Detection and Quantitation of Malignant Cells in the Peripheral Blood of Multiple Myeloma Patients

By Daniel Billadeau, Lynn Quam, William Thomas, Neil Kay, Philip Greipp, Robert Kyle, Martin M. Oken, and Brian Van Ness

One of the distinguishing features of multiple myeloma (MM) is the proliferation of plasma cells that home to the bone marrow (BM). However, there still remains some uncertainty concerning the presence of related malignant cells in the peripheral blood of myeloma patients. Using consensus oligonucleotide primers, we amplified the third complementary determining region (CDR3) of rearranged immunoglobulin heavy chain alleles from MM marrow samples by polymerase chain reaction (PCR). From the sequences of the products, we derived allele-specific oligonucleotides (ASO), and these were used in subsequent amplification reactions to detect malignant clones in the peripheral blood of myeloma patients. This method is highly specific and sensitive to 1 malignant cell in the background of 10⁸ normal cells. Using this method we detected circulating malignant cells in 13 of 14 previously untreated MM patients. Furthermore, by applying ASO-PCR to artificial titrations of initial BM DNA sample into normal peripheral blood lymphocyte (PBL) DNA we were able to generate standard curves and quantitate the amount of tumor in the patient PBL. We observed a wide variation in the amount of circulating tumor between patients. In addition, we found that the incidence of circulating tumor cells was independent of BM tumor burden and stage of disease. The detection and quantitation of circulating tumor cells in the PBL of MM patients may offer an alternative assessment of the disease and may be an important consideration in the use of peripheral stem cells in bone marrow transplantation.

MULTIPLE MYELOMA (MM) is a disease characterized by the clonal proliferation of plasma cells that characteristically home to the bone marrow (BM). However, there still remains some uncertainty concerning the presence of related malignant cells in the peripheral blood lymphocytes (PBL). This is an important issue, because detection of malignant cells in the blood may offer unique clinical insight into the course of disease and the response to therapy, and also because some autologous transplant protocols use peripheral stem cells in the treatment of myeloma. Therefore, the detection and quantitation of myeloma cells in the peripheral blood may be important as a prognostic indicator and to assess contamination of cells used in transplantation.

A variety of methods have been used to detect cells related to the malignant marrow cells in the blood of myeloma patients, including immunologic detection by monoclonal antibodies, dual parameter flow cytometry analysis of DNA and cytoplasmic Ig, and gene rearrangement. The conclusions reached by these studies suggest that circulating tumor cells can be detected, but the frequency of detection and quantitation remains controversial. In addition, each of these methods has limitations in sensitivity, and in some cases, specificity. Gene rearrangement studies can detect specific gene rearrangements, but require 2% to 5% monoclonal cells, whereas dual parameter analysis claims detection as low as 0.1%, but only detects Ig* aneuploid cells.

Recently, a number of laboratories have described application of polymerase chain reaction (PCR) techniques to significantly increase the sensitivity and specificity of clonal B-cell detection. We recently described an approach to the analysis and quantitation of B-lymphoid malignancies using allele-specific PCR. Consensus oligonucleotide primers were used to amplify the complementary determining region 3 (CDR3) of rearranged heavy chain alleles from lymphoid tumor samples. We have found consensus amplification to be successful in approximately 70% of the cases studied and that 1.0% initial plasma cell tumor content is sufficient to obtain a discreet amplified product. From the sequence of the amplified products, allele-specific (tumor-specific) primers were synthesized and used directly in PCR amplification reactions to detect the malignant clone. This method was highly specific and sensitive to 1 malignant B cell in a background of 10⁸ normal cells. In addition, by using titrations of malignant target cells, standard curves were generated, allowing comparisons and quantitation of a wide range of malignant cell targets. This approach was used in a sequential analysis of BM from myeloma patients, some of whom were in clinical remission but had clearly detectable residual malignant cells.

