CLINICAL TRIAL

A virosomal formulated Her-2/neu multi-peptide vaccine induces Her-2/neu-specific immune responses in patients with metastatic breast cancer: a phase I study

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Abstract We have previously shown in mice that vaccination with three Her-2-peptides representing B-cell epitopes of the extracellular domain of Her-2/neu induces Her-2/neu-specific IgG antibodies with strong anti-tumor activity in vitro and in vivo. We have now finalized a phase I clinical trial with an anti-Her-2/neu vaccine-construct of immunopotentiating reconstituted influenza virosomes with the three peptides in patients with metastatic breast cancer (MBC). Ten MBC patients with low protein overexpression of Her-2/neu of MBC (+ or ++ upon immunohistochemistry, FISH negative) and positive hormone receptor status were enrolled in a single center phase I study. The virosomal formulated vaccine, consisting of 10 μg/peptide, was intramuscularly applied three times on days 1, 28, and 56. The primary endpoint of the study, which lasted 12 weeks, was safety, the secondary endpoint immunogenicity. Local erythema at the injection site was the only vaccine-related side effect occurring in four patients. In 8 of 10 patients an increase in peptide-specific antibody titer measured by ELISA was found. Importantly, the induced antibodies were also directed against the native Her-2/neu protein. Cellular immune responses, as measured by in vitro production of IL-2, IFN-γ, and TNF-α of PBMCs showed a marked increase after vaccination in the majority of vaccinees. Notably, the number of CD4+CD25+Foxp3+T regulatory cells, which were significantly increased compared to healthy controls prior to vaccination, was markedly reduced following vaccination. In all, the immunological responses after vaccination indicated that the patients in stage IV of disease were immunocompetent and susceptible to vaccination. The Her-2/neu multipeptide vaccine was safe, well tolerated and effective in overcoming immunological tolerance to Her-2/neu. The induction of anti-Her-2-specific antibodies could result in clinical benefit comparable to passive anti-Her-2 antibody therapy.

Keywords Her-2/neu · B-cell peptides · Vaccination · Virosomes · Antibodies · T cell · T regulatory cells · Phase I trial

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Introduction

The Her-2/neu protein is overexpressed in 15–20% of breast cancers. Its primarily detrimental prognostic potential including rapid progression from early to metastatic breast cancer, the formation of metastases of mainly

visceral origin and rapid disease progression to death [1, 2] has been reversed by the introduction of trastuzumab in the late 1990's and the first decade of the current century. Trastuzumab is a monoclonal antibody directed against the extracellular domain of the Her-2/neu protein. Its clinical use has resulted not only in significant prolongation of disease-free and overall survival in early breast cancer, but also in the significant prolongation of overall survival in patients with metastatic breast cancer overexpressing the Her-2/neu protein. Trastuzumab toxicity mainly consists of the induction of congestive heart failure which occurs only in a very small percentage of patients [3].

The therapeutic administration of such monoclonal antibodies as trastuzumab, cetuximab, and rituximab targeting various proteins on the tumor cell membrane has brought about a milestone in the treatment of certain malignant diseases [4, 5]. Although of high therapeutic efficacy, the passive antibody administration suffers from some drawbacks including the need of frequent administration of the drug in varying, but usually rather short time intervals, the necessity for a prolonged duration of drug delivery and the impossibility of application in a prophylactic manner in high-risk patients. All these difficulties could be circumvented by the generation of an active immune response aimed at the identical target induced by vaccination with an appropriate substance. However, clinical studies on the efficacy of various vaccines used in cancer patients have resulted in limited results due to cancer- or treatment-related anergy with low seroconversion rates, HLA-restriction of vaccine-induced cytotoxic T cells and, finally, inappropriate immunologic targeting [6, 7].

