localized diffuse large cell lymphomas, the occurrence of MALT lymphomas as a distinct group of lymphomas with homing properties of lymphoma cells, and a paucity of follicular lymphomas. Homing of lymphocytes refers to a controlled pattern of traffic that directs specific lymphocytes to a specific lymphoid tissue in the body. The trafficking of lymphocytes plays a major role in the physiology of the immune system and the implementation of immune mechanisms. Mature lymphocytes have the ability to recirculate continuously between the blood and the lymph. Lymphocyte recirculation is an essential component of the functional immune system. Adhesive interactions between recirculating lymphocytes and the high endothelial venules are mediated by lymphocyte homing receptors (integrins) that recognize tissue-specific molecules expressed on the endothelium (addressins). Homing mechanisms have been implicated in the biology of primary extranodal lymphomas of the skin and gastrointestinal tract, which have several characteristic features that might relate to their trafficking properties.[17] Most significant is a marked inclination to spread or recur in the skin or at mucosal gastrointestinal sites, respectively, which is best explained by tissue-specific homing. The identifica-

tion of the mucosal homing receptor $\alpha_4\beta_7$ integrin that binds to mucosal addressin cellular adhesion molecule (MAdCAM-1), a vascular recognition addressin selectively expressed on mucosal endothelium, supports the unique character of MALT lymphomas.[18]

Although lymphomas that occur in some sites have nearly always identical histology, e.g. diffuse large cell in testis, in other sites a full spectrum of histologic disease entities is seen.[4,5,10,18] For example, in intestine in addition to diffuse large B-cell lymphoma, enteropathy-associated T-cell lymphoma, IPSID, mantle cell lymphoma, and follicular lymphoma occur. Another site with a spectrum of histologies is the breast. Primary lymphoma of the breast occurring in young women and associated with pregnancy is commonly a high grade Burkitt-like lymphoma, while later in life MALT lymphomas and diffuse large cell lymphomas occur. The full spectrum of lymphomas also occurs in the skin where low grade T-cell lymphomas, Ki-1 positive large cell lymphomas, low grade B-cell lymphomas, peripheral T-cell lymphomas, and large B-cell lymphomas are all seen.[19] Histologic type is the main determinant of prognosis in both nodal and extranodal lymphomas,[4–6,10,18] but the specific presenting site is also of paramount importance in extranodal lymphomas. For example, in spite of the other factors being equal, the prognosis of brain lymphoma, or testis lymphoma, is worse than that of bone lymphoma.[20-23]

In the majority of reports primary lymphomas affecting the stomach and Waldeyer's ring are the most common,[18] Waldeyer's ring lymphomas, however, are often considered to be nodal presentations, and therefore are not included in all extranodal lymphoma statistics. A recent registry-based report from the USA found stomach, skin, intestine, and brain to be the most common sites of extranodal lymphoma.[3] A population-based study from Denmark found stomach, intestine, skin, and bone to be the most common sites of presentation. Referral patterns may affect institutional experience with primary extranodal lymphoma.[25] In the experience of a Department of Radiation Oncology at Princess Margaret Hospital in Toronto, Canada, tonsil lymphoma and stomach lymphoma were consistently the most common extranodal sites over the last 30 years.[18,26] Less common localizations include salivary gland, paranasal sinus lymphoma, gingiva, nose, orbit, thyroid, breast, lung, female genital tract, genitourinary tract, and soft tissue lymphomas. The least common sites of primary extranodal lymphoma include heart, muscle, kidney, pleura, adrenal gland, liver, and dura.

The characteristics of patients with nodal and extranodal lymphomas are similar, although patients with primary extranodal presentation tend to be older and have a lower male to female ratio.[18] Signs and symptoms at presentation depend largely on the localization; generally, patients with extranodal lymphomas tend to present B symptoms less often than do patients suffering from lymphomas arising in the nodal regions.[4]

Whether or not patients with extranodal lymphomas have an overall survival similar to that of patients with nodal types as a whole remains a matter of controversy. Some reports found no difference while others showed a better survival in the nodal cases. However, considering the many variables which influence site-specific outcome in extranodal lymphomas, it is questionable whether such an overall difference has clinical relevance.[4] Survival rates vary among all of the specific sites of primary extranodal lymphomas. In fact, the primary organ of origin represents the most significant discriminatory factor among extranodal lymphomas. This is partially due to differences in natural history, but mainly to differences in management strategy which are related to organ-specific problems.[4]

Testis and thyroid lymphomas are seen almost exclusively in patients over 50 years old, while the incidence of hepatic and intestinal lymphomas is significantly higher in younger patients. Salivary gland and thyroid lymphomas are significantly more common in females, while intestinal and pulmonary lymphomas are more often found in males. NHL of the stomach, salivary glands and thyroid are more frequently localized, whereas extranodal lymphomas of the lungs, liver, bones and testes are mostly widespread. Aggressive histologic subtypes are predominant in NHL of CNS, testes, bone, liver.

The pronounced heterogeneity of outcome of the various primary extranodal presentations is one of the main justifications for the design of clinical studies addressing the specific site-related treatment problems of these lymphomas. Further investigation of extranodal lymphomas at different sites will provide opportunities to learn more about the host factors and mechanisms involved in lymphoma development. This is the aim of the several trials launched by the International Extranodal Lymphoma Study Group (*http://www.ielsg.org/*), which, it is to be hoped, this effort will also lead to improvements in clinical management.

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Follicular Lymphoma. Grey-zone: Grading and Transformation

Chairman: A. Lister

Prognostic factors in follicular lymphoma

Follicular lymphoma (FL) represents the second most common non-Hodgkin's lymphoma (NHL) subtype after diffuse large B-cell lymphoma (DLBCL).[1] FL is characterized by a significant degree of clinical heterogeneity as evidenced by the shape of the typical survival curve in this disease. The simple observation that almost 15% of patients die in the first 2 years following diagnosis, while other patients are still alive 25-30 years following diagnosis provides ample evidence for the spectrum of clinical behavior. Molecular heterogeneity is also a feature of FL. Cytogenetic studies of diagnostic biopsies in FL reveals a markedly variable pattern of karyotypic alterations ranging from cases with an isolated t(14;18) through to patients with more than 20 different karyotypic alterations.[2,3] Thus, clinical and molecular heterogeneity are characteristic findings in FL. Clinical variables predictive of outcome in FL have recently been described in the form of a clinical prognostic model known as the Follicular Lymphoma International Prognostic Index (FLIPI).[4] The variables included in this index are age, stage, hemoglobin level, number of nodal sites and serum lactate dehydrogenase (LDH) concentration. An individual's risk is assessed as low (FLIPI = 0-1), intermediate (FLIPI = 2) or high (FLIPI 3) with corresponding 10-year overall survival estimates of 70.7%, 50.9% and 35.5%, respectively. Using this model patients can be divided into three nearly equal sized groups. Overall, the FLIPI performs better in FL than does the IPI, originally designed for aggressive NHL.

Biological predictors of outcome in FL are not currently used to stratify risk in newly diagnosed patients with FL. To some extent this is due to a lack of consistent results in the literature regarding what constitute important biomarkers in FL, how they should be measured and how they should be incorporated into existing clinical prognostic models. An international effort is currently underway (the Lunenburg Lymphoma Biomarker Consortium or LLBC) with the goal of defining biomarkers in FL and determining their role in treatment planning and risk-stratification. Biomarkers in FL will be discussed under the following headings: histopathology, immunophenotype, molecular markers, cytogenetics and finally, gene expression profiling. Factors implicated as possible prognostic variables in FL are listed in Table 2.

Table 1. World Health Organization classification of FL.

Follicular Lymphoma: grading & variants
Grade 1, 0-5 centroblasts/HPF
Grade 2, 6-15 centroblasts/HPF
Grade 3, > 15 centroblasts/HPF
3a, > 15 centroblasts, but centrocytes are still present
3b, centroblasts form solid sheets with no residual centrocytes
Variants
Cutaneous follicle center lymphoma
Diffuse follicle center lymphoma
Grade 1, 0-5 centroblasts/HPF
Grade 2, 6-15 centroblasts/HPF

Table 2. Prognostic factors in FL.

Factor	*Affect on Outcome*	*Reference(s)*
Increasing cytologic grade	Unfavorable	5
Diffuse areas	Unfavorable	5, 7
Bcl-6 protein expression	Favorable	12
Bcl-2 protein expression	Unfavorable	10, 11
Chromosomal gains of +7, +12, +18	Unfavorable	28-32
Chromosomal losses of 6q, 17p13	Unfavorable	3, 28
Bcl-X_L expression	Unfavorable	13
BCL6 translocation	Favorable	21, 22
Host immune response	Favorable	15, 16*

** Dave et al., manuscript submitted.*

Histopathology

A number of morphologic features have been associated with outcome in FL, including cytological grade, proliferation rate, and presence of marginal zone differentiation and diffuse areas in otherwise typical FL. The current WHO classification of FL and grading scheme are shown in Table 1.[5] FL are divided into three grades based on the number of centroblasts per high-power field (HPF) within the malignant lymphoid follicles. Grade 3 FL is further subdivided into 3a and 3b, with 3a characterized by > 15 centroblasts/HPF with some residual centrocytes, while 3b reveals only centroblasts without admixed centrocytes. Data suggest that grade 3b FL may be more akin to *de novo* DLBCL. Reproducible grading of FL represents a problem for pathologists and suffers from both inter-observer and intra-observer inconsistencies. This fact alone casts doubt on the validity of distinguishing grades of FL and interpreting the clinical impact of the distinction. Additionally, reporting of diffuse areas in FL is incon-

sistently done, due in part to the failure to use immunostains for follicular dendritic cells (FDC) that would contribute significantly to this assessment. Unfortunately, these immunostains are infrequently used.[6,7] There is no uniform approach or criteria for dealing with variable grades in a lymph node biopsy. For example, if half of the lymph node contains grade 1 follicles and the other half grade 2 follicles, how should the case be classified? Moreover, unusual cytological variants of FL such as small centroblasts and large centrocytes are occasionally encountered, but are not consistently reported with respect to grade. All of these problems contribute to difficulty in interpreting the prognostic relevance of cytological grade in patients with FL.

That said, the weight of previously published data would support that overall survival decreases slightly with increasing grade. Cytogenetic data, discussed in more detail below, suggest that grade 1 FL through grade 3a FL represents both a clinical and biological continuum, with a common immunophenotype and the consistent presence of the t(14;18). Previous clinical studies of follicular large cell lymphoma consisted of a heterogeneous mixture of grade 3a and grade 3b cases, as well as some cases with a predominance of large centrocytes. This fact may underlie some of the controversy regarding survival in this subset of FL. The implication from the literature is that grade 3b cases may behave more like DLBCL and thus contribute to the potential for a plateau on the progression-free survival curve. However, a recent study failed to demonstrate a survival difference comparing grade 3a FL vs grade 3b7. Nonetheless, further studies are required to address this important clinical question. Proliferative activity (Ki-67/MIB-1) and gene expression profiles change with FL grade, but neither has been convincingly shown to affect survival. A component of marginal zone differentiation is a feature of approximately 10% of newly diagnosed FL. Although an initial report suggested that this differentiation was associated with inferior survival, several subsequent studies have not shown a clinical impact.[8] So-called blastoid or blastic variants of FL are encountered rarely and this morphological feature appears to be associated with an aggressive clinical course.[9]

Immunophenotype

Most FLs share a signature phenotype with expression of the pan-B-cell antigens CD19, CD20, CD22 and CD79a. Most cases are surface immunoglobulin positive and reveal light chain restriction. Most cases express Bcl-2 protein, characteristically a result of the t(14;18). Grade 1-3a FL cells usually express two markers of the germinal center, CD10 and Bcl-6 protein. However, the expression of CD10 is reported to be positive in only 50% of grade 3b FL6. Very few immunophenotypic markers have shown any correlation with outcome in FL. One study showed that lack of Bcl-2 protein expression was associated with improved overall survival in FL, but complete multivariate analysis was not performed.[10,11] In a recent study of FL a semi-quantitative analysis of Bcl-6 expression suggested that cases with lower expression levels did worse.[12] Over-expression of Bcl-XL has recently been shown to decrease survival in FL, however these studies did not measure protein by immunohistochemistry, despite the existence of paraffin-reactive antibodies.[13] The level of Bcl-XL also correlated with the apoptotic index, suggesting a functional role for this anti-apoptotic molecule.

Thus, beyond this very short list of examples of biomarkers that may have prognostic significance in FL, there are no other bona fide examples. The hope is that new genes discovered through expression profiling can be adequately assessed using antibody reagents with activity against formalin-fixed epitopes. Such approaches will serve to validate mRNA expression results, allow a determination of the specific cell(s) producing the protein and may in fact be the technique used to translate this new knowledge into the routine clinical laboratory. Future immunohistochemical assays of this nature will be made simpler if tissue microarray construction becomes an integral part of all clinical trial designs. This strategy would facilitate the determination of important biomarkers in FL and allow a comparison of multiple prognostic factors using the same clinical cohorts.

The roles of follicular dendritic cells and reactive $CD4^+$ T cells in affecting clinical outcome in FL have been incompletely studied, but are worthy of future studies based on recent gene expression profiling analysis discussed in detail below.[14-16]

Molecular genetics

The majority of grade 1-3a FL are characterized by the t(14;18)(q32;q21). Previous studies had suggested that specific breakpoints involved with this translocation had prognostic relevance. For example, a previous analysis of FL cases suggested that patients without a polymerase chain reaction (PCR)-positive major breakpoint region (mbr) or minor cluster region (*mcr*) *BCL2* oncogene translocation, corresponding to those with a germline PCR result, had inferior survival.[17] However, more recent data have questioned these findings and in fact, data from the BC Cancer Agency would favor the opposite conclusion that patients lacking the t(14;18) appear to do better.[18,19] *BCL6* gene rearrangements have been shown to be present in about 10-15% of FL cases. In the author's experience, these can be found coincident with the t(14;18) in grade 1 through 3a FL, but appear to be mutually exclusive of *BCL2* translocations in grade 3b FL.[20] In two separate reports, *BCL6*

translocation-positive cases were associated with improved survival in FL.[201,22] These studies need to be repeated using larger patient cohorts and importantly can be accurately assessed using locus-specific FISH assays on paraffin sections. Translocations and/or mutations of the MYC oncogene, loss of the tumor suppressor p53 and loss of p16 have all been associated with inferior survival in FL. Typically these events occur during clonal evolution and have been associated with histologic transformation of FL to DLBCL.[23-25] However, uncommonly they may be present at the time of diagnosis and herald a more aggressive clinical course that need not be accompanied by transformation. Mutations in the open reading frame of *BCL2* and translocations involving *BCL6* as determined using long distance-inverse PCR, have been associated with risk of transformation.[26,27] The latter finding requires conformation, as these results are distinctly different from those of other studies that suggest an association of *BCL6* rearrangements with improved survival.

Cytogenetic studies

A small number of cytogenetic studies have demonstrated similar findings, and identify a number of recurrent alterations associated with prognosis in FL.[3,10,21,28-30] These studies include investigations employing comparative genomic hybridization (CGH) as the tool used to determine genome-wide alterations in FL. In summary, findings associated with inferior survival include del6q, del17p, +12 and more specifically, +12q13-14 and +7.[3,28,31,32] In addition, a higher percentage of abnormal metaphases as well increasing cytogenetic complexity have also been associated with prognosis in FL. The majority of these alterations are also correlated with karyotypic changes that are associated with histologic transformation. Loss of cytogenetic material at chromosomal region 1p36 and extra copies of chromosome 18 have been associated with inferior survival.[30] Importantly, most of the studies attempting to analyze the clinical impact of cytogenetic alterations in FL have studied diagnostic biopsy samples. Thus, these changes define the clone at presentation, but also represent the background on which further clonal evolution is occurring in FL.

Gene expression analysis

Very few studies of molecular profiling have been reported in FL. In an initial analysis, 9 cases of FL were examined using Atlas arrays and flow purified B cells, revealing a moderate number of genes that were either up-regulated or down-regulated in comparison to those in normal germinal center B cells.[33] No conclusions regarding clinical impact could be drawn from 9 cases. However, these gene expression data were correlated with high-resolution cytogenetic data and some conclusions regarding the pathogenesis of FL could be made.[30] Firstly, the deregulated gene expression that characterizes FL is strongly correlated with copy number alterations, either chromosomal gains or losses. Secondly, these data suggest that following the acquisition of the t(14;18) in FL, most of what contributes to pathogenesis in FL and leads to altered gene expression results not from balanced translocations, but rather from chromosomal gains or regions of loss. Thirdly, these chromosomal alterations may follow specific pathways, first characterized by chromosomal gains and finally culminating in a common pathway associated with chromosomal losses.[34] A large study conducted by the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) consortium was presented at the recent ASH meeting in San Diego (*Dave et al., manuscript submitted*). This group studied 191 FL samples at diagnosis using Affymetrix U133 oligonucleotide arrays and correlated the patterns of gene expression with survival of the patients. An outcome predictor could be formulated that was characterized by 3 dominant gene expression signatures. One related to B cell differentiation and was likely derived from the malignant B cells, but the other 2 were characteristic of host immune response cells and were clearly shown to be derived from infiltrating non-neoplastic cells (mostly T cells, tissue-based macrophages and follicular dendritic cells). The latter 2 signatures, referred to as immune response 1 and 2, were not simply surrogates for the numbers of host immune cells, but in stead were likely specifically addressing the function of these cells. Importantly, these profiles were present at the time of diagnosis and, to a large degree, dictated the aggressiveness of the disease and the survival of the patient, suggesting that the stochastic acquisition of oncogenic abnormalities after diagnosis does not appear to have a major overall impact on survival. Although not conclusively established, these data suggest a dominant role for the immune response in FL.

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Stem cell transplantation for follicular lymphoma

In contrast to patients with relapsed aggressive non Hodgkin's lymphoma (NHL), fewer patients with follicular lymphoma (FL) have undergone high dose therapy followed by autologous bone marrow or stem cell transplantation (ASCT). The lower interest in ASCT for FL has largely been based upon the belief that this is a disease with a very long natural history, in which excessive treatment-related toxicities associated with aggressive therapy would not be acceptable. As an additional complication, many of these patients had disease resistant to conventional therapy prior to transplantation, a situation that is known to reduce the effectiveness of transplants in other malignancies. The high frequency of overt bone marrow and peripheral blood infiltration has been a major obstacle, resulting in the reinfused marrow or peripheral blood stem cells being contaminated by with viable tumor cells. Accordingly, a number of maneuvers have been tried in order to circumvent this problem, including use of *in vivo* as well as *in vitro* tumor cell purging techniques. An alternative to ASCT has been allogeneic transplantation (alloBMT). A number of studies have been reported on alloBMT for patients with indolent NHL. The concern over mortality from transplant-related complications and graft-versus-host disease (GVHD) has limited this approach to patients with disease refractory to chemotherapy and those with extensive bone marrow involvement. This paper will review studies om ASCT involving B-cell purging and allogeneic transplantation for FL.

