Idiotype vaccination in patients with myeloma reduced circulating myeloma cells (CMC)

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Background: Circulating myeloma cells (CMC), exhibiting the same immunoglobulin heavy-chain gene rearrangements as the plasma cell clone, are part of the myeloma clone. In this study, we evaluated the effect of idiotype (Id) vaccination on CMC.

Patients and methods: Eleven patients were immunized with the autologous Id in combinations with granulocyte–macrophage colony-stimulating factor and interleukin 12, and followed for CMC by quantitative real-time allele-specific PCR. Id-specific T cells were monitored by proliferation assay, enzyme-linked immunospot (Interferon-γ) assay, and quantitative real-time PCR for cytokines. Regulatory T (Treg) cells were analyzed by flow cytometry.

Results: CMC were detected in 9 of 11 patients at start of vaccination. In four patients, CMC declined and two had a complete molecular remission. Further two patients had stable levels of CMC during follow-up, while in three patients CMC progressively increased. Six patients had a vaccine-induced Id-specific T-cell response. A significant correlation was observed between reduced/stable levels of CMC and the Id-specific T cells (P < 0.02). The frequency of Treg cells was decreased in immune responders, but increased in immune nonresponders (P < 0.05). No significant change in the serum M-protein concentration was, however, observed in any patient.

Conclusion: Id vaccination reduced CMC, which correlated with vaccine-induced Id-specific T cells. Further studies are warranted to analyze the clinical significance of CMC and clinical effects of Id vaccination.

Key words: circulating myeloma cells, idiotype vaccination, multiple myeloma

introduction

The characteristics of the B cells belonging to the malignant clone in multiple myeloma (MM) are a matter of debate. An early B lymphocyte lineage cell with the ability to replicate and differentiate into the malignant myeloma plasma cell has been postulated as the precursor myeloma cell [1–3]. Precursor myeloma cells might be relatively resistant to chemotherapy and may be responsible for recurrence of the disease [4–6]. Those cells might be detected in the blood [2, 3, 7].

Clonal expansion of B lymphocytes during maturation is accompanied by rearrangement of genes encoding the variable (V) regions of the immunoglobulin (Ig) heavy (H) and light (L) chains forming the idiotypic structure harboring the antigenic determinants. In MM, the tumor cell clone encompasses a differentiating B-cell population which shares identical IgVH and IgVL gene rearrangements including terminally differentiated plasma cells expressing the idiotypic structures [2, 7].

The idiotypic structure is a unique target for immunotherapy [8] and has extensively been used in vaccine trials [9]. In MM, idiotype (Id) vaccination induced Id-specific T cells. Clinical effects measured as reduction of the serum M-protein concentration [9] and prolongation of time to progression (TTp) [10] have been noted. Id-reactive T cells may target Id-peptide/major histocompatibility complexes on myeloma cells and lyse the cells [11, 12]. Circulating myeloma cells (CMC) expressing the Id might be a target for the specific T cells.

A previous pilot study from our group showed that CMC might be reduced by Id vaccination [13]. In the present study, those preliminary observations are extended and related to the induction of Id-specific T cells and regulatory T (Treg) cells. Our data show that vaccine-induced reduction of CMC correlated significantly to vaccine-induced Id-specific T cells.

material and methods

patients

Eleven patients with stage I–II IgG MM (Table 1) were included. The median age was 70 years (range 51–87 years). All patients were immunized with the autologous Id protein together with granulocyte–macrophage colony-stimulating factor (GM-CSF) alone, interleukin (IL)-12 alone, or a combination of GM-CSF and IL-12 as adjuvant cytokines [10, 14]. The median time for maintained Id vaccination was 46 weeks (range 4–126 weeks). The studies were approved by the Medical Product Agency and the local ethics committee. Four of the 11 patients were previously analyzed for...
One patient (no. 3) received the Id vaccine plus 75 l continued at weeks 30, 46, 62, 78, 94, and 110 (maintenance phase). Was repeated after 2, 4, 6, 8, and 14 weeks (induction phase) and daily at the vaccine site, days 1 to 4 [10]. The vaccination procedure described above plus 75 l patients (no. 5, 6, 8, 9, and 11) were given the Id vaccine and IL-12 as Wyeth-Ayerst Research, Cambridge, MA) subcutaneously [10]. Five

