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Detection and characterization of circulating clonal cells in human multiple myeloma

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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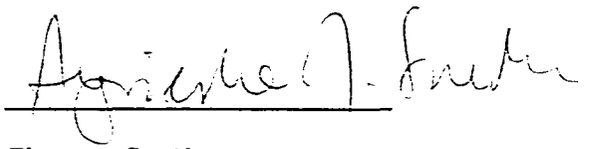

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *"Detection and characterization of circulating clonal cells in human multiple myeloma"* submitted by Agnieszka Justyna Szczepek in partial fulfillment of the requirements for the degree of *Doctor of Philosophy.* in Medical Sciences.

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ABSTRACT

Multiple myeloma (MM) is an incurable lymphoproliferative malignancy of the B cell lineage. The cause of MM is presently unknown. MM is characterized by clonotypic plasma cells (PC) producing monoclonal antibody in the bone marrow (BM). A variety of evidence suggests that the regenerative and spreading potential in myeloma can be assigned to the circulating B-lymphocytes. This study explores composition of the circulating malignant clone in myeloma. We examined the presence, numbers and maturation stage of the circulating clonotypic B cells. To ascertain the clonality of cells we used as a tag the mRNA encoding immunoglobulin heavy chain (IgH) specific for each patient. Three methods were developed to derive the sequence of the expressed allele of the rearranged immunoglobulin heavy chain and to establish the numbers of circulating clonal cells. The results show that a large compartment of clonotypic B cells exists in the circulation of MM patients (average of 66% of total B cells). Pre- and post-switch clonotypic B cells were detected in the blood, bone marrow and in the G-CSF-mobilized blood (MPB) obtained from MM patients that were undergoing autologous stem cell transplantation. Three patterns of expression of pre- and post-switch clonotypic IgH were identified in association with the progression of the disease. Some of the pre-switch clonotypic transcripts obtained from MPB exhibited intraclonal diversity suggesting presence of antigenic selection. This study provides evidence to show that clonotypic MM B cells are frequent in circulation, that they are heterogeneous and that infrequent pre- and post-switch members of the circulating clone may play a role in the pathology of the disease.

Abbreviations

Ab	antibody
BM	bone marrow
BMC	bone marrow cells
Bp	base pair
BSA	bovine serum albumin
BCR	B-cell receptor
cDNA	complementary DNA
DNA	Deoxyribonucleic acid
CDK	cyclin-dependant kinase
CDR	complementarity determining region
dNTP	Dinucleotide triphosphate
DIG	Digoxygenin
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FDC	follicular dendritic cells
FGFR3	fibroblast growth factor receptor 3
FISH	fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
FR	framework of Ig
GC	germinal center
G-CSF	granulocyte-colony stimulating factor

HLA	human leukocyte antigen
IL	interleukin
IF	immunofluorescence
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
mAb	monoclonal antibody
mRNA	messenger RNA
OD	optical density
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PC	plasma cell
pRB	retinoblastoma protein
MPB	mobilized peripheral blood
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
SKY	spectral karyotyping
VAD	combined chemotherapy: Vincristine, Adriamycin and Dexamethasone

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I. INTRODUCTION

A. Multiple myeloma – history and characteristics of the disease

Multiple myeloma – history

MULTIPLE MYELOMA (MM) is the most common lymphoproliferative malignancy affecting B cell lineage and accounting for about 10% of all hematological tumors.

In the fall of 1845, Dr. Henry Bence Jones, a London physician and chemist, discovered an oxide of albumin that dissolved upon boiling in the urine sample of a 44-year-old patient. He concluded that this modified albumin was a specific finding for a disease called *mollities ossium*. The patient, Thomas Alexander McBean, suffered from pain and multiple fractures in his bones, his liver was enlarged and he was experiencing attacks of severe diarrhea. Despite an intensive treatment that included bleeding, leeches, steel and quinine therapy, Mr. McBean died a few months later. The autopsy revealed that his ribs were very soft and filled with a red, gelatin-like substance formed by large nucleated cells [1].

At present, we recognize "Mr. McBean's disease" as multiple myeloma (MM). The oxide of albumin described by Bence Jones was to be later identified as a light chain of a clonal antibody produced by tumor cells. Rusitzky introduced the term "multiple myeloma" in 1873 based on his finding of cells with eccentric nuclei in 8 separate tumors of bone marrow in an autopsied patient. In 1875 Waldeyer introduced the term "plasma cell" and

in 1890 Ramon y Cajal for the first time described accurately plasma cells[2]. Novel immunoelectrophoresis techniques developed in 1939 facilitated the diagnosis of MM [3]

Multiple myeloma is classified as a lymphoproliferative malignancy of mature B cell lineage. The other related lymphoproliferative disorders of B lymphocytes include Waldenstrom's macroglobulinemia, plasmacytomas (extramedullary, solitary) and the monoclonal gammopathy of undetermined significance (MGUS). Waldenstrom's macroglobulinemia is characterized by an infiltration of bone marrow by B cells or plasmocytoid cells which produce IgM monoclonal antibody usually below the concentration of 30g/L [3]. Solitary plasmacytoma of bone differs from myeloma by the containment to a single space in the bone while extramedullary plasmacytoma develops in the soft tissues [4]. MGUS is characterized by the proliferation of clonal plasma cells consisting of less than 10% of BM nucleated cells; presence of the monoclonal antibody (MoAb) at a concentration less than 30g/L; and an absence of osteolytic lesions, anemia or hypercalcemia [5]. A significant proportion of patients with MGUS (about 20%) develops MM. The mechanism of this transformation is presently unknown [6 7].

Multiple myeloma –incidence and symptoms

The incidence of MM cases increases with age with a median age at diagnosis of 68 years. This disease is rarely found in patients under the age of 40. The yearly incidence is 1/25000 [8]. Approximately 10% of patients have an indolent course of the disease; another 15% die within first 3 months from diagnosis; and the remaining patients have a median survival time of about 30 months [3].

The symptoms in MM can be divided into two groups. The first is a direct result of plasma cells occupying space in the bone marrow; the second is a consequence of a high concentration of monoclonal immunoglobulin (called also MoIg, M-protein or MoAb) in serum. Osteolytic lesions that are associated with plasma cell accretion and the activation of osteoclasts cause bone pain, the most common symptom in MM [9]. The lesions provoke bone brittleness and increase the possibility of fracture. Bone resorption by osteoclasts causes hypercalcemia that contributes to kidney dysfunction. Renal failure can also occur due to excretion of light chain [10]. Immunodeficiency linked with MM creates a high susceptibility to bacterial infections that over three-quarters of MM patients are suffering from [11]. Anemia occurs in about 80% of patients due to multiple mechanisms [12]. The main cause for anemia is the suppression of erythropoiesis due to deficient production of ertyhropoietin and the displacement of bone marrow space by malignant plasma cells [13] [14]. M-protein can be as high as 95% of all protein in serum causing increased serum viscosity. adds to the symptoms of MM.

Multiple myeloma – etiology and prognosis

MM is believed to develop in a multistep process in which the MM precursor cell is first immortalized and then transformed by sequentially acquiring mutations and chromosomal aberrations [15]. It has been speculated, that the malignant transformation occurs in a late stage B cell precursor, after the immunoglobulin gene rearrangement [16] [17] [18]. Genetic factors are probably important in the susceptibility of B cells for the transformational event.

Genetic factors

The incidence of MM is as twice as high in men as in women and twice as high in blacks than in whites [19]. Japanese and Chinese populations have the lowest incidences of MM cases [20]. A large population-based study determined an increased frequency of major histocompatibility complex (located to chromosome 6) HLA types Bw65, Cw2 and DRw14 for black patients, while for white patients there was a higher frequency of A3 and Cw2 [21]. Overrepresentation of the Cw2 allele in both racial populations suggests that this allele or a gene close to the Cw2 loci confers susceptibility to the development of MM. The occurrence of multiple cases of malignancy in a family without a Mendelian pattern of inheritance suggests as a cause the environmental hazard exposure [22 23,24]. Alternatively, if the affected family members have inherited identical HLA haplotypes this suggests again a link between HLA and susceptibility to develop MM. [25 23 22]

Environmental factors

Several investigators studied the occurrence of MM in groups exposed to radiation: atomic bomb survivors, nuclear industry workers and soldiers that participated in nuclear weapon tests [26 27 28]. The overall conclusions were that the risk of developing MM after exposure to radiation is not increased.

The connection between farming and MM was demonstrated in several studies. A large cohort study implicated that farmers who work with pigs or poultry but not on other types of farms are in greater risk for developing MM [29]. Similarly, printers but not rubber

manufacturers were shown to have higher incidences of MM. [30] [31]. No association was established for benzene, petroleum and other organic solvents [32] [33].

Chronic antigenic stimulation

Chronic antigenic stimulation can generate B cell clones that may become a target for neoplastic transformation. A number of groups have found an association between rheumatoid arthritis and MM [34] [35] [36]. Monoclonal gammopathy of undetermined significance (MGUS) that is believed to involve chronic antigenic stimulation precedes MM in about 20% of MGUS cases [37] [38] [7] [6].

Infectious agents

Rettig et al. found DNA of the Kaposi sarcoma-associated virus (HHV-8) in the bone marrow dendritic cells of 15 MM patients. They postulated that HHV-8 could induce MM cell growth in an indirect fashion through the production of viral interleukin-6 (v-IL-6) that may stimulate other BM cells to secrete IL-6 [39]. IL-6 is a well characterized growth and anti-apoptotic factor for MM cells [40] [41]. This theory is controversial because other groups were unable to detect the virus in MM-derived specimens [42] [43] [44] [45].

Oncogenes and Tumor Suppressor Genes

C-*myc*, an oncogene controlling proliferation, differentiation and apoptosis was shown to translocate to a switch region of the IgH locus t(8;14) in 5% of MM cell lines tested by using cytogenetics, FISH and a Southern blot assay [46]. Expression of the c-*myc* protein was increased in plasma cells from MM patients with no apparent karyotypic

abnormalities [47]. Later studies associated the enhanced translation of c-*myc* with a mutation found in the 5'untranslated region of the gene [48]. Another family of oncogenes, *ras,* encodes G-proteins that participate in signal transduction. In a study of 56 MM patients, mutations involving the N- or K-*ras* genes were detected in 27% of cases at diagnosis and 46% of cases after treatment. Moreover, the presence of mutated *ras* correlated with a poorer response to therapy [49] [50]. The protein encoded by the p53 tumor-suppressor gene activates transcription of the gene encoding p21, which then suppresses cell proliferation by inhibiting Cdk's at the G1->S phase of cell cycle. Point mutations of the p53 gene were detected in samples from 9.8% of primary MM cases and were associated with an advanced stage of the disease implying that it may be a late event [51]. Thirty-two percent of plasma cells from newly diagnosed MM patients carry deletion p53. That number increases with the progression of the disease to up to 55% [52]. FGFR3 (fibroblast growth factor receptor 3) translocation to the Ig locus has been recently described in 5/21 MM cell lines and 3/11 primary tumors [53]. Expression of this growth factor was postulated to provide a growth advantage for MM PC through paracrine and anti-apoptotic pathways. Cyclin D1 phosphorylates pRB (retinoblastoma protein) which allows for progression of the cell cycle. In the studied MM cell lines, 30% had a translocation t(11,14) resulting in overexpressed cyclin D1 protein [54]. Another MM cell line studied (U226) was shown to aberrantly insert a portion of the excised IgH switch region to chromosome 11q13 adjacent to the cyclin D1 oncogene, resulting in overexpression of cyclin D1 [55]. The *RB* tumor suppressor gene has been shown to be deleted in 34.7% of primary MM specimens and is associated with the disease progression [51].

Many of the studies researching translocations or aberrant expression of oncogenes. were done using MM cell lines. The lack of conclusive evidence from patient samples is mainly due to the low proliferative activity of MM plasma cells [56 57]. It is not clear, if the translocations detected in MM cell lines contribute to the primary neoplastic transformation or if they are acquired in the later stages of the disease. A large karyotype analysis done on the bone marrow from 151 MM patients (117 at diagnosis) showed that only two patients had detectable translocations – none of them involving chromosome 14 [58]. Nonetheless, as postulated by Bergsagel et al. the conventional karyotyping methods may sometimes not be sufficiently sensitive to detect chromosomal abnormalities [46]. New approaches in cytogenetics involving chromosome painting and multicolor spectral karyotyping (SKY) have increased the resolution in analysis of translocations. SKY enables analysis of methaphase spreads by hybridization of chromosomes with fluorescently labeled chromosome-specific probes. Rao et al. performed SKY on 10 MM cell lines and MM 8 BM aspirates [57]. The authors found 79 translocations (2 recurring) in cells lines and 6 non-recurring translocations in 2 patients samples. However. lack of MM plasma cells in metaphase was and still is the major limitation in applying that technique to the bone marrow biopsies.

Another indicator of genetic instability is chromosomal aneuploidy. Previous reports indicated aneuploidy for at least one chromosome in 50 to 80% of MM cases [59 60 61]. Recent studies using interphase fluorescence *in situ* hybridization (interphase FISH) and DNA probes specific for chromosomes 3, 7 and 9 indicated that for 18 MM patients,

aneuploidy was a universal event [62]. Moreover, an identical gain in chromosomes specific for respective patients was found not only in plasma cells but also in myeloid cells and in lymphoid cells. The results strongly suggested that PC, lymphoid and myeloid cells are pathologically related and that the neoplastic transformation could may have occurred during early maturation of B cells. However, the authors did not perform experiments that would indicate clonality of tested cells.

B. The cell of origin in multiple myeloma

The nature of the cell from which multiple myeloma develops remains unclear. For many years, it was believed that the disease originates from the plasma cell population. This should be reflected by an increased proliferation of malignant cells. To address this notion, Mellstedt and his colleagues investigated the mitotic activity of bone marrow cells from 3 MM patients and 2 healthy controls [63]. The authors have shown that only 2.5-5% of the purified BM MM plasma cells had incorporated radiolabeled tritium (1 hour pulse incubation *in vitro*) while BM lymphocytes labeled in the range 11.5-14%, as measured using autoradiography combined with May-Grunwald-Giemsa staining. This suggested rather low proliferative activity within the plasma cells and increased mitosis within the small lymphocyte fraction. In one patient, the mitotic activity was also observed in lymphocytes from peripheral blood suggesting that this may be the compartment that differentiates into malignant PC [63]. The authors complemented the study by using idiotype-specific antibodies to detect the monoclonal patient-specific Ig. Although the results indicated the presence of monoclonal plasma cells and lymphocytes in both blood and bone marrow, the actual data were not shown. In light of Mellstedt's findings, the attention of researchers has shifted to the lymphoid population that precedes plasma cell in maturation.

Anti-idiotype antibodies that recognize patient-specific monoclonal immunoglobulin produced by MM plasma cells were used in an elegant study by Kubagawa et al [64]. Within B cells isolated from blood of two MM patients, 22% and 3.1%, respectively,

stained positive for surface expression of MM idiotype. Interestingly, during analysis of the bone marrow cells, apparent pre-B cells showing a morphology distinct from plasma cells were shown to express the clonal idiotype in cytoplasm, suggesting very early commitment to the MM lineage. Moreover, a proportion of the circulating clonal B cells expressed heavy chain isotypes that were different from plasma cells (IgM, IgD, IgG and IgA) implying heterogeneity within that population. Although very carefully controlled, the studies were done using fluorescent microscopy and only a limited number of cells could be analyzed. Based on obtained results, Kubagawa et al. proposed an attractive theory that assumes that MM originates from pre-B cells. However, at that time molecular or immunocytochemical confirmation of the identity of the clonal population as pre-B cells could not be done, which makes the conclusions of Kubagawa et al. debatable. A study by other group using MM specific antiserum verified the observations by Kubagawa et al. regarding circulating clonal cells. The authors have shown that 0.5-44% of blood B lymphocytes from 5 MM patients bear the same idiotype as the respective plasma cells [65].

Multiple lines of evidence from Dr. Pilarski's laboratory suggested that clonal MM B cells have malignant properties. They have the phenotype of large, monocytoid, activated lymphocytes [66], DNA aneuploidy [67], the ability to migrate on hyaluronan [68] multidrug resistance [69 70 71 72 73] and an abnormal expression of the surface markers CD10, PCA-1, CD11b, RHAMM, CD34 [66 74 75]. These findings proved that MM B cells may exhibit invasive behavior consistent with spread of the disease and, unlike the plasma cells, are resistant to chemotherapy [72]. Based on their results, Pilarski's group has postulated that

MM constantly regenerates from its circulating B cell compartment that homes to bone marrow [76]. Supportive evidence came from functional studies, where plasma cell-free MM PBMC cultured with cytokines IL-3 and IL-6 yielded antibody-producing plasma cells [77]. Nonetheless, Tokumine et al. were unable to repeat the above studies, perhaps because the cytokines they used came from a different source [78].

With time, the phenotypic and functional studies were complemented by the molecular biology techniques that enabled more sophisticated analysis of the immunoglobulin gene rearrangement. The detection of clonal rearrangements of chromosomal DNA involved DNA purification, restriction enzyme digestion, size fractionation by agarose gel electrophoresis, blotting and hybridization with immunoglobulin-specific probes, all components of Southern blot technology. Palumbo et al (1989) using Southern blotting observed clonotypic rearrangements in cells isolated from peripheral blood of MM patient that did not have circulating plasma cells [79]. Baldini et al (1991) using the same approach detected clonotypic rearrangements in only 2 out of 11 MM blood specimens [80]. Chiu et al. demonstrated the presence of the clonal Ig gene in the blood of 10 of 28 MM patients [81]. At the same time another was unable to detect the clonotypic marker in 21 patients [82]. The publication by Clofent et al. raised a discussion about the technical approach to detect clonal cells in blood. Berenson and Lichtenstein have pointed to two major issues that might have accounted for Clofent et al.'s negative results: a different protocol for Southern blot and extensive depletion of adherent cells that were shown to contain clonal B lymphocytes [83] [84]. However, it rapidly became clear that the Southern blot technique is not sufficiently sensitive and specific for these analyses. Because they

were unable to detect monoclonal Ig rearrangements in blood that showed 10% contamination with malignant plasma cells, Humphries et al. challenged the use of Southern blot technology for the detection of clonotypic rearrangements in MM and other B cell malignancies [85].

New methods in molecular biology that replaced Southern blot emerged in the late eighties. The polymerase chain reaction was a major break-through increasing the sensitivity and specificity in the detection of clonotypic Ig rearrangements. Pioneering work where the traditional approach was compared to the novel one was published by Trainor et al. [86] [87]. Side-by-side comparison of results obtained by these two techniques showed that in MM, all 10 analyzed cases were positively identified by Southern blot, but only 6 by PCR. It was indeed one of the first attempts to use PCR to amplify IgH DNA and the authors concluded, that the method in their hands was less sensitive than Southern blotting. However, because of it is cost-effectiveness, they recommend its use in diagnostics. Trainor et al.'s work was followed by Billadeau et al. [88]. These authors for the first time used the patient-specific CDR3 portion of clonotypic Ig DNA as a template for primers in the semi-quantitative PCR to demonstrate the presence of clonotypic cells in circulation. With this procedure Billadeau et al. detected clonal circulating B cells in blood [89]. Subsequent studies by other groups revealed clonality of B cells in the circulation and bone marrow [17] [90] [18].

Efforts to quantitate clonotypic B cells produced a broad range of values. Experiments by Billadeau et al. showed that circulating clonal B cells range from 0.00088% to 32% of

total MM peripheral blood mononuclear cells [89]. The numbers obtained did not correlate with the percentages of PC in the bone marrow suggesting that the circulating cells do not represent the plasma cell population. In a later study, the same group analyzed 13 other MM patients, and detected clonal B cells in range from 0.01% to 3.6% of total PBMC [91]. Using the same technical approach, only 0.34% clonotypic cells were seen in the CD19+ MM fraction by Epstein's laboratory [92]. In the study by Brown et al. (1998), an *in situ* hybridization protocol was used with patient-specific fluorescent probes. The results showed that the numbers of circulating clonal cells changed during the course of therapy, initially dropping in response to the treatment followed by an increase with time to 0.1% – 23% of PBMC [93]. These non-direct or relatively insensitive approaches for quantitation of clonotypic B cells left this issue unresolved and controversial, but supported the idea that sometimes large numbers of circulating clonal cells were detectable.

The characterization and sequencing of germline DNA segments encoding human immunoglobulin were completed and published recently [94]. The comparison of the IgH V-region in MM PC with the germline sequences revealed that the MM PC accumulate somatic hypermutations representative of antigen selection in germinal centers (discussed in detail later). In MM plasma cells, the pattern of somatic mutations is highly conserved [95 96 97]. If some of the MM cells remained subject to antigenic pressure, part of the clone would branch out to produce higher affinity antibody. This would be reflected by a different pattern of somatic hypermutation in the V-region (termed intraclonal diversity). To clarify that issue, which could reflect the differentiation status of clonotypic circulating B cells, an analysis of hypermutational patterns was performed. Sequencing of

amplified Ig VhDJh from MM B cells showed a pattern identical to that found in respective plasma cells indicating a lack of intraclonal diversity [98]. This implied that the analyzed cells passed through the antigen-driven differentiation and no longer are under antigenic pressure.

The somatic hypermutations are independent of isotype class switching which usually happens after antigen-driven selection [99,100] [101]. B cells at their earlier differentiation stages use μ or δ genes while the more differentiated B cells switch to γ or α. In MM, malignant plasma cells usually express a post-switch isotype. IgD and IgM-type myelomas are very rare and in the IgD-type myelomas the plasma cells have actually undergone the switching event deleting the chromosomal μ segment [102]. To find the maturation status of clonotypic B cells, the usage of the constant chain was analyzed. The sequencing analysis of clonotypic transcripts showed that the pre- and post-switch isotypes were present in blood B cells [18] suggesting heterogeneity in the population of clonal cells. Sorted bone marrow CD45+ CD38- B cells were also analyzed to yield similar results [17]. In both experiments, the transcripts encoding other than clinical isotype could only be detected by using nested PCR, implying their low frequency. Other groups that located pre-switch isotypes in bone marrow, failed to find the pre-switch clonotypic IgM transcripts in blood [103]. However, the strategy that was used combined a single round of PCR with Southern blotting and radioactive hybridization, which might not have been sensitive enough to detect infrequent transcripts. Berenson's group, which addressed the same issue, failed to demonstrate the presence of pre-switch μ clonotypic transcript in blood [104]. The authors were using single-round PCR with CDR1 and constant chain

primers followed by colony hybridization with CDR3 probe. Although some of the colonies gave positive results, sequencing revealed that the analyzed species were not clonal. These results put in question the use of the CDR1 region as a template for the PCR primers. Moreover, in the light of data obtained by Bakkus and Billadeau it is evident that two, not one round of PCR amplification is needed to detect infrequent clonotypic pre-switch transcripts [18] [17].

To establish whether or not the cells expressing non-clinical isotype were under antigenic selection their V-region was analyzed for the presence of intraclonal diversity. Sequencing of clonotypic pre- and post-switch IgH DNA and RNA samples derived from bone marrow and blood B cells revealed intraclonal homogeneity [17] [18]. This suggested that the antigenic pressure no longer affected the clone. The malignant status of pre- and post-switch clonal cells and their location, numbers and regenerative potential remained unclear.

In the past decade, autologous bone marrow transplantation has become an important component in the therapy of MM [105] [106]. The hematopoietic progenitor cells are obtained for this procedure by a process termed mobilization. Patients first undergo extensive chemotherapy, and the clinical symptoms such as the concentration of monoclonal protein in blood and urine, BM PC count and the level of calcium in serum are monitored. Once the clinical indicators reach satisfactory low levels, hematopoietic progenitors are mobilized by injection of cytokine, granulocyte-colony stimulating factor (G-CSF) or granulocyte/macrophage-colony stimulating factor (GM-CSF), combined

with high dose chemotherapy. This results in migration of hematopoietic progenitor cells from BM to blood. Mobilized PBMC are collected, cryopreserved and reinfused to patients [105 107 108]. Although a substantial reduction in BM tumor load is achieved after chemotherapy and autologous transplantation, most patients still relapse. Contamination of the mobilized PBMC harvests with tumor cells may account for the recurrence of MM. To explore this notion, PBMC obtained from MM mobilized blood were scrutinized for the presence of clonotypic cells. A majority of researchers found that the blood was contaminated with clonotypic cells [109 110 111 112]. To dissect the MM-related population of cells from the hematopoietic progenitors, surface marker typing was used. It was established, that CD34+ Lin- Thy+ cells purified by five-parameter sorting are free of contamination with tumor cells, as measured by PCR [113]. However, if the population of hematopoietic progenitor cells was only enriched in CD34+ by column fractionation, virtually all samples (29/30) tested positive for the presence of clonotypic Ig in patient-specific PCR [114]. On the other hand, Berenson's group did not detect clonotypic cells in CD34+ fraction from MM mobilized blood [115]. As a cell source for the graft, the majority of hospitals use unpurified leukapheresis product from mobilized blood, introducing clonal cells back to the patient. Some of the transplantation clinics use different selecting methods to enrich for CD34+ cells. However the results of these clinical studies are inconclusive and so far show no benefit in the post-transplantation survival time as compared to controls [116]. Another group, in a study of 183 patients, has demonstrated no difference in response rate or survival between the group that received CD34+ selected cells and those receiving unfractionated cells [108]. Recent data from Pilarski's laboratory

showed that mobilized blood contained MM progenitor cells that colonized xenotransplanted mice and generated myeloma [117].

Based on the presented evidence, the MM progenitor cell may be located in the B cell population from blood, bone marrow and from mobilized blood. Information about the maturation status of clonotypic B cells as well as their numbers and malignant potential remains controversial and needs to be clarified in order to design a curative treatment.

C Immunoglobulins in healthy system and in myeloma

Structure of an antibody

Antibodies are glycoproteins produced exclusively by B cells. They are composed from two heavy (IgH) and two light (IgL) chains held together by disulfate bonds (Fig.1.1) [118]. There are five immunoglobulin classes also called isotypes that differ in the properties of their heavy chain C terminus (IgM, IgD, IgG, IgA and IgE). In myeloma, the most commonly expressed heavy chain is IgG (~60%), followed by IgA (~20%). The IgM, IgD and IgE myelomas are very rare and constitute less than 2% of the cases [3]. Each of the Ig heavy chains associates with one of the two types of light chain, either lambda (λ) or kappa (κ). Within both heavy and light chains, one can distinguish two regions: variable (V) and constant (C). The variable region determines the ability of antibody to bind antigens, while the constant region is characteristic for a given immunoglobulin class or subclass. Different isotypes mediate specific effector functions in the immune response. Within the V-regions of both heavy and light chains, there can be distinguished specific domains called complementarity-determining regions (CDR) that directly contact the antigen. There are three CDR's on each heavy and light chain. The most variable region is CDR3 of the heavy chain, composed of three different mini-gene segments (5' end of Vh, D and Jh).

Immunoglobulin heavy chain rearrangement

The heavy chain is encoded by a set of genes located on chromosome 14q32 in humans [119 94]. The variable region is encoded by three segments: Vh, D and Jh; the constant

region is encoded by one of 11 segments. There are about 200 Vh, 30 D and 7 Jh segments [94]. Only one of each type of these mini-genes is used to form a gene encoding functional antibody. In the process of immunoglobulin gene rearrangement, first the segments Dh and Jh and next the Vh are brought to proximity by the recombinatory enzymes RAG1 and RAG2 (Fig.1.2[120 121 122]. Terminal transferase (TdT) activated during B cell maturation randomly adds nucleotides (N-nucleotides) between the joining ends. The process of rearrangement and the activity of TdT create great diversity in the antibody repertoire [123 124 118 125 126 127]. Only the B lymphocyte is capable of rearranging the immunoglobulin genes from the germline conformation. Because the rearrangements are unique for each B cell and its clonal progeny, they are often used as clonal markers in immunoproliferative disorders. In fact, the first recombined V_hDJ_h genes were isolated from myelomas and plasmacytomas [128 129].

The immunoglobulin heavy chain can be expressed as a peptide only if the transcript is kept in the open reading frame. If one of the Ig alleles fails to generate a productive rearrangement, then the second allele is used. However, only one of the alleles can be expressed, which is a phenomenon mediated by a complex, multi-enzyme process of allelic exclusion [130 131].

Somatic hypermutations in variable region of immunoglobulin heavy chain

Further diversification and specificity of an antibody is achieved by somatic hypermutation where point mutations are introduced into the V-region sequences [132 123 133]. This process specifically affects the rearranged V-region of IgH and IgL after

interaction of B cell with antigen [134] [135]. In vitro studies showes that somatic hypermutation process can be induced in naïve B cells after cross-linking surface immunoglobulin and co-culture with activated T cells [136]. The evidence from a mouse animal model indicates that the hypermutation process is activated only in the germinal centers [137]. However, genetically manipulated mice (lymphotoxin-α knock-outs) that are unable to form germinal centers, are still capable of activating the hypermutational process, proving that the presence of a germinal center might not be mandatory for hypermutations to occur [138]. Mutations often cluster around the regions that encode CDR domains and change the amino acid composition, and thus the conformation and affinity of immunoglobulin. The accumulation of mutations to the CDR's is indicative of positive antigenic selection and passage through the germinal center. In MM, analysis of mutations in over 100 sequenced Vh genes showed the mutations clustering in the CDR regions, which implied a previous response to antigenic pressure [95] [97] [139].

Class switching

The class of an antibody and therefore its biological function depends on the constant region of the IgH. Because the V region is retained, the specificity of the antibody remains the same but the effector function changes. A non-random process of class switching mediates the change in the isotype of the antibody. One of the chromosomal DNA segments encoding constant chains is joined to the IgH V-region while the region between V and C genes is deleted as a "switch circle" (Fig.1.3) [140] [141]. The B cell can switch the Ig isotype upon antigen stimulation in the presence of T helper cells [142] but also through stimulation *via* CD40 and cytokine receptors [143] [144]. Cytokines have a

pivotal role in selecting the isotype class to be expressed, the best example being interleukin-4 and interleukin-13 that mediate expression of IgE [145] [146] [147] [148]. Class switching is a process separate from that of somatic hypermutations but both usually occur at the same time. The autonomy of these two processes was demonstrated by stimulation of naïve IgM+IgD+ B cells *via* CD40/CD40L in the presence of cytokines. This resulted in isotype switching while the Vh was still in the germline conformation [100] [101].

Based on the advancement of chromosomal rearrangements and somatic hypermutations V-regions can be an indicator of B cell maturation and clonality [149].

The development of normal B cells

All human lymphocytes are derived from a hematopoietic progenitor cell. Born in the bone marrow, they undergo a series of selective events committing them to become either B or T lymphocytes. B-lymphocytes took their name from Bursa of Fabricius - an anatomical organ found in birds where the B cells mature [150] [151]. In humans, B lymphocytes develop in the bone marrow [152]. The B lymphocyte is responsible for recognition, binding *via* B cell receptor (BCR), and production of antibodies against an antigen. The BCR is an antibody of the same class and specificity as the secreted one but it is bound to the cell surface and associates with additional molecules mediating signaling [153].

Maturation in the bone marrow

B cell maturation is reflected by the chromosomal changes in the Ig locus [118,154]. Lymphocyte becomes committed to a B cell lineage after the primary Dh to Jh rearrangement takes place (Pro-B cell). Once the entire IgH locus is rearranged, the lymphocyte is called a Pre-B cell [155]. The heavy chain is expressed as a protein on a cell surface at first with a surrogate light chain and later with the rearranged light chain [156]. Immature B lymphocytes that have completed rearrangement of both light and heavy chains, express surface IgM isotype, and migrate from the bone marrow to the circulation and the secondary lymphoid organs [157].

