

Idiotype Vaccination in Multiple Myeloma Induced a Reduction of Circulating Clonal Tumor B Cells

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Abstract

Myeloma cells express the idiotype (Id)-specific antigen that may be targeted by idiotype vaccination. Six stage I IgG myeloma patients were immunized with the autologous purified M-component together with the adjuvant cytokines IL-12 alone or in combination with GM-CSF. The effect of Id vaccination on circulating clonal tumor B cells was monitored by a real-time allele-specific oligonucleotides PCR method. No other treatment was given. Blood tumor mass reduction was observed in 4/6 patients, with one patient achieving a complete molecular remission in blood. In 3 of these 4 patients an idiotype specific T cell response was induced. In the remaining two patients with an unchanged level of blood tumor cells, one patient mounted a T cell response while the other did not. No significant change in the serum M-protein was noted. Id vaccination may target clonal B cells suggesting that this strategy might be complementary to achieve tumor control. The clinical significance of these findings remains to be established.

Introduction

Although a high response rate is achieved in patients with multiple myeloma (MM) by high dose chemotherapy, the management of MM patients remains a problem. Complementary therapeutics are needed to cure or control the disease. The idiotype (Id) of the myeloma immunoglobulin (Ig) is regarded a tumor specific antigen and a target for immunotherapy.

Natural occurring MHC restricted myeloma Id-specific T-cells have been identified.¹ Such T-cells may target Id Ig peptide/MHC complexes on myeloma cells and might lyse tumor cells.^{2,3} Idiotype vaccination may therefore be a treatment approach to induce a therapeutic idiotype specific T cell response.^{2,3,4} The concept of idiotype immunization in multiple myeloma was already introduced about 30 years ago.⁵ The idiotype seems however to be a weak immunogen and to evoke a strong response adjuvant cytokines should be added. GM-CSF augments the functional capacity of antigen presenting cells and IL-12 amplifies the immune response and directs towards a type I T cell response.⁴ A Th₁ type cellular response seems to be of importance for tumor regression, while a Th₂ T cell response might provide a microenvironment conducive to disease progression.⁶

Idiotype vaccination in malignant lymphomas induced idiotype specific T cells and complete blood molecular remission.⁷ In MM, idiotype vaccination evoked an idiotype specific T cell response and occasionally clinical effects as reduction of serum M-component and disease stabilisation.⁸

The clonal cell compartment in myeloma represents an ongoing differentiating cell population⁹ including pre-switch cells.^{10,11} The number of pre-plasma cells seems to

be related to disease burden and clinical outcome.¹² Clonal pre-plasma cells express MHC class I and II molecules and plasma cells mainly MHC class I molecules with idiotypic Ig peptides in the groove and may thus be targeted by idiotype specific T cells.

In this study, we monitored peripheral blood (PB) tumor cells by a real-time allele-specific oligonucleotides (ASO) PCR method in myeloma patients undergoing Id vaccination and could show that immunization, using the autologous myeloma Id, induced PB tumor mass reduction.

Materials and methods

Patients with multiple myeloma stage I and an increasing serum M-component concentration were included. Ten patients were considered for the study. In 6/10 patients we were able to monitor the level of clonal cells using complementarity determining region 3 (CDR3) specific PCR assays, which 6 patients are included in this report.

The M-component was purified as described.⁴ The patients were immunized with the autologous Id together with IL-12 with or without GM-CSF as follows: i) in the IL-12/GM-CSF group 0.5 mg of the autologous M-component in 0.5% aluminiumphosphate (SBL-Vaccin, Solna, Sweden) was given i.c. on day 1 together with 75 µg/day of GM-CSF (Leucomax, Schering-Plough, Kenilworth, NJ, USA) i.c. at the same site, days 1-4, and 30 ng/kg of IL-12 s.c. day 1 (Wyeth Ltd, London, UK); ii) in the IL-12 group 0.5 mg of the M-component was given i.c. on day 1 and 30 ng/kg of IL-12 s.c. day 1. Immunizations were done weeks 0, 2, 4, 6, 8, 14 and 30.

