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Guest Editor: Paolo Coser

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Welcome from the German-Speaking Section of the European School of Oncology

Hans-Jörg Senn

Chairman of ESO-D Scientific Committee, ZeTuP, St.Gallen, Switzerland

It is my pleasure to welcome you, on behalf of the Scientific Committee of the German-Speaking Section (ESO-D) of the European School of Oncology, which has its "headquarters" at the Center for Tumor Detection + Prevention (ZeTuP) in St.Gallen, Switzerland, to this ESO-D-course on *Multiple Myeloma: Update 2003* in Bozen, Südtirol. The German-Speaking Section is in its 7th year of operation since 1997 and encompasses all of the usually 14-15 annual German-speaking ESO-D-courses for physisicans, oncology nurses and multi-professional audiences in Austria, Germany, Switzerland as well as in South Tyrol in north-east Italy.

According to our cultural and language-based ESO — "*Constitution*", even this present myeloma-event in Bolzano, Italy should have been organized in the German language, but in the interest of comprehensiveness of the specific hemato-oncologic topic and in the view of the many excellent and indispensable speakers from various other parts of Europe — especially from Italy — we have declared this important myeloma-course to be the exception, which proves the rule! However, as you can see from the programme, the lectures will be simultaneously translated in German and Italian to respect the multi-cultural nature of the region, which over 1500 years ago was a Räto-Roman province and - like its neigboring Swiss Canton of Graubünden (or Grisons) — has maintained the Ladin-language as a third option of communication in some of the mountain areas.

Over the years, Professor Paolo Coser has transformed the large Regional Hospital in Bolzano/Bozen into a center of excellence in hemato-oncology, which offers its sophisticated diagnostic potential and its highly developed therapeutic services of intensive chemotherapy and bone marrow transplantation to the whole province of South Tyrol — or Alto Adige — and to its neighboring province of the Trentino as well.

We congratulate Professor Coser and his very active team for organizing this interesting *Update 2003* on multiple myeloma and, on behalf of the ESO-D here in Bolzano/Bozen, we wish him, the invited faculty and the audience two days of fruitful professional exchange about this prognostically still very challenging disease.

Introduction to multiple myeloma

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Multiple myeloma is the malignant counterpart of activated B-cells, which during the secondary immune response undergo IgH somatic mutation and isotype switch recombination. The disease is thought to be disseminated by circulating clonogenic cells which selectively "*home*" to the bone marrow, where they receive proliferation, differentiation and osteoclast activation signals from interleukin (IL)-6 and IL-1, tumor necrosis factor (TNF) and other cytokines.

The risk factors of multiple myeloma pointed out in a recent population-based case control NCI study,[1] are correlated with occupational exposure. Exposure to pesticides, herbicides and fungicides and subjects who lived or worked on a farm where sheep were raised had a modestly increased risk (Odds Ratios 1.3, 1.5, 2.3, 1.7, respectively), while a significantly increased risk was observed among pharmacists, therapists, roofers, heating equipment operators, hand molders and casters (Odds Ratios 6.1, 6.1, 3.3, 4.7, 3.0, respectively)

Until 10 years ago, the median survival of myeloma patients treated with conventional chemotherapy did not exceed 30-35 months, as reported by the South West Oncology Group in numerous publications comparing different drug-combinations. In 1996, for the first time in the world, the *Intergroupe Francais du Myelome* demonstrated in a randomized trial, that autologous transplantation was superior to conventional chemotherapy in patients with newly diagnosed myeloma, with a complete remission (CR) rate of 22% versus 5%, respectively, and 5-years overall survival and event-free survival (EFS) of 52% vs 12% and 28% vs 10%, respectively.[2] Three years later, Barlogie[3] published his results obtained with *tandem peripheral blood stem cell* (PBSC) *transplantation*, with which he obtained an increase of CR (51%) and higher 5-year OS and EFS (58% and 42%). An historical comparison of 152 patients treated with tandem transplantation and 152 closely matched patients treated with standard SWOG therapy showed 10-year OS and EFS of 33% versus 15% and 16% versus 5% respectively.[4]

The emerging questions in these last years are whether double transplantation is better than single transplantation and whether melphalan/total body irradiation (TBI) is better than melphalan alone as the conditioning regimen for the PBSC transplantation. Recently, a final analysis of a prospective randomized study of the *Intergroupe Francophone du Myelome* showed that the 7-year survival and EFS are better among patients who had undergone tandem transplantation (42% vs 20% and 20% vs 10%, respectively)[5] and that melphalan alone offers faster hematologic recovery of blood cells, shorter hospitalization and higher survival (66% vs 45% at 45 months).[6]

Compared to the conventional chemotherapy, autologous transplantation produces a high rate of CR and a longer OS and EFS but it does not cure this disease and the continuous relapses are the major problem. Negative prognostic factors and minimal residual disease must be more carefully considered when taking therapy decisions.

Evaluating the CR after autologous and allogeneic transplantation, using molecular technology, Corradini *et al.*[7] demonstrated that although 50% of patients had clinical CR, only 7% also had a molecular remission and that the relapses were correlated with positive polymerase chain reaction of bone marrow.

Cytogenetics play the most important role as a prognostic factor in newly diagnosed myeloma patients and the 13q-deletion is the most powerful indicator of chemotherapy response and overall survival.

In a recent overview of 1,000 patients treated with high dose therapy, only 20% of those with 13q-del obtained CR compared to 62% of those without this cytogenetic alteration and the OS at 5 years was 0% versus 61%.[8]

In another study,[9] in which FISH detection was used, the 13q14 deletion in newly diagnosed myeloma patients was present in more than 50% of patients and was correlated with advanced stage of disease, lower response rate, higher labeling index and lower OS (14.9 months versus 30.8).

The new microarray technology offers the possibility to evaluate the expression of genes profiles. In multiple myeloma, 120 genes differentiate normal plasma cells from myeloma cells; 6 out of these 120 genes differentiate monoclonal gammopathy of unknown significance (MGUS) from myeloma, predict response to chemotherapy, predict deletion of chromosome 13 and could indicate future personalized strategies.[10]

At present, allogeneic transplantation is the only therapy able to cure myeloma patients. Unfortunately, transplant-related mortality (TRM), although decreased in

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recent years, remains high (20-30%) and this compromises the OS despite the improvements recently achieved.[11]

With the intent to reduce the toxicity and mortality of allogeneic transplantation, as well maintain the graft-versus-myeloma (GVM) effect, which is probably the major determinant of cure, low intensity conditioning regimens have been designed. These produce sufficient immunosuppression to allow the donor graft to be established rather than cytoreduction, minimizing toxicity and allowing the GVM.

Limited publications are now available on this topic but the long-term results on survival and relapse rate still need to be explored in randomized trials in order to determine the relative benefits of tandem autologous/non-ablative allografting compared to tandem autologous transplantation.

The issues to be examined in myeloma patients who lack a suitable donor (with the intent of obtaining a molecular remission, which is very rare after autologous transplantation) are *purging* of the apheresis product and more efficient maintenance therapy with chemotherapy, bisphosphonates, immunotherapy, idiotype vaccination, and use of new drugs.

The role of *purging* is presently controversial. The apheresis products, which are mostly used for autologous stem cell support, are practically always contaminated by myeloma cells. In our experience,[12] using polymerase chain reaction (PCR) amplification of patient specific CDR III DNA sequences, myeloma precursor cells were detected in 85% of the PBSC collected: the reinfusion of these cells may contribute to disease relapse. Positive stem cell selection using antigen not expressed by myeloma cells, such as CD34, was employed for *purging* apheresis products before transplantation, but no difference was observed with respect to EFS and OS.[13-15] Loss of T lymphocytes and the contamination by clonal CD34 cells documented in some studies[16,17] could be responsible for the failure.

In contrast, two randomized studies,[18,19] in which a negative selection was employed, such that the B-cells of the PBSC were captured and removed by specific monoclonal antibody and immunomagnetic beads, showed a significant increase of pregression-free survival and OS in the purged patients.

Aiming to target the minimal residual disease after tandem transplantation, acute lymphoblastic leukemia (ALL)-type intensive consolidation chemotherapy was performed and the results evaluated in a matched pair analysis, but no difference was shown for OS/EFS between the patients given the ALL-infusional chemotherapy and controls.[20] In contrast, in another study,[21] patients treated with two or more cycles of dexamethasone, cyclophosphamide, etoposide and cisplatin (DCEP) as consolidation chemotherapy after tandem melphalan-based high dose therapy, obtained a significantly higher OS/EFS than did historical controls (p=0.01/p=0.006).

Bisphosphonates are widely used in myeloma patients and pamidronate and zolendronic acid, compared to clodronate, were shown to improve not only bone pain and progression of lytic lesions but also to reduce the rate of new pathologic fractures. Current *American Society of Clinical Oncology Practice* guidelines recommend intravenous bisphosphonates to prevent skeletal complications in myeloma patients with evidence of osteolytic bone destruction.[22] Moreover, the new nitrogen-containing bisphosphonate zolendronic acid, showed antiproliferative and pro-apoptotic activity on bone marrow stromal cells, myeloma cell lines and osteoclasts,[23] anti-angiogenetic activity and suppression of IL-6 production.

Interferon-α 2b (IFN) has also been widely used in myeloma maintenance therapy, but with controversial results. The Oxford meta-analysis has shown that IFN as maintenance therapy significantly prolongs both OS/EFS, although only by 6 to 7 months and that this benefit needs to be weighed against effects on quality of life and the drug's cost.[24]

The high remission rate obtained in myeloma patients after sequential chemotherapy and tandem transplantation opens the possibility of active vaccination strategies to raise immunity against tumor cells and control minimal residual disease. The rationale is based on the fact that the specific antigenic determinants of Ig variable regions, known as idiotypes, produced by a single B-cell clone are unique and represent truly tumor-specific antigens as they are present on all tumor cells and absent from all normal B-cells. The specific anti-idiotype immunologic response is therefore directed only to malignant B-cells. The methods used involve subcutaneous vaccination with idiotype and low dose of IL-2 or GM-CSF, vaccination with idiotype-pulsed or transfected autologous dendritic cells, DNA vaccination in which the foreign gene encoding the tumor antigen is cloned under regulation by eukaryotic or viral elements into an expression cassette, which is then injected in solution either intramuscularly or subcutaneously. Although feasible and exciting, these procedures are not easy to achieve for all patients and, to date, very few experiences have been published and only in small group of patients.

In recent years the knowledge of cellular interactions and different cytokines influencing the proliferation of myeloma cells has stimulated the drug industry to develop specific molecules able to influ-

ence the microenvironment in order to modulate and/or reset these malignant cells to a normal level of activity.

These new or revived molecules include thalidomide, PS341, arsenic trioxide, 2-methoxiestradiol, PTK 787/ZK 222 584, farnesyltransferase inhibitors, histone deacetilase inhibitors, NF-κB inhibitors, PS1145, IKB kinase inhibitors, P38 MAPK inhibitors, agent targeting cell surface receptors and others which act on different levels of the biological activity of myeloma cells. The role of these novel therapies targeting myeloma cells and their microenvironment is to induce G1 growth arrest, cause cell apoptosis, inhibit adhesion of myeloma cells to bone marrow stromal cells, inhibit bioactivity and/or secretion in myeloma cells or bone marrow stromal cells of cytokines, inhibit angiogenesis, inhibit 26 S proteosome activity, and induce T-cell and NK-cell anti-myeloma immunity. Numerous studies are ongoing to evaluate the efficacy of these drugs used alone or in combination and the results are very promising.

All the topics mentioned in my introduction to multiple myeloma will be widely developed during the meeting by speakers who have been selected from among European experts on this malignant disease.

In this last decade, the history of multiple myeloma has changed profoundly and autologous and allogeneic transplantation has significantly prolonged the survival of myeloma patients, improved their quality of life and in some cases cured them. Better knowledge of the prognostic factors, the genetic heterogeneity in multiple myeloma, plasma-cell immunobiology and bone disease pathophysiology, the progress in allogeneic transplantation, vaccination strategies and the development of new drugs and targeting therapies should have a further positive impact on the course of this disease.