Maturation and selection in secondary lymphoid organs

Somatic hypermutation and isotype class switching are events that happen upon antigenic stimulation [155,158]. Naive B cells migrate to germinal centers (GC) in the secondary lymphoid organs (lymph nodes, spleen, Peyer's patches) where they encounter an antigen [159,160]. Studies in mice facilitated the understanding of how GC are formed [161]. GC's are active structures created in response to infection or immunization. They are composed of B cells, follicular dendritic cells (FDC) and T cells distributed in light and dark zones [160]. GC provides a microenvironment within which affinity maturation of the humoral response takes place [134]. During affinity maturation, B cells proliferate in the dark zone of GC and accumulate somatic hypermutation in the chromosomal DNA encoding Ig V-region. Subsequently, B cells are selected in the GC light and dark zones for an increased affinity for antigen [161]. As a result of affinity maturation, B cells produce an antibody that

has better "fit" with an antigen. The isotype switching takes place after selection, upon activation *via* CD40 and in the presence of GC T-cell-derived cytokines [100] [101].

Plasma cells and memory B cells

B cells that had successfully completed maturation in GC's become committed to one of two populations. Some of the cells go on to become PC's that secrete antibodies. These cells are short-lived, with a large cytoplasmic compartment. PC require continuous stimulation via adhesion molecules expressed on stromal cells to survive [162]. Other B cells become non-secretory, long-lived memory cells that makeup about 0.1% of circulating B cells [163] [157]. The mechanism of these differentiation pathways is not fully understood [164].

Immunoglobulin structure

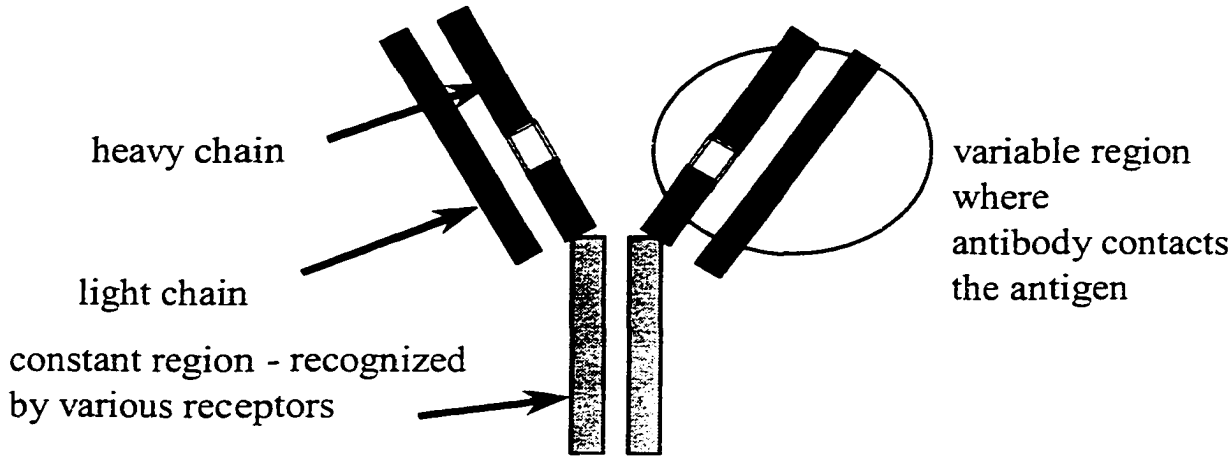


Figure 1.1 Immunoglobulin composition

Two identical heavy and two identical light chains are bound together by sulfate bridges. The constant region mediates the effector function of Ab, the variable region is specific for an antigen.

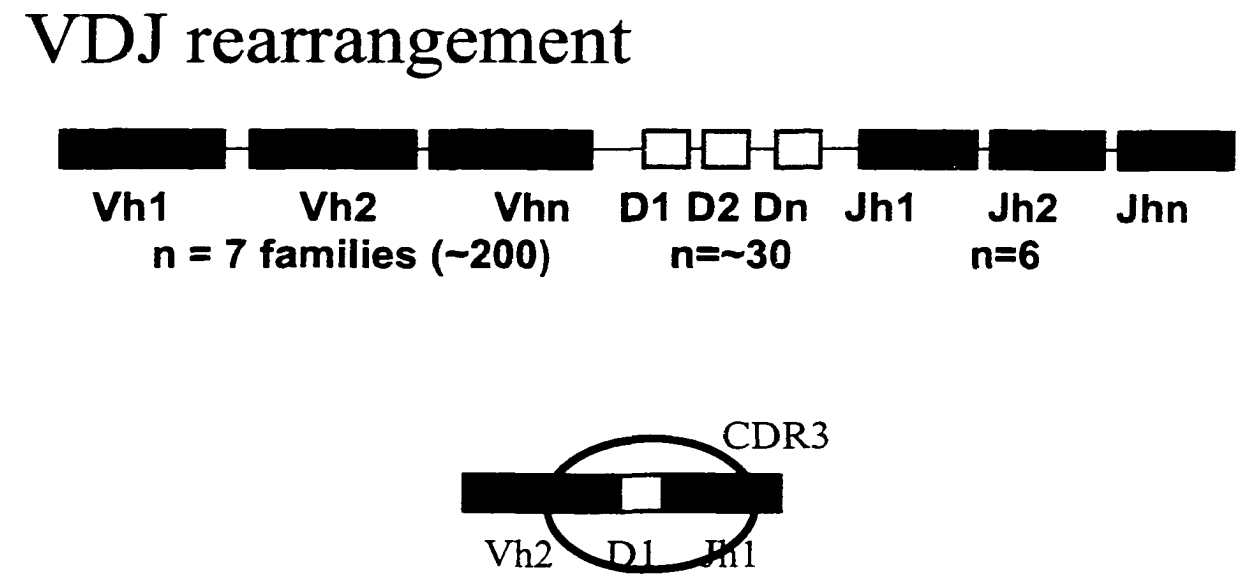


Figure 1.2

Rearrangement of the Vh, D and Jh segments cerates a DNA sequence unique for each B cell.

The VhDJh rearrangement happens sequentially: the first step is joining random Dh with Jh, the next step is joining one of the Vh to the DhJh tandem. The fragments of DNA in between are excised.

Isotype class switch

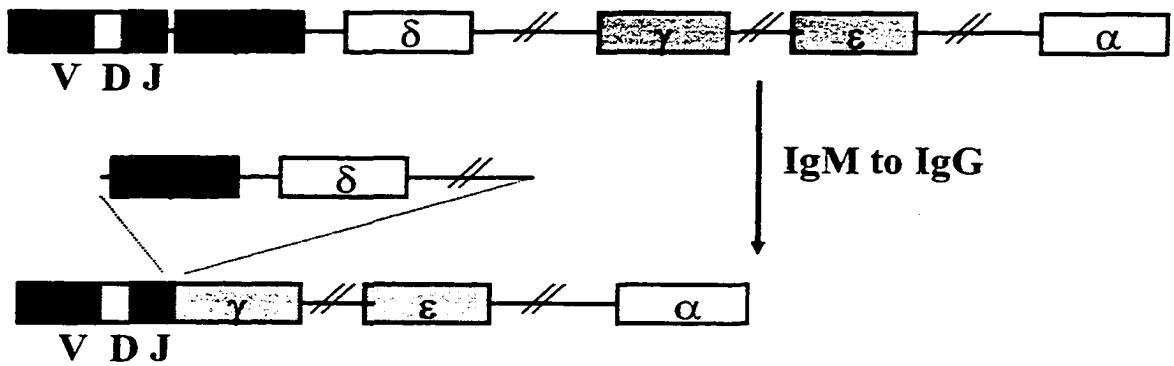


Figure 1.3

Isotype switching

The class switching brings VhDJh to proximity with the gene segment encoding specific constant chain while excising chromosomal DNA in between (looping-out).

B cell maturation

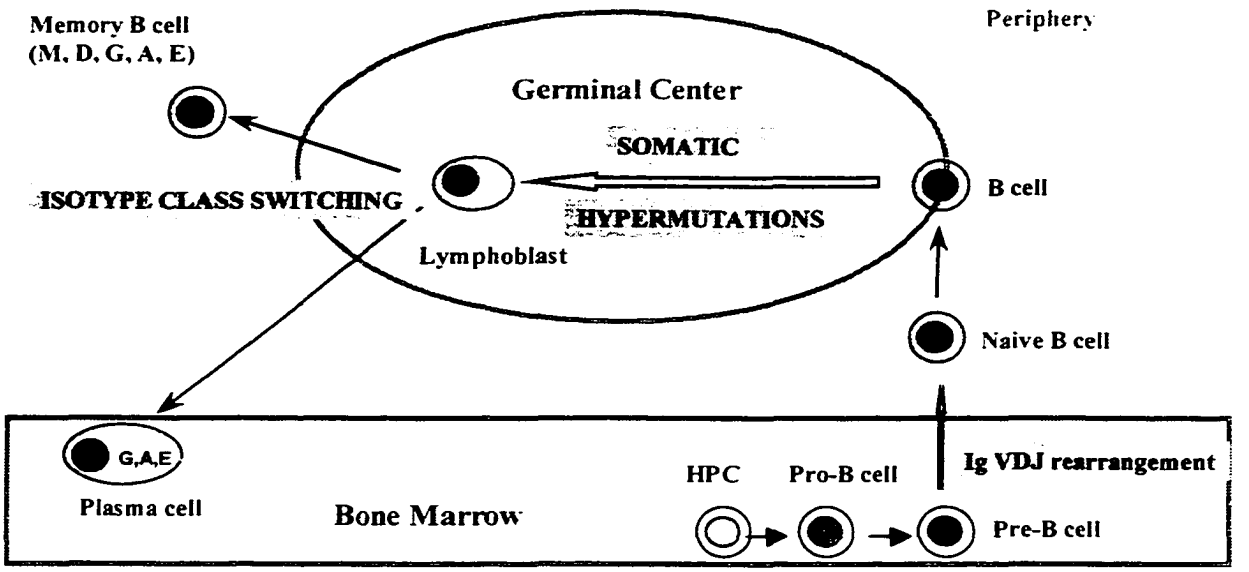


Figure 1.4

B cell maturation model

Three important events in B cell maturation are highlighted: VhDJh rearrangement in the bone marrow and the somatic hypermutations and isotype switching in the germinal centers.

HPC= hematopoetic progenitor cell

II. OVERALL OBJECTIVE, HYPOTHESES, APPROACH AND AIMS

A. Overall objective

The overall objective was to detect and characterize the B cells in various stages of development that express the clonal immunoglobulin transcripts within the MM clone.

B. Hypotheses

The circulating MM clone includes clonotypic cells of B lineage at different maturation stages. Clonotypic. isotype-distinct cells persist throughout duration of disease.

C. Overall approach

We used the clonal immunoglobulin transcript to track all the cells clonally related to MM in blood and BM of patients. The heavy chain variable region is assembled at the very early, pre-B cell stage. Although it is a target for somatic hypermutations, the basic rearrangement structure remains unchanged. Each B cell contains a single copy of the Ig gene that when transcribed generates many copies of the transcript making the mRNA an accessible target for molecular typing. If the B cells have rearranged both Ig alleles but only one rearrangement was successful, the investigator needs to distinguish between the productive and non-productive alleles when working with DNA. Because the allelic exclusion process inhibits transcription of the non-productive allele, the mRNA encoding immunoglobulin within that cell will be homogenous.

Multiple myeloma is incurable. Treatment targets the bone marrow PC; but does not eradicate the disease. Multiple lines of evidence suggested that B cells clonally related to PC have the regenerative and spreading potential. The B cell population in MM is heterogeneous and includes normal, polyclonal and abnormal monoclonal lymphocytes. The clonotypic B cells are at different stages of differentiation [74] and it is not known if all members of the clone are neoplastically transformed.

Research described in this dissertation was stimulated by conflicting evidence about the numbers and characteristics of circulating clonotypic B cells in MM. The lack of direct quantitation techniques prompted the development of a reliable strategy to derive the Ig sequence from clonotypic PC and to quantify clonotypic cells based on their surface marker expression in combination with molecular detection of clonotypic IgH VhDJh transcripts.

Moreover, we tested for the presence of heterogeneous cells within the MM clone in PBMC that were freshly isolated from blood, BM and from the G-CSF-MPB used for autologous transplantation. We also conducted long term observations to detect the pre- and post-switch clonotypic B cells. These findings were analyzed and linked with the response of the patients to treatment.

D. Specific Aims

1. To identify the sequence of the expressed allele for the IgH variable region from patients, design patient/tumor-specific primers, and check their specificity.

2. To develop sensitive methods based on single cell analysis to quantitate circulating clonotypic cells.

3. To analyze the presence of clonotypic pre- and post-switch isotypes at diagnosis.

4. To analyze possible intraclonal diversity within the clonotypic pre- and post-switch cells.

5. To analyze the expression of clonotypic pre- and post-switch cells in relation to the clinical symptoms.

III. MATERIALS AND METHODS

A. Patients

Blood and BM were obtained from 53 MM patients and 30 healthy individuals (List of patients below). Blood samples were obtained during routine visits. BM samples were taken at diagnosis, during relapse and prior to the hematopoietic progenitor cell transplantation.

PATIENT CODE	MM Ig isotype
ACZ	IgA
AKU	IgA
ASZ	IgG
AUM	IgG
BBE	IgG
CWH	IgG
DAL	IgG
DOR	IgG
EKA	IgA
ELF	IgG
ERS	IgG
EVP	IgG
FRE	IgG
GEH	IgA
GEL	IgG
GEO	IgA/IgG
GEV	Light chain MM
GLM	IgG
GML	IgG
GPO	IgG
HSP	IgG
IAP	IgG
IRS	IgG
JAB	IgA
JHP	IgA
JHW	IgG
JLE	IgD
JPL	IgG
JOD	IgG

PATIENT CODE	MM Ig isotype
JOK	IgG
JUW	IgA
KAI	IgG
KFE	IgG
LAB	IgG
LAL	IgA
LAR	IgA
LWA	IgG
MCO	IgG
MFO	IgA
MKA	IgG
MRM	IgG
NIS	IgG
OBO	IgG
ORS	IgA
PAC	IgG
PEB	IgG
PEF	IgA
PHM	IgA
RAM	IgG
REH	IgG
ROT	Light chain MM
STH	IgA
STK	IgG

B. Molecular biology techniques

Total RNA purification

To isolate RNA from cells we used TRIZOL reagent (GIBCO-BRL) containing guanidine thiocyanate and phenol. Cells (10^6-10^7) were lysed in 1 ml of TRIZOL at room temperature. Next, 0.2 ml of chloroform was added, samples were shaken for 20 seconds and centrifuged for 30 minutes at 12,000g. The aqueous phase was collected into fresh tube and RNA was precipitated with 0.6 ml of isopropanol at room temperature for 10 minutes, followed by centrifugation for 30 minutes at 12,000g. The RNA pellet was washed twice with 70% ethyl alcohol dried and resuspended in ultrapure water (SIGMA). To facilitate that process, samples were heated for 10 minutes at 70°C in a water bath. Next, an aliquot was taken to measure optical density at 260-nm wavelength and to calculate the RNA concentration.

First strand cDNA synthesis

cDNA was synthesized by priming 1 μg of total RNA with 100ng universal primer oligo-dT_{15} in ultrapure water (SIGMA, Mississauga, Ontario) at 70°C for 10 minutes. Next, 5x First Strand Buffer (Gibco BRL), 0.1M DTT, 0.25mM dNTPs and 200U Superscript reverse transcriptase were added and the reaction was conducted for 1 hr at 42 °C and terminated at 99 °C for 3 minutes.

Polymerase chain reaction (PCR)

Each PCR reaction was performed with equivalent amounts of cDNA in a 50 µl volume containing Ultrapure water (SIGMA), 10x PCR buffer, 200 µM of each deoxynucleotide (dNTP), 2mM $MgCl_2$, 10 µM of each primer and 1 unit/reaction tube of TAQ polymerase (GIBCO/BRL).

In the studies of intraclonal diversity high fidelity (HiFi) Platinum TAQ polymerase (GIBCO/BRL) was used to avoid PCR-generated mutations and mispriming.

For the generation of the patient-specific IgH sequence, 30 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 45 seconds at 72°C followed by 25 cycles with nesting primers under the same cycling conditions were performed on the PCR Thermal Cycler Perkin Elmer 9600 (Perkin Elmer ABI. Mississauga, Ontario) with the set of consensus degenerate primers.

For the detection of clonal sequences in total RNA 35 cycles were used.

Primers used for PCR

Histone 5' CCACTGAACTTCTGATTCGC

Histone 3' GCGTGCTAGCTGGATGTCTT

IgH FR2 5' TATGAATTCGGAAAGGGCCTGGAGTGG

IgH JH1 3' *ACGGGATCC*ACCTGAGGAGACGGTGACC*

IgH JH2 3' *ACGGGATCC*GTGACCAGGGTNCCTTGGCCCCAG*

FR1c AGGTGCAGCTG(G/C)(A/T)G(G/C)AGTC(G/A/T)GG

Jh-consensus ACCTGAGGAGACGGTGACCAGGGT

*These primers contain restiction cloning sites (represented in italics).

Vh family-specific primers [165]

Vh1 CCTCAGTGAAGGTCTCCTGCAAGG

Vh2 TCCTGCGCTGGTGAAGCCACACA

Vh3 GAAGATCTCTCCTGTGCAGCCTCTGG

Vh4 GTGGATCCTGTCCCTCACCTGC(A/G)(T/C)TG

Vh5 GAAAAAGCCCGGGGAGTCTCTGA

Vh6 GAAGATCTCTCACTCACCTGTGCCATC

Vh leader family-specific primers:

Vh1 leader: ATGGACTGGACCTGGAG

Vh2 leader: CTTTGCTCCACGCTCCTG

Vh3 leader: GA(A/G)TT(G/T)GGGCTGAGCTGG

Vh4 leader: ATGAAACACCTGTGGTTCTTC

Vh5 leader: TGGGGTCAACCGCCATC

Constant chain primers [17]

CμA GGGTTGCCGAAGAAGCCGCCGCGGGGTGGG

CμB GACGGAATTCTCACAGGAGAC

CδA CCCAGTTATCAAGACTGCCAGGAC

CδB TGGGTGTCTGCACCCTGATAT

CγA TC(T/C)GAATTCAGGG(T/C)GCCAGGGGGAGAC

CγB GGGGAAGACCGATGGGCCCT

CαA GGGATTCGTGTAGTGCTTCACGTG

CαB GAGGCTCAGCGGGAAGACCTT

Agarose gel electrophoresis

Products of PCR were resolved on 2% agarose gels. Gels containing ethidium bromide were molded in a Kodak horizontal electrophoresis apparatus. Ten µl of PCR samples were mixed with 2 µl of loading buffer containing Ficoll 9000 and bromophenol blue and loaded onto the gel. Electrophoresis conditions were 10V/cm for 90–120 minutes. Gels were scanned with a VRC camera on UV box and images were stored as TIF files.

Single cell RT-PCR

Using the ELITE flow cytometer with an Autoclone Cell Deposition Unit (Coulter, Hiliah, FL), single cells were sorted into 0.2 ml PCR tubes containing 8 µl of RT-Lysis Buffer (SuperScript first strand buffer, 0.5% NP-40 (v/v), 0.01M DTT, 0.25mM dNTPs, 0.006mM dT16, 200U of RNAse inhibitor (Gibco BRL)). All samples were processed, sorted and frozen within 3-4 hours after being drawn. Immediately after the sort, tubes were centrifuged for 1 minute and then frozen at -80°C. After thawing, samples were placed on ice, and 2µl (100U) of reverse transcriptase SuperScript (Gibco/BRL) was added into each tube followed by incubation at 42°C for 60 minutes. The reaction was stopped by heating at 99°C for 3 minutes. All of the obtained single cell cDNA was then used in a two step nested PCR as previously described (6). Briefly, PCR mix (0.2mM dNTPs, 10mM Tris-HCL pH 8.3, 0.2µM each of sense and antisense primers, 2mM $MgCl_2$ and 2U of TAQ polymerase) up to 50µl final volume was added to tubes, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 52°C and 1 minute at 72°C. 4% (v/v) of this PCR amplified mixture were transferred into a secondary PCR mix with

2mM $MgCl_2$, FR2 and JH2 primers and cycled as previously for 35 cycles. Finally, 20% (v/v) of the product was analyzed on 2% agarose gels (Gibco/BRL).

Patient-specific amplification (PSA):

For amplification of patient-specific sequences, primers to the CDR2 and the CDR3 regions of the rearranged IgH VhDJh from individual BM plasma cells were designed and used for *in situ* RT-PCR. The size of the PCR product varied from patient to patient in a range from 120 to 180 bp. The optimal primer sequences in CDR2 and CDR3 were chosen based on computerized analysis as described below, and gave a discrete single band when mRNA from autologous BM plasma cells was analyzed. The IgH VhDJh sequence used in PSA of PBMC subsets, was confirmed to be expressed by >80% of individual BM plasma cells using single cell and *in situ* RT-PCR. For all patients, the specificity of the patient-specific amplification was confirmed by testing the primers using RNA isolated from PBMC B cells of healthy donors, $CD38^{hi}cIg^{+}$ BM plasma cells of healthy donors, and unrelated myeloma B and plasma cells as negative controls.

In situ RT-PCR:

In situ reverse transcriptase-polymerase chain reaction (RT-PCR) was used for quantitation of the expressing clonal IgH VhDJh rearrangements. PBMC and BMC from myeloma patients were stained in single or double direct immunofluorescence with mAb to CD19, or with mAb to CD38 and Ig, and fixed for 18 hours in 10% formalin/PBS prior to sorting directly onto slides using the ELITE flow cytometer with an Autoclone cell deposition unit (Coulter). Rapid processing prior to the fixation step was essential to

preserve mRNA (particularly for B cells which have fewer IgH mRNA transcripts than do plasma cells). All blood specimens were processed within 3-4 hours after being drawn. Samples were placed in three spots, at 10.000 cells per spot, on In Situ PCR glass slides (Perkin Elmer) and air-dried. Cells were permeabilized using 2 mg (5000 U) pepsin (Boehringer Mannheim, Laval, QB) per ml of 0.01N HCl for 35-45 minutes, followed by a 1 minute wash in DEPC-treated water and a 1 minute wash in 100% ethanol. Overnight digestion at 37°C with 1000U/ml of DNAseI (RNAse-free) (Boehringer Mannheim) in the In Situ PCR System (Perkin Elmer) thermal cycler, removed genomic DNA from the negative control and the test sample prior to reverse transcription. DNAseI was inactivated by a 1 minute wash in DEPC-treated water followed by a 1 minute wash in 100% ethanol. *In situ* reverse transcription was performed for 60 minutes at 37°C only for the test samples under standard conditions recommended by the manufacturer using SuperScript (Gibco-BRL), 1X SuperScript buffer (Gibco/BRL), 0.5 μM dT15 and 1μM dNTPs, and 0.01 M DTT. After washing with water and ethanol, a cycling mix containing 1X PCRII buffer, 0.01 mM DIG-11-dUTP (Boehringer Mannheim), 0.19 mM dTTP, 0.2 mM of each dATP, dCTP, dGTP, 4 mM $MgCl_2$, 20μM each of patient-specific primers and 200 U/ml of AmpliTAQ (Perkin Elmer) was applied to all 3 spots. After 25 cycles (94°C for 1 minute, 60°C for 1.5 minutes and 72°C for 1.5 minutes) slides were washed in 2XSSC for 5 minutes, blocked at 45°C in 0.2% bovine serum albumin (Sigma, Mississauga, ON) dissolved in 0.1 X SSC, equilibrated for 10 minutes in buffer 1 (0.15 M NaCl, 0.1 M TRIS HCl pH 7.5) and incubated with anti-DIG Fab conjugated with alkaline phosphatase (Boehringer Mannheim) for 30 minutes at room temperature. Excess antibody was washed out with buffer 2 (0.15 M NaCl, 0.1 M TRIS

HCl pH 9.5, 0.05 M $MgCl_2$, followed by incubation with the chromogen NBT/BCIP substrate solution (Boehringer Mannheim). Submerging slides in water stopped the reaction. After air drying, slides were mounted in CrystalMount (BIOMEDA Corp. Foster City, CA) and examined microscopically. Only those slides with acceptable positive and negative control spots were read and counted. Slides were scored visually and for approximately one third of all the slides, the reader was blinded to their identity.

Limiting dilution assay

For the limiting dilution assay (LDA) we prepared lysis buffer containing 375 µl of DEPC treated water, 200 µl of 5X 1st Strand Buffer (supplied with the SuperScript (GIBCO/BRL), 5 µl of the detergent NP-40 (SIGMA), 100 µl of 0.1 M DTT (supplied with the SuperScript), 50 µl of10 µM dT15 universal primer, 50 µl of 10 mM dNTPs and 20 µl of RNAse Inhibitor (GIBCO/BRL) up to total volume 800 µl. Cell suspension was prepared in PBS at 1, 3, 10, 30, 100, 300 and 1000 cells/1 microliter. One µl from each dilution was added into 8 µl of the lysis buffer, in triplicates. SuperScript reverse transcriptase was diluted 4 times before use with the DEPC treated water up to the concentration of 50U/µl and 1µl of the diluted enzyme was added per sample up to 10 µl final volume. Samples were centrifuged and incubated at 42°C for 1 hour. Heating at 99°C for 3 minutes stopped the reaction. The entire sample was amplified in PCR reaction. Ten microliters of PCR product from each triplicate were analyzed by 2% agarose gel electrophoresis. The positive samples were scored as containing at least one clonotypic cell.

Cloning

Cloning of PCR products was performed with TA TOPO cloning kit (Invitrogen, Carlsbad, CA). Two μl of PCR product was mixed with 2 μl of ultrapure water (SIGMA) and 1 μl of TA TOPO cloning vector and incubated for 5 minutes at room temperature. Placing samples on ice stopped the reaction.

Transformation of competent bacteria

Bacteria used for transformation were part of the TA TOPO cloning kit. After thawing on ice bacteria were mixed with plasmid and incubated on ice for 30 minutes followed by heat shock at 42°C for 30 sec. Transformed bacteria were spread on LB agar plates containing 50 μg of amplicillin/ml for selection and X-gal for differentiation. Only the bacteria that contained plasmid encoding the ampilicillin resistancy gene could grow on the plates. Single white colonies were selected for screening by sequencing.

Sequencing

Sequencing was done with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. DNA samples were purified from 40 μl of PCR reaction by centrifuging in Millipore spin columns at 5000 rpm for 20 minutes and resuspending them in Ultrapure water (Sigma). One microliter of DNA was mixed with 4 μl of the sequencing mix, 3 μl of Ultrapure water and 2 μl of T7 sequencing primer (3.2 pM/μl). Twenty-five PCR cycles were used at following conditions: 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. The sequencing product was purified by precipitation with 25μl of 95% ethyl alcohol and 1 μl of 3M sodium acetate on ice for 10 minutes, centrifugation at 12,000 g

for 30 minutes and two washes with 200 µl of 75% ethyl alcohol. Next, samples were dried briefly in a Speedvac at room temperature, resuspended in 15 µl of the template suppression reagent (TSR, Perkin Elmer). After denaturation at 95°C for 3 minutes followed by brief vortexing, incubation on ice for 10 minutes and brief centrifugation samples were transferred into ABI310 analysis tubes and placed in the ABI310 capillary sequencer (Perkin Elmer).

Analysis of clonotypic IgH sequences and primer design

The obtained IgH sequences were analyzed using GCG version 8.1 Wisconsin Sequence Analysis Package (GCG, Madison, WI). Sequences were arranged under stringent conditions to generate high-homology alignment. This approach usually resulted in a majority of, or even all sequences identified as homologous and few that were polyclonal. The consensus sequence that was generated from the over-represented subclones was then analyzed using the V-base sequence directory (http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html) and DNAPLOT engine (http://www.mrc-cpe.cam.ac.uk/imt-doc/DNAsearch.html). The V-base identifies Vh, D and Jh segments in V-region of the antibody, aligns the sequence against the closest representative of germline family and highlights the hypermutations (Figure 3.1). The hypermutated sites are important in the primer design because they will provide specificity during annealing step in PCR. Primers were designed either manually or by using the WWW-based program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Oligonucleotides were synthesized on ABI PCR mate 391 (Perkin Elmer ABI, Mississauga, ON).

C. Immunological techniques

Purification of mononuclear cells from blood and bone marrow

Blood and bone marrow were obtained after informed consent. Bone marrow aspirates contained 11-90% PC as identified morphologically and phenotypically. Bone marrow cells (BMC) were purified using Ficoll Paque (Pharmacia, Dorval QB). Peripheral blood was drawn into heparinized tubes, diluted 1:1 (v/v) with PBS and layered over Ficoll Paque. The peripheral blood mononuclear cells (PBMC) were collected from the upper fraction of Ficoll, washed twice with PBS and counted. All samples were purified immediately after being drawn, and were stained and fixed within 3-4 hours after collection, to preserve mRNA.

Antibodies and Reagents:

FMC63 (CD19) was conjugated to FITC. Leu17-PE (CD38) was from Becton Dickinson (San Jose, CA). Ig2aPE, anti-human Ig $F(ab)_2$ fragments coupled to FITC and $F(ab)_2$ fragments of goat-anti-mouse PE were purchased from Southern Biotech (Birmingham, AL).

Immunofluorescence (IF) and Sorting:

Staining for surface phenotype utilized one or two color IF with CD19-FITC or CD38-PE/anti-HumanIg-FITC. All experiments included controls with isotype matched monoclonal antibodies (mAbs). PBMC and BMC subsets of PBMC were sorted using the ELITE (Coulter, Hialeah, FL). BMC were stained with CD38-PE and anti-human Ig-

FITC followed by sorting of $CD38^{hi}$ Ig^{+} BMC with high forward and side scatter. PBMC were sorted for $CD19^{+}$ cells with no gates set on scatter beyond those used to exclude red and dead cells. Sort gates were set to include only those cells with staining brighter than the relevant isotype controls, as previously described (6). For single cell experiments, individual $CD19^{+}$ PBMC or $CD38^{hi}Ig^{+}$ large BMC were sorted directly into 0.2 ml thin walled PCR tubes or onto slides. On reanalysis, sorted populations had a purity of 96% or greater for the defining phenotype. PBMC had no detectable contamination with any peripheral plasma cells as defined by their relatively low cIg content (6). Less than 1% of morphologically identifiable plasma cells were observed in cytospins of sorted subsets, in cytospins of PBMC or in smears of patient blood. To avoid any contamination between samples, and in particular to avoid contamination of blood cells with BM plasma cells, blood samples were always sorted prior to the bone marrow samples, and tubing in the flow cytometer was always washed with bleach between sorts.

