Long-term Idiotype Vaccination Combined with Interleukin-12 (IL-12), or IL-12 and Granulocyte Macrophage Colony-Stimulating Factor, in Early-Stage Multiple Myeloma Patients

Lotta Hansson,[1,2,3] Amir Osman Abdalla,[2,3] Ali Moshfegh,[2,3] Aniruddha Choudhury,[2,3] Hodjattallah Rabbani,[2,3] Bo Nilsson,[2,3] Anders Osterborg,[1,2,3] and Håkan Mellstedt[1,2,3]

Abstract

Purpose and Experimental Design: Twenty-eight patients with immunoglobulin G myeloma stages I to II were immunized i.d. over 110 weeks with autologous M protein combined with interleukin-12 (IL-12; $n = 15$) or with IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF; $n = 13$). Idiotype-specific T-cell responses were assessed by [^{3}H]thymidine incorporation, enzyme-linked immunospot assay, and delayed-type hypersensitivity reaction.

Results: Based on these three assays, idiotype-specific immune responses were noted in 5 of 15 (33%) patients in the IL-12 group and in 11 of 13 (85%) patients in the GM-CSF/IL-12 group ($P < 0.01$). Immune response was seen only in patients with M-component concentration of <50 g/L. Three of 16 (19%) responders showed a gradually increasing idiotype-specific T-cell response, whereas 11 of 16 (69%) patients showed initial response, which then disappeared rapidly; the latter pattern was frequently associated with subsequent progressive disease. Immune nonresponse was associated with an increase in the numbers of $CD4^+/CD25^+$ cells (regulatory T cells), which was absent in responding patients. Median time to progression for immune responders ($n = 16$) was 108 weeks compared with 26 weeks for nonresponders ($n = 12$; $P = 0.03$).

Conclusions: These results indicate that idiotype immunization of myeloma patients with GM-CSF and IL-12 may induce specific T-cell response more frequently than with IL-12 alone and that immune response may correlate with time to progression and nonresponse with increased numbers of regulatory T cells.

Multiple myeloma is a lymphoproliferative disorder characterized by a clonal expansion of B cells, producing a monoclonal immunoglobulin (Ig) that can be detected in serum and/or urine. The idiotypic Ig is a unique tumor antigen of B-cell malignancies, which may be presented as complete Ig molecules on the tumor cell surface (1) and as peptides in the groove of the MHC molecules (2). The tumor-derived idiotype may spontaneously elicit a humoral and cellular anti-idiotypic response (3), which may control the growth of the myeloma B-cell clone. Idiotype-specific T cells are cytotoxic for human multiple myeloma cells (4, 5). Immunizing patients with the autologous tumor-derived idiotype is known to evoke an anti-idiotype response, which may be associated with tumor regression in non–Hodgkin's lymphoma (6) and multiple myeloma (7).

The aim of the present study was to improve idiotype vaccination in early-stage multiple myeloma patients. Immunity induction can potentially be enhanced through the use of adjuvants, such as granulocyte macrophage colony-stimulating factor (GM-CSF), which augments the activity of antigen presenting cell (8). GM-CSF is an important cytokine for the induction of antitumor immunity in humans as well as in animal models (9, 10). Interleukin-12 (IL-12) is another adjuvant cytokine mainly produced by dendritic cells. It is necessary for the generation of a Th1 response and for the augmentation of CTL activity (11). IL-12 has been used to boost protective as well as therapeutic tumor immunity in animal models (12) as well as in patients (13, 14).

In one previous study, we immunized myeloma patients with idiotype protein without any cytokine adjuvant (15). Immune responses were seen in a subpopulation of the vaccinated patients without any clinical effect. In a subsequent study, we immunized with idiotype protein along with GM-CSF as a cytokine adjuvant (7). Anti-idiotype immune responses were noted in all the patients in this study, with a stable decrease in

Authors' Affiliations: [1]Department of Haematology and [2]Department of Oncology (Radiumhemmet) and CancerCentreKarolinska, Karolinska University Hospital; [3]Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden
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Note: L. Hansson, A. Osterborg, and H. Mellstedt coordinated the study; B. Nilsson was responsible for statistical analysis; and A.O. Abdalla, A. Moshfegh, A. Choudhury, and H. Rabbani conducted the immunologic analysis.

Requests for reprints: Anders Osterborg, Department of Oncology, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden. Phone: 46-8-517-755-08; Fax: 46-8-31-83-27; E-mail: anders.osterborg@karolinska.se.

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circulating M protein in one patient. In a continued attempt to improve clinical and immune responses, we have added IL-12 as well as prolonged the immunization period in the present study. Additionally, we have also investigated the role of regulatory T (T_{reg}) cells in influencing the immune response following anti-idiotype vaccination.

Materials and Methods

Patients. Twenty-eight patients with multiple myeloma of the IgG type were included. The median age was 65 years (range, 46-82). All patients had slowly progressive stage I (n = 20) or asymptomatic stage II disease (n = 8), with no need for ongoing chemotherapy (16). Patients were previously untreated (n = 24) or were in a stable unmaintained response/plateau phase (>1 year; n = 4) following chemotherapy or radiotherapy. The study was approved by the local ethics committee and the Swedish Medical Products Agency. Written informed consent was obtained from each patient.