Two methods were applied to detect idiotype specific T cells: a proliferation assay (^3H -thymidine incorporation) and ELISPOT (γ -interferon). The techniques have been described in detail elsewhere⁴. Proliferation assay: Briefly, peripheral blood mononuclear cells (PBMC) were stimulated with purified $\text{F(ab}')_2$ -fragments of the autologous monoclonal IgG (1 pg/ml to 100 $\mu\text{g/ml}$) as well as with 3 allogeneic monoclonal isotype matched IgG from other myeloma patients and cultured for 6 days. ^3H -thymidine was added during the last 18h. Tests were run in triplicates, and mean reactivity was calculated for each triplicate. For each testing time, the highest mean value of a triplicate obtained from the 5 different concentrations of the idiotype was used. A stimulation index (SI) was calculated by dividing the mean radioactivity for a triplicate of stimulated cells by that of unstimulated cells. γ -IFN ELISPOT: Briefly, wells of nitro-cellulose bottomed microtiter plates were coated with a mouse monoclonal anti-human γ -IFN (Mabtech, Stockholm, Sweden) at 4°C over night. After removal of the coating solution, the plates were washed twice in PBS. Subsequently, 100 μl aliquots of freshly prepared PBMC (10^6 cells/ml) were added to each well. Cells were incubated with $\text{F(ab}')_2$ -fragments of the autologous or isotypic monoclonal IgG (1 pg/ml – 100 $\mu\text{g/ml}$) for 30 h. To visualize spots corresponding to single γ -IFN secreting cells, the cells were washed with PBS and the wells were incubated with a rabbit monoclonal anti-human γ -IFN at 37°C for 2 h. After washing, biotinylated anti-rabbit IgG (Vector Lab, Burlington, CA, USA) was added, followed by avidin-biotin-peroxidase complex (ABC, Vectastin elite kit, Vector Lab) for 50 min. After peroxidase staining using the substrate 3-amino-9-ethyl-carbasol (Sigma St Louis., MA, USA), the number of spots corresponding to cells secreting γ -IFN was quantified using an automatic device (Zeiss-Kantron, Jena,

Germany). The results are expressed as mean number of spots (cells) of duplicate wells after subtraction of the mean number of spots of unstimulated cells. The highest value obtained by any of the concentration is used. A patient was considered to have developed idiotype specific T cells if a response was observed at two different time points in at least one of the two test (read-out) systems. To establish a cut-off level for an idiotype specific T cell response, lymphocytes of the myeloma patients were stimulated with purified irrelevant M-components. A stimulation index of ≥ 3.1 [$1.21 + 1.88$ (mean + 2 SD) of 131 control experiments] was considered a positive response. In the γ -IFN ELISPOT assay ≥ 10 cells/ 10^5 PBMC was considered a positive response [$1.16 + 7.76$ (mean + 2 SD) of 213 control experiments]. Both cut-off levels are in line with our previous results.^{1,4} The patients were tested at weeks 0, 4, 8 or 10, 14 or 16 and 30 respectively.

For the generation of ASO primers and probes, the V_H gene sequence was determined by RT-PCR using consensus V_H family primers.¹³ Patient-specific quantitative real-time PCR analyses were performed as previously described.¹⁴ Briefly, real-time PCR reactions contained a DNA sample, 10 x PCR Buffer A (5 μ l), $MgCl_2$ (optimized for each ASO assay) 200 μ M dATP, dCTP, dGTP (each), 400 μ M dUTP, 1.25 Units Ampli Taq Gold DNA polymerase, 0.5 Units AmpErase uracil *N*-glycosylase (UNG), concentration of ASO primer, J_H primer (5'-ACCTGAGGAGACGGTGACCAG-3') and ASO TaqMan probe were optimized for each ASO assay. For the β -actin assay, forward and reverse primer concentrations were 0.3 μ M (each) and the β -actin probe concentration was 0.2 μ M. Reaction volumes were adjusted to 50 μ l with dH₂O. For generation of a standard curve, the TaqManTM DNA Template Reagents Kit containing DNA of known concentrations was used. All PCR