Table 1. Clinical characteristics of the myeloma patients vaccinated with the autologous idiotype in combination with adjuvant cytokines

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gender</th>
<th>Age</th>
<th>Clinical stage</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>51</td>
<td>IA</td>
<td>Untreated</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>69</td>
<td>IIA</td>
<td>Treated*</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>85</td>
<td>IA</td>
<td>Untreated</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>69</td>
<td>IA</td>
<td>Untreated</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>60</td>
<td>IA</td>
<td>Untreated</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>77</td>
<td>IIA</td>
<td>Untreated</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>85</td>
<td>IA</td>
<td>Untreated</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>88</td>
<td>IIIA</td>
<td>Untreated</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>67</td>
<td>IA</td>
<td>Untreated</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>87</td>
<td>IA</td>
<td>Untreated</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>70</td>
<td>IA</td>
<td>Untreated</td>
</tr>
</tbody>
</table>

*CIB (cyclophosphamide, interferon-α, betamethasone) ended 3 years before sampling. F, female; M, male.

CMC [13] but reevaluated by the modified real-time allele-specific oligonucleotide (ASO) PCR technique as described below and followed for a longer time.

immunization protocol and immune testing times

The individual Id vaccines were prepared as described [10, 14]. Five patients (no. 1, 2, 4, 7, and 10) received 0.5 mg of the autologous Id vaccine intracutaneously together with 2 µg IL-12 (Genetics Institute, Inc./Wyeth-Ayerst Research, Cambridge, MA) subcutaneously [10]. Five patients (no. 5, 6, 8, 9, and 11) were given the Id vaccine and IL-12 as described above plus 75 µg of GM-CSF (Schering-Plough, Kenilworth, NJ) daily at the vaccine site, days 1 to 4 [10]. The vaccination procedure was repeated after 2, 4, 6, 8, and 14 weeks (induction phase) [14] and continued at weeks 30, 46, 62, 78, 94, and 110 (maintenance phase). One patient (no. 3) received the Id vaccine plus 75 µg of GM-CSF as above but only repeated after 2, 4, 6, 8, and 14 weeks (induction phase). Patients were tested for cellular immune responses before vaccination and at weeks 4, 8, 10, 14, 16, 30, 32, 46, 48, 62, 64, 78, 80, 94, 96, 110, 112, 126, 142, and 158 or until progressive disease.

isolation of peripheral blood mononuclear cells and bone marrow mononuclear cells

Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMC) were isolated using Ficoll/Hypaque (Amersham-Bioscience, Uppsala, Sweden) density-gradient centrifugation as described [10].

preparation of monoclonal IgG and F(ab′)2 fragments for in vitro tests

The procedure has been described in detail [14]. Briefly, serum was applied to a sterile mabTrapG column (Pharmacia, Uppsala, Sweden). Isoelectric focusing (Pharmacia Phast system) showed that >90% of the IgG was monoclonal. The monoclonal IgG was dialyzed against sterile NaCl and filtered through a Millipore filter (0.20 mm). F(ab′)2 fragments were prepared by pepsin digestion. F(ab′)2 fragments of the monoclonal IgG from other myeloma patients were used as controls.

proliferation assay

The method has been described in detail [14]. Briefly, PBMC were stimulated with purified F(ab′)2 fragments of the idiotype IgG and allogeneic monoclonal isotype-matched IgG F(ab′)2 fragments as controls. Unstimulated cells and cells stimulated with purified protein derivate (PPD) (Statens Seruminstitut, Copenhagen, Denmark) and phytohemagglutinin (PHA; Sigma-Aldrich, Stockholm, Sweden) were used as controls. Cells were cultured for 6 days and [3H]-thymidine (Amersham, Life Sciences, Amershams, UK) was added during the last 18 h of culture. Tests were run in triplicates. Mean thymidine incorporation was calculated for each triplicate. Stimulation index (SI) was calculated by dividing mean thymidine incorporation of antigen-stimulated cells with that of unstimulated cells. Mean ± SD of SI induced by isotypic monoclonal IgGs and polyclonal IgG (n = 2183 experiments) was 2.78. An SI cut-off level of ≥3.00 was used to indicate the presence of an Id-specific cellular response [10].