In the present article, we report on a phase I clinical study in patients with metastatic breast cancer, using a vaccine directed against three peptides derived from the extracellular domain of the Her-2/neu protein. These peptides were preselected by computer-aided prediction to search for B-cell epitopes able to induce anti-Her-2/neu antibody responses [8]. In previously published animal studies, we have shown that these peptides, when coupled to tetanus toxoid and used for immunization in mice, induced anti-Her-2/neu antibodies that were able to inhibit growth of Her-2/neu overexpressing SKBR-3 cells in vitro via direct proliferation inhibition, antibody-dependent cellular cytotoxicity as well as complement-dependent cell lysis [8]. Using a c-neu transgenic mouse model we further demonstrated that immunization with this multi-epitope vaccine led to delayed tumor growth onset and reduced tumor growth progression in vivo [9]. To enhance the antigenicity against self-antigens for a human phase I study, we have chosen to couple these peptides to immunopotentiating reconstituted influenza virosomes (IRIV), which have been shown to exert impressive adjuvant characteristics in the context of vaccines directed at

different targets associated with infectious diseases [10, 11, 12]. Here, we report on the primary endpoint of this phase I trial consisting of safety and tolerability of the used vaccine, but also on secondary endpoint which included immunogenicity of the multi-peptide vaccine. It is shown that the vaccine yielded a specific immune response in eight out of ten individuals with metastatic breast cancer and exerted only very mild, local side effects, thus making it a good candidate for further clinical phase II and phase III trials.

Patients and methods

Her-2/neu peptide-based vaccine

The peptide-based vaccine contains three immunogenic, putative B-cell peptides derived from the extracellular domain of Her-2/neu: P4 (378–394), P6 (545–560), and P7 (610–623) as previously reported [9]. Peptides were associated in a defined fashion to immunopotentiating reconstituted influenza virosomes (IRIV) [10]. These are spherical, unilamellar vesicles consisting of a mixture of natural and synthetic phospholipids and membrane glycoproteins. Native inactivated viruses were first dissolved in detergent and the viral nucleocapsids were removed. Her-2/neu peptides coupled to phosphatidylethanolamine and hemagglutinin, originating from the influenza A/Singapore/86 strain, were then integrated into the virosome membrane during reconstitution process. The final vaccine formulation (PEV 6) contained 10 μg of each of the three peptides incorporated into the virosomes. The vaccine was manufactured by Pevion Biotech Ltd (Bern, Switzerland) according to the rules of Good Manufacturing Practices including tests for sterility, pyrogenicity, and stability. The vaccine was supplied in 1-ml ready to use syringes for each vaccination.

Patients

Patients with metastatic breast cancer were eligible for the study. Measurable disease was not required as study inclusion criteria, because metastatic breast cancer patients with bone only disease were also included in the study. These patients had to present elevated tumor markers (CA 15-3), and levels of tumor markers were used for response evaluation.

Furthermore, patients were required to have positive hormone receptor (estrogen and/or progesterone receptor) and low Her-2/neu overexpression (+ or ++ upon immunohistochemistry, IHC, by HercepTest; DAKO, Carpinteria, CA—and negative by fluorescence in situ hybridization, FISH). Previously administered chemotherapy was allowed,

Table 1 Patients' characteristics are listed with respect to age, onset of disease, Her-2/neu expression, prior chemotherapy, and current hormone treatment

Pat. number	Age (years)	Met. disease since	Her-2 status	Prior chemotherapy	Current antihorm. therapy
1	55 a	Oct. 2006	++	No	Anastrozol
2	66 a	May 2004	+	Yes (1 adj)	Fulvestrant
3	84 a	Mar. 1999	++	No	Anastrozol
4	79 a	Sept. 2003	++	No	Anastrozol
5	67 a	Apr. 2004	++	No	Fulvestrant
6	69 a	Sept. 2004	+	No	Anastrozol
7	60 a	Aug. 2002	+	Yes (3 met)	Fulvestrant
8	76 a	Apr. 1999	+	No	Fulvestrant
9	63 a	Jun. 2006	+	Yes (1 met)	Exemestan
10	70 a	Apr. 2008	+	NO	Anastrozol

but no concomitant chemotherapy was permitted for inclusion of patients into the study protocol. All patients received endocrine therapy (tamoxifen or aromatase inhibitors) for treatment of their disease. Patients with positive results of Her-2/neu testing upon IHC (+++) or FISH were excluded. Ten patients were enrolled in the study. Characteristics of the patients are shown in Table 1.