consumables except the oligonucleotides mentioned in the previous section were supplied by PE Applied Biosystems, (Foster City, CA.). The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C followed by 45 cycles of 95°C for 0.15 min and 58°C for 1 min. All reactions were performed in the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). Collection and analysis of data were done with the Sequence Detector Software version 1.6 (PE Applied Biosystems). Blood samples obtained at weeks 0, 8, 14 and 30 for the assessment of the levels of clonal B cells. For each sample, 6 aliquots with approximately 1 µg of DNA were subjected to analysis, 3 samples for quantitation of β-actin and 3 samples for clonal cells. Nested ASO RT-PCR including approximately 0.5 µg of total RNA were performed as described previously.¹³

Results and Discussion

For the development of an idiotype vaccination strategy in MM patients we are focusing on stage I based on the observation that early stage MM patients may naturally exhibit mainly type I Id-specific T cells whereas in late stage type II Id-specific T cells seemed to prevail.¹ In the present study, half of the patients had an idiotype specific proliferative T cell response pre-vaccination, which is in agreement with our earlier observations.¹⁵ In stage I, the general T cell functions also seems to be well preserved (data to be published).

In 8/10 of the stage I patients considered for the study, we were able to identify the myeloma specific immunoglobulin heavy chain gene (IgH) DNA sequence. For these 8 patients we designed a patient-specific ASO primer and a patient-specific dual-labeled fluorogenic probe (ASO TaqMan probe) corresponding to CDR3 (Table 1). The CDR3 primers were tested on a panel of DNA from different patients, ensuring that the primers

amplified only an IgH sequence from the patient of interest. The forward CDR3 primer was designed to span the V-D junction, and the CDR3 TaqMan probe was designed to span the D-J junction giving maximum specificity (Table 1).

Table 1. The nucleotide specific IgH sequences of eight myeloma patients.

Patient	Patient specific IgH sequences
MM-1	<u>CTGTGCGAAA...GTAGTGGTGGACTATGATAGTAGTGGAGGCTATGGCTCC...TGGGGTCAG</u>
MM-2	TGTGCGAAA...TCGTCATACAGTGGCTACGATTATGTCCTCTCGGGGCAC... TGGGGCCAGGGAA
MM-3	TGTGCGAGA...GTCGGGACTAGTGCTTCTTACTACTACTACTACTACATAGACGTC... TGGGGCGAAGGGA
MM-4	GAGGACACAGCCGTCTATTTTTGTACCACC... <u>CTCTTTGACTAC...TGGGGCCAGGGA</u>
MM-5	TGTGCGAGA...TGGGGGACTTCGGAGGC CTTTGACTCC...TGGGGCCAGGGA ACC
MM-6	<u>TGTGCGAGA...GGGCCGTTGGGTTGGGACTAC...TGGGGCCAGGGA</u> ACCC
MM-7	TGTGCGCGC...GTCCCAAACGCCTATGCCAGTT CGGTTTGGGGCTGACGAC...TGGGGCCAG
MM-8	TGTGCGCGA...GCGATCTCGCGGCCGTTGTTCCGAGAATT GGTACAGCACGGTTTGGACGTC...TGGGGCC

CDR3 sequences is borderlined by ..., forward ASO primers is shown as underlined and ASO probes is shown as bold underlined.

Sequential analyses of circulating blood myeloma B cells were performed (Figure 1). In 2/8 patients, circulating clonal tumor cells were detectable by nested ASO RT-PCR, but below reliable quantitation by real-time ASO PCR. The sensitivity of the real-time ASO PCR is in the range of $1:1 \times 10^5 - 5 \times 10^5$ and $1:10^6$ for nested ASO RT-PCR.^{12,13} Of the remaining 6 patients, 3 patients were immunized with the idiotypic immunoglobulin together with IL-12 alone (MM-1,3 and 6) and 3 patients received a combination of the adjuvant cytokines IL-12 and GM-CSF (MM-2,4 and 5). No other treatment was given during the study period.