total RNA extraction and first-strand complementary DNA synthesis

Total RNA was extracted from PBMC by the guanidium thiocyanate–phenol–chloroform extraction technique using RNXzol B (AMS Biotechnology Europe, Stockholm, Sweden). First-strand complementary DNA (cDNA) synthesis was carried out according to standard protocols [15] using the First-Strand cDNA Synthesis Kit (Amersham-Pharmacia Biotech, Uppsala, Sweden).

genomic DNA preparation

Genomic DNA was extracted from PBMC using the QRAprep Spin Miniprep Kit (Qiagen®, Helden, Germany).

quantitative real-time PCR for cytokines and cytotoxic proteins

Details of the method have been described [15]. Briefly, PBMC were incubated with F(ab′)2 fragments of the idiotype- or the isotype-matched (control) monoclonal IgG for 48 h. Cells were also incubated with medium alone and PHA as controls. Cells incubated for 48 h were analyzed for the expression of granzyme B and perforin. MgCl2 concentration was optimized for the individual primers to obtain maximum efficiency. The enzyme-linked immunospot (ELISPOT) assay for identification of interferon-γ (IFN-γ)-secreting cells was carried out as described [10]. Briefly, PBMC were incubated with F(ab′)2 fragments of the idiotype or the isotypic (control) monoclonal IgG for 48 h in humidified air with 5% CO2 at 37°C. PBMC were also stimulated with medium alone, PPD, and PHA. Spots-forming units (SFU) corresponding to cells secreting IFN-γ were quantified using an automated computer-assisted video imaging analysis system (Axioplan2) (Carl Zeiss Vision, Jena, Germany). The number of SFU was obtained by subtracting the number of spots in cultures incubated with medium alone from that of stimulated cultures. Results are expressed as number of SFU/106 PBMC. Mean ± SD of SFU/106 PBMC induced by the isotype-matched IgG F(ab′)2 (control) (n = 513 experiments) was 60. A cut-off level of >70 SFU/106 PBMC was used to indicate the presence of an Id-specific cellular response [10].

criteria for a vaccine-induced Id-specific T-cell response

A vaccine-induced Id-specific T-cell response was considered to be present when all the following three criteria were met: (i) an Id-specific SI, SFU, or messenger RNA (mRNA) cytokine gene expression ratio above the
corresponding cut-off levels (3, 70, and >1, respectively); (ii) an Id-specific SI, SPU, or mRNA cytokine expression ratio more than twice the respective pre-vaccination baseline value; and (iii) an Id-specific cellular response in at least two of the three analyses detected at least two different testing times after start of vaccination [10].

The cellular immune response was defined as a Th1 response when only Th1 cytokine genes (IFN-γ and/or TNF-α) were expressed and as a Th2 response when only the Th2 cytokine genes (IL-4 and/or IL-5) were noted. When both types of cytokine genes were found, the immune response was considered a mixed Th1/Th2 with a predominance of Th1 cells when Th1 cytokine genes’ fold increase ratios were higher or expressed at more testing times than the Th2 cytokine genes. The immune response was considered a mixed Th1/Th2 response with a predominance of Th2 cells when the Th2 cytokine genes’ fold increase ratios were higher or expressed at more testing times than Th1 cytokine genes [10, 16].

ASO probes and primers
The gene encoding the variable heavy chain (VH) of each patient was determined by reverse transcription (RT)–PCR using consensus VH family primers [17] and a consensus gamma constant Cc region primer [18] using BMMC. The dominant VH gene family (tumor clone) was identified as the primer amplified only the VH gene from the patient of interest. The complementary-determining regions (CDR) II and III sequences were identified for all patients. For the cDNA assay, patient-specific ASO sense primers corresponding to the CDRIII regions were designed for each patient to obtain maximum specificity (Table 2). A Cγ antisense primer (GGAAGTACTCCTGACGAC) and probe (CCTGCCAACAGGGCCCATCG) were used for all patients. For the genomic DNA assay, a patient-specific sense primer corresponding to the VH 3 or 1 region, ASO (CDRIII) antisense primer, and ASO (CDRII) probe were used (Table 2). The VH 3/1, CDRIII, and CDRII primers were tested on a panel of DNA from different patients to ensure that the primer amplified only the VH gene from the patient of interest.