Because allele-specific oligonucleotide PCR (ASO-PCR) is rapid, sensitive, highly specific, and quantifiable, we applied this approach to the examination of PBL of myeloma patients. We determined the CDR3 sequence from the malignant cells of the BM obtained from 14 patients with various stages of MM, and synthesized an allele-specific (tumor-specific) oligonucleotide primer for each patient sample. Using the quantitative ASO-PCR technique, we can detect and quantify the cells in the peripheral blood containing the tumor-specific sequence. This has important application to the evaluation of disease.
progression or response, and the determination of contaminating cells in autologous transplants for MM.

MATERIALS AND METHODS

Patient samples. Myeloma patients received no previous treatment and were enrolled on the Eastern Cooperative Oncology Group (ECOG) clinical trial, EST 9486. All patients received 2 months of vincristine, bis-chloronitrosourea (BCNU), melphalan, cyclophosphamide, and prednisone (VBMCP) before randomization to different clinical therapies. Myeloma bone marrow specimens were prepared by NH4Cl isotonic lysis to remove the red blood cells. Peripheral blood lymphocytes were prepared by Ficoll-Hypaque (Sigma) density centrifugation.

DNA blot hybridization and quantitation of initial B-cell myeloma population by IgH gene rearrangement. DNA was extracted from BM samples using the Applied Biosystems Automated Nucleic Acid Extractor Model 340A (Foster City, CA). Ten micrograms of genomic DNA was then digested with HindIII, electrophoresed through a 0.7% agarose gel, and transferred to nylon membranes as described.16 The blots were hybridized with a JH probe (6kb BamHI-HindIII fragment) and heavy chain gene rearrangements were detected by autoradiography. The signal intensities of germ-line and rearranged hybridization signals were determined by videodensitometry, and the fractional tumor population was calculated as previously described.15,17

Consensus amplification and sequence of the CDR3 region from myeloma bone marrow DNA. PCR was performed essentially as described by Saiki et al,18 using consensus primers to VH (framework 3) and JH. The sequences of the consensus primers and all allele-specific primers are shown (see Fig 2). Consensus primers were synthesized on a Milligen Biosearch Model 8750 DNA Synthesizer (Novato, CA) and the allele-specific primers were synthesized on an Applied Biosystems DNA/RNA Synthesizer Model 394. Conditions for consensus PCR amplification of BM myeloma DNA were as previously described.15 Normal PBL and no template reactions were included as negative controls in each experiment. The PCR products were analyzed by electrophoresis through an 8% polyacrylamide gel and visualized by UV light following staining of the gel with ethidium bromide. Consensus amplified CDR3 products were excised from polyacrylamide gels and were sequenced.15

Allele-specific PCR of patient PBL and BM samples. Allele-specific oligonucleotide (ASO) primers were designed from the sequence information of CDR3 amplified products and are shown (see Fig 2). Quantitation analysis of PCR products was performed as previously described.15 Briefly, allele-specific linear dilution curves were generated from analysis of initial patient marrow samples (ECOG on-study samples) after serially diluting the patient DNA into sterile ddH2O in 10-fold decrements, followed by the addition of an appropriate amount of normal peripheral blood DNA to yield a total target concentration of 1 μg DNA/100 μL reaction. ASO-consensus PCR was performed as previously described15 with annealing temperatures ranging from 50°C (M22) to 64°C (M27) using the half-denaturation temperature (Tm) of the ASO primer. The number of cycles varied from 25 to 40 depending on the linear range required. To establish the linear range required for quantitation, several independent titrations were performed and found to be highly reproducible. Along with the diluted samples, 1 μg of DNA from on-study PBL and/or 2-month PBL and/or 2-month BM were run in triplicate or duplicate. PCR products were radiolabeled by addition of 32P-dCTP to the reaction, separated on an 8% polyacrylamide gel, dried, and then exposed to x-ray film at room temperature for 1 to 18 hours. The signal intensities of the radiolabeled PCR products were determined by videodensitometry.16