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Clinical significance of chromosomal abnormalities in multiple myeloma

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Cytogenetics defines two major entities of MM

Cytogenetic and molecular genetic investigations of multiple myeloma (MM) cells has provided evidence that virtually all cases of MM have chromosomal abnormalities. Karyotypes from MM cells are usually very complex, but careful analyses of large series have demonstrated that MM can be subdivided into two cytogenetic categories. The hypodiploid/pseudodiploid category (which also includes the near-tetraploid karyotypes) and the hyperdiploid category. The hyperdiploid subtype is defined by the presence of multiple trisomic chromosomes (most commonly chromosomes 3, 5, 7, 9, 11, 15, and 19) associated with a gain of DNA, which is detected as DNA-aneuploidy by flow-cytometry. With respect to the presence of structural abnormalities, the number of structural abnormalities per cell was lower in the hyperdiploid group (average 5.1) than in the hypodiploid group (average 9.1); the type of abnormalities was similar in both groups. Furthermore, analyses by interphase fluorescent *in situ* hybridization (FISH) have shown that translocations of 14q32 are relatively infrequent in hyperdiploid MM (<40%), whereas such translocations occur in 84% of non-hyperdiploid MM. Recognition of hypodiploid MM is also of clinical significance, since MM patients in this category have a particularly unfavorable prognosis.

IgH-translocations in MM and their molecular consequences

One of the most frequent structural abnormalities observed in MM karytypes involves the Ig heavy-chain (IgH) gene locus on 14q32, which is usually part of a translocation. Unlike the physiological process in which Ig gene sequences are brought together during switch recombination, 14q32 translocations in MM are characterized by juxtaposition of IgH gene sequences with non-Ig DNA sequences (so-called illegitimate switch rearrangements). These translocations are an almost universal event in MM cell lines. By interphase FISH analyses, it was reported that IgH translocations are present in about 50% of patients with MGUS, in 60-75% of patients with MM, and in more than 80% of patients with plasma cell leukemia Heterogeneous translocation partners have been described, with 11q13,

4p16.3, 16q23, and 6p21 being recurrently involved in 14q32 translocations of primary MM tumor specimens. These 4 types of IgH-translocations, which are mutually exclusive, comprise about 60% of all IgH-translocations, and are mediated primarily by errors during IgH switch recombination. These reciprocal translocations result in activation of oncogenes because they come under the influence of enhancer regions at the IgH gene locus (11q13: *cyclin D1*, 4p16.3: *fgfr 3* and *mmset*; 16q23: *c-maf*; 6p21: *cyclin D3*).

In the remaining 40% of MM tumors with evidence of a 14q32 translocation, the translocation partner has remained unidentified. Several translocations have been described in cell lines [e.g. t(6;14)(p25;q32)], but there is lack of evidence that these abnormalities are recurrent translocations in primary MM specimens.

Prognostic implications of IgH translocations

The analysis of recurrent IgH translocations in the context of the clinical data strongly suggests that distinct subgroups of MM patients can be defined according to their genomic alterations: presence of a t(4;14) and t(14;16) is indicative of a poor prognosis whereas a t(11;14) is associated with a rather favorable outcome. Patients with an IgH translocation with an unknown translocation partner form an intermediate prognostic group, which can be further subdivided according to the status of chromosome 13q (favorable outcome with normal chromosome 13, poor prognosis with deletion 13q).

Deletion of chromosome 13q

Partial or complete loss of chromosome 13q has been observed to be the most frequent chromosomal region which is recurrently deleted in MM karytypes. By metaphase cytogenetics, a chromosome 13q abnormality can be found in about 15% of MM patients at diagnosis, whereas interphase FISH studies have shown a higher frequency of 13q deletions in MM, occurring in 39-54% of newly diagnosed cases. This abnormality has gained considerable interest since several studies have reported a strong association between deletion 13q and an unfavorable prognosis of MM patients in the setting of both standard-dose and high-dose therapy. However, the negative effect on prognosis may not only be limited to loss of chromosome 13, because it has also been found with loss of other chromosomes, as reflected by a hypodiploid karyotype.

Information regarding candidate genes, which are lost

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as a consequence of the deletion, is still limited. In the majority of cases with a 13q deletion, large proportions of the 13q arm are deleted indicating loss of the entire chromosome arm or even monosomy 13. However, interstitial deletions mainly involving band 13q14 as well as dual loss at 13q14 and 13q34 with an intact intervening region have also been observed recurrently. In a recent study, it has been suggested that a common deleted region including the *D13S319* locus is located at 13q14 between the *RB-1* and *D13S25* gene loci. This genomic region encompasses an area rich in expressed sequence tagged sites and contains *DLEU1*, *DLEU2*, and *RFP2* genes.

A chromosome 13 abnormality may be associated with specific 14q translocations. Data obtained thus far indicate that there are significant associations between t(4;14) and a deletion 13q (> 80%) as well as t(14;16) and deletion 13q (100% in the few reported cases). In contrast, patients lacking any 14q translocation displayed significantly less frequent abnormalities of chromosome 13q (about 25% of cases). No correlations were found between t(11;14) and deletion 13q; likewise, there is no apparent association between a 14q32 translocation with an unknown partner chromosome and deletion 13q.

Conclusions

Classical and molecular cytogenetic studies should be part of the diagnostic evaluation of MM patients. According to the cytogenetic pattern, MM entities with significantly different survival times can be defined. Future work will be directed towards a molecular classification of MM, and it is anticipated that MM patients will be treated according to their individual risk profile.

DNA microarrays in hematology

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Biological and biomedical research has reached a meaningful turning point, characterized by two recent acquisitions: the first draft of the sequence of the human genome[1,2] and development of new technologies to study it.[3] Unfortunately, the plain knowledge of human sequences does not, alone, provide information on how genes act, how cells work, how organisms are formed, the physio-pathology of diseases and how to develop new drugs. The possibility of manipulating raw data to get real insights is the task of functional studies, whose efforts are aimed to understand the inter-relations between biological components that end up in the formation of living cells and organisms. Oligonucleotide and cDNA arrays are among the most efficient and versatile tools available to research.[4,5] This technology has presented scientists with an image of gene expression so far only dreamt of, that is the expression of thousands of genes simultaneously, as close as possible to the total abundance and variety of mRNA inside a cell.[6] The set of genes transcribed from the human genome is referred to as an *expression profile* or *transcriptome* and characterizes the phenotype and the function of a cell. Unlike the genome, the transcriptome is a highly dynamic entity that changes dramatically in response to every environmental change or during events such as DNA replication, cell division, differentiation, apoptosis or neoplastic transformation. Every change in the expression profile offers the possibility of understanding regulatory mechanisms, functions and biochemical pathways. Moreover, gene expression studies help in determining causes and consequences of diseases, the mechanisms of action of drugs and identifying which genes may become targets for therapy. It must, however, be pointed out that mRNAs are only an intermediate product on the path leading to protein expression. However, to date, protein studies are not as advanced as DNA and RNA studies and they are not as sensitive. The mRNA level gives a measure of the state of the cell and, for the majority of the genes, an increase of mRNA corresponds to a greater amount of protein.

Analytical protein chemistry, or proteomics as it is now commonly known,[7] has a vital role in the daunting task of identifying, through mass spectometry, post-transla-

tional modifications such as phosphorylation, glycosilation, protein-protein interactions and the super-structures consequently formed.[8] DNA arrays and proteomics are undoubtedly complementary. Genome sequencing and its analysis show that between 30,000 and 40,000 genes are transcribed but, out of them, more proteins are expressed as a consequence of alternative splicing mechanisms. The comparative analysis of different cell contexts through DNA microarrays will deepen the knowledge of fundamental biological processes such as apoptosis, proliferation, differentiation and neoplastic genesis. Historically, functional studies have always examined one gene at a time, but the advent of this new technology makes it possible to collect information on thousand of genes in one experiment, allowing a wider gene expression map to be drawn and one that might be able to suggest new mechanisms of gene regulation.[9] For instance, many interesting biological contexts have been examined: a) differential gene expression in the different phases of the cell cycle or in cells undergoing differentiation;[10-13] b) molecular mechanisms underlying transformation and neoplastic progression;[14-18] c) identification of genes regulating the pro-apoptotic program;[19,20] d) modifications of gene expression during viral infections;[21] and e) discovery of genes involved in drug metabolism.[22,23] Clustering and expression analysis in different cell contexts will provide plenty of information on the complexity of many gene programs and will help to identify the genetic alterations underlying many diseases.[9,24] More than 200 types of cancer have been listed and each one of them can be considered as a unique cellular context. Such considerations makes classification, through microarray analysis, an outstanding tool in the struggle against cancer. An advantage in studying normal and leukemic hematopoietic cells is that they can be quite easily collected and purified in order to separate the various sub-populations. Different types of blood cells have a common origin in the hematopoietic stem cell that resides in the bone marrow. This maturation process, known as hematopoiesis, is a valid model for studies concerning the organization and execution of gene programs and the alterations of hematopoietic homeostasis typical of leukemia. In fact, among the unsolved problems, understanding the molecular mechanisms underlying stem cell differentiation raises the strongest interest. Stem cells have two main characteristics: self-renewal and the capacity to differentiate.[25] In the bone marrow, blood cells are closely connected to other cell types responsible for the production of

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cytokines and hemopoietic growth factors. It will be important to evaluate whether these extra-cellular signals push the stem cells to maturate into precursors or whether they just play a support role allowing proliferation and survival.[26] The second hypothesis seems to have gained more credit. Recently, it has been reported that the transition from stem cell to multiple or single lineage precursor could be controlled by transcriptional modulators that antagonize hematopoiesis.[27] In the last few years, the molecular phenotype of mice-derived stem cells has been characterized through analysis of gene expression profiles:[28] the authors of this research described a fair number of gene products and deepen the knowledge about the mice stem cell thanks to a functional classification. After this initial work, many other publications have described transcriptome changes in different types of normal hematopoietic stem cells, both in the mouse and human, trying to characterize the genetic programs underlying self renewal, commitment, plasticity till to molecular characterization of stemness, i.e. the stem cell signature. Similar studies have been carreid out on hematopoietic stem cells purified from patients with hematologic diseases.[29-38] The molecular mechanisms underlying the terminal differentiation hematopoietic cells are partially known, and this knowledge derives from the study of fusion proteins that arise during leukemogenesis from specific or recurrent translocations.[39-41] DNA microarray technology has also been used to study the gene expression profile of myeloid cell lines induced to differentiate via treatment with drugs.[42-48] A recent study analyzes the profiles of 60 neoplastic cell lines of different origin, including hematopoietic ones, to compare them with the original tumors.[59] Moreover, microarray analysis can be used as a tool for molecular diagnosis of hematologic cancers,[50] for new molecular phenotyping of myeloid/lymphoid,[51] classification of large B-cell lymphomas,[52] characterization of the changes in gene expression profile of different acute myeloid leukemias carrying trisomy 8,[53] and sub-classification of pediatric acute lymphoblastic leukemia[54] and acute myeloid leukemias both in adults and children.[55,56] Studies using DNA microarray technology are underway to define the differential gene expression between malignant and normal plasma cells,[57,58] to study the multistep transformation of monoclonal gammopathy of unknown significance to myeloma,[59] to identify different sub-groups of plasmocytomas carrying different genetic abnormalities,[60-64] to identify intercellular signaling genes in malignant plasma cells[65-67] and to perform pharmaco-genomics investigations.[68-73] One of the most relevant aspects of microarray technology is the data analysis. In fact, every experiment generates thousands of data, i.e. information on thousands of mRNAs, and a whole study needs to be validated by programming more than one experiment. To analyze and organize such a *heap* of information, algorithms and bioinformatic tools have been developed that allow *clustering* analysis and correlation of the expression data with the biological functions of the expressed messengers.[74-77]

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Immunophenotypic evaluation of plasma cells: a useful tool for minimal residual disease evaluation in patients with multiple myeloma

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In multiple myeloma (MM), the use of high-dose chemotherapy followed by autologous stem cell transplant (ASCT) is apparently superior to conventional chemotherapy, as shown by the higher complete remission (CR) rate and prolonged relapse-free (RFS) and overall survival (OS).[1] However, most patients ultimately relapse due to the persistence of residual malignant cells — minimal residual disease (MRD) — after transplantation. Analysis of MRD, below the detection limit methods conventionally employed to define CR, may be of clinical relevance in order to predict impending relapses. Moreover, in acute leukemias, accumulating evidence exists that MRD techniques also contribute to stratifying patients at different risks of relapse, through the quantitative measurement of tumor load depletion.[2] Multiparametric flow cytometry immunophenotyping represents an attractive approach for the analysis of the bone marrow (BM) plasma cell (PC) compartment in patients with MM, since it discriminates between myelomatous (my) and normal PC (nPC), even when both populations coexist in the BM.[3,4] This is based on the presence of phenotypic aberrations in the former PC population — which are absent in the nPC — and which could be considered as a *tumor-associated markers*. The limit of detection of residual myPC by this technique ranges between 10^{-4} and 10^{-5}. For identification of residual myPC, patient-specific quadruple monoclonal antibody (MoAb) combinations adapted to the aberrant antigenic profile observed in the PC at diagnosis were designed (i.e. CD38/CD56/CD19/CD45). A highly sensitive two-step acquisition procedure was performed in order to screen at least 3,000 PC per test. Firstly, acquisition of 20,000 cells corresponding to the total BM cellularity was assessed, and secondly, phenotypic information of those events included in a *live-gate* drawn in the CD38+++ fraction — where PC are located — was recorded. In all cases, the percentage of myPC as well as nPC referred to the total cellularity, and the proportion of nPC within the total PC (Prn) were calculated.