Figure 3.1 Alignment of the IgH V-region sequence against the closest germline sequences for one representative patient (REH) using the V-base program

```
      1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16
REH   TAG GTG CAG CTG GTG GAG TCT GGG GCT GAG GTA CAG AAG CCT GGT TCC
DP-10 C-- --- --- --- --- C-- --- --- --- --- --G A-- --- --- --G --- -
2M27/ C-- --C --- --- --- C-- --- --- --- --- --G A-- --- --- --G --- -
DP-88 C-- --- --- --- --- C-- --- --- --- --- --G A-- --- --- --G --- -
VHGL1 C-- --C --- --- --- C-- --- --- --- --- --G A-- --- --- --G --- -
RR.VH1C-- --C --- --- --- C-- --- --- --- --- --G A-- --- --- --G --- -

17  18  19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34
TCG GTG AAG GTC TCC TGC AAG ACT TCT GAA GAC ACC TTC AGT GAC TAT ACT ATC
--- --- --- --- --- --- --- G-- --- -G- -G- --- --- --C AG- --- G-- ---
--- --- --- --- --- --- --- G-- --- -G- -G- --- --- --C AG- --- G-- ---
--- --- --- --- --- --- --- G-- --- -G- -G- --- --- --C AG- --- G-- ---
--A --- --- --- --- --- --- G-- --- -G- -G- --- --- --C AG- --- G-- ---
--- --- --- --- --- --- --- G-- --- -G- -G- --- --- --C AG- --- G-- ---

35  35a 35b 36  37  38  39  40  41  42  43  44  45  46  47  48  49  50
AGT ... ... TGG CTG CGA CAG GCC CCT GGA CAA GGT CTT GAG TGG TTG GGA GGC
--C ... ... --- G-- --- --- --- --- --- --- --G --- --- --- A-- --- --G
--C ... ... --- G-- --- --- --- --- --- --- --G --- --- --- A-- --- --G
--C ... ... --- G-- --- --- --- --- --- --- --G --- --- --- A-- --- --G
--C ... ... --- G-- --- --- --- --- --- --- --G --- --- --- A-- --- --G
--C ... ... --- G-- --- --- --- --- --- --- --G --- --- --- A-- --- --G

51  52  52a 52b 52c 53  54  55  56  57  58  59  60  61  62  63  64  65
ATC ATC CCT ... ... CGC TTT GGT ACA GCG AAC TAC GCA CAG AAG TTC CCG GGC
--- --- --- ... ... AT- --- --- --- --A --- --- --- --- --- --- -A- ---
--- --- --- ... ... AT- --- --- --- --A --- --- --- --- --- --- -A- ---
--- --- --- ... ... AT- --- --- --- --A --- --- --- --- --- --- -A- ---
--- --- --- ... ... AT- --- --- --- --A --- --- --- --- --- --- -A- ---
--- --- --- ... ... AT- --- --- --- --A --- --- --- --- --- --- -A- ---

66  67  68  69  70  71  72  73  74  75  76  77  78  79  80  81  82  82a
AGA ATC GCT CTT ACT GCG GAC GAA CCG ACA AAC ACA GTC TAC ATG GAG CTG CGC
--- G-- A-G A-- --C --- --- --- T-C --G -G- --- -C- --- --- --- --- A--
--- G-- A-G A-- --C --- --- --- T-C --G -G- --- -C- --- --- --- --- A--
--- G-- A-G A-- --C --- --- A-- T-C --G -G- --- -C- --- --- --- --- A--
--- G-- A-G A-- --C --- --- --- T-C --G -G- --- -C- --- --- --- --- A--
--- G-- A-G A-- --C A-- --- --- T-C --G -G- --- -C- --- --- --- --- A--

82b 82c 83  84  85  86  87  88  89  90  91  92  93  94
GAC CTC AGA TCT GAA GAC ACG GCC GTG TTT TAC TGT GCG AGA
AG- --G --- --- --G --- --- --- --- -A- --- --- --- ---
AG- --G --- --- --G --- --- --- --- -A- --- --- --- ---
AG- --G --- --- --G --- --- --- --- -A- --- --- --- ---
AG- --G --- --- --G --- --- --- --- -A- --- --- --- ---
AG- --G --- --- --G --- --- --- --- -A- --- --- --- ---
```

```
REH score   TGTGCGAGAGCCCGCTATATGGGTGGGACTGGGGGCTTCTACGACTACTG
DK1 34      _____________________....ATA.A.T....A.G.TT..
DA4 27      _____________________________________T......A.TAACTA
DA1 27      _____________________________________T......A.TAACTAC

The D segment D21-10 is used in reading frame 2

                                                 <----------

REH score TGTGCGAGAGCCCGCTATATGGGTGGGACTGGGGGCTTCTACGACTACTGGGGCCAGGGAA
JH4b 186  _____________________________________A..A..TT...................
JH4d 177  _____________________________________...A..TT..............A..G.
JH4a 177  _____________________________________A..A..TT..............A....


CCACGGTCACCGTCTCCTCAGG
..CT..................
..CT..................
..CT..................

      01234567890123456789012345678 90
REH   CARARYMGGTGGFYDYWGQGTTVTVSSGKPN
In frame rearrangement
```

Alignment of consensus MM IgH sequence for patient REH against germline sequences Assigned are the Vh. D and Jh segments used by MM plasma cells; the hypermutations are identified. The amino acid sequence of the joining between Vh D and Jh was deduced to ensure that the message is kept in frame. Highlighted are parts of the sequence used for the primer design. Arrows indicate the direction of PCR primers.

IV. ESTABLISHING THE PATIENT-SPECIFIC DNA SEQUENCES FOR THE CLONAL IMMUNOGLOBULIN.

(The majority of the data presented in this chapter have been published in an article entitled "A high frequency of circulating B cells share clonotypic IgH VhDJh rearrangements with autologous bone marrow plasma cells in multiple myeloma, as measured by single cell and in situ RT-PCR" [166]

A. Introduction

The rearranged immunoglobulin gene is a footprint for each B cell or its clonal progeny. Since the malignant cells in MM represent the B cell lineage, they can be characterized based on immunoglobulin gene rearrangement. All MM cells have an identical VhDJh as opposed to the normal, polyclonal B cells that use a highly diversified repertoire of VhDJh.

The clonotypic properties of MM IgH have been used in diagnostics and research. At first, to detect the clonal rearrangements, consensus radioactive probes were used together with the Southern blot [87]. The detection means changed with the development of sequencing, molecular cloning, PCR and synthetic oligonucleotides technologies [86,88]. To derive a clonotypic sequence for MM clone, the consensus primers annealing to Vh and Jh portions of the V-region were used in PCR. The DNA was purified from BM cells and used as a template for PCR. The success rate in characterization of MM immunoglobulin rearrangement by PCR was only 40-50%, and sometimes 70%, as described in literature

[165] [88]. Using a variety of the consensus primers can increase the rate of Ig identification [165].

We reasoned that the most accurate method for deriving the tumor-specific Ig sequences would be based on an analysis Ig transcripts in single cells. The degenerate consensus primers for IgH used in PCR with the heterogeneous templates, preferentially anneal to the template with which they are most homologous and not necessarily to the one that is overrepresented. The MM bone marrow contains not only MM PC but also normal B lineage cells that may do belong to the malignant clone. Total RNA isolated from such a heterogeneous population will therefore contain polyclonal Ig transcripts. Using the RNA derived from a single cell limits the type of Ig templates to one. That is why our method of choice for determining the genetic sequence of clonal Ig transcripts became single cell sorting combined with direct cell lysis RT-PCR. Sometimes, however, in the absence of other starting material, manual dilutions of BM cells were used or total RNA was isolated from cells immediately lysed in TRIZOL after purification on Ficoll and stored at –80C.

Moreover, our strategy in deriving patient-specific IgH sequence included a procedure to check the accuracy of the sequence identified. Previously published reports did not include these confirmation steps. The authors assumed that VhDJh IgH sequence belongs to the malignant clone based only on numbers of homologous clones derived from consensus PCR [88] [97]. Many cells of B lineage are present in the bone marrow and consequently the RNA isolated from BM contains heterogeneous population of Ig transcripts. Consensus primers anneal to the most compatible Ig cDNA, not necessarily to

the MM Ig. During PCR, this non-MM sequence can be selected and amplified generating many identical products. It is imperative to confirm the identity of the derived sequence as clonotypic because if the non-MM sequence will be used to design and synthesize primers, it will result in false-negative data. Here, we apply a multi-step confirmation strategy to ensure that Ig sequence was derived from clonal malignant MM PC.

At the time when this project was undertaken, there was no report in the literature about the percentage of malignant PC in BM as measured by the expression of clonotypic Ig. Chen and Epstein used a semi-quantitative assay to estimate the numbers of clonal PC. The results showed lack of significant difference between the unpurified BM containing 40% PC and highly purified PC [92]. This implied that the semi-quantitative approach is not suitable for enumeration of clonotypic cells. In a diagnostic laboratory, quantitation of malignant PC is routinely done by the histochemical staining of a BM aspirate and counting cells of the appropriate morphology (large nucleoli, irregular nuclear configuration, variation in cell size) [3]. Sometimes, immunohistochemical staining with anti-light chain antibodies is performed. Nevertheless, no patient-specific marker is used and only small numbers of cells are analyzed. Here, we have combined surface marker staining for CD38 and Ig with the molecular analysis of each single PC. Our method enables to identify and enumerate the clonotypic PC. It also allows the investigation to check for the accuracy of primers and to count the numbers of clonotypic PC present in the bone marrow.

B. Results

Processing time affects the integrity of Ig mRNA

We have tested if the sample processing time affects the results of RT-PCR. A blood sample was obtained from a patient. One aliquot was processed immediately by purification on Ficoll and dissolving PBMC in TRIZOL. Other samples were processed in several time intervals. After RNA isolation, RT-PCR with the housekeeping gene primers and with Ig consensus primers were performed. We showed that when blood is left for more than 4 hours there is a significant decrement in a PCR signal, most likely due to decomposition of mRNA by an endogenous ribonuclease. Others in our laboratory confirmed this observation (Dr.B.Taylor, personal communication).

Single plasma cell sorting combined with RT-PCR yields tumor-specific immunoglobulin sequences.

Figure 4.1 shows the typical FACSCAN profile of BM cells. The sorter was set up to select for cells that are characteristic of PC (CD38 positive, weakly Ig positive, large and granular) and these cells were individually sorted into PCR vials. Subsequently, the samples were lysed, and subjected to nested RT-PCR with the consensus IgH primers. Figure 4.2 shows agarose gel electrophoresis visualizing the final product of representative RT-PCR from single PC's. The expected size of amplicons was about 250 bp, depending on the Vh family usage. The PCR product was cloned directly into TA vector. Based on β-galactosidase activity, the white transformants were selected for T7 sequencing. At least 24 colonies were analyzed for each patient. Sequencing data was

analyzed by BLAST and V-base alignments. The most represented Ig sequence was chosen. Primers were designed based on CDR2 and CDR3 regions, preferably ending on the hypermutated portion. During the course of this study, primers for 53 patients were successfully designed (Table 4.1)

Confirmation of primer specificity

– Single plasma cell RT-PCR with patient-specific primers

To confirm the specificity of primers they were used in single cell PCR with the product from the first-stage, consensus RT-PCR. Product of the anticipated size (between 170-190 bp) was analyzed by agarose gel electrophoresis. Figure 4.3 shows a scan of a representative gel. The patient-specific product is expressed by majority of plasma cells. However, the number of single cells that can be tested this way is limited. To analyze greater numbers of plasma cells, *in situ* RT-PCR with sorted plasma cells was used. Figure 4.4 shows typical processed slides visualizing cells that expressed clonotypic VhDJh transcripts. On average, 84% of the viable plasma cells were positive (Table 4.2).

- Patient-specific primers used in RT-PCR generate product only from the respective patient's samples but not from the controls.

To establish that the primers will only anneal to the patient specific target and not to other VhDJh cDNA, total RNA isolated from BM of different MM patients was subjected to RT-PCR. Amplification with the CDR2/CDR3-based primers generated a product only if RNA from respective patient was used but not from an unrelated MM patient (Figure 4.5). In a few cases, if non-specific amplification was observed, a new set of primers was

designed and tested as described above. Similar confirmation was done on cells isolated from peripheral blood.

Instances of misidentification of Ig transcript for a clonotypic one

Several problems were encountered in deriving clonotypic Ig sequences in cases when only total RNA was available for starting material. RT-PCR often generated products that contained several different Ig transcripts without predominant species. To identify the clonal transcript, we sequenced several additional clones. However, primers designed based on the most frequent homologous sequences often did not generate a product in PCR amplification. Alternatively, very weak PCR product from BM but not from blood could be observed on agarose gel. In both cases, another frequent sequence was selected and new set of primers was designed. Occasionally this procedure had to be repeated several times. In one case (JOD), it took over a year to identify the clonotypic Ig. Several sequential RNA samples isolated from blood and BM were subjected to RT-PCR, cloning and sequencing. One infrequent, but persistent transcript was identified in all samples. Based on a sequence of this transcript, a set of patient-specific primers was designed. Subsequent *in situ* RT-PCR on sorted PC from BM obtained during relapse confirmed clonality of this sequence.

In summary, deriving the clonotypic Ig sequence from total RNA has proven to be difficult and labor- and time-intensive. Quantitation of the clonotypic B cells depended on a correctly identified sequence and accurately designed patient-specific primers. Only the single cell assays (single cell RT-PCR and *in situ* RT-PCR) could validate the identity

of obtained Ig sequence as the one belonging to myeloma clone. Further studies relied on identyfying the correct Ig sequence.

Distribution of the heavy chain V families in MM matches the pattern in healthy individuals.

During the study, the clonotypic Ig sequences from 53 MM patients were analyzed for Vh family usage. The most frequently used segment was Vh3, followed by Vh1, Vh4, Vh2 and Vh5. There was no Vh6 or Vh7 segment identified within MM specimens. We compared our results to those obtained by Demaison and Brezinschek, where CD19+ B cells from healthy individuals were analyzed [167] [168]. The results showed some differences between all three groups. However, the main trend in using different Vh mini-genes was similar (Fig. 4.6)

Single case of non-secretory myeloma with major aberrations in variable region of IgH

In case of one light chain myeloma (GEV), transcripts encoding IgH were sequenced and analyzed as described above. This transcript was shorter than anticipated and was composed of three Vh segments of the same family joint directly to Jh segment without Dh present (Fig.4.7). It has also contained a stop codon that accounts for the heavy chain not being translated. However, the V-region sequence was successfully used to design patient-specific primers and to employ them in RT-PCR. The abbreviations in V-region are suggestive of an aberrant homologous recombination that took place during the B cell maturation. Under normal selective conditions, this cell should have undergone apoptosis. The fact that it survived and gave rise to myeloma suggests following possibilities:

a. The aberrant recombination took place post- negative selection process
b. The apoptotic machinery in the cell was inactive or downregulated during the selective events
c. The other, non-expressed allele might be the one that is properly rearranged but there is a transcriptional preference for the aberrant allele.

To explain this particular case, further analysis of chromosomal DNA together with research of the apoptotic mechanism would be required.

C. Discussion

This study concentrated on detection and characterization of clonotypic IgH sequence expressed by the majority of individual BM plasma cells in patients. This type of quantitation is possible only at the single cell level. Published protocols applied to quantitate BM PC did not, for instance, distinguish between 40% and 100% of plasma cell content [92] demonstrating a need for improvement of the detection methods.

Methods described in this chapter were developed to meet the need for a reliable, high fidelity strategy to identify clonotypic VhDJh rearrangements in MM cells. The previous success rate in deriving clonotypic VhDJh sequences in MM was relatively low, as described in literature (40-70%) [88,165]. We reasoned, that using multiple sets of primers [165] with excellent starting material would increase the success ratio of characterizing clonal Ig in MM. Unlike other groups, we used single plasma cells to derive the patient-specific VhDJh sequence. The presence of homogenous templates in each individual tube prevented annealing of the consensus primers to non-clonotypic cDNA. In the single cell analysis, the polyclonal B cells represented a minority against the monoclonal PC, reflecting the proportions from MM bone marrow. This method also enabled quantitation of clonotypic plasma cells in the bone marrow. Examination of IgH transcripts instead of chromosomal DNA automatically focuses on an expressed allele and provides much higher quantities of nucleic acid, an important factor in single cell analysis.

We showed that deriving the clonotypic VhDJh sequence from single sorted plasma cells is a reproducible method with a high success rate. The only drawback is that not all

analyzed cells give positive results in the amplification. This might be due to the sorting conditions in which single cell has to travel a substantial distance between the sorter and the PCR vial. For the experiment to be successful, each cell must be deposited precisely in the buffer. The buffer volume in the PCR tube is only 5 μl, and slightest imprecision in the sorting set up will result in the cell drying out on the walls of the vial. The freshness of the sample and short processing time are also important factors since both affect stability of the mRNA.

In some instances, (e.g. when there was no sorting facility available) the clonotypic Ig transcript had to be amplified from RNA isolated from unfractionated BM cells. In most of these cases, analysis of IgH transcripts showed high frequency of polyclonal Ig species. This extended the time of analysis and made it laborious and expensive, because more clones had to be sequenced. We found that using Vh-family leader-specific primers together with constant chain-specific primers and applying low numbers of PCR cycles (25-30) usually generated better results than when using consensus primers. The leader segment is conserved and unlike the rest of V-region does not accumulate somatic hypermutation, which lowers the chance of mispriming. However, at least 12 clones representing each Vh family had to be analyzed, which increased the cost and time of the experiments. When compared to single cell-based method, deriving the clonotypic Ig sequence from total RNA was less effective and increased the possibility of an error.

In two cases when only total RNA was available, we were unable to derive the clonotypic sequences. This might be due to the high rate of somatic hypermutations in the region

targeted by the consensus primer(s) used in PCR, resulting in lack of annealing to clonotypic cDNA. Another explanation might be that there were only few malignant plasma cells in a background of polyclonal B cells present in these samples. The consensus Ig primers could have greater homology with the polyclonal transcripts than with the underrepresented clonotypic template. All of these reasons emphasize the need for single cell analysis.

Immune disorders that involve autoaggression have been postulated in the etiology of MM. Vh4-34 (Vh4-21) is a segment commonly represented in the repertoire of autoreactive B cells [169.170]. Data published on the Vh repertoire in healthy individuals confirmed the absence of usage of Vh4-34 by normal, non-autoreactive blood B cells [167] [168]. It has been suggested that the MM plasma cells do not use this particular Vh mini-gene implying that MM does not originate from an autoreactive disease [171.172]. We identified Vh4-34 in one case (PEF) out of the 53 analyzed. The clinical history of this patient did not indicate previous autoimmune disorder.

Analysis of Vh-family segment usage by MM cells did not show major differences in distribution between MM patients and healthy individuals. The Vh pattern was compared to the data published by two different groups who analyzed CD19+ circulating blood cells using different approaches [168] [167]. Brezinschek et al. applied single cell sorting combined with PCR analysis, while Demaison screened a cDNA library derived from purified CD19+ B lymphocytes. We compared these Vh distribution patterns with our own results because of a lack of such analysis in the healthy mature plasma cells. In

addition, the number of patients with identified Vh family segments (53) was larger than the numbers of healthy individuals analyzed in above publications (1 and 3), but smaller that total number of analyzed clones (117), which might account for some discrepancies on the distribution pattern. However, the frequency of the use of Vh3, Vh4, Vh1 and Vh2 in their studies was similar implying that there is no selective usage of a specific Vh segment in MM.

In summary, to identify clonotypic MM VhDJh rearrangements we analysed immunoglobulin transcripts from single plasma cells. This method identifies the expressed Ig allele, enables quantitation of clonal cells and ensures the specificity of the detected transcript for clonotypic BM plasma cells.

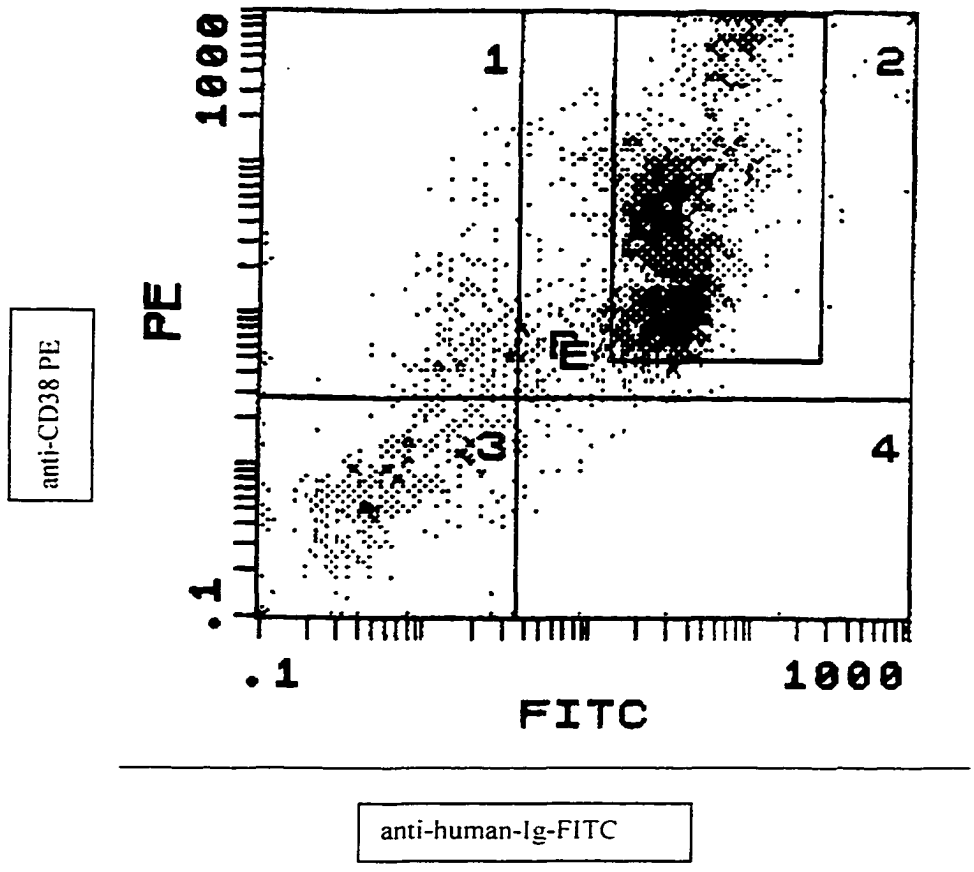


Figure 4.1 Flow cytometry profile of sorted plasma cells

Plasma cells isolated from BM of patient ORS were stained with anti-CD38PE and anti-human-IG monoclonal antibodies. Double positive cells (field E) were gated and sorted individually into PCR vials.

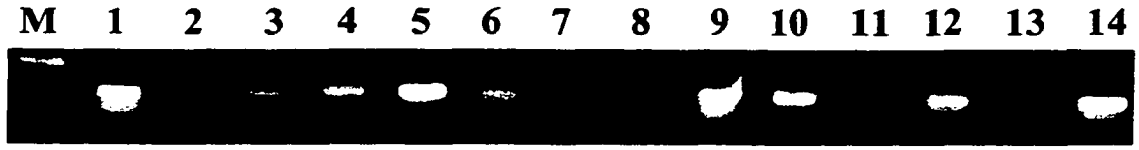


Figure 4.2 Nested RT-PCR with consensus VhDJh primers derives product from single sorted plasma cells.

Single plasma cells isolated from the BM of patient JHW were sorted into PCR vials, reverse transcribed and amplified in two rounds of PCR with consensus primers. The product was visualized on 2% agarose gel stained with ethidium bromide. M – 100 bp molecular weight marker, lines 1-14 – RT-PCR product from individual plasma cells from a representative MM patient.

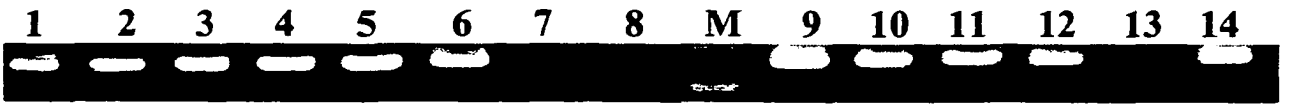


Figure 4.3 Single plasma cell RT-PCR confirms the accuracy of patient-specific primers.

Single plasma cells isolated from the BM of patient JHW were sorted into PCR vials, reverse transcribed and amplified in two rounds of PCR first with consensus and next with patient-specific primers. The product was visualized on 2% agarose gel stained with ethidium bromide. M – molecular weight marker, lines 1-14 – RT-PCR product from individual plasma cells.

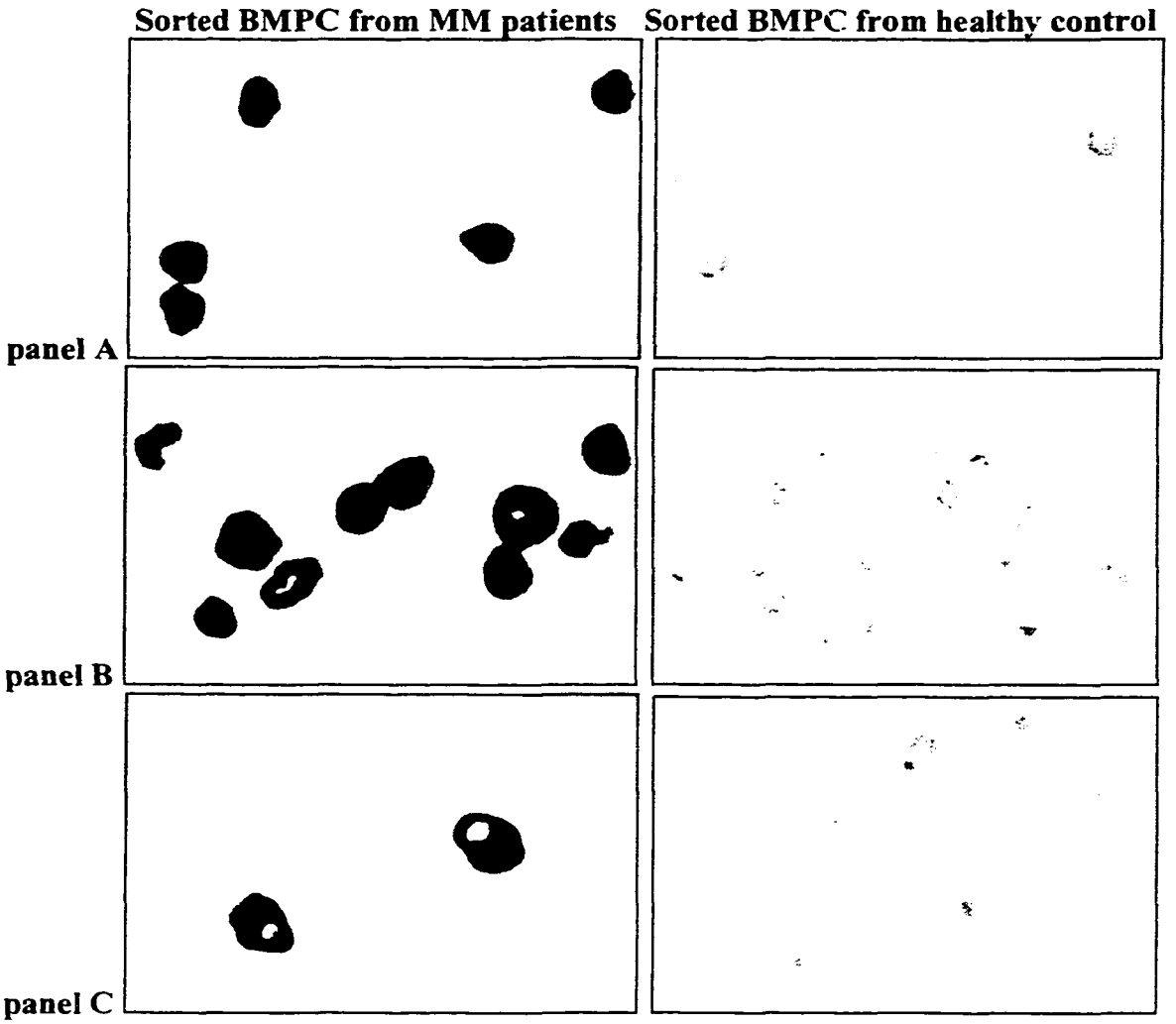


Figure 4.4 *In situ* RT-PCR with the patient-specific primers on sorted plasma cells

Panel A: Plasma cells sorted from BM of MM patient EVP and a healthy donor underwent *in situ* amplification with patient A CDR2/CDR3-IgH-specific primers.

Panel B: Plasma cells sorted from BM of MM patient CWH and a healthy donor underwent in situ amplification with patient B CDR2/CDR3-IgH-specific primers.

Panel C: Plasma cells sorted from BM of MM patient JOD and a healthy donor underwent in situ amplification with patient C CDR2/CDR3-IgH-specific primers.

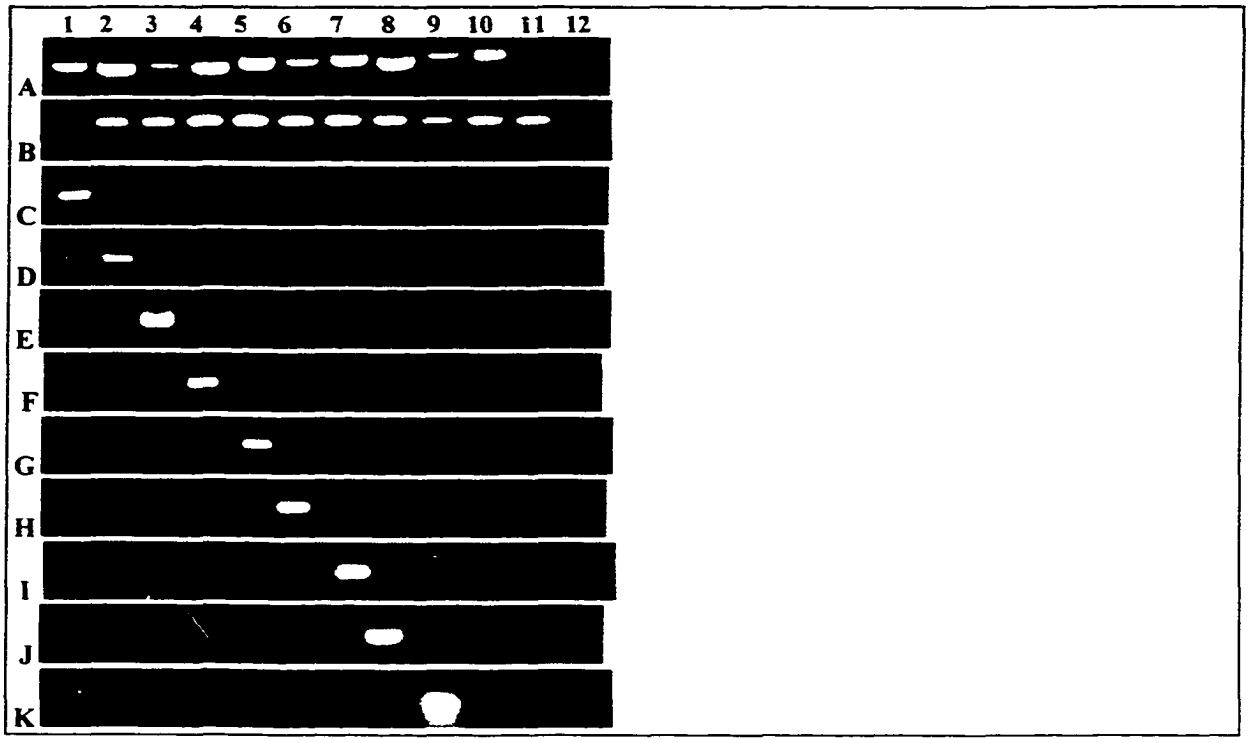


Figure 4.5 Primer specificity tested on total RNA isolated from BM of MM patients.

Clonotypic transcripts are detected only in BMC of the patient for whom the primers were generated. The letters indicate experiments where different primer sets were used; the numbers indicate samples from respective patients.

One microgram of total RNA isolated from MM BMC was reverse transcribed followed by amplification with patient-specific primers. The following primer pairs were used in PCR of reverse transcribed RNA: Row A: consensus IgH VhDJh. Row B: histone (housekeeping gene); Row C: primers for CDR2/CDR3 of patient ASZ. Row D: primers for CDR2/CDR3 of patient JHW Row E: primers for CDR2/CDR3 of patient JUW. Row F: primers for CDR2/CDR3 of patient LAR Row G: primers for CDR2/CDR3 of patient LWA. Row H: primers for CDR2/CDR3 of patient NIS Row I: primers for CDR2/CDR3 of patient PEB. Row J: primers for CDR2/CDR3 of patient PHM Row K: primers for CDR2/CDR3 of patient ROT. Column 1: ASZ, Column 2: JHW, Column 3: JUW, Column 4: LAR, Column 5: LWA, Column 6: NIS, Column 7: PEB, Column 8: PHM, Column 9: ROT, Column 10 and 11: irrelevant BM, Column 12 - water control.