Statistical methods. The band intensities of ASO-PCR products from serially diluted BM samples were determined by videodensitometry of autoradiograms, giving an optical density (OD, arbitrary units).16 Least squares was used to fit a linear regression equation for ln (OD) as a function of ln (target DNA concentration) for each patient. The estimated concentration of DNA, ie, the relative PBL content, and its 95% Scheffe’s confidence interval were computed on the natural-log scale by standard methods,19 then transformed to the original scale (relative DNA concentration). The absolute PBL tumour percentage was estimated by the product of the relative PBL content (as a fraction of the initial BM tumor DNA content) and the initial bone marrow tumor burden (percent of total lymphocytes), as determined by gene rearrangements. Confidence limits for the estimate were found by multiplying the endpoints of the interval for relative PBL content by the amount of initial BM tumor burden.

Fig 1. Outline of the experimental method used to generate allele specific oligonucleotides (ASO) for patient specific PCR. (A) Schematic representation of the rearranged heavy chain locus depicting the CDR3 region amplified by the consensus primers. Also shown are events that lead to the sequence diversity observed within this region. (B) Ethidium bromide stained polyacrylamide gel showing consensus amplified PCR products from six different myeloma BM samples. PBL, normal peripheral blood; NT, no template. (C) Representation of patient-specific PCR of the CDR3 region using ASO and consensus J region oligonucleotides.

From www.bloodjournal.org by guest on October 22, 2017. For personal use only.
RESULTS

IgH consensus PCR and sequencing of amplified CDR3 regions from myeloma bone marrows. As described in Fig 1A, we have used consensus primers to the VH region (framework 3) and to the 5' portion of the JH region to amplify junctional sequences representing the CDR3 region. We have performed consensus amplification on DNA from 14 myeloma BM samples in different stages of disease, that showed prominent clonal malignancies based on immunohistochemical staining and as detected by DNA blot hybridization with a JH region probe (not shown). All of these myelomas produced a discrete amplified product that could be detected by ethidium bromide staining after electrophoresis through an 8% polyacrylamide gel. Six representative samples are shown in Fig 1B, along with normal PBL, which typically yields a faint smear because of the heterogeneity of the rearranged IgH genes within the sample. Consensus amplified products show considerable diversity of size as a result of the random deletion and insertion events that occur during V-D-J rearrangement (Fig 1A). The sizes vary typically between 65 and 130 bp. Although some of the BM DNA samples show two gene rearrangements by blot hybridization (not shown), we generally observe only one consensus amplified PCR product. The failure to amplify both rearranged alleles could be the result of (1) extensive nucleotide trimming into the region complementary to the primers, (2) one of the alleles having undergone only a D-J recombination, or (3) inefficient annealing of our consensus primers to some alleles because of somatic mutations or sequence diversity observed within the framework 3 region of the V gene families. It has been shown that four nucleotide mismatches within the consensus VH primer is enough to abolish amplification.20

The 14 myeloma consensus PCR products were sequenced (as described in Materials and Methods), and the regions corresponding to the V-D and D-J junctions are shown in Fig 2. Based on published sequence,21,22 J gene and D gene usage could be determined, as well as the extent of nucleotide deletion, insertion, and somatic mutations within these gene segments. It is clearly shown from these sequences that there is extensive sequence heterogeneity among the junctional regions, and that primers designed from this region can provide a unique genetic marker of the clonal malignancy.

Quantitation of tumor fraction by DNA blot hybridization and ASO-PCR. DNA blot hybridization of initial BM DNA samples and subsequent densitometry of the autoradiogram enabled us to quantify the fraction of tumor within the BM (see Materials and Methods). ASOs derived from the CDR3 sequences (underlined regions in Fig 2) were synthesized and paired with the consensus JH primer to specifically amplify the tumor-specific DNA within the patient samples (Fig 1C). Alpha 32P-dCTP was included in the reaction, and the PCR products were separated on 8% polyacrylamide gels and visualized by autoradiography. The JH consensus primer was preferentially used in the ASO-PCR because we have typically obtained better amplification by ASO-JH versus ASO-VH reactions (data not shown). This is likely because the consensus JH primer maintains specific annealing to the limited number of J segments under the more stringent conditions used in the ASO-PCR. Specificity was also confirmed by the size of the ASO-PCR product, which could be accurately predicted by the sequence derived from the initial amplification of the BM myeloma DNA (Fig 2).