Using this approach, we have previously shown that ASCT is more efficient than conventional chemotherapy in reducing tumor load: ASCT produced a significantly higher reduction in the number of residual my-PC and, simultaneously, there was a higher recovery of the normal PC population. The level of recovery of non-involved

immunoglobulins correlated with the number of nPC after treatment. Moreover, the proportion of patients who achieved an immunophenotypical remission after ASCT was also significantly higher than it was after conventional chemotherapy. Regarding the influence of MRD on RFS, the cut-off level of % n PC/total PC ≥30% showed the highest predictive value for discriminating patients who were at different risk of relapse from among those with MM. However, higher cut-off levels of %nPC/total PC might be more accurate for the specific assessment of patients undergoing ASCT. Rawstron *et al.*[4] obtained similar results in a series of 45 transplanted patients, showing that detectable neoplastic PC at three months post-transplant predicts an earlier relapse than in those with phenotypically normal PC.

At present, we are analyzing the impact of MRD transplanted patients with MM (n=113) treated according to the current GEMM multi-center protocol. All received 6 courses of alternating cycles of VBCMP/VBAD and subsequently underwent ASCT conditioned with melphalan 200 mg/m^2 or BUMEL (12 mg/kg busulphan-140 mg/m^2 melphalan). Stem cells were collected after the fourth cycle of chemotherapy. Patients who achieved immunological CR after ASCT went into maintenance therapy while patients in partial response (PR) received a second transplant (either autologous or mini-allogeneic transplant). MRD was evaluated 3 months after the first ASCT and only in those patients achieving CR (n=87). In 31 of these 87 cases, MRD was subsequently evaluated at two or more consecutive time-points post-ASCT (median: 3 studies/case; range: 2 to 8 studies). All these 87 patients showed < 5% BMPC at all morphologic examinations post-ASCT. CR was defined as the absence of monoclonal component on electrophoresis; 75% of the patients also displayed negative immunofixation (IFE) (CR$_1$ response) while the remaining 25% were electrophoresis negative but IFE positive (CR$_2$ response). The median follow-up from diagnosis was 22 months. Phenotypically aberrant PCs were detected at 3 months after ASCT in 37 out of 87 CR patients (42%) at a median level of 0.035% myPC (range: 0.002% to 3.18%). Comparing the level of MDR between IFE positive and IFE negative cases, we observed a significantly lower level of myPC in IFE negative cases (myPC$_{median}$: 0%; range: 0% to 3.18%) than in IFE positive case (myPC$_{median}$: 0.008%; range: 0% to 0.38%)(p=0.041) together with a higher recovery of nPC (nPC$_{median}$: 0.18% vs 0.25%)(p=0.029) and a superior number of cases in immunophenotypic remission

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(77% cases vs 46%, *p*=0.028). Follow-up studies showed MRD tests became negative in six cases and became positive in four other cases.

Finally, although the follow-up is stil too short to reach firm conclusions, we have explored the impact on RFS of MRD in the BM obtained 3 months after ASCT within 87 patients in eletrophoretic CR. Preliminary data showed that patients in whom ≥85% of the total BMPC displayed a normal phenotype had a longer PFS than did patients with <85% Prn (32 months vs 20 months, *p*=0.03). In addition, follow-up studies indicate that patients who remained MRD positive or became positive had a significantly worse outcome than the MRD negative cases. In summary, investigation of MRD by immunophenotyping may be a useful tool for disease monitoring in MM patients.

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T-cell receptor excision circles: a novel prognostic parameter for the outcome of transplantation in multiple myeloma patients

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Despite the progress achieved with single or double peripheral stem cell transplantation (PBSCT), multiple myeloma remains an incurable disease. Different prognostic factors have been identified, the most important being elevated β_2 microglobulin and C-reactive protein levels, as well as monosomy or deletion of chromosome 13. The presence of a T-cell receptor β gene rearrangement and idiotype-reactive T cells have been correlated with the prognosis of the patient. The aim of this study was to investigate, whether T cell receptor excision circles (TRECs), known to be a surrogate marker for recent thymic emigrants (RTEs), measured at different time points of therapy, have a prognostic impact on the clinical outcome of myeloma patients after autologous PBSCT. A total of 25 patients (12 male, 13 female, median age 51 years) were enrolled in this study. The stage of myeloma was III A in 19 cases and IIIB and IIA in three cases each. All patients received 2-4 cycles of debulking VAD therapy, one cycle of IEV for PBSC mobilization and 1 cycle of EDAP therapy for consolidation. The patients were then transplanted twice within 3 months. DNA was extracted from periperal blood after isolation of mononuclear cells by density gradient centrifugation. TRECs were quantified by real-time polymerase chain reaction (PCR) analysis, using the Taqman assay. Values were expressed as TREC copies/10^5 PBMCs and measured at the following 7 time-points: at study enrollment, after conventional therapy, between the first and second trans-plantation and 3, 6, 12 and 24 months after the second transplantation. Each sample was run in triplicate and the mean value was used for data analysis. Clinical response was evaluated according to the criteria published by Blade *et al.* Statistical analyses were performed using Spearman's rank correlation coefficient and the Wilcoxon-Mann-Whitney test. *p* values are two-tailed and are considered statistically significant when *p* was < 0.05.

A high variation of TREC levels was found among the patients at diagnosis (median TREC level 136/10^5 PBMCs; range 1-1729), suggesting individual differences in thymic output of naive T cells. The median TREC value was lowest after the first PBSCT (52/10^5 PBMCs) and reached baseline 12 months after the second transplantation. After a follow up of 36 months, patients with more than 136 TRECs/10^5 PBMCs at diagnosis had a statistically significant better overall survival (*p*=0.05) and event free survival (*p*=0.045), while low baseline TREC levels correlated with a higher incidence of infectious complications. Correlation of TREC levels 3 months after the second transplantation and event-free survival was highly statistically significant (*p*=0.008). This was independent of β_2-microglobulin and C-reactive protein levels.

These data suggest that TRECs are an independent prognostic factor for event-free and overall survival in transplanted myeloma patients.

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Impact of negative selection (B cell depletion) in multiple myeloma autologous transplantation. Final analysis of a prospective comparative trial of the Bolzano-Münich Study Group

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High-dose chemotherapy, followed by autologous or allogeneic transplantation of hematopoietic stem cells, represents the therapy of choice today for patients affected by multiple myeloma. Autologous transplantation with peripheral blood stem cells (PBSC) improves the survival and the quality of life of myeloma patients. Nevertheless, relapse remains the major problem, and can be explained by the persistence of myeloma cells after high-dose chemotherapy and/or by reinfusion of clonal cells with the leukapheresis product. To reduce the risk of relapse after autograft, positive selection of CD34+ cells and negative selection with B-cell depletion are the most useful techniques for stem cell purging. Until recently positive selection of CD34+ cells did not show any correlation with improved overall or event-free survival. In our study, we compared the outcome of patients transplanted with or without ex vivo purged stem cells using an immunomagnetic approach of B cell depletion (negative selection) in an attempt to obtain tumor-free products.

Between 1995 and 2000, 110 consecutive patients, median age 53, median serum β_2 microglubulin 2.7 mg/L (range 1.4-14.3) in advanced stage of disease, were treated with sequential chemotherapy and tandem transplantation, according to the "*total therapy*" concept of Barlogie. This therapy consists of 3 × VAD cycles (vincristine, doxorubicin, dexamethasone), 1 × IEV + G-CSF (Ifosfamide 2500 mg/m^2 iv days 1-3, epirubicin 100 mg/m^2 iv day 1, Etoposide 150 mg/m^2 iv days 1-3) administered on an outpatient basis with MESNA and hydration support therapy for mobilizing the PBSC, 1 × EDAP (etoposide, dexamethasone, Ara-C, cisplatin) and then tandem high-dose therapy with melphalan 200 mg/m^2 iv within 3-6 months followed by autotransplantation of peripheral blood stem cells. Immunomagnetic *ex vivo* B-cell purging of PBSC, using a cocktail of CD10, CD19, CD20, CD22, CD37 antibodies and immunomagnetic beads (MaxSep System Baxter, Unterschleißheim, Germany) was applied in 53 patients out of 110 in order to remove clonogenic B cells. Whether the PBSC autograft of a patient was purged or not depended on the availability of the purging technique at the treatment center, but not on individual patient characteristics. Contamination of the apheresis products and minimal residual disease (MRD) where controlled by Gene Scan Analysis of CDR III- and CDR I-polymerase chain reaction (PCR) products.

Out of 110 patients 32 were in stage II and 78 in stage III. The purging efficacy using a panel of 3, 4 or 5 anti-B monoclonal antibodies did not differ (3 log) and engraftment after the first transplantation (unmanipulated) and the second transplantation (purged) was identical (10 days for 0.5×10^9/L neutrophils, 11 days for 20×10^9/L platelets and 15 vs 17 days for 50×10^9/L platelets). One patient had a transitory graft failure due to reactivation of CMV infection after the second transplant. The treatment-related mortality for all patients was 3.6%. With PCR analysis of the CDR III and CDR I region, we documented that 86% of the collections of immunomagnetic bead B-cell fractions isolated from the apheresis products were contaminated by myeloma precursor cells. The outcome of the transplanted patients was correlated with the clonal pattern of the removed B-cell fraction. Patients showing a predominantly monoclonal B-cell population in their apheresis products had an event-free survial (EFS) of 20% at 40 months, in comparison to 50% for patients showing a polyclonal or oligoclonal pattern. Complete remission (bone marrow, Bence Jones, IF: negative) and partial remission were obtained in 48% and in 37%, respectively. The impact of purging was favorable: After a median follow-up of surviving patients of 53 months, the median EFS for patients transplanted with purged PBSC was 3.5 years versus 1.9 years for the patients transplanted with unmanipulated PBSC (p=0.011) calculated from the time of first transplant. The median overall survival was 6.4 years for B-cell purged transplantation versus 6.0 years for patients receiving an unmanipulated transplant (p=0.21).

Our results confirm that a tandem transplantation procedure represents a favorable therapy for multiple myeloma patients and that transplant purging by negative selection of B cells can improve the EFS of the patients.

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Autologous stem cell transplantation in multiple myeloma. The Intergroupe Français du Myelome experience

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Autologous stem cell transplantation (SCT) versus conventional chemotherapy (CC)

The Intergroupe Français du Myelome (IFM) was the first group to conduct a randomized trial showing the superiority of high dose therapy (HDT) with autologous bone marrow transplantation (ABMT) over CC.[1] The IFM 90 trial showed that HDT significantly improved the response rate since 38% of patients enrolled in the HDT arm achieved a complete remission (CR) or a very good partial remission (VGPR) (90% reduction of the M-component) whereas only 14% of patients enrolled in the CC arm did so (p <0.001). An updated analysis of this study confirms that, with a median follow-up of 7 years, HDT significantly improves event-free survival (EFS) (median 28 months versus 18 months, 7-year EFS 16% versus 8%, p=0.01) and overall survival (OS) (median 57 months versus 44 months, 7-year OS 43% versus 25%, p=0.03). These results, published 7 years ago, were recently confirmed by the MRC7 trial.[3]

How to improve the results of autologous stem cell transplantation?