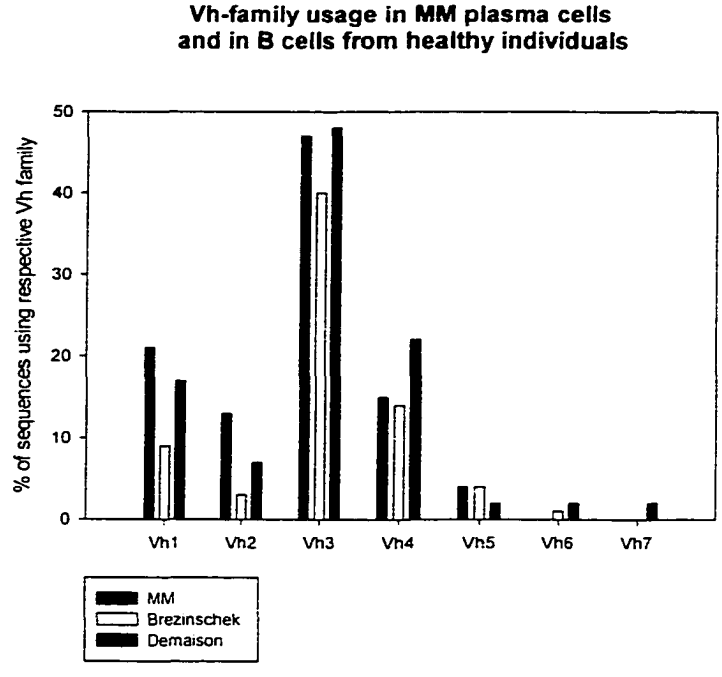


Figure 4.6 The usage of Vh family segments by clonotypic plasma cells from MM patients and by CD19+ normal B cells.

The lack of Vh6 and Vh7 segments that are very infrequently used by normal B cells might be due to number of samples analyzed (Vh7 was also absent in the study by Brezinschek et al).

Figure 4.6 The usage of Vh family segments by clonotypic plasma cells from MM patients and by CD19+ normal B cells.

The lack of Vh6 and Vh7 segments that are very infrequently used by normal B cells might be due to number of samples analyzed (Vh7 was also absent in the study by Brezinschek et al).

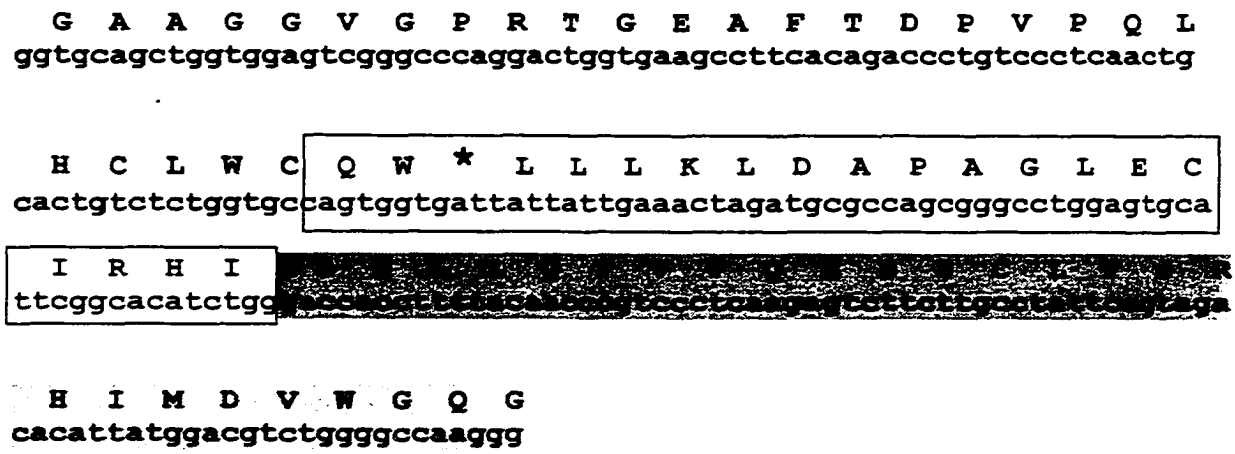


Figure 4.7 An aberrant rearrangement of GEV IgH V-J.

Shown in small letters is the cDNA sequence of IgH: shown in caps is the deduced amino acid sequence.
Fragments of three different Vh minigenes belonging to the same Vh4 family were used this aberrant heavy chain. There is no D segment; Jh is directly joined to Vh.1
The STOP codon is designated by an asterisk.

Vh germlines that have the closest homology with segments used in the IgH.

DP-65 83-6F2 DP-68 Jh6a

PATIENT CODE	Expressed IgH isotype	Vh family
ACZ	IgA	2
AKU	IgA	1
ASZ	IgG	2
AUM	IgG	5
BBE	IgG	3
CWH	IgG	5
DAL	IgG	3
DOR	IgG	3
EKA	IgA	3
ELF	IgG	4
ERS	IgG	1
EVP	IgG	3
FRE	IgG	3
GEH	IgA	3
GEL	IgG	3
GEO	IgA/IgG	4
GEV	Light chain MM	3
GLM	IgG	1
GML	IgG	4
GPO	IgG	2
HSP	IgG	4
IAP	IgG	1
IRS	IgG	3
JAB	IgA	3
JHP	IgA	3
JHW	IgG	2
JLE	IgD	4
JPL	IgG	3
JOD	IgG	2
JOK	IgG	3
JUW	IgA	3
KAI	IgG	2
KFE	IgG	4
LAB	IgG	3
LAL	IgA	1
LAR	IgA	3
LWA	IgG	1
MCO	IgG	1
MFO	IgA	3
MKA	IgG	2
MRM	IgG	1
NIS	IgG	3

OBO	IgG	3
ORS	IgA	3
PAC	IgG	3
PEB	IgG	1
PEF	IgA	4
PHM	IgA	3
RAM	IgG	3
REH	IgG	3
ROT	Light chain MM	1
STH	IgA	3
STK	IgG	4

Table 4.1 Expression of the Ig heavy chain isotype and Vh family usage in plasma cells from characterized MM patients.

Table 4.2 The percentage of CD38+ BM plasma cells with clonotypic transcript detected using *in situ* RT-PCR with patient-specific CDR2/CDR3 primers.

Patient number	% of plasma cells that were positive by *in situ* RT-
1	90.6
2	92.4
3	89.6
4	90.6
5	92.4
6	98.4
7	82.4
8	84.4
9	76.6
10	90.8
11	59.4
12	77.8
13	81.3
14	83.9
15	13.6
16	41.2
17	85.7
18	88.5
19	89.8
20	43.3
21	66.3
22	43.3
23	66.3
24	91.0
Average	*75.8*
Mean +/- SE	*84.1 +/- 4.3*

In the majority of cases over 80% of PC expressed MM clonotypic IgH. Patients #15, #16, #20 and #22 displayed unusually low numbers of clonal cells reflecting technical difficulties with the method at that particular time period. It was not always possible to perform *in situ* RT-PCR with the housekeeping gene primers to estimate viability of sorted cells. The accuracy of primer design was also confirmed using single cell sort.

V. ENUMERATION OF CIRCULATING CLONAL B CELLS

(The majority of the data presented in this chapter have been published in an article entitled "A high frequency of circulating B cells share clonotypic IgH VhDJh rearrangements with autologous bone marrow plasma cells in multiple myeloma, as measured by single cell and in situ RT-PCR" [166])

A. Introduction

MM affects multiple sites of the skeletal system. Spread of the disease from one location in bones to another must happen *via* blood. However, circulating MM plasma cells are not common until terminal stages of the disease. In addition, few MM PC proliferate [63]. In a healthy immune system, all plasma cells are derived from less differentiated B cells. Likewise in MM, malignant plasma cells develop from less mature B lymphocytes. These less differentiated clonotypic B cells have been postulated to be a precursor cell population in MM [64 173 174]. However, the quantities of myeloma-related B cells in the circulation and the role of these cells in the development of the disease remain controversial issues.

Various techniques that differed in sensitivity and specificity have been used to detect circulating B cells in MM. Initially, the clonotypic cells were detected by using idiotype-specific antibodies raised against monoclonal immunoglobulin from patients with MM [64]. These studies identified large numbers of such cells (up to 45% in B cell population). A similar approach taken by Pettersson et al. [65] detected monoclonal B-lymphocytes in blood in a range from 0.5 to 44% of all the B cells in MM.

More recently, many groups have studied clonotypic MM cells in blood by using molecular tracking of clonally rearranged DNA [91 88 18 90]. Some of these groups attempted to quantitate clonal circulating B cells [89 91 92]. Using analyses of serially diluted DNA followed by mathematical calculation of cell equivalents per DNA concentration. Although sensitive, these methods were indirect and involved numerous manipulations of cells and DNA that might have affected the outcome. For example Chen and Epstein showed no difference inbetween specimens that contained 100% and those that contained only 40% clonotypic cells [92]. The supposedly semi-quantitative, PCR-based assays gave conflicting results by estimating the number of circulating clonotypic B cells at 0.34% of all PBMC in one study [92] and from 0.001% to 32% of PBMC in another study [89].

Data obtained in Dr. Pilarski's laboratory suggested that in MM CD34+CD19+ and CD11b+CD19+ cell populations have many unusual properties suggesting that they may be part of the malignant clone [67 76 73 175 75]. CD11b+CD19+ cells in MM exhibited multidrug resistance mediated by P-glycoprotein 170 [70]. Furthermore, these cells had adhesive and motile properties that may be responsible for spread of disease [176]. The majority of CD11b+CD19+ cells overlapped with CD34+CD19+ population, as measured by surface marker expression [73]. Here, we tested these two cell subsets in a quantitative way for the expression of clonotypic IgH.

A direct approach was used to quantitate clonotypic B cells by applying three methods developed in our laboratory based on PCR using patient-specific CDR2/CDR3 primers.

The first method used single cell RT-PCR performed on sorted CD19+ MM cells in individual PCR tubes. In the second method, cells were stained for CD19+ followed by fixation and sorting onto slides for *in situ* RT-PCR. The third method was designed to estimate numbers of clonal cells among PBMC using direct cell lysis RT-PCR.

B. Results

Sorted CD19+ cells are B cells as shown by expression of IgH and CD19 transcripts

B lymphocytes are the only cell type that rearranges and transcribes Ig genes. To confirm that sorted cells CD19+ were B cells, we performed *in situ* RT-PCR with consensus VhDJh primers and with CD19-specific primers to detect respective transcripts. On average, 89.6% of sorted CD19+ cells transcribed the Ig message (Table 5.1) and 84.6% transcribed CD19 message (Table 5.2). Because the number of CD19+ cells expressing the housekeeping gene (histone) was on average 87%, a majority of viable cells contained mRNA for Ig and CD19+ proving their identity as B cells.

Quantitation of clonal B cells by single-cell sorting combined with RT-PCR

To quantitate B cells that express clonotypic VhDJh transcripts, sorting of single CD19+ cells was combined with nested RT-PCR using patient-specific primers. Cells were stained with anti-CD19 monoclonal antibody and deposited automatically by the ELITE flow cytometer with an Autoclone Cell Deposition Unit directly into RT lysis buffer. The respective isotype controls were always included. Samples were processed immediately after the sort by adding reverse transcriptase to synthesize cDNA. We observed that blood had to be processed in less than 4 hours after obtaining it from the patient, otherwise the integrity of RNA was affected as measured by the housekeeping gene expression. After two rounds of PCR amplification, first with consensus primers and next with patient-specific primers, samples were loaded on agarose gel and resolved by electrophoresis. A representative scan of the gel is shown in Fig. 5.1. The results indicate involvement of clonotypic B cells in the CD19+ population. The single B cell RT-PCR

proved to be more difficult to perform than on PC because of the smaller copy number for the clonotypic Ig transcripts in B cells than in PC. The long processing time needed for sorting affected the stability of mRNA and augmented the detection problems. Consequently, to detect the clonotypic VhDJh, more PCR cycles had to be performed (35-38) which often generated non-specific, concatamerized PCR product. Because most of these problems were due to loss of mRNA during sample preparation, we complemented or sometimes substituted the single B cell RT-PCR with limiting dilution assay (LDA) that has much shorter processing time.

Quantitation of clonal B cells by in situ RT-PCR

In situ RT-PCR analysis was used to estimate frequency of the clonotypic cells within a few hundred cells at a time from 17 MM patients. After staining with anti-CD19+ antibody, fixation and washes, the CD19-positive cells were sorted directly onto in situ slides. During the amplification step, a reporter molecule DIG-11-dUTP was incorporated into the amplicons and later detected by immunocytochemistry (Fig.5.2). The slides were examined under the light microscope using 100X magnification with the immersion oil. Several controls were included in the experiment, as described in methods. Slides with sorted B cells from healthy individuals and/or irrelevant MM patients were always included in the PCR to control for the specificity of patient-specific primers. The ultimate results indicate that 9-90% (mean/SE 66% +/-4) of sorted circulating MM PBMC B cells were clonotypic (Table 5.3).

Quantitation of clonal B cells by limiting dilution assay

The limiting dilution assay (LDA) was used to evaluate the frequency of clonotypic cells within unfractionated PBMC. Giemsa staining of PBMC cytospins to estimate the percentage of circulating plasma cells complemented the assay. Scan of a typical gel is shown in Figure 5.3. LDA was used when the sorting facility was not available or as a back-up assay that complemented the other two described above. Table 5.4 summarizes results obtained from the analysis of PBMC from 10 patients. The range of circulating clonotypic cells varied from 0.1% to 30% of PBMC. During these studies, multiple time points were analyzed for four patients indicating persistent presence of the clonotypic cells among PBMC. In one case (ELF-6) PBMC were diluted up to one cell/well and 96 cells were analyzed. The standard LDA was performed as well. Both methods generated similar results indicating that 24% of PBMC (as per single cell dilution) or 30% PBMC (as per serial dilutions) from the respective patient were clonotypic (Figure 5.4). The identities of PCR products were confirmed as clonotypic by subcloning and sequencing each of the single cell-generated products. LDA does not identify cells included in the analysis in a qualitative fashion. However, the histological analysis of Giemsa-stained cytospins showed presence of less than 1% PC in the preparation, consequently at least 23% of clonotypic cells must have represented circulating MM B lymphocytes.

Clonotypic cells are present in the CD34+CD19+ and CD11b+CD19+ subsets

We tested the CD34+CD19+ and CD11b+CD19+ subsets for the presence of clonotypic cells. PBMC were stained with the respective monoclonal antibodies and the double positive populations were fixed and selected by sorting onto slides. Next, the specimens

were subjected to *in situ* RT-PCR with patient-specific primers. The data are presented in the table 5.5. We found that the majority of cells within CD11b+CD19+ (mean 66.5%+/-5.8) and CD34+CD19+ (mean 77.2+/-6) populations expressed clonotypic transcript in contrast with the CD34-CD19+ cells representing healthy polyclonal B cells, where very few clonotypic cells were present.

C. Discussion

This research was designed to identify and quantify circulating clonotypic MM B cells. One part of the experiments addressed the identity of the CD19+ cells as B lymphocytes. In previous studies, Pilarski's group showed that in MM, B cells displayed unusual phenotypic and morphological properties [76]. One of these properties included the monocytoid appearance, which was also characteristic of activated lymphocytes. Standard procedures, used by the majority of flow cytometry laboratories, excluded this population when analyzing lymphocytes in MM. Dr. Pilarski's results indicated presence of monotypic light chain in the cytoplasm of CD19+ monocytoid MM cells, demonstrating that they belong to B cell lineage [177]. To further ascertain the identity of monocytoid CD19+ cells, we subjected them to *in situ* RT-PCR analysis with Ig-consensus (detecting the polyclonal and monoclonal Ig) and CD19-specific primers. We demonstrated that the viable cells expressed transcripts for Ig and for CD19, unequivocally proving that these are B cells.

The second part of the research in this chapter was dedicated to quantifying clonotypic circulating B cells. Three methods were applied: single cell RT-PCR, *in situ* RT-PCR and limiting dilution assay (LDA). All methods were based on a direct approach to distinguish the clonotypic cells from the polyclonal ones by using patient-specific primers. Using all three methods we demonstrated that the range of CD19+ B cells bearing the clonotypic VhDJh rearrangements in MM was 9-90% with a mean of 66±4 in a B-lymphocyte population and 14%±2% within the PBMC. When performing LDA, the

histological screening of cytospins was done to avoid the false-positive results due to the possible presence of plasma cells in the blood. Our results are in accordance with the early data obtained by Kubagawa and by Pettersson, where anti-idiotypic staining was used to detect clonally-related B cells in blood in a range from 0.5% to 44% of all B lymphocytes [64 65].

Although within a range first described by Billadeau (range 0.001% to 32‰ of PBMC) [89] and by Brown (range 0.1% - 23%) [93] our results are in contrast to those published by others (0.34%) [92]. This can be explained by the different approaches used. The other laboratories used a method whereby DNA extracted from blood and from BM was serially diluted in 10-fold decrements, and then amplified in radioactive PCR with patient specific CDR3-based and consensus Jh primers. The PCR products were separated on polyacrylamide gels, blotted and exposed to an X-ray film. Finally, video densitometry was used to estimate the density of the bands and converted by mathematical analysis into cell equivalents. Unlike the other authors, we directly quantitated CD19+ cells that expressed clonotypic transcript, avoiding the numerous technical manipulations that could contribute to loss of nucleic acid. This direct approach enabled quantitation of positive versus negative cells and did not require mathematical calculations used by other groups. The other important difference was that we used freshly obtained blood samples from MM patients while other groups analyzed cryopreserved specimens. We observed that clonal transcripts detectable before cryopreservation may became undetectable post-cryopreservation. This may reflect loss of clonotypic cells or reduction in numbers of clonotypic transcripts.

The high frequency of clonotypic B cells in blood suggests that they represent an important part of the MM clone. The clonal relationship of CD19+ B cells and BM PC implies a sequential relationship that may explain how the disease regenerates and disseminates. The clonotypic B cells can migrate trough the circulation to the bone marrow where they may differentiate into clonotypic PC. Evidence from Pilarski's laboratory shows that in contrast to normal MM PC, MM CD19+ cells resist chemotherapy, express the RHAMM oncogene and have DNA aneuploidy [71 178 70 67 73 179]. These characteristics, in combination with the expression of clonotypic IgH VhDJh, are suggestive of malignant potential within circulating MM B cells.

In summary, we have identified clonotypic B cells in blood from MM patients as positive for CD19 transcript and surface protein. They are also characterized by abnormal expression of CD11b and CD34 surface molecules. On average, they represent 66% of the total B cells or 14% of the total PBMC. Clonotypic B cells persist throughout the duration of the disease, as shown in multiple time point analysis, suggesting that they should become targets in anti-myeloma therapy.

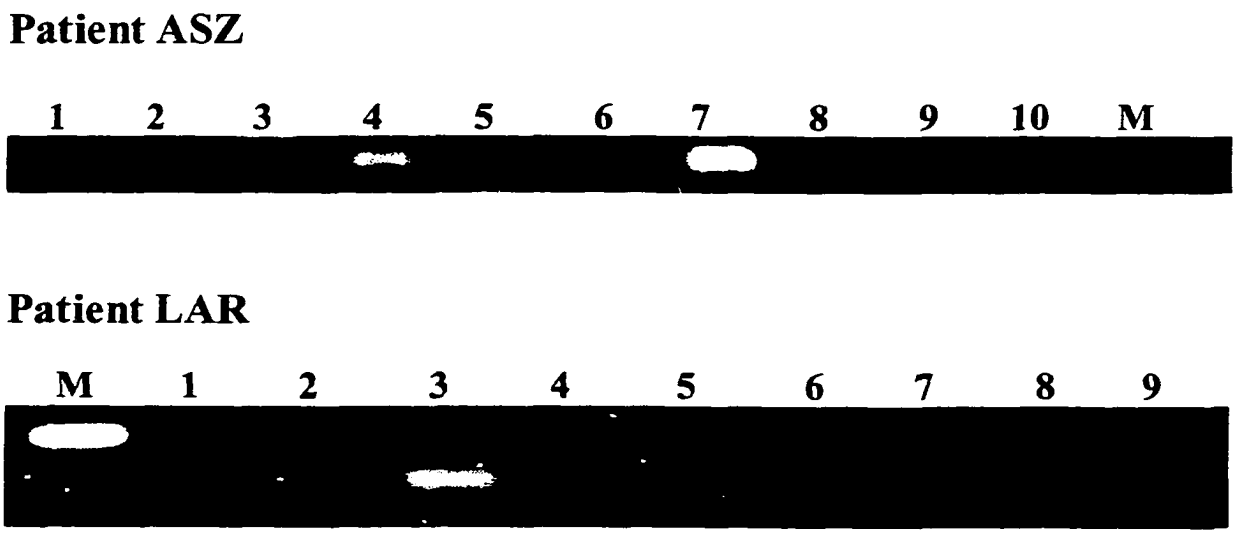


Figure 5.1 Single CD19+ patient-specific PCR.

Single CD19+ cells were individually sorted into the PCR vials with 8µl of lysis buffer. After the reverse transcription step, cDNA was amplified in nested PCR, first with consensus and then with patient-specific primers. The anticipated and obtained size of PCR product was 160 bp.

Patient A – lines 1-8 – single sorted cells; lines 9 and 10 – negative controls (no cells)
Patient B – M= DNA marker, lines 1-8 single sorted cells, line 9 – negative control (no cells)

The single sorted RT-PCR proved to be more difficult to perform on B cells than on plasma cells, because of the smaller copy number of the clonotypic Ig transcripts in B cells than in PC. The long processing time needed for sorting protocol and affecting the stability of mRNA augmented the detection problems. To detect the clonotypic VhDJh more PCR cycles had to be performed, which has often generated non-specific, concatamerized PCR product.

Because most of these problems were due to loss of mRNA during sample preparation, we complemented or sometimes substituted the single B cell RT-PCR with LDA.

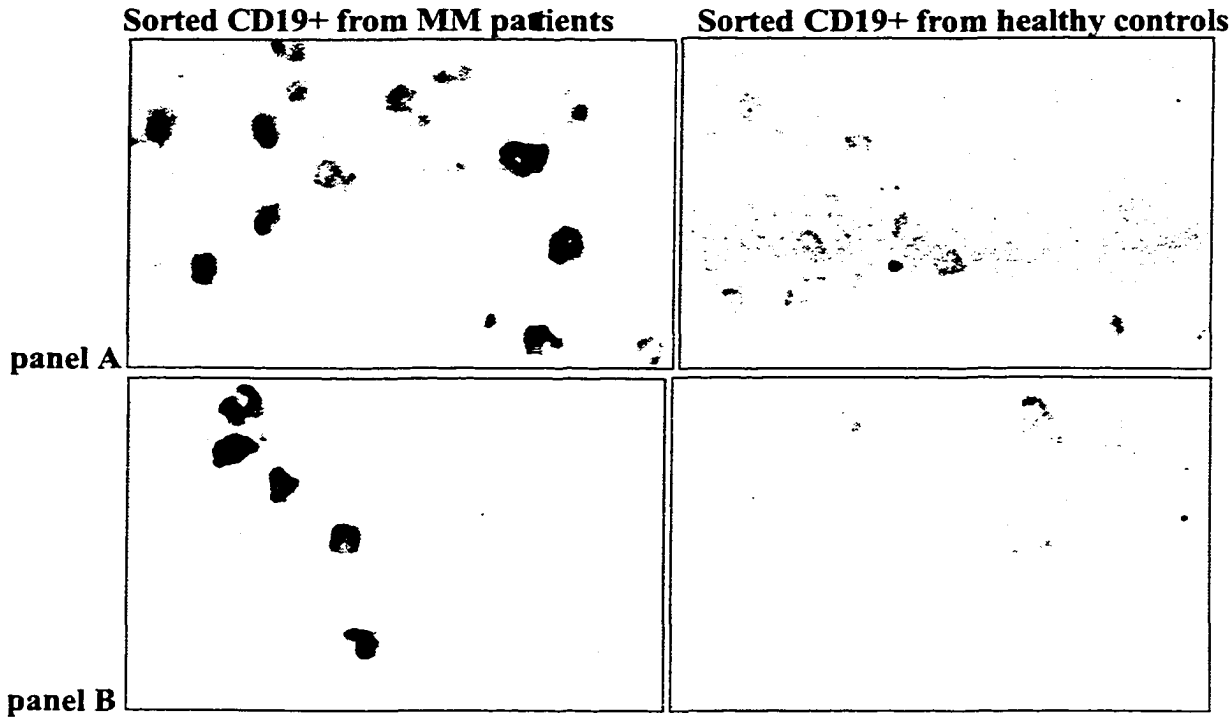


Figure 5.2 *In situ* RT-PCR with the patient-specific primers on sorted CD19+B cells

Panel A: CD19+ sorted from a blood of MM patient JAB and a healthy donor underwent *in situ* amplification with patient A CDR2/CDR3-IgH-specific primers.

Panel B: CD19+ sorted from a blood of MM patient JOD and a healthy donor underwent *in situ* amplification with patient B CDR2/CDR3-IgH-specific primers.

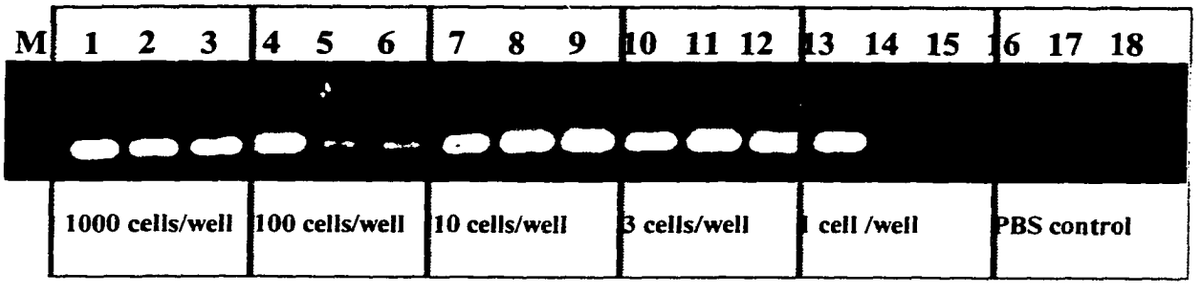


Figure 5.3 Limiting dilution analysis of MM PBMC using patient-specific primers

PBMC freshly isolated from MM patient (ELF) were immediately diluted in PBS at concentrations of $1x10^6$, $1x10^5$, $1x10^4$, $3x10^4$ and $1x10^4$ cells per ml. One microliter from each dilution was added to PCR tubes containing 8µl of RT-lysis buffer, reverse transcribed and amplified in PCA with patient specific primers. Lanes 1-3 are 1000 cells/well, lines 4-6 are 100 cells/well, lines 7-9 are 10 cells/well, lines 10-12 are 3 cell/well, lines 13-15 are 1 cell/well. Lines 16-18 show the PBS controls. The clonotypic product was detected at all the dilutions. Out of three tubes containing one cell, one was positive and two were negative suggesting an approximate frequency of the clonotypic cells in the blood of 30%.

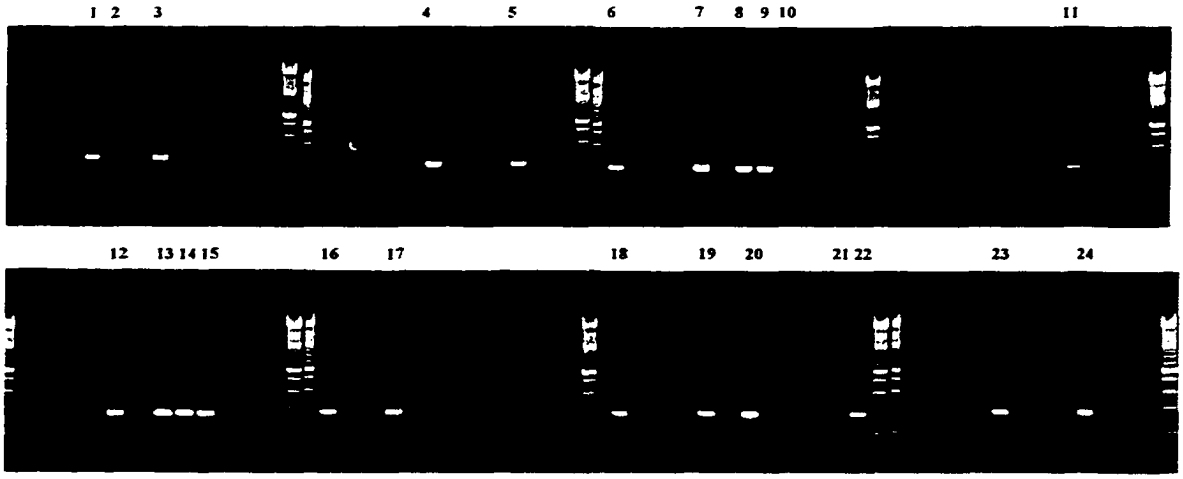


Figure 5.4 Single cell dilution RT-PCR with patient-specific primers

PBMC freshly isolated from MM patient (ELF) were resuspended in PBS at a concentration of $1x10^4$ cells per ml. One microliter of the cell suspension was added to PCR tubes containing 8µl of RT-lysis buffer, followed by the reverse transcription and amplification with patient specific primers. Out of 96 tested cells, 24 cells contained clonotypic transcripts

Patient code	% of Ig-transcript-positive CD19+ cells
ASZ-01	95.0
DOR-01	78.9
GEO-02	93.2
JHW-01	82.1
JPL-01	96.4
JUW-06	97.9
LAR-06	96.5
LWA-05	67.9
MAG-05	78.3
NBL9-02	91.8
NKI-04	96.5
PEB-03	91.6
PHM-21	96.5
RAM-11	83.8
ROT-01	86.1
JUW-06	94.9
BC0-08	96.6
Mean (+/-SE)	*89.6 (+/-2.1)*

Patient code	% of Ig-transcript-positive T cells
GEO-02	0.0
NKI-04	0.0
RAM-11	0.0
THYMOCYTES	0.0

Table 5.1 Results of in situ RT-PCR to detect Ig transcript in CD19+cells.

Individual CD19+ cells were sorted on slides and subjected to in situ RT-PCR with the consensus IgH VhDJh primers as described in methods. The positive cells were scored and the results are shown as a percentage of all cells counted.
For the controls, sorted MM T cells and thymocytes were used. None of the analyzed samples with T cells scored positive showing the specificity of reaction only for the B cells that express surface CD19+.

Patient code	% of CD19-transcript-positive CD19+ cells
ASZ-08	78.5
ASZ-10	94.3
BC0-08	91.7
BTA-22	84.8
GEL-01	80.7
GEO-02	88.5
JAB-01	92.1
JHW-01	55.2
JHW-05	96.5
JOD-06	89.4
JPL-01	64.7
JUW-02	87.2
JUW-06	97.3
JUW-06	94.8
JUW-13	42.2
LAR-06	94.4
LAR-11	87.8
LWA-05	94.3
LWA-08	84.3
MAG-05	84.7
NIS-02	85.9
NIS-06	88.2
NKI-04	78.3
PEB-03	90.6
PHM-21	96.6
RAM-11	88.2
RAM-21	78.9
RAM-22	64.7
RAM-23	88.9
RAM-23	94.1
Mean (+/-SE)	*84.6 (+/-2.3)*

Patient code	% of CD19-transcript-positive T cells
GEO-02	0.0
JOD-06	4.0
MAG-05	0.0
NKI-04	0.0
RAM-11	0.0
THYMOCYTES	0.0

Table 5.2 Results of the in situ RT-PCR detecting CD19 transcript in sorted CD19+cells.

Individual CD19+ cells were sorted on slides and subjected to in situ RT-PCR with the CD19-specific primers as described in methods. The positive cells were scored and the results are shown as a percentage of all cells counted.

For the controls, sorted MM T cells and thymocytes were used. One out of 6 analyzed samples with T cells showed 4% of positive cells. It can be explained by the contamination of T cells with CD19+ cells, because the purity of sorting was 95%.