Study design

We report on a single center, non-randomized phase I study. Primary endpoint was to demonstrate the safety and tolerability of the virosomal formulated Her-2/neu multi-peptide vaccine. Secondary endpoint was to determine the immune response against the three virosomal formulated Her-2/neu-derived peptides. The vaccine was the virosomal formulation of 10 µg/peptide administered in patients intramuscularly on days 1, 28, and 56. The total duration of the study for each patient was 12 weeks. This phase I study was approved by the Ethical Review Board of the Medical University Vienna and the General Hospital Vienna and was monitored by four independent outside reviewers who were assembled as an independent data safety monitoring board (DSMB).

Safety assessments

Clinical examination and laboratory assessment of blood counts, clinical chemistry, and liver function were carried out at screening, before each of the three vaccinations, at final assessment, and ad hoc as clinically indicated. Left ventricular ejection fraction (LVEF) was assessed by echocardiography at screening and final assessment. Assessment of health status, including local vaccination reactions were furthermore performed 2 and 7 days after each vaccination. Adverse events were assessed every cycle for the duration of the trial and graded according to

the National Cancer Institute Common Toxicity Criteria (NCI CTC), version 2.0. Data on serious adverse events (SAEs) were collected throughout the study.

Evaluation of response

Although not defined as a goal in this phase I study, patients had to undergo CT scans as part of their routine clinical observance. CT scans were performed routinely every three months or earlier in the case of a change in the clinical course of the disease. Responses were defined according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Sample collection for immunological assays

Blood samples (40 ml) were taken prior to vaccination and 4 weeks after the last immunization. Serum was stored at −20°C until analysis. Sera from healthy volunteers ($n = 16$) were used for background determination in ELISA.

PBMC were separated on Ficoll-Paque density gradients as described elsewhere [13] and cryopreserved until evaluation. In addition, PBMC were isolated from eight age- and gender-matched healthy volunteers prior and after vaccination with a hepatitis A vaccine (HAVRIX 1440) [13].

Peptide-specific responses

Ninety-six-well microtiter plates (Nunc-Immuno Plate, Nalge Nunc International, Denmark) were coated with 5 µg/ml Her-2/neu peptides conjugated to KLH (synthesised and coupled by PiChem, Austria) or 5 µg/ml KLH (SIGMA, St. Louis, MO, USA) in 100 mM carbonate buffer, pH 9.6 overnight at 4°C. Plates were washed with PBS and non-specific binding sites were blocked for 5 h with PBS containing 2% milk powder (Roth-Lactan, Austria). Serial dilutions of sera 1:100, 1:200, 1:400, 1:800,

1:1,600, and 1:3,200 were prepared in PBS-tw (0.05% v/v Tween 20) containing 0.5% milk powder and incubated overnight at 4°C. After washing with PBS-tw, HRP-labeled anti-human IgG (SIGMA, St. Louis, MO, USA) diluted 1:60,000 in PBS-tw containing 0.5% milk powder were added to the plates and incubated for 2 h at room temperature. Color development was performed with tetramethylbenzidin (TMB) substrate (Chemicon, Temecula, CA, USA) and was stopped with H_2SO_4 after 10 min. Absorbance was measured with TECAN Genios reader. The resulting OD of each serum dilution was calculated by subtracting the OD obtained on KLH-coated wells from the OD values of the peptide-KLH coated wells. Assay control was performed with defined patient's serum and defined negative serum pool.

Titer was defined as serum dilution to obtain an $OD = 1.0$ over the background derived from healthy donors. Pre- and post-vaccination titers were calculated.

Her-2/neu-specific responses

Recombinant human ErbB2/Fc Chimera (R&D Systems, MN, USA) consisting of the extracellular domain, six-amino acid linkage, and the human IgG1 Fc fragment was used as coating antigen. Plates were coated with 1 µg/ml ErbB2/Fc Chimera in carbonate buffer overnight at 4°C. Control plates were coated with the analogous dilution of the reconstitution buffer for ErbB-2-Fc Chimera containing 0.1% BSA. Plates were blocked with 5% milk powder in PBS for 5 h before serial dilutions of sera as listed above were added and incubated overnight at 4°C. Bound Igκ and Igλ antibodies were detected with HRP-labeled anti-human κ and anti λ chain IgG (SIGMA, St. Louis, MO, USA), respectively, diluted 1:10,000 in PBS-tw/0.5% milk. Herceptin® (Roche, Switzerland) titration curve 0.05–0.003125 µg/ml was used as control in assays with Igκ detection and serum obtained from patient with Her-2/neu+++ positive tumor for the Igλ detection. ELISA was developed with TMB substrate and stopped after 20 min. Titer was calculated as described above. The ratio post-vaccine titer/pre-vaccine titer represents titer increase. Ratio greater than 1 was regarded as Her-2/neu-specific titer increase.