Preparation of monoclonal IgG and F(ab′)$_2$ fragments. The procedure has been previously described in detail (17). Briefly, patient sera were fractionated using a sterile MabTrapG column (Pharmacia, Uppsala, Sweden). IgG was eluted with 0.1 mol/L glycine-HCl (pH 2.7). Isoelectric focusing (Pharmacia PhastSystem) indicated that >90% of the IgG was monoclonal. The monoclonal IgG was dialyzed against sterile NaCl followed by filtration through a Millipore (Bedford, MA) filter (0.20 μm). F(ab′)$_2$ fragments were obtained following pepsin digestion of the IgG. F(ab′)$_2$ fragments of different myeloma patients prepared in the same way as the autologous F(ab′)$_2$ were used as controls in the immunologic assays.

Preparation of idiotype vaccine. Equal volumes of sterile-filtered monoclonal IgG and alum solution (0.5% aluminium phosphate; SBL Vaccin AB, Stockholm, Sweden) were mixed under aseptic conditions and adjusted with sterile 0.9% NaCl to a final IgG concentration of 1 mg/mL as described (7, 15). Tests for sterility, pyrogens, and viruses were done and determined to be negative.

Immunization protocol. Patients received 0.5 mg of the autologous idiotype on day 1 i.d. in the left arm. One group of patients (n = 15) also received 2 μg of the adjuvant cytokine IL-12 (Genetics Institute, Inc./Wyeth-Ayerst Research, Cambridge, MA) s.c. in the contralateral arm (IL-12 group). Another group of patients (n = 13) was given the autologous idiotype and IL-12 as described above, with the addition of 75 μg GM-CSF (Schering-Plough, Kenilworth, NJ) i.d. at the vaccine (idiotype) site daily (days 1-4; GM-CSF/IL-12 group). The complete vaccination procedure was repeated after 2, 4, 6, 8, and 14 weeks (induction phase) and continued at weeks 30, 46, 62, 78, 94, and 110 (maintenance phase).

Proliferation assay (DNA synthesis). Details of the assay have been previously described in detail (18, 19). Briefly, peripheral blood mononuclear cells (PBMC) were stimulated with purified F(ab′)$_2$ fragments of the autologous monoclonal IgG (1 pg/mL to 100 μg/mL) and polyclonal IgG (Octapharma AB, Stockholm, Sweden) as well as with three allogeneic monoclonal isotype-matched IgG F(ab′)$_2$ fragments (purified from other myeloma patients) and cultured for 6 days. Thymidine (1 μCi/well; specific activity, 5 Ci/mmol; Amersham Biosciences, Buckinghamshire, United Kingdom) was added during the last 18 h. Concanavalin A (40 μg/mL; Amersham Biosciences, Uppsala, Sweden) and purified protein derivative of tuberculin (2.5 μg/mL; Statens Serum Institut, Copenhagen, Denmark) were used as positive controls. Mean radioactivity incorporation of triplicate cultures was calculated. For each testing time, the highest mean incorporation obtained with five different concentrations of idiotype was used (18). Stimulation index was calculated by dividing mean radioactivity of a triplicate of stimulated cells by that of unstimulated cells. Mean + 3 SD of stimulation index induced by isotypic monoclonal IgGs and polyclonal IgG (n = 2,183 experiments) was 2.87. The threshold was set to ≥3.00, which is similar to our previous reports (18, 19). The patients were pretested before the first immunization and considered to have a preexisting idiotype-specific T-cell immunity if the proliferation assay value was above the threshold. Tests were done at weeks 0, 4, 8, 10, 14, 16, 30, 32, 46, 48, 62, 64, 78, 80, 94, 96, 110, 112,126, 142, and 158.

Reverse enzyme-linked immunospot assay. The enzyme-linked immunospot (ELISPOT) assay for identification of IFN-γ–secreting cells was used as described (18). Briefly, PBMCs (1×10^5 per well) were incubated with F(ab′)$_2$ fragments of the autologous or isotype (control) monoclonal IgG (1 pg/mL to 100 μg/mL) for 48 h in humidified air with 5% CO_2 at 37°C. Cells incubated with medium alone, concanavalin A (40 μg/mL), purified protein derivative of tuberculin (2.5 μg/mL), tetanus toxoid (50 mg/mL; SBL Vaccin AB), and phytohemagglutinin (10 μg/mL; Sigma, St. Louis, MO) were used as controls. The number of spots corresponding to cells secreting IFN-γ was quantified using an automated computer-assisted video imaging analysis system (Axioplan 2; Carl Zeiss Vision, Jena, Germany). The results were expressed as spot-forming units (SFU; IFN-γ–secreting cells)/10^6 PBMC. The SFU was determined as the total number of spots minus the number of SFU in cultures with medium only. Mean + 3 SD of SFU/10^6 PBMC induced by isotype-matched (control) IgG F(ab′)$_2$ fragments (n = 513 experiments) was 60 and ≥70 SFU/10^6 PBMC was considered as the threshold. The patients were tested before immunization and considered to have a preexisting idiotype-specific T-cell immunity if the ELISPOT assay was above the threshold. Tests were done at weeks 0, 4, 8, 10, 14, 16, 30, 32, 46, 48, 62, 64, 78, 80, 94, 96, 110, 112,126, 142, and 158.

