Idiotype protein vaccination in combination with adjuvant cytokines in patients with multiple myeloma - evaluation of T-cell responses by different read-out systems

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ABSTRACT

Anti-idiotypic T cells were analyzed in myeloma patients (n=18) vaccinated with idiotypic protein together with the adjuvant cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or interleukin-12 (IL-12). In the group given IL-12/GM-CSF, 78% developed idiotype specific T cells as compared to 22% in the group given only IL-12 (proliferation/ELISPOT assays) ($p<0.05$). The percentage of immune-responding patients increased when quantitative real time polymerase chain reaction assays for cytokines were included. A predominance of a Th_1 (IFN-γ/TNF-α) immune response was noted in the IL-12 group while a Th_2 (IL-5) response prevailed in the IL-12/GM-CSF group ($p=0.053$). Application of multiple read-out systems improved the characterization of the immune response.

Key words: idiotype, vaccination, myeloma, T-cell response.

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The idiotype (Id) is a B-cell tumor antigen, and a unique target for immunotherapy of multiple myeloma (MM). A detailed characterization of a vaccine-induced T-cell response is crucial for the evaluation of the efficacy of a vaccine and the assessment of even a low frequency of antigen-specific T cells might be of value. A T-cell response against tumor antigens, which are often weak self-antigens, such as the Id, might be enhanced by adjuvant cytokines.[1] Granulocyte-macrophage colony-stimulating factor (GM-CSF) augments the activity of antigen presenting cells and interleukin-12 (IL-12) favors a Th_1 polarized immune response,[2] which is of importance for an anti-tumor effect. The main objectives of this study were to characterize in detail the anti-Id T-cell response in MM patients vaccinated with idiotypic protein in combination with GM-CSF and/or IL-12 and to evaluate the potential of different read-out systems for the detection of Id-specific T cells.

Design and Methods

Patients

Eighteen patients with stage I-II MM[3] and a median age of 65 years (range 46-82) from an ongoing Id vaccination trial[4] were enrolled.

Immunization protocol and immune testing

The Id vaccine was prepared as previously described.[4] Patients were allocated to receive 0.5 mg of the Id vaccine i.c. on day 1 in the left arm, and 2 μg IL-12 (Genetics Institute, Inc., Wyeth-Ayerst Research, Cambridge, MA, USA) s.c. in the contralateral arm (IL-12 group) (n=9) or the Id vaccine and IL-12 as described above plus 75 μg of GM-CSF (Schering-Plough, Kenilworth, NJ, USA) i.c. at the vaccine site daily, days 1 to 4 (IL-12/GM-CSF group) (n=9). The vaccination was repeated at weeks 2, 4, 6, 8, 14 and 30. Patients were tested for cellular immune

responses before vaccination and at weeks 4, 8, 10, 14, 30, and 32.

Immune monitoring

Id-specific T cells were evaluated by several read-out systems. Peripheral blood mononuclear cells were isolated from blood and stimulated with F(ab')$_2$ fragments of the autologous or isotype matched monoclonal IgG (1 pg/mL to 100 μg/mL).[5] Proliferation assay, ELISPOT for interferon γ [IFNγ] polymerase chain reaction, quantitative real time PCR (QRT-PCR) for cytokines (IFN-γ, tumor necrosis factor-α [TNF-α], IL-5) and cytotoxic proteins (granzyme B, perforin) and cytokine flow cytometry for intracellular IFN-γ were performed as described elsewhere.[6,7] The CBA (cytometric bead array) Human Th$_1$/Th$_2$ Cytokine kit (Becton-Dickenson, Mountain View, CA, USA) was used for analysis of secreted cytokines (IFN-γ, TNF-α, IL-5).

Criteria for a vaccine-induced Id-specific T-cell response, Th$_1$/Th$_2$ profiling, and clinical response

A vaccine-induced Id-specific T-cell response was considered to be present when (i) the autologous Id-specific response was greater than the isotype-matched one; (ii) above the corresponding cut-off level; (iii) more than twice the pre-vaccination value; (iv) and detected at two or more separate times. A cellular response was defined as Th$_1$ when only Th$_1$ cytokine genes (IFN-γ and/or TNF-α) were expressed and as Th$_2$ when only the IL-5 gene was expressed. When all three cytokine genes were expressed the response was considered as mixed Th$_1$/Th$_2$ with a Th$_1$ predominance when the IFN-γ and/or TNF-α gene fold increase ratio was higher or expressed at more testing times than IL-5 and as a mixed Th$_1$/Th$_2$ with a Th$_2$ predominance when the IL-5 gene fold increase ratio was higher than that of IFN-γ and/or TNF-α. A clinical response was defined according to the Myeloma Subcommitte of the European Group for Blood and Marrow Transplantation (EBMT).[8]

Statistical methods

The χ^2 test was used to compare differences between groups and Spearman's rank correlation test to estimate correlations between assays.