Follicular lymphoma

Studies using in vitro tumor cell purging. The evidence supporting the efficacy of anti-B cell monoclonal antibody *in vitro* purging in FL remains controversial. The Dana-Farber Cancer Institute treated 153 patients with FL in sensitive relapse or incomplete first remission with cyclophosphamide/ total body irradiation conditioning and anti-B-cell monoclonal antibody-purged autologous bone marrow transplantation between 1985 and 1995.[1] Disease-free survival and overall survival at eight years following autologous bone marrow transplantation were 42 and 66%, respectively. Those patients whose bone marrow was purged of tumor cells and found to be polymerase chain reaction (PCR) negative for t(14;18) had a significantly longer freedom from recurrence than patients who were re-infused with bone marrow which remained PCR-positive after purging.

Investigators at St. Bartholomew's Hospital treated 99 patients with relapsed FL with anti-B1 monoclonal antibody-purged autologous bone marrow transplantation with a cyclophosphamide/ total body irradiation conditioning regimen.[2] In subgroup analysis, there was no difference in outcome between patients whose graft was purged negative. The overall survival (OS) and disease-free survival (DFS) were 68% and 63% at 5.5 years, respectively. Colombat *et al*. examined the role of autologous stem cell transplantation with *in vitro* antibody purging as first line therapy in 29 patients with poor risk FL.[3] Initial therapy consisted of VCAP. Patients achieving a complete response (CR) or partial response (PR) after three cycles then went on to autologous transplantation. After bone marrow harvesting, but before conditioning, patients received ifosfamide, etoposide, and methotrexate. The preparative regimen for transplant consisted of total body irradiation and high dose cyclophosphamide. The purging technique for most patients consisted of immunomagnetic beads or complement-mediated lysis. Twelve patients had measurable t(14;18) by PCR at the onset of therapy. Despite the intensive induction chemotherapy, only one patient became PCR-negative after initial treatment. Two of the eleven other patients' grafts became PCR-negative after *in vitro* purging. For the trial as a whole, overall survival was 64% and event-free survival 53% at 8 years. Too few patients had effective purging to comment on the procedure's efficacy.

The only randomized trial comparing conventional chemotherapy to autologous stem cell transplantation with purged or unpurged bone marrow is the European CUP trial.[4] In this study, 89 patients with relapsed or progressive FL received 3 cycles of CHOP. Those patients who achieved a CR or PR and had less than 20% paratrabecular bone marrow involvement with FL were randomized to 3 further cycles of CHOP chemotherapy or to high dose therapy and autologous stem cell support with immunomagnetic anti-B-cell antibody purging or high dose therapy and autologous stem cell support without purging. At a median follow-up of 69 months, the overall 5-year survival for all registered patients was 50% but the median survival had not yet been reached for the subset of patients treated with high dose therapy and autologous stem cell transplantation (either purged or unpurged). Both progression free and overall survival favored the transplantation arms but there was no discernible difference between those patients receiving a purged autograft and those receiving an unmanipulated graft. The authors attributed the lack of a difference between the purged and un-purged groups to small sample size, intercenter variability in purging, and relatively high level of residual disease in patients undergoing transplant.

Many transplantation series in FL have utilized bone marrow as the source for stem cells. However, peripheral blood collection has become the preferred

source for stem cells. Very few series have examined the efficacy of anti-B cell antibody purging in peripheral blood. One recent series was published by Friedberg *et al*.[5] The authors enrolled patients with grade 1-3 FL in second or further remission with minimal disease (<20% bone marrow involvement and < 5 cm tumor masses). Patients were mobilized with cyclophosphamide and granulocyte colony-stimulating factor (G-CSF). The subsequent stem cell harvest was then purged with anti-B cell monoclonal antibody coated high-density microparticles. A total of 29 patients were enrolled and 21 were available for analysis. Only 4 of 17 patients had a detectable t(14;18) pre-purge, a number that is significantly lower than expected. This low number may have occurred because most patients had received rituximab. There was no correlation between PCR status and subsequent risk of relapse. There was a trend suggesting that increased log depletion of B cells correlated with a decreased risk of relapse following transplantation (p=0.06).

Lazzarino *et al*. published a data from a series of 70 patients with FL, small lymphocytic lymphoma, and mantle cell lymphoma, the majority of whom (61 of 70) were treated with first line therapy followed by autologous peripheral blood stem cell transplantation.[6] Before treatment, a PCR-amplifiable Bcl-2 rearrangement was detected in 75% of FL patients. At the time of harvest, a PCR-negative graft was obtained in 54% of FL patients. The median molecular follow-up in this trial was 75 months. The rate of relapse in patients never achieving a molecular remission was 88% compared to only 8% in those achieving a molecular remission (p<0.005). At a median clinical follow-up of 90 months, 74% of patients in the group as a whole were alive, with an estimated 12-year overall-survival projection of 76%. This study emphasizes the importance of achieving a molecular remission and the importance of achieving a PCR negative autograft. Although the treatment plan for this study did not include *in vitro* purging, the authors did design the intensive chemotherapy regimen with the intent of achieving *in vivo* purging. Thus, the study suggests that effective purging techniques could have an impact upon survival, particularly in FL.

Overall, most studies of autologous stem cell transplantation in FL suggest a continuous pattern of relapse. Patients undergoing autologous bone marrow transplantation do appear to have significantly longer disease-free survivals than those receiving standard therapy.[7] Whether anti-B cell monoclonal antibody purging affects disease-free and overall survival remains unclear. Most available data suggest that achieving a PCR-negative status is important in achieving prolonged survival with FL. The data also suggests that purging can effectively render autografts PCR-negative for evidence of FL. Unfortunately, a randomized European trial comparing purged to un-purged autografts was closed early due to limited accrual and therefore had an insufficient sample size to answer the question definitively.

Rituximab plus chemotherapy for in vivo purging prior to autologous stem cell transplantation for follicular lymphoma

Several investigators have examined the role of in vivo purging with rituximab in FL. Voso *et al*. published data on a series of 18 patients with advanced stage mantle cell (3 patients) and follicular (15 patients) lymphoma treated with a sequential regimen of chemotherapy and rituximab followed by high dose therapy and autologous stem cell support in responding patients.[8] Initial therapy consisted of CHOP followed by mobilization with one dose of rituximab followed by cytarabine and mitoxantrone with G-CSF support. After stem cell collection, patients then underwent a second course of rituximab and cytarabine/mitoxantrone followed by a preparative regimen of sequential total body irradiation and high dose cyclophosphamide. Another dose of rituximab was given prior to TBI and prior to cyclophosphamide. Seven of the patients with FL in this study had a t(14;18) detectable by PCR in the peripheral blood. Of these, six became PCR-negative after one cycle of rituximab, mitoxantrone, and cytarabine. All seven patients had a PCR-negative leukapheresis product.

In a similar approach, with rituximab given after mobilization chemotherapy, the peripheral blood stem cells harvested from rituximab recipients were PCR-negative for bcl2/IgH in 93% of cases compared to in 40% of patients who did not receive rituximab.[10] Furthermore, clinical and molecular remission was obtained in all 14 evaluable rituximab recipients compared to in 70% of controls after high dose therapy.

These results were confirmed by Galimberti et al. who compared patients with FL treated with CHOP followed by either high-dose therapy or high-dose therapy then rituximab prior to ASCT.[11] In this series, 86% of grafts obtained from patients treated with rituximab had no PCR evidence of FL compared to 14.3% in those not treated with rituximab. The 5-year progression free survival in the group receiving a PCR-negative graft was 100% compared to 41% in those receiving a graft with PCR evidence of residual lymphoma, though this difference was not statistically significant. Overall survival did not differ between the groups. The number of patients in this series was small and some patients (including most of those receiving a graft with evidence of FL) never received prior rituximab. Given that rituximab has been demonstrated to prolong progression-free sur-

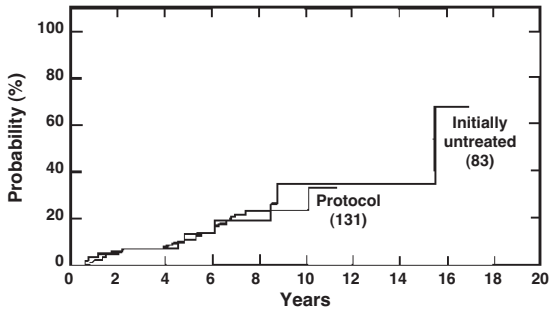

Figure 1. Risk of histologic transformation in patients with stage III-IV indolent lymphoma. The rates of transformation were identical whether patients were initially managed with a watch and wait approach (n=83) or were treated at the time of diagnosis with chemotherapy or radiotherapy (protocol therapy, n=131). Reproduced with permission from Horning and Rosenberg, N Engl J Med 1984;311:1471).

vival in FL, the PFS advantage in the rituximab group in this study could be attributed to the drug itself rather than from actual purging of the marrow graft.

Allogeneic transplantation

Following alloBMT, relapse rates are lower than those noted after ASCT. However, treatment-related mortality is higher at approximately 30%.[12] These differences were highlighted in a report from the International Bone Marrow Transplant Registry.[13] In 176 patients with FL, 67% of whom had chemosensitive disease at the time of transplantation, Kaplan-Meier estimates of treatment-related mortality, recurrence rates, and overall survival at five years following alloBMT were 30, 21, and 51%, respectively. During this same period of time, 597 patients with FL received unpurged autologous transplantation (ABMT); 82% were chemosensitive at the time of transplant. Kaplan-Meier estimates of treatment-related mortality, recurrence rates, and overall survival at five years following unpurged ABMT were 8, 58, and 55%, respectively.

In an attempt to decrease transplant-related morbidity and mortality, T-cell depletion as a means of GVHD prophylaxis was performed on allografts in 16 patients with indolent lymphoma at the Medical College of Wisconsin[14] At five years, DFS was 62%, with a relapse rate of 13%. A very low treatment-related mortality following T-cell depletion has been observed at the Dana-Farber Cancer Institute in patients with relapsed indolent lymphoma, with DFS of 50%.[15] Longer follow-up is needed to determine whether or not T-cell depletion in patients with NHL will interfere with a putative graft-versus-leukemia effect.

Another strategy to decrease transplant related mortality involves the use of non-myeloablative conditioning regimens. In a phase II study, 20 patients with recurrent follicular or small lymphocytic lymphoma were treated with fludarabine and cyclophosphamide, with or without rituximab, followed by an allogenic transplant from an HLA-indentical sibling donor.[16,17] All achieved complete remission with mixed chimerism and prompt hematologic recovery. One patient developed grade III acute GVHD and two patients developed extensive chronic GVHD. The actuarial probability of survival at two years was 84%.

A second series of non-myeloablative allogeneic transplantation involved 188 patients, including 52 previously treated patients with indolent NHL, 85% of whom had chemosensitive disease; 29% had received a prior autologous transplant.[18] Non-myeloablative transplantation yielded a 100-day treatment-related mortality of 11 percent and an estimated two-year overall survival of 65%. Predictors for an adverse outcome included chemoresistant disease and age >50 years.

Conclusions

The role of *in vitro* purging in FL remains uncertain. Several trials indicate that re-infusion of a PCR-negative graft improves progression-free survival. As techniques improve and more grafts can be effectively purged, the clinical benefit of purging may become more apparent. The one randomized trial that was initiated to define the value of current *in vitro* purging techniques - the CUP trial - was closed early due to poor accrual. *In vivo* purging with the anti-CD20 monoclonal antibody rituximab appears promising. However, despite the increased rate of eliminating PCR-detectable disease from autologous grafts with this technique there remains no proven survival advantage. The same impact on disease progression may be achievable, for instance, by giving rituximab as maintenance to patients in CR after an un-purged autologous transplant. The role of allogeneic transplantation remains similarly unclear. Despite a lower relapse rate with alloBMT, significant treatment related morbidity and mortality persists even in non-myeloablative transplantation. With additional study and refinement of techniques and supportive care, the role of these modalities may become better defined in the treatment of FL.

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The role of monoclonal antibodies and radioimmunotherapy in the treatment of patients with transformed non-Hodgkin's lymphomas

Transformation of non-Hodgkin's lymphoma (NHL) is defined as the histologic conversion of indolent (*low grade*) disease to aggressive lymphoma.[1] One classic form of transformation occurs when diffuse large B-cell lymphoma (DLBCL) develops in a patient with chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL), a metamorphosis called Richter's transformation. In a similar fashion, follicular lymphoma (FL) often degenerates into DLBCL by acquisition of additional somatic mutations associated with alterations in gene expression profiles.[2] The frequency with which histologic transformation of NHL has been reported varies widely from study to study[3-6] Simon followed 1153 NHL patients for a median of 11.2 years and reported histologic transformation in 20-30% of patients followed clinically and in 70% at the time of death.[3] Bastion reported that transformation occurred more commonly in patients with adverse prognostic factors or in those who did not achieve complete response after initial treatment.[6] On the other hand, Horning documented histologic transformation in ~40% of 224 patients with indolent NHL after 8 years follow-up regardless of whether they were followed with an expectant *watch and wait* approach or treated with chemotherapy or radiotherapy soon after diagnosis.[5]

Nearly all authorities concur that the prognosis is poor following histologic transformation with a median survival after transformation of 7 to 22 months.[6] In one series, transformation accounted for 44% of all deaths in patients with a diagnosis of follicular lymphoma.[6] Most patients with transformation to DLBCL are treated with doxorubicin-based regimens such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) plus rituximab but outcome is poor despite aggressive therapy. Consequently, many authorities recommend high dose chemoradiotherapy and autologous stem cell transplantation for transformed lymphomas.[7] A recent review summarized the findings of eight studies of autologous transplantation for transformed lymphoma and concluded that approximately one-third of patients remain disease-free after five years.[7] Therefore, it is apparent that new treatment approaches are required for this clinical entity. In the remainder of this manuscript, we will review the available data assessing the utility of unconjugated and radiolabeled monoclonal antibodies for the treatment of patients with transformed NHL.

Use of Rituxan™ in transformed non-Hodgkin's lymphoma

There are no published clinical trials exclusively devoted to the treatment of transformed lymphomas with monoclonal antibody monotherapy. Furthermore, data addressing this topic are difficult to extract from the existing literature. However, a careful analysis of published trials reveals data on nine patients with transformed lymphoma treated with rituximab. Witzig reported on four patients with transformed NHL enrolled in the phase III randomized trial of rituximab vs zevalin. Three of these four patients achieved objective remissions, demonstrating the therapeutic utility of a standard course of rituximab (375 mg/m^2 weekly for four weeks) in this setting. On the other hand, Faderl reported the cases of two patients with Richter's transformation of CLL to DLBCL treated with rituximab and alemtuzumab; neither patient obtained a remission.[8] Byrd described a single case of a patient with refractory follicular lymphoma which had transformed to DLBCL with 77,000 circulating malignant leukocytes. This patient experienced serious infusional toxicities with rituximab and failed to respond.[9] Maloney described a patient with DLBCL transformed from follicular lymphoma who experienced complete resolution of adenopathy with a single infusion of rituximab, but subsequently had disease progression in bony sites.[10] Finally, Kirchner published a case report of a patient with Hodgkin's disease which transformed to DLBCL who achieved a complete remission lasting 14+ months after rituximab monotherapy. In summary, five of nine patients (56%) treated with antibody therapy alone achieved meaningful clinical remissions.

Zevalin™ for the treatment of transformed non-Hodgkin's lymphoma

Bartlett *et al*. reported on a total of 15 patients originally diagnosed with follicular B-cell lymphoma that had transformed to a higher grade (4 diffuse mixed, 11 diffuse large cell) treated in clinical trials with ^{90}Y-ibritumomab tiuxetan.[11] All patients had < 25% bone marrow involvement as confirmed by biopsy, WHO performance status < 2, neutrophil counts > 1,500/mm^3, platelets > 150,000/mm^3, no prior myeloablative therapy or radioimmunotherapy, no prior radiation to >25% of active bone marrow, and total bilirubin or serum creatinine < 2.0 mg/dL. The median age was 60 years (range 35 to 76 years). Thirty-three percent had bone marrow involvement, 27% had splenomegaly, and 20% had extranodal disease in more than 2 sites. The patients had received a median of two prior regimens (range 1 to 5). Eighty percent had tumors > 5 cm and 20% had tumors > 10 cm. The overall response rate (ORR) for these 15 patients was 53% with 13% achieving a complete response and 40% achieving a partial

response. The median time to progression in responders was 7.1 months (range 2.1 to 51.1 months). The median time to next anti-cancer therapy was 4.2 months (range 1.2 to 51.7 months). Adverse events were primarily hematologic. Two patients were hospitalized with febrile neutropenia on study day 39 and 43. Both patients recovered. Grade 4 thrombocytopenia was reported in 20% and grade 4 neutropenia was reported in 40%. Nonhematologic adverse events of grade 1 or 2 occurred in 67%. These events included asthenia, nausea, vomiting, chills, fever, headache, and throat irritation.

Bexxar™ for the treatment of transformed non-Hodgkin's lymphoma

Zelenetz identified 71 patients with transformed NHL who were treated with tositumomab/iodine I-131-tositumomab (Bexxar™) in five clinical trials; 47 of these cases had the histologic transformation confirmed on an independent pathologic review.[12] According to the International Working Formulation, which was being employed at the time of these studies, 64% of these 47 patients had disease transforation to DLBCL (including immunoblastic lymphomas) at the time they were treated with Bexxar, 9% had diffuse mixed lymphoma, 2% had diffuse small cleaved cell lymphoma, and 26% had follicular large cell lymphoma. The median age of these patients was 58 years and they were treated with Bexxar a median of 18 months after histologic transformation. These patients had previously been treated with a median of four prior chemotherapeutic regimens before receiving Bexxar. The patients had many high-risk features including an elevated lactate dehydrogenase level in 50% of cases, bulky disease (>5 cm) in 65%, and marrow involvement in 28%. In this unfavorable group of patients, the overall response rate was 40%, including 23% complete responses. The median time to progression after Bexxar was 4.3 months for all patients and 16.1 months for the responding patients.