The 7-year EFS in the IFM 90 trial was only 16% for patients enrolled in the HDT arm and the survival curves showed no plateau. Therefore strategies to improve these results were clearly warranted. Since the achievement of CR or VGPR in this trial was significantly associated with a prolongation of survival, the aim of subsequent studies was to increase the CR rate.

Conditioning regimen

Improving the conditioning regimen could be one way to attain this objective. The combination of total body irradiation (TBI) plus high dose melphalan (HDM) 140 mg/m³ yields CR rates ranging from 20 to 50%. This conditioning regimen was used in the IFM90 trial and could, therefore, be considered as the standard regimen. However, in newly diagnosed patients, the Royal Marsden Group reported an impressive 70% CR rate with HDM 200 mg/m², with a low extramedullary toxicity.[4]

In 1995 the IFM initiated a randomized study comparing HDM 200 mg/m³ and HDM 140 mg/m³ plus TBI in 282 patients with newly diagnosed multiple myeloma.[5] In this study, HDM 200 mg/m³ was significantly

less toxic (shorter duration of neutropenia and thrombocytopenia, lower incidence of grade $\geq$ 3 mucositis, no toxic death versus 5 in the TBI group). Although the response rate and the EFS were identical, OS was superior in the group receiving HDM 200 mg/m³ apparently because of a better salvage after relapse. Therefore, HDM 200 mg/m³ should be preferred to HDM 140 mg/m³ plus TBI as the conditioning regimen for ASCT in MM.

Impact of tandem transplants

Another way to increase the CR rate could be to repeat intensive treatments. Thanks to autologous transplantation of peripheral blood progenitor cells (PBPC) and to hematopoietic growth factors, the sequential use of two courses of HDT is feasible and appears to increase the CR rate.[6] However, the actual impact of tandem transplantations on EFS and OS needed a randomized comparison with less aggressive strategies.

In 1994, the IFM initiated such a randomized trial (IFM94). From October 1994 to March 1997, 403 untreated patients under the age of 60 years were enrolled by 45 centers. At diagnosis the patients were randomized to receive either a single ASCT prepared with HDM (140 mg/m³) and TBI (8 Gy) or a double ASCT: the first prepared with HDM (140 mg/m³) and the second prepared with melphalan (140 mg/m³) and TBI (8 Gy).

Overall 399 patients were evaluable. Out of 199 patients assigned to the single ASCT arm, 177 (85%) actually received the planned transplant and there were 3 toxic deaths. Out of 200 patients randomized to the double ASCT arm, 156 (78%) actually received both transplants and there were 5 toxic deaths.

The results are presented in Table 1. There is no significant difference in the CR rate between single and double ASCT recipients. However with a median follow-up of 6 years the median EFS and the OS are superior in the double ASCT arm.

Prognostic factors

The Little Rock Group analyzed prognostic factors in a large series of patients treated with this tandem auto-transplants and showed that several initial parameters are associated with a poor outcome: high β2 microglobulin or LDH levels, low albumin level, hypodiploidy or presence of chromosome 13 abnormality as detected by conventional cytogenetics.[7-9] A subgroup of

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Table 1. Results of the IFM94 trial (intention to treat analysis).

	Single ASCT N = 199	p value	Double ASCT N = 200
CR (%)	34	ns	35
CR + VGPR (%)	42	0.15	50
EFS median (months)	25	<0.03	30
7-year EFS (%)	10		
OS median (months	48	<0.01	58
7-year OS (%)	21		42

patients with a very poor prognosis can be identified by combining these factors. Patients with high β2 microglobulin (or low albumin) and with chromosome 13 deletion (or hypodiploidy) have a short survival even when managed with tandem ASCT. For these patients new therapies are clearly needed.[7,8] On the other hand, patients with none of these unfavorable prognostic factors may enjoy prolonged EFS and OS after double ASCT with a plateau of the EFS curve for 45% patients.[9]

In a retrospective analysis of 110 patients treated with HDT in 2 IFM centers, the detection of chromosome 13 abnormalities (-13, 13 q-) by FISH was the most powerful adverse prognostic factor.[10] The combination of FISH analysis, β2 microglobulin and IgA isotype produced a very powerful staging system in the context of HDT. Again, patients with a high β2 microglobulin level and chromosome 13 abnormalities had a very poor prognosis.

Based on this analysis, the IFM99 trial was designed according to a risk-adapted strategy. At diagnosis, all patients were screened for the presence of adverse prognostic factors (β2 microglobulin level > 3 mg/L, chromosome 13 abnormalities by FISH analysis). All patients received VAD chemotherapy as induction treatment. Patients with 0 or 1 adverse prognostic factor received tandem ASCT (the 1st being prepared with HDM 140 mg/m² and the 2nd being prepared with HDM 200

mg/m²). The patients were then randomly assigned to one of 3 maintenance therapy arms (control, pamidronate, pamidronate + thalidomide) [IFM99-02]). Patients with 2 adverse prognostic factors first received HDM 200 mg/m³ plus ASCT. Patients with an HLA identical sibling were allocated to allogeneic BMT with a reduced intensity conditioning regimen. All other patients were randomized between 2 conditioning regimens prior to a 2nd ASCT (HDM 200 mg/m² ± anti-IL6 antibody).

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Autologous stem cell transplant in the elderly patient

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Conventional chemotherapy has been the treatment of choice for multiple myeloma patients since 1960. It continues to play an important role in patients over 70, who represent about 50% of the whole MM patient population at presentation.

The superiority of high–dose chemotherapy has been definitively demonstrated for patients below 60 years old. The best strategy for patients between 60 and 70 still needs to be defined: the available high-dose regimens are probably still too toxic, and new and less toxic approaches should be evaluated.

We tested a novel strategy in patients aged between 60 and 70 years old. We administer tandem melphalan at the dose of 100 mg/m^2 (MEL100), followed by peripheral blood progen itor cell (PBPC) support. To ensure the clinical advantage of this procedure, a prospective randomized trial comparing MEL100 versus methylprednisolone (MP) has been carried out among several Italian Hematology Departments (*Italian Multiple Myeloma Study Group*). MEL100 was well tolerated in elderly myeloma patients; it produced a higher response rate, and longer event-free survival and overall survival than did the MP arm. We analyzed the group of 73 patients who were older than 65 years. The probability of survival for 3 years was 63.4% after MP and 75.4% after MEL100 (*p*=0.015). In patients younger than 65, the magnitude of these improvements was identical. In the multivariate analysis of these elderly patients, the administration of MEL100 was the first factor affecting survival (*p*<0.05). The incidence of toxicities after MEL100 was not age-related.

MEL100 is superior to MP, but the efficacy of MEL100 in comparison with melphalan at the dose of 200 mg/m^2 (MEL200) remains unclear in a patient population between 60 and 70 years old. To address this issue, a case-matched control analysis was performed. Ninety patients at diagnosis were treated with tandem MEL100 courses between 1994 and 2001. Their clinical outcome was compared with that of a control group of 90 pair mates matched for serum β2 microglobulin levels and Durie and Salmon clinical stage, and treated at diagnosis with tandem MEL200 courses.

Complete remission (CR) was achieved in 35% after MEL100, and in 48% after MEL200 (p =0.08). Median event-free survival (EFS) was 32 months for patients treated with MEL100, and 42 months for those treated with MEL200 (p<0.005), but overall survival (OS) was not different. Transplant-related mortality was not significantly different. Hematologic and extra-hematologic toxicity was significantly less after MEL100.

Despite a significant age difference, tandem MEL100 was less toxic than tandem MEL200, MEL100 was inferior to MEL200 in terms of EFS but not in terms of OS. The intensified non myeloablative MEL100 regimen is an effective first line treatment.

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Double versus single autologous stem cell transplantation as primary therapy for multiple myeloma

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The *Bologna 96* clinical study was designed to prospectively compare a single autologous transplantation (arm A) versus double autologous transplantation (arm B) as primary therapy for symptomatic multiple myeloma (MM). In both arms of the study, the treatment plan consisted of the following phases: 1) conventional remission induction chemotherapy with vincristine, doxorubicin and dexamethasone (VAD) administered at 4-week intervals, for a total of 4 courses; 2) mobilization and collection of peripheral blood stem cells (PBSC) with high-dose cyclophosphamide (HD-CTX) (7 g/m^2) and granulocyte colony-stimulating factor (G-CSF); 3) PBSC-supported high-dose therapy (Tx-1) with high-dose melphalan (MEL) (200 mg/m^2); 4) maintenance therapy with recombinant alfa interferon (IFN) following the completion of autologous transplantation. In patients randomized to double autologous transplantation, a second course of high-dose therapy (Tx-2) with melphalan and busulfan (Mel-Bu) (120 mg/m^2 and 12 mg/kg, respectively) was planned to be administered within 90 to 180 days after Tx-1. Primary end-points of the study were response rate, time to relapse/progression (TTR), overall survival (OS) and event-free survival (EFS). Curves for OS, EFS and TTR were plotted according to the method of Kaplan and Meier starting from the time of initiation of VAD chemotherapy and were compared by the log-rank test. The study was closed in December 2001; an analysis of the first 220 patients who were enrolled from January 1996 to December 1999 was performed and the results are reported here. Comparison of the presenting clinical and hematologic characteristics between the two groups of patients (arm A: 110 patients) (arm B: 110 patients) revealed that they were well balanced with respect to the most common variables presumed to have prognostic relevance. The probability of receiving VAD x 4, HD-CTX and Tx-1 for patients randomized to arm A of the study was 89%, 83% and 80%, respectively. The corresponding figures for patients randomized to arm B were 96%, 86% and 85%, respectively. Thirty-six percent of patients who were assigned to receive double autologous transplantation failed to complete their assigned treatment program (15% for non-medical reasons). Among patients who actually received Tx-2 the median interval between Tx-1 and Tx-2 was 4 months. The median number of PBSC collected following HD-CTX was 10.4×10^6 CD34$^+$ cells/kg. The most frequent non-hematologic toxicity associated with high-dose therapy was mucositis, which was graded III-IV (WHO criteria) in 14% of patients receiving MEL and in 13% of those treated with Mel-Bu. On an intention-to-treat analysis, the probability of attaining complete remission increased with the progression through VAD, HD-CTX and high-dose therapy up to a final rate of 31% in arm A and 43% in arm B (*p*=not significant). Complete remission rates for patients who actually received a single autologous transplantation or double autologous transplantation were 36% and 52%, respectively (*p* = 0.004). With a median follow-up of 39.5 months from the start of VAD therapy, median OS was 62.5 months for Tx-1 vs. 74+ months for Tx-2 (*p*=not significant). Three percent of patients died of treatment-related causes in group A and 4.9% in group B. Compared to group A, patients assigned to the the double autologous transplantation arm of the study had a significantly longer TTR (median, 23.5 months vs. 39.5 months, respectively; *p*=0.002) and extended EFS (median, 21 months vs. 31 months, respectively; *p*=0.05). Analysis of subgroups of patients revealed that the gain offered by double autologous transplantation in terms of extended OS, EFS and TTR was particularly relevant for patients who were initially refractory to VAD and for those who ultimately failed to attain complete remission following autologous transplantation. In contrast, curves of OS, EFS and TTR for patients who entered complete remisssion after either a single or double autologous transplantation were almost identical. It is concluded that double autologous transplantation as primary therapy for MM could be opportunely performed in slightly less than two thirds of patients aged below 60 with a risk of treatment-related mortality that did not exceed 5%. Non-hematologic toxicity of sequential high-dose therapy consisting of MEL and Bu-Mel was minimal and no cumulative toxicity was observed in the Tx-2 arm of the study compared to in the Tx-1 arm. On an intent-to-treat basis, double autologous transplantation significantly prolonged EFS and TTR in comparison with Tx-1. Longer follow-up is required to form a definitive assessment of definitely assess the impact of two sequential courses of PBSC-supported high-dose therapy on the ultimate outcome of MM.