Patient status-Apr97	Number of sequential time points	Percent of B cells that are clonotypic	Percent of PBMC that are clonotypic	Percent of clonotypic cells x 10^9/L of blood
ASZ Tr	5	69, 90, 90, 90, 62	24, 3, 14, 10, 18	0.1, 0.35, 0.09, 0.05, 0.11
CWH Deceased	2	74, 74	19, 31	0.17, 0.15
EKA Tr	2	71, 54	14, 9	0.22, 0.12
LAR Allo Tsp	4	45, 46 ,46 ,62	9, 9, 10, 19	0.12, 0.08, 0.06, 0.11
NIS Off	3	90, 40, 73	18, 8, 16	0.24, 0.1, 0.21
GML Tr	3	60, 77, 90	10, 10, 50	0.08, 0.03, 0.1
IRS Deceased	1	90	12	0.06
GEH Deceased	1	9	0.9	0.01
JHW Tr	1	52	7	0.09
JAB Unt	2	65, 64	10, 6	0.08, 0.06
JOD Auto Tsp	2	46, 64	15, 7	0.16, 0.13
JHP Tr	1	73	12	0.18
BBE Tr	2	28, 90	8 ,21	0.03, 0.08
HSP Tr	2	66, 90	11, 13	0.17, 0.13
LWA Tr	1	56	13	0.17
DAL Tr	1	53	20	0.42
AUM Tr	2	68, 49	20, 18	0.61, 0.39
ELF Unt.	1	50	21	0.27
Mean		**66±4**	**14±2**	**0.15±.02**

Table 5.3 Summary of the in situ quantitation of the circulating clonotypic B cells
In situ analysis of sorted CD19+ MM cells for the presence of clonotypic Ig transcript. The results of quantitation of clonotypic B cells at individual time points are listed in the table as a percentages within all B cells, percentages within PBMC and the absolute percent within white blood cells.
Abbreviations:
Tr – patient during treatment

Off – patient off treatment
Unt – patient untreated
Allo – patient has undergone an allogeneic transplantation
Auto - patient has undergone an autologous transplantation

Patient code	Estimated % of clonotypic cells detected among PBMC
ACZ-03	9.5
ACZ-06	0.1
ASZ-11	30
AUM-19	8.3
BBE-63	0.1
BBE-64	1.1
DOR-06	3
DOR-16	0.1
EKA-24	0.3
ELF-02	3
ELF-05	16
ELF-06	30
JAB-03	9
JUW-13	0.3
LWA-10	0.1
REH-16	0.1
Range	***0.1 – 30***
Median (+/- SE)	***7 (+/-2.5)***

Table 5.4 Results from the limiting dilution assay with MM PBMC.
PBMC were suspended in PBS, serially diluted and subjected to LDA, as described in methods. The estimated frequency of clonotypic cells is shown as a percentage of PBMC.

VI. ANALYSIS OF THE IMMUNOGLOBULIN CONSTANT HEAVY CHAIN EXPRESSION IN MULTIPLE MYELOMA PATIENTS INDICATES ISOTYPE SWITCHING EVENTS

(The majority of the data presented in this chapter have been submitted for publication in an article entitled "Analysis of the immunoglobulin constant heavy chain expression in multiple myeloma patients indicates isotype switching events.")

This chapter is presented in paper format.

Abstract

This study was designed to characterize the stages of differentiation within multiple myeloma (MM) related population of B cells. We identified clonotypic cells from peripheral blood, bone marrow and G-CSF mobilized blood of MM patients. The nucleic acid sequence of the monoclonal immunoglobulin heavy chain (IgH), which is unique for each patient, was used as an indicator of clonality. Clonotypic transcripts were detected in nested RT-PCR with patient Ig-specific primers. The IgH isotypes designating each maturation stage of B cells were identified with constant chain-specific primers. Our experiments revealed the full spectrum of post- and pre-switch clonotypic Ig transcripts in the blood and bone marrow at diagnosis in 10/13 patients. Non-clinical pre- and post switch transcripts were infrequent, as indicated by sequencing results from four patients. For 7/9 patients, their G-CSF-mobilized blood was positive for pre- and post-switch clonotypic isotypes. For untransplanted patients, analyses of blood samples taken at multiple time-points revealed associations between progression of MM and expression of clonotypic transcripts in the full spectrum of isotypes. For 11 patients that underwent

autologous transplantation and in one that underwent allogeneic transplantation, three patterns of clonotypic transcript expression were observed in the blood post-transplantation. Pattern #1, when there was no clonotypic message expressed during stages of clinical remission. Pattern #2, in which only the clinical isotype was expressed, was also associated with periods of remission. Pattern #3, when clinical and non-clinical clonotypic isotypes were expressed, occurred during relapse and progression of the disease. Sequence analysis detected pre-switch transcripts that showed intraclonal diversity in 1/2 mobilized peripheral blood (MPB) samples and in 1/2 of hematopoietic progenitor-enriched MPB fractions. In the diversified transcripts, the majority of replacement mutations accumulated in the CDR1 and CDR2 regions suggesting antigenic stimulation and selection. Detection of diversified, clonally related cells implies the persistent presence in G-CSF mobilized blood of an antigen-stimulated clone from which MM has originated. Pre- and post-switch clonotypic isotypes may originate from a common clonotypic precursor that undergoes switching events or alternatively, from isotype-specific clonotypic memory cells.

Introduction

Multiple myeloma is an incurable cancer affecting the B lymphocyte lineage. The ultimate neoplastic plasma cell (PC) is located in the bone marrow and produces monoclonal antibody of a fixed isotype (here referred to as the clinical isotype) found in serum and sometimes in urine of MM patients. MM may arise in a multi-step process of accumulation of mutations leading to neoplastic transformation [15]. The population of circulating clonotypic B cells has been postulated to contain the MM precursor [180 98 174]. However, the maturation stage of the hypothetical progenitor B cell in MM remains a point of debate and investigation. Here, we investigated B cells from the circulation of MM patients for expression of the clonotype in cells in the different stages of B cell maturation.

During differentiation of a normal B cell from a hematopoietic progenitor cell into a mature, antibody producing plasma cell, several sequential events occur at the chromosomal, transcriptional and translational levels. Initially, germline formation of multiple chromosomal segments encoding immunoglobulin Vh, Dh and Jh are rearranged and then translated as a variable region (V-region) joined with the μ constant chain. B cells producing functional antibody are positively selected and migrate to the secondary lymphoid tissues. Upon exposure to an antigen, the naïve B lymphocyte proliferates in germinal centers. During cell division, the IgH V-region undergoes somatic hypermutation, followed by antigen-mediated selection for higher affinity binding. The hypermutational pattern of the IgH V-region becomes a part of the clonotypic signature

for the clonal progeny of any positively selected and expanded post-germinal center B cell. Different patterns of somatic hypermutation within clonal VhDJh rearrangements are referred to as intraclonal diversity. One of the five main constant chain subtypes (IgM, IgD IgG, IgA and IgE) can be joined to the V-region by gene switching. The pre-switch isotypes are IgM and IgD classes, while the post-switch isotypes are IgG, IgA and IgE. Switching results in permanent deletion of genomic DNA between V and C segments. The IgM and IgD isotypes are usually expressed by differential RNA splicing. However, some of IgD expressing cells have been shown to delete the IgM segment on the chromosomal level, generating a post-switch cell [102]. After positive selection, B cells differentiate into short-lived effector plasma cells or long-lived memory cells that upon secondary activation become plasma cells.

In B cell malignancies, detection and analysis of the IgH VhDJh rearrangement is used to identify and enumerate the clonotypic cells and to assess their differentiation stage at the time when neoplastic transformation took place. Molecular analysis of PBMC in MM demonstrated circulating B cells that are clonally related to plasma cells [89 181]. Single cell analysis showed that on average 66% of all circulating MM B cells are clonotypic [166]. Molecular studies of MM B-lymphocytes revealed isotype switching, implying the potential for differentiation within the MM clone [17 182 18]. However, no intraclonal diversity was observed in the variable regions of clonotypic cells [37]. Based on these findings, the MM progenitor may be a circulating B cell that is neoplastically transformed after maturation in the germinal center and no longer under antigenic selection [139].

In the last decade, autologous transplantation of hematopoietic progenitors became part of treatment in MM. Stem cell harvests obtained for autologous transplantation were shown to contain clonotypic MM cells [183] [113] [184] [114]. Moreover, clonotypic circulating cells were shown to be CD19+ B cells that co-express CD34, a stem cell marker [75]. However, the hematopoietic progenitor cell population from MM patients has not previously been examined for the presence of diversified or isotype-switched clonotypic transcripts.

In this work, we identify and characterize stages of differentiation in B cells belonging to the MM clone isolated from blood, bone marrow and G-CSF-mobilized blood. We also analyze malignant potential within pre- and post-switch cells using a xenograft model. PCR with patient-specific primers detecting MM IgH mRNA was used to track clonotypic cells. The patterns of somatic hypermutation in the IgH V-region combined with the isotype usage provided additional information about the maturation stage of clonotypic B cells. Using constant-chain-specific and patient-specific RT-PCR, we examined all specimens for the presence of pre- and post-switch, clinical and non-clinical clonotypic isotypes. Analysis of blood and BM was performed at diagnosis and at multiple time-points over an extended time period in patients that underwent chemotherapy only, or chemotherapy combined with autologous or allogeneic transplantation. We have also analyzed clonotypic transcripts in different isotype classes for intraclonal diversity.

Materials and methods

Cells

Mononuclear cells were obtained from a heparinized peripheral blood (PBMC) and bone marrow (BM) of 22 MM patients (Table 6.1). Thirteen samples of matching pairs of PBMC and BM were obtained at the time of diagnosis. Other PBMC and BM specimens were taken during multiple time-points (on average, over a 3-year period). G-CSF mobilized blood (MPB) was obtained from 9 MM patients. Mobilization procedure followed previously published protocol [108]. Two of the MPB samples were subjected to progenitor cell enrichment procedure described below.

RNA isolation, reverse transcription and PCR

Cell pellets were collected and total RNA was extracted using the TRIZOL method following the manufacturer's protocol (Gibco/BRL, Burlington, ON). Following quantification of isolated RNA by spectrophotometry, cDNA was synthesized by priming with 100 ng universal primer oligo-dT_{15} in ultrapure water (SIGMA, Mississauga, Ontario), 5x First Strand Buffer (Gibco BRL), 0.1 M DTT, 0.25 mM dNTPs and 200 U Superscript reverse transcriptase per 1 μg of total RNA in 20 μl volume. Each PCR reaction was performed equivalent amounts of cDNA in 50 μl volume containing Ultrapure water (SIGMA), 10x PCR buffer, 200 μM of each deoxynucleotide (dNTP), 2 mM MgSO4, 10 μM of each primer and 1 unit/reaction tube of high fidelity HiFi Platinum TAQ (GIBCO/BRL, Burlington, Ontario). Primers used for Vh family leader-specific:

Vh1 leader: ATG GAC TGG ACC TGG AG

Vh2 leader: CTT TGC TCC ACG CTC CTG

Vh3 leader: GA(A/G) TT(G/T) GGG CTG AGC TGG

Vh4 leader: ATG AAA CAC CTG TGG TTC TTC

Vh5 leader: TG GGG TCA ACC GCC ATC

Sequences for the primers specific for the constant heavy chain regions were previously published by Billadeau et al. [17]. Primers used to derive patient-specific sequences were published by Aubin et al. [165]

Sequences for CDR2 and CDR3 specific-patient primers are listed in Table 6.1.

We used three strategies for amplification of PCR product (Fig.6.1):

Strategy A *was used for an estimation of relative frequency of clonotypic transcripts within a specific isotype class.*

30 cycles of PCR with Vh-leader specific primer and any of the constant chain primers.

Strategy B *was used to determine whether any of clonotypic transcripts are detectable within a given isotype class.*

30 cycles of PCR with Vh-leader specific primer and any of the constant chain primers followed by reamplification of 2% (v/v) of primary PCR product in 25 cycles of PCR with patient-specific CDR2 and CDR3 primers.

Strategy C *was used to analyze intraclonal diversity of the IgH V-region.*

30 cycles of PCR with Vh-leader specific primer and any of the constant chain primers followed by reamplification of 2% (v/v) of primary PCR product in 25 cycles of PCR with Vh-leader specific and patient-specific CDR3 primers.

All PCR products of appropriate sizes were detected in 2% agarose electrophoresis containing ethidium bromide.

Cloning and sequencing

We cloned PCR products using the INVITROGEN (Carlsbad, California) TA TOPO cloning system accordingly to the manufacturer's directions. The transformants were analyzed by a direct-lysis PCR with a standard M13 primer set. PCR products of an appropriate size were purified on Millipore spin columns and subjected to sequencing with a fluorescent di-deoxy terminator sequencing kit (Perkin Elmer, Mississauga, Ont.) and T7 universal primer. The sequencing products were purified by precipitation in 95% ethanol, resuspended in template suppression reagent (TSR) (Perkin Elmer, Mississauga, Ont.), denatured for 3 minutes at 95oC, chilled quickly on ice and analyzed on an ABI310 capillary genetic analyzer (Perkin Elmer, Mississauga, Ont.).

Analysis of the DNA sequences

The primary analysis was performed with a V-Base WWW based program to establish the type of variable region and the pattern of somatic hypermutations against the germline. The secondary analysis was done by an alignment of the variable regions against the plasma cell-derived sequence, respectively for each patient. For this, the AutoAssembler (Perkin Elmer) program was used.

Mobilization of hematopoietic progenitor cells with high-dose chemotherapy and granulocyte-colony stimulating factor (G-CSF)

Patients with MM received 10 mg/kg/day of G-CSF (Amgen, Canada Inc. Mississauga) in addition to 2.5g/m^2 cyclophosphamide to mobilize progenitor cells. The collection of

leukapheresis product was done between days 9 and 13 from the initial mobilization (protocol obtained from Dr. A. Belch, Cross Cancer Institute, Edmonton, Alberta).

Progenitor cell-enrichment procedure

Progenitor cell enrichment was done on StemSep columns (Stem Cell Technologies Inc, Vancouver, BC) according to the manufacturer's directions. Briefly, $5x10^8$ MPB cells were incubated on ice for 30 minutes with StemSep antibody cocktail containing anti-CD2, -CD3, CD14, CD16, CD19, CD24, CD56 and CD66b. Next, magnetic colloid was added to the cell/antibody mix and after 30 minutes incubation on ice, cells were loaded into the StemSep column. The negatively selected, enriched hematopoietic progenitor fraction was collected and counted. To check for purity, eluted cells were labeled with anti-CD34 antibody and the staining profile was analyzed on FACSCAN (Becton-Dickinson).

Human myeloma xenografts

NOD/LtSz-SCID (NOD SCID) mice at 6-8 weeks of age were irradiated (300-340Gy) and injected *via* the intracardiac route with patient's PBMC [117]. Mice were sacrificed after they developed symptoms of illness and for all three mice described here vertebral tumor masses were macroscopically detected at autopsy. Cells were isolated from the tumor masses, femoral BM and from spleens. RNA was purified as described above.

Results

Circulating MM cells frequently express the clinical isotype of the MM clonotypic Ig

The relative frequency of pre- and post-switch clonotypic isotypes in blood and bone marrow was estimated by sequencing the cloned product of RT-PCR. RNA isolated from peripheral blood and bone marrow of patient ELF was subjected to RT and the respective cDNA was amplified in single stage PCR using strategy A (Figure 6.1), which should amplify all transcripts of the Vh3 family, regardless of clonotype. The experiment of Figure 6.2 shows that Vh3-family (ELF) of transcripts are expressed in conjunction with μ, δ, γ and α constant chains. To assess diversity within each isotype, PCR products were subcloned and sequenced. The examination of IgH sequences revealed that for both blood and BM within the analyzed 142 species of IgH Vh3 family (from ELF), clonotypic transcripts could be found only within the clinical (IgG) isotype while in non-clinical isotypes (IgM, IgD and IgA) only heterogeneous polyclonal Ig transcripts could be found (Table 6.2). For the clinical isotype, all species of Vh3 transcripts were clonotypic (24/24 for blood and 12/12 for BM). No intraclonal diversity was detected among the clonotypic transcripts.

At diagnosis, MM bone marrow and peripheral blood contain infrequent clonotypic cells expressing non-clinical isotypes

To detect pre- and post-switch clonotypic isotypes, we used a more permissive system of nested RT-PCR in which isotype-specific transcripts were amplified in the first step and the clonotypic template in the second step PCR. RNA from peripheral blood and bone

marrow of 13 MM patients was analyzed at the time of diagnosis (Table 6.3). Patient-specific transcripts were amplified in nested PCR from cDNA using strategy B. The results indicated that for 10/13 patients, clonotypic transcripts were expressed in the full spectrum of isotypes in both blood and bone marrow, although the non-clinical clonotypic isotypes were detected only when using the nested PCR strategy. In one case of solitary plasmacytoma (STK) that later developed into MM, the only expressed isotype was the clinical one; it was present in the bone marrow but not in the blood. In two cases, bone marrow contained a full spectrum of clonotypic isotypes but the blood contained only clinical isotype (ORS) or clinical (IgG) plus clonotypic IgA (HSP), but not pre-switch clonotypic isotypes.

Isotypically distinct clonotypic transcripts are expressed in blood throughout the course of disease

Three MM patients were followed longitudinally for three years to characterize the spectrum of clonotypic isotypes in blood and bone marrow. RNA samples were obtained at multiple time points. The samples were subjected to reverse transcription and nested patient-specific PCR using strategy B. Our results (Table 6.4) show that the clonotypic cells of the clinical isotype persist in blood and bone marrow over the course of disease, as expected. For patient MCO, a long-term survivor diagnosed in 1991 who responded well to treatment, at most time points the only detectable clonotypic cells were of the clinical isotype. At two time-points, we detected clonotypic IgM and IgA. During this period, the patient was progressing and the clinical M-protein value was increasing. Presently, this patient is off treatment having obtained a further remission. Patients REH

and JHP had persistent clonotypic cells of all isotypes throughout their disease course. Neither patient responded well to treatment. JHP, diagnosed in 1997, after 7 cycles of VAD had achieved some symptomatic response but the hemoglobin value remained low (97), M-protein was high (34 g/L) and shortly after the last VAD cycle the patient relapsed and is presently progressing. Patient REH, diagnosed in 1997, was treated with 11 cycles of melphalan/prednisone chemotherapy. This patient achieved partial clinical response but shortly after the last chemotherapy cycle the M-protein value rose and symptoms worsened. Presently REH is on maintenance chemotherapy. Overall, expression in the blood of the clonotypic transcripts in the complete spectrum of isotypes appears to be associated with clinical and symptomatic progression of MM.

G-CSF-mobilized blood contains clonotypic cells of the clinical isotype

The relative frequency of clonotypic isotypes in MPB was estimated by using RT-PCR and sequencing the cloned product. RNA purified from patients BBE, ELF, SBA and RAM mobilized blood was subjected to RT-PCR using strategy A to amplify all transcripts of the Vh family specific for the respective patients. The product was subcloned and sequenced. Twelve-24 clones representing each isotype were analyzed for each of the four patients (Table 6.5). As for the blood or bone marrow of previously analyzed patients, using the strategy A RT-PCR, the only detectable clonotypic Ig was of the clinical isotype. The IgH VhDJh sequences for non-clinical isotypes were polyclonal. Within the clinical isotype where all or the majority of transcripts were clonotypic, no intraclonal diversity was observed.

G-CSF mobilized blood contains infrequent clonotypic cells expressing non-clinical isotypes

To detect any presumed infrequent pre- and post-switch non-clinical clonotypic transcripts in mobilized blood, RNA isolated from the freshly collected blood samples of nine G-CSF-mobilized patients was subjected to RT-PCR using strategy B (nested RT-PCR). The results summarized in Table 6.6 show the full spectrum of clonotypic isotypes in 7 out of 9 tested specimens while in one case, only the clinical isotype was seen (ORS) or clinical and IgM isotypes (JLE). For two cases (JOD, ATM) we also tested the same samples of MPB after cryopreservation. Although the full spectrum of clonotypic isotypes was detected at the time of mobilization, after cryopreservation we observed expression of only clinical isotype (JOD) or the clinical and IgM isotypes (ATM). This suggested that freezing compromises detection of mRNA encoding clonotypic isotypes.

Some pre-switch clonotypic transcripts isolated from mobilized blood show intraclonal diversity

After G-CSF mobilization, hematopoietic cells from the BM migrate to the peripheral blood. Because this unique cell population may contain B cells at an early stage of maturation, we analyzed clonotypic cells in G-CSF mobilized blood to detect any intraclonal diversity. RNA purified from mobilized blood of two patients (JOD, ATM) was subjected to nested patient-specific RT-PCR using strategy C. The final product spanning the entire Vh and part of Dh regions was subcloned, sequenced and analyzed. The majority of clonotypic transcripts in all isotype classes lacked intraclonal diversity (Table 6.7). However, in patient JOD, a few of the clonal transcripts in the pre-switch μ

and δ class showed an altered pattern of somatic hypermutations. Figure 6.4A presents the DNA sequences of V-regions from the germline, plasma cells and clonotypic pre-switch transcripts. Some of the patient-specific hypermutation pattern was retained, but there were new replacement and silent mutations, or some mutations were absent. The new mutations occurred mainly in the CDR2 region suggesting they were selected in response to antigenic pressure; there was also one replacement mutation in the FR2 region. In two diversified clones, the FR1 and CDR1 regions were not hypermutated, conserving the germline sequence. The deduced developmental pathway for clonotypic cells is shown in Figure 6.4C.

Pre- and post-switch clonotypic transcript in the enriched hematopoietic progenitor fraction of mobilized blood

To determine whether the clonotypic MM cells co-purify with enriched hematopoietic progenitors, we analyzed MPB cells eluted from StemSep columns. Post-column fractions from freshly obtained MPB of 2 MM patients (ATM, PEF) were processed as described above (Figure 6.3). For the enriched progenitor fraction from ATM, we amplified clonotypic IgM, IgD and IgG but were unable to detect clonotypic IgA. Sequencing analysis in ATM revealed lack of intraclonal diversity for all clonotypic transcripts in the detectable pre- and post-switch isotype classes (Table 6.7). For patient PEF, we detected all four pre- and post-switch clonotypic isotypes in enriched hematopoietic progenitor cells. We observed a modified pattern of somatic hypermutations in some of the pre-switch clonotypic IgM and IgD transcripts but not in clonotypic IgG or IgA (the clinical isotype). Analysis of intraclonal diversity of pre-

switch sequences as compared to the germline and autologous plasma cell sequences showed accumulated replacement mutations in the CDR1 region suggesting a response to antigenic selection (Figure 6.4B). The deduced developmental pathway for clonotypic cells is shown in Figure 6.4D.

Expression of clonotypic isotypes in blood of MM patients pre- and post- transplantation

The extent to which the clonotypic isotype persists after hematopoetic transplantation was investigated. We analyzed RNA samples obtained at several time points from blood and bone marrow of 11 MM autologous and one allogeneic transplant patients. RNA was subjected to RT-PCR according to strategy B. Our results (Table 6.8 A, B and C) indicate that there are three major expression patterns for clonotypic isotypes. The first one is represented by an absence of clonotypic message in the blood post-transplantation (Figure 6.5A.Table 6.8A). We have seen that pattern in two cases post-autologous transplantation (JLE. ORS both transplanted in May 1998) and the single case of post-allogeneic transplantation (LAR transplanted in Oct.1996). To date. all three patients are in full remission. The second pattern is expression of only the clinical clonotypic isotype post-transplantation (Figure 6.5B,Table 6.8B). Three patients: ATM (Feb.1999), SBA (Oct.1998) (off chemo, full remission) and IAP (Jun 1998) (remission) exhibit that profile. The last pattern is when all clonotypic isotypes are expressed in addition to the clinical isotype. Six patients, of whom three recently deceased (BBE, GEV and RAM) and three others are in clinical relapse (PEF, JOD, and ELF), comprise this group (Figure 6.5C, Table 6.8C). Consistent with the pattern for non-transplanted patients, the appearance of the full spectrum of clonotypic isotypes is associated with progression of

MM. The time of autologous transplantation and VAD/Melphalan therapy (in clinical responders) associates with loss or diminished expression of non-clinical isotypes.

Expression of clonotypic pre- and post-switch isotypes in xenotransplanted human myeloma

We have established an animal model that supports development of primary human MM xenotransplants [117]. This system was used to investigate the clonogenic potential of cells expressing clonotypic isotypes. The GEV-11 blood used for injections to mice was found to express all clonotypic isotypes (Table 6.8 sample GEV-11). RNA from the bone marrow, spleen and tumor mass of mice previously injected with blood from patient GEV was isolated and subjected to nested RT-PCR as described in strategy B. We detected the full spectrum of clonotypic Ig isotypes present in the tumor mass for 2/2 mice (mice #1 and #2). BM cells from one mouse (#3) showed expression of clonotypic IgM and post-switch clonotypic IgG and IgA. For BM cells from mouse #2 the pre-switch clonotypic IgM was detectable. BM cells from mouse #1 had no clonotypic transcripts detectable. For spleen cells from two mice (#2 and #3) only the clonotypic pre-switch IgM was detectable and spleen cells from mouse #1 lacked detectable clonotypic transcripts (Figure 6.6).

Discussion

This study describes the presence of pre- and post-switch circulating MM B cells in the blood, BM and mobilized blood from nearly all MM patients. Transcripts encoding the clinical post-switch clonotypic isotype were frequent. Those encoding non-clinical pre- and post-switch clonotypic isotypes were relatively infrequent since they were detectable only by nested PCR. Some of the pre-switch transcripts from enriched and unfractionated mobilized blood showed intraclonal diversity suggesting that an antigen-responsive component of the malignant clone may persist after the onset of frank malignancy. In long-term studies on patients before and after autologous transplant, the expression of pre-switch clonotypic isotypes was associated with the progression of MM. In our earlier work we detected and enumerated a B cell population in MM blood, that was clonally related to the neoplastic BM plasma cells. On average, 66% of B lymphocytes in MM have the clonotypic VhDJh rearrangement [166]. Functional and phenotypic analysis showed that they are drug resistant [72 73], exhibit migratory behavior [68] and express abnormal surface markers [174]. These properties strongly suggest that MM B cells may be a reservoir for the spread and regeneration of the disease. To further examine this population on a molecular level, we have analyzed the expression of the pre- and post-switch clonotypic isotypes as an indicator of the B cell developmental stages in patient PBMC and BM. In all cases, only freshly isolated cells were characterized.

First, we addressed the relative frequency of transcripts encoding non-clinical pre- and post-switch clonotypic isotypes as compared to clinical clonotypic isotypes in MM

PBMC and BM at diagnosis. Our sequencing results show the predominant presence of the clinical isotype within the tumor-specific Vh family of Ig transcripts in both PBMC and BM. The differentiation status for clonotypic B cells can be determined based on pattern of somatic hypermutations acquired during antigen-driven maturation. If that pattern were altered, it would be suggestive of antigen-dependant selection of IgH VhDJh variants. We compared clonotypic transcripts against each other and found them 100% homologous implying that the cells expressing them are not responsive to antigenic pressure.

Next, we used more sensitive, nested RT-PCR to identify any presumably infrequent, non-clinical isotypes present in the MM PBMC and BM at the time of diagnosis. In BM, for all 13 patients tested, we detected the clinical isotype; for 12/13, the full spectrum of pre- and post-switch clonotypic isotypes was present. In PBMC, 12/13 patients expressed the clinical isotype; 10/13 expressed the full spectrum. An IgA-MM patient (HSP) had detectable clonotypic IgG and IgA but not IgM or IgD in his PBMC. In one case (ORS), we detected only the clinical clonotypic isotype at diagnosis. A lack of pre- and post switch clonotypic cells might reflect particular stages of the disease or may represent different types of MM. In addition, the clonotypic, isotype distinct cells may be localized to tissues other than blood or bone marrow. In addition, if the IgH mRNA copy numbers are very low, as might be the case in the pre-switch B cells, the assay sensitivity might have been insufficient to identify clonotypic isotypes. Our observations confirm the earlier data of others [18] [17] [103] indicating that within the population of MM-related cells, some express clonotypic IgH VhDJh in context of μ, δ, γ, and α constant chains.

Billadeau et al [17] established that in the BM, clonotypic isotypes are expressed by the CD38-CD45+ B cells but not by the plasma cells. In our earlier work [166], we detected high numbers of abnormal, clonotypic B cells in blood. Although the majority of these are post-switch cells based on our ability to detect clonotypic transcripts in the first stage PCR, our results here show that infrequent pre-switch cells are detectable in both blood and bone marrow of MM patients.

We investigated the presence of non-clinical isotype-expressing clonotypic B cells post-diagnosis. The expression of clonotypic isotypes in PBMC of 3 MM patients was monitored over an extended period of time. Our analysis suggests that the appearance of non-clinical clonotypic isotypes may be increased during disease progression and decreased during clinical response to treatment.

G-CSF mobilized blood contains a population of cells that upon cytokine-induced mobilization has migrated into the blood. The clinical isotype was detected in all analyzed MPB specimens and the pre- and post-switch non-clinical clonotypic isotypes in 7/9 specimens. To determine whether diversification had occurred within the clonotypic cells, we analyzed the sequences of clonotypic RNA transcripts. For one patient, intraclonal diversity was detected within the CDR regions of IgM and IgD transcripts. This indicated the presence of clonotypic cells that had undergone distinct hypermutation presumably during affinity maturation. MPB from another two patients were enriched in hematopoietic progenitors on StemSep columns and analyzed for co-purified clonotypic cells. The pre-and post-switch clonotypic isotypes were detected for both patients. After

sequencing of clonotypic transcripts, in one patient we found diversified pre-switch clonotypic mRNA (IgM and IgD). The pattern of somatic hypermutations found in the pre-switch clonotypic cells matched in large percentage that found in autologous MM plasma cells. Nonetheless, some of the mutations were missing and a germline sequence was used instead, suggesting branching off the main differentiation pathway during early stages of the somatic hypermutation process. New mutations were also present, mostly limited to the CDR1 and CDR2, but they were found in the framework regions as well. The replacement mutations, however, accumulated to a specific "hot spots" (Fig.6.3A, 6.3B). Since the CDR3 region was used as a priming site, any additional mutations accumulated there might cause mispriming during PCR and consequently a lack of product, leading to an underestimate of the total intraclonal diversity.

The presence of pre- and post-switch clonotypic transcripts among enriched hematopoietic progenitor cells suggested that they might give rise to clonal progeny after transfusion. To monitor the presence of clonotypic isotypes pre- and post-transplantation we analyzed sequential blood samples. Multiple time-point studies revealed presence of three patterns. The first two (lack of clonotypic transcript or expression of only the clonotypic clinical isotype) associated with remission of MM. The third one, characterized by presence of non-clinical pre- and post-switch clonotypic isotypes, in addition to the clinical isotype corresponded with disease progression and relapse. Another group has recently examined pre- and post-autologous transplantation specimens of MM BM and has showed that the post-transplantation samples lack non-clinical clonotypic isotypes [185]. However, cryopreserved samples were used, only one time-point

post-transplantation was analyzed, and a different PCR approach was used which might explain the discrepancy in results between our groups. Our results showed that non-clinical isotypes often become undetectable after cryopreservation.

Xenotransplants of primary human MM were used to analyze the clonogenicity of MM cells expressing the pre-switch clonotypic isotypes. Human cells from vertebral tumor masses in 2/2 transplanted mice tested, expressed the full spectrum of clonotypic isotypes. In the lymphoid tissues, although BM from one engrafted mouse was positive for the pre- and post-switch clonotypic transcripts, BM from a second mouse and spleens from 2 engrafted mice tested positive only for clonotypic pre-switch IgM. This implies that the pre-switch clonotypic IgM may preferentially colonize the spleen and BM of engrafted mice. It appears that a large concentration of human MM cells may be a prerequisite for the expression of all pre- and post-switch isotypes, as detected in the tumor mass. The lack of detectable post-switch clonotypic isotypes may reflect a lower frequency of post-switch MM precursors in mouse lymphoid tissues as compared to the vertebral tumor. Alternatively, a high concentration of MM cells may be needed to maintain and/or to generate cells of post-switch clonotypic isotypes, perhaps reflecting production of human cytokines and a requirement for cell-cell interaction.