High dose radioimmunotherapy with autologous stem cell transformation for transformed lymphomas

Because of the poor prognosis of patients with transformed lymphomas, our group has investigated the use of myeloablative doses of ^{131}I-tositumomab either alone or in combination with etoposide (60 mg/kg) and cyclophosphamide (100 mg/kg) followed by autologous stem cell transplantation in this setting.[13-16] Four patients were treated with ^{131}I-tositumomab alone followed by ASCT. One of these four patients achieved a complete remission and has remained alive and disease free for more than twelve years since he was treated. One other patient achieved a partial remission lasting 28 months. The other two patients progressed during treatment and died. Ten other patients were treated with ^{131}I-tositumomab, etoposide, cyclophosphamide, and ASCT. Seven of nine patients with sufficient follow-up to be evaluable were rendered disease free (78%) and eight are alive (88%). Five patients are currently alive and disease-free (56%) for up to seven years following high dose radioimmunotherapy.

Conclusions

Transformed lymphoma remains a difficult clinical situation in need of novel treatments. Unmodified monoclonal antibodies such as rituximab and radiolabeled antibodies such as Zevalin and Bexxar show encouraging clinical activity and some patients appear to be cured by myeloablative radioimmunotherapy with autologous stem cell transplantation.

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Mantle Cell Lymphoma. Are All Aggressive Lymphomas?

Chairman: W. Hiddemann

Mantle cell lymphoma: molecular pathology

Mantle cell lymphoma (MCL) is a lymphoproliferative disorder derived from a subset of naive pre-germinal center cells characterized by a monomorphic proliferation of small to medium sized lymphoid cells with irregular nuclei, These cells have a B-cell phenotype, co-expressing CD5.[1] MCL is genetically characterized by 11q13 translocations and bcl-1 rearrangement leading to a constant overexpression of cyclin D1 which plays an important pathogenetic role in the development of the tumor. These genetic and molecular aberrations have been crucial in recognizing MCL as a distinct disease entity and defining precise diagnostic criteria which, in turn, have allowed a broader spectrum of morphological and clinical manifestations to be identified in MCL patients.[2] As said, MCL cells express CD5. Human CD5-positive B cells are present in normal fetal lymphoid tissues and blood, and decrease with age. In normal adults, small numbers of CD5-positive B cells circulate and localize in primary follicles and the mantle area of secondary follicles. However, CD5 expression in MCL is very high, its intensity resembling that observed in fetal B-cells, in contrast to the low levels detected in the subset of adult follicular mantle cells.[3] Mantle cell lymphomas also maintain the expression of different genes normally expressed in naïve and follicular mantle zone cells such as TCL-1,[4] tyrosine phosphatase SHP1,[5] and the polycomb group member BMI1.[6,7] Immunological analyses and the identification of no or very little somatic mutations in immunoglobulin genes of MCL have confirmed its origin in pregerminal center cells.[8,9] In addition, analyses of the JH/bcl-1 breakpoints have suggested that this translocation is predominantly generated during a primary DH-JH rearrangement in early B cells unlike the JH/bcl-2 in the t(14;18) that seems to occur during secondary rearrangements at a later stage of B-cell differentiation.[10,11] However, recent studies indicate that 20-30% of MCL may show somatic hypermutations in the immunoglobulin genes and also that there is a biased use of the V_H4-34 and V_H3-21 genes in the rearrangements, suggesting that these tumors may originate from specific subsets of B cells[12] However, contrary to the experience in chronic lymphocytic leukemia, this mutational status does not correlate with ZAP-70 expression or the survival of the patients.

t(11;14) translocation

The t(11;14) translocation juxtaposes the Ig heavy chain joining region in chromosome 14 to a region on 11q13 designated *bcl-1* (B-cell lymphoma/leukemia 1).[13] Other breakpoints far away from the original cloned region have also been identified (Figure 1).[14] Most rearrangements (30 to 55%) occur in a region known as the major translocation cluster (MTC), whereas up to 10-20% of cases may have breakpoints in additional distal regions.[14] The MTC breakpoints occur within a relatively small region of around 80 bp on chromosome 11 and in the 5' area of one of the Ig JH regions on chromosome 14, 15;16 making it possible to detect this translocation by polymerase chain reaction (PCR) techniques.[16,17]

Cyclin D1 oncogenic mechanisms

Cyclin D1 may function as an oncogene, co-operating with other oncogenes, generally *c-myc* and *ras*.[18] However, the oncogenic mechanisms of cyclin D1 are not well understood. Cyclin D1 participates in the control of the G1 phase by binding to the cyclin dependent kinases (CDK) 4 and 6.[19] The complexes phosphorylate retinoblastoma (*Rb*) leading to the inactivation of its suppressor effect on cell cycle progression.[20] Hyperphosphorylation of *Rb* releases important transcription factors, such as E2F, that participate in the regulation of other genes involved in cell cycle progression. Cyclin D1 also binds physically to *Rb* and may overcome the growth suppressing effect of *Rb* independently of its phosphorylation.[21] In MCL, *Rb* seems to be normally expressed in all cases and no mutations in its functional domains have been detected.[22] However, it is hyperphosphorylated, particularly in blastic variants with high proliferative activity.[23] Concordantly, E2F overexpression is detected in a high number of MCL.[24] These findings suggest than cyclin D1 may play a role in these tumors by overcoming the suppressive effect of *Rb*.

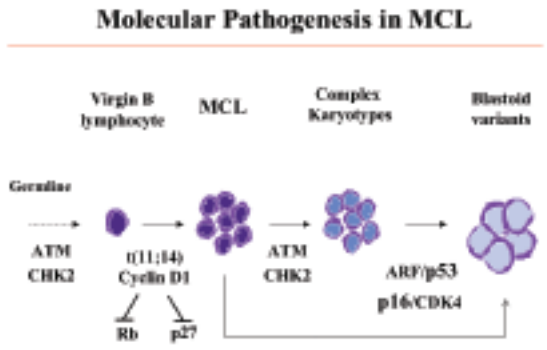
There may also be impaired control of late G1 phase and G1 to S phase transition in MCL. This step of the cell cycle is regulated by the cyclin E/CDK2 complex and the cyclin kinase inhibitor $p27^{Kip1.}$ In non-Hodgkin's lymphomas other than MCL, $p27^{Kip1}$ protein expression is inversely related to the proliferativeactivity of the tumors.[25] However, immunohistochemical detection of $p27^{Kip1}$ is lost in typical MCL but it is paradoxically positive in blastic variants. No structural alterations of the $p27^{Kip1}$ gene have been found. The mechanisms for this peculiar detection of $p27^{Kip1}$ in MCL is not clear but may include both increased p27 protein degradation by the proteasome pathway,[26] and sequestration by the overexpressed cyclin D1, rendering it inaccessible to antibody detection. p27 Kip1 inhibits the complexes between CDK2 and cyclin E at the end of the G1 phase. Increased degradation and/or blocking p27 Kip1 by cyclin D1 would release the activation of these complexes and allow the cell to progress to the following cell cycle phases.[25] All these observations indicate that cyclin D1 deregulation plays an important role in the development of MCL, probably overcoming the suppressive effect of Rb and p27 Kip1. (Figure 1). In addition to these mechanisms, Cyclin D1 may have also an important oncogenic potential independently of its catalytic function by acting as a transcriptional modulator of multiple genes.[27]

DNA damage response genes: ataxia-telangiectasia mutated (ATM) and CHK2

MCL is genetically characterized by a very high number of chromosomal alterations suggesting that these tumors may harbor alterations in DNA damage response pathways and mitotic checkpoints.[28] One of the most frequent secondary chromosomal aberrations in MCL is deletion of the 11q22-23 region, including a common deleted area where the *ATM* gene is located.[28] ATM plays an important role in DNA damage response pathways, connecting DNA damage sensors to different downstream effector elements regulating cell cycle, cell death, and DNA repair.[29] *ATM* mutations have been detected in 40-75% of MCL.[30-32] *ATM* inactivation in typical MCL has been associated with a high number of chromosomal aberrations suggesting that this gene may be involved in the chromosomal instability of these tumors.[31] Occasional MCL patients carry an *ATM* mutation in the germline.[31,32] This finding is similar to that in patients with chronic lymphocytic leukemia in whom *ATM* mutations in germline and in cells prior to lymphoid differentiation have been detected,[33,34] raising the possibility that *ATM* mutations are a very early predisposing event in these neoplasms. *CHK2* is a putative tumor suppressor gene downstream of *ATM* also involved in the DNA damage response pathway. Inactivation of this gene by protein downregulation and occasional germline mutations have been described in MCL patients,[35,36] suggesting that, similarly to ATM, alterations in this gene may participate in the pathogenesis of MCL (Figure 1).

p53, INK4a/ARF, CDK4 and other cyclin-dependent kinase inhibitors

One of the major differences between typical and blastoid MCL variants is the proliferative activity of the tumors. *p53* and $p16^{INK4a}$ located at 17p13 and 9p21, respectively, are key elements in two different cell cycle regulatory pathways frequently disrupted in a broad range of human tumors. *p53* mutations occur in around 30% of blastoid variants with high proliferative activity and shorter survival of the patients.[37-39] In addition, $p16^{INK4a}$ inactivation by homozygous deletions has been found in aggressive variants of MCLs.[40-43] Interestingly, inactivation of this gene occurs in cases with wild type *p53* indicating that alteration of these genes may be alternative mechanisms in the



progression of these tumors.[14] The genomic locus where *p16*^{INK4a} is located also encodes for a second gene named ARF (alternative reading frame). This gene is encoded by the same exon 2 used by *p16*^{INK4a} but a different exon 1 called exon 1α, centromeric to the first exon of *p16*^{INK4a}. The *ARF* gene stabilizes p53 protein by blocking its MDM2-mediated degradation. Therefore, the *INK4a/ARF* locus is a common genomic regulatory link between p53 and *p16*^{INK4a} pathways. Deletions of this region in B-cell lymphomas usually include both genes, leading to a simultaneous inactivation of both pathways.[40,44,45] Although the number of cases examined is limited, it seems that inactivation of these two pathways by homozygous deletions of the *INK4a/ARF* locus in MCL is associated with a more aggressive clinical behavior than is the isolated inactivation of p53 gene.[44] Inactivation of other CDK inhibitors such as *p15*^{INK4b}, *p18*^{INK4c}, and *p21*^{Waf1} hasve been found in occasional blastic MCLs.[40,46]

CDK4 amplifications have been detected in occasional cases with 12q13 gains.47 These amplifications occur in highly proliferative variants and are associated with mRNA and protein overexpression. Interestingly, *CDK4* aberrations seem to be alternative to *INK4a/ARF* deletions but may occur simultaneously with *p53* alterations. Patients with *INK4a/ARF* deletions or simultaneous aberrations of p53 and *CDK4* had a significant shorter median survival than patients with isolated alterations of *p53* or *CDK4* and patients with no alterations in any of these genes. These findings suggest that *CDK4* aberrations may cooperate with *p53* inactivation in determining cell proliferation and survival in MCL and may act as an alternative mechanism to *INK4/ARF* inactivation.

Other oncogenic events

The high number of genetic alterations observed in MCL has promoted the search for additional potential targets of these chromosomal aberrations involved in the pathogenesis of the tumors. *c-myc* is overexpressed in 38% of the tumors with a slightly higher frequency in blastoid variants than in typical ones.[48] Although occasional *c-myc* gene amplification and translocations have been observed in blastoid MCL variants, these alterations seem to be relatively uncommon.[47-51] *BMI-1* is a putative oncogene of the Polycomb group family involved in murine lymphomagenesis and seems to participate in cell cycle regulation and senescence by acting as a transcriptional repressor of the *INK4a/ARF* locus.[52] This gene is located at chromosome 10p13, a region amplified in occasional MCL.[47] Gene amplification and overexpression occurs in a subset of MCL with the wild type *INK4a/ARF* locus.[6] Other frequently altered chromosomal regions in MCL are 1p22, 2pter, 3q27-29, and 18qter. However, virtually no alterations of *bcl-10*, *N-myc*, *bcl-6*, and *bcl-2*, located in these chromosomal regions, have been observed in these tumors.[47,53]

Mantle cell lymphoma expression profile

Different studies have analyzed the global expression profile of MCL and showed a specific expression pattern different from that of other B-cell neoplasms.[54-57] Overexpression of cell cycle-related genes is a constant finding in most of these studies and the quantification of a small subset of these genes is the strongest survival predictor allowing groups of patients with markedly different survival to be defined.[55] MCL also seem to have a relatively high expression of genes related to multidrug resistance.[56] Interestingly, a possible small group of *true* cyclin D1 negative MCL has been identified. Cyclin D1 was not detected in these tumors despite them having identical morphology, phenotype, survival, and global expression profile as conventional cyclin D1-positive tumors.[55]

In summary, MCL is distinctive clinicopathological entity molecularly characterized by a primary oncogenic deregulation of cyclin D1 that may participate in the pathogenesis of this tumor by overcoming the suppressor effect of the cell cycle G1 phase regulators retinoblastoma and p27. Frequent *ATM* inactivation may allow accumulation of additional chromosomal aberrations. *ATM* and *CHK2* gene mutations in germline raise the possibility that these genetic alterations are a very early event in these neoplasms. Aggressive variants of these tumors are associated with high proliferation that may be the result of different oncogenic events targeting other cell cycle and senescence regulators including *BMI1*, the *INK4a/ARF* locus, *p53* and *CDK4*.

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The morphology of mantle cell lymphoma. The Würzburg Experience

Mantle cell lymphopma (MCL) is a disease of middle-aged to elderly people. Most patients present in stages III and IV with the tumor tending to involve lymph nodes, Waldeyer's ring and the spleen. The bone marrow is often infiltrated, and some patients have prominent splenomegaly and peripheral blood involvement. The gastrointestinal tract is quite often infiltrated (lymphomatous polyposis). Sometimes, a high peripheral leukocyte count is found (mantle cell leukemia). Blastic transformation may occur (blastoid MCL).

Mantle cell lymphoma, in the majority of cases, presents with vaguely nodular and/or diffuse infiltrates of monomorphic small to medium-sized cells with a scanty, barely recognizable cytoplasm and irregular or cleaved nuclei. The chromatin is slightly dispersed and inconspicuous nucleoli are found. In rare cases, the cells are nearly round, making B-cell chronic lymphocytic leukemia a differential diagnosis. In other cases, the tumor cells may be slightly larger than *typical* centrocytes and harbor a broad pale cytoplasm giving the cells an appearance of marginal zone cells. A paraimmunoblastic MCL variant has also been described.

Two blastoid variants have been recognized, one with medium-sized nuclei, finely dispersed chromatin resembling lymphoblasts and a high proliferative index (blastoid or lymphoblastoid type). Another variant variously termed as *anaplastic*, *large cell*, or *pleomorphic* is characterized by rather large, cleaved cells with sometimes light or slightly basophilic cytoplasm and a coarse chromatin distribution. This type had been included in the Kiel classification as the *centrocytoid* variant of centroblastic lymphoma.

In some cases, the infiltrate in mantle cell lymphoma may be found predominantly surrounding (partially) preserved germinal centers giving the impression of a *mantle zone* pattern or may form sometimes vaguely circumscribed nodules.

In a thorough analysis of cytology and growth pattern of 226 cases of MCL from the Würzburg Lymph Node Reference Center, we found MCL to display a continuum of cytomorphologic features ranging from small-cell to blastoid and pleomorphic types rather than a well-reproducible segregation into classical and blastoid types (Table 1).

It is interesting to note that larger and blastic MCL variants were significantly associated with a predominantly diffuse growth pattern that has been claimed to indicate a worse prognosis (Tables 1 and 2).

Table 1. Cytomorphologic differences in 226 cases of MCL.

Variable	*Total*	*C1*	*C2*	*all C*	*C2L/P*	*L/P*
Number of cases	*226*	*121*	*33*	*154*	*9*	*30*
Growth pattern						
Diffuse (%)		66 (55)	26 (79)	92 (60)	8 (89)	24 (80)
Nodular (%)		42 (34)	5 (15)	47 (30)	0 (0)	5 (17)
Perifollicular (%)		13 (11)	2 (6)	15(10)	1 (11)	1 (3)

C: classical type; C1: small cells; C2: slightly larger cells, but not blastoid; L: (lympho-) blastoid; P: pleomorphic; C2L/P: indeterminate between classical and blastoid/pleomorphic.

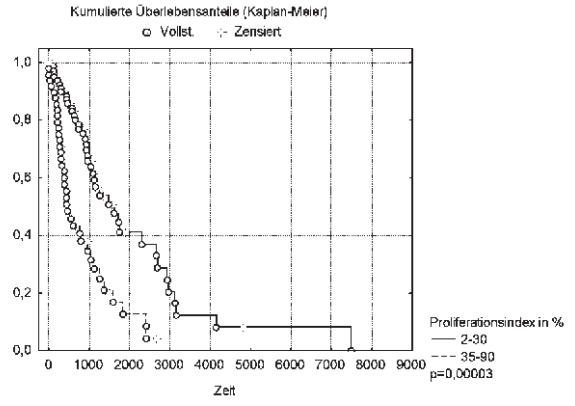

Figure 1. Proliferation index and survival in MCL.

Table 2. Association between cytology and growth pattern.

Diagnosis	*C1 vs. C2*	*All C vs. blastic types*
Diffuse growth pattern in	66/121 (55%) C1 vs. 26/33 (79%) C2	92/154 (60%) vs. 32/39 (82%)
p value	<0.05	<0.05

C:classical type; C1:small cells; C2:slightly larger cells, but not blastoid. All C: (Lympho-) blastoid, pleomorphic and indeterminate types.

Table 3. Morphology and proliferation.

	Classical types		*Blastic types*	
Proliferation (% Ki67+ cells)	*C1*	*C2*	*C2L/P*	*L/P*
Range	5-90	20-90	15-80	20-90
Mean	25	44	46	65
Median	20	40	50	60
(SD)	(18)	(21)	(21)	20
Proliferation Index >30%	25/139 18%	18/33 54%	6/9 66%	27/30 90%

Table 4. Association of mitotic and proliferative indices with p53 overexpression.

	Proliferation Index		*Mitotic Index*	
	0-40%	41-90%	0-40/10HPF	41-160/10HPF
P53>30%	10/134 (7%)	16/69 (23%)	11/137 (8%)	15/65 (23%)
P value	<0.001		<0.001	

Association with biological features

The tumor cells of MCL are characterized by the expression of CD5 and CD43 in the absence of CD10 and CD23. Notably, the characteristic overexpression of cyclin D1 can be recognized by suitable antibodies. It has been shown that the classical variant of MCL displays — in contrast to other non-Hodgkin's lymphomas with comparably low proliferative indices — a marked down-regulation of nuclear p27 expression, a feature which is highly characteristic of and favors mantle cell lymphoma. More recently, a concept of cyclin D1-negative mantle cell lymphomas has been established.