Western blot

SK-BR-3 cells were lysed as described elsewhere [8]. The protein content was quantified with BCA Protein assay kit (Thermo scientific Rockford, IL USA). Her-2/neu was immunoprecipitated from a total of 16 mg protein with 200 µg Herceptin® (Roche, Basel, Switzerland) and 150 µl protein A + G agarose (Merck-Calbiochem, Darmstadt, Germany) in 0.1 M TBS over night at 4°C. Pellet was washed twice in 0.1 M TBS pH 7.4 and twice in 0.1 M TBS with 0.5% Nonidet P40. Precipitate was separated on 8% SDS PAGE and blotted on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Strips were blocked with 2% milk in 0.1 M TBS buffer and then incubated over night with sera diluted 1:25 in 0.1 M TBS-0.05% v/v Tween-20 containing 0.5% milk. Rabbit anti-Her-2/neu antibody (Zymed, San Francisco, CA, USA) was used as positive control and serum obtained from healthy volunteers were as negative controls. After three washing steps, bound human IgG was detected by AP-labeled anti-human IgG (SIGMA, Saint Louis, Missouri, USA) and subsequently stained with NBT/BCIP substrate mixture. Rabbit IgG was detected by AP-swine anti-rabbit antibody (DAKO A7S Denmark).

Hemagglutination-inhibition assay

The analysis of the serum samples for neutralizing antibody levels against influenza were performed according to standard methodology [14]. Two serial twofold dilutions of the sera were prepared. The diluted sera were incubated with either the hemagglutinin antigen of the Influenza strain A/Sing/86 or with the solvent. Thereafter a suspension of chicken erythrocytes was added. Hemagglutination occurred with virus antigen in the absence of sufficient inhibiting antibodies. The hemagglutination inhibition titer is the reciprocal value of the highest dilution which inhibits hemagglutination. The titer assigned to each sample is the geometric mean of two independent determinations (EMEA criteria in Appendix 13.3). A fourfold increase (≥4) in the titers after vaccination was taken as positive vaccine response.

Cellular responses specific to virosomal antigens

Peripheral blood T cell responses were assessed by cytokine production in vitro upon stimulation with virosomes. PBMC were cultured at 1×10^6/well in medium (RPMI with 10% heat inactivated human AB serum) containing virosomes with total hemagglutinin content of 8 µg/ml or 1 µg/ml streptococcal enterotoxin B (SIGMA, Saint Louis, Missouri, USA) as positive control antigen and in the absence of any antigens (at least three wells per antigen). The virosome formulation was kindly provided by Pevion Biotech Ltd (Bern, Switzerland). The final culture volume was 200 µl/well, culture conditions were 37°C and 5% CO_2. Supernatants were harvested after 48 h and stored frozen until measurement of IFN-γ, TNF-α, and IL-2 by ELISA.

Commercially available ELISA kits were used for measurement of TNF-α (R&D, MN, USA) and IL-2

(ENDOGENE/Thermo Scientific, IL, USA). Assays were carried out according to manufacturer's instructions.

For IFN-γ levels determination plates were coated with 0.5 μg/ml mouse anti-human IFN-γ (Calbiochem, NJ, USA) in carbonate buffer over night at 4°C. After blocking with 4% BSA (bovine serum albumin) in PBS, 50 μl of undiluted samples were added at room temperature for 1 h. Recombinant human IFN-γ (ENDOGENE/Thermo Scientific, IL, USA) in concentration ranging from 8 to 500 pg/ml was used to generate a calibration curve. Plates were subsequently washed with PBS-tw. Biotin-labeled mouse anti-human IFN-γ (Thermo Scientific, IL, USA) at a concentration 0.9 μg/ml was applied, followed by peroxidase-conjugated streptavidin (Thermo Scientific) 1:15,000 in PBS-tw/4% BSA for 30 min. The assay was developed with TMB substrate. Cytokine levels represent results after subtraction of unstimulated medium values.