Broadly speaking, there are two potential explanations for the persistence of pre-switch clonotypic isotypes in post-transplant patients and in xenotransplanted MM: 1. There are distinct pre- and post-switch precursor MM B cells in the circulation, or 2. The MM progenitor cell may be a long-lived, IgM+ B cell that undergoes class switching. The first

possibility assumes that the B cell which underwent somatic mutation generates long-lived, clonotypic cells of pre- and post-switch isotypes. They may continuously produce the pre- and post-switch progeny. The growth may occur at different rates, favoring cells of the clinical isotype. The second possibility assumes the existence of IgM+ post-germinal center, clonotypic B cells confirmed here to be an infrequent compartment in MM blood and BM. These pre-switch clonotypic progenitors may generate a population of cells bearing the same VhDJh rearrangement and conserving an identical pattern of hypermutation, but able to express diverse constant chains. The final neoplastic event may occur during IgH switching to the clinical isotype as postulated by Bergsagel et al. [46]. Alternatively, the IgM+ precursor could be already transformed and the growth and switching rate of neoplastic cells may depends on microenvironmental conditions (e.g. cytokine stimulation).

The evidence presented here suggests that MM might in fact originate from a persistent, antigen-driven clonotypic B cell. That cell during somatic mutation may give rise to a few diversified cells of which one is neoplastically transformed and becomes a progenitor for the MM clone. The exact stage at which the transformation takes place remains to be established. The progenitor cell may persistently generate pre- and post-switch clonotypic lymphocytes. Their association with the disease progression indicates the clinical importance of the pre- and post-switch clonotypic cells.

Table 6.1 List of MM patients, their clinical isotype, Vh family and patient-specific primers

patient	Expressed heavy chain	Vh family	Patient-specific primer sequences (5'=CDR2; 3'=CDR3)s
ATM	IgG	1	5'CTGATGGGAGTACCACAGAG 3'CATACCATAATGGTATACATGGA
BBE*	IgG	3	5'TGGAGTGGGTGGCTTCTTAT 3'TCCCGTAGTCACCGTAGTCA
EKA	IgA	3	5'AAAGCAAAATTGATGGTGGG 3'TCTGAGGTCCTTGACCCTGT
ELF	IgG	3	5'CCATCAACTACATTGCTGACA 3'GCAGCCACCTCCATTACAA
EVP*	IgG	3	5'TTAGTTGGAATAGTGGGAACATAGA 3'GCCCGACTTTTGCACAGTAAT
GEV*	(lambda)	4	5'GTCTCTGGTGCCAGTGGTG 3'ACTGAATAGGCAAGAAGACTC
GML	IgG	3	5'TGGAAGCCAAAAATACTACGG 3'GGGATAACAGCCACTCCAAA
HSP*	IgG	1	5'GCCAATGGCAACACAAGATA 3'ATCGTAGCCACGAAATCCAA
IAP	IgG	3	5'AAGGGCCTGGAGTGGGTCTC 3'CCGGTCACCACTAAACCCGA
JHP	IgA	3	5'TTTATAGCGGTGGTAGGACATT 3'GTACCAGCACGCCACCAC
JLE	IgD	4	5'GGTCCATCTCCTACACTGGCG 3'AAAGCCACCGTAATCGGGGT
JOD	IgG	2	5'GGGATGATGATCAGCGCTAC 3'GCTGGGAAGACCACAATAGG
JOK	IgG	1	5'CGCTGCCAGTGGTAACACAAA 3'GGAGTCGATCCAATTTCGATTTTT
JUW	IgA	3	5'AGACTCAATGAAGGCCGAT 3'CCAGTATGAATAACTCCCTGAACC
LAB	IgG	5	5'TGGGTGGGAATCATCTATCC 3'CCGATCGGTAACCGTGATAA

LAR	IgA	4	5'ACTTCTACGACAATGGCGAAAC 3'CCCTGGGAGGACGTGGTG
MCO	IgG	1	5'CGTACTACGCACAGAAGTTCCA 3'CCGAAGTCGAAGTAAGTGGC
MFO	IgA	3	5'CTACGCAGACTCCATGAGG 3'CCAGACGTCCAGACCATAAT
ORS	IgA	1	5'GGATCATCCCTATCTTTGGTACAGCA 3'GACAAGGTAGTTGTAAAAGCAG
PEF	IgA	4	5'GAAGAACCGACTACAACCCG 3'CCCCATGTAGCAGCAACG
RAM*	IgG	3	5'GTGGTGGGACAACAGACTCC 3'GCTCACTGAACGCTGTGGTA
REH	IgG	1	5'ACTACGCACAGAAGTTCCCG 3'AGTCGTAGAAGCCCCCAGTC
SBA	IgG	3	5'TTCATATGATGGAAGCAATCAATT 3'TACCGTCACGGTGTTCCCA
STK	IgG	4	5'TAGTGGGACTACCCATTACAACC 3'CACGGAGTTACCACCGTACA

*) – PATIENT DECEASED

Table 6.2 Relative frequency of clonotypic isotypes in the blood and bone marrow of a MM patient.

RNA source	primers used	number of sequences analyzed	status
Blood	Vh3 leader/μ	28	none clonotypic
Blood	Vh3 leader/δ	18	none clonotypic
Blood	Vh3 leader/γ	24	all clonotypic
Blood	Vh3 leader/α	24	none clonotypic
Bone marrow	Vh3 leader/μ	8	none clonotypic
Bone marrow	Vh3 leader/δ	16	none clonotypic
Bone marrow	Vh3 leader/γ	12	all clonotypic
Bone marrow	Vh3 leader/α	12	none clonotypic

Table 6.3 Detection of pre and post-switch clonotypic isotypes in the blood and bone marrow of MM patients at diagnosis

		Blood				Bone marrow			
Patient code	Clinical isotype	IgM	IgD	IgG	IgA	IgM	IgD	IgG	IgA
EKA	IgA	+	+	+	+	+	+	+	+
EVP	IgG	+	+	+	+	+	+	+	+
JOK	IgG	+	+	+	+	+	+	+	+
LAB	IgG	+	+	+	+	+	+	+	+
REH	IgG	+	+	+	+	+	+	+	+
JHP	IgA	+	+	+	+	+	+	+	+
IAP	IgG	+	+	+	+	+	+	+	+
GEV	BJ	+	+	+	+	+	+	+	+
PEF	IgA	+	+	+	+	+	+	+	+
ELF	IgG	+	+	+	+	+	+	+	+
HSP	IgG	-	-	+	+	+	+	+	+
ORS	IgA	-	-	-	+	+	+	+	+
STK	IgG	-	-	-	-	-	-	+	-

Table 6.4 Expression of pre- and post-switch clonotypic isotypes post diagnosis in MM patients

Sample	Source	Treatment	Date	IgM	IgD	IgG*	IgA
MCO-30	BLOOD	Off	21-Mar-97	-	-	+	-
MCO-35	BLOOD	On	27-Aug-97	-	-	-	-
MCO-35	BLOOD	Off	24-Sep-97	+	-	+	+
MCO-38	BLOOD	Off	18-Mar-98	-	-	+	-
MCO-40	BLOOD	Off	7-Aug-98	+	-	+	+
MCO-41	BLOOD	On	18-Sep-98	-	-	+	-
MCO-42	BLOOD	On	16-Oct-98	-	-	+	-
MCO-44	BLOOD	On	11-Dec-98	-	-	+	-
MCO-45	BLOOD	On	8-Jan-99	-	-	+	-
MCO-46	BLOOD	On	5-Feb-99	-	-	+	-
MCO-47	BLOOD	Off	5-Mar-99	-	-	+	-
MCO-48	BLOOD	Off	11-Jun-99	-	-	+	-
Sample	Source		Date	IgM	IgD	IgG*	IgA
REH-1	BM	Unt	12-Mar-97	+	+	+	+
REH-1	BLOOD	Unt	12-Mar-97	+	+	+	+
REH-2	BLOOD	On	17-Apr-97	+	+	+	+
REH-4	BLOOD	On	6-Aug-97	+	+	+	+
REH-7	BLOOD	On	10-Sep-97	+	+	+	+
REH-8	BLOOD	On	8-Oct-97	+	+	-	+
REH-9	BLOOD	On	5-Nov-97	+	+	+	+
REH-13	BLOOD	On	4-Mar-98	+	+	+	+
REH-15	BLOOD	Off	3-Jul-98	+	+	+	+
REH-16	BLOOD	Off	25-Sep-98	+	+	+	+
REH-17	BLOOD	Off	15-Nov-98	+	+	+	+
REH-19	BLOOD	Off	17-Mar-99	+	+	+	+
REH-20	BLOOD	Off	14-Apr-99	+	+	+	+
Sample	Source		Date	IgM	IgD	IgG	IgA*

JHP-1	BLOOD	Unt	11-Jun-97	+	+	+	+
JHP-1	BM	Unt	11-Jun-97	+	+	+	+
JHP-2	BLOOD	On	8-Aug-97	+	+	+	+
JHP-3	BLOOD	On	3-Oct-97	+	+	+	+
JHP-4	BLOOD	On	6-Jan-98	+	+	+	+
JHP-5	BLOOD	Off	4-Mar-98	+	+	+	+
JHP-6	BLOOD	Off	23-Jun-98	+	+	+	+
JHP-9	BLOOD	Off	19-Aug-98	+	+	+	+
JHP-10	BLOOD	Off	16-Sep-98	+	+	+	+
JHP-11	BLOOD	Off	14-Oct-98	+	-	-	+
JHP-12	BLOOD	Off	10-Nov-98	+	-	-	+
JHP-13	BLOOD	Off	9-Dec-98	+	+	+	+
JHP-14	BLOOD	Off	6-Jan-99	+	+	-	+
JHP-15	BLOOD	Off	3-Feb-99	+	-	+	+
JHP-16	BLOOD	Off	3-Mar-99	+	+	+	+
JHP-17	BLOOD	Off	31-Mar-99	+	+	+	+
JHP-18	BLOOD	Off	28-Apr-99	+	+	+	+
JHP-19	BLOOD	Off	26-May-99	+	+	+	+

*= clinical isotype

Table 6.5 Relative frequency of clonotypic isotypes in the mobilized blood of MM patients.

Patient code	Clinical isotype	clonotypic transcripts/ # of IgH transcripts analyzed			
		IgM	IgD	IgG	IgA
BBE	IGG	0/12	0/12	12/12	0/12
ELF	IGG	0/12	0/12	5/12	0/12
SBA	IGG	0/24	0/24	20/26	0/24
RAM	IGG	0/25	0/25	25/25	0/25

Table 6.6 Expression of clonotypic isotypes in G-CSF-mobilized blood of MM patients

Patient code	Clinical isotype	IgM	IgD	IgG	IgA
BBE	IgG	+	+	+	+
ATM	IgG	+	+	+	+
ATM cryopreserved	*IgG*	+	-	+	-
PEF	IgA	+	+	+	+
SBA	IgG	+	+	+	+
RAM	IgG	+	+	+	+
JOD	IgG	+	+	+	+
JOD cryopreserved	*IgG*	-	-	+	-
MFO	IgA	+	+	+	+
JLE	IgD	+	+	-	-
ORS	IgA	-	-	-	+

Table 6.7 Some diversified pre-switch clonotypic sequences are detectable in mobilized blood and progenitor-enriched fraction

Patient code	# of diversified transcripts/ # of clonotypic transcripts			
	IgM	IgD	IgG	IgA
JOD	1/24	5/34	0/24	0/24
ATM	0/25	0/25	0/23	0/25

A unfractionated MPB

Patient code	# of diversified transcripts/ # of clonotypic transcripts			
	IgM	IgD	IgG	IgA
PEF-enr	2/20	6/19	0/24	0/24
ATM-enr	0/23	0/25	0/23	n.d.

B progenitor-enriched fraction of MPB

Table 6.8 A Expression of pre- and post-switch clonotypic isotypes at multiple time-points in MM patients pre and post-transplant (pattern #1)

Sample	Source	Date	treatment	IgM	IgD*	IgG	IgA
JLE-01	BLOOD	31-Jan-96	Unt	+	+	+	+
JLE-02	BLOOD	28-Feb-96	On	+	+	-	+
JLE-03	BLOOD	3-Apr-96	On	-	+	-	-
JLE-04	BLOOD	10-May-96	On	+	+	-	-
JLE-05	BLOOD	14-Jun-96	On	-	-	-	-
JLE-06	BLOOD	12-Jul-96	On	-	-	-	-
JLE-07	BLOOD	6-Sep-96	Off	-	-	-	-
JLE-07	BM	6-Sep-96	Off	+	+	+	-
JLE-08	BLOOD	10-Jan-97	Off	-	+	-	-
JLE-09	BLOOD	2-May-97	Off	-	+	-	-
JLE-10	BLOOD	26-Sep-97	Off	+	+	-	-
JLE-11**	BLOOD	12-Nov-97	Off	+	+	-	-
JLE-12	BLOOD	9-Jan-98	On	+	+	-	-
JLE-13	BLOOD	13-Feb-98	On	+	+	-	-
transplantation	June 1998						
JLE-17	BLOOD	21-Aug-98	Off	-	-	-	-
JLE-18	BLOOD	20-Nov-98	Off	-	-	-	-
JLE-19	BLOOD	30-Apr-99	Off	-	-	-	-

** clinical relapse

Sample	Source	Date	Treatment	IgM	IgD	IgG	IgA*
LAR 06	BM	17-May-96	On	+	+	+	+
transplantation	Oct. 1996						
LAR 13	BLOOD	9-Jul-97	Off	-	-	-	-
LAR 19	BLOOD	2-Jul-98	Off	-	-	-	-
LAR 21	BLOOD	19-Oct-98	Off	-	-	-	-
LAR 22	BLOOD	16-Nov-98	Off	-	-	-	-
LAR 23	BLOOD	11-Dec-98	Off	-	-	-	-
LAR 24	BLOOD	1 Feb-99	Off	-	-	-	-
LAR 25	BLOOD	1-Mar-99	Off	-	-	-	-
LAR 27	BLOOD	30-Apr-99	Off	-	-	-	-

Sample	Source	Date	Treatment	IgM	IgD	IgG	IgA*
ORS-01	BLOOD	26-Mar-97	Unt	-	-	-	+
ORS-02	BM	10-Oct-97	On	+	+	+	+
ORS-03	BLOOD	3-Dec-97	On	-	-	-	+
ORS-07	BM	5-May-98	On	-	-	-	+
transplantation	May 1998						
ORS-11	BLOOD	6-Jan-99	Off	-	-	-	-
ORS-12	BLOOD	5-May-99	Off	-	-	-	-

Table 6.8 B Expression of pre- and post-switch clonotypic isotypes at multiple time-points in MM patients pre and post-transplant (pattern #2)

Sample	Source	Date	Treatment	IgM	IgD	IgG*	IgA
ATM-01	BLOOD	7-Aug-98	Unt	+	+	+	+
ATM-04	BLOOD	23-Feb-99	On	-	-	+	-
ATM-04	BM	23-Feb-99	On	+	+	+	+
transplantation	Feb. 1999						
ATM-06	BLOOD	5-Mar-99	Off	-	-	+	-
ATM-07	BLOOD	10-Mar-99	Off	-	-	+	-
ATM-08	BLOOD	26-Mar-99	Off	-	-	+	-
ATM-09	BLOOD	23-Apr-99	Off	-	-	+	-

Sample	Source	Date	Treatment	IgM	IgD	IgG*	IgA
IAP-01	BLOOD	9-Oct-97	Unt	+	+	+	+
IAP-03	BM	13-Nov-97	Unt	+	+	+	+
IAP-04	BLOOD	10-Dec-97	On	-	-	+	-
IAP-06	BLOOD	11-Feb-98	On	-	-	+	-
IAP-07	BLOOD	13-Mar-98	On	-	-	+	-
transplantation	June 1998						
IAP-09	BLOOD	17-Jun-98	Off	-	-	+	-
IAP-14	BLOOD	26-Mar-99	Off	-	-	+	-

Sample	Source	Date	Treatment	IgM	IgD	IgG*	IgA
SBA-02	BLOOD	27-May-98	Unt	+	+	+	+
SBA-04	BLOOD	22-Jul-98	On	+	+	+	+
SBA-06	BLOOD	16-Sep-98	On	+	+	+	+
SBA-09	BLOOD	18-Nov-98	On	+	+	+	+
transplantation	Nov. 1998						
SBA-12	BLOOD	1-Dec-98	Off	-	-	+	-
SBA-13	BLOOD	8-Dec-98	Off	-	-	+	-
SBA-14	BLOOD	14-Dec-98	Off	-	-	+	-
SBA-15	BLOOD	6-Jan-99	Off	-	-	-	-
SBA-16	BLOOD	7-Apr-99	Off	-	-	+	-

Table 6.8 C Expression of pre- and post-switch clonotypic isotypes at multiple time-points in MM patients pre and post-transplant (pattern #3)

Sample	Source	Date	Treatment	IgM	IgD	IgG*	IgA
BBE-59	BLOOD	23-Sep-96	On	+	+	+	-
BBE-60	BLOOD	30-Dec-96	On	+	+	+	-

BBE-62	BLOOD	15-Jan-97	**On**	+	+	+	+
BBE-63	BLOOD	12-Mar-97	**On**	+	+	+	+
transplantation	Apr. 1997						
BBE-66	BLOOD	30-May-97	**Off**	+	+	+	+
BBE-67	BLOOD	2-Jul-97	**Off**	-	-	-	-
BBE-69	BLOOD	19-Nov-97	**Off**	-	+	-	-
BBE 72	BLOOD	16-Apr-98	**Off**	+	+	+	+
BBE-73	BLOOD	21-May-98	**Off**	+	+	+	-
BBE-74	BLOOD	22-Jun-98	**Off**	+	+	+	-
BBE-76	BLOOD	28-Oct-98	**Off**	+	+	+	-
BBE-77	BLOOD	20-Jan-99	**Off**	-	+	+	-

Sample	Source	Date	Treatment	IgM	IgD	IgG	IgA
GEV-01	BM	8-Jul-97	**Unt**	+	+	+	+
GEV-01	BLOOD	8-Jul-97	**Unt**	+	+	+	+
GEV-02	BLOOD	1-Aug-97	**Unt**	+	+	+	+
GEV-03	BLOOD	29-Aug-97	**On**	+	+	+	+
GEV-04	BLOOD	1-Oct-97	**On**	+	-	+	+
GEV-05	BLOOD	5-Nov-97	**On**	+	-	+	+
transplantation	Mar 1998						
GEV-09	BLOOD	20-May-98	**Off**	+	+	+	+
GEV-11	BLOOD	-98	**Off**	+	+	+	+

Sample	Source	Date	treatment	IgM	IgD	IgG*	IgA
JOD-02	BM	4-May-90	**On**	-	-	+	-
JOD-06	BLOOD	26-Oct-96	**On**	-	-	+	-
JOD-09	BLOOD	24-Apr-97	**On**	-	-	+	-
JOD-09	BM	24-Apr-97	**On**	+	+	+	+
transplantation	Aug. 1997						
JOD-16	BLOOD	8-Sep-97	**Off**	+	-	+	+
JOD-17	BLOOD	3-Oct-97	**Off**	-	-	+	-
JOD-18	BLOOD	17-Apr-98	**Off**	-	-	+	-
JOD-19	BLOOD	6-Nov-98	**Off**	+	-	+	+
JOD-20	BLOOD	23-Apr-99	**Off**	+	+	+	+

Sample	Source	Date	Treatment	IgM	IgD	IgG	IgA*
PEF-01	BLOOD	23-Mar-98	**Unt**	+	+	+	+
PEF-01	BM	23-Mar-98	**Unt**	+	+	+	+
PEF-02	BLOOD	5-Jun-98	**On**	+	+	+	+
PEF-03	BLOOD	5-Aug-98	**On**	+	+	+	+
PEF-03	BM	5-Aug-98	**On**	+	+	+	+

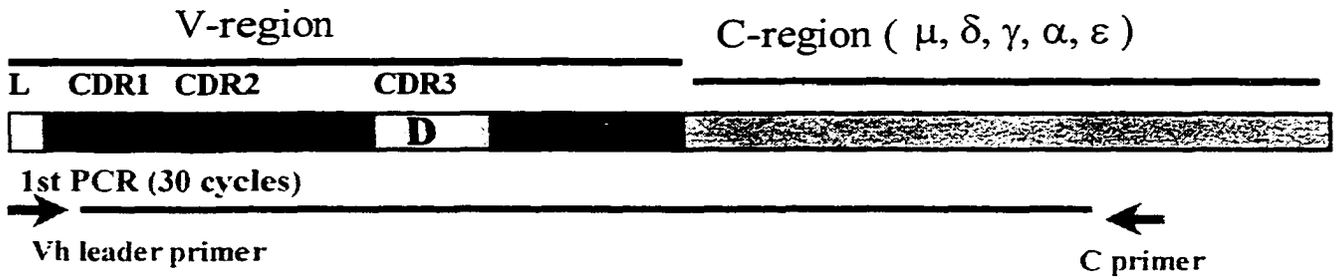
transplantation	Aug.1998						
PEF-05	BLOOD	11-Sep-98	Off	+	+	-	+
PEF-06	BLOOD	23-Apr-99	Off	+	+	+	+

Sample	Source	Date	Treatment	IgM	IgD	IgG*	IgA
RAM-12	BLOOD	12-Apr-96	Off	+	+	+	+
RAM-13	BLOOD	10-May-96	Off	+	+	+	+
RAM-14	BLOOD	19-Jun-96	On	-	+	+	+
RAM-20	BLOOD	13-Dec-96	On	+	+	+	+
RAM-20	BM	13-Dec-96	On	+	+	+	+
transplantation	Dec. 1996						
RAM-24	BLOOD	25-Jun-97	Off	+	+	+	-
RAM-26	BLOOD	3-Oct-97	Off	+	+	+	+
RAM-27	BLOOD	14-Jan-98	Off	+	+	+	+
RAM-29	BLOOD	12-Aug-98	Off	+	+	+	+
RAM-32	BLOOD	2-Oct-98	Off	+	+	+	+
RAM-33	BLOOD	30-Oct-98	Off	+	+	+	+
RAM-35	BLOOD	21-Jan-99	Off	+	+	+	+
RAM-36	BLOOD	26-Mar-99	Off	+	+	+	+
RAM-36	BM	26-Mar-99	Off	+	+	+	+
RAM-38	BLOOD	19-May-99	Off	+	+	+	+

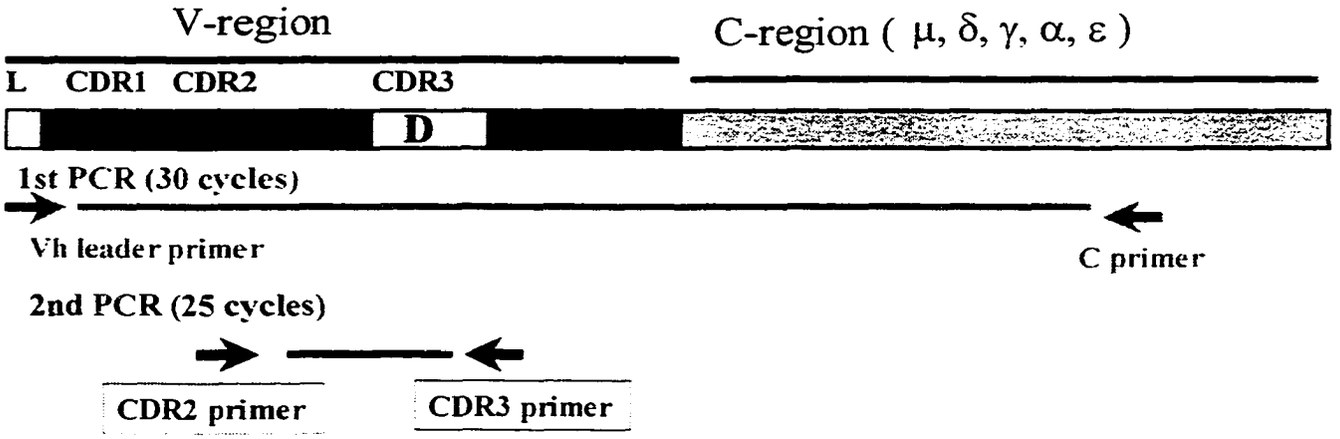
Sample	Source	Date	Treatment	IgM	IgD	IgG*	IgA
ELF-1	BLOOD	23-May-97	Unt	+	+	+	+
ELF-1	BM	23-May-97	Unt	+	+	+	+
ELF-2	BLOOD	04-Jul-97	On	+	+	+	+
ELF-3	BLOOD	01-Aug-97	On	+	+	+	+
ELF-4	BLOOD	29-Aug-97	On	+	+	+	+
ELF-5	BLOOD	26-Sep-97	On	+	+	+	+
ELF-6	BLOOD	17-Oct-97	On	+	+	+	+
ELF-6	BM	17-Oct-97	On	+	+	+	+
ELF-7	BLOOD	06-Jan-98	On	+	+	+	+
transplantation	Jan-98						
ELF-8	BLOOD	11-Feb-98	Off	+	+	+	+
ELF-9	BLOOD	15-Apr-98	Off	-	-	+	-
ELF-10	BLOOD	24-Jun-98	Off	-	-	+	-
ELF-11	BLOOD	26-Aug-98	Off	-	-	+	-
ELF-12	BLOOD	13-Jan-99	Off	+	+	+	+

* - clinical isotype
Unt - untreated

Strategy A



Strategy B



Strategy C

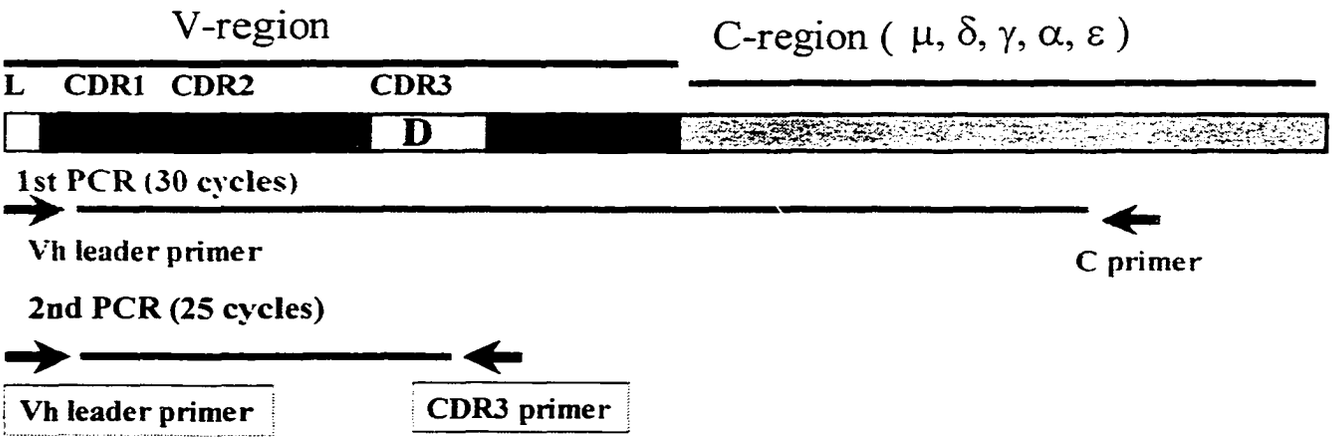


Figure 6.1 RT-PCR strategies used for amplification

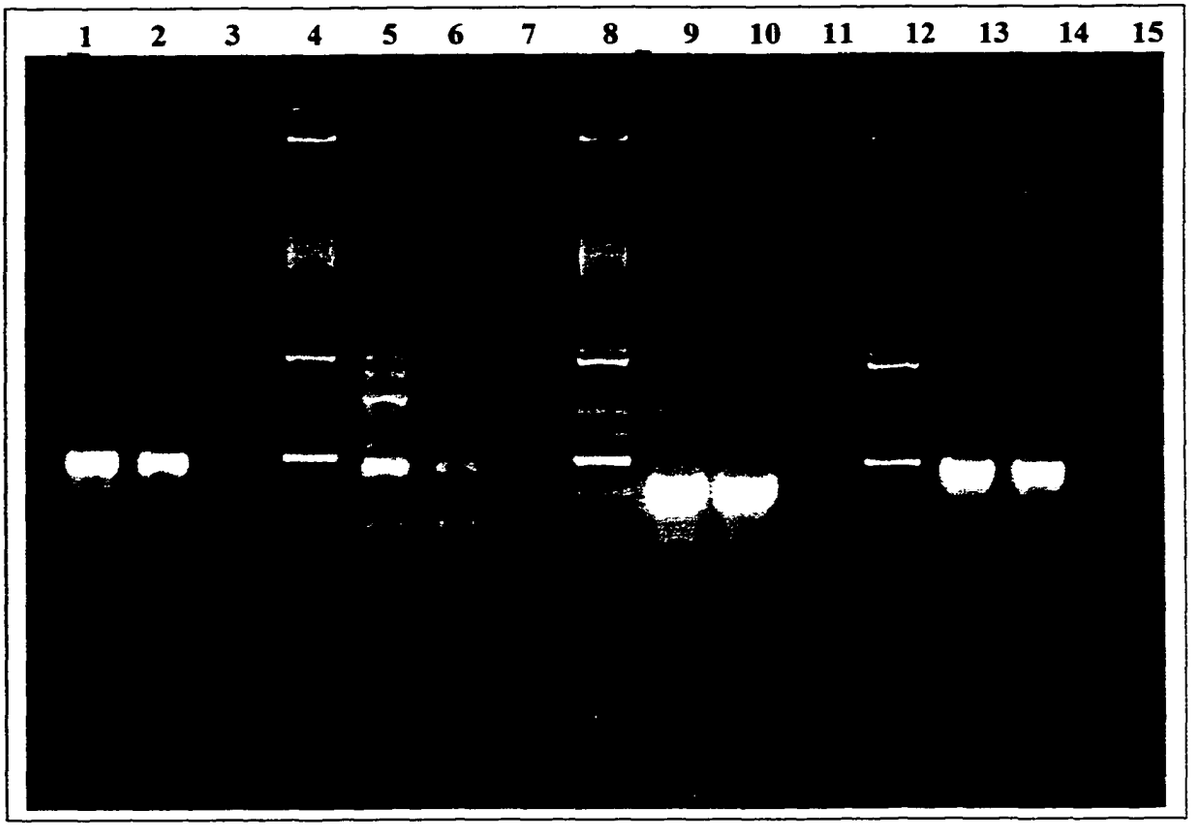


Figure 6.2 RT-PCR amplification of clonotypic isotypes

RNA was isolated from blood and bone marrow samples of patient ELF at the time of diagnosis, reverse transcribed and amplified with Vh4-specific leader primer together with each of the constant chain-specific primers. Shown here are PCR products resolved on 2% agarose gel stained with ethidium bromide. ; Line 1: blood/Vh3-leader/ IgM, Line 2: BM/Vh3-leader/ IgM, Line 3: neg. control, Line 4: DNA marker, Line 5: blood/Vh3-leader/ IgD, Line 6: BM/Vh3-leader/ IgD, Line 7: neg. control, Line 8: DNA marker, Line 9: blood/Vh3-leader/ IgG, Line 10: BM/Vh3-leader/ IgG, Line 11: neg. control, Line 12: DNA marker, Line 13: blood/Vh3-leader/ IgA, Line 14: BM/Vh3-leader/IgA, Line 15:neg control.

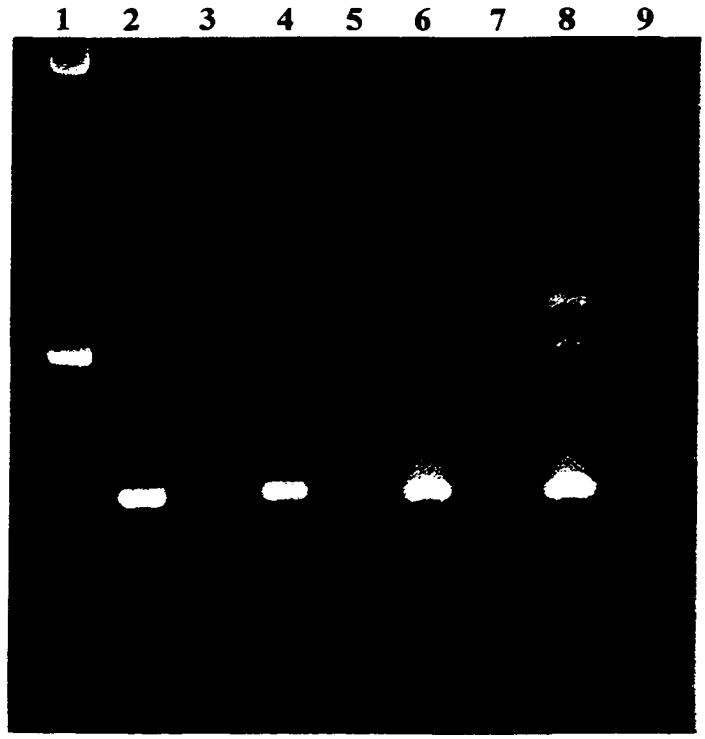


Figure 6.3 Nested RT-PCR amplification of clonotypic transcripts obtained from the progenitor -enriched fraction of MPB from MM patient (PEF).