Alterations in T_{reg} cells. Differences in levels of T_{reg} cells ($CD4^+/CD25^+$) were analyzed at various time points in the study. Cell surface markers were analyzed by staining with fluorochrome-conjugated monoclonal antibodies against CD3, CD4, and CD25 as well as with isotype controls (BD Biosciences-PharMingen, San Diego, CA). A commercial kit was used for intracellular staining of the cells with FoxP3 (eBiosciences, San Diego, CA). An antigen presenting cell conjugated antibody was used for surface staining of glucocorticoid-induced tumor necrosis factor receptor (R&D Systems, Oxon, United Kingdom). Appropriate concentrations of the antibodies were added to the cells (5×10^5 per tube) in 150 μL fluorescence-activated cell sorting buffer containing PBS, 1% bovine serum albumin, and 0.1% NaN_3 and incubated for 30 min on ice in the dark. After final washing twice, the cells were resuspended in PBS, 1% paraformaldehyde, and 0.1% NaN_3. The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software. Twenty thousand total events were collected by live gating on forward and side scatter. Flow cytometry gating was used to detect and separate lymphocytes by their different light scattering properties and to analyze the T cells ($CD3^+$ cells). To analyze the relative change in T_{reg} cells, the percentage of $CD4^+/CD25^+$ T cells (isotype mouse IgG1 control subtracted) at each time point was divided by the level of $CD4^+/CD25^+$ T cells before the start of vaccination (week 0). The results were pooled from all idiotype-responding and nonresponding patients, respectively (see criteria below), and presented as mean + SE relative change.

Delayed-type hypersensitivity. Delayed-type hypersensitivity (DTH) was determined at week 10 only. Sterile autologous monoclonal IgG (0.1 mg) or pooled human polyclonal IgG used as a control (0.1 mg; Gammaglobulin Kabi, 165 mg/mL; Pharmacia) was injected i.d. Induration and erythema were measured after 24, 48, and 72 h (20). A positive reaction was defined as an induration of ≥10 × ≥10 mm (18, 19).

Criteria for an overall idiotype-specific T-cell response. A patient was considered to have developed an idiotype-specific T-cell response if all of the following three criteria were fulfilled: (*a*) an idiotype-induced stimulation index and/or SFU value equal or more than the respective cutoff level (see above), (*b*) an idiotype-induced stimulation index and/or SFU equal or more than twice the baseline value, and (*c*) a positive test (proliferation, ELISPOT, or DTH) at a minimum of two different time points.

Clinical antitumor effect and side effects. Serum M-component concentration, blood cell counts, liver enzymes, and electrolytes were analyzed at weeks 0, 14, 30, 46, 62, 78, 94, 110, 126, 142, and 158 and then every 3rd month until disease progression. Side effects were graded according to the WHO toxicity scale.[4]

Partial response was defined according to the Blade (21) criteria for evaluating disease progression and response as ≥50% reduction of the M-component and minor response as 25% to 49% reduction of the M component. Progressive disease was attained when one or more of the following criteria were fulfilled: (*a*) an increase in the serum M-component concentration by at least 25% of pretreatment or response value, (*b*) >25% increase in 24-h urinary light chain excretion, (*c*) >25% increase in plasma cells in bone marrow, (*d*) development of hypercalcemia, and (*e*) development of new bone lesions or progression of osteolytic lesions (21). Patients were withdrawn from the study if the disease progressed.

Statistical methods. The number of patients to be enrolled was set as per Gehan's design (22). According to this two-stage design, the frequency of clinical responses in the first 14 patients determines the number of additional patients to be recruited. This enables the estimation of the response frequency with a SE of ±10%. Maximum number of patients to be included in each group was 25 (22). However, due to discontinued production of IL-12 as well as GM-CSF, the study had to be closed after recruitment of 15 patients in the IL-12 group and 13 patients in the GM-CSF/IL-12 group. The χ^2 exact test for independent variables was applied to compare differences between the groups. This test was also used to compare differences in immune response between patients with a preexisting immunity and patients with a non-preexisting immunity. Time to progression was defined as time from start of vaccination to progressive disease, death, or follow-up. The Wilcoxon (Gehan) statistics was used to test differences in time to progression between vaccination groups and between immune overall responders and nonresponders, respectively. The Mann-Whitney *U* test was used to test differences in levels of T_{reg} cells following immunization compared with baseline (before start of vaccination) in responding and nonresponding patients.

Results

Twenty-eight patients were included in the study. Fifteen patients were allocated to the IL-12 group and 13 patients to the GM-CSF/IL-12 group on an alternating basis. All patients received at least three vaccinations (≥4 weeks). Two patients did not complete the whole induction vaccination schedule (14 weeks) due to progressive disease. Thirteen patients were withdrawn from the study at various time points during the maintenance immunization phase: 12 patients due to progressive disease (6 in the IL-12 group and 6 in the GM-CSF/IL-12 group) and 1 patient due to a stroke not related to vaccination (GM-CSF/IL-12 group).

Preexisting idiotype-specific cellular immunity. Six patients in the IL-12 group and six in the GM-CSF/IL-12 group had a preexisting idiotype-specific cellular response in the proliferation and/or ELISPOT assay (Table 1A and B).