Flow cytometry

Immunofluorescent staining of cell surface markers on PBMC was performed on patients and eight healthy age- and gender-matched volunteers. Cryopreserved cells were washed with PBS/0.5% BSA/0.5% sodium azide, diluted to 5×10^5 cells per micronic tube, blocked with 20% human AB serum for 20 min and subsequently stained with directly conjugated mAbs at predetermined optimal concentrations for 30 min at 4 °C in the dark. Antibodies used: αCD3-PE-Cy5, αCD19-FITC (IgG1 k), αCD27-PE, αCD4-FITC (IgG2 k), αCD4-PE-Cy5 (IgG1 k), αCD28-PE-Cy5 (IgG1 k), αCD8-APC (IgG1 k), αCD45R0-PE (IgG2a k), αCD62L-FITC (IgG1 k), and αCD25-PE (IgG1 k). All antibodies were purchased from BD Pharmingen, CA, USA. Antibody binding to PBMC was analyzed on a FACS CALIBUR flow cytometer (BD Biosciences) by gating on cells with forward and side light scatter properties of lymphocytes, using BD CellQuest software. Isotype matched negative control reagents were used to verify the staining specificity of the experimental antibodies.

CD4+/CD25+/Foxp3+ regulatory T cells were analyzed using Foxp3 staining kit (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. 1×10^6 PBMC were incubated with anti-CD4/CD25 cocktail for 30 min. Cells were then washed with cold staining buffer, resuspended in 1 ml fixation/permeabilization buffer and incubated for 30 min. After another washing step and blocking with 2% normal rat serum, intracellular staining with anti human Foxp3-APC antibody (PCH101) and isotype control Ab (rat IgG2a) was performed for 30 min. Finally cells were washed with permeabilization buffer, resuspended in staining buffer, and measured as described above. Results are depicted as percent of CD25+Foxp3+ of CD4+ T cells.

Statistical analysis

As detailed above, anti-peptide- and anti-Her-2/neu antibodies were measured by ELISA using six serum dilutions in twofold steps. In simultaneous assays the pre-immunization serum and serum obtained 4 weeks after the third vaccination were measured. Because there is no standard antibody sample against which the obtained OD values can be expressed, a parallel line procedure was chosen and the post-vaccination antibody concentration was expressed as fold-increase above the pre-vaccination serum. The procedure was as follows: in a simultaneous logistic regression analysis all OD values from the pre- and post-vaccination sera were related to the log dilution and a parameter for the time of blood withdrawal (pre- or post-vaccination). The anti-log of the shift from pre- to post-vaccination regression lines is the fold increase of antibody concentrations. Confidence intervals were computed based on Fieller's theorem. The fit of the model was excellent with pseudo-R^2 values above 0.98 in all cases.

Statistical comparison of Her2/neu peptide vaccinees with the control group of age and sex matched anti-HAV vaccinees was done by matched sample ANOVA with repeated measurements. Differences between groups with respect to the trend from pre- to post-immunization sera were tested based on the interaction source of variance. Fractions of lymphocytes from FACS analyses were arcsine transformed prior to analysis to obtain normality of residuals that was assessed by Lilliefors' corrected Kolmogorov–Smirnov tests. Differences between cytokine concentrations in pre- and post-immunization sera were tested by Wilcoxon's matched-pairs tests. For all analyses a P-value below 0.05 was considered significant. No correction for multiple endpoints was applied.

Results

Recruitment

Ten patients have been recruited. All participants have successfully finished the schedule of three vaccinations.

Evaluation of safety

No local or systemic grade 3 or 4 side effects were observed. Four patients showed a grade 1 local vaccination reaction (Table 2). In particular serological evaluation revealed no signs of hepatic or nephrologic toxicity. With special regard to a possible cardiotoxicity comparable to that seen in the treatment of breast cancer patients with trastuzumab all patients had to undergo measurement of LVEF at screening and at the end of study. No decrease in

Table 2 Safety evaluation

Pat. number	Local vacc. reaction grade	Systemic grade 3/4 toxicity
1	1	No
2	0	No
3	0	No
4	1	No
5	1	No
6	0	No
7	0	No
8	0	No
9	1	No
10	0	No

Occurrence of local reactions and systemic side reactions (grades 1–4) were evaluated

LVEF higher than 10% was documented in study patients during the study duration.