The above-mentioned cytomorphologically defined groups show distinct differences in the number of Ki67-positive cells (p <0.05) (Table 3). The proliferation and mitotic indices are positively correlated with distinct biological features, e.g. with an overexpression of p53 (Table 4).

Association with clinical features

Several morphologic and biological features have been associated with worse prognosis in MCL, such as a diffuse growth pattern, blastic morphology, and p53 mutation/overexpression. The most important variable today, however, seems to be the proliferation index as measured by Ki67 staining or the determination of the *proliferation signature*.

Association with genetic features

The cytogenetic hallmark of MCL is the chromosomal translocation t(11;14)(q13;q32), joining the *Ig* heavy chain locus and the *bcl-1* locus and resulting in overexpression of the cell-cycle protein, cyclin D1. It should be noted, however, that t(11;14)/cyclin D1 deregulation is not absolutely specific to mantle cell lymphomas, having also been described in plasmacytoma/multiple myeloma. Mantle cell lymphomas, in addition, show characteristic secondary aberrations, many of them targeting other cell cycle-related genes such as *p16*, *p53*, and the *ATM* gene. Interestingly, deletions in 1p, 1p and in 9p (the site of the *p16* gene) are correlated with high proliferation indices.

Particular clinical presentations

Although MCL predominantly presents as a systemic disease affecting lymph nodes, some particular clinical presentations are well recognized in MCL, the most frequent being isolated gastrointestinal disease (lymphomatous polyposis). There are, however, some other conditions that merit attention such as *primary splenic* MCL (with or without overt leukemic presentation) or some clinicopathologic particularities in MCL patients who remain are alive with the disease for long periods.

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Conventional treatment of mantle cell lymphoma

In spite of deeper insights into the molecular biology of mantle cell lymphoma (MCL) this disease still remains the lymphoma subtype with the poorest prognosis.[1,2] The purpose of this review is to summarize the current treatment strategies in MCL with a special focus on recent improvements.

Conventional treatment

Radiation in early stages. Only 10-15% of patients with MCL are diagnosed in limited Ann Arbor stages I-II. Potentially these patients may be cured by modified extended or involved field radiation. A recent study suggested that sequential radiochemotherapy could be advantageous.[3] In contrast, in advanced stages III-IV, the benefit of radiation therapy in addition to chemotherapy was not been proven and thus radiation should not be applied outside of clinical trials.

Conventional chemotherapy

Anthracycline-containing regimens. Different studies have investigated the efficacy of anthracycline-containing regimens (Table 1). In the only randomized study published so far, Meusers *et al.*[4] compared the COP (cyclophosphamide, vincristine and prednisone) and the CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) regimens. No advantage from CHOP was detectable. The overall response rate was 84% following COP as compared to 89% after CHOP. Similarly, no differences in the relapse-free (10 months vs. 7 months) or overall survival (32 months vs. 37 months) were detectable. In contrast, in a retrospective study, Zucca claimed that anthracycline-containing regimens were superior with regard to the complete response rate, failure-free survival and overall in the group of MCL patients at low risk according to the International Prognostic Index (IPI).[5,6] Although data from clinical studies are controversial, CHOP-like regimens are broadly applied.

Purine analogs

The use of purine analogs (e.g. fludarabine or cladribine) in MCL has been investigated by various groups. Single-agent fludarabine showed only moderate activity with response rates of approximately 30-40%.[7,8] In contrast, combinations with alkylating agents or anthracyclines were able to achieve higher remission rates.[9-11] In a study by Flinn *et al.*, the combination of fludarabine and cyclophosphamide achieved an overall response rate of 80%.[9] Similarly, in a study by Cohen and colleagues, fludarabine and cyclophosphamide were highly effec-

tive in newly diagnosed as well as in relapsed or refractory disease.[10] Previously untreated patients had a remarkable overall response rate of 100% (70% complete and 30% partial remissions) with a progression-free survival of 28.1 months.

Encouraging results were also achieved in another study, in which cladribine was combined with mitoxantrone. A remarkable complete response rate of 44% was achieved and the overall response rate was 100%,[12] confirming the suggested synergism of purine analogs and anthracyclines.

Other chemotherapy regimens

Various phase II studies of high-dose cytarabine (Ara-C) have also produced encouraging data. Lefrere *et al*. reported a complete remission in over 80% of patients following a sequential CHOP-DHAP regimen (dexamethasone, high-dose cytarabine and cisplatin).[13] Similarly, high response rates of more than 90% were achieved by a dose-intensified approach of the M.D. Anderson Cancer Center, applying an alternating regimen of Hyper-CVAD (fractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone) with high-dose cytarabine and methotrexate in elderly patients not suitable for stem cell transplantation.[14] As these data suggest that high-dose cytarabine has a high efficacy in MCL, this concept is currently being investigated by the *European MCL Network*.

Interferon-α

Two phase III studies investigated the efficacy of interferon-α maintenance (IFNα) following induction therapy.[15,16] Both analyses showed a tendency towards a prolonged progression-free survival with IFNα. However, the number of investigated patients was too low to determine definitely to what extent IFNα might prolong progression-free survival.

Monoclonal antibodies

Another encouraging approach is the use of the anti-CD20 antibody, rituximab. Rituximab is a chimeric murine/human monoclonal antibody which binds the B-cell specific antigen CD20. *In vitro* studies demonstrated that rituximab lyses CD20+ cells by complement activation or antibody-dependent cell-mediated cytotoxicity.[17,18] In the past few years, various studies have investigated the efficacy of rituximab in MCL. Rituximab, as a single-agent, showed only moderate activity with partial response rates of approximately 20-40%.[19-21] In contrast, in a non-randomized phase II study combined immuno-chemotherapy with rituximab and CHOP achieved remarkably high overall and complete response rates (96% and 48% respectively; Table 2).[22]

These results were confirmed by a recent randomized trial of the German Low Grade Lymphoma Study Group (GLSG) which investigated the efficacy of R-CHOP in comparison to CHOP alone in previously untreated patients.[23] The overall response rate in patients receiving CHOP alone was 75%, with only 7% complete remissions detectable. In contrast, in the R-CHOP study arm 94% of patients achieved a complete or partial remission (34% and 60%, respectively). Although the time to treatment failure was significantly longer in the immuno-chemotherapy arm, no plateau in the overall survival was observed, indicating the absence of durable remissions (Table 2). Thus, R-CHOP may become the standard therapeutic approach in the first-line therapy of MCL but multimodal consolidation concepts are warranted to

Table 1. Anthracyclines in the therapy of MCL.

Author	*n*	*Regimen*	*CR/OR*
Meusers *et al.* 1989[4]	37	COP: Cyclophosphamide 400 mg/m²/d×5 Vincristine 1.4 mg/m²/d ×1 Prednisone 100 mg/m²/d× 5	41%/84%
	26	CHOP: Cyclophosphamide 750 mg/m²/d ×1 Doxorubicin 50 mg/m²/d× 1 Vincristine 1.4 mg/m² × 1 (maximum 2 mg) Prednisone 100 mg/m²/d× 5	58%/89%
Hiddemann et al. 1996[30]	20	COP: Cyclophosphamide 400 mg/m²/d× 5 Vincristine 1.4 mg/m²/d× 1 Prednisone 100 mg/m²/d× 5	5%/80%
	19	PmM: Prednimustine 100 mg/m2/d x 5 Mitoxantrone 8 mg/m2/d x 2	27%/80%
Zinzani et al. 2000[11]	18	Fludarabine 25 mg/m²/d × 3 Idarubicin 12 mg/m²/d× 1	33%/61%
	11	Fludarabine 25 mg/m²/d× 5	27%/73%

n, number of patients; CR, complete remission; OR, overall response.

Table 2. Combined immuno-chemotherapy in MCL.

Author	*n*	*Regimen*	*CR/OR*
Howard *et al.* 2002[22]	40	R-CHOP	48%/96%
Hiddemann et al. 2003[24]	24	R-FCM	33%/62*
Hiddemann et al. 2003[23]	62	R-CHOP	34%/94%*

*n: number of patients; CR: complete remission, OR, overall response. *Significant improvement in comparison to chemotherapy alone.*

translate this high response rate into long-term remissions.

More encouraging results were recently published by the GLSG.[24] In a randomized trial, the combination of FCM chemotherapy (fludarabine, cyclophosphamide and mitoxantrone) was compared to the combination of rituximab and FCM in refractory and relapsed MCL. The addition of rituximab resulted in significantly improved complete and overall remission rates (33% vs. 0% and 62% vs. 43%), clearly indicating the superiority of combined immuno-chemotherapy in MCL (Table 2). However, even more importantly, after a median follow-up of 19 months a significantly improved overall survival was detectable in the R-FCM study arm.

Another innovative approach is the application of radio (^{131}iodine or ^{90}yttrium) labeled anti-CD20 antibodies.[25,26] Different phase II studies achieved remarkably high and long lasting remissions in relapsed or refractory MCL patients and thus this strategy is currently being evaluated in phase III trials.

New therapeutic agents

A new molecular targeting agent in the treatment of MCL is the specific inhibitor of the cyclin-dependent kinase (CDK)4-cyclin D1 complex flavopiridol. Several small studies investigated the efficacy of this agent.[27,28] However, the efficacy in relapsed or refractory MCL was limited. As cell culture experiments suggest a chemosensitizing effect, flavopiridol might be more effective in combination with chemotherapy. The proteasome inhibitor bortezomib (Velcade) represents another molecular targeted approach in the treatment of MCL. After being highly effective in MCL-derived cell lines and SCID mouse models, bortezomib showed its efficacy in a recent phase II study of the M.D. Anderson Cancer Center.[29] More than 50% of previously heavily pre-treated patients responded to bortezomib therapy at a dose of 1.5 mg/m^2.

Conclusion and future directions

In summary, MCL remains incurable by conventional chemotherapy. So far, CHOP-like combinations have been the standard therapeutic approach. However, recent randomized trials have confirmed the benefit of combined immuno-chemotherapy with rituximab both in newly diagnosed and in relapsed or refractory MCL. However, even after combined immuno-chemotherapy, the vast majority of patients will eventually relapse. Thus, new therapeutic strategies are urgently needed to improve the prognosis of MCL.

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Diffuse Large B-cell Lymphoma. Is It Useful to Identify Different Subsets?

Chairman: R. Dalla Favera

Molecular diagnosis, outcome prediction, and therapeutic target identification in cancer by gene expression profiling

In our laboratory we study the molecular pathogenesis of human lymphoid malignancies and have three primary goals: to establish a new molecular diagnosis of human lymphoid malignancies using gene expression profiling, to elucidate the oncogenic pathways that result in malignant transformation of normal B lymphocytes, and to identify molecular targets for the development of novel therapeutics for these cancers.

To provide a molecular basis for the diagnosis of human lymphoid malignancies, we are exploiting DNA microarray technology to profile gene expression in these cancers on a genomic scale. A novel DNA microarray, the *Lymphochip*, has been created in the laboratory. This DNA microarray is enriched in genes that are expressed in and/or function in lymphocytes.[1] We have used Lymphochip microarrays to profile gene expression in diffuse large B-cell lymphoma (DLBCL),[2,3] chronic lymphocytic leukemia (CLL),[4,5] mantle cell lymphoma,[6] follicular lymphoma, and in a wide variety of normal lymphoid subsets. Recently, we have also profiled these tumors using Affymetrix U133 oligonucleotide arrays, with results that are strikingly comparable to those obtained with Lymphochip DNA microarrays. The central goal of these studies is to relate gene expression to clinical outcome, thereby establishing useful prognostic indicators and identifying potential targets for new therapies.[7]

Definition of molecularly and clinically distinct diseases within diffuse large B-cell lymphoma

DLBCL has long been enigmatic in that 40% of patients can be cured by combination chemotherapy whereas the remainder succumb to this disease. By gene expression profiling, the laboratory discovered that DLBCL is actually two different diseases that are indistinguishable by current diagnostic methods.[2] One subgroup of DLBCL, termed germinal center B-cell-like (GCB) DLBCL, expresses genes that are hallmarks of normal germinal center B cells and thus most likely arises from B cells at this stage of differentiation. By contrast, the other DLBCL subgroup, termed activated B cell-like (ABC) DLBCL,

expresses genes that are induced during mitogenic stimulation of blood B cells[2] as well as genes that are expressed in plasma cells.[8] The cell of origin of ABC DLBCL has not been fully elucidated, but may be a plasmablastic B cell that is poised to exit the germinal center. In support of this hypothesis is the observation that both GCB and ABC DLBCL have somatically mutated immunoglobulin genes, a hallmark of B cells that have traversed the germinal center.[9] GCB DLBCL have ongoing somatic hypermutation of their immunoglobulin genes, in keeping with their derivation from germinal center B cells. By contrast, ABC DLBCL have a fixed complement of immunoglobulin gene mutations, suggesting that the somatic hypermutation machinery has been inactivated as occurs normally during plasmacytic differentiation.

The scope of our laboratory's gene expression profiling analysis of the lymphoid malignancies has been significantly expanded with the creation of the Lymphoma/Leukemia Molecular Profiling Project (*http://llmpp.nih.gov*). This international collaborative project involves eight groups that have maintained biopsy samples and clinical data from hundreds of lymphoma patients. In the initial study by the LLMPP consortium, we profiled gene expression in 240 biopsy samples of DLBCL,[3] and confirmed the existence of the GCB and ABC subgroups of DLBCL. Importantly, two recurrent oncogenic abnormalities in diffuse large B-cell lymphoma, BCL-2 translocation and c-rel amplification, were only detected in GCB diffuse large B-cell lymphomas, demonstrating that these gene expression subgroups of diffuse large B-cell lymphoma are pathogenetically distinct diseases.[3,10]

To deliver gene expression-based diagnoses to cancer patients, we developed a method to determine the probability that a patient's tumor belongs to a particular cancer subgroup.[8] Bayesian statistics was used determine the probability that a DLBCL biopsy sample belonged to the GCB or ABC subgroup. Although this method was developed using gene expression data from Lymphochip microarrays, it was also able to classify an independent set of diffuse large B-cell lymphoma samples that were profiled using Affymetrix microarrays.[11] The GCB and ABC subgroups of DLBCL identified by this Bayesian predictor had distinct survival rates following chemotherapy of 59% and 31%, respectively. Only 14 genes are needed for this Bayesian predictor, which should hasten its clinical application.

Molecular diagnosis of primary mediastinal B-cell lymphoma

Recently, we and others developed a molecular diagnosis of a third subgroup of DLBCL, termed primary mediastinal B-cell lymphoma (PMBL).[12,13] PMBL cannot be reliably distinguished from other types of DLBCL by current clinical criteria. PMBL was readily distinguished from GCB and ABC DLBCL by the expression of hundreds of genes, and we were able to develop a molecular diagnosis of PMBL using the Bayesian predictor method described above. Clinically, PMBL patients were younger (median age 33) than patients with GCB or ABC DLBCL (median age over 60). PMBL lymphomas frequently extended from the mediastinum into other thoracic structures, but did not involve the extrathoracic sites that are typical of other DLBCL. The 5-year survival rate of PMBL patients treated with current regimens was 64%, which was superior to that of ABC DLBCL and marginally better than that of GCB DLBCL.

A striking relationship was found between PMBL and Hodgkin's lymphoma by gene expression profiling. Over one third of the genes that were characteristically expressed in PMBL were also expressed in Hodgkin's lymphoma cell lines. We confirmed the expression of several of these genes in primary Hodgkin Reed Sternberg cells from nodular sclerosing Hodgkin's lymphoma. In the light of this strong molecular resemblance, it is particularly notable that PMBL and Hodgkin's lymphoma share numerous other clinical and pathological features, including a prevalence in young patients, especially women, presentation in the mediastinum, and prominence of sclerotic reactions pathologically.

The gene that best discriminated PMBL from other DLBCL was *PDL2*, which encodes a regulator of T-cell activation. This gene was also highly expressed in Hodgkin lymphoma cells. Interestingly, the genomic region harboring PDL2 was amplified in over half of PMBL and in Hodgkin's lymphoma cell lines. By contrast, this locus was amplified in only 4% of cases derived from the other DLBCL subgroups. PMBL and many cases of Hodgkin's lymphoma arise in the mediastinum, possibly from a rare B-cell subpopulation in the thymus. It is therefore conceivable that PDL2 functions to modulate the response of thymic T cells to the lymphoma cells.

Molecular targets in cancer discovered by gene expression profiling

The subdivision of DLBCL into gene expression subgroups has also identified new molecular targets in these diseases. Our laboratory discovered that a critical molecular difference between the GCB and ABC DLBCL subtypes is the activation of the NF-κB pathway.[14] ABC DLBCL, but not GCB DLBCL, express a number of know target genes of the NF-κB transcription factors, including IRF-4 and cyclin D2. ABC DBLCL show constitutive activation of the IkB kinase, which leads to the phosphorylation and degradation of IkB, an inhibitor of the NF-κB pathway. GCB DLBCL, in contrast, have little if any activity of the NF-κB pathway, in keeping with the fact that normal

germinal center B cells do not express NF-κB target genes appreciably.[15] Inhibition of the NF-κB pathway in ABC DLBCL cells is lethal whereas the survival of GCB DLBCL does not depend upon NF-κB. These results validate the NF-κB pathway as a molecular target in ABC DLBCL. More recently, PMBL was shown to express NF-κB target genes, presumably due to the presence of NF-κB in the nucleus of PMBL cells.[12,13] Whether PMBL, like ABC DLBCL, depends on the NF-κB pathway for survival is not yet known. A new clinical trial at the NCI is now open for patients with relapsed DLBCL in which an inhibitor of the NF-κB pathway, Velcade/PS-341, will be combined with chemotherapy. Gene expression profiling of tumor biopsies from the patients in this trial will help us understand the molecular basis of responsiveness to this new regimen.

A molecular predictor of survival following chemotherapy for diffuse large B-cell lymphoma

The subdivision of DLBCL into distinct subgroups of the basis of gene expression accounts, in part, for the differences in survival of these patients following multiagent chemotherapy. However, further scrutiny of the gene expression profiling data revealed many additional genes whose expression patterns were associated with the length of survival. These predictive genes reflected different biological features of the tumors that influenced the ability of chemotherapy to cure these patients. The relationship of these predictive genes to particular biological processes was revealed by assigning them to *gene expression signatures*.[15] A gene expression signature is operationally defined as a group of coordinately regulated genes that are characteristically expressed in a particular cell type or stage of differentiation, or are expressed during a particular cellular response to internal or external stimuli.