As we have previously shown,15 titration of a specific BM myeloma DNA into normal PBL DNA, followed by ASO-PCR and densitometry can produce a linear standard curve from which subsequent, patient-derived ASO-PCR densities can be quantified. Whereas the linearity of the PCR titration is dependent on both target concentration and cycle number, the linear PCR response can span at least

```
Veal
ACACGGCCCGCTTTATTCTCTG
M21
ACTCTGCAGAGA
AGCGAGAGATGCGGT
CTATGACTA
JH consensus primer was preferentially used in the ASO-PCR because we have typically obtained better amplification by ASO-JH versus ASO-VH reactions (data not shown). This is likely because the consensus JH primer maintains specific annealing to the limited number of J segments under the more stringent conditions used in the ASO-PCR. Specificity was also confirmed by the size of the ASO-PCR product, which could be accurately predicted by the sequence derived from the initial amplification of the BM myeloma DNA (Fig 2).

As we have previously shown,15 titration of a specific BM myeloma DNA into normal PBL DNA, followed by ASO-PCR and densitometry can produce a linear standard curve from which subsequent, patient-derived ASO-PCR densities can be quantified. Whereas the linearity of the PCR titration is dependent on both target concentration and cycle number, the linear PCR response can span at least

```
two logs of target DNA concentration.\textsuperscript{15,23} Therefore, ASO-PCR titrations were individually designed to encompass the region of linearity within which the intensity of experimental PCR products lies.

An example of a linear titration generated to quantify the amount of tumor in patient peripheral blood is shown in Fig 3 (patient M22). The JH probed autoradiogram of the BM DNA from which the initial tumor burden was calculated is shown at the left. The upper autoradiograph was derived from ASO-PCR of serially diluted M22 BM DNA into normal PBL DNA. Densitometric scanning shows a linear titration that spans approximately 2 logs of the dilution and was used to generate the standard curve and confidence intervals (see Materials and Methods). ASO-PCR of the same patient’s PBL DNA showed a product of the same size. The average OD value of the ASO-PCR products obtained from the PBL sample can then be plotted on the standard curve to give a DNA concentration value as shown in Fig 3, with 95\% confidence intervals. Because the fraction of tumor in the initial BM sample can be determined by densitometry of the JH probed autoradiogram, the fraction of tumor within the peripheral blood and sequential samples can be calculated (see Materials and Methods). The results presented in Fig 3 and those in Fig 4 show the ability to generate tumor-specific standard curves that can be used to quantitate subsequent patient samples, with a sensitivity of detection equivalent to 1 malignant cell in the background of 5 \times 10^6 normal cells.

Quantitative ASO-PCR analysis of myeloma patient PBL and sequential samples. Based on the sequences presented in Fig 2, we synthesized oligonucleotides that provide tumor specificity in ASO-PCR for each of 14 different myeloma samples. We then generated linear, target concentration-dependent standard curves from each of the myeloma BM DNA samples (M20-M33) obtained before therapy. In addition, the initial narrow sample from 12 of the patients was analyzed by DNA blot hybridization to quantitate initial BM tumor burden based on the Ig heavy chain gene rearrangement. As previously noted\textsuperscript{15} the PCR analysis agreed quite well with the morphologic examination of plasma cell content in the marrow for most cases. The initial tumor burden from two patients, M25 and M28, were below our detection limit by blot hybridization. The initial tumor burden of sample M25 was obtained from histocchemical staining,\textsuperscript{13} whereas the initial tumor burden of M28 was estimated by comparing ASO-PCR product intensity of 1 \mu g of the initial BM sample, with ASO-PCR product titrations from BM DNA tumor samples with known tumor burdens that share the same J gene (36).