RNA obtained from MPB cells was subjected to reverse transcription and semi-nested PCR. The first PCR was done with Vh-specific and constant chain-specific primers. In the secondary PCR Vh-specific and CDR3-patient specific primers were used. Shown here is a scan of 2% agarose gel electrophoresis. It visualizes final PCR products of the appropriate size. ; Line 1: DNA marker, Line 2: Vh3-μ-CDR3, Line 3: neg. control, Line 4: Vh3-δ-CDR3, Line 5: neg. control, Line 6: Vh3-γ-CDR3, Line 7: neg. control, Line 8: Vh3-α-CDR3, Line 9: neg. control

```
                                        -CDR1-                  ------CDR2------
.....1.......10........20........30..........40........50........60....
GL    QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALEWLALIYWDDDKRYGPSL
PC    .....M....A....tE.............TtN.................l.V.F.N..Q.....
IgD1  .....M....A....tE.............TtN.................Rl.V.F.N..Q.....
IgD3  .....M....A....tE.............TtN.................l.V.F.N..Q....E
IgD2  ..................................................l...F.N..Q....E
IgM11 ..................................................l.V.F.N..Q.....
IgD4  .....M....A....tE.............TtN.................V.F.N..Q.....
IgD21 ..........................................................N..Q.....
IgD14 ..............................N...................V.F.N..Q.....
IgD10 ...........................TtN...................V.A.N..Q.....

....70........80..............94
KSRLTIKDTSKNQVVLTMTNMDPVDTATYYCAH
NrV.i......n...l......A.....yy..Y
NrV.i......n...l......A.....yy..Y
NrV.i......n...l......A.....yy..Y
NrV.i......n...l......A.....yy..Y
NrV.i......n...l......A.....yy..Y
NrV.i......n...l......A.....yy..Y
NrV.i......n...l......A.....yy..Y
NrV.i......n...l......A.....yy..Y
N...i......n...l......A.....yy..Y
```

JOD

Figure 6.4A Deduced amino acid sequences of the Vh regions of the plasma cell-derived and mobilized blood-derived clones from MM patient JOD.

Uppercase – replacement mutations, lowercase – silent mutations, highlighted are the differences between tumor-derived (PC) sequence and individual clones

```
                                          -CDR1-                  ------CDR2------
      1.......10.........20.........30.........40.........50.........60.....
GL    QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGRTNYNPSLKS
PC    ..q.....................V.H.....N.F.....................P..S.D.......
IgD11 ..............................NNV.....................P..S.D.......
IgD17 K.q.....................V.H.....g.F.....................P..S.D.......
IgD14 ..q.................................N.....................P..S.D.......
IgD6  ...lRES.PV.A..........V.H.....g.F.....................P..S.D..p....
IgD4  ..............................ND..........R.....................
IgM14 ..q.....................V.HS....N.F.....................p..S.D.......
IgM1  ..q............s.......V.H.....N.F.....................p..S.D.n.....

...70.........80.........87.....94
RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
.....v..s...I..R....A.....L....S
.....v..s...I..R....A.....L....S
.....v..s...I..R....A.....L....S
.....v..s...I..R....A.....L....S
.....v..s...I..R....A.....L....S
r.S..A.........Rls.......aA.F..S
.....v..s...IF.R....A.....L....S
.....v..s...I..R....A.....L....S
```

PEF

Fig. 6.4B Deduced aminoacid sequences of the Vh regions of the plasma cell-derived and mobilized blood-derived clones from MM patient PEF.

Uppercase – replacement mutations, lowercase – silent mutations, highlighted are the differences between tumor-derived (PC) sequence and individual clones.

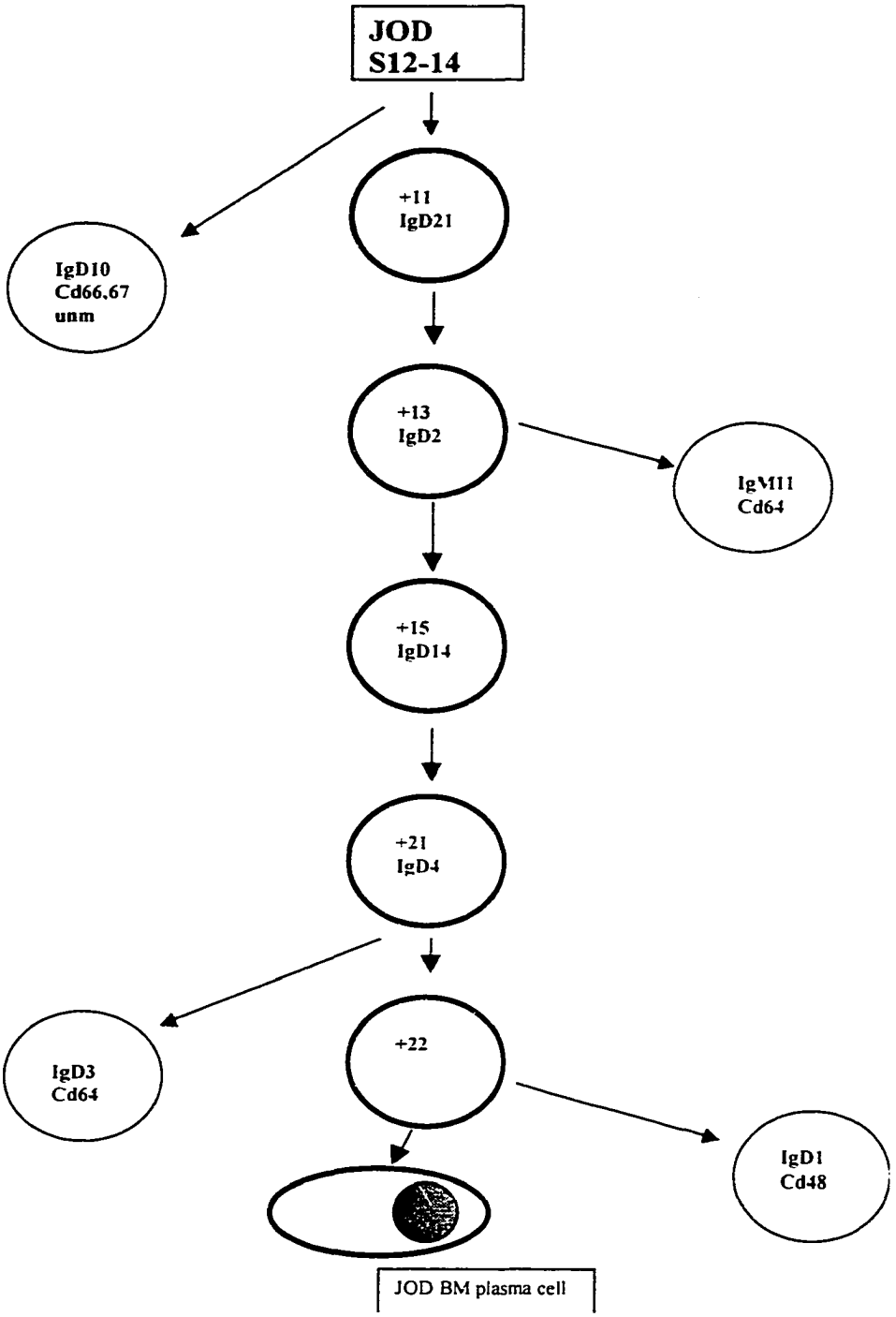


Fig. 6.4C Intraclonal diversity in JOD.

Genealogical trees for the cells exhibiting intraclonal diversity within mobilized blood and hematological progenitor-enriched MPB populations.
Vh used by the MM cells had the closest homology to the germline segment designated Vh2 (S12-14)

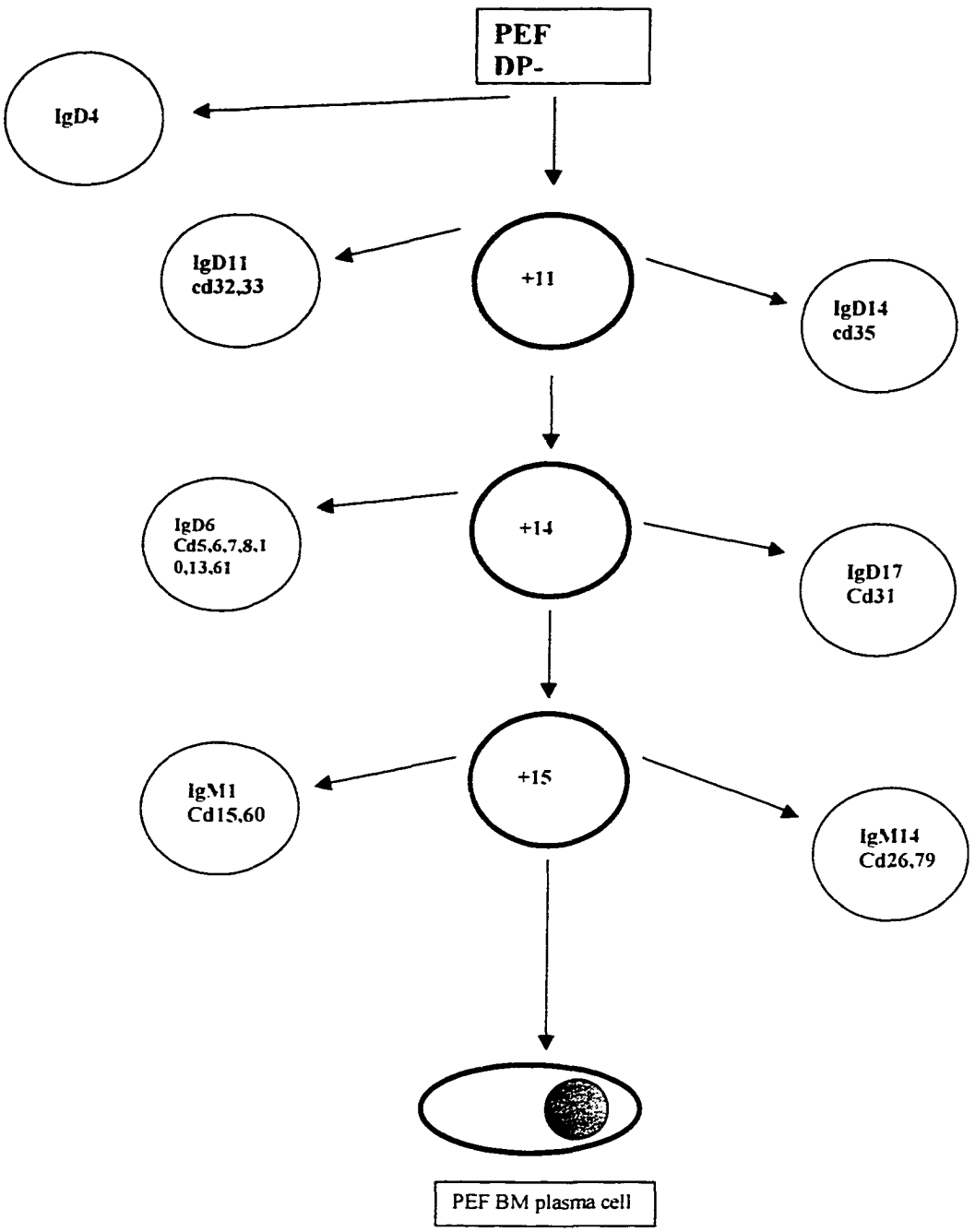


Fig. 6.4D Intraclonal diversity in PEF.

Genealogical trees for the cells exhibiting intraclonal diversity within mobilized blood and hematological progenitor-enriched MPB populations.
Vh used by the MM cells had the closest homology to the germline segment designated Vh4 (DP63, Vh4.34).

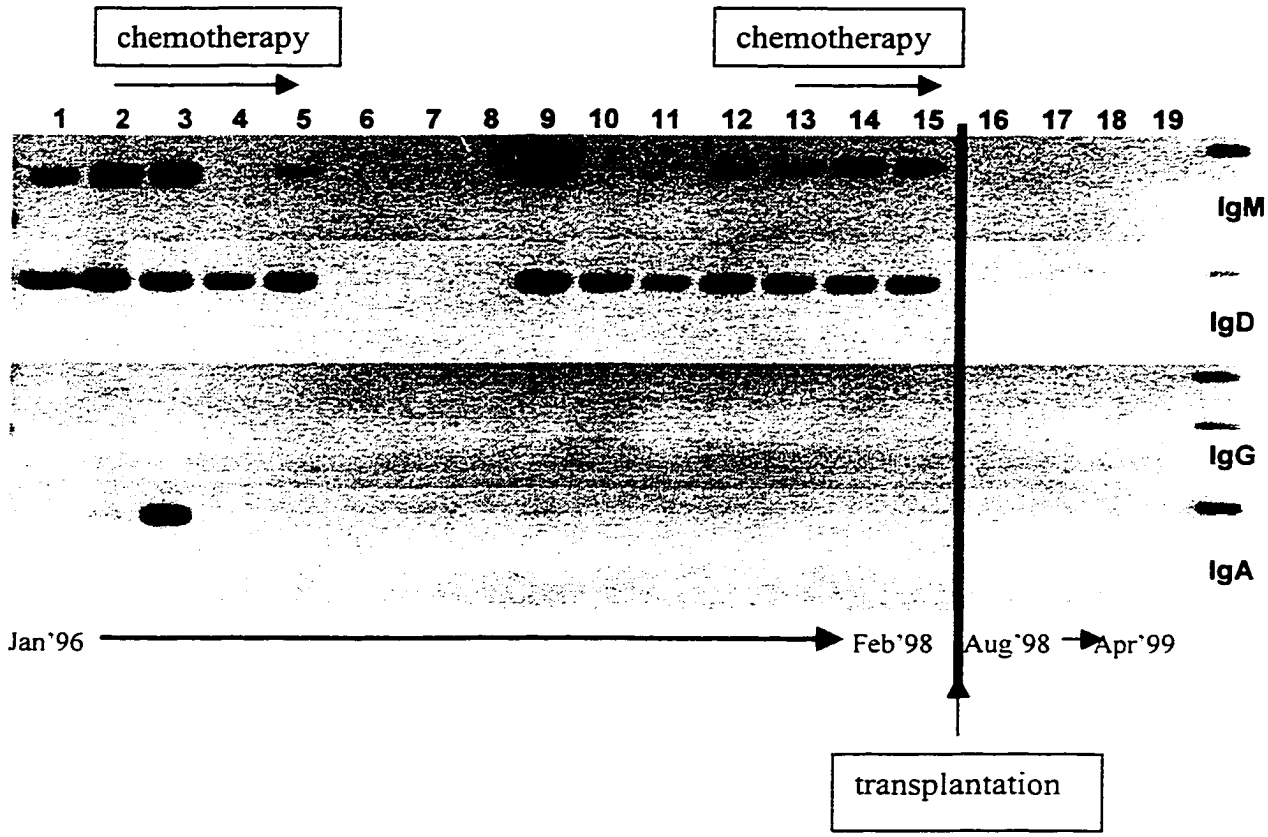


Figure 6.5A Expression of pre and post-switch clonotypic transcripts in blood of patient JLE as a representation of pattern 1

(time points as listed in table 6.8A). Line 19 - negative control.

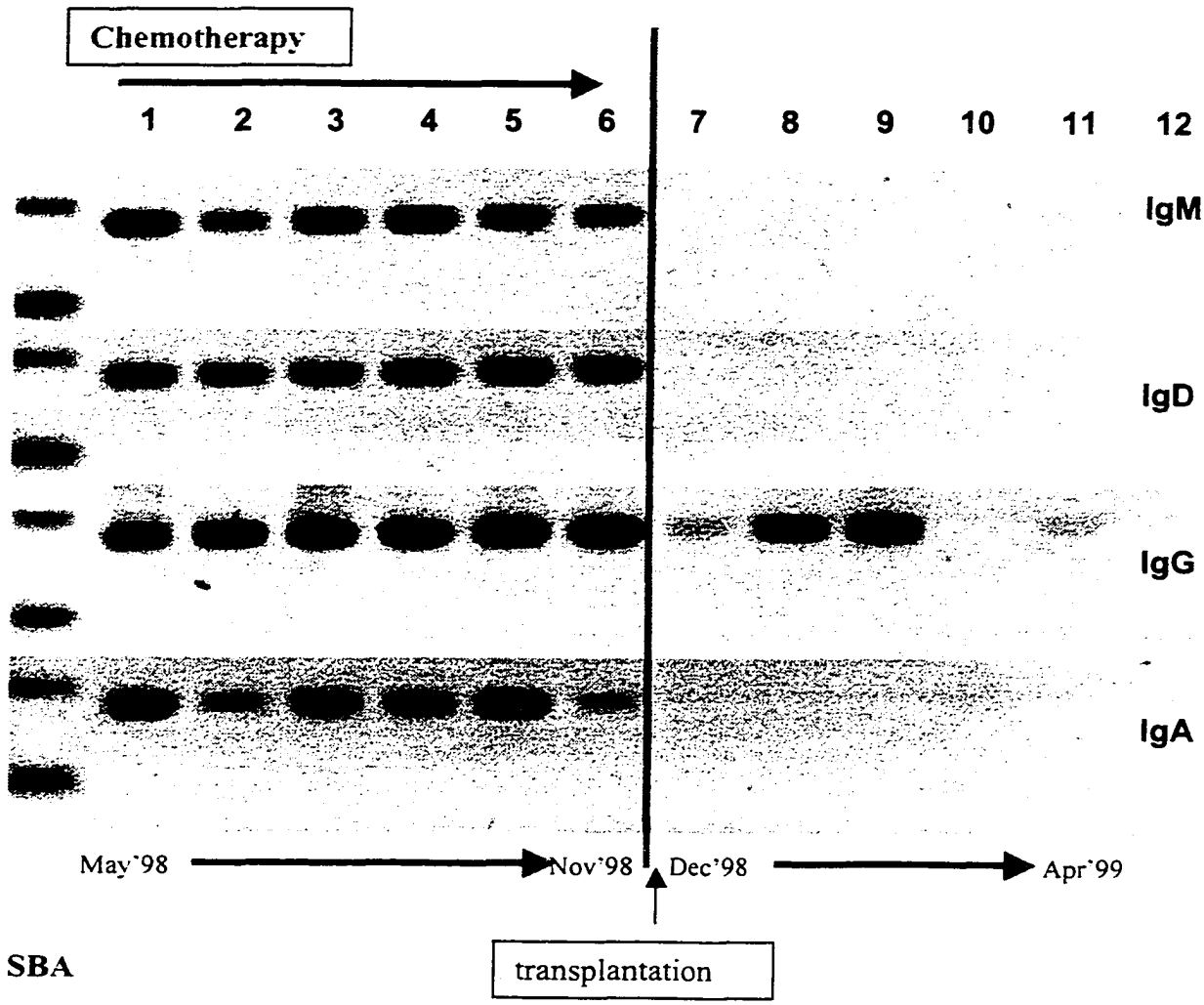


Figure 6.5B Expression of pre and post-switch clonotypic transcripts in blood of patient SBA as a representation of pattern 2

(time points as listed in table 6.8B). Line 12 - negative control.

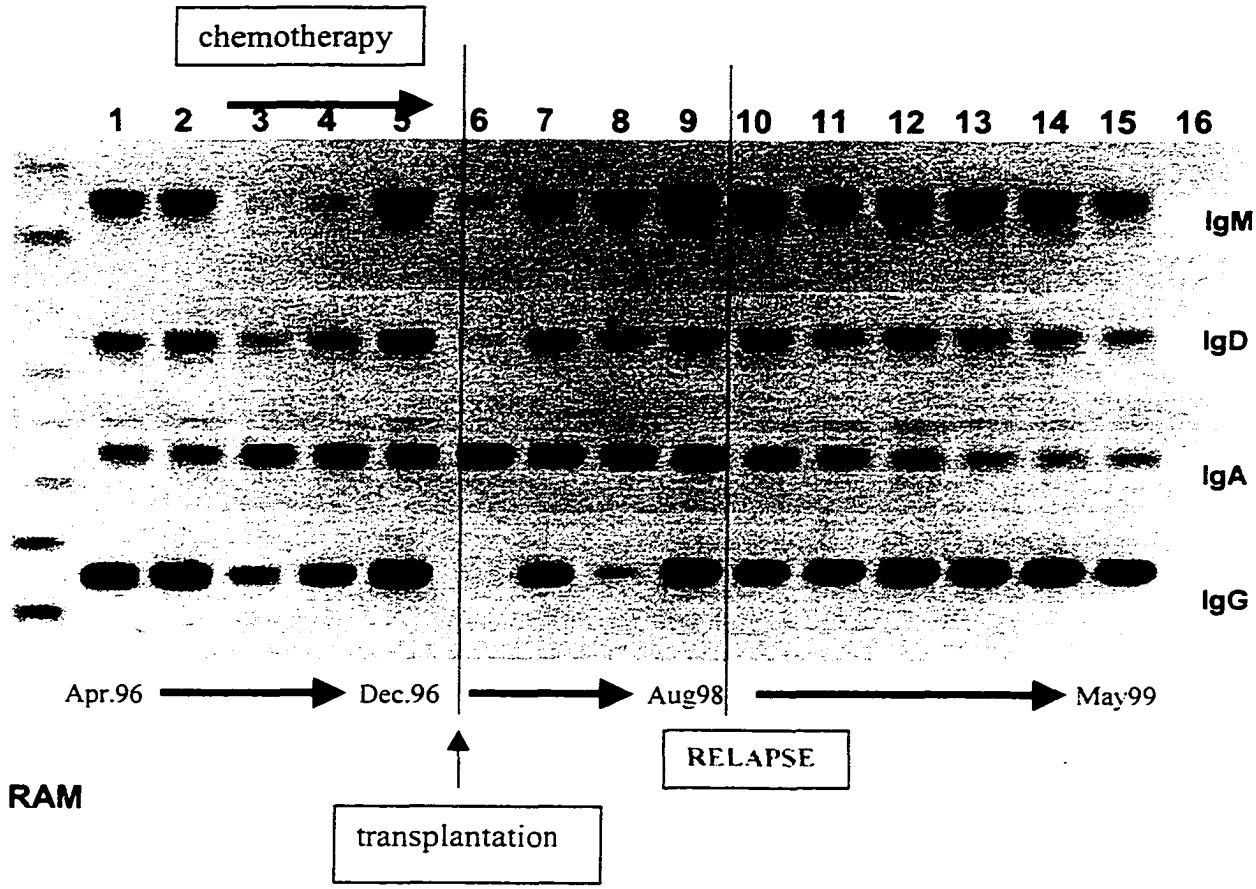


Figure 6.5C Expression of pre and post-switch clonotypic transcripts in blood of patient RAM as a representation of pattern 3

(time points as listed in table 6.8C). Line 16 - negative control.

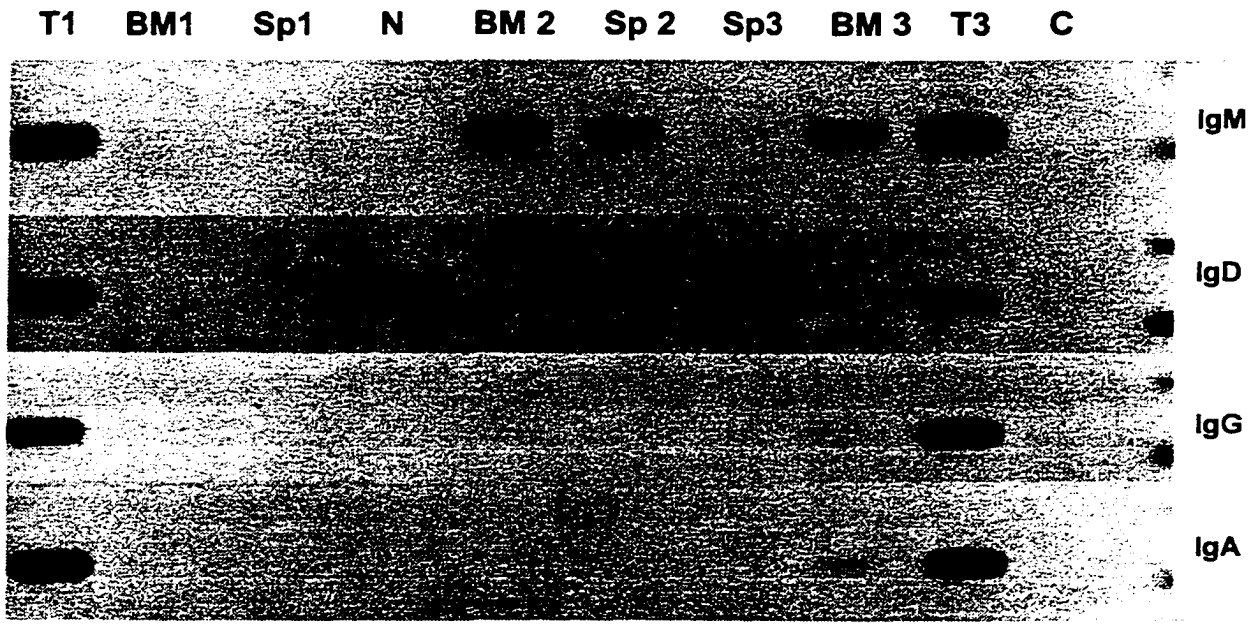


Figure 6.6 RT-PCR amplification of clonotypic isotypes in xenotransplanted animal model of human MM.

RNA was isolated from the tissues of three mice that have been injected with unfractionated PBMCs from patient GEV, and from one control mouse. After reverse transcription, cDNA was first amplified with Vh4leader and constant chain-specific primers and next with CDR2/CDR3 patient specific primers. Shown here is the analysis of PCR product on 2% agarose gel stained with ethidium bromide

T = tumor

Sp = spleen

N = normal mouse

C = no RNA control

VII. GENERAL DISCUSSION AND FUTURE DIRECTIONS

Multiple myeloma is a malignancy affecting cells of the B-lymphocyte lineage. There has been little improvement in 5-year survival over the past 40 years [186]. The cause of MM is still unknown. Despite radiotherapy, chemotherapy and bone marrow transplantation MM remains an incurable disease. Although the terminally differentiated lymphocyte, the plasma cell, is morphologically identified as the malignant cell in MM, research has focused on the plasma cell precursors that circulate in peripheral blood.

The work described in this dissertation was intended to identify and characterize circulating B cells related to malignant plasma cells determined by expression of monoclonal Ig gene.

The notion that clonotypic B cells may not in a major way contribute to pathology of MM [92] was mainly based on conflicting evidence about their numbers in blood [64 89 92 93]. Early studies by Kubagawa and Mellstedt using patient-specific antibodies raised against the respective MM monoclonal proteins showed that almost half of the B lymphocytes express the clonal immunoglobulin [64 65]. The use of Southern blotting for the detection of clonally rearranged Ig genes from MM PBMC generated a wide scope of data, ranging from the complete absence of clonal DNA in blood [82] to its almost universal presence [83]. The semi-quantitative PCR applied for the enumeration of clonotypic B cells has not resolved the issue but generated even more conflicting evidence, in some instances detecting as many as 32% of mononuclear cells as clonal [89] or as low as 0.34% [92]. The factors most likely responsible for these discrepancies are the handling of samples (fresh

or frozen), different technical approaches in defining the clonotypic sequence and varying criteria for selection of B cells based on surface marker expression and morphology.

The first part of this dissertation concentrated on establishing reliable methods to characterize the clonal VhDJh IgH from MM plasma cells. We showed that obtaining the Ig sequence from single bone marrow PC improves the overall outcome of the consensus RT-PCR reaction. The sequencing analysis of single PC RT-PCR product reflects the true proportion of the clonotypic versus normal cells. Our success rate in deriving patient-specific clonotypic Ig was 96%. The patient-specific primers that anneal to the CDR2 and CDR3 portions of IgH V-region greatly increase the specificity of PCR. We felt that it was of critical importance to assure that the derived Ig sequence was clonotypic and represented MM clone and not another frequent B cell population (for instance present in response to an infection). For that reason, several check points were designed and implemented. The primers were used in RT-PCR in the liquid phase single PC and *in situ* RT-PCR to check if a majority of cells contained the transcript. The specificity of primers was confirmed by absence of product amplification from irrelevant MM and healthy control cDNA. Using this rigorous strategy, the clonotypic sequences were identified for 53 patients.

As the research progressed, a set of patient-specific primers annealing to the clonotypic transcript was used to quantitate circulating B cells from 18 patients. The clonotypic cells consisted on average of 66% of B lymphocytes and 14% of PBMC, which was in agreement with the data obtained by other groups [64 65 89]. The opposing results obtained

by Chen and Epstein [92] can be explained by the fact, that the authors analyzed DNA purified from a very small quantities of cells, which usually results in great loss of nucleic acid; that only one of the primers used was patient-specific and finally, that the derived Ig sequence was never confirmed as a clonotypic one.

Further studies indicated that the clonotypic population of B cells was not homogenous and included cells at different maturational stages. Infrequent pre- and post-switch clonal transcripts of non-clinical isotype constant chain expressed together with clonotypic V-region were identified in blood and in the bone marrow at diagnosis and in G-CSF-mobilized blood. These cells persisted throughout the course of disease and their presence was associated with the progression of MM. Three distinct expression patterns of the pre- and post-switch clonotypic transcripts were identified in the patients that had undergone stem cell transplantation. The first pattern, in which no clonotypic transcript was detected in blood after transplantation was associated with the total remission. The second pattern, also associated with clinical remission, included expression of only the clinical isotype. The third pattern, where the pre- and post-switch clonotypic transcripts were identified (including the clinical isotype), corresponded with MM progression, clinical relapse and sometimes death. Our observations confirm the earlier findings by Kubagawa et al. and Mellstedt et al., where infrequent, idiotype positive cells were detected within IgM, IgD, IgG and IgA-expressing B lymphocytes, regardless of the clinical isotype [64 63]. Likewise, they are in agreement with the molecular data obtained by Billadeau et al. and Bakkus et al., where the pre- and post-switch clonotypic transcripts were amplified, cloned and sequenced [17 18]. The inability of Berenson's and Corradini's

groups to find the pre-switch clonotypic specimens in MM blood may be explained by their apparent low frequency and the fact that the authors were using only one round of PCR cycling, while we and the others used two rounds of nested PCR [103 104].

Based on the above findings, two hypothetical models of maturation within the MM clone can be proposed. For both models, clonally rearranged B cells undergo affinity maturation and acquire somatic hypermutations in the V-region. During this process, some of the clonally rearranged cells may accumulate mutations of different patterns resulting in intraclonal diversity. All of the analyzed transcripts obtained from blood or bone marrow were 100% homologous. However, G-CSF-mobilized blood contained infrequent diversified clonotypic species of mRNA in the pre-switch IgM and IgD fraction. We speculate that these transcripts are expressed by the remnants of the original B cell clone that was underwent clonal diversification and expansion during the normal response to an antigen. In this model, one of the clonal cells became a founder for MM cells.