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Appendix

The following physicians actively participated in the "Bologna 96" study:

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A new conditioning regimen involving total marrow irradiation, busulfan and cyclophosphamide followed by autologous stem cell transplantation evaluated in a phase I/II study and in comparison to tandem melphalan in a phase III study

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The overall survival of patients with advanced multiple myeloma (MM) undergoing high-dose chemotherapy and autologous stem cell transplantation (SCT) depends mainly on the quality of response. Thus, to improve the response rate, a new intensified high-dose chemoradiotherapy regimen was evaluated in a phase I/II study. After induction chemotherapy 89 patients (median age 51, range 32-60 years) with stage II/III MM received a conditioning regimen with total marrow irradiation (9 Gy), busulfan (12 mg/kg) and cyclophosphamide (120 mg/kg) (TMI/Bu/Cy) followed by SCT. Regimen-related toxicity according to WHO criteria and response rates defined by EBMT/IBMTR were analyzed. The main toxicity was grade III/IV mucositis in 76%, and grade > I fever in 75% of patients. Three patients developed reversible veno-occlusive disease. Two patients died of transplant-related causes. Among patients with *de novo* and pretreated MM, the complete response rate was 48% and 41%, respectively.

With a median follow-up of 45 months, the actuarial median duration of EFS and OS after transplant was 29 and 61 months, respectively, for the whole group, and 36 and 85 months for patients with *de novo* MM. Administration of this intensified conditioning regimen was associated with a tolerable toxicity, a high response rate and long EFS and OS. Thus, conditioning therapy with TMI/Bu/Cy was compared to tandem melphalan followed by autologous SCT in a European multicenter phase III study involving 49 centers and enrolling 294 patients. Patients received 4 cycles of induction therapy and stem cell mobilization (IEV and G-CSF 5 mg/kg). The 215 patients who completed the induction therapy, achieved at least stable disease and mobilized > 4×10^6 CD34$^+$ cells /kg underwent randomization to either receive TMI/Bu/Cy or tandem high-dose melphalan therapy. Preliminary data from this study will be presented at the meeting.

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Allogeneic transplantation in multiple myeloma – the EBMT experience

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The European Group for Blood and Marrow Transplantation (EBMT)

The rationale for performing allogeneic transplantation in multiple myeloma[1] is firstly that the myeloma cell is sensitive to irradiation and many cytotoxic drugs, secondly that high dosages of irradiation or alkylating agents have proven to give a higher frequency of complete remissions than do conventional low dosages[2] and thirdly that there is proof that the graft has an antimyeloma effect.[3] Recent results indicate that the graft-versus-myeloma effect may be more important for sustained response than was hitherto acknowledged.[4]

Early results of allogeneic sibling donor transplantation indicated that long-term survival and long-term progression-free survival could be obtained in a small fraction of patients, but that the transplant- related mortality was high.[5,6] However, recent results from the European Group for Blood and Marrow Transplantation (EBMT) indicated a dramatic improvement in overall survival of patients receiving an allogeneic transplant performed during the period 1994 – 1998 as compared to transplants performed during the period 1983 to 1993.[1] During the earlier period the 4-year overall survival was 32% but improved to 50% during the later time period. The reason was reduced transplant-related mortality. Early transplant-related mortality decreased from 38 % to 21%, and the total treatment-related mortality decreased from 46 % to 30 % from the earlier to the later time period, respectively. These decreases in transplant-related mortality were, in turn, due to earlier transplantation (10 months from diagnosis during the later period versus 14 months during the earlier one), fewer pre-transplant lines of cytotoxic drug treatment, and better supportive treatment.

Since 1994, an increasing number of hematopoietic stem-cell transplants with peripheral blood stem cells (PBSC) have been performed in multiple myeloma.[1] However the improved survival during the later period was not due to increased use of PBSC. In a first comparison of patients transplanted with bone marrow or PBSC no significant difference was found between the two groups in overall survival, transplant-related mortality or relapse rate. However in a later follow-up a border line significantly (*p*=0.05) better survival was found in patients who had received bone marrow (n=297) than in those who had received PBSC (n=224). There was no significant difference in acute graft-versus-host disease (GVHD) or relapse rate but the rate of chronic GVHD was significantly higher in those who had received PBSC.

Prognostic parameters have been extensively studied by the EBMT.[6,7] Females do better than males, IgD myeloma seems to have a worse prognosis than do other subtypes and younger patients do better than older ones. Early transplantation is better than late and those patients who have received several treatment regimens before their transplant fare worse than those who receive only one regimen. A female donor with a female recipient is the best combination while a female donor and a male recipient is the worst.

Non-myeloablative transplantation has been performed in an increasing, but still limited number of patients with multiple myeloma. Recently Crawley *et al*.[8] made a survey among EBMT centers. Results on 256 transplants from 40 centers were collected. Of these, 194 were matched sibling donor transplants, 40 unrelated donor transplants and 9 were from mismatched or other related donors. At transplant 61% of patients were in partial or complete remission (CR, 7.6%) and 29 % had evidence of disease progression. The median time to transplant was 20 months. Conditioning regimens were considered non-myeloablative by the reporting centers and included fludarabine, cyclophosphamide, melphalan, busulphan, and Campath/ATG. Total body irradiation was used in 29% at a median of 2.0 Gy. Most patients received PBSC. The overall survival at 2 years was 52%. Acute GVHD (grade II-IV) occurred in 31%, and grade III-IV in 11.9%. Chronic GVHD occurred in 43% of evaluable patients and was extensive in 22.7 %. It was encouraging that although overall treatment-related mortality was 23% at one year, it was only 9% in patients transplanted within 1 year of diagnosis. Thus although it is too early to evaluate long-term survival and relapse rate, the results appear promising considering the relatively low transplant-related mortality.

Relapse following allogeneic transplantation may respond to donor lymphocyte transfusions.[9,10] Previous studies indicate that up to 30% of relapsing patients may respond. However, the duration of remission has so far been relatively short.

The EBMT has started a prospective phase-II study comparing non-myeloablative allogeneic transplantation following autologous transplantation to single or

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double autologous transplantation. The study is based on genetic randomization, i.e. the availability of a matched sibling donor. Conditioning for the autologous transplantation is provided by melphalan 200 mg/m^2 and the non-myeloablation is performed with total body irradiation 2 GY and fludarabine 30 mg/kg for 3 days. It is hoped that this study will help to define the role of non-myeloablative transplantation in multiple myeloma.

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Allogeneic transplantation from unrelated donors: the GITMO experience

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Allogeneic bone marrow transplantation from HLA identical family donors is not yet a standard or routine treatment for multiple myeloma; the use of matched unrelated donors is even more controversial.[1] We report on a study of allogeneic transplantation in multiple myeloma (MM), from unrelated donors, which was started by the Italian group for bone marrow transplantation (GITMO) in 1999, after approval by the general Assembly, is ongoing and planned to end patient accrual by 2003.

Study design

This is a phase II, prospective, multicenter trial, aimed at testing the feasibility of unrelated transplants in MM. Considering the perplexities about such a procedure — the trial was discussed between 1988 and 1999 — it was decided that it was of fundamental importance not to expose the patients to unnecessary risks; for this reason, it was decided a) that a full HLA-identity should be present between patient and donor and b) antithymocyte globulin (ATG) be incorporated in the preparative regimen (see details later). The primary end-points are transplant-related mortality (TRM) at 100 days and response rate at 6-9 months. The secondary end-points were rates and grades of acute and chronic graft-versus-host disease (GVHD), overall survival, and event-free survival.

The number of patients to be accrued was decided according to the primary-end points.

For the day 100 TRM it was decided to perform a two-stage trial in order to stop the trial in case of excessive toxicity (day 100 TRM > 70%).

The preliminary phase entails an accrual of 15 patients: if the number of patients alive at 100 days is ≤ 5 (TRM rate > 69%), the trial will be stopped because of excessive toxicity. If the number of patients alive exceeds 5, the trial will continue with the complementary phase to accrue a total of 48 patients.

This method — Simon's optimal design[2] — has the scope to expose the minimum number of patients to a potentially dangerous therapy. The evaluation of the efficacy of the transplant will be conducted with the assessment of the rates of complete + partial responses 6-9 months after transplant.

Assuming that p0 (proportion of responses below which the treatment is ineffective and does not deserve further study) is 40%, while p1 (the opposite of p0) is 60%, that potency is 80%, that the two-tail probability and error is 0.05%, that success is defined as the number of complete + partial responses, and that failure is the number of non-responses + progressions, the number of patients needed to be accrued was 48.[2,3] The treatment will be considered effective if the number of successes is >27.

Inlusion criteria

The criteria necessary for inclusion in the trial are: age: 18-45 yrs, MM of any stage, responsive or refractory to 2 lines of therapy, but not in progression, absence of general contraindications to an allotransplant procedure, and Informed consent.

HLA typing

Donors and recipients must have full HLA class I and II compatibility, as assessed by molecular techniques. For class I, HLA-A, B, C compatibility must be confirmed by at least low molecular resolution techniques. For class II, identity of HLA DRB1, DRB3, DRB4, DRB5, DQA1, DQB1 must be assessed by high-resolution techniques. No HLA DP identity is required.

Preparative regimen

Two preparative regimens are possible. The first regimen, Regimen A, is based on total body irradiation (TBI) and consists of TBI, fractionated or in a single dose, from a linear accelerator or a Co 60 source, cyclophosphamide, 120 mg/kg day, melphalan 120 mg[2] and low-dose rabbit ATG. The second, chemotherapy based regimen, regimen B, consists of busulfan, 16 mg/kg, melphalan, 140 mg/m^{2}, and rabbit ATG.

GVHD andf infection prophylaxis

GVHD prophylaxis is cyclosporine A 1-3 mg/kg i.v from day −1 to oral realimentation, then orally for 9 months after transplant, and methotrexate, 15 mg/m^{2} on day 1, 10 mg/m^{2} on days 3, 6, and 11. The prophylaxis against infection is Acyclovir, until the ninth month after BMT, Co-trimoxazole, for 6 months and CMV monitoring, weekly for 4 months, with pre-emptive therapy in case of positivity.

Table 1. data from first 15 patients.

Sex, F/M		10/5
Age, median	40 yrs	(32-49)
β2 microglobulin	3.5	(0.47-17.53)
Stage at diagnosis	III	9
	II	3
	I	2
	Not evaluated	1
Interval dg-tx median	19 months	(3-75)
Status at BMT	8	chemoresistant
	7	chemoresponsive
N° previous lines, median	3	(1-4)
N° pts previous auto-transplant	8	(double 3)
β2 microglobulin at transplant	2.2	(1.1-16.7)
Stage at BMT	III	8
	II	5
	I	1
	Not known	1
Preparative regimen	TBI based	(double 3)
	Busulfan based	(1.1-16.7)
Disease response	CR	4
	PR	4
	No response	2
	Not evaluable	5
Acute GVHD	NO	5
	I	4
	II	4
	III	1
	IV	1

Develoment of the study

The 100-day TRM rate of the first 15 patients in the preliminary phase was 5/15 (33%); the study could thus continue, the mortality being half than the limit set for stopping the trial. The causes of death were: GVHD 1; infections 2; multiorgan failure 1; myocardial infarction 1. During the complementary phase, amendment was made to the upper age limit. The upper age limit was raised to 50 yrs, during the 2001 General Assembly, on the grounds of the results of the preliminary phase and the general trend to extend the upper age of transplants in the unrelated setting.

Patient accrual

The number of patients transplanted was 2 in 1999, 5 in 2000, 7 in 2001, 7 in 2002 and 4, so far, in 2003. Since the accrual has been somewhat slower than anticipated, it has become apparent that the target of 48 transplants is unrealistic. It was therefore decided, at the 2003 GITMO annual assembly, that setting the p0 at 35% and p1 at 60%, the number of pts to enrol will be 28. This number should be reached by the end of 2003.

Patients' characteristics

Twenty-five patients have been transplanted so far. Data of the first 15 patients belonging to the preliminary phase are shown in the Table 1.