RT-ASO-PCR and real-time ASO-PCR
Patients were first tested for the presence of the malignant clone by RT-ASO-PCR using patient-specific ASO primers. All positive patients were then subjected to quantitative real-time ASO-PCR analyses as described [20] with minor modifications. Briefly, PCR was carried out in a 25-μl reaction volume containing 1× TaqMan Buffer A (Perkin Elmer), MgCl2 (optimized for each ASO assay), and 3 mM MgCl2 for the β-actin assay (Perkin Elmer), 0.5 mM dNTPs (deoxyribonucleoside triphosphates) (Perkin Elmer, Stockholm, Sweden), 5.0 pmol forward (CDR3) ASO primer (Cybergene AB, Stockholm, Sweden), 5.0 pmol reverse (Cγ) primer (Cybergene AB), 2.5 pmol (Cγ) TaqMan probe (Cybergene AB), 0.5 units Ampli-Taq Gold, and 0.1 units uracil-N-glycosylase (Perkin Elmer). Two microclones of cDNA were included in each reaction volume except for the negative controls. In two patients, the test was additionally run on genomic DNA for comparison. For generation of standard curves, serial dilutions of known concentrations of the patient-specific VH and β-actin plasmid DNA were used. For each sample, at least four or six aliquots (when material allowed) were used, two to three aliquots for quantitation of myeloma B cells and three aliquots for β-actin. Aliquots were amplified for an initial period of 2 min at 50°C and 10 min at 95°C followed by 40 concurrent cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. ABI PRISM 7700 Sequence Detection System (Perkin Elmer) was used for online quantification.

The mean gene copy number of the patient-specific VH was divided by that of the corresponding β-actin copy number for normalization. The value of each sample before vaccination was set to 100%. The values during

### Table 2. Allele-specific oligonucleotide (ASO) primers and probes

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>ASO CDRIII primer (sense) (cDNA assay)</th>
<th>VfH 3/1 families, ASO CDRIII and CDRII primers and probes (genomic DNA assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAGAGCCCAAGGACCACAGTAAC</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>ACGAGAGCCGGTGCTGTATTAG</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>ACAGCCTATAGCATTGTTGCT</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>CATAGACGTTCTGGGGCGGA</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>AAATGGACGGAGTGGGGTTG</td>
<td>GGTTACACCTTTACCCGACTATG (sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAAAGCCCTTCCGCCATATACA</td>
</tr>
<tr>
<td>6</td>
<td>CGCCCCTCCCGATATAACA</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>ATAGTAGCTGGAGGCTATGCGGTCC</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>GTCCCTCTGGGGCAGCTG</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>GCAAGGGAGGCTCCTGAGCTCA</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>GGGTGGGACTCAGGGG</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>GAGTGGGAGTTGGCCGACATCT</td>
<td>ND</td>
</tr>
</tbody>
</table>

cDNA, complementary DNA; CDR, complementary-determining regions; ND = not done.
follow-up were presented as percent of the pre-vaccination value. When genomic DNA was analyzed, the absolute gene copy number was plotted against testing times and compared with the corresponding gene copy number in the cDNA real-time ASO-PCR assay.

**Treg cells**

Treg cells were analyzed using the Human Regulatory T cell Staining Kit (eBiosciences, Inc., San Diego, CA). Frozen PRBCs were thawed and subjected to Ficoll gradient centrifugation to obtain viable cells. Cell viability was >98%. Cell surface markers were analyzed by fluorescein-conjugated monoclonal antibodies against CD4 and CD25. FoxP3 was assessed intracellularly by anti-FoxP3 monoclonal antibody. Cells incubated with irrelevant isotype-matched antibodies were used as controls. For each patient, a minimum of 10⁶ CD4⁺ cells were acquired. The CD4⁺/CD25⁺/Foxp3⁺ subset was analyzed using FACSCalibur (BD, Mountain View, CA). The data were analyzed by the Flowjo® Software program (Tree Star, OR) and presented as the frequency of triple positive (CD4⁺/CD25⁺/Foxp3⁺) cells within the CD4⁺ population.