Overall idiotype-specific T-cell response. When combining the results of the proliferation assay, ELISPOT, and DTH, 16 (57%) patients fulfilled the criteria for having developed an overall idiotype-specific T-cell response (see Materials and Methods): 5 (33%) patients in the IL-12 group and 11 (85%) patients in the GM-CSF/IL-12 group ($P < 0.01$; Table 1A and B). Three of the 16 (19%) responding patients showed a gradually increasing idiotype-specific T-cell response during the study, whereas 11 of 16 (69%) patients showed an initial response, which then disappeared rapidly; the latter pattern was frequently associated with subsequent progressive disease with an increase in the M-component level by >25% in the majority of patients. The two remaining patients showed a variable immune response pattern over time.

Idiotype-specific proliferative T-cell response. Eleven patients mounted an idiotype-specific proliferative T-cell response: 4 in the IL-12 group and 7 in the GM-CSF/IL-12 group. Maximum individual proliferative responses are shown in Table 1A and B. Stimulation indices in responding patients ranged between 3 and 179 (median, 15.7). All proliferative responses were seen in patients where the serum M-component concentration was <50 g/L. The time kinetics of the idiotype-specific proliferation varied considerably between patients. The peak response was noted within 8 weeks after the start of vaccination and then gradually declined in most patients despite continuous immunization. This is further visualized in Fig. 1A and B where the time kinetics of the mean + SE idiotype-induced proliferative T-cell response among responding patients in the IL-12 group ($n = 5$; Fig. 1A) and in the IL-12 + GM-CSF group ($n = 11$; Fig. 1B) are shown. A peak in the mean proliferative response was observed during the induction phase in the IL-12 group; this response was transient and did not reappear later during the study period. In the IL-12 + GM-CSF group, the kinetics of the proliferative response varied to a larger extent (Fig. 1B).

IFN-γ response (ELISPOT). Nine patients developed an IFN-γ response: two in the IL-12 group and seven in the GM-CSF/IL-12 group. Maximum individual IFN-γ responses are shown in Table 1A and B. SFU/10^6 cells ranged between 80 and 2,650 (median, 420). The response pattern varied. The individual IFN-γ responses over time in relation to the M-component concentrations are shown in Fig. 2. All IFN-γ responses were also seen in patients where the serum M-component concentration was <50 g/L (Fig. 2). However, there seem to be a difference in the time kinetics of the IFN-γ response in comparison with the proliferative response. The IFN-γ response appeared later and was sustained for a longer time. In Fig. 1A and B, time kinetics of the mean + SE idiotype-induced IFN-γ–secreting T cells among responding patients in the IL-12 group ($n = 5$; Fig. 1A) and in the IL-12 + GM-CSF group ($n = 11$; Fig. 1B) are shown. A considerable variability was seen within patients and between patients. In both vaccination groups, the response varied to a larger extent and a slight increase was noted at the end of the study period.

Changes in T_{reg} cells. Twenty patients, 11 in the IL-12 group and 9 in the GM-CSF/IL-12 group, were analyzed for relative changes in the proportion of T_{reg} cells ($CD4^+/CD25^+$) during vaccination. There was no significant difference in the proportion of T_{reg} cells ($CD4^+/CD25^+$) between the IL-12 group and the GM-CSF/IL-12 group at baseline. Moreover, the mean percentage at baseline in immune responders was 0.67% (range, 0.38-0.89) compared with 2.01% (range, 1.25-3.66) in immune nonresponders. The difference was not statistically significant. Similar patterns at baseline were also noted with other T_{reg} markers, such as FoxP3 and glucocorticoid-induced tumor necrosis factor receptor. The mean percentage at baseline of $CD4^+$ cells that were $CD25^+FoxP3^+$ was 0.41% (range, 0.31-0.56) in the responders and 1.21% (range, 0.68-1.79) in the nonresponders. For glucocorticoid-induced tumor necrosis factor receptor expression, the mean percentage at baseline

[4] http://www.fda.gov/cder/cancer/oncrefto.htm

Table 1. Response in patients receiving adjuvant cytokine IL-2 and response in patients receiving adjuvant cytokines GM-CSF/IL-2