Discussion

The analysis of the efficacy of the study (see statistical considerations) will be performed by mid 2004, 6-9 months after the entry of the last patient. No preliminary analyses are planned. While the results of allogeneic bone marrow transplant in multiple myeloma can be appreciated only after several years,[4,5] and this applies to our study also, a few methodological points deserve some consideration. First, the choice to look for perfectly HLA-matched donors, which had been pursued in a GITMO study of volunteer unrelated donor transplants for thalassemia,[6] has certainly reduced the number of possible transplants; recent results from a multicenter study[7] indicate that differences at the C locus can be accepted; however, in that study, the preparative regimen was of reduced intensity. Second, our study was intended for patients with advanced disease: indeed many had refractory or progressive disease and had received several lines of therapy, including autotransplants. Such a population of patients, which we think should be treated as maximally as possible,[4] would nowadays be treated by many centers with a *mini* transplant and indeed this has happened: in fact, during 2002, 4 patients enrolled in this program received a transplant with reduced-intensity conditioning instead, thus contributing to the slower accrual. This *competition* from an emerging, alternative, form of treatment could not have been foreseen at the time of designing the study.

The slowness in the accrual and the long course of multiple myeloma do not allow a definitive evaluation of the study at present.

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Rationale for allogeneic hematopoietic cell transplantation with reduced conditioning in patients with multiple myeloma

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Autologous stem cell transplantation (HCT) has shown to be effective in the treatment of multiple myeloma.[1,2] Recurrence of the underling disease, however, is generally observed even after tandem autologous HCT3. In contrast, long-term progression-free survival is obtained in patients after allogeneic HCT.[4-7] Recent results indicate that the graft-versus-myeloma effect is the main mechanism responsible for sustained responses.[8] Its full potential, however, is not realized yet because of high transplant related mortality after related and even more unrelated allogeneic HCT. Despite dramatic improvements during the last years, transplant related mortality remains the major obstacle for successful allogeneic HCT in patients with multiple myeloma.[4] In addition, the majority of patients with multiple myeloma are to old to undergo HCT limiting the application of this treatment.

Recently, reduced-intensity preparative regimens have been developed to decrease treatment related mortality and to extend the curative effect of allogeneic HCT to older or infirm patients. These protocols aim at obtain donor engraftment with regimens conveying different degrees of myelosuppression ranging from minimal to severe and at use graft-versus-tumor effects to eradicate underlying malignancies. The regimens involve fludarabine and myelosuppressive drugs like melphalan, busulfan or cyclophosphamide or a merely immunosuppressive treatment with minimal hematopoietic and overall toxicities using 200 cGy total body irradiation before and cyclosporine and mycofenolate mofetil after HCT. Lately, autologous HCT and reduced intensity allogeneic HCT have been combined to maintain the benefits of both, cytoreduction following high dose therapy and the graft-versus-myeloma effect. A protocol using autologous HCT in combination with an immunosuppressive conditioning has been developed within the Seattle consortium.[9] Mortalities not related to relapse of 2% after autologous and 15% after allogeneic HCT were observed. The 100-day transplant-related mortality after the allografts was 0%. Using this approach, 81% of patients showed tumor responses, which is noteworthy as 48% of patients had relapsed or refractory disease before HSCT.

Other groups treated myeloma patients with intermediate dose melphalan (100 g/m²) using matched sibling HSCT following one or two prior autografts.[10,11] Overall 61% achieved a CR or near CR.

Kröger *et al.* have also utilized tandem auto/allografts using unrelated or mismatched related donors and conditioning with fludarabine, melphalan and antithymocyte globulin.[12,13] Day 100 TRM was 11%, and the estimated 24 month disease-free survival 56%.

Optimizing the graft-versus-myeloma effect in this two-step procedure remains a critical future research objective. Interestingly the time to reach CR was longer than in patients with other hematological diseases like CML suggesting a gradual increasing graft-versus-myeloma effect. This effect was noted in combination with but also without GvHD, suggesting either a subclinical graft-versus-myeloma effect or a tumor-specific immune reaction. Protocols using donor lymphocyte infusion, but also more sophisticated therapies such as NK-cell infusions, applications of in vitro cultured T-cells with specificity for myeloma cells and the use of idiotype vaccination have been passed the planning stage and are now applied in phase I studies.

In conclusion high-dose autologous HSCT and graft-versus-myeloma effects of HCT after reduced or minimal conditioning might reduce transplant related mortality and safely extend the benefits of allogeneic transplantation to older patients and hopefully achieve substantial and permanent cure rates of multiple myeloma.

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Stem cell transplantation from related and unrelated donors in multiple myeloma after reduced intensity fludarabine/melphalan conditioning

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Dose-reduced regimens based an fludarabine and melphalan allowed stable engraftment of allogeneic stem cells from related and unrelated donors in patients with hematologic diseases including multiple myeloma.[1,2] We report the results of two multicenter phase I/II studies investigating the feasibility of a fludarabine-melphalan dose-reduced intensity regimen followed by stem cell transplantation in patients with advanced multiple myeloma. Our program is focusing on four issues: (i) auto-allo tandem approach in newly diagnosed patients and those with less advanced disease (melphalan 200mg/m² plus auto-PBSC followed after 3 months by melphalan 100mg/m² and allo-PBSC);[3] (ii) allo-transplant in patients who have relapsed after a prior autograft (melphalan 140mg/m²); (iii) stem cell transplantation from unrelated donors (MUD) (within both protocols);[4] (iv) effect of donor lymphocyte infusion for persistent or relapsed disease.

The reduced intensity conditioning regimen consists of fludarabine (150mg/m²), melphalan (100-140mg/m²) and antithymocyte globulin (ATG: 3×10-20mg/kg).

So far 64 patients with a median age of 52 years (range 31-64) have been included in the two protocols: 40 males and 24 females. All patients had advanced stage II (n=21) or stage III (n=43) disease.

The median number of prior chemotherapies was 5 (range: 2-26). A prior autograft had been performed in 63 patients: 34 of 63 had experienced relapse to an autograft while in 29 patients the autograft was part of the auto-allo tandem approach. No graft failure was observed and the median time to absolute neutrophil count (ANC)>1.0×10⁹/L and platelet >20×10⁹/L was 16 (range 11-23) and 43 days (range 12-22), respectively. Acute graft-versus-host disease (GvHD) grade II-IV was noted in 38% (MUD 55% vs related 31%; *p*=0.05). Severe grade II/IV acute GvHD was seen in 15%. Chronic GvHD was observed in 37%, while only 10% experienced extended cGvHD.

The 1 year transplant-related-mortality (TRM) was 22% (MUD: 25% vs related: 17%, *p*=n.s.). Prior to

transplantation the disease status was: CR (n=5), PR (n=33), MR (n=1), NC (n=10), PD (n=15). After allografting 45% of the patients achieved a complete remission with negative immunofixation. The estimated overall and progressive-free survival at two years was better in patients treated with the auto-allo
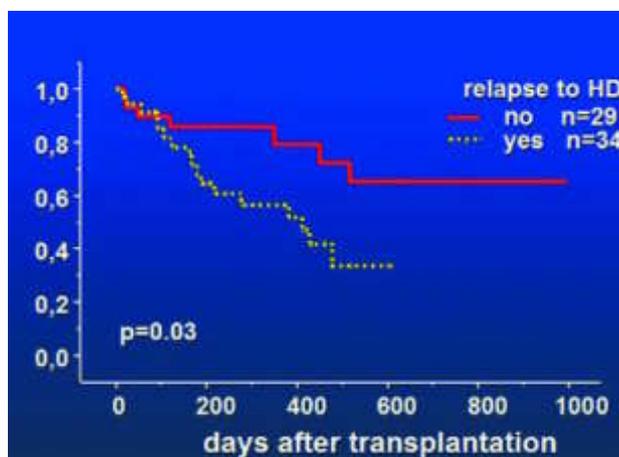
Figure 1. Overall survival after dose-reduced Melphalan-Fludarabin in multiple myeloma according relapse after a prior autograft.
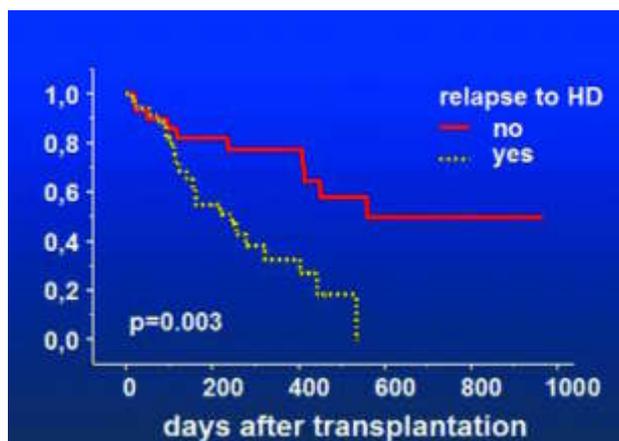
Figure 2. Event-free survival according relapse to a prior autograft.

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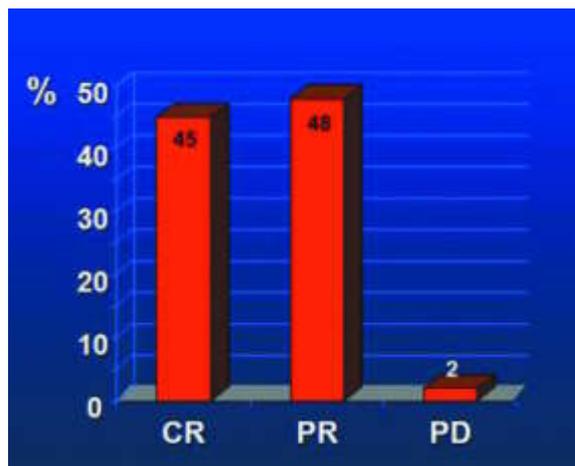
Figure 3. Response to allogeneic stem cell transplantation.

approach than in patients who had already relapsed after a prior autograft: 65% vs 38% (p=0.03) and 48% vs 25% (p=0.06), respectively. In a multivariate analysis bone marrow as stem cell source (HR 1.98: 95% CI: 1.08-3.64; p=0.03) is the most important factor predicting TRM, while female sex of the donor and relapse after autograft are significant for overall survival (HR 2.17; 95% CI: 1.36-3.45; p=0,001 and HR 2.38: 95% CI: 1.43-3.96; p=0.0008) and for event-free survival (HR 1.69; 95% CI: 1.18-2.44; p=0.005 and HR 2.02; 95% CI: 1.35-3.03; p=0.0007), respectively.

Twenty-one patients received donor lymphocyte infusions (DLI) because of persistent disease (n=6) or progressive disease (n=15). The median CD3+ cell dose for MUD (n=10) was 1×10^6/kg and for related donors (n=11) 5×10^6/kg. Acute GvHD grade II-IV was seen in 25%, but one patient experienced fatal grade IV GvHD. Despite the lower cell dose the probability of developing acute GvHD was higher after MUD-DLI than after related DLI (p=0.01). The response rate was 42% (3 CR and 4 PR).

Melphalan/fludarabine-reduced conditioning with pre-transplant antithymocyte globulin, followed by related or unrelated stem cell transplantation provides rapid and sustained engraftment with durable complete donor chimerism, and low one-year treatment-related mortality. Donor lymphocyte infusions are effective in patients with relapse or persistent disease. Because of the better outcome in patients without prior failure of an autograft, allogeneic stem cell transplantation should be performed at an earlier phase of the disease. Randomized studies comparing dose-reduced allografting after an autograft with a second autograft in high-risk patients are ongoing in Germany and within the EBMT.