The majority of the genes that predicted outcome following chemotherapy could be classified into one of four gene expression signatures. Expression of genes in the *germinal center B-cell signature* predicted a favorable outcome, a finding that mirrors the DLBCL subgroup distinction described above. Two gene expression signatures that reflect the immune response to the tumor were favorable prognostic features. One of these, the *MHC class II signature*, may reflect the ability of tumor cell antigens to be presented to T lymphocytes. The other, termed the *lymph node signature*, reflects an intense fibrotic response to the tumor cells in the lymph nodes of some patients which includes infiltration by macrophages and NK cells. The favorable prognosis associated with this gene expression signature suggests that the innate immune response contributes to curative responses to chemotherapy. The *proliferation gene expression signature* contained many of the genes that predicted poor outcome. This signature includes genes that are expressed at high levels in dividing cells and at lower levels in quiescent cells. Roughly one sixth of all genes are regulated in this fashion, including many novel genes of unknown function. All together, 55% of the genes that predicted outcome could be classified into one of these four gene expression signatures.

A composite molecular predictor of survival was created using only 17 genes and was used to divide the DLBCL patients into four equal groups with widely differing 5-year survival rates of 73%, 71%, 36% and 15%. This molecularly-based outcome predictor may prove useful in the clinical management of patients with DLBCL. On the one hand, this predictor can identify patients who are likely to be cured by current chemotherapy regimens, including patients who present with clinically aggressive and advanced disease. On the other hand, the DLBCL patients assigned to the least favorable risk group might specifically benefit from alternative therapies such as bone marrow transplantation or might be candidates for clinical trials involving novel therapeutic agents.

Development of a molecular predictor of survival following diagnosis of mantle cell lymphoma

Mantle cell lymphoma accounts for roughly 8% of all non-Hodgkin's lymphomas, but contributes disproportionately to the number of deaths from lymphoma since there is no curative chemotherapy. Currently, the diagnosis of mantle cell lymphoma requires the expression of cyclin D1, which is a consequence of the t(11;14) translocation. By gene expression profiling, mantle cell lymphoma could be readily distinguished from DLBCL and from small lymphocytic lymphoma.[6] This molecular diagnosis led to the identification of a novel subgroup of mantle cell lymphoma that is cyclin D1-negative. This subgroup, which accounted for ~6% of mantle cell lymphomas, was indistinguishable from cyclin D1-positive mantle cell lymphomas by histology and by gene expression but lacked the t(11;14) translocation. Cyclin D1-negative and cyclin D1-positive mantle cell lymphomas were also indistinguishable in terms of length of survival following diagnosis.[6]

Survival times in patients with mantle cell lymphoma range from less than 1 year to more than 10 years following diagnosis. DNA microarrays were used to correlate gene expression in 91 cyclin D1-positive mantle cell lymphoma biopsies with length of survival following diagnosis.[6] Genes associated with cellular proliferation were more highly expressed in tumors of patients with short survival. A molecular predictor of survival based on the expression of these proliferation signature genes

could stratify patients into 4 prognostic groups with median survival times of 0.8 years, 2.3 years, 3.3 years, and 6.7 years. This survival prediction could be made based on as few as 4 genes.

Tumor proliferation rate was found to be quantitatively influenced by levels of cyclin D1 mRNA expression in the tumors.[6] Differential utilization of cyclin D1 isoforms accounted for differences in cyclin D1 mRNA expression. The presence or absence of deletions in the INK4A locus also influenced the tumor proliferation rate. The proliferation gene expression signature thus serves as a quantitative integrator of multiple oncogenic events in mantle cell lymphoma.

Identification of ZAP70 as the gene that best predicts clinically distinct subgroups of chronic lymphocytic leukemia

Two clinically distinct subgroups of chronic lymphocytic leukemia (CLL) can be distinguished by the presence or absence of immunoglobulin variable gene (*IgVH*) mutations in the leukemic cells.[16,17] Patients whose leukemic cells have unmutated *IgVH* genes have a median survival of 6-10 years whereas patients whose leukemic cells have mutated *IgVH* genes have a median survival of greater than 20 years.[18,19] By gene expression profiling, ZAP-70 was identified as the gene whose expression best discriminated these two subtypes of CLL.[5] Among 107 cases of chronic lymphocytic leukemia, the expression of ZAP70 predicted the dichotomy between the two CLL subgroups with 93% accuracy.[4] Since *IgVH* sequencing is impractical in a clinical diagnostics laboratory, mRNA or protein-based assays for ZAP70 expression should be of clinical benefit.

Molecular diagnosis and prognosis in the clinic

The various examples cited above suggest that gene expression profiling should become a consistent part of the routine diagnosis of cancer patients. This technology can identify clinically distinct subgroups of cancer that are not apparent by current diagnostic methods. Further, the prognostic information derived from gene expression profiling could help guide clinical management of these patients. The effective clinical translation of these findings will require a method that is quantitative, sensitive, and inexpensive. Custom DNA microarrays that measure the expression of several hundreds of genes hold considerable promise in this regard. In contrast to immunohistochemistry, DNA microarrays are quantitative and enable the expression of many genes to be assessed in a high-throughput fashion. Although quantitative polymerase chain reaction (PCR) is a sensitive and accurate technology, it is considerably more expressive than DNA microarrays if more than a few genes need to be assayed. Importantly, DNA microarrays are amenable to automation and deliver highly reproducible results.

Clinical trials in cancer should include genomic-scale gene expression profiling as a necessary component. Only in this way will we understand the heterogeneous response of patients to therapy. Furthermore, we will be able to accurately compare the patients enrolled in different clinical trials based on the gene expression profiles of their tumors. This approach to clinical trials will lead to a molecular definition of cancer that is relevant to the currently available therapeutic agents, and will identify which cancer patients need new therapeutic strategies in the future.

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Conventional treatment of diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLCL) is a systemic disease at the time of diagnosis; therefore, chemotherapy is the mainstay of treatment. Although the standard chemotherapy regimen has not significantly changed over 25 years, the relatively recent incorporation of monoclonal antibody therapy into the standard treatment program is improving overall survival for the majority of patients with this disease.

Early stage disease (Stages I and II non-bulky)

Abbreviated CHOP chemotherapy + involved field radiation therapy is excellent therapy for patients with low risk, non-bulky early stage DLCL.[1] Patients with poor prognostic features, such as advanced stage, tumor bulk, or high lactate dehydrogenase (LDH), remain at considerable risk of disease progression, and current clinical trials are designed to improve outcome in this group of patients.

Advanced stage disease (stages II bulky, III, and IV)

The most definitive study was an Intergroup trial conducted by SWOG and ECOG, in which 1138 previously untreated patients with stages II bulky, III, and IV disease with intermediate or high grade histology were randomized to one of four treatment arms: CHOP, m BACOD, ProMACE CytaBOM, or MACOP B.[2] Eight hundred and ninety-nine patients were eligible for the study. Each of the regimens was administered exactly as had been described in the prior phase II studies. The median age of patients was 54 years, with 25% of the patients being older than 64. With a median follow-up of 49 months, no differences were observed among the four treatment arms with respect to complete remission (CR) or overall response rates. Forty three percent of all eligible patients were estimated to be alive without disease at 3 years. By treatment arm, 43% in the CHOP arm, 43% on the m BACOD arm, 44% in the ProMACE CytaBOM arm, and 40% in the MACOP B arm are projected to be alive without disease at 3 years ($p = 0.35$). The projected overall survival (OS) at 3 years for all eligible patients is 52%; 49% in the MACOP B arm, 51% on the m BACOD arm, 53% in the ProMACE CytaBOM arm, and 55% in the CHOP arm ($p = 0.90$). However, when fatal and life threatening reactions were combined, significant differences were found between regimens, with CHOP and ProMACE CytaBOM being less toxic than m BACOD and MACOP B ($p < 0.001$). This study established CHOP as the standard therapy throughout the world although with a projected disease-free survival (DFS)

rate of 43%, it is obvious that it is far from the ideal therapy, and there is clearly a need for better treatment approaches.

Attempts have been made to improve the response to CHOP by combining it with the monoclonal antibody, rituximab. The GELA group randomized 399 previously untreated patients with diffuse large-B-cell lymphoma, 60 to 80 years old, to receive either eight cycles of CHOP every three weeks or eight cycles of CHOP plus rituximab given on day 1 of each cycle.[3] With a median follow-up of two years, the addition of rituximab to the CHOP regimen increased the complete-response rate (76% vs. 63%, p=0.005) and prolonged both event-free and overall survival in these patients, without a clinically significant increase in toxicity.

A larger (N=632) intergroup United States Study randomized a similar population of patients to CHOP vs. CHOP with rituximab given using a different schedule[35] as described in follicular lymphoma.[4] Responding patients were then randomized to receive either rituximab maintenance therapy (4 doses q 6 months × 2 years) or no maintenance. Preliminary results suggest that the addition of rituximab is of benefit to progression-free survival, however no overall survival benefit is yet apparent. There was no clear benefit to rituximab maintenance when rituximab was incorporated into the initial treatment regimen. Using a weighted analysis, an overall survival benefit was apparent when rituximab was combined with CHOP chemotherapy. Finally, preliminary results from an international trial evaluating the combination of CHOP and rituximab in patients younger than age 60 analyzed 326 patients.[5] Patients receiving rituximab with chemotherapy had a significantly longer time to teatment failure (84% vs. 62.5%) than those given chemotherapy alone. Based largely upon the published results from GELA, CHOP with rituximab therapy (all therapy administered on day 1) has emerged to become the standard initial treatment for advanced stage diffuse large B cell lymphoma in the United States. An unplanned subgroup analysis of the GELA trial demonstrated that the benefit of rituximab appeared limited to patients with lymphoma that overexpressed bcl-2 on immunohistochemistry.[6] Ongoing research will better define groups of patients with large cell lymphoma, if any, who do not benefit from the addition of monoclonal antibody therapy to chemotherapy.

Therapy of de novo large B cell lymphoma

Most patients with advanced stage diffuse large B cell lymphoma are not cured with conventional therapy. Hence, each treating physician must recognize the inadequacy of current therapy and urge all eligible patients to participate in well designed clinical trials. The best therapy remains to be defined, and therefore the best management for the patient is an experimental approach designed to improve our ability to cure the disease. If a patient is not eligible or does not wish to participate in a clinical trial, CHOP with rituximab is now the gold standard against which all new therapy must be compared.

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Peripheral T-cell Lymphoma. A Still Uncurable Disease?

Chairman: G. Delsol

Peripheral T-cell lymphoma

The family of peripheral T-cell lymphomas (PTCL) includes tumours derived from peripheral T-lymphocytes and NK cells.[1,2] With only three exceptions (mycosis fungoides, CD30+ lymphoproliferative disorders of the skin and large granular lymphocyte leukaemia), all PTCL behave aggressively.[3-7] They can roughly be subdivided into specified and unspecified forms (Table 1).[1,2] The former correspond to a series of rare, but distinct clinico-pathologic entities, while the latter — much commoner — gather an array of morphologically heterogeneous neoplasms characterized by poor response to therapy irrespectively of their nodal or extra-nodal presentation.[4,7] The WHO classification also included an *NK blastic lymphoma* within PTCL:[2] recent studies have shown that this process does not stem from lymphocytes and, according to its actual origin, should be termed *dendritic cell precursor tumor* (*Facchetti, et al 2003*).

In principle,[1,2,7] PTCL consist of more or less polymorphic elements of variable size, provided with irregularly shaped nuclei and a rather wide rim of cytoplasm, more frequently clear at Giemsa staining. Neoplastic cells can be admixed with reactive elements, such as follicular dendritic cells (FDC), histiocytes, eosinophils, plasma cells and epithelioid elements. The latter can at times obscure the neoplastic population (so-called Lennert's lymphoma). High-endothelium venules are comprised within the tumor, being prominent in the angio-immunoblastic lymphadenopathy (AILD)-type. In the lymph node, the lymphomatous growth selectively involves the paracortex, at times sparing reactive follicles (so-called T-zone pattern). On immunohistochemistry, PTCL show phenotypic aberrations consisting in CD4/CD8 restriction, loss or co-expression and/or loss of one or more of the following T-cell associated antigens: CD2, CD3, CD5, CD6, and CD7.[2,7] They can express CD30 irrespectively of anaplastic morphology as well as cytotoxic markers, including TIA-1, granzyme B, perforin, CD56 and CD57.[2,7] Interestingly, TIA-1, granzyme B and perforin are absent in mycosis fungoides and most nodal PTCL, while they are usually detected in extra-nodal PTCL and ALCL.[2,7] Finally, aberrant expression of CD15, CD20 and/or CD79a has at times been reported.[9-12]

Since an exhaustive description of PTCL is beyond the scope of this review, we will focus on the peculiar morphologic and phenotypic features of the specified forms.[1,2]

T-PLL includes four morphologic variants: prolymphocytic (75% of cases), small-cell (20%), cerebriform and hairy cell-like, all characterized by clinical aggressiveness. On phenotypic grounds, T-PLL can show either CD4 or CD 8 restriction or even CD4/CD8 co-expression: the latter two events along with protein S-100 positivity are associated with a worse prognosis. In addition, the process is characterized by CD52 expression (allowing *in vivo* administration of the Campath-1 monoclonal antibody)[11,12] and may be positive for CD25 and CD38.

Aggressive NK-cell leukemia is a rare condition that is sustained by small-medium sized elements with twisted/notched nuclei, moderately dispersed chromatin, small nucleoli, and a wide rim of cytoplasm containing azurophilic granules. On immunohistochemistry, neoplastic cells express CD2, $CD3_{cyt}$, CD56, TIA-1, granzyme B, and perforin.

ATLL represents an endemic tumor in the southern part of Japan, in the Caribbean islands, and in some small areas of Mexico and USA: it is pathogenetically related to HTLV-1 infection. The lymphomatous cells show extreme polymorphism with frequent floret-, embryo- or mulberry-like nuclei. Reed-Sternberg-like cells can be seen as well as large blasts containing either deeply basophilic or clear cytoplasm. Reactive elements (e.g. plasma cells, eosinophils and macrophages) are frequently encountered. The phenotypic profile is characterized by almost regular positivity for CD2, CD3, CD5, and CD25. CD4, CD8 and CD30 are variably expressed. CD7 and cytotoxic markers are absent.

Nasal/nasal-type T-cell lymphoma is more commonly observed in some geographic areas (Asia, Central and South America) and/or immunocompromised patients (especially following solid organ transplant): it is regularly associated with EBV infection, as shown by *in situ* hybridization with EBER1&2 probes and immunostaining for the latent membrane protein 1 (LMP-1). The tumoral growth - highly polymorphic - is typically angio-centric and angio-invasive, thus producing extensive necrosis and destruction of the involved anatomic area, which is most often the nose and central facial structures (fulfilling the criteria for being a *lethal midline granuloma*), but localizations at other parts of the body can be observed: the process is then termed *nasal-type*. The neoplastic population carries the characteristics of NK/T-cells with positivity for CD2, CD3cyt, CD8, CD56, TIA-1, granzyme B, and perforin. CD43,

CD45R0, HLA-DR, CD25 and CD95 are commonly expressed.

Enteropathy-type T-cell lymphoma is a rare condition that may be or not associated with celiac disease. If present, the latter likely represents a tumor precursor, as suggested by molecular studies showing oligoclonality or even monoclonality. The process is sustained by a rather monomorphic medium-sized population of cells that infiltrate the entire intestinal wall producing ulcers and perforations. At times, the lymphomatous elements resemble those of ALCL and are associated with a huge amount of inflammatory cells. In the past, this led the tumor to being misdiagnosed as *idiopathic inflammatory bowel disease*.[13] At immunohistochemistry, intestinal T-cell lymphoma usually displays the following profile: CD103$^+$ (typically expressed by normal intestinal T cells), CD2$^+$, CD3$^+$, CD5$^-$, CD7$^+$, CD4, CD8$^\pm$, CD30$^\pm$. *Hepato-splenic T-cell lymphoma* is an even rarer tumor characterized by selective infiltration of the liver and spleen at the sinusoidal level with preservation of the portal tracts and Malpighi's corpuscles, respectively. The lymphomatous elements, with medium-sized and overt cytological atypia, have the following phenotype: CD2$^+$, CD3$^+$, TIA-1$^+$, CD56$^\pm$, CD5$^-$, CD4$^-$, CD8$^-$, and perforin$^-$. They generally express the γ/δ dimer of the T-cell receptor (TCR), although occasional α/β-positive cases have been reported.[14]

Panniculitis-like T-cell lymphoma can develop either *de novo* or within the context of a previous inflammatory process. It involves the subcutis, sparing the septae and the overlying dermis and epidermis. It is sustained by a frankly atypical population of cells that surround individual adipocytes like a rim and cause extensive apoptosis and tissue necrosis. Reactive histiocytes are numerous and may produce a fatal hemophagocytic syndrome. On phenotypic grounds, lymphomatous elements express CD8, TIA-1, granzyme B, and perforin. The search for EBV is regularly negative.

AILD is characterized by diffuse effacement of the lymph node structure, a low cell density, abundant arborizing epitheliod venules and possible *burnt-out* (i.e. sclero-hyalinotic) residual follicles. Lymphomatous cells have different sizes, irregularly shaped nuclei and a wide rim of clear cytoplasm. They are admixed with EBV-infected B-immunoblasts, eosinophils, plasma cells and histiocytes. At immunohistochemistry, neoplastic cells show usual CD4 restriction, frequent CD10 expression[15] and moderate Ki-67 marking. Within this context, there is an abundant FDC component (CD21$^+$/CD23$^+$/CD35$^+$) that contributes to produce the low cellular density. B-immunoblasts may undergo neoplastic transformation and give rise to an unrelated DLBCL.[16]

ALCL is the most frequent type of *specified* PTCL.

Table 1. Peripheral T-cell lymphomas (categories listed in the REAL/WHO Classification).