**statistical methods**

Fisher’s exact test was used to compare the kinetics of CMC in immune responders and immune nonresponders. The chi-square test was amplified against testing times and compared with the corresponding gene copy number in the cDNA real-time ASO-PCR assay. The data were analyzed by the Flowjo® Software program (Tree Star, OR) and presented as the frequency of triple positive (CD4⁺/CD25⁺/Foxp3⁺) cells within the CD4⁺ population.

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**Table 3. Vaccine-induced idiotype-specific T-cell responses and relation to circulating myeloma cells (CMC) responses**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Adjuvant cytokine(s)</th>
<th>Proliferation assay (SI)</th>
<th>ELISPOT (IFN-γ) (SFU/10⁶ cells)</th>
<th>QRT-PCR for multiple cytokines (fold increase ratio)</th>
<th>Overall vaccine-induced T-cell response</th>
<th>Overall response of CMC during follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IL-12</td>
<td>—</td>
<td>—</td>
<td>IFN-γ: 32 (30)</td>
<td>b</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>IL-12</td>
<td>—</td>
<td>—</td>
<td>IL-5: 128 (16)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>GM-CSF</td>
<td>3.4 (16), 3.2 (30), 16.3 (48)</td>
<td>90 (30), 320 (62)</td>
<td>TNF-α: 44415 (16), 4 (30); GRZMB: 2 (8), 23 (10)</td>
<td>(Th1)¹ ³</td>
<td>Complete remission</td>
</tr>
<tr>
<td>4</td>
<td>IL-12</td>
<td>—</td>
<td>3850 (48)</td>
<td>TNF-α: 14972 (14); GRZMB: 4.7 (14)</td>
<td>(Th1)¹ ³</td>
<td>Complete remission</td>
</tr>
<tr>
<td>5</td>
<td>IL-12/GM-CSF</td>
<td>3.6 (4), 4.4 (14)</td>
<td>—</td>
<td>IFN-γ: 1820 (8); TNF-α: 13308 (8); IL-5: 5 (4) 6580 (8), 3 (16); perforin: 50 (30)</td>
<td>(Mixed with a Th1 predominance)³</td>
<td>Decline</td>
</tr>
<tr>
<td>6</td>
<td>IL-12/GM-CSF</td>
<td>—</td>
<td>110 (30)</td>
<td>IL-5: 10 (32); GRZMB: 5 (8), 4 (10); perforin: 3 (10), 5 (14)</td>
<td>(Mixed with a Th2 predominance)³</td>
<td>Decline</td>
</tr>
<tr>
<td>7</td>
<td>IL-12</td>
<td>7.5 (4), 179 (8), 24.2 (10), 63.9 (14)</td>
<td>120 (8), 780 (10), 110 (14), 100 (96)</td>
<td>IL-5: 16 (4); IFN-γ: 9 (14); TNF-α: 18 (14); GRZMB: 1.5 (16), 1.5 (30)</td>
<td>(Mixed with a Th1 predominance)³</td>
<td>Stable</td>
</tr>
<tr>
<td>8</td>
<td>IL-12/GM-CSF</td>
<td>90.5 (4), 43.5 (8), 40 (10), 35.3 (14)</td>
<td>—</td>
<td>IL-4: 80684 (16)</td>
<td>(Th1)³</td>
<td>Stable</td>
</tr>
<tr>
<td>9</td>
<td>IL-12/GM-CSF</td>
<td>—</td>
<td>—</td>
<td>ND</td>
<td></td>
<td>Increase</td>
</tr>
<tr>
<td>10</td>
<td>IL-12</td>
<td>—</td>
<td>—</td>
<td>TNF-α: 4 (8)</td>
<td></td>
<td>Increase</td>
</tr>
<tr>
<td>11</td>
<td>IL-12/GM-CSF</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>Increase</td>
</tr>
</tbody>
</table>

¹Figure within bracket indicates week for the response. Only positive responses are shown.
²No detectable CMC at any time.
³A T-cell response according to criteria (see ‘material and methods’).