Figure 4 shows ASO-PCR amplifications generated from 14 initial myeloma BM DNA samples that were individually titrated into normal PBL DNA. In each case, conditions of the PCR amplification were adjusted (ie, cycle number and dilution series), so that intensities of the ASO-PCR products from the PBL DNA and sequential samples were within the linear range of the titration. Quantitation of the fraction of tumor in the PBL and sequential samples were determined from the linear regression of the BM data points, and are shown as the percent of the total peripheral blood lymphocytes (Fig 5). In each case, the ASO-PCR product obtained from the PBL and sequential samples were determined to be myeloma specific because (1) the ASO primer was designed to be specific to the unique junctional sequence of the BM tumor, (2) the ASO-PCR

Fig 3. Generation of a standard curve from an ASO-PCR titration. Shown to the left of the plot is a JH probed Southern blot of DNA from BM sample M22: G, germline; R, rearranged allele. Above, a linear titration of on-study BM DNA was made by dilution into normal PBL. PCR cycle conditions and titration target concentrations were adjusted to ensure that the ASO-PCR product of the on-study PBL DNA (run in triplicate) was within the linear range of the titration. The titration points as tumor equivalent DNA input (in picograms) 1 \times 10^{-4} to 5 \times 10^{-5} were plotted versus the respective PCR product OD in an In/In plot. Linear regression and confidence intervals were calculated as described in Materials and Methods. The average OD of the PBL ASO-PCR product is indicated by the intersection of the dotted line on the vertical axis and the equivalent tumor DNA content can be determined (arrow) with 95\% confidence limits.
products from the PBL DNA samples were identical in size to those from the initial BM myeloma sample, (3) normal PBL DNA was not amplified by ASO-PCR, and (4) ASO-PCR did not amplify myeloma DNA samples for which it was not specific (not shown).

As shown in Fig 4, we were able to detect and quantify ASO-PCR products in all but one of the on-study PBL DNA samples (sample M21). Sample M21 DNA did amplify with an N-ras primer pair (not shown), but because it was negative by ASO-PCR, the malignant cells could be below our limits of detection (0.0001%). The percent of tumor in the peripheral blood samples is shown in Fig 5 with confidence intervals generated from linear regression of the on-study BM titration data points. Correlation coefficients (R² values) for the standard curves are generally greater than 0.95, although the calculated 95% confidence intervals typically span about 1 log of tumor content. The total analysis shows that tumor content in the PBL can vary over 6 logs. For example, the quantity of tumor within the on-study peripheral blood samples varies from 32% (M29) to 0.00088% (M22). However, in most PBL samples (11 of 14) the level of circulating tumor falls between 0.001% and 1.0%. Our data shows that the percent of circulating malignant cells in the peripheral blood is independent of both stage of disease and the percent of tumor in the untreated BM. This lack of correlation is highlighted by the fact that although the sample with the highest tumor burden in the BM has the largest population of circulating tumor cells, the sample with the second highest BM tumor burden has the fewest circulating tumor cells (compare M22 and M29). Interestingly, samples such as M23, M28, and M29 show significant levels of circulating cells that amplify with the tumor-specific ASO primer (Fig 5), yet differential blood analysis did not show significant numbers of circulating plasma cells. This apparent discrepancy highlights the possibility that there may be genetically identical but more immature tumor cells in the circulation.

In some selected examples (M23, M26, M27, and M28) we show the detection of malignant cells in PBL even after the first cycle (2 months) of VBMCP therapy (Fig 4 and
MALIGNANT CELLS IN BLOOD OF MYELOMA PATIENTS

Fig 5. Myeloma tumor burden in BM and peripheral blood samples. The sample numbers are given in the first column, followed by the status of the Ig heavy chain gene as determined by DNA blot hybridization with a JH probe (G, germline; R, rearranged). The third column indicates the patients on study disease stage. Column 4 represents the percent of tumor in the BM samples as determined by densitometry (see Materials and Methods). Column 5 shows percent of tumor in PBL with statistically generated confidence intervals (see Materials and Methods). (●) On-study peripheral blood samples; (○) samples following 2 months of therapy. The arrow in sample M21 indicates that detection of the ASO-PCR product in the PBL was below our limits of detection and could lie within the area indicated. ND, not detected.

shown as open circles in Fig 5). Quantitation of these sequential samples shows that three samples had at least a log decrease in the percent of tumor in the PBL, whereas one sample showed an increase in the amount of circulating tumor (M27). This data shows that changes in tumor content in PBL can be monitored during the course of therapy.