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Reduced intensity - non myeloablative conditioning regimens for allografting In multiple myeloma

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High dose therapy with autologous hematopoietic cell support has been show to confer survival advantages over those produced by conventional chemotherapy. The Intergroup Francais du Myélome 90 Trial reported a 7-year event-free survival of 16% and an overall survival of 43% in multiple myeloma (MM) patients treated with autografting compared with 8% and 25%, respectively, in patients treated with conventional chemotherapy.[1] Despite this aggressive approach, patients invariably relapse or progress failing the attempt to eradicate the disease. Allografting provides a tumor-free hematopoietic cell source and graft-versus-myeloma immune effects, which currently remain the only genuinely curative treatment for MM.[2-3] However, the curative potential of conventional allografting for MM patients has not been fully elucidated because of the associated high transplant-related mortality (TRM), primarily as a consequence of infection and graft-versus-host disease (GVHD).[4-7] Moreover, as it is performed in younger patients, usually under 50 years of age, this transplant procedure becomes feasible only for a minority of MM patients. To date, the best reported analysis, by the EBMT registry, showed a TRM of 1% at 6 months with a 55% 3-year survival for patients transplanted between 1994-1998.[8] Of note, the median age in this cohort was only 44 (range 18-57) years whereas the median age of MM patients at diagnosis is approximately 65-70. These findings prompted investigators to design novel approaches employing reduced intensity conditioning regimens in the attempt to reduce TRM and increase the eligible age for transplant. Badros *et al.* reported a series of 31 high risk MM patients treated with a melphalan based conditioning regimen.[9] After a median follow-up of 6 months, 61% of patients reached complete remission (CR) or near CR. Median overall survival was 15 months. Of note, previous chemotherapy and disease status at transplant appeared to influence outcome significantly. Significantly longer event-free survival and overall survival were observed when allografting was performed after only one rather than two or more autologous transplants. Importantly, only 2 out of 17 patients transplanted with progressive disease remained alive and progression free at 7 months post allografting. Kröger *et al.* used a reduced intensity regimen consisting of fludarabine, melphalan and antithymocyte globulin to condition 21 patients with advanced MM for transplant from unrelated donors.[10] At a median follow-up of 13 months, the 2-year estimated overall and progression-free survival rates were 74% and 53%, respectively. CR and partial remission (PR) rates were 40% and 50% respectively. A better outcome was observed in patients in whom a prior autologous transplant had not failed. More recently, the Seattle group reported a multicenter trial on 52 patients employing a tandem *auto-allo* transplant approach in newly diagnosed MM patients.[11] After induction chemotherapy, patients underwent GCSF mobilized autografting with high dose melphalan (200 mg/m^2) followed, 2-4 months later, by non myeloablative low dose (2.0 Gy) total body irradiation, peripheral blood hematopoietic cell infusion from HLA-identical siblings, and immunosuppression with mycophenolate mofetil and cyclosporin.

The rationale of this study was that of combining an allogeneic graft-versus-myeloma effect with high dose autologous hematopoietic cell rescue. With a median follow-up of 552 days post-allografting, survival was 78% with an overall response of 83% including 57% CR and 26% PR. In order to confirm these promising data on a larger series of patients, we are conducting an Italian multicenter trial, on behalf of Gruppo Italiano Trapianto di Midollo Osseo (GITMO), employing a similar tandem transplant approach for newly diagnosed stage IIA-IIIB MM patients up to the age of 65.[12] To date, 64 patients (median age 55, range 34-65) from 13 Italian Transplant Centers have entered the study. Fifty-seven patients have so far completed both transplant procedures.

Allografting was carried out at a median of 76 (range 44-195) days after autografts. All patients readily achieved sustained T-cell donor engraftment. After a median follow up of 317(36-1207) days post-allografting, overall survival is 84% (48/57). The overall response rate evaluated on 52 patients, with a follow-up of at least 84 days, is 83%, with 58% (30/52) CR and 25% (13/52) PR. Overall, disease recurrence was observed in 2/52 (4%) patients who showed a positive immunofixation at 2 years post-allografting, after obtaining a CR.

Remarkably, in 42/52 (80%) patients who were not in CR at allografting, 19/42 (45%) patients attained CR at a median of 90 days (range 28-180) showing an effective graft-versus-myeloma effect. Grade II acute GVHD

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and grade III-IV GVHD developed in 25% and 11%, respectively. In 49 patients with a follow-up longer than 120 days, chronic GVHD requiring therapy developed in 31% (15/49). Overall, TRM was 16% (9/57), day 100 TRM 2% (1/57). The main causes of death were steroid-refractory GVHD, infectious complications and HUS-TTP syndrome. These findings confirm that non myeloablative low dose TBI based allografting is feasible in older MM patients providing higher response rates and lower TRM than does conventional allografting. In conclusion, the clinical results, recently reported, with non-myeloablative/reduced intensity conditioning regimens have been encouraging and have kindled new interest in allografting in MM.

However, all these procedures should be offered to patients only in the context of controlled clinical trials. Of note, large controlled studies are needed to evaluate if, in the presence of a potential donor, allografting should be proposed up front to all patients or only to those with poor prognostic factors. Moreover, longer follow-up will determine the impact of chronic GVHD on disease response, quality of life, and survival.

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Appendix

The following Divisions of Hematology are currently contributing to the GITMO study:

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Growth and differentiation signalling in myeloma cells

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Multiple myeloma is a cancer of plasma cells leading to bone destruction. It is an incurable disease whose biology is still elusive. Thus, there is no unified pathogenesis for multiple myeloma. Plasma cell differentiation appears to be quite complex and depends on pathways which are not completely defined so far. This complexity, which dominates plasma cell generation, makes it difficult to unravel the molecular bases of multiple myeloma. However, considering comparatively normal versus neoplastic plasma cells may guide us through a number of issues related to regulation of B cell differentiation.

Growth and terminal differentiation in B cells: molecular events

From a molecular point of view, evidence suggests that at least three levels of regulation are involved in plasma cell differentiation: (i). In the microenvironment, cytokines and growth factors such as IL-4, CD30L, CD40L, IL-6, IL-10 and EGF-related molecules drive the expansion of B/plasmacytoid cells.[1] Chemokines such as CXCL12 are involved in plasma cell homing to specific tissues,[2] but they also elicit a number of cell activities, including growth signals; ii). A further level of regulation is related to the suppressor activity mediated through BLIMP-1. This latter switches off B specific factors such as PAX-5 and Oct-2 and, together with XBP-1, it takes control of plasma cell differentiation.[3-5] Moreover, BLIMP-1 influences the cell cycle regulation in B/plasmacytoid cells, inhibiting BCL-6, E2F and cMYC.[6,7] Finally, a third level of regulation is associated with CDK activities.[8,9] The BLIMP-1- and CDK-dependent control levels are highly complex and eventually responsible for the apoptotic clearance of the plasma cells. These interplaying levels as well as apoptotic signals appear to be dysregulated in myeloma cells.

Growth and terminal differentiation in multiple myeloma: molecular events

Typically, myeloma is characterized by accumulations of malignant plasma cells in the bone marrow, though they can be also observed in other anatomical sites. Normal plasma cells appear to migrate to lymph node and bone marrow niches depending, at least in part, on their membrane expression of CCR7/CXCR5 and CXCR4,

respectively. A decrease of CCR7/CXCR5, increase of CXCR4 and bone marrow stromal cell expression of CXCL12 regulate plasma cell homing to the bone marrow.[10,11] Myeloma cells seem to derive from long-lived bone marrow plasma cells which are dominated by BLIMP-1 and XBP-1. CXCL12 (which also leads to release of EGF-related factors), and IL-6 activities seem to be relevant during the first events of neoplastic transformation. According to some experimental models, neoplastic immortalization is mainly supported by the ectopic expression of an oncogene responsible for CDK dysregulation[8,9] followed by the expansion of an established myeloma clone. In a number of cases, cyclin D1 dysregulation seems to depend on bone marrow signals. The survival advantage of the myeloma clone following CDK dysregulation is mainly sustained by transduction pathways involving JAK/STAT3 (anti-apoptotic functions), ERK-1, ERK-2 and Ras (growth functions)[12] activated by both IL-6R and EGFR-related molecules. As a whole, this is considered to lead to a quite restricted growth compartment producing putatively differentiating plasma cells, which accumulate in the bone marrow.[10,11] Myeloma cells, on the other hand, release factors able to induce bone marrow microenvironment modifications associated with symptoms typically present in myeloma patients. Some experimental data, however, are hard to fit into this scenario.

Network biology of normal and neoplastic plasma cells

An interesting way around this biological complexity is to analyze growth and differentiation pathways in plasma cells in terms of computational networks.[13] Cell structures respond to environmental signals, switch each other on and off, and dismantle and rebuild their own shape and function in a way which shares impressive similarities with computational networks. The unifying principles instructing the topology of a network, its evolution, and its dynamics may shed light on cell signaling and functions, though some risks are inherent in this approach based on the assumption that software, not hardware, provides the right level of abstraction.[14] In network terms, the plasma cell regulatory levels reported above are dynamic systems interacting with each other to build an extended, coherent program usually called plasma cell differentiation. Some specific losses of network coherence may lead to multiple myeloma. However, to date little is known about this putative network. If plasma cell regulator factors build

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up a computational network, it would be possible to identify its topology, where the most important nodes (factors) are characterized by the highest number of connections to other nodes. Cyclin D, JAK/STAT3, BLIMP-1, XBP-1, CXCR4[2-12] are examples of highly connected nodes. We assume that these nodes represent the keys to plasma cell differentiation and that their power in the cellular context must be tested. This approach may highlight nodes crucial to network maintenance and thus contribute to define normal and pathogenetic models as well as therapeutic targets, and may even suggest the relevance of novel molecules which turn out to be necessary according to the logic inherent in the computational network of interest.[14,15]

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Angiogenesis in multiple myeloma

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Three decades ago, Folkman introduced the concept of angiogenesis in solid tumors.[1] In solid tumors, angiogenesis was shown to be required for invasive tumor growth and metastasis. In the absence of angiogenesis, tumors cannot grow beyond 1–2 mm in size. Tumors make an *angiogenic switch* in order to grow and metastasize by perturbing the local balance of pro-angiogenic and anti-angiogenic factors. In the last years, angiogenesis has been shown to be increased not only in solid tumors but also in a large variety of hematological malignancies.

Vacca *et al.* were the first to show that bone marrow angiogenesis is increased during active in comparison to during inactive multiple myeloma.[2] To study bone marrow angiogenesis, endothelial cells were visualized by immunohistochemistry using monoclonal antibodies against antigens expressed by endothelial cells, e.g. CD34, and microvessel density (MVD) was quantified as a measure of bone marrow angiogenesis. Among hematologic malignancies, multiple myeloma was the first in which increased bone marrow angiogenesis was shown to be an independent prognostic factor for survival by Rajkumar *et al.* and our group.[3,4] Using the Mayo Clinic classification, Rajkumar *et al.* recently reported an increase of bone marrow angiogenesis in active myeloma in comparison to in smoldering multiple myeloma in a very large population of patients.[5] There was a further increase in angiogenesis in relapsed myeloma. In line with these results, angiogenesis was found to be significantly increased in stage II-III myeloma in comparison to in stage I, using the Durie and Salmon staging system.[6] Thus, the degree of angiogenesis progressively increases along the various stages of myeloma progression and it is possible that the angiogenic switch is, at least in part, responsible for the progression of early to advanced multiple myeloma.[5] Additionally, in stage II-III myeloma, we found a highly significant correlation between bone marrow angiogenesis and both serum β2-microglobulin levels and plasma cell infiltration in the bone marrow.[6] In contrast, no such correlation was found in early myeloma. Furthermore, a positive correlation between the absolute number of circulating plasma cells and the microvessel density was found in multiple myeloma.[7] This relationship was independent of the disease activity and of the plasma cell burden in the marrow. It was suggested that the increased angiogenesis may promote plasma cell proliferation and migration into the circulation. Some investigators reported that microvessel density in patients who achieved a complete or partial remission after chemotherapy decreased significantly in comparison to their pretreatment values.[8] In myeloma patients who did not achieve a remission, no significant change in the bone marrow microvessel density could be detected. These findings show similarities to those observed in acute myeloid leukemia.[9] Thus, even conventional chemotherapeutics used in cancer treatment may have direct or indirect effects on tumor angiogenesis.[10]

Myeloma cells were shown to secrete a number of angiogenic cytokines, e.g. vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF).[11-13] Studies of the effects of VEGF on the secretion of interleukin-6 (IL-6) in bone marrow stroma suggested paracrine interactions between myeloma and bone marrow stromal cells, triggered by VEGF and IL-6.[14] Furthermore, bFGF may also play an important role in angiogenesis in myeloma. Patients with multiple myeloma had significantly higher serum bFGF levels than controls and serum levels significantly increased parallel to the progression of myeloma from stage I to III.[15] Recently, serum bFGF and HGF levels were shown to predict survival in multiple myeloma patients.[16] These data lend additional support to the assumption that angiogenic cytokines are involved in the growth and progression of multiple myeloma. Beside the paracrine action of angiogenic cytokines on endothelial and stromal cells, recent data also suggest autocrine effects.[17]

The research on angiogenesis in multiple myeloma was also inspired by Barlogie's group showing that thalidomide induces remissions in refractory myeloma patients, although this drug also has some other properties beside its anti-angiogenic action.[18] Thalidomide inhibits endothelial cells extracted from bone marrow of patients with active multiple myeloma.[19] A number of novel antimyeloma drugs, such as proteasome inhibitors and new analogs of thalidomide have been shown to have antiangiogenic properties, which may add to their effectiveness. A number of inhibitors of angiogenesis pathways have recently become available and are being evaluated in multiple myeloma models or in clinical trials.