Patient no.	Preexisting immunity	DTH	Proliferation*	ELISPOT*	Overall response*
A. Maximum induced idiotype-specific T-cell response in patients receiving the adjuvant cytokine IL-12					
1	+	ND	3.7 (wk 8)	3850 (wk 48)	–
2	+	NE	2.1 (wk 16)	0	–
3	–	–	3.97 (wk 126)	300 (wk 158)	+†
4	–	–	179 (wk 8)	780 (wk 10)	+
5	–	–	4.1 (wk 96)	25 (wk 4 + wk 110)	–
6	+	–	6.16 (wk 8)	715 (wk 32)	+
7	+	–	14.2 (wk 10)	2,650 (wk 110)	+
8	–	–	1.25 (wk 4)	4 (wk 16)	–
9	+	ND	3.3 (wk 10)	1,160 (wk 46)	+
10	–	ND	1.75 (wk 16)	40 (wk 10)	–
11	–	–	1.46 (wk 8)	20 (wk 16)	–
12	+	ND	1.09 (wk 4)	675 (wk 4)	–
13	–	–	1.66 (wk 62)	40 (wk 46)	–
14	–	–	2.5 (wk 4)	130 (wk 8)	–
15	–	–	1.0 (wk 10)	65 (wk 14)	–
Total response frequency		0% (0/10)	27% (4/15)	13% (2/15)	33% (5/15)
B. Maximum induced idiotype-specific T-cell response in patients receiving the adjuvant cytokines GM-CSF/IL-12					
16	+	+	72.4 (wk 62)	1085 (wk 96)	+
17	–	+	3.3 (wk 4)	37.5 (wk 10)	+
18	+	+	101.1 (wk 10)	1,185 (wk 142)	+
19	+	–	83.6 (wk 10)	700 (wk 16)	+
20	–	–	90.5 (wk 4)	60 (wk 30)	+
21	+	NE	4.3 (wk 4)	725 (wk 30)	+
22	–	–	48.4 (wk 112)	1,950 (wk 110)	+
23	–	–	2.67 (wk 48)	175 (wk 32)	+
24	–	–	2.45 (wk 30)	110 (wk 30)	–
25	–	–	4.18 (wk 8)	280 (wk 16)	+
26	+	–	4.36 (wk 14)	955 (wk 30)	+
27	–	ND	2.8 (wk 4)	40 (wk 4)	–
28	+	–	15.7 (wk 30)	185 (wk 14)	+
Total response frequency		30% (3/11)	54% (7/13)	54% (7/13)	85% (11/13)

Abbreviations: ND, not done; NE, not evaluable.
*A positive response in the proliferation assay, the ELISPOT assay, and an overall response, respectively, is defined as a response at two different time points above the threshold and at least twice that of prevaccination baseline.
†A response positive in proliferation and ELISPOT assays at only one time point each (as defined in Materials and Methods).

was 1.01% (range, 0.6-1.07) in the responders and 4.93% (range, 3.4-5.99) in the nonresponders.

No relative change in the levels of T_{reg} cells was found among immune-responding patients (n = 12) in either vaccination arm. In contrast, a relative increase in T_{reg} cells was seen weeks 2 to 14 (induction phase) in the nonresponding patients (n = 8). The increase in T_{reg} frequency in the nonresponders during the induction phase was statistically significant compared with responders ($P < 0.001$; Fig. 3).

Idiotype-specific DTH response. Three patients (all in the GM-CSF/IL-12 group) mounted an idiotype-specific DTH response (Table 1B). All these three patients exhibited also an *ex vivo* T-cell response.

Clinical effects. In one patient (no. 3; IL-12 group), a partial response was induced. The clinical response occurred late, during the unmaintained follow-up phase (18 months after the last immunization), and was characterized by a gradual reduction of the M-component concentration (from 23 to 8 g/L). Partial response remained for 10 months, and then, the M-component concentration gradually increased to 20 g/L. Partial response was accompanied by an idiotype-specific proliferative as well as IFN-γ T-cell response. Another patient (no. 5), also in the IL-12 group, developed late a minor response [i.e., a 35% decrease of the M-component concentration (from 17 to 11 g/L) 21 months after the last immunization]. In this patient, no idiotype-specific T cells could be detected. None of these patients received bisphosphonates or any other clinical intervention.

Median time to progression was 96 weeks (range, 4 to 371+) in the IL-12 group and 63 weeks (range, 9-368+) in the GM-CSF/IL-12 group (not significant). Median time to progression for all immune responders (n = 16) was 108 weeks (range, 29 to 371+) compared with 26 weeks (range, 4 to 330+) for immune nonresponders [n = 12; P = 0.03; Wilcoxon (Gehan) statistics; Fig. 4].

Adverse effects associated with the vaccination. In the IL-12 group, six patients had a transient local skin reaction at injection site. Four of the patients had grade 1 erythema and two grade 2 (including edema and/or itching). In the GM-CSF/IL-12 group, all patients had local skin reactions. Three patients had grade 1 reaction and 10 patients had grade 2 reaction. The reactions declined gradually following each immunization and had disappeared after 1 month. Systemic adverse effects were minimal in the IL-12 group. One patient had fever (38.5°C) after approximately each vaccination during the induction phase, which resolved spontaneously within 24 h.

In the GM-CSF/IL-12 group, general symptoms were more common. Myalgia occurred in 8 of 13 patients (grade 1 in 6 patients and grade 3 in 2 patients). Grade 1 fever and chills were observed in five patients and nausea in three patients. Headache (grade 1) was noted in one patient. Fatigue was reported by four patients in the GM-CSF/IL-12 group and by one patient in the IL-12 group. Two patients in the GM-CSF/IL-12 group had an episode of syncope 6 h after vaccination (at weeks 4 and 14, respectively). Both patients recovered promptly and a causal relationship could not be established.

Discussion

Multiple myeloma is yet a disease with a poor prognosis. High-dose chemotherapy with autologous stem cell rescue has improved the prognosis in patients below the age of 65 years, but the 7-year survival is not >20% (23). Thus, there is an imperative need for new and nontoxic therapeutic approaches.