Results and Discussion

There was a considerable variation between individual patients in the three different assays [^{3}H-thymidine incorporation, ELISPOT (IFN-γ), QRT-PCR for cytokine genes] used to evaluate all 18 patients for Id-specific T cells (Tables 1, 2). Nine of the patients (50%) developed an Id-specific T-cell response in the proliferation and/or ELISPOT assays. Seven of the responding patients were in the IL-12/GM-CSF group (78%) while only two were in the IL-12 group (22%) (p<0.05). Vaccine-induced immunity was noted more frequently in patients with a pre-vaccination Id-specific immunity (7/9) than in those without (2/9) (proliferation/ELISPOT) (p<0.02). A vaccine-induced response at the gene level (QRT-PCR) was noted in five patients in the IL-12 group and six in the IL-12/GM-CSF group (Table 2). There was a strong trend towards a Th$_1$ polarized response in the Il-12 group (4/5) whereas this type of response was less common in the IL-12/GM-CSF group (2/8) in which Th$_2$ immunity prevailed (p=0.053) (Table 1). By adding determination of the Id-specific response at the gene level, the percentage of overall Id-specific responses increased from 50% (9/18) to 78% (14/18) (Table 1).

In five selected patients (no. 6, 7, 8, 12, and 16), the Id-specific T-cell response was further evaluated by cytokine flow cytometry for intracellular IFN-γ and cytokine bead array for secreted cytokines. The results are summarized in Figure 1. An Id-specific proliferative T-cell response was noted in two patients while Id-specific IFN-γ protein production was observed in three patients in ELISPOT and four by cytokine bead array. Id-specific IFN-γ gene expression (evaluated by QRT-PCR) was noted in three patients. Id-specific intracellular IFN-γ protein was detected by cytokine flow cytometry in four patients. The frequency of specific T cells detected by cytokine flow cytometry varied between 1.3% and 2.6% and the T cells were of both CD4 and CD8 phenotypes (*data not shown*). Assessment of the Id-specific T-cell response by cytokine bead array measuring two additional cytokines (TNF-α and IL-5) showed that both Th$_1$ (IFN-γ and/or TNF-α) and Th$_2$ (IL-5) cytokines were secreted (Figure 1). No correlation was noted between the expression of these cytokines (measured by cytokine bead array) and the corresponding genes (measured by QRT-PCR). A statistically significant correlation was only seen comparing the Id-specific IFN-γ gene and IFN-γ protein expression determined by cytokine flow cytometry (r=0.79, p<0.0001).

Induction of T-cell immunity against tumor antigens is the common goal of cancer vaccines. There are different opinions about which read-out system might be optimal for monitoring an immune response.[9,10] Multiple read-out systems are recommended.[11] In this pilot study we applied several read-out assays to characterize Id-specific T-cell responses. The results indicate a synergism between GM-CSF and IL-12 with regard to the frequency of induction of an Id-specific T-cell response, as shown in animals.[1] GM-CSF increases the magnitude of a response,[12] while IL-12 promotes Th$_1$ immunity[2] and augments the frequency of memory T cells.[13]

In the present study IL-12 seemed to favor a Th$_1$ polarized response, while the combination of IL-12 and GM-CSF favored Th$_2$ immunity. The combination of the two cytokines has also been reported to down-regulate the magnitude of the immune response as compared to that

Table 1. Individual idiotype-specific T-cell responses assessed by the proliferation and ELISPOT assays as well as the estimated overall vaccine induced response based on these assays and QRT-PCR as defined in Design and Methods.