**results**

**vaccine-induced Id-specific T cells**

The Id-specific T-cell responses are shown in Table 3. Six patients (no. 3, 4, 5, 6, 7, and 8) mounted a vaccine-induced Id-specific T-cell response with a predominance of Th1 cells. Five of the six immune responders had Id-induced expression of the genes for the cytotoxic proteins granzyme B and/or perforin. The remaining five patients did not meet the criteria for mounting vaccine-induced T cells. No patients had a pre-vaccination Id-specific T-cell immunity (data not shown).

**effect of Id vaccination on CMC**

A representative experiment showing the patient-specific IgVH_{11} cell clone by RT-ASO-PCR during follow-up is depicted in Figure 1 (patient no. 3). The patient showed complete molecular remission of CMC lasting for >1 year.

All RT-ASO-PCR-positive patients were subjected to quantitative real-time ASO-PCR to determine the kinetics of CMC. Patients who mounted a vaccine-induced Id-specific T-cell response (n = 6) either showed a reduction of CMC over time (no. 3, 4, 5, and 6) (Figure 2A) or maintained a similar level of CMC (no. 7 and 8) (Figure 2B). Two of the patients (no. 3 and 4) achieved a complete molecular...
remission in blood and remained negative for CMC during long-term follow-up. Patients who did not mount an Id-specific T-cell response (no. 9, 10, and 11) showed a progressive increase in CMC (Figure 2C). (Patients 1 and 2 had no detectable CMC at any time.) The reduction of CMC in patients with an Id-specific T-cell response was statistically significant compared with the increase in CMC seen in patients who failed to mount a vaccine-induced Id-specific T-cell response ($P < 0.02$). It should, however, be noted that no significant change in the serum M-protein concentration was observed in any patient.

The kinetics of CMC were similar, when CMC were analyzed by genomic DNA or cDNA, as exemplified in Figure 3A and B (patient no. 6). However, the gene copy numbers in the cDNA assay were much higher than the corresponding numbers in the genomic DNA assay, which is to be expected as a single-gene activation may yield multiple mRNA transcripts.

**Treg cells**

Due to lack of material, Treg cells were only analyzed before and at a median time of 4.5 weeks (range 2–19 weeks) after the last Id immunization in six patients (no. 4, 5, 6, 9, 10, and 11). Three patients (no. 4, 5, and 6) were immune responders and three (no. 9, 10, and 11) immune nonresponders. The frequencies (%) of Treg cells before and after immunization in immune responders were 4.6, 6.1, and 6.2 and 2.4, 3.7, and 3.8, respectively, while in immune nonresponders the corresponding figures were 8.2, 0.75, and 4.5 and 7.3, 2.7, and 5.7, respectively. The change in the frequency of Treg cells comparing immune responders and immune nonresponders was statistically significant ($P < 0.05$) (Figure 4). Three of the four patients with a reduction of Treg cells were immune responders and had a reduction of CMC, while the two patients with an increase in Treg cells were immune nonresponders and had an increase in CMC.

**discussion**

Circulating tumor cells have been described in several tumor types. The primary tumor lesion might shed tumor cells into the circulation even at an early stage [21–23]. The number of blood tumor cells correlated with progression-free and overall survival in patients with metastatic breast cancer [24, 25]. In patients with high-risk uveal melanoma, the presence of circulating tumor cells was an independent prognostic factor for development of distant metastases [26].

In myeloma, the presence of CMC at the time of diagnosis was associated with aggressive disease and a poor prognosis [27]. CMC in the stem-cell harvest for autologous stem-cell transplantation was an independent prognostic factor predicting early relapse regardless of the initial response to treatment [22].

In spite of the initial high response rates achieved by current anti-myeloma therapies, myeloma remains an incurable disease. Thus, there is a need for complementary therapies to overcome the inevitable disease recurrence. Id-induced immunity was found to be associated with a significant increase in disease-free survival of patients with follicular lymphoma [28] and molecular remission in blood by eliminating circulating lymphoma cells [29]. Id vaccination in myeloma patients has been shown to induce a tumor-specific T response and prolonged TtP [9, 10].