Induction of tumor cell–specific immunity is considered an attractive therapeutic alternative. In B-cell malignancies, the tumor-derived idiotypic Ig is a suitable vaccine candidate. Idiotype immunization in follicular lymphoma has induced tumor regressions (6). A hallmark of a tumor antigen that is suitable as a vaccine antigen is its ability to spontaneously stimulate immune responses (24, 25). In multiple myeloma, naturally occurring idiotype-specific type 1 T cells have been shown (3, 19) especially in stage I disease (19), whereas idiotype-specific T-cell reactivity was down-regulated and/or shifted toward a type 2 T-cell response in advanced disease (26). In an animal model, idiotype-specific T cells were deleted at high concentrations of myeloma protein (50 μg/mL; ref. 27). Furthermore, T-cell function becomes increasingly aberrant with advancing stage in multiple myeloma (28). This corroborates with the present study in which immune responses were observed only in patients with M-component concentration of <50 g/L (Fig. 2), indicating that idiotype vaccination should preferentially be explored in patients with a low tumor burden (i.e., nonprogressive indolent multiple myeloma patients or patients in complete response or with minimal residual disease following chemotherapy). Moreover, patients with a preexisting T-cell immunity might respond better to vaccine treatment as it might be easier to boost than to induce *de novo* immunity against weak self-antigens (24, 25). This hypothesis was at least partly supported by the present study, in which 12 patients (6 in each group) had a preexisting T-cell immunity before vaccination. A numerically higher frequency of patients with a preexisting immunity developed an idiotype-specific T-cell response (75%) compared with patients with a non-preexisting immunity (44%), but the difference was not statistically significant.

In this study, 28 IgG multiple myeloma patients stages I to II were included. Fifteen patients received the idiotype together with the adjuvant cytokine IL-12 and 13 patients received a combination of GM-CSF and IL-12. In the GM-CSF/IL-12 group, 85% of the patients developed an idiotype-specific T-cell response but only 33% in the IL-12 group. In the present study, multiple assays have been used for measuring anti-idiotype responses because no single test can accurately reflect the diverse immunologic variables (19, 29). In the IL-12 group, one partial response and one minor response were noted compared with none in the GM-CSF/IL-12 group. It is also noteworthy

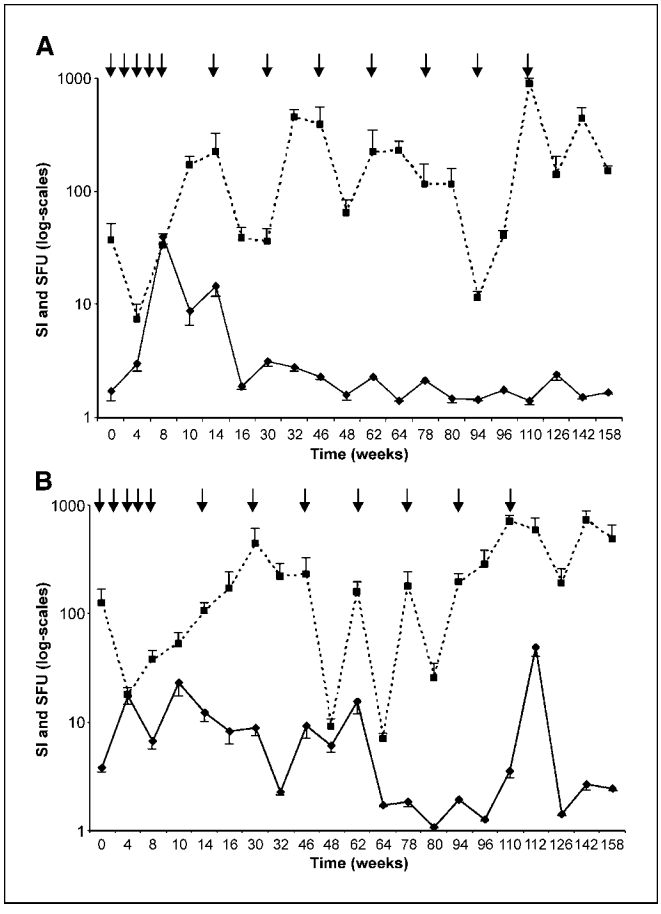

Fig. 1. Time kinetics of the mean + SE idiotype-induced proliferative T-cell response ([^{3}H]thymidine incorporation; *solid lines*) or mean + SE idiotype-induced IFN-γ–secreting T cells (ELISPOT; *broken lines*) among responding patients in the IL-12 group ($n = 5$; *A*) and in the IL-12 + GM-CSF group ($n = 11$; *B*). Arrows, time of immunizations. SI, stimulation index.

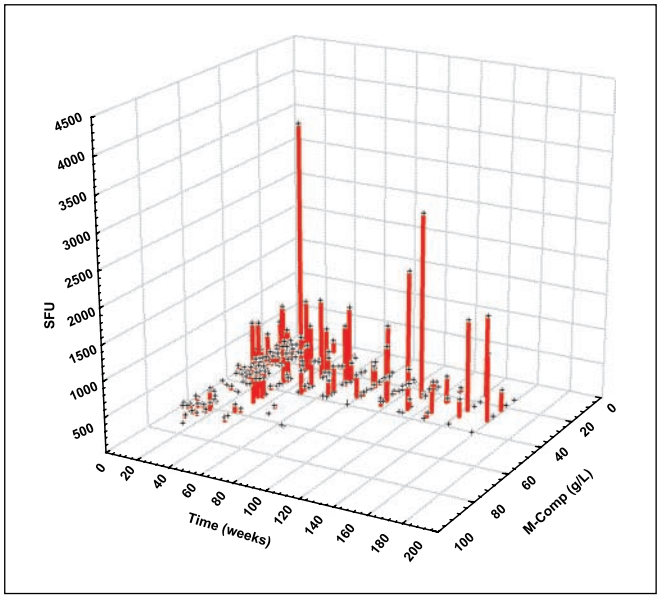

Fig. 2. Time kinetics of idiotype-induced IFN-γ–secreting T cells (ELISPOT) in relation to M-component concentration in all patients (both vaccination groups).