Unspecified forms
Nodal
Extranodal (rarer)
Specified forms
Leukemic/disseminated
T-prolymphocytic leukemia (T-PLL)
Large granular lymphocyte leukemia
Aggressive NK-cell leukemia
Adult T-cell lymphoma/leukemia (ATLL)
Cutaneous
Mycosis fungoides
Sezàry syndrome
CD30$^+$ lymphoproliferative disorders of the skin
Other extranodal
Nasal/nasal-type T-cell lymphoma
Enteropathy-type T-cell lymphoma
Hepato-splenic T-cell lymphoma
Panniculitis-like T-cell lymphoma
Nodal
Angioimmunoblastic T-cell lymphoma (AILD)
Anaplastic large cell lymphoma (ALCL)

In the present review, attention will be focused on systemic ALCL, by omitting the primary cutaneous lesions that are now included among *CD30$^+$ lymphoproliferative disorders of the skin* (*see above*).[2] In its commonest form, systemic ALCL is predominantly composed of very large cells (so-called hallmark cells) with an eccentric kidney-shaped nucleus, a rod-shaped nucleolus and a wide rim of basophilic cytoplasm with a clear paranuclear area.[17-22] The tumor tends to grow cohesively and to spread through sinuses, a fact that in the past led to a misdiagnosis of metastatic undifferentiated carcinoma or malignant histiocytosis.[22] Morphologic variants of the process have been described: giant-cell rich, lympho-histiocytic, small cell, mixed, Hodgkin's-like and, more rarely, sarcomatoid, signet-ring cell-like, and epitheliod cell-rich.[18,23, 20,21] In reality, ALCL is always characterized by a morphologic spectrum: from small cells to hallmark cells.[17,24,23,21] However, usually one cytotype predominates over the others, thus producing different patterns. Notably, divergent cytotypes may be observed in biopsies taken from the same patient at different anatomic sites, either simultaneously or sequentially (e.g. at presentation and relapse). On phenotypic grounds, the tumor shows regular CD30 expression, common epithelial membrane antigen (EMA) positivity, frequent pre-

sentation of cytotoxic markers (TIA-1, granzyme B, and perforin), possible lack of the leukocyte common antigen/CD45, occasional CD15 staining, and a T- or null-phenotype.[17,24,20,21] This profile might make the distinction between ALCL and classical HL difficult. On this respect, the search for BSAP is of paramount importance: in fact, this molecule is regularly absent in ALCL, while it is usually expressed in Hodgkin and Reed-Sternberg cells of classical HL.[25] Sixty to ninety per cent of systemic ALCL express anaplastic large cell lymphoma kinase (ALK). This molecule — which is not detected in normal lymphocytes, or cells from classical HL and DLBCL with anaplastic morphology[26] — is thought to be involved in the pathogenesis of the process[24] and may be present in different locations: in the nucleus and/or cytoplas.[24,21] ALK positivity corresponds to translocations involving the *ALK* gene and different partners which lead to the formation of hybrid genes that encode for a series of chimeric proteins as shown below:

t(2;5)(p23;q35) = fusion gene and chimeric protein NPM/ALK (nuclear and cytoplasmic location);
t(1;2)(q25;p23) = fusion gene and chimeric protein TMP3/ALK (cytoplasmic location);
inv(2) = hybrid gene and chimeric protein ATIC/ALK (cytoplasmic location);
t(2;3)(p23;q21) = fusion gene and chimeric protein TGF/ALK (cytoplasmic location);
t(2;11;2)(p23;p15;q31) = hybrid gene and chimeric protein CARS/ALK (cytoplasmic location);
t(2;17)(p23;q23) = fusion gene and chimeric protein CLTL/ALK cytoplasmic location);
t(2;17)(p23;q25) = hybrid gene and chimeric protein ALO17/ALK (cytoplasmic location);
t(2;19)(p23;p13) =fusion gene and chimeric protein TPM4/ALK (cytoplasmic location);
T(2;22)(p23;q11.2) = hybrid gene and chimeric protein MYH9/ALK (cytoplasmic location);
T(2;X)(p23;q11-12) = fusion gene and chimeric protein MSN/ALK (cytoplasmic location).

Notably, the cases carrying these translocations respond extremelyt well to therapy, with the exception of the leukemic ones; they preferentially occur in the first two decades of life.[17,23] By contrast, the ALK- anaplastic tumors have a much worse prognosis (5 year overall survival 30% vs. 85%) and occur in the elderly.[17,24] This has led to question whether or not the term ALCL should be applied to large cell tumors with anaplastic morphology and T/null phenotype, but lacking ALK protein. Finally, ALK positivity contributes — along with BSAP negativity — to identify the rare forms of ALCL with Hodgkin's-like features and to differentiate them from cytologically aggressive classical HL.[25,24,21]

Recent advances

During the last few years, gene expression profiling techniques have aroused great interest, as they allow the identification of deregulated genes that take part in the development and response to therapy of specific tumor categories. In the field of malignant lymphomas, they have extensively been applied to diffuse large B-cell lymphoma,[27-29] mediastinal (thymic) large B-cell lymphoma , follicular lymphoma,[30,31] mantle cell lymphoma,[32] hairy cell leukemia,[33] and B-cell chronic lymphocytic leukemia.[34] In addition, they have allowed the genomic investigation of Hodgkin-derived cell lines.[35,36]

So far, only two studies have focused on T-cell lymphomas.[37,38] One of these was restricted to mycosis fungoides,[38] while the other compared neoplasms derived from precursors and peripheral elements.[37] The latter, based on the evaluation of 42 T-cell tumors, found significant differences between the peripheral and precursor cell forms, which included a deregulation of the NF-κB pathway. A set of 17 genes with different expression between tumors and normal T cells was also detected, as was a series of genes correlated with proliferation, response to therapy, stage of disease and survival of patients.

Perspectives

A gene expression profiling study on PTCL — integrated by the determination of key molecules on tissue micro-arrays — was recently carried out by the authors. The results of this study will be presented at the Symposium.

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Morphologic and phenotypic features and molecular anomalies of anaplastic large cell lymphomas

Our understanding of the heterogeneous group of large cell lymphoid neoplasms was significantly advanced in 1985 by Stein and co-workers[1] with the discovery of the Ki-antigen (later clustered at CD30 - lymphoid activation antigen). Initially, almost all nodal and extra-nodal large cell tumors expressing the CD30 antigen were referred to as *Ki-1 lymphoma* with ill-defined morphologic criteria, and irrespective of B, T or null cell phenotype. However, since the classifications (REAL, WHO) of malignant lymphomas were based on morphology and not on antigen expression, the term *Ki-1 lymphoma* was replaced by *anaplastic large cell lymphoma* (ALCL), and so incorporated in the REAL and WHO most classifications.[2] A few years after their description, it appeared that ALCL were associated with a unique chromosomal abnormality, the t(2;5)(p23;q35), thought initially to be characteristic of malignant histiocytosis.[3-5] In 1994, Morris *et al*. showed that the (2;5) translocation fuses part of the nucleophosmin (NPM) gene on chromosome 5q35 to a portion of the ALK (anaplastic lymphoma kinase) receptor tyrosine kinase gene on chromosome 2p23, resulting in the expression of a unique chimeric NPM-ALK protein. A dramatic progress in the diagnosis and understanding of ALCL lymphoma ensued with the availability of antibodies detecting the NPM-ALK and variant ALK fusion proteins.[6-9] Since most pathologists are likely to encounter these tumors infrequently, the following description is constructed from experience of approximately 270 cases acquired in-house or referrals.

Morphologic spectrum of anaplastic large cell lymphoma

The range of morphologic features in ALCL is wider than was initially described[1] as reflected by subsquent published descriptions of at least eight putative subtypes.[10-16] This range becomes even more evident after immunostaining with ALK antibody, which is suitable for paraffin sections and can be used on archival material.[6] Some cases show predominance of small cells that would meet the criteria for a pleomorphic T-cell lymphoma, but all cases show a variable population of large cells with characteristic morphology of eccentric horse-shoe or kidney shaped nuclei with often an eosinophilic region near the nucleus (*hallmark cells*). Large cells with *crown-like* nuclei are present in most cases. The common denominator is the strong reactivity for CD30, and the staining is surface membrane related associated with the perinuclear (Golgi) area.[16] We have identified the following seven cytomorphologic variants in our material. Only the three first are recognized by the WHO classification.

ALCL common type.[16] This is the most frequently seen ALCL in lymph nodes, populated by large cells with the aforementioned horse-shoe shaped nuclei and abundant cytoplasm. Within this category there re a few cases with a predominant population of cells with round nuclei (monomorphic variant). A sinusoidal growth pattern of malignant cells is seen in most cases. Of the 167 cases in our archives, expression of ALK protein was found in 149 cases (90%). Staining is charactistically diffuse in the cytoplasm and the nucleus, and usually extends to the nucleolus, but in some cases, the staining is limited to the cytoplasm.

ALCL lymphohistiocytic variant (23 cases).[11] In these cases, malignant cells are difficult to detect because they are admixed among large numbers of reactive histiocytes. Malignant cells are highlighted by CD30 staining and often seen around vessels. Approximately 80% of cases express ALK protein.

ALCL small cell variant: (11cases).[13] These are usually misdiagnosed as pleomorphic T-cell lymphoma on conventional examination. They consist of a predominant population of small to medium sized cells with irregular nuclei. *Hallmark cells* are often concentrated around the vessels, but this perivascular pattern is better seen after immunostaining for CD30, epithelial membrane antigen (EMA) or ALK. All our cases are positive for ALK but the small cell population is only variably positive and the staining is restricted to nuclei.

ALCL giant cell variant (6 cases). These are relatively rare and contain large numbers of pleomorphic giant cells with a characteristic immunophenotype.

ALCL mixed variant (28 cases of which 23 cases (80%) were positive for ALK). These 28 cases could not be classified because features of more than one ALCL variant were found in a single biopsy. These tumors were diagnosed as ALCL- *mixed*.[16] For example, in some cases areas suggesting lymphohistiocytic variant are associated with areas consistent with small cell variant ALCL. Furthermore, in four cases, a repeat biopsy at the time of relapse revealed morphologic features which differed from those seen initially.

ALCL unclassifiable. Twenty cases, of which 16 were positive for ALK, were either cases in which only a small biopsy specimen was available or tumors which showed unusual morphologic features. ALCL showing sarcomatous features, as described by Chan *et al*.,[12] associated with common type areas, and *signet ring* tumour cells, fall in this category.[15] We have not seen cases described as *neutrophil-rich* anaplastic large cell lymphoma as described by Mann and co-workers,[14] even when a neutrophil infiltrate

was clearly present in some cases.

Hodgkin's-like ALCL (12 cases). This is a variant recognised by the presence of vaguely nodular fibrosis associated with capsular thickening and a significant number of tumor cells resembling classical Reed-Sternberg cells. However, in our cases malignant cells (unlike classical Reed-Sternberg cells) were CD15-negative and lacked evidence of EBV (i.e. EBER). In addition, in all cases, malignant cells were positive for EMA and expressed one or more T-cell markers. Only 4 cases were found to be reactive for ALK.

Extranodal involvement by anaplastic large cell lymphoma

The primary systemic form of ALCL affects lymph nodes and extranodal sites. Skin involvement is frequent and found in approximately 30% of our cases. Bone marrow involvement by ALCL is considered to be uncommon. However, such an involvement is difficult to detect by routine morphologic examination alone. In studying 42 patients with ALC lymphoma, we analyzed the usefulness of immunohistochemistry in detecting minimal bone marrow involvement on routinely processed biopsy specimens. On conventional examination, 7 cases out of 42 (17%) were found to have bone marrow infiltration at diagnosis. Interestingly, almost one third (29%: 10/35 patients) of bone marrow specimens which were considered to be uninvolved on conventionnal examination showed scattered malignant cells only detectable with immunohistochemistry using Ber-H2 (CD30) antibody.[17]

Immunophenotype of anaplastic large cell lymphoma

Virtually all cases of ALCL are positive for EMA and we consider the co-expression of CD30 and EMA as highly indicative of ALCL.[18,19] In a previous study, H and Y blood group-related antigens, detected by antibody BNH.9, were found in a significant proportion of ALCL.[20] CD15 was not expressed, except for four cases showing a small proportion of CD15-positive neoplastic cells that stained.[16]

The great majority of cases of ALCL, whatever their morphological features, appear to be of T-cell origin (75%). However, CD3 staining of malignant cells is usually weak and observed in only one third of cases. The best T-cell marker (although not T-cell restricted) is CD43/MT1 since two thirds of ALCL are positive for these markers. In addition, most ALCL are developed from cytotoxic T lymphocytes and are positive for proteins associated with cytotoxic granules such as TIA1, perforin and granzyme B. Characteristically ALCL are negative or weakly positive for bcl-2 protein.

Differential diagnosis of anaplastic large cell lymphoma

Even if morphologic features of most ALCL suggest the diagnosis, the definitive diagnosis cannot be made without immunohistochemistry. The latter is critical in the diagnosis of some variants of ALCL. In our experience, one of the most difficult differential diagnoses is peripheral T-cell lymphoma (unspecified) consisting of a predominant population of large cells, some infiltrating lymphatic sinuses. Some of these tumors express CD30 strongly, sometimes even in association with EMA. In contrast to ALCL however, these tumors are usually strongly positive for CD3 and bcl-2 protein, and we have now a tendency to consider CD30-positive T-cell proliferation consisting of large cells as peripheral T-cell lymphoma unspecified.

There are some rare large cell lymphomas that show a predominant sinusoidal growth pattern and simulate ALCL. Two types of tumors deserve the most attention. The most frequent are the recently described large B-cell lymphomas expressing ALK protein.[21,22] Morphologically, these tumors consist of monomorphic large immunoblast-like cells with large central nucleoli, and they have a tendancy to invade lymphatic sinuses. Superficially they resemble anaplastic large cell lymphoma but they lack CD30. These lymphomas express EMA (as do ALCL) but also contain intracytoplasmic IgA of a single light chain type and the plasma cell associated marker (CD138 and VS38). They lack lineage-associated leukocyte antigens (CD3, CD20, CD79a), with the exception in some cases of CD4 and CD57. They are labeled by ALK1 antibodies and most cases show a characteristic cytoplasmic granular staining highly indicative of the t(2;17) and the expression of CLTC-ALK fusion protein.[22] A few cases show cytoplasmic nuclear and nucleolar ALK staining pattern and are associated with the t(2;5) and NPM-ALK protein as seen in ALCL. Most patients are male and the disease follows an aggressive course. Beside ALK-positive large B-cell lymphoma, some rare large B-cell lymphomas (sometimes referred to as microvillous lymphoma) may also invade lymphatic sinuses mimicking a metastatic malignancy. However, these tumors are relatively easy to recognize by immunohistochemistry since they express B-cell antigens (CD20, CD79a) and are ALK negative and variably positive for CD30 antigen.

ALCL *small cell variant* are commonly misdiagnosed as pleomorphic T-cell lymphomas. *Hallmark cells* are present but difficult to detect among small to medium sized cells. Although, the majority of small to medium sized lymphoid cells are malignant cells they usually express CD30 and EMA weakly which makes the diagnosis even more difficult. By contrast, large cells strongly express CD30 and EMA

and are localized around blood vessels. ALK staining is of critical diagnostic value in these cases.

Hodgkin's-like variant of ALCL, mimicking nodular sclerosis, does exist but is extremely rare. It is probable that most tumors previously diagnosed as Hodgkin's-like ALCL are cases of neoplastic cell-rich Hodgkin's disease (i.e. *anaplastic large cell lymphoma-like Hodgkin's disease* rather than *Hodgkin's-like anaplastic large cell lymphoma*. We suggest that, in ALK-negative cases, the diagnosis of Hodgkin's-like ALCL should be confined to cases showing not only morphologic features consistent with Hodgkin's disease but also an antigen profile characteristic of ALCL, i.e. positive staining for CD30, EMA, CD3, CD43 in conjunction with negative staining for EBV-associated markers (LMP1 and EBER) and B-cell antigens (both CD20 and CD79a). In these difficult cases, a morphologic feature which may be of value in differentiating anaplastic large cell lymphoma from anaplastic large cell lymphoma-like Hodgkin's disease is the perivascular pattern of neoplastic cell infiltration. We found this feature in more than half of our cases. It appears never to occur in Hodgkin's disease. We suspect that some cases of neutrophil-rich anaplastic large cell lymphoma described by Mann and co-workers[14] were cases of Hodgkin's disease. ALK-positive non lymphoid tumors may be responsible for diagnostic difficulties. Overall, ALK expression can be considered as highly indicative of ALCL. However, as originally reported by Morris *et al*.[5] rhabdomyosarcomas can occasionally express the full-length ALK protein (200 kD).[7] More recently, some myofibroblastic tumors associated with ALK gene rearrangement at 2p23[23] have been reported. Some neuroblastomas can also expressed full-length ALK protein but, in contrast to ALCL, the staining is weak.[9]

Primary cutaneous CD-30 positive T-cell lymphoproliferative disorders

Skin involvement by systemic ALCL can cause diagnostic problem with other CD-30 positive T-cell lymphoproliferative disorders. Three types of primary cutaneous CD-30 positive T-cell lymphoproliferative disorders are distinguished. These include anaplastic large cell lymphoma of primary cutaneous type; lymphomatoid papulosis; and borderline lesions. The distinction between these disorders is sometimes difficult and requires combined assessment of histological, clinical and phenotypic features. The prognosis of these lesions seems to be favorable, but further studies are needed.

Genetic anomalies associated with anaplastic large cell lymphoma

Recurrent chromosomal translocations play an important role in many human lymphoid tumors and are often responsible for the deregulation of genes involved in the control of normal cell proliferation. Originally, anaplastic large cell lymphoma was considered to be strongly linked with the t(2;5)(p23;35).[4,3,24,8] However, recent studies have shown that other translocations can be associated with this clinicopathological entity such as t(1;2) (q25;p23),[9] t(2;3)(p23;q35),[25,26] inv(2)(p23q35)[27] and t(2;17)(p23;q11.2).[28] All these translocations involve the ALK gene at 2p23 and are responsible for the aberrant expression of ALK. The t(2;5)(p23;q35) translocation is found in approximately two thirds of ALK-positive ALCL (Table 1).[4,3,24,8] This translocation involves the *ALK* gene at 2p23 and the *NPM* gene at 5q35.[5] The *ALK* gene encodes a tyrosine kinase receptor belonging to the insulin growth factor receptor superfamily, which is normally silent in normal lymphoid cells).[5,8] The nucleophosmin gene (NPM) fuses with a receptor tyrosine kinase gene, called ALK (anaplastic lymphoma kinase) to produce a chimeric protein in which NPM is linked to the intracytoplasmic portion of ALK.[5]

Interestingly, virtually all cases associated with the t(2;5) show a cytoplasmic, nuclear and nucleolar staining. This staining pattern is the most commonly observed in ALK-positive anaplastic large cell lymphoma (Table 1). Reports of ALK-positive ALCLs associated with the presence of a t(1;2)(q25;p23)[8] translocation suggested that a gene on chromosome 1 could contribute a promoter for the expression of the ALK catalytic domain, comparable to NPM pro-

Table 1. Genetic abnormalities in ALK-positive lymphoma which create fusion genes.