Vaccination group	*Pat. no.*	*Proliferation assay (SI)****	*ELISPOT (IFN-γ) (SFU/10^6 cells)*****	*Overall vaccine-induced idiotype-specific T-cell response*	
				Prol./ELISPOT	*Prol./ELISPOT/QRT-PCR*
IL-12/GM-CSF	1	3.3 (4)*	–	–	+ (Th_1)
	2	4.5 (0), 83.6 (10), 80.8 (14), 57.9 (16), 53.5 (30)	700 (16), 245 (30)	+	+ (Th_2)
	3	90.5 (4), 43.5 (8), 40 (10), 35.3 (14), 4.9 (16)	–	+	+ (**)
	4	5.2 (4), 3.3 (32)	270 (14), 420 (16), 1050 (30), 760 (32)	+	+ (Th_2)
	5	3.7 (0), 4.3 (4)	90 (0), 725 (30), 450 (32)	+	+ (Th_2)
	6	3.6 (4), 4.4 (14)	570 (0), 108 (8), 180 (10), 435 (14), 955 (30), 105 (32)	+	+ (Th_2)
	7	–	110 (30)	–	+ (Th_2)
	8	10.1 (8), 14.7 (10), 15.7 (30)	75 (0), 185 (14), 80 (16)	+	+ (Th_1)
	9	4.4 (0)	80 (8), 160 (14)	+	+ (Th_2)
IL-12	10	6.2 (8), 3.5 (14)	100 (0), 715 (32)	+	+ (Th_1)
	11	3.3 (0)	–	–	–
	12	–	–	–	+ (Th_1)
	13	3.6 (0), 9.1 (8), 14.2 (10)	625 (14), 480 (32)	+	+ (Th_1)
	14	–	–	–	+ (Th_1)
	15	3.3 (10)	–	–	+ (Th_2)
	16	–	–	–	–
	17	14 (0)	–	–	–
	18	–	–	–	–

**Number within brackets indicates week of testing. — = no detectable immune response during the whole testing period, (**) = QRT-PCR negative). ***cut-off level ≥3.0. ****cut-off level ≥70/10^6 cells.*

Table 2. Individual idiotype-specific mRNA gene expression values (fold increased ratios) of different cytokines in relation to testing times and the estimated Th_1/Th_2 immune response as well as overall vaccine-induced cytokine gene response.**

Vaccination group	*Pat. no.*	*IFN-γ*	*TNF-α*	*IL-5*	*Granzyme B*	*Perforin*	*Overall vaccine-induced cytokine gene response (Th_1 or Th_2)*
IL-12/ GM-CSF (n=9)	1	–	2.2 (14)*	–	–	11474 (14), 6 (16)	Th_1
	2	–	–	1859 (14)	–	–	–
	3	–	–	–	–	–	–
	4	–	–	1.3 (0), 15 (16)	–	1.5 (0), 5 (30)	Th_2
	5	–	–	25 (10)	–	–	–
	6	1820 (8)	13308 (8)	5 (4), 6580 (8), 3 (16)	–	50 (30)	Mixed, Th_2 predominance
	7	–	1.4 (0)	10 (32)	5 (8), 4 (10)	3 (10), 5 (14)	Mixed, Th_2 predominance
	8	2 (10), 5 (14)	–	1.8 (0)	–	5 (32)	Th_1
	9	898 (4)	5670 (4)	308 (4), 4 (8)	6 (0), 47 (4)	ND	Mixed, Th_2 predominance
IL-12 (n=9)	10	11 (4), 7 (8), 6 (10), 3.3 (16)	5 (4)	2.2 (4), 36 (8), 3.3 (10), 3.2 (16)	4 (4), 18 (10)	7 (16)	Mixed, Th_1 predominance
	11	–	14972 (14)	–	5 (14)	–	–
	12	–	343 (14)	12 (0)	–	16 (16)	Mixed, Th_1 predominance
	13	156 (4), 2 (30), 12 (32)	5 (8), 3 (14)	–	3.3 (32)	1.7 (0)	Th_1
	14	–	6985 (14)	–	–	7 (16)	Th_1
	15	1.4 (0), 4 (30)	–	24 (14)	3 (4),2 (16)	-	Mixed, Th_2 predominance
	16	32 (30)	–	–	–	1.8 (0)	–
	17	–	4 (8)	1.8 (0)	–	1.5 (0)	–
	18	36 (0), 13147 (8)	1910 (8)	13007 (8)	–	ND	–