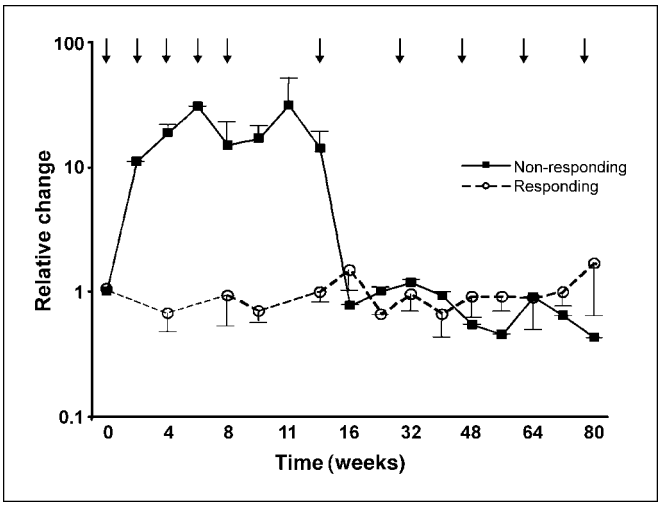

Fig. 3. Relative change in T_{reg} cells ($CD4^+/CD25^+$) on time. At each time point, the mean value + SE of responding ($n = 12$; ○) or nonresponding ($n = 8$; ■) patients in both vaccination groups are shown. Arrows, time of immunizations. The frequency of T_{reg} in the nonresponders during the induction phase (weeks 2-14) was statistically significant compared with responders ($P < 0.001$).

that immune-responding patients had a statistically significant ($P = 0.03$) longer time to progression than nonresponding patients (Fig. 4). These results suggest that idiotype-specific T cells may restrain the growth of the neoplastic B-cell clone by preventing or delaying disease progression. On the other hand, immune responsiveness may simply reflect better disease prognosis and preservation of the immune system. The present study does not unequivocally establish this issue but provides the premise for randomized trials to answer whether idiotype vaccination may delay disease progression in multiple myeloma, similar to ongoing phase III idiotype-based vaccine trials in low-grade follicular lymphoma.

In our first idiotype vaccination study, we used idiotype alone (precipitated in alum). A weak transient idiotype-specific T-cell response was observed in two of five stage I to II patients. No clinical response was seen (15). In our subsequent study, idiotype was used together with GM-CSF in five stage II multiple myeloma patients. All patients developed an idiotype-specific T-cell immunity, and a clinical response (>50% reduction of M-component level) was observed in one patient (7). Massaia et al. (30) vaccinated patients with minimal residual disease following high-dose chemotherapy using the idiotypic protein with keyhole limpet hemocyanin and a low dose of the adjuvant cytokines GM-CSF or IL-2. An idiotype-specific T-cell response (proliferation or DTH) was documented in eight (75%) patients.

In an attempt to augment the induction of an idiotype-specific T-cell response, autologous dendritic cells pulsed with the idiotype have been applied. In a study by Lim and Bailey-Wood (31), six multiple myeloma patients were immunized and a minor response was observed in one patient. Liso et al. (32) reported that 4 of 26 (15%) multiple myeloma patients, in partial response or complete response following high-dose chemotherapy, developed an idiotype-specific T-cell response on vaccination with dendritic cells pulsed with idiotype but without concomitant cytokine administration. Titzer et al. (33) used idiotype-pulsed dendritic cells together with GM-CSF and could show that 4 of 10 (40%) with advanced disease developed a specific cellular response. Lacy et al. (34) vaccinated 17 patients with idiotype-pulsed dendritic cells, without the addition of GM-CSF, after high-dose chemotherapy treatment. Five patients developed idiotype-specific response (proliferation). Of these 17 patients, the same 5 patients improved their clinical response during vaccination: 3 patients entered complete response and 2 patients entered partial response. Yi et al. (35) vaccinated five patients in partial response following high-dose chemotherapy using idiotype-pulsed dendritic cells and IL-2. An idiotype-specific T-cell response was elicited in four (80%) patients. A 50% reduction in serum M-component concentration was observed in one patient.

These studies indicate that GM-CSF or IL-2 may be important adjuvant cytokines, which is further supported by a murine myeloma model (36). Dendritic cells generated *ex vivo* may be a superior adjuvant and induce a better clinical response compared with a non–dendritic cell-based idiotype vaccination (37).