DISCUSSION

In this study we have used ASO-PCR to detect and quantitate circulating malignant cells in the PBL of MM patients. Compared with previously reported techniques, this method provides an increase in both specificity and sensitivity. Our initial quantitation of the BM was determined by the fractional intensity of clonal rearrangements observed by DNA blot hybridizations. Quantitation of the PBL samples was based on ASO-PCR product intensities relative to those obtained from titrations of the initial BM sample. Although myeloma may represent a homogeneous infiltration of the marrow, deviations in tumor burden within the marrow are common. Therefore, to improve monitoring of the course of disease it may be worthwhile to use peripheral blood because it is a more homogeneous mixture of cells.

As shown in Fig 5, the range in which we were able to quantitate tumor cell content spans 6 logs. Although our correlation coefficients, derived from the standard dilution curves, are generally greater than .95, the 95% confidence intervals typically span almost a log. This shows an important limitation to interpretation of quantitative PCR methods: logarithmic amplification can generate expanded confidence intervals. This is not surprising because any errors will be logarithmically amplified. Nevertheless, quantitative PCR offers an evaluation of tumor burden at levels unattainable by any other method. The real value of this method may lie in the ability to evaluate a single patient sequentially, as a predictor or indicator of therapeutic response.

The frequency with which we were able to detect circulating tumor cells suggests that peripheral blood involvement occurs at a greater incidence than previously reported. Furthermore, in contrast to Cassel et al, we found that the detection of circulating tumor was independent of the percent of tumor cells in the BM and the stage of disease. However, the patients we chose for analysis were all newly diagnosed, with limited time for sequential analyses or long-term assessments. It will be important to consider such parameters as response to therapy, patterns of tumor involvement, and long-term survival in light of the levels of circulating malignant cells detected by our quantitative PCR.

Previous studies have shown circulating lymphocytes that express plasma cell antigens in myeloma patients. Indeed, reports have suggested that the detection of circulating plasma cells is a useful marker of disease activity in patients with plasma cell proliferative disorders. By virtue of the allele-specific sequences our results show genetic identity between circulating malignant cells and the marrow tumor. However, it is important to note that, from this analysis, we cannot distinguish malignant plasma cells that escape from the marrow from possible circulating precursor cells that may continually feed the malignant population of the marrow by homing and maturing there. This may lead to possible discrepancies between our methods and others in correlating tumor cell burden with disease stage or response to therapy. For example, we found significant levels of circulating tumor cells in some patients despite the fact that there was not a correlation with high levels of circulating plasma cells. With the availability of specific DNA probes that identify genetically related cells, it may be possible to address this in the future.

One of the potential uses of PCR blood studies may be in the detection of tumor in nonsecretory myeloma or where changes in myeloma protein no longer reflect changes in tumor load. The use of autologous blood stem cell transplantation following intensive chemotherapy has been implemented as a treatment for MM. Although the contamination of tumor cells from the peripheral blood may be small after high-dose chemotherapy or purging techniques, the presence of any tumor, be it plasma cell or precursor cell in the autograft, may promote reemergence of the disease. The ability to specifically detect malignant populations in the pools used for engraftment could also provide an important assessment of the transplant.

ACKNOWLEDGMENT

We thank Annette Miller and Nancy Bone for excellent technical assistance in processing clinical samples.
REFERENCES

Detection and quantitation of malignant cells in the peripheral blood of multiple myeloma patients

D Billadeau, L Quam, W Thomas, N Kay, P Greipp, R Kyle, MM Oken and B Van Ness