Chromosomal anomaly	*ALK partner*	*M Wt of ALK hybrid protein*	*ALK staining pattern*	*(%)*
t(2;5)(p23;q35	NPM	80	Nuclear, diffuse cytoplasmic	70
t(1;2)(q25;p23)	TPM3	104	Diffuse cytoplasmic with peripheral intensification	18
Inv (2)(p23q35)	ATIC	96	Diffuse cytoplasmic	4
t(2;3)(p23;q21)	TFGxlong	113	Diffuse cytoplasmic	1
	TFG_{long}	97	Diffuse cytoplasmic	
	TFG_{short}	85	Diffuse cytoplasmic	
t(2;17)(p23;q11ter)	CLTC	250	Granular cytoplasmic	1
t(2; X(p23;q11-12)	MSN	125	Membrane staining	1
t(2;19)(p23;p13.1)	TPM4	nd	Diffuse cytoplasmic	1
t(2;17)(p23;q25)	ALO17	nd	Diffuse cytoplasmic	1
t(2;22)(p23;q11.2)	MYH9	220	Diffuse cytoplasmic	1
Others*	?	?	Nuclear or cytoplasmic	3

*ALK: Anaplastic Lymphoma Kinase gene; NPM: nucleophosmin gene; TPM3: Non Muscular Tropomyosin gene; TFG: TRK-fused gene; ATIC: amino-terminus of 5-aminoimidazole-4- carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase gene; CLTCL: clathrin heavy polypeptide-like gene; MSN: moesin gene; ALO17:ALK lymphoma oligomerization partner; MYH9: myosin heavy chain 9 gene (present study); nd: not determined (%): percentage of these variants in a personal series of 270 cases of ALK-positive ALCL; *It is likely that further genes will be identified as rare partners involved in novel translocations with ALK.*

moter in cases associated with t(2;5). In cases associated with the t(1;2) translocation, which express the TPM3-ALK protein, (tropomyosin-ALK fusion protein) the staining is restricted to the cytoplasm and is associated with a membrane reinforcement.

Other ALK gene rearrangements have been described (Table 1). In the t(X;2) (*MSN-ALK*) (*Tort et al 2001*) ALK staining is restricted to the cell membrane. We have described two cases of ALCL showing a unique granular cytoplasmic ALK staining pattern. The cloning of the breakpoint showed that the *ALK* gene was fused to CLTCL gene which encodes the clathrin heavy chain (CLTC-ALK protein). More recently, we showed that few cases of ALCL are associated with the t(2;22) and expressed the new chimeric MYH9-ALK protein.

Prognostic implication of ALK protein expression

In our series of 267 cases of anaplastic large cell lymphoma (excluding cases of primary cutaneous CD30-positive tumors, all ALK negative), the male:female ratio showed a slight excess (1.4:1) of male subjects. Clinical data including age at onset, sex and follow up were available for 154 patients. The median follow-up time was 36 months (1-218 months). The average age of patients with ALK-positive tumors was 18 years, whereas it was 33 years for those with ALK-negative tumors ($p<0.01$). The overall ten-year survival for the 154 patients with follow-up was 72.3%. Survival based on the expression of ALK protein (including children and adults) showed a favorable prognostic significance for ALK expression (10 year-survival of 78.5% for ALK-positive patients compared to 44.4% for ALK-negative ones; $p<0.001$). Late relapses are rare since only one patient with ALK-positive tumor died of disease 150 months after the diagnosis. No significant difference was found between survival and histopathologic patterns.

Concluding remarks

ALK-positive tumours show a broad spectrum of morphologic features, ranging from small cell neoplasms, to cases in which very large cells predominate. The link between these morphologic subtypes of anaplastic large cell lymphoma is further reinforced by the presence in all cases of a highly characteristic large cell (*hallmark cell*). As emphasized in the REAL and WHO (*Delsol et al. 2001*) classifications, these tumors are of T or null phenotype and co-express CD30 and EMA. Rare cases of large B-cell lymphomas are positive for ALK.

The Hodgkin's-like form of anaplastic large cell lymphoma is still the subject of controversy. However, it is probable that most tumors previously diagnosed as Hodgkin's-like ALCL are cases of neoplastic cell-rich Hodgkin's disease (i.e. *anaplastic large cell lymphoma-like Hodgkin's disease* rather than *Hodgkin's-like anaplastic large cell lymphoma*).

Significant progress has been made in the diagnosis of ALCL with the availability of antibodies detecting the ALK tyrosine kinase domain. However, even though positive staining for ALK is most commonly associated with the hybrid NPM-ALK protein, ALCL can be associated with other fusion proteins. These variants are associated with different ALK staining patterns. In addition to its diagnostic value, ALK expression is an independent prognostic factor and ALK-positive cases are associated with a better survival than are ALK-negative tumors. Thus, it is likely that in the group of ALCL, only ALK-positive tumors represent a distinct clinicopathologic and genetic entity and should be designated as *ALKoma*.

Preliminary results (unpublished) using analysis of gene expression by microarrays suggest that anaplastic large cell lymphomas of common type have a different molecular signature than the other morphological variants. Furthermore, patients with stage 1 or 2 also have a molecular signature different from that of patients with stage 3 or 4.

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Chemotherapy

The classification of T/NK neoplasms as described by the REAL and the WHO classifications is based upon principles outlined by Lukes and Collins in the United States and Lennert in Europe in the 1970s. The Working Formulation (WF), devised in the 1980s, did not recognize T/NK lymphomas so that many diseases of different biology were *lumped* together. The present day classification is an evolving one, which identifies diseases as distinct entities according to clinical, morphologic, immunophenotypic, and genotypic data when available.

Peripheral T-cell and NK-cell neoplasms have been difficult to classify and to treat for multiple reasons.

The optimal therapy for T/NK neoplasms is an area of controversy due to the rarity of the diseases, their variable clinical course, and the lack of randomized trials. Although the intergroup trial comparing CHOP to three newer regimens established CHOP as the standard for WF intermediate grade lymphoma, there were few data available regarding the impact of immunophenotype. The assumption is that PTCL is a subset of the WF groups in the trial, and no regimen would be superior to CHOP. Despite this assumption, there are numerous reports of small series of rare subsets of T/NK neoplasms, such as hepatosplenic and nasal types, which have had a poor outcome despite anthracycline-based therapy. Anaplastic large cell lymphoma (ALCL) associated with the t(2;5) has been highly chemosensitive, while primary cutaneous ALCL, which lacks the t(2;5), has often had an indolent course for which combination chemotherapy may not be warranted. In the subsequent paragraphs, we will review the clinical spectrum and therapy of ALCL, nodal PTCL, subsets of extranodal T/NK neoplasms, and potential novel therapeutic strategies, including therapy for mycosis fungoides (MF) and adult T-cell leukemia/ lymphoma (ATLL) and the role of transplantation.

Anaplastic large cell lymphoma

ALK+ ALCL tends to respond better to chemotherapy than ALK- systemic ALCL. In three series there was an approximately two-fold or more increase in survival in ALK+ ALCL than in ALK- ALCL.[1-3] Other series, however, have not confirmed an improved survival for ALK+ ALCL.[4] Differences among studies could be due to variable percentages in adverse prognostic factors between groups and due to the younger age of ALK+ ALCL patients; however, an improved survival has been reported for ALK+ ALCL over ALK- ALCL in patients less than 30 years of age.[1]

The management of localized lesions may be watchful waiting, with approximately one quarter

regressing, local excision with or without radiation, or radiation alone.[5] Patients with disseminated skin disease or localized skin disease with nodal extension may benefit from combination chemotherapy, but these patients tend to relapse.[6] Lymphomatoid papulosis usually occurs as small crops of papules, regresses spontaneously, and rarely develop into a lymphoma. Low dose weekly methotrexate can control the lesions of lymphomatoid papulosis.[7]

Nodal peripheral T-cell lymphoma

The natural history of PTCL seems to have been unchanged by the use of second- and third-generation chemotherapy regimens[8] and 5-year overall survival still remains between 25% and 47%.[9-15] Although high-dose sequential chemotherapy followed by autologous hematopoietic stem cell transplantation (ASCT) has been successfully performed in 2 small series of patients,[16,17] others,[18,19] in a large cohort of high-risk NHL treated with high dose sequential chemotherapy followed by ASCT, have definitely demonstrated no benefit of autologous bone marrow transplantation in the subset of T-cell lymphomas.[16]

Mycosis fungoides

The traditional treatment of mycosis fungoides/Sézary's symdrome (MF/SS) includes topical and systemic therapies, alone or in combination.[20] Psoralen and ultraviolet A radiation (PUVA) is effective in early-stage MF/SS, inducing complete remission in most patients.[21-23] PUVA may also be combined with low doses of interferon to treat stage I and II disease.[24-26] Early aggressive therapy with radiation and chemotherapy does not improve the prognosis.[27] Local radiotherapy or total-skin electron-beam irradiation (TSEB) has been used with success to control advanced skin disease.[28,29] Extracorporeal photopheresis may also be used successfully but is not generally available.[30,31] Once the disease becomes refractory to topical therapy, interferon-α, bexarotene, single-agent chemotherapy, or combination chemotherapy may be given, but the duration of response is often less than 1 year, and ultimately all patients have relapses and the disease becomes refractory.[20,32-36] Response rates following combined modality therapy with TSEB and chemotherapy/interferon-α appear to be similar to those of other therapies.[37]

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Immunotherapy

Immunotherapy may be an approach to improve the treatment of peripheral T-cell lymphoma (PTCL). The definition of immunotherapy is not clear but this paper will focus mainly on antibody treatment and allogeneic stem cell transplantation.

The success of the-antiCD20 antibody (rituximab) in the treatment of B-cell lymphomas has inspired hoping of finding a similar substance for T-cell lymphomas. The basic requirement for successful antibody therapy is to find a suitable antigen. As a consequence of the remarkable variety of T-cell differentiation and antigen expression in PTCL this is a much harder task than in B-cell lymphomas. Clinically chimeric or humanized antibodies against CD52 and CD4 have been studied in phase 2 trials but many antibodies, such as anti-CD3, anti-CD7 and anti-CD25, still await this clinical testing.

Allogeneic stem cell transplantation has been associated with a high mortality, especially in immunocompromised patients. Patients with T-cell lymphoma are often severely immunocompromised and thus a risk group for this treatment. The development of reduced-intensity conditioning will probably reduce this risk and such regimens are becoming more popular.

Cutaneous PTCL (mycosis fungoides/Sézary's syndrome)

One of the problems in the choice of a systemic treatment for MF/Sézary's syndrome is very number of treatment options such as chemotherapy, α-interferon, bexarotene, denileukin diftitox and extracorporeal photopheresis which all produce temporary remissions in 30-50% of the patients. A novel approach have includes the use of the antiCD52-antibody alemtuzumab (MabCampathR) which is directed against a pan-lymphocyte antigen thus hitting both B and T-lymphocytes. In a Swedish trial,[1] patients with MF/Sézary's syndrome who required treatment, with a documented failure to respond to PUVA and a history of up to five previous systemic therapy regimens were eligible. Twenty-two patients were included. Alemtuzumab 30 mg was administered three times weekly, for up to 12 weeks. An overall response rate of 55%, with 32% complete responses (CR), was achieved. The response seemed to be better among patients with erythroderma than among those with large tumors and for those who were not heavily pretreated. The median time to treatment failure was, however, rather short, being only 12 months. The activity of alemtuzumab in MF/Sézary's syndrome has been confirmed in case reports and in two smaller studies with heavily pretreated patients.[2,3] In these

two smaller studies, including 16 patients together, a lower remission rate was found. Furthermore, cardiac toxicity was reported.

MF/Sézary's syndrome is most often CD4+ and this characteritic has been exploited in its treatment. A chimeric anti-CD4 antibody was administered intravenously as a single dose to eight patients with MF2. Seven of the eight patients responded to treatment with an average freedom from progression of 25 weeks. A fully humanized antiCD4-antibody (HumaxCD4) has recently been studied in a phase II study and was presented at the SID-meeting in April 2004. The dose was escalated from 280 mg/week to 560 mg/w in early stage disease to 980 mg/week in advanced stage of disease and the duration of response for those patients who did respond was 17 weeks. The response was evaluated by a physician´s global assessment using a grading scale including the different parameters: erythema, scaling, plaques elevation and pigmentation. In the 560 mg group (early stage) 9/14 patients showed remission while 3/4 in the 980 mg group (advanced stage) did so.

Allogeneic stem cell transplantation has successfully been used in a few patients with MF but the experience is still limited. With the development of reduced-intensity conditioning the number of patients treated will probably increase and the value of allogeneic stem cell transplantation will be able to be evaluated better.

Leukemic PTLC

Among the rare leukemic T-cell lymphomas, T-PLL is the most common and the largest clinical problem. The response to conventional chemotherapy is generally poor, with a median survival of only 6-8 months and only a few patients achieve CR. The data on the use of alemtuzumab are encouraging. In a study[5] of alemtuzumab 30 mg, administered three times weekly, for up to 12 weeks in 39 patients with T-PLL, 23 (59%) achieved CR and 6 (15%) had a partial remission. The remission duration was, however, short and the median survival from start of treatment was only 10 months.

Allogeneic bone marrow transplantation has been reported to induce long-term remissions in patients with T-PLL Consequently many authors recommend allogeneic stem cell transplantation early in first remission, in order to consolidate the response to alemtuzumab.

Nodal and other extra-nodal PTLC

Enblad *et al*[6] reported on the use of antibody therapy with alemtuzumab (antiCD52-antibody) 30 mg 3/weeks for 12 weeks. This phase II study, in 14 relapsed or refractory patients, achieved 3 CR and 2 PR, but the remissions were of short duration. The accrual was stopped after 14 patients because of treatment-associated toxicity consisting of opportunistic infections with 5 treatment-related deaths.

The humanized monoclonal antiCD4-antibody (HumaxCD4) was used at a rather low dose (280 mg/week) in one patient with relapsed angioimmunoblastic lymphadenopathy. A short remission was obtained without any severe side-effects (*unpublished data*).

The largest series of patients with nodal PTLC treated with allogeneic bone marrow transplantation was recently published.[7] The patients, whose median age was 41 years, had primary refractory disease or had relapsed after chemotherapy and received salvage chemotherapy followed by reduced-intensity conditioning consisting of thiothepa, fludarabine and cyclophosphamide. At a median follow-up of 28 months after receiving the allogeneic stem cells, 12 of 17 patients (64%) were still in CR, one patient had PR and one had stable disease. The toxicity was low, being fatal in onlyone case.

Conclusions

Immunotherapy with monoclonal antibodies against different T-cell antigens has the potential to be an important treatment modality, especially in combination with chemotherapy. One of the most interesting antigens at the moment is CD4. Immunotherapy with allogeneic bone marrow transplantation, using a reduced-intensity conditioning schedule, is a promising management strategy.

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Hodgkin's Lymphoma Why 30% of the Patients is Still Uncurable?

Global gene expression analysis of Hodgkin/Reed-Sternberg cell lines: identification of autocrine activated receptor tyrosine kinases in Hodgkin's lymphoma

The presence of large, mononucleated Hodgkin and multinucleated Reed/Sternberg (HRS) cells is typical and of diagnostic relevance for Hodgkin's lymphoma (HL). HRS cells are the tumor cells in HL and usually comprise less than 1% of the cellular infiltrate in the lymphoma tissue. Single cell investigations have provided detailed information about the origin and biology of HRS cells. Polymerase chain reaction (PCR) amplification and analysis of rearranged immunoglobulin (Ig) genes from single HRS cells revealed that the tumor cells are derived from pre-apoptotic germinal center B cells.[1] In addition, in a small proportion of HL (about 3% of cases) the HRS cells carried no Ig but T-cell receptor gene rearrangements showing their T cell derivation.[2]

An attractive method to get new insights into the biology of malignant lymphomas is the global analysis of gene expression. This analysis is based on microarray platforms or high throughput serial sequencing (SAGE). Such techniques were applied to several B-cell non-Hodgkin lymphomas (B-NHL) and led to the identification of different subtypes within lymphoma entities, of genes or gene sets as prognostic factors and of potential novel therapeutic approaches. However, array technologies are difficult to use for the analysis of HL because large amounts of tumor cells (about 10^6) with purities of at least 80% or higher are needed in order to obtain tumor-specific information not significantly masked by expression data originating from admixed reactive cells such as T and B cells, histiocytes, macrophages and eosinophils. With available techniques it is difficult to isolate sufficient HRS cells for gene expression experiments, and thus, so far HRS cell lines have been used as models in microarray studies.[3,4]

Large-scale gene expression profiling was performed to compare the transcriptome of HRS cell lines with that of purified normal B-cell subsets and the major B-NHL. Although HRS cell lines may differ from primary HRS cells in the lymphoma tissue in several aspects, especially regarding interactions with the microenvironment, this approach was nevertheless useful for identifying HRS-specific genes as well as B-cell specific genes which are not expressed in HRS cells although HRS cells are B-cell derived. For most of the genes for which validation studies were performed, the array data could be confirmed by immunohistochemistry or with reverse transcription PCR using RNA from HRS cells obtained by laser micromanipulation (PALM).

Unsupervised hierarchical clustering identified the HRS cell lines as a distinct group among the normal B cells and B-NHL. Interestingly, three of the HRS cells lines (KMH2, L428 and L1236) are of B-cell origin and one is of T cell origin (HDLM2). Clustering of the four cell lines indicated that the particular HRS-cell-specific gene expression profile may be independent of their cellular derivation. In addition, it was shown that the HRS cell line gene expression profiles were most similar to those of EBV-transformed peripheral blood B cells and of a subtype of diffuse large B-cell lymphoma. Unexpectedly, although HRS cells are germinal center B-cell-derived, the gene expression data showed greatest similarity to the activated B-cell subtype of diffuse large B-cell lymphoma and not to the germinal center subtype or to other germinal center B-cell-derived lymphomas. This may be due to the loss of expression of germinal center specific B-cell markers and could be related to the general loss of the B lineage phenotype in HRS cells, a finding correlating well with the previously known immunohistological properties of HRS cells, which are usually negative for the B-cell or germinal center markers CD20, CD19, CD79a, CD10, Bcl-6 and Oct-2. The reason for this loss of B-cell and germinal center cell markers is unknown. It may be speculated that it is associated with transforming events, which are not yet identified. So far, knowledge about HRS cell pathogenesis is rather limited. Constitutive NFKB activity, caused in a considerable fraction of cases by mutations of its inhibitor IKBα, seems to be important.[5] EBV, a virus capable of immortalizing B cells, also seems to be relevant for the malignant transformation of HRS cells, at least in about 40% of EBV-associated HL cases. We used two different approaches to identify genes involved in HRS cell pathogenesis. One approach was to apply antibodies to proteins with well known functions in cellular signaling to histological sections of HL to screen HRS cells for activated signaling pathways.[6] The other approach was to analyze the microarray data of HRS cell lines for aberrant expression of signaling molecules.