Myeloma patients with early-stage disease or minimal residual disease after treatment most likely have preserved T-cell functions as compared with patients with advanced disease [30]. Such patients may be suitable candidates for immunotherapy. We have previously reported, in a small pilot study [13], that in early-stage myeloma, CMC were reduced following Id vaccination. In the present study, where all patients had stage I or asymptomatic stage II disease, we substantiated those findings. Seven of the patients (64%) had Id-specific T cells before vaccination (data not shown), which are known to be more frequently found in myeloma patients.
with a low tumor burden [31]. The presence of spontaneously induced tumor-specific T cells is considered a favorable sign for response to vaccine treatment as it might be easier to boost than to induce an immune response against an auto-antigen [9]. Six of the 11 patients (55%) mounted or boosted an Id-specific T-cell response during vaccination and all of them showed a reduction and/or stable level of CMC. A blood complete molecular remission was observed in two patients, which lasted for 4 and 14 months, respectively. The decline in CMC was noted quite late and after multiple vaccinations indicating the importance of repeated immunizations. Since no treatment other than the Id vaccine was given, the observed clinical effect is likely to be vaccine induced. No significant change in the serum myeloma protein was, however, noted. The serum M-component reflects the myeloma tumor mass and is secreted mainly by the myeloma plasma cell population. CMC is regarded a spill over of myeloma bone marrow cells including the precursor B lymphoid myeloma cells, which are relatively resistant to chemotherapy and may be responsible for recurrence of the disease [4–6]. The presence of CMC in peripheral blood is an unfavorable prognostic sign [27]. Elimination of CMC might be of therapeutic benefit. The lack of correlation between the levels of CMC and M-component concentration might be explained by that CMC is not the major Ig-producing cell fraction and these cells are a minor fraction of the total myeloma population in patients with a serum M-component. Elimination of CMC might thus not induce a detectable reduction of the M-protein in patients with overt disease. No plasma cells evaluated by morphology were found in peripheral blood (data not shown). Although real-time ASO-PCR analysis using genomic DNA reveals the actual number of CMC, the number of CMC might be too few to be detected [13]. A real-time ASO-PCR assay on the basis of cDNA is more likely to detect VH gene copy numbers of a very small CMC population. Therefore, we carried out real-time ASO-PCR analysis on cDNA to reduce the risk of false-negative results that might occur using genomic DNA. This sensitive technique may also explain why we noticed a reduction in CMC but not in the

**Figure 2.** Kinetics of circulating myeloma cells (real-time allele-specific oligonucleotide PCR) in (A) patients (no. 3, 4, 5, and 6) who mounted an idiotype-specific T-cell response during vaccination or (B) patients (no. 7 and 8) with stable levels and (C) in patients (no. 9, 10, and 11) who did not show a vaccine-induced idiotype T-cell response. Baseline for each patient was set to 100%. The values during follow-up are given as percent of the initial value. Arrows indicate time of idiotype vaccination.

**Figure 3.** Representative experiment comparing the kinetics of circulating myeloma cells by (A) genomic DNA and (B) complementary DNA (patient no. 6).
M-component concentration, i.e. vaccine therapy might only be able to eliminate a small tumor cell population but not a large to bring about reduction of the M-component. A Cy antisense primer and probe were used to avoid the amplification of any contaminating genomic DNA. The two assay systems were shown to yield similar results with regard to the kinetics of CMC.

Another interesting preliminary observation was the reduction of T\(_{reg}\) cells in immune responders and increase in immune nonresponders. There are ample indications in both animals and cancer patients that an increase in both natural and induced T\(_{reg}\) cells is associated with decreased tumor-specific immunity and disease progression. Reduced T\(_{reg}\) levels seem to be a requirement for an effective tumor immunity and disease regression [32].

The results of the present study confirm and extend our previous observation, indicating that long-term Id vaccination in MM patients might reduce/eliminate CMC. Similar to the results in follicular lymphoma, vaccine-induced anti-Id immunity may be associated with a long-lasting molecular remission in blood. Further studies are warranted to establish the role of CMC in the biology of MM and in particular if analyzing such cells might be a valuable surrogate readout system for the evaluation of novel therapeutic approaches.

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**references**