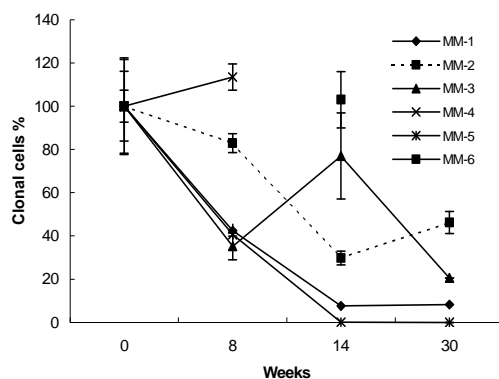


Fig. 1 Blood clonal myeloma tumor B cell levels measured by real-time ASO PCR (see Materials and Methods) in 6 MM patients immunized with the autologous Id during a 30 weeks of follow-up. Arrows indicate immunization times. Three patients received IL-12 alone (MM-1,3 and 6) and 3 patients IL-12 plus GM-CSF (MM-2,4 and 5) as adjuvants. The number of IgH copies detected at initiation of vaccination was set to 100%. The values during follow-up are given as percent of the initial sample. Mean \pm SEM for 3 measurements are shown.

No hematological toxicity was observed in any of the patients and no abnormalities in kidney and liver function tests were noted (data not shown). Patient MM-2 experienced transient dyspnoea, weight on the chest and shivering starting 2h following the sixth vaccination. The symptoms disappeared spontaneously within 1h. Patient MM-3 had fever (up to 38.5°C) for about 24 h after each vaccination and an NCI grade II local skin reaction. Patient MM-4 had an NCI grade II local skin reaction at the first 5 vaccination times. Patient MM-5 developed at the last vaccination petechial bleedings of the lower legs considered to be due to capillary leakage.

One patient (MM-5) achieved a complete peripheral blood molecular remission (below 1:10⁶, PCR negative using both ASO real-time and nested RT-PCR). Three patients had a reduction of 92%, 79% and 54% respectively of the clonotypic B cells (MM-1,3 and 2 respectively). Two patients (MM-4 and 6) had unchanged levels of clonal B cells. In no patient a significant change of the serum M-component concentration was observed during the study period (Table 2).

Table 2. Idiotype specific T cell response (proliferation/cytokine production) and M-component conc. in myeloma patients immunized with the autologous tumor derived idiotypic immunoglobulin in combination with the adjuvant cytokines IL-12 ± GM-CSF

Patients	Week 0			Week 4††		Week 8/10			Week 14/16			Week 30		
	Prol.*	E**	M [§]	Prol.	E	Prol.	E	M	Prol.	E	M	Prol.	E	M
MM-1 (IL-12)§§	Neg. †	Neg.	25	8	Neg.	179	78	ND	64	11	35	Neg.	Neg.	34
MM-2 (IL-12/GM)	Neg.	Neg.	22	91	Neg.	44	Neg.	ND	36	Neg.	26	Neg.	Neg.	29
MM-3 (IL-12)	Neg.	Neg.	34	Neg.	Neg.	Neg.	Neg.	ND	Neg.	Neg.	30	Neg.	Neg.	37
MM-4 (IL-12/GM)	18	Neg.	17	71	Neg.	101	Neg.	ND	11	Neg.	17	Neg.	ND	16
MM-5 (IL-12/GM)	Neg.	Neg.	30	Neg.	Neg.	84	Neg.	ND	81	70	28	54	25	44
MM-6 (IL-12)	14	Neg.	20	Neg.	Neg.	Neg.	Neg.	ND	Neg.	Neg.	21	ND	ND	ND

* Prol. = proliferation assay expressed as stimulation index: ≥ 3.1 was considered a positive response. ** E = ELISPOT (γ -IFN production) expressed as number of spots/10⁵ PBMC: ≥ 10 was considered a positive response. § M = serum M-component concentration

(g/l). §§ indicates the adjuvant cytokines. † negative indicates a value below the cut-off level. ND = not done. †† M-component concentration was not analysed at week 4