**Number within brackets indicates week of testing. - = no response during the whole testing period. ND: not done. All cytokines were measured at each testing time (weeks 0-32) unless indicated ND. For the definitions of Th_1, Th_2 and mixed Th_1/Th_2 with either Th_1 or Th_2 predominance immune responses see Design and Methods. **the relative increase of a gene induced by the autologous idiotype compared to the isotype-matched control.*

produced by IL-12 alone.[14] The impact of GM-CSF on a Th_1/Th_2 response is not clear. We have previously reported that GM-CSF may favor a Th_1 response,[5] while others have observed an unbiased mixed cellular response.[15] The timing of GM-CSF administration might be of importance. The administration of GM-CSF 3 days before an HIV-DNA vaccine induced mainly a Th_2 response whereas simultaneous administration evoked both Th_1 and Th_2 responses. When GM-CSF was injected 3 days after the vaccine, a Th_1 response predominated.[16] In this study, patients with spontaneous Id-specific immunity more frequently mounted Id-specific immunity compared to those with no pre-vaccination immunity. This indicates that the Id antigen has the potential to break tolerance and might be a good candidate vaccine. These results support those of previous studies showing that patients with a pre-exist-

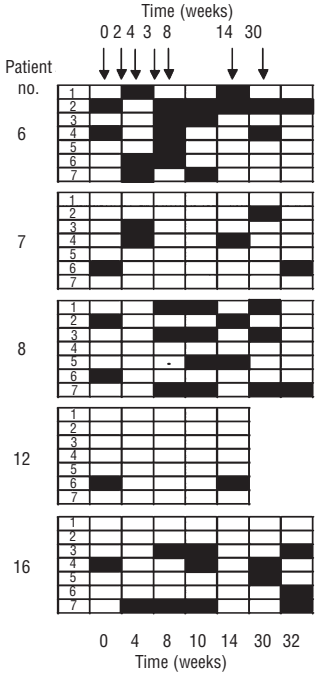

Figure 1. Schematic presentation of immune responses of the individual assays in five myeloma patients vaccinated with the idiotypic protein. Arrows indicate vaccination time points. Filled boxes indicate immune responses above the cut-off level and empty boxes responses below the cut-off level. 1: proliferation assay, 2: ELISPOT (IFN-γ), 3: cytokine bead array (IFN-γ), 4: cytokine flow cytometry (IFN-γ), 5: QRT-PCR (IFN-γ), 6: QRT-PCR (TNF-α, IL-5), and 7: cytokine bead array (TNF-α, IL-5).

ing T cell immunity are more prone to mount an immune response than are tumor antigen immune naïve patients.[17]

Antigen-specific T cells consist of a polyclonal population of cells producing various cytokines. Measuring only IFN-γ, as in ELISPOT, might underestimate the frequency of antigen-specific cells.[18] By adding QRT-PCR to evaluate multiple cytokines, T-cell subsets producing different cytokines might be detected. Indeed, when this assay was included, the frequency of immune responders increased and functional capability could be predicted. The addition of granzyme B and perforin assays indicated that the majority of the patients had Id-specific T cells with cytolytic activity.

A significant correlation was noted between the Id-specific expression of the IFN-γ gene (measured by QRT-PCR) and the protein as detected by cytokine flow cytometry. A strong correlation has previously been reported between QRT-PCR and cytokine flow cytometry findings for IFN-γ.[19] In our study there were, however, testing times when the IFN-γ protein was detected but not the corresponding gene transcript. The time point selected for analyzing cytokine gene expression after antigen activation might be critical. We previously showed that 18 h was an acceptable time of antigen activation for the detection of IFN-γ against a recall antigen, but there was a great inter-individual variation.[6] The time kinetics of cytokine gene expression might vary for different antigens and gene expression might be missed, despite the presence of the corresponding protein, if evaluations are conducted at only a few activation times.

More than 20 Id vaccine trials have been performed in MM patients. Roughly 55% of the patients mounted an Id-specific response, but only 12% had a clinical response. Clinical responders were also noted among patients having no detectable Id-specific response.[20] In the present study, during the first 32 weeks of follow-up, none of the patients had a clinical response regardless of whether they were immune responders (n=14) or not (n=4). A clinical benefit might, however, be noted late during follow-up and a longer observation period is needed. Based on the present limited material, firm recommendations on the choice of assays for immune monitoring cannot be made. Extended vaccine trials are needed. However, the application of multiple read-out assays seems to improve the detection and characterization of a weak immune response.

Author Contributions

AOA: performed all the real time PCR assays and analysis and wrote the manuscript. LH: analyzed proliferation and ELISPOT assays and defined vaccine induced responses by these assays. IE, BN-G: carried out the proliferation, ELISPOT, CBA, and flow cytometry assays and contributed to the analysis; EDR: put out the cut-off level for flow cytometry analysis; HR: contributed to the design of project and real time PCR probes and primers; HM: group leader and provided all the necessary supervision, study design and corrections; AO: senior co-supervisor and contributed to planning the project arranging material and correcting various versions of the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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