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Physiopathology of bone disease in multiple myeloma

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Multiple myeloma (MM) is a plasma cell malignancy characterized by a marked capacity to induce bone destruction. Almost all patients with MM have osteolytic bone lesions that mainly result from increased bone resorption related to stimulation of osteoclast recruitment and activity. However, the biological mechanisms involved in the pathogenesis of bone disease in MM are not completely understood.

In the lasts years the RANKL system has been identified as critical in the regulation of bone resorption. It was demonstrated that stromal and osteoblastic cells express the critical osteoclastogen factors, RANKL, that induce osteoclast formation and activation. Osteoblastic cells also produce the soluble receptor osteoprotegerin (OPG) that blocks the interaction of RANKL with its recepètor RANK, inhibiting the bone resorption. Extensive studies have shown that RANKL and OPG exert a pivotal coupled control of bone resorption. On the basis of this evidence we have investigated the potential role of RANKL in the physiopathology of MM-induced bone disease.

Our data indicate that human MM cells or human myeloma cell lines do not directly express RANKL but that they are able to up-regulate RANKL in bone marrow (BM) stromal cells/osteoblastic cells and to inhibit its soluble antagonist OPG through cell-to-cell contact involving a VLA-4/VCAM-1 interaction.

Immunohisochemistry, performed on BM biopsies of MM patients, confirmed that patients with osteolyisis and nodular plasma cell infiltration have a higher RANKL/OPG ratio in BM stromal cells than do patients without bone lesions or normal subjects.

In addition, since growing evidence suggests that also T cells may regulate bone resorption through the cross-talk between RANKL and interferon (IFN)-γ, which strongly suppresses osteoclastogenesis, we investigated the potential effect of human myeloma cells on T-cell RANKL and IFN-γ production. Using a co-culture transwell system we found that human myeloma cell lines increased the expression and secretion of RANKL in activated T lymphocytes and similarly purified MM cells stimulated RANKL production by autologous T lymphocytes. Moreover we found that the release of IFN-γ by T lymphocytes was reduced by the presence of both human myeloma cell lines and purified MM cells. The finding of high RANKL mRNA expression in BM activated T lymphocytes of MM patients with severe osteolytic lesions than in those without skeletal involvement supports these observations.

In conclusion our results suggest that myeloma cells induce the critical osteocloastogenetic factor RANKL in both BM stromal cells and T lymphocytes and that RANKL is critically involved in MM-induced bone destruction.

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Novel therapeutic approaches in multiple myeloma

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Until recently treatment of multiple myeloma was based on the use of a few cytostatic drugs and glucocorticosteroids. During the last few years significant advances have been made in the development of new drugs, some of which have already been proven as effective treatment while others have presently entered or will soon enter clinical testing.

Thalidomide

Thalidomide was introduced in 1998 as a treatment for relapsing or refractory patients with multiple myeloma and has shown a 30% response rate as a single agent in this category of patients.[1] The optimal dose depends on the sensitivity of the individual patient's tumor clone to thalidomide. Some patients respond to doses as low as 50 mg per day, whereas in others doses of up to 600 mg seem to be required. Two studies indicate a dose-response relationship, particularly in patients with poor prognostic features.

Combining thalidomide with dexamethasone increases response rates up to 70% in newly diagnosed patients.[2] Similar results have been reported with thalidomide-chemotherapy combinations. Responses usually occur rapidly after initiation of treatment and may last for months or even years. The median remission duration has not been defined. Major thalidomide toxicities are fatigue, drowsiness, constipation, peripheral sensory neuropathy, arrhythmias and an increased rate of thromboembolic complications, particularly when thalidomide is used in combination with dexamethasone and/or chemotherapy. Presently, several trials are ongoing to evaluate the efficacy of thalidomide-dexamethasone first line therapy in relation to standard chemotherapy.

Proteasome inhibitors

The proteasome is an intracellular enzyme system for the breakdown of key proteins such as cyclins or falsely arranged proteins. Recently, a potent inhibitor of the proteasome (Bortezomid, Velcade®) has been introduced into clinical use. Bortezomid inhibits the activation of NF κ B, which controls genes that encode interleukin(IL)-6, tumor necrosis factor(TNF)-α, and other cytokines and growth factors. In addition, Bortzomid exerts anti-angiogenic activity, sensitizes tumor cells to chemotherapy and radiation and inhibits the crosstalk of myeloma cells with bone marrow stroma cells.

Bortezomid has been studied in patients with relapsing and/or refractory myeloma and shown to induce responses in about 35% of pretreated patients with some complete responses being observed even in patients who have received 5 or more lines of prior therapy.[3] The most common side effects are thrombocytopenia and leukopenia, particularly in heavily pretreated patients. In addition, peripheral neuropathy may be dose-limiting. Some patients complain of fatigue, mild diarrhea and nausea.

Immunomodulatory drugs (ImiDs)

Immunomodulatory drugs are derivates of thalidomide and are much more potent than the parent drug. They exert pleiotropic activity and stimulate T- and NK-cells as well as the production of several cytokines such as IL-2, interferon (IFN)-α and IL-10. Furthermore, they reduce the expression of TNF-α and IL-1β in bone marrow stroma and inhibit angiogenesis. Direct interaction between ImiDs and myeloma cells enhances apoptosis, reduces proliferation and vascular endothelial growth factor (VEGF) secretion and adhesion of myeloma cells to bone marrow stroma cells. ImiDs seem also to exert synergistic activity with other anti-myeloma drugs. One of their major advantages is their high enteral absorption rate, which makes the use of an oral formulation feasible.[4]

Preliminary clinical data indicate a response rate of up to 60% in heavily pre-treated patients and disease stabilization in up to 79%. ImiDs are also active in patients resistant to thalidomide and do not cause neuropathy, somnolence or constipation. Large phase III trials are currently underway on both sides of the Atlantic.

Arsenic trioxide

Arsenic trioxide is an approved treatment for acute promyelocytic leukemia and is currently being tested as salvage therapy for relapsed or refractory patients with multiple myeloma. It inhibits myeloma proliferation and induces apoptosis by the production of reactive oxygen species and mitochondrial damage. Arsenic trioxide increases expression of p21, cyclin-dependent kinase inhibitor protein and of caspase-3 inhibitor while Bcl-2 expression is downregulated. Other important effects are the inhibition of myeloma stroma cell bind-

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ing and of IL-6 effects on myeloma cells as well as inhibition of angiogenesis and the increase of leukocyte activated killer (LAK)-cell mediated killing of myeloma cells by upregulation of adhesion molecules and their ligands on tumor and LAK cells. Up to now, only limited clinical data are available. Minor responses have been observed in 20-40% of pre-treated patients. Complete responses have not, as yet, been reported.[5,6]

The anti-tumor activity of arsenic trioxide can be potentiated by reducing intracellular glutathione levels, which can easily by achieved by addition of ascorbic acid.[7] Other *in vitro* data indicate a potential synergy of arsenic trioxide with steroids, thalidomide, chemotherapy and bortezomid. Trials have been initiated in order to evaluate its impact in myeloma therapy.

Other drugs currently in phase II trials

Several promising drugs are currently in phase II trials in patients with myeloma. Some of the most promising are: 2-methoxyestradiol (angiogenesis inhibitor), Genasense (bcl-2 antisense facilitates apoptosis), Glivec (blocks autophosphorylation), SU5416 (inhibits VEGF tyrosine kinase) and Osteoprotegerin (inhibits RANKL and thereby osteoclastogenesis).

Autologous transplantation followed by non-myeloblative allogeneic transplantation

Non-myeloablative transplantation is associated with a reduced transplant-related mortality, but yields similar rates of graft-versus-host disease and infectious complications and is presently being tested in several institutions. Another interesting approach is sequential auto-allotransplantation. Patients are first submitted to autologous transplantation in order to reduce tumor load and improve the performance status before they are subjected to allogeneic transplantation. This procedure is less risky, better tolerated and associated with a high response rate. Preliminary results look promising but available clinical data are still limited.[8]

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Idiotype vaccination against multiple myeloma in patients with progressive disease or partial remission after high dose therapy. First results of a Phase I study

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Multiple myeloma (MM) is a malignant disease commonly affecting elderly individuals and is characterized by accumulation of mature plasma cells in the bone marrow. Although it is possible to produce a period of disease remission using various forms of chemotherapy, the disease remains largely incurable. In order to develop immunotherapeutic treatment strategies in MM a clinical phase I idiotype vaccine trial was performed in patient with progressive disease or partial remission after high dose therapy. The study was designed as dose escalation study in order to determine the feasibility and safety of idiotype vaccination in patients after high dose therapy. Patients received a total of 6 intradermal/ subcutaneous (i.d./s.c) immunizations of idiotype vaccines at day 1, 7, 14 and at week 4, 8 and 12. The dose started from 0.25 mg for the first 5 patients and was escalated to 1.25 mg and finally to 2.5 mg. Each vaccine contained 0.2 mg GM-CSF (Leukomax®) as adjuvant and 0.2 mg KLH (Immucothel®) as control antigen. In addition, GM-CSF was administered s.c. close to the injection site for 3 consecutive days after vaccination. Status of MM disease was determined 1 week before and 2-4 weeks after vaccination.

Currently, 15 patients were enrolled into the study and 12 patients have already completed the full treatment cycle of 6 immunizations at 3 different dose levels. Toxicity could be evaluated for 14 patients. Up to now, all vaccines were tolerated well and caused only minor or transient side effects like skin irritations at the injection site and flu-like symptoms. Most notably, no dose-limiting toxicity was observed. On order to explore the clinical potential of idiotype vaccine formulation for immunotherapy of MM, paraprotein levels and anti-idiotypic immune response was monitored.

Strikingly, a decrease of paraprotein levels could be noted during the course of the first 3 weekly immunizations in 7/8 patients with plasmacytoma of IgG1 isotype. 3 months after starting the treatment, 1/3 patients of the lowest dose group but 4/5 of the middle dose group exhibited stable paraprotein levels or even reductions.

Remarkably, all of these patients raised antibody titers against the idiotype, although the patients exhibiting considerable levels of paraprotein, implying an extraordinary immunogenicity of the Idiotype vaccine formulation used. More detailed analysis revealed that the Id-specific antibodies were mainly antibodies of the immunoglobulin subtype IgG1 and to a lesser amount IgG3, thereby suggesting that a Th1-like T cell response is elicited by idiotype vaccines. This finding is of importance, since human antibodies of the IgG1 and IgG3 isotypes can potentially support the effector functions of antibody-dependent cellular toxicity (ADCC) and complement-dependent toxicity (CDC).

For examination of the cellular immune response, a delayed type hypersensitivity (DTH)-test was performed, which represents the primary measure for the induction of a cellular immune response in immunotherapy. With this test the cellular response against different defined antigens, including the myeloma Id-protein and KLH was determined. So far, all patients immunized demonstrated a strong reaction against the idiotype vaccine formulation and KLH 24-48 h after application. The reaction evoked against purified myeloma Id-protein was apparently weaker, compared to the other antigens, but was unequivocally positive in these patients, who responded to the idiotype vaccine by reduction of their paraprotein levels and by induction of antibodies against the idiotype.

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