Patients MM-1, MM-2, MM-4 and MM-5 mounted an idiotypic specific T cell response, while in patients MM-3 and MM-6 no idiotypic specific T cell response was detected (Table 2). Thus, 3 patients mounting an idiotypic specific T cell response had tumor cell reduction while one patient developing an anti-idiotypic cellular response showed no decrease in circulating tumor B cells. However, one patient (MM-3) had a tumor mass reduction without a detectable T cell response. A similar phenomenon has been described in melanoma patients immunized with MAGE-3 peptides. Partial and complete remissions were obtained without detectable MAGE-3 specific T cells.¹⁶

Massaia and co-workers¹⁷ immunized 2 MM patients with minimal residual disease using the idiotypic but were not able to detect a reduction of circulating tumor cells by PCR. The reason for the discrepancy is not clear but may be due to the small number of patients, a different vaccination schedule and that the patients probably exhibited a pronounced T cell dysfunction due to high-dose chemotherapy. Moreover, in that study GM-CSF or IL-2 was used as adjuvant cytokines while all our patients received IL-12. It was recently shown in an animal model that immunization with myeloma cells transfected both with the GM-CSF and IL-12 genes induced proliferative as well as cytotoxic T cell responses directed against the secreted monoclonal Ig and protected >90% of the animals against tumor challenge.¹⁸ It is not likely that IL-12 alone contributed to the blood tumor cell reduction observed in our patient as the adjuvant dose of IL-12 was very low, only 1% of the dose used for treatment of patients with malignancies. However, in our patients no major tumor reduction was noted, as measured by serum M-component conc., which was stable over time in all patients, indicating that myeloma specific T cells might only be able to eradicate a minimal tumor burden. This is

the first study in myeloma showing that idiotype immunization is able to induce reduction at the molecular level of circulating tumor cells similar to the observation in follicular lymphomas where immunotherapy could induce molecular remission after chemotherapy induced clinical remission using idiotype vaccination⁷ as well as monoclonal antibody therapy (rituximab)¹⁹.

In conclusion, Id vaccination in MM patients may induce tumor specific T cells which might target circulating clonal tumor B cells. Idiotype vaccination may preferentially be applied when the tumor load is low, i.e. early during the course of the disease or after chemotherapy. In the latter case measures should be taken to augment T cell functions as otherwise an effective tumor specific immune response may not be induced.

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References

1. Yi Q, Osterborg A, Bergenbrant S, Mellstedt H, Holm G, Lefvert AK. Idiotype-Reactive T-Cell Subsets and Tumor Load in Monoclonal Gammopathies. *Blood*. 1995;86:3043-3049.
2. Li Y, Bendandi M, Deng Y, Dunbar C, Munshi N, Jagannath S, Kwak LW, Lyerly HK. Tumor-specific recognition of human myeloma cells by idiotype-induced CD8(+) T cells. *Blood*. 2000;96:2828-2833.
3. Wen YJ, Barlogie B, Yi Q. Idiotype-specific cytotoxic T lymphocytes in multiple myeloma: evidence for their capacity to lyse autologous primary tumor cells. *Blood*. 2001;97:1750-1755.
4. Osterborg A, Yi Q, Henriksson L, Fagerberg J, Bergenbrant S, Jeddi-Tehrani M, Rudén U, Lefvert AK, Holm G, Mellstedt H. Idiotype Immunization Combined With Granulocyte-Macrophage Colony-Stimulating Factor in Myeloma Patients Induced Type I, Major Histocompatibility Complex-Restricted, CD8- and CD4-Specific T-Cell Responses. *Blood*. 1998;91:2459-2466.
5. Lynch RG, Graff RJ, Sirisinha S, Simms E, Eisen NH. Myeloma proteins as tumor specific transplantation antigens. *PNAS, USA*. 1972;69:1540-1544.