Course of disease/clinical outcome

One patient died one month after the last vaccination due to disease progression. An extensive evaluation of the course of disease including the last episode of this patient by the investigator as well as by an independent data safety monitoring board (DSMB) did not suggest any vaccine-related event, but disease progression to be responsible for the patient's death. Another patient died 6 months after the last vaccination with progressive disease while receiving concurrent chemotherapy. At time of study closure (December 2008), the remaining 8 patients were alive, 5 were stable for a period of up to 12 months, 1 showed a partial remission according to RECIST, and 2 had disease progression after 5 and 7 months, respectively.

Immunogenicity of the virosomal-formulated peptide-based vaccine

The vast majority of study participants (8 of 10) produced peptide-specific antibodies to all peptides after the 3rd vaccination, as shown in Fig. 1a. Two patients (patient 06 and 10) did not respond to any of the peptides. A moderate titer increase was measured in one patient with existing pre-immunization titer. Baseline levels obtained with serum samples from healthy donors were significantly below the post-immunization titers in the patients (data not shown).

Immunization with Her-2/neu peptides generated Her-2/neu-specific antibodies

Figure 1b demonstrates antibody titers specific to the extracellular domain of the Her-2/neu protein determined by ELISA. Her-2/neu-specific IgG, κ and/or λ-chain were detected in 7 of 10 patients after immunization with the multi-peptide vaccine. The ELISA results were verified by Western blot analysis confirming the presence of Her-2/neu-specific IgG in 5 of 10 patients. High pre-existing antibody levels against the extracellular domain of Her-2/

Fig. 1 Humoral responses after immunizations with virosomally formulated Her-2/neu peptides. ELISA analysis was performed with serum samples taken at baseline and 4 weeks after the last immunization. **a** IgG-specific titers to peptides P4, P6, and P7. **b** Her-2/neu-specific κ- and λ-light chain IgG. Scatter graphs represents the ratio post-/pre-vaccination titer. **c** Western blot analysis demonstrating increase of Her-2/neu-specific IgG post-vaccination in patient 04. Serum prior (lane 3) and after completed vaccinations (lane 4) was analyzed. Polyclonal rabbit anti Her-2/neu antibody was used as positive control (lane 1), serum of a healthy volunteer as negative control (lane 2)
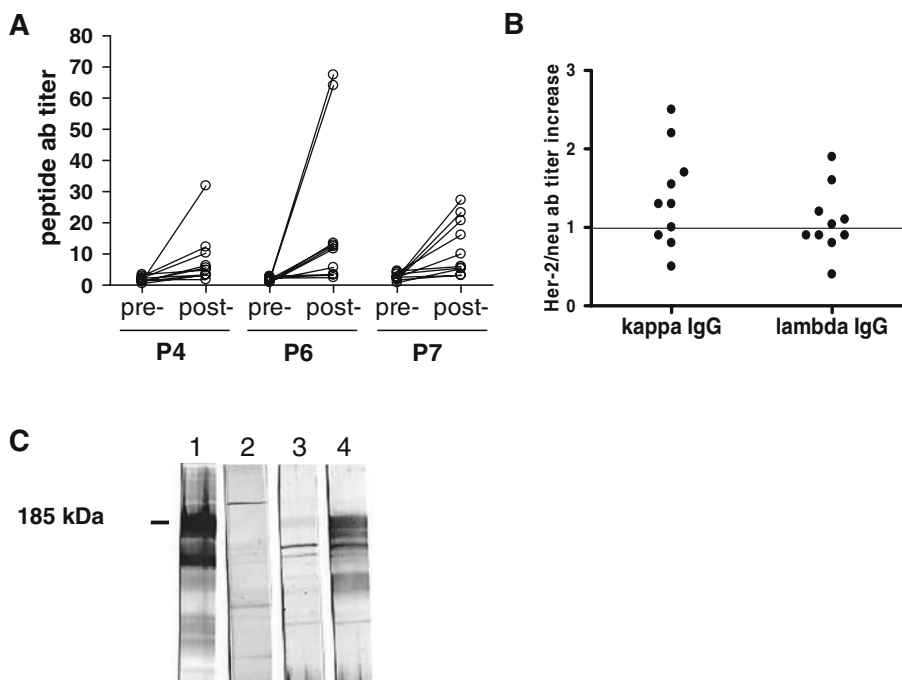
neu was found in one patient; in this case, no increase of Her-2/neu antibody level was achieved during vaccination when measured by ELISA. Qualitative analysis of pre- and post-vaccination serum by Western blot, as shown in Fig. 1c, demonstrates that the Her-2/neu-specific responses to linear epitopes significantly increased after immunizations with peptides.