The role of IL-12 as a adjuvant in vaccine trials is not clear (11). Melanoma patients vaccinated with melanoma-associated peptides in combination with IL-12 mounted tumor-specific CTLs, but the clinical responses were limited (14, 38). In a phase I trial where recombinant human IL-12 plus low-dose IL-2 were given twice weekly to renal cell carcinoma ($n = 14$) and melanoma ($n = 9$) patients, four of seven assessable patients with renal cell carcinoma treated at or above the maximum tolerated dose (500 ng/kg of IL-12 and 3×10^6 units/m^2 of IL-2 had stable disease for three to six cycles). Tumor responses were observed in three patients with melanoma (13). In the present study, IL-12 induced a lower frequency of patients mounting a specific T-cell response compared with IL-12 in combination with GM-CSF. However, objective tumor regressions were only noted in the IL-12 alone group. Notably, both clinical responses were seen late, 18 and 21 months after the last immunization. A late clinical effect has also been observed in melanoma patients vaccinated with melanoma-specific antigens (39). Clinical antitumor effects of the idiotype vaccination are further supported by our findings that myeloma-related clonal B cells

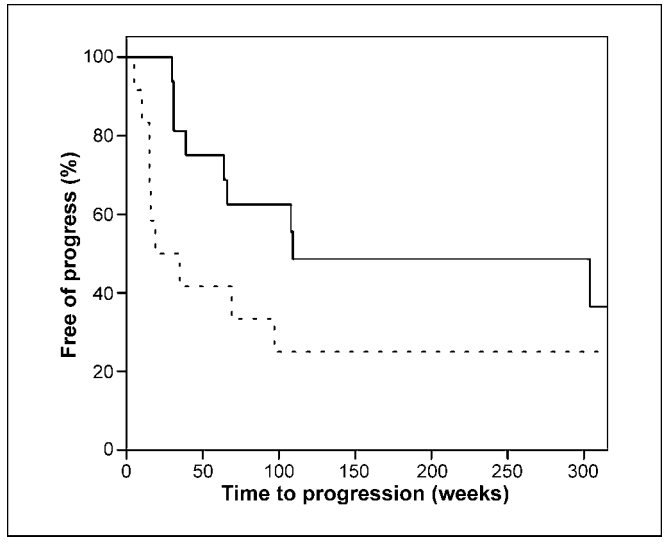

Fig. 4. Time to progression. Time to progression (weeks) following vaccination comparing immunologically responding (*solid lines*) and nonresponding patients (*broken lines*). The difference is statistically significant ($P = 0.03$).

were reduced or eliminated from blood during vaccination (40). An interim analysis on early immune effects in 6 of the 28 patients reported in the present article was also included in that report (40).

Idiotypes are low-avidity antigens (41), which induce a weak cellular response. To break unresponsiveness and maintain a long-lasting immunity using low-avidity self-antigens, repeated immunizations might be necessary (42–44). In the past, repeated immunizations with self-antigens in general have not been considered likely to induce tolerance. This concept was applied in the present study and patients received immunizations for up to 110 weeks. In 19% of the responding patients, the specific immune response was gradually boosted. However, in the majority (69%) of the immune responders, a T-cell response was observed during the induction phase, which subsequently disappeared. The reason is not clear. This particular response pattern was associated with progression of the disease. The tumor clone may produce immune-suppressive factors (45, 46), which may hamper the immune system. Another explanation might be induction of T_{reg} cells, which has been noted in other vaccine trials. T_{reg} cells suppressed the specific immune response (47). In human cancer, T_{reg} cells ($CD4^+/CD25^+$ T cells) have been shown to exist at the local tumor site and contribute to tumor growth *in vivo* and have also been shown to be associated with a poor prognosis (48, 49). Increased numbers of T_{reg} cells were recently described also in patients with multiple myeloma (50). In our study, a discernible increase in T_{reg} cells was observed in immune-nonresponding patients during the induction phase. It may be assumed that the increase in T_{reg} cells might contribute to the inability of these patients to develop an immune response following idiotype vaccination; further studies on the role of T_{reg} cells are warranted in multiple myeloma and related tumors (51, 52).

Repeated immunizations may also induce anergic T cells/clonal deletion. Antigens with a high binding affinity (i.e., other than idiotypes) may induce clonal deletion of T cells rather than T-cell expansion during an immune response (53). It cannot be excluded that high-affinity epitopes of the idiotype may have induced tolerance during repeated immunizations, although the most majority of T-cell epitopes within the V region of the idiotypic Ig are in the low to intermediate range (41).

It is important to understand the mechanisms of down-regulation of the immune response so that adequate measures can be taken to boost the immune response. There are also studies indicating that the induction of high numbers of tumor-specific T cells is of significance for a clinical response (24), further underlining the importance to understand mechanisms involved in suppressing a vaccine-induced immune response. Idiotypes might be acceptable antigens expressing mainly low to intermediate T-cell epitopes (41). In an experimental system, it has been shown that the CTL repertoire against high-affinity self-epitopes was partially tolerized, whereas that against low-affinity epitopes was composed of frequent CTLs with high avidity. High-affinity epitopes were not able to protect from lethal tumor challenge, although mice developing CTL responses against low-affinity epitopes exhibited potent anti-tumor immunity (54).

When an optimal vaccination approach has been defined, randomized clinical trials are needed to establish the clinical efficacy of idiotype vaccination in relation to time to treatment failure with the aim to develop a progression-preventive therapeutic vaccination approach similar to the ongoing phase III idiotype vaccination trials in patients with low-grade follicular lymphomas.

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