Such an analysis indicated aberrant expression of several receptor tyrosine kinases (RTK), which are frequently involved in cellular transformation, in the HRS cell lines, and Western blot analysis confirmed the expression of six RTK (PDGFRA, DDR2, TRKA, TRKB, RON and EPHB1 in the HRS cell lines.

Using antibodies specific for these six RTKs, we analyzed their expression patterns in normal lymphatic tissues, various B-NHL (mantle cell lymphoma, chron-

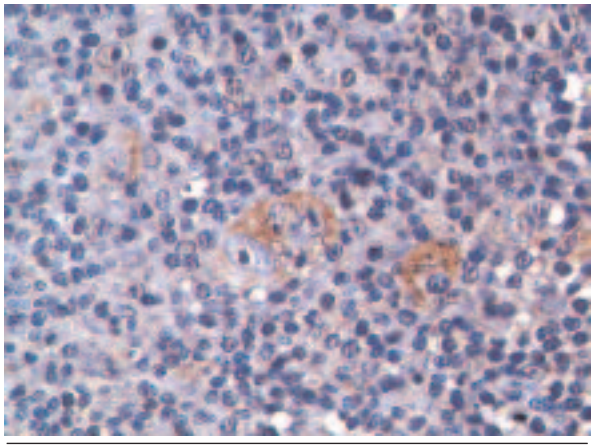

Figure 1. Positive Immunostaining for PDGFα receptor in two Reed-Sternberg cells.

ic lymphocytic leukemia, Burkitt´s lymphoma, follicular lymphoma, diffuse large B-cell lymphoma) and primary HL. None of these kinases was detected in normal B cells by immunohistochemistry. Expression was only occassionally observed in B-NHL, with 80% of the 86 cases analyzed expressing none of the RTK and the most frequently expressed RTK being found in 7% of cases. In contrast, each of the RTK was aberrantly expressed in a considerable fraction (30-75%) of classical HL. Expression was most pronounced in the nodular sclerosis subtype, that all 18 cases analyzed expressed at least one RTK, and on average about 4 kinases were expressed per case; in mixed cellularity HL 6 of 21 cases expressed none of the RTK and on average less than 2 RTK per case were expressed. Immunohistochemical positivity for RTKs was less frequent in lymphocyte-predominance HL, in that 9 of 23 cases were negative for all RTK tested and there was an average of less than 1 RTK per case. For PDGFRA, the aberrant HL-specific expression among lymphomas derived from mature B cells was further demonstrated to be HRS-cell specific with two composite follicular/Hodgkin lymphomas, both with common precursor clones.[7,8] In both of these cases only HRS cells expressed PDGFRA.

RTK activation could be demonstrated with antibodies specific for phosphorylated RTKs (PDGFRA, TRKA and TRKB). Furthermore, pan-phospho-tyrosine antibodies showed that HRS cells often contain phospho-tyrosine levels comparable to those in tumors with activated tyrosine kinases, such ann anaplastic large cell lymphoma and gastrointestinal stroma tumor. Aberrantly high phospho-tyrosine levels were more pronounced in nodular sclerosis than mixed cellularity HL and correlated with the numbers of expressed RTK.

RTK in tumors are frequently activated by mutations. However, when cDNA encoding the six RTK expressed in HRS cell lines was sequenced, no potentially activating aberrations were detected. Immunohistochemistry for RTK ligands revealed that the RTK for which analysis was possible are likely activated by paracrine or autocrine mechanisms. Thus, collagen 1, the high affinity DDR2 ligand, is present in the sclerotic bands branching into the tumor infiltrate in nodular sclerosis HL, and in many cases cells expressing the TRKA ligand NGF were more frequently present in HL infiltrates than in normal lymphatic tissues. Furthermore, in most of the cases expressing PDGFRA, the PDGFA ligand was also expressed in the HRS cells (14 of 15 cases). Likewise, in cases expressing EPHB1, co-expression of the EphrinB1 ligand was frequently observed.

In conclusion, these data indicate that aberrantly expressed and paracrine or autocrine activated RTK are involved in the pathogenesis of HRS cells and may be potential targets for therapeutic intervention.

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New insights into the molecular biology of Hodgkin's lymphoma

Hodgkin and Reed/Sternberg (HRS) cells represent the malignant cells in classical Hodgkin's lymphoma (HL). Their immunophenotype cannot be attributed to any normal cell of the hematopoietic lineage, but molecular studies established their derivation from germinal center (GC) B cells in most cases.[1] Only a few cases of classical HL show a T-cell derivation.[2,3]

Based on the recognition of the GC B-cell origin of HRS cells, it became reasonable to perform analyses of differential gene expression between these cells and normal mature B cells as well as other types of B-cell non-Hodgkin's lymphomas (B-NHL). Since it was initially not technically feasible to create large scale gene expression profiles from the rare primary HRS cells, we generated gene expression profiles (approx. 9,000 genes) of four HL-derived cell lines (L428, L1236, KMH2, HDLM2) and compared them to those of the main subsets of normal mature B cells and various types of B-NHL.[4] In an independent analysis, the gene expression profile of the HL line L1236 (with confirmed origin from the HRS cells of the respective patient)[5] was compared to that of GC centroblasts by SAGE (serial analysis of gene expression).[6]

Focusing on the expression of B lineage markers, the analysis revealed decreased mRNA levels for nearly all established B lineage-specific genes.[7] In particular, multiple components of signaling pathways active in B cells, including B-cell receptor (BCR) signaling, were severely affected. Notably, HRS cells in classical HL presumably derive from a peculiar subset of pre-apoptotic GC B cells that fail to express high-affinity BCR.[1] Such cells would normally quickly undergo apoptosis within the GC, because GC B cells are stringently selected for expression of a high-affinity BCR. We propose that the lost B lineage identity in HRS cells may explain their survival without BCR expression, by allowing these cells to escape the stringent selection for BCR expression, and reflect a fundamental defect in maintaining the B cell differentiation state in HRS cells.

In unsupervised analysis of the microarray data, a T-cell-derived HL line (HDLM2) clustered together with the three B-cell-derived HL lines, suggesting that HRS cells share a common gene expression pattern, independent from their cellular origin from either B or T cells.[4] The microarray study also reveled a number of genes that were highly and consistently expressed by the four HL lines, but at much lower or undetectable levels by the normal B cell subsets and the other B cell lymphomas. The list of these aberrantly expressed genes included a few genes known to be expressed by HRS cells (e.g. fascin and TARC), but most of the genes were previously not known to be expressed by HRS cells. These genes included several transcription factors (e.g. *GATA-3, ABF1*) and kinases (e.g. Fer kinase), that may play roles in the pathogenesis of HL. For some of the genes, transcription was confirmed also in primary HRS cells in the tumor by reverse transcription polymerase chain reaction (RT-PCR) with pools of microdissected HRS cells.[4] Somewhat surprisingly, most of the genes tested by RT-PCR were also found to be expressed by L&H cells microdissected from cases of lymphocyte-predominant HL. We are now beginning to test the functional role of some of the aberrantly expressed genes in HL lines by modulating their expression. As the number of HL cell lines is limited and since the cell lines most likely do not retain the features of primary HRS cells in all important aspects, we plan to study differential gene expression of microdissected HRS cells using gene expression profiling. Small cell samples of HL cell lines and microdissected lymphoma cells were used to isolate RNA and subsequently amplify it. We first tested various RNA isolation methods to obtain RNA with high quality and yield from small cell samples isolated by laser microdissection and pressure catapulting (LMPC) from tissue sections (P.A.L.M. Microlaser Technologies). In our hands, the best RNA isolation method tested was a modified protocol for the Purescript RNA isolation Kit (Gentra). To obtain enough labeled aRNA for hybridization to Affymetrix microarrays, total RNA of approximately 1000 cells had to be amplified. We tested two T7 RNA polymerase-based amplification kits and a PCR-based amplification method. The T7 RNA polymerase-based RiboAmp RNA Amplification Kit (Arcturus) in conjunction with the ENZO BioArray High Yield Transcript Labeling Kit with modifications turned out to give the best aRNA yield after two rounds of *in vitro* transcription. The methods showed good reproducibility. The results indicate that total RNA can be efficiently obtained from LMPC-isolated HRS cells and can be reproducibly amplified by *in vitro* transcription for microarray analysis.

In rare cases a combination of HL and B-NHL occurs in the same patient. These composite lymphomas are frequently clonally related, as demonstrated by the detection of the same *IgV* gene rearrangements in the two lymphomas.[8-13] Hence, such cases represent interesting models to study the multistep transformation process in the two lymphomas and the development of two distinct tumors from a shared precursor.

We analyzed six clonally related composite lymphomas for shared and distinct transforming events (manuscript in preparation). HL were combined in two cases with follicular lymphomas (FL), in one case with B-cell chronic lymphocytic leukemia (B-CLL), in one case with splenic marginal zone lymphoma, in one case with mantle cell lymphoma (MCL) and in one case

with diffuse large B-cell lymphoma (DLBCL).[9-13] In the two HL/FL combinations and in the HL/MCL case, the hallmark *IgH*-associated translocations (*bcl-2/IgH* and *bcl-1/IgH*, respectively) were detected in both the HL and the B-NHL. Furthermore, mutational analysis of the tumorsuppressor genes *CD95*, *IκBα*, *p53* and *ATM* was performed on laser microdissected HRS cells and/or whole tissue DNA of the NHL. In none of the cases, were *CD95*, *IκBα* or *ATM* gene mutations detected. In the composite lymphoma composed of HL and DLBCL, clonal replacement mutations of the p53 gene on both alleles were found exclusively in the DLBCL cells. In the composite HL and MCL, Epstein-Barr virus (EBV) was detected only in HRS cells, but not in the MCL. Interestingly, only a fraction of the HRS cells was EBV-infected, and these cells were distinguished from the EBV-negative HRS cells by a slightly distinct *IgV* gene mutation pattern.[11] This provides strong evidence that EBV infection was a late event in HL pathogenesis in this case and likely happened in a subclone of the HRS cell (precursor) in the GC. In conclusion, we show at DNA sequence level that HRS cells can harbor *IgH*-associated translocations and we conclude that such translocations involving *bcl-2* or *bcl-1* can represent an early step in the pathogenesis of composite HL/FL or HL/MCL. The restriction of p53 mutations to the DLBCL in another composite lymphoma and the EBV infection of a subclone of the HRS cells in a composite HL/MCL exemplify late transforming events. The p53 mutations might have affected the fate of the common lymphoma precursor towards the outgrowth of the DLBCL.

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Ten years after REAL classification: Hodgkin's lymphomas in the European Perspective

The advent of the REAL classification, now used as the revised WHO classification, has had an enormous impact on the harmonization of globally accepted diagnostic tools for recruiting patients with malignant lymphomas into clinical trials and, moreover, has led to a better understanding and comparability of outcome results of prospective multicenter trials on both sides of the Atlantic.

Unlike the situation in non-Hodgkin's lymphomas, the decision on how to treat a patient with classical Hodgkin's lymphomas (HL) depends more on clinical stage and biological and clinical risk factors than on histology. There is one exception: nodular lymphocyte predominant HL, a subtype separated from the classical HL subtypes and treated less aggressively, because of its more benign course.

Previous lymphoma classifications subdivided HL according to the preponderance or scarcity of lymphocytes, the prevalence of fibrosis and sclerosis and the mixture of reactive cells surrounding the pathognomonic Hodgkin-Reed-Sternberg HRS-cells. The old Jackson and Parker classification described three types: the paragranuloma, the granuloma and the sarcoma.

The Lukes and Butler classification of HL, later leading to the Rye Classification, first defined six subtypes, and then later 4: nodular lymphocyte-predominant HL, nodular sclerosis HL, mixed cellularity HL and lymphocyte-depleted HL.

The REAL classification added, to the commonly used panoptic cytochemical and histological criteria, immunological, molecular genetic as well as cytogenetic data in order to define subentities more precisely that might have clinical and prognostic significance.

The major change was the differentiation between the classical HL subtypes with the typical CD30, CD15 positive and mostly CD20 negative HRS cells and the nodular lymphocyte predominant (NLP-HL)-subtype with CD20 positive, CD30, CD15 negative lymphohistiocytic *lacunar-cells.* The other major advent in the REAL classification was the identification of the lymphocyte- rich classical subtype (LRC-HL), similar to the NLP-HL, but comprising HRS cells.

The clinical phenotype of this newly described LRC-HL is not yet completely known, but preliminary data from studies by the GHSG show a close similarity to the NLPHL subtype with mainly stages I-II, rarely B-symptoms and an excellent prognosis, although after treatment using protocols designed for the other subtypes of HL (nodular sclerosis, mixed cellularity and lymphocyte-depleted).

Eligibility of patients with HL for clinical trials and outcome results in Europe

There is marked heterogeneity of treatment protocols in the various countries of the European Union and between co-operative groups. Most study groups in Europe use clinical and biological risk factors to define treatment strategies. Histological, cytological, immunological and cytogenetic markers have never had or have lost prognostic impact due to the more aggressive, risk- and failure-adapted treatment strategies used in most European countries.

The only treatment-related discrimination on the basis of histological subclassification is the use of less aggressive protocols for patients with the NLPHL- subtype and more aggressive, risk tailored protocols for patients with classical HL- subtypes. The inclusion of the LRC-HL subtype in this category needs to be re-examined in the future when more detailed analyses on initial clinical presentation and outcome data of this special subgroup are available.

Prognostic subgroups

The EORTC and the GHSG have defined treatment-related prognostic subgroups based on the amount and site of initial anatomical disease involvement, categorized into clinical stages (CS) according to the Ann Arbor Classification, and biological and laboratory parameters.

The commonly used prognostic groups, used for determining treatment, are:

- early favourable subgroup: CS I-IIA,B without risk factors;
- early unfavourable (intermediate) subgroup: CS I-II, with risk factors;
- advanced subgroup: CS IIB with bulky disease (>10 cm and LMM), III, IV;

Risk factors are: B-symptoms, bulky disease (<10 cm), >3 lymph node areas, E-stage (GHSG), age >50 years (EORTC).

Treatment protocols and outcome

Early favorable subgroup. Most groups today use 2-4 courses of ABVD (C-MOPP/ABV) + 20-36 Gy of involved field radiotherapy (IF-RT). The results are excellent with a progression (or event)-free survival of more than 95% and an overall survival >90% after 5-10 years. The rates of acute and late toxicity are low, and in particular, the rate of secondary leukemia or myelodysplastic syndrome is <1.0%; the rate of solid tumors is also low, but the observation time is short. Studies are underway to determine the minimum amount of chemotherapy adriamycin/vincristine (AV) necessary in combination with the lowest dose of radiotherapy in the IF-RT setting (20-30 Gy). The EORTC plans to undertake a study to compare pure chemotherapy (4-6 courses of ABVD) with a combined modality of chemotherapy i.e. 4-6 cours-

es of ABVD + IF-RT.

Early unfavorable (intermediate) subgroup. In this patient cohort most European co-operative groups use combined modality treatment with 4-6 courses of ABVD (C-MOPP/ABV) + 20-36 IF-RT. The results are not as good as in the early favorable group with progression-free survival rates of 85-87% and overall survival rates of <90% at a median of 5 years, meaning that 13-15% of patients have early progression or relapse and need salvage therapy with a prospective prognosis not better than patients with treatment failures in the advanced stage group (*unpublished observation of the GHSG*). Neither the REAL/WHO classification criteria nor the in vogue molecular genetic markers obtained from gene profiling or tissue microarray) techniques have been able to discriminate a subgroup of patients with a worse prognosis within this cohort. It is, therefore, still of pivotal importance to improve the chances for this group of HL patients by defining the subgroup with an adverse prognosis and escalating treatment intensity or intensifying therapy for the whole group until valuable and reproducible adverse prognostic factors other than bulky and advanced disease become available. The GHSG has started to compare 2 courses of escalated BEACOPP + 2 ABVD+ 30 Gy IF-RT with 4 ABVD+ 30 Gy IF-RT(HD-14 study).

Advanced subgroup (CS IIB bulk (>10 cm), LMM, CS III-IV)

The eligibility of patients with advanced disease for specific treatments depends on anatomic spread of disease (stage IV), tumor size (bulk,LMM), systemic symptoms, low hemoglobulin and albumin levels, age >50 years, and lymphocytopenia (International Prognostic Score = IPS).

The REAL/WHO classification does not add to risk discrimination by defining the known classical subcategories, nodular sclerosis, mixed cellularity, lymphocyte dominant, LRC-HL. More subtle molecular insight is needed to define risk factors at diagnosis that drive a more malignant biological strain of tumor and identify molecular targets which have to be modulated to reverse the malignant status of the transformed cells either by allowing the cell to undergo apoptosis or inhibiting pro-proliferative transcription pathways. Ongoing research in our and other laboratories is being used to investigate these pathways and molecular errors in the hope of finding less toxic strategies which selectively hit the malignant target and not the host.

As far as the present status of therapeutic strategies in advanced HL patients in Europe is concerned, most large cooperative study groups use 6-8 courses of anthracycline-containing polychemotherapy ± IF-RT, i.e. ABVD, C-MOPP/ABV or more recently, as in an increasing number of centers, baseline or escalated BEACOPP±IF-RT with a considerable advantage for the escalated BEACOPP as far as progressio-free and overall survival are concerned, in spite of a higher number of secondary leukemias/myelodysplastic syndromes which amounted in the recently concluded HD-12 study to about 0.8% after a median observation time of 3 yers.

Conclusions

Unlike for the non-Hodgkin's lymphomas, the REAL/WHO classification has not made considerable changes to the day to day clinical practice for the diagnosis and treatment of patients with Hodgkin's lymphomas.

The most important addition is the precise discrimination of the NL-PHL and the classical subtypes of HL, which is to the great advantage of patients with the more benign NLPHL who need only mild therapy or none at all.

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