In general, the κ-light chain-IgG responses were found in the majority of patients and the magnitude of this isotype was higher than of the λ-chain-isotype. Interestingly, only λ-chain light-IgG response was induced in one of the patients (05).

Overall, in 8 of 10 patients induction/increase of Her-2/neu-specific antibody levels was observed after the three-dose vaccination schedule.

Influenza-specific responses/neutralizing antibodies

Hemagglutination-inhibition test (HI) was performed to measure antibody levels against virosome-associated hemagglutinin. Patients' sera were analyzed at baseline and 4 weeks after the three-dose vaccination schedule. Neutralizing antibodies against A Sing 86 specific-hemagglutinin were detectable in 7 of 10 patients already prior to vaccination. Vaccination with PEV6 elicited a significant HI titer increase ($\geq$4-fold) in 6 of 10 patients. In four patients the HI titer was not significantly influenced by the multi-peptide vaccination (Table 3).

Cytokine release after in vitro stimulation with virosomes

Cellular immune responses against the virosomal antigen hemagglutinin were analyzed in PBMC obtained prior and after completion of the vaccination schedule. In 7 of 10

Table 3 Neutralizing antibody titers against influenza associated antigens

Patient	Pre-immune	Post-immune
01	80	160
02	40	640
03	640	320
04	20	160
05	20	160
06	60	60
07	<10	1280
08	<10	160
09	<10	160
10	80	80

HI titers prior and after the three dose vaccination schedule with PEV6

patients an increase of cytokine production was observed after completion of the vaccination schedule. IL-2 production increased significantly ($P = 0.023$), also TNF-α production was markedly higher ($P = 0.055$) as well as IFN-γ production (n.s.). In one patient, high cytokine levels already existed in cultures prior to vaccination and remained at similar level after the third vaccination. In cell cultures of another patient, cytokine concentrations before and after vaccination were below detection limit. A decrease of pre-vaccination cytokine secretion was observed in PBMC of one patient. Of note, this patient displayed also insufficient humoral responses to peptides and influenza antigens (Fig. 2).

Analysis of surface marker expression on lymphocytes

We assessed various subpopulations of PBMC in all study patients prior and after completion of vaccinations as well as in eight age- and gender-matched healthy volunteers. Cell surface marker expression on B cells (CD19), memory B cells (CD19/CD27), T cells (CD4, CD8), memory subpopulations (CD45R0), as well as CD25 (IL-2 receptor α chain), and L-selectin (CD62L) expression on CD4 lymphocytes were evaluated (Table 4). A significant decrease of CD3 T lymphocytes ($P = 0.017$) compared to healthy controls was noticed after completion of the vaccination series. The B cell lymphocyte population tended to increase in the study group post-vaccination, similarly as in the healthy donor group. CD4+ and CD8+ T cells did, however, not significantly differ before and after vaccination between the study patients and the healthy donor group. The L-selectin (CD62L) expressing subpopulation declined in 6 of 10 study patients after vaccination (Table 4).

The median value of CD4+/CD25+Foxp3+ regulatory T cells in circulating PBMC of the breast cancer patients prior to vaccination was 4.86 [3.18–6.54] and significantly declined after vaccination to 4.02 [2.74–5.95], $P = 0.007$ (Fig. 3). In the healthy control group the median values of CD4+CD25+Foxp3+ T reg cells prior (3.98 [2.90–3.66]) and after vaccination (2.69 [2.63–3.10]) were significantly ($P < 0.033$) lower than in the tumor patients (Fig. 3).