6. Tatsumi T, Kierstead LS, Ranieri E, Gesualdo L, Schena FP, Finke JH, Bukowski RM, Mueller-Berghaus J, Kirkwood JM, Kwok WW, Storkus WJ. Disease-associated bias in T helper type 1, (Th1)/Th2 CD4⁺ T cell responses against MAGE-6 in HLA-DRB1*0401⁺ patients with renal cell carcinoma of melanoma. *J Exp Med.* 2002;196:619-628.
7. Bendandi M, Gocke CD, Kobrin CB, Benko FA, Sternas LA, Pennington R, Watson TM, Reynolds CW, Gause BL, Duffey PL, Jaffe ES, Creekmore SP, Longo DL, Kwak LW. Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nat Med.* 1999;5:1171-1177.
8. Adelchi Ruffini P, Neelapu SS, Kwak LW, Biragyn A. Idiotypic vaccination for B-cell malignancies as a model for therapeutic cancer vaccines: from prototype protein to second generation vaccines. *Trends Hematol Oncol.* 2002;87:989-1001.
9. Rasmussen T, Jensen L, Johnsen HE. The Clonal Hierachy in Multiple Myeloma. *Acta Oncol.* 2001;39: 765-770.
10. Billadeau D, Ahmann G, Greipp P, Van NB. The bone marrow of multiple myeloma patients contains B cell populations at different stages of differentiation that are clonally related to the malignant plasma cell. *J Exp Med* 1993;178:1023-1031.

11. Corradini P, Boccadoro M, Voena C, Pileri A. Evidence for a bone marrow B cell transcribing malignant plasma cell VDJ joined to C mu sequence in immunoglobulin (IgG)- and IgA-secreting multiple myelomas. *J Exp Med.* 1993;178:1091-1096.
12. Reiman T, Seeberger K, Taylor BJ, Szczepek AJ, Hanson J, Mant MJ, Coupland RW, Belch AR, Pilarski LM. Persistent pre-switch clonotypic myeloma cells correlate with decreased survival: evidence for isotype switching within the myeloma clone. *Blood.* 2001;98: 2791-2799.
13. Rasmussen T, Jensen L, Honore L, Andersen H, Johnsen HE. Circulating clonal cells in multiple myeloma do not express CD34 mRNA, as measured by single-cell and real-time RT-PCR assays. *Br J Haematol.* 1999;107:818-824.
14. Rasmussen T, Poulsen TS, Honore L, Johnsen HE. Quantitation of minimal residual disease in multiple myeloma using an allele specific real-time PCR assay. *Exp.Hematol.* 2000;28:1039-1045.
15. Österborg A, Yi Q, Bergenbrant S, Holm G, Lefvert A-K, Mellstedt H. Idiotype-specific T cells in multiple myeloma stage I: an evaluation by four different functional tests. *Br J Haematol.* 1995;89:110-116.

16. Marchand M, van Baren N, Weynants P, Brichard V, Dréno B, Tessier M-H, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Liénard D, Beauduin M, Dietrich P-Y, Russo V, Kerger J, Masucci G, Jäger E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, Van der Bruggen P, Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer*. 1999;80:219-230
17. Massaia M, Borrione P, Battaglio S, Mariani S, Beggiato E, Napoli P, Voena C, Bianchi A, Coscia M, Besostri B, Peola S, Stiefel T, Even J, Novero D, Boccadoro M, Pileri A. Idiotype vaccination in human myeloma: generation of tumor-specific immune responses after high-dose chemotherapy. *Blood*. 1999;94:673-683.
18. Galea HR, Cogné M. GM-CSF and IL-12 production by malignant plasma cells promotes cell-mediated immune responses against monoclonal Ig determinants in a light chain myeloma model. *Clin Exp Immunol*. 2002;129:247-253.
19. Rambaldi A, Lazzari M, Manzoni C, Carlotti E, Arcaini L, Baccarani M, Barbui T, Bernasconi C, Dastoli G, Fuga G, Gamba E, Gargantini L, Gattei V, Lauria F, Lazzarino M, Mandelli F, Morra E, Pulsoni A, Ribersani M, Rossi-Ferrini PL, Rupolo M, Tura S, Zagonel V, Zaja F, Zinzani PL, Reato G, Foa R. Monitoring of

minimal residual disease after CHOP and rituximab in previously untreated patients with follicular lymphoma. *Blood*. 2002; 99:856-828.