According to the first hypothetical model, an IgM memory cell is generated during the antigen-dependant phase of differentiation. Upon restimulation (perhaps by a persistent antigen or CD40/CD40L and cytokines) the IgM memory cell gives rise to clonal progeny of different isotypes that also include memory cells of different isotypes. One of the clonotypic memory cells becomes neoplastically transformed and continuously generates clonotypic B lymphocytes that upon migration to the bone marrow ultimately differentiate to the plasma cell stage (Fig.7.1). The infrequent, pre- and post-switch

clonotypic B cells we detected would represent the MM memory cells that reside in the body as a part of the normal immune system.

In the second model, the early carcinogenic event happened after the cell passed the affinity maturation stage and was committed to the IgM memory lineage. The transformed IgM memory cells generate the clonotypic cells of all isotypes. During or after switching to the clinical isotype, perhaps an additional mutation promotes growth, dissemination and maturation to the plasma cell stage in clonotypic B cells (Fig.7.2).

The main difference between the two models is the neoplastic status of the pre- and post-switch cells expressing non-clinical isotype. The first model assumes that these cells represent normal compartment of the humoral memory while the second model implies that all pre-and post-switch cells are transformed.

The neoplastic transformation (for the first model) or tumor progression (for the second model) could have taken place during the class switch from IgM to the clinical isotype. The chromosomal Ig locus is very unstable during the isotype switching event [140] and consequently a translocation of oncogenes may occur. However, the data published on translocations in MM patient-derived material (unlike in MM cell lines) does not support the notion that translocations are a universal event in MM [58] [57] [187]. Moreover, translocations are just one of many known mechanisms that can lead to neoplastic transformation.

In the first model, it is difficult to explain why the pre- and post-switch memory cells associate with the disease progression if they are not transformed. In a healthy system, the mechanism behind the maintenance of a long-term humoral memory is unclear. It has been proposed that either the memory cells require contact with low amounts of the persistent antigen or that the long-term memory cells are antigen-independent [163]. In MM, the antigen against which the monoclonal antibody is produced, is unknown. Thus, it is not possible to correlate antigen concentration with the presence of pre- and post-switch clonotypic cells. The status of the clonotypic memory cells in MM may be clarified once the biology of memory maintenance is fully understood.

The second model creates a more attractive hypothesis regarding the cell of origin in MM. The presence of pre- and post-switch clonotypic transcripts may indicate the activity of transformed IgM memory cells that generate clonal, multi-isotype progeny. The cells expressing clinical isotype have a growth advantage over non-clinical ones. This dominance in expansion can be explained by a model proposed by Nowell [188]. According to his theory, tumor progression happens in a step-wise fashion by initiation of carcinogenic events that create a population of cells having some selective growth advantage. As the tumor progresses, there is a sequential selection of subpopulations that increasingly gather abnormalities. Based on one mathematical model, for MM to occur, eight different mutations have to accumulate in the affected cell [189]. One can speculate, that the IgM memory cell and the pre- and post-switch cells have seven or perhaps less of these mutations giving them advantageous neoplastic properties (or at least survival

advantage) and that the clinical isotype-expressing cell acquires the last few mutations necessary for the uncontrolled clonal expansion and differentiation into MM plasma cell.

The data obtained from xenotransplantation of human MM cells into mice showed that the most frequently represented clonotypic isotype is IgM while other isotypes are expressed preferentially at the apparent tumor site. PBMC that were used for injections contained, however, the full spectrum of isotypes. This observation supports the second model and the idea that the other cells originate from the persistent clonotypic IgM cell.

To prove our observations and explore the hypothetical models, more experiments are needed in the long-term follow-up of MM patients and in xenografting MM cells in respect to the expression of pre- and post switch clonotypic transcripts.

The following questions have been generated by the research presented in this dissertation:

1. **Do the pre- and post-switch cells of non-clinical isotype contribute to the disease pathology or are they just bystanders?**
2. **What are the phenotypic properties of the hypothetical IgM memory cell in MM?**
3. **Where, besides blood and bone marrow, does the memory compartment reside?**
4. **Does continuous stimulation with an antigen persist in MM?**

5. **What genomic or metabolic aberrations can be detected in the circulating clonotypic cells?**

Although we have detected the pre- and post-switch clonotypic transcripts, the cells expressing them remain unidentified. It would be very attractive to use an approach similar to that of Kubagawa et al. as described earlier [64] and combine it with current molecular biology techniques. For instance, we could raise the anti-idiotype antibodies in a specific manner, by using peptides synthesized based on the cDNA sequence from cloned the V-region of MM IgH. These mAb could be used together with the anti-constant chain antibodies to identify the clonotypic cells and to extensively characterize their cell surface and molecular properties. Particularly fascinating would be the experiments involving detection of the oncogenes in clonotypic circulating cells as compared to those of the plasma cells.

In this dissertation, all the specific aims have been met and the working hypothesis was confirmed. Although all closely related by the expression of clonotypic immunoglobulin, the circulating cells within MM clone are at various maturation stages, as determined by constant chain expression and intraclonal diversity. The biological meaning of these findings awaits more research, which in the future may contribute to better treatment and perhaps a cure of multiple myeloma.

Figure 7.1 Hypothetical model of maturation of the MM clone from an isotype-specific memory cell (MODEL-1)

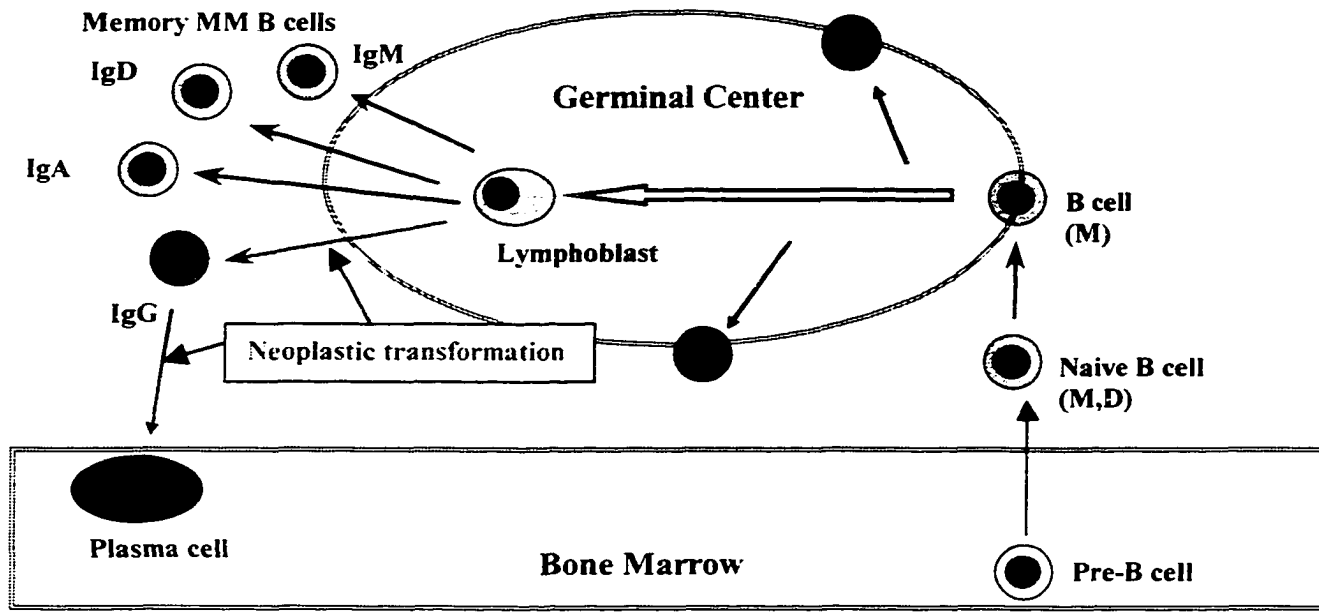
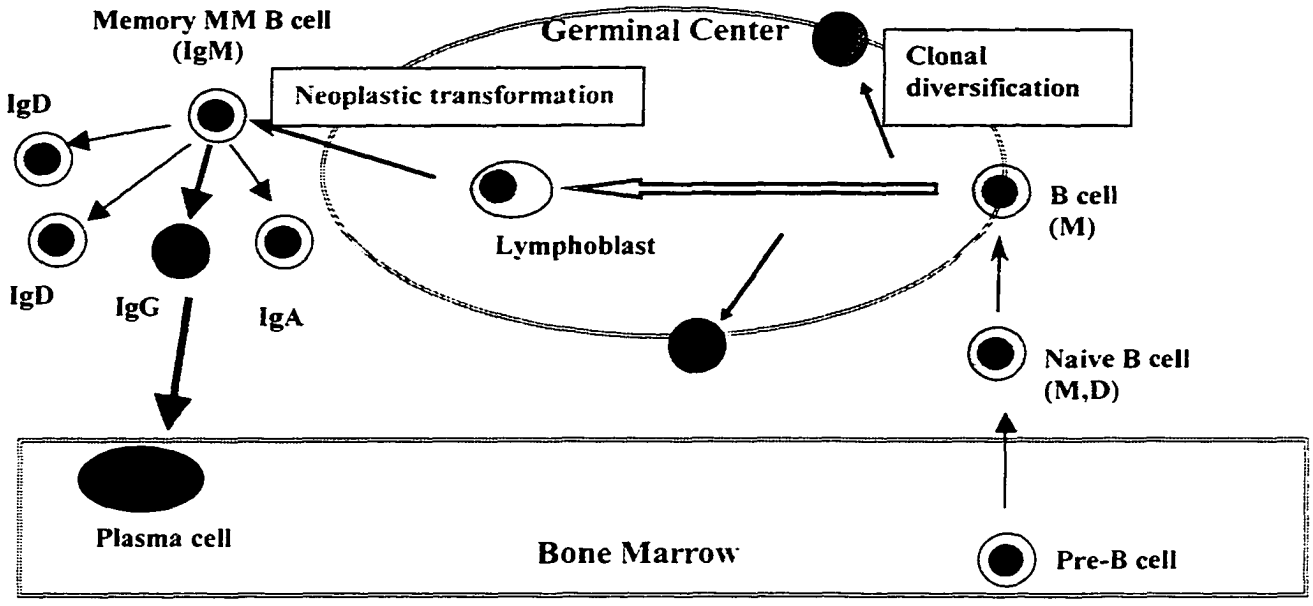


Figure 7.2 Hypothetical model of maturation of the MM clone from an IgM memory cell (MODEL-2)



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IX. APPENDIX A

IL-6 AND IL-6 RECEPTOR EXPRESSION BY CIRCULATING CLONAL MM B CELLS

Interleukin-6 network in multiple myeloma

Cytokines are small polypeptides or glycoproteins. They exert regulatory functions by binding to specific receptors expressed on cells. Interleukin-6 (IL-6) is a pleiotropic cytokine produced by lymphoid and non-lymphoid cells and tissues. IL-6 is a glycoprotein of a size ranging from 21 kDa to 28 kDa. The IL-6 receptor consists of two subunits: α chain (gp80) specific for the cytokine and a common β chain (gp130) responsible for the signal transduction [40 190 191]. The biological functions of IL-6 include between others B and T cell differentiation, hematopoietic cell growth and induction of acute-phase protein synthesis. In malignancies, IL-6 promotes growth of plasmacytomas, T cell lymphoma, renal cell carcinoma and MM cells [40 190].

In vitro studies showed that IL-6 is a growth and anti-apoptotic factor for freshly isolated MM plasma cells and for the MM derived cell lines [192 193]. Increased IL-6 concentration in blood is attributed to secretion from malignant plasma cells, BM stromal cells and T cells [194 195]. Molecular studies confirmed the transcription and translation of IL-6 from MM BM PC [196]. Plasma cells not only produce the interleukin but also have a potential to upregulate IL-6 secretion from other cell types e.g. osteoblasts, creating a paracrine loop [197]. Serum levels of IL-6 in MM patients are significantly elevated in correlation with progression of the disease [198]. IL-6 was implicated an important anti-apoptotic factor used

by PC in a paracrine and autocrine fashion. Monoclonal antibodies that block the IL-6 receptor and the monoclonal antibodies against IL-6 inhibited *in vitro* growth of MM cell lines demonstrating that IL-6 and IL-6-transduced signal support growth and survival of MM PC [199 200].

Interleukin-6 (IL-6) was first described as a B-cell stimulatory factor that was ultimately identified as a multifunctional cytokine produced by a variety of cells including B and T lymphocytes, endothelial cells, fibroblasts and many tumors including myelomas, sarcomas and carcinomas. IL-6 binds to its receptor consisting of two subunits: an 80kDa IL-6 specific alpha subunit designated CD126 and a 130kDa signal transducing protein (gp130) thereby exerting numerous functions. In MM, IL-6 acts an antiapoptotic agent for MM plasma cells and a stimulatory one for osteoclasts. Taken all together the IL-6 functions in MM contribute to biology and severity of the disease.

In MM, one of the sources of IL-6 is MM plasma cells [196]. However, nothing was known about the contribution to the IL-6 network by circulating clonal B cells. Many features, such as histological appearance, expression of adhesion molecules and spontaneous mobility exhibited *ex vivo* suggests that the circulating MM B cells are activated. The state of activation usually corresponds with cytokine production. We hypothesized that circulating clonal MM B cells may produce IL-6 and possess a receptor for that cytokine. Here, we looked for an expression of transcript and protein for IL-6 and for the presence of IL-6 receptor on freshly isolated MM B cells.

A. Materials and methods

ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of IL-6 and IL-6 soluble receptor in the 24hr tissue culture supernatants. Quantikine (R&D Systems, Minneapolis, MN) kits were used, following the manufacturer's protocol. The supernatants were stored at –80 C immediately after the collection and kept there until 1 hour before test. For the read-out, UV-max ELISA plate reader (Molecular Devices, CA) was used at the wavelength 450 nm with 570 nm reference filter.

Tissue culture:

Cells were stained with anti-CD19 mAB coupled with FITC and soretd into CD19+, CD19- and CD19+/CD19- populations. After sorting were incubated in a 96 well U bottom plate (Costar) in humidified incubator at 37 °C with 5% $C0_2$ at a concentration of $1x10^5$ cells/well/200µl. The media used was DMEM (GIBCO/BRL) supplemented with 5% fetal bovine serum and antibiotics (Penicillin and Gentamycin both at 50 µg/ml). After 24 hr, supernatant was collected and frozen immediately at -80 °C until the ELISA assay was performed.

B. Results

IL-6 transcript is expressed by MM B cells ex vivo

The expression of IL-6 transcript was studies in PBMC from MM and healthy controls. RNA isolated from peripheral blood and BM of MM patients and healthy controls was subjected to reverse transcription and subsequent PCR with IL-6 specific primers. We detected IL-6 transcript in all samples tested. Because the analysis of total RNA is not quantitative, to find what proportion of cells express IL-6 message we used cell sorting followed by *in situ* RT-PCR. Cells were sorted based on CD19 surface marker expression. as described earlier. On average, 47% of all MM B cells and only 6% of control B cells contained IL-6 transcript (Figure A.1).

IL-6 is produced spontaneously by CD19+MM cells in short term cultures

We examined freshly isolated MM PBMC for the IL-6 secretion. Purified PBMC from healthy controls and from MM patients were sorted into 96 well U-bottom plates containing tissue culture media. After 24 hr, the supernatant was collected and the concentration of IL-6 was measured by ELISA. Figure A.2 presents the ELISA results. On average, the IL-6 levels spontaneously produced in MM PBMC cultures were 5 fold higher than in healthy controls. To establish which cell population produces the majority of IL-6 in MM PBMC cultures, the cells were sorted into CD19+ and CD19- subsets and subsequently incubated at 37°C for 24hr in the tissue culture media at the density of $1x10^5$ cells/well/200µl. The ELISA results with CD19+ and CD19- supernatants are shown in figure A.3 and A.4. The statistical analysis suggests that CD19+ cells isolated from MM blood are the major IL-6 producers amongst PBMC (Fig.A.5).

IL-6 receptor is expressed on the cell surface of CD19+ MM cells

We tested the CD19+ cells for the presence of the IL-6 α chain subunit (gp80) by using cell surface staining with the specific monoclonal antibodies and flow cytometry for the analysis.

Our results show that on average 50% of MM B cells and 22% of control B cells express the IL-6 receptor (Fig.A.6). This finding is supported by the RT-PCR results demonstrating the presence of a respective transcript.

Concentration of soluble IL-6-receptor in serum of MM patients is not elevated as compared to healthy controls.

The concentration of soluble IL-6 receptor (sIL-6R) was measured in serum of MM patients and healthy controls by using commercial ELISA sIL-6R kit. There was no statistical difference in levels of sIL-6R between two groups.

C. Discussion

In a healthy system, IL-6 drives the differentiation from a late stage B cell into a plasma cell. In MM, a similar process of maturation is most likely taking place. On average, at least half of the circulating MM B cells are clonotypic. Since normal B cells mature to plasma cells it is logical to speculate that the clonotypic B cells are able to give rise to clonal plasma cells perhaps stimulated by differentiation agents. The maturation process of a B cell requires much more than a presence of one cytokine. However, the IL-6 knock-out mouse shows impaired antibody responses clearly demonstrating the need for this cytokine in humoral responses [201]. In addition, IL-6 transgenic mice show increased production of polyclonal immunoglobulin in serum [190,202].

In this work, we tested the ability of circulating clonotypic MM B cells to produce and to bind IL-6. First, we have established that the IL-6 transcript is detectable in both MM and control PBMC. Quantitative *in situ* RT-PCR showed that within the sorted CD19+ population 47% of MM B cells and only 6% of control B cells are transcribing IL-6 message.

PBMC from thirteen patients and seven healthy controls were tested for the secretion of IL-6. The IL-6 ELISA test, conducted on supernatants collected from short term cultures revealed that MM PBMC secrete IL-6 protein in 5 times higher quantities than the PBMC isolated from healthy individuals. To establish if MM B cells are a major source of IL-6 among circulating cells or alternatively if all of the PBMC equally activated, we sorted and cultured CD19+ and CD19- subsets. Unlike the control, the MM CD19+ B cells

turned out to be active secretors of IL-6. We have also investigated the presence of CD126, IL-6 specific alpha subunit of IL-6 receptor on the B cell surface. Our results indicate that on average half of the MM B cells and only 20% of the control B cells stain positive for CD126.

Thus, we found that MM B cells are potent spontaneous secretors of IL-6 and have capacity to bind this cytokine. When homing to the bone marrow, B cells may create an IL-6-rich environment that is thought to characterize MM [40,196]. Further experiments are required to establish if B cells cultured on autologous stromal BM cells will indeed differentiate into the plasma cells and if that differentiation is IL-6 dependant. Tests involving blocking IL-6 receptor and blocking the secretion of IL-6 from B cells may help in answering these questions. Interleukin-6 may be an important factor in the maturation process of MM B cells. Other cytokines and cell surface molecules most likely contribute to the final development into an antibody-producing cell.

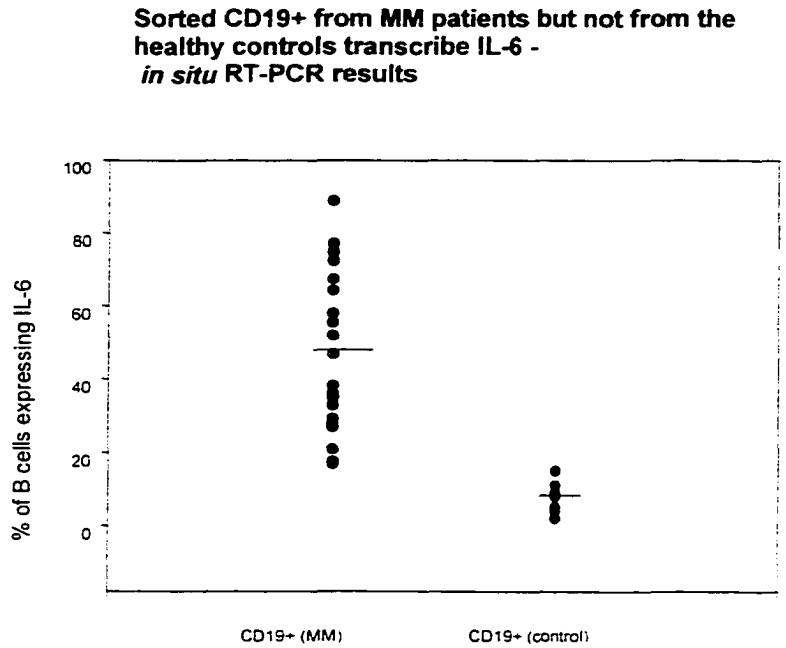

Figure A.1 Sorted CD19+ from MM patients but not from the healthy controls transcribe IL-6 *in situ* RT-PCR results

PBMC from MM patients and from healthy controls were stained, fixed and sorted based on CD19 expression onto *in situ* slides. Next, slides were subjected to *in situ* RT-PCR with IL-6-specific primers, as described in methods. The slides were scored and the results were expressed as a percentage of positive cells. In MM CD19+, on average 47% of B cells expressed IL-6 mRNA and in controls 6% of B cells expressed IL-6 mRNA.

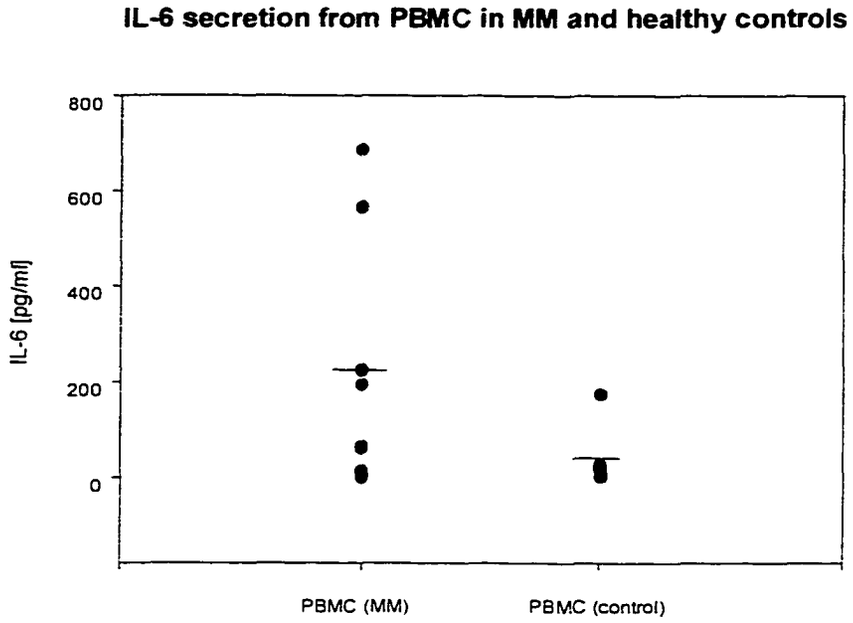

Figure A.2 IL-6 secretion from PBMC in MM and healthy controls

PBMC were isolated from MM patients and healthy controls and cultured at $1x10^5$ cells/well in 100 µl of media. Supernatants were collected after 24 hours and assayed for IL-6. MM derived supernatants contained on average 141 pg/ml of IL-6 (range 1-686 pg/ml) while the controls contained 33 pg/ml (range 2-175pg/ml).

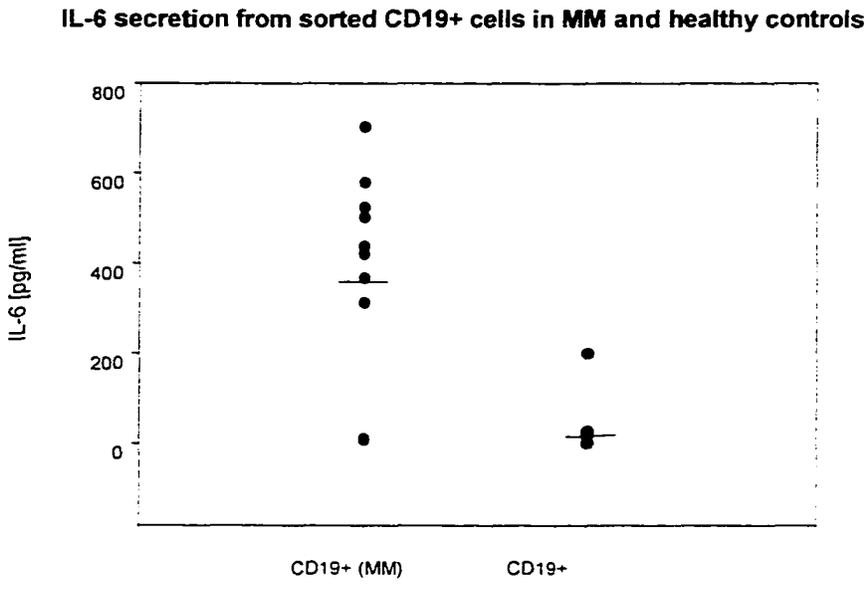

Figure A.3 IL-6 secretion from sorted CD19+ cells in MM and healthy controls

PBMC were isolated from MM patients and healthy controls, stained for CD19 antigen. CD19+ cells were sorted and cultured at $1x10^5$ cells/well in 100 µl of media. Supernatants were collected after 24 hours and assayed for of IL-6. MM derived supernatants contained on average 299 pg/ml of IL-6 (range 7-703 pg/ml) while the controls contained 36 pg/ml (range 1-200pg/ml).

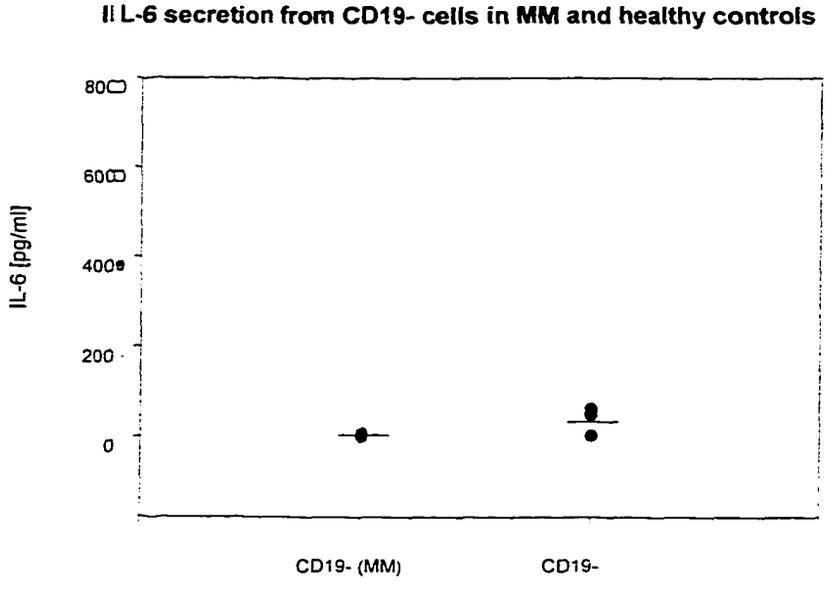

Figure A.4 IL-6 secretion from CD19- cells in MM and healthy controls

PBMC were isolated from MM patients and healthy controls, stained for CD19 antigen. $CD19^-$ cells were sorted and cultured at $1x10^5$ cells/well in 100 µl of media. Supernatants were collected after 24 hours and assayed for the presence of IL-6. MM derived supernatants on average contained 3.4 pg/ml of IL-6 (range 0-7 pg/ml) while the controls on average contained 31 pg/ml (range 0.2-62/ml).

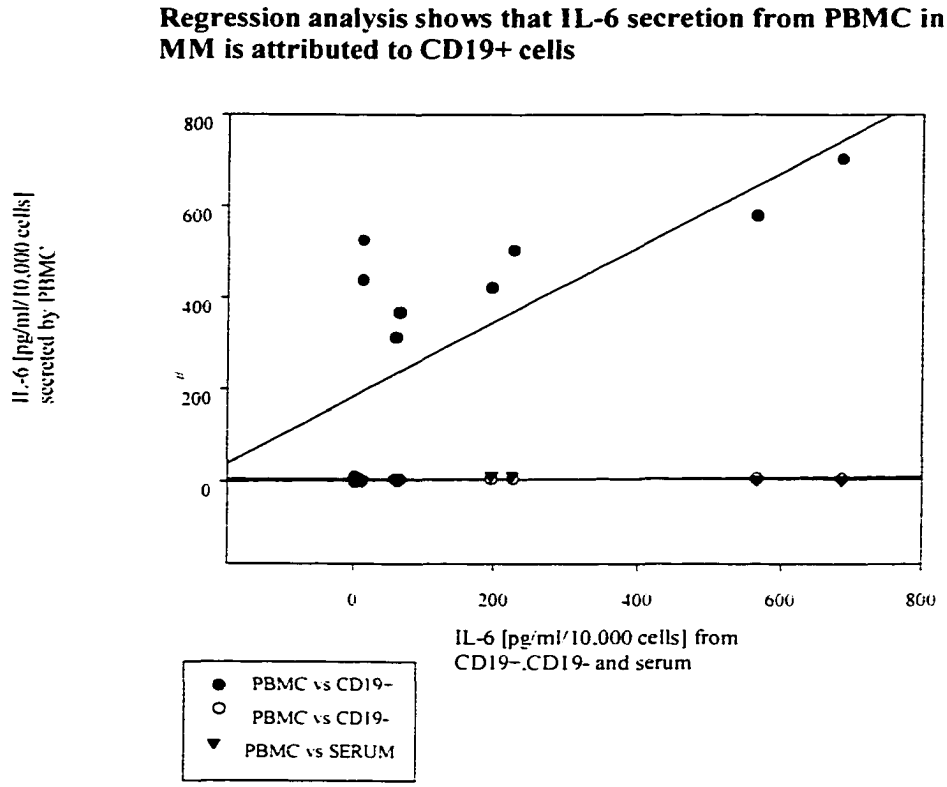

Figure A.5 Regression analysis shows that IL-6 secretion from PBMC in MM is attributed to CD19+ cells

Regression analysis was used to search for an association between the secretion of IL-6 from CD19+, CD19- cells and from PBMC. The results suggest that the high concentration of IL-6 present in the media from MM PBMC is mainly due to secretion from CD19+ cells. $r^2 = 0.85$

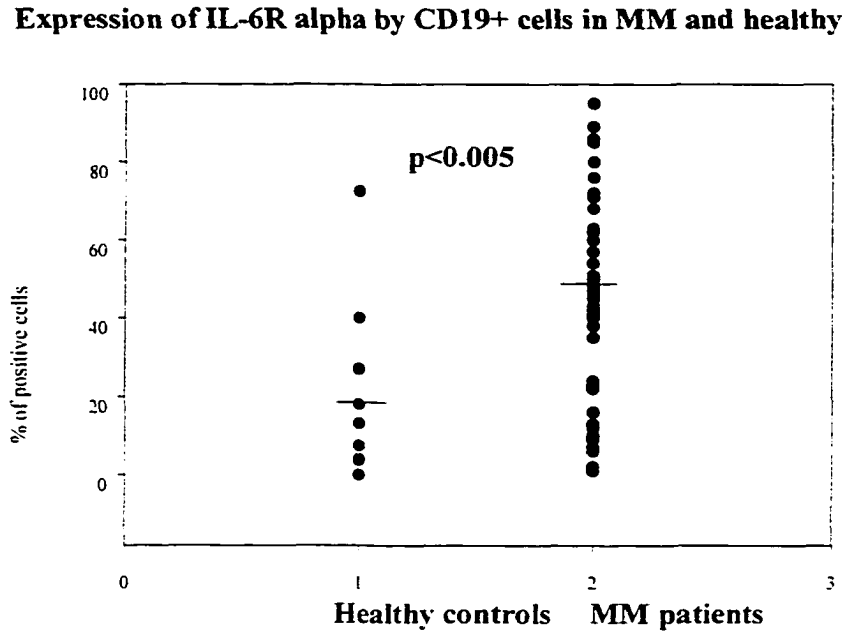

Figure A.6 Expression of IL-6R alpha by CD19+ cells in MM and healthy controls

PBMC isolated from MM patients and healthy controls were stained with anti-CD126 (IL-6-receptor) and anti-CD19 monoclonal antibodies. Using FACSCAN, the population gated on CD19+ was analyzed for the expression of CD126. On average, 50% of B cells in MM and 22% in control samples express CD126 on their surface.

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FIGURES

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