Discussion

Several studies in experimental models including our own preclinical data [8, 9] have demonstrated that successful reduction of Her-2/neu overexpressing tumors after vaccination requires both humoral and cellular immune responses. Here, we demonstrate the results of a single center phase I clinical trial showing that a Her-2/neu multi-peptide vaccine is safe and immunogenic concerning B-
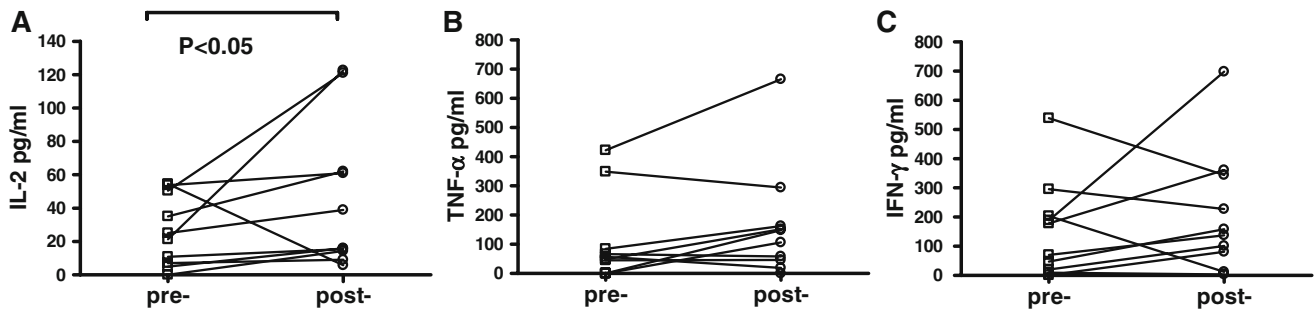
Fig. 2 Cellular responses to influenza antigens in vitro. PBMC isolated at baseline and 4 weeks after the last vaccination were stimulated with virosomes containing hemagglutinin. Cytokine production in supernatants was measured by ELISA. Data represent values after subtraction of unstimulated background

Table 4 Characterization of PBMC by surface marker expression analyzed by flow cytometry

| Cell type | CD marker | Study patients $n = 10$ | | Normal donors $n = 8$ | | Pre-values* | Trend[‡] |
		Pre-	Post-	Pre-	Post-	P	P
T lymphocytes	CD3	66.5 [60.2–73.3]	62.1 [54.9–64.4]	73.6 [63.4–75.9]	73.6 [66.5–78.4]	0.332	0.017
B lymphocytes	CD19	7.9 [5.9–12.5]	12.4 [10–14.5]	8.9 [6.6–14.8]	9.3 [8.2–13.1]	0.643	0.100
T cells/memory	CD4	44.2 [38.7–52.3]	43.0 [37.0–46.0]	53.8 [38.5–57.0]	49.1 [37.0–55.3]	0.678	0.667
	CD4/CD45R0	36.6 [30.6–41.6]	32.9 [27.4–37.6]	31.1 [29.3–32.9]	31.0 [28.0–32.0]	0.102	0.367
	CD8	20.5 [18.6–26.0]	21.3 [18.2–24.5]	21.8 [17.8–31.3]	25.3 [17.8–28.2]	0.822	0.360
	CD8/CD45R0	11.5 [10.2–13.0]	10.3 [8.1–10.7]	9.2 [6.8–15.7]	12.6 [6.8–16.0]	0.980	0.075
Peripheral lymph node homing receptor	CD4/CD62L	16.7 [6.4–22.9]	8.9 [5.0–14.6]	13.9 [11.5–27.6]	16.5 [12.8–22.3]	0.757	0.265
High affinity IL-2 receptor	CD4/CD25	17.3 [11.3–21.4]	15.6 [7.7–19.3]	16.5 [10.7–21.5]	13.7 [10.2–23.3]	0.897	0.198

* P-values for comparison of preimmune cell populations in patients and normal donors

[‡] P-values for comparison of trend (pre-immune–post-immune cell populations) between patients and normal donors

Fig. 3 a Frequency of regulatory T cells assessed by flow cytometry in tumor patients and healthy controls. Data depict percentage of CD4+ T cells expressing CD25 and Foxp3 before and after vaccinations with the Her-2/neu peptide vaccine in tumor patients as well as in healthy age- and gender-matched donors prior and after vaccination with a hepatitis A vaccine. **b** Scatter plots of two representative tumor patients and one healthy control. Cells were gated on the CD4+ population of lymphocytes, the numbers represent percent of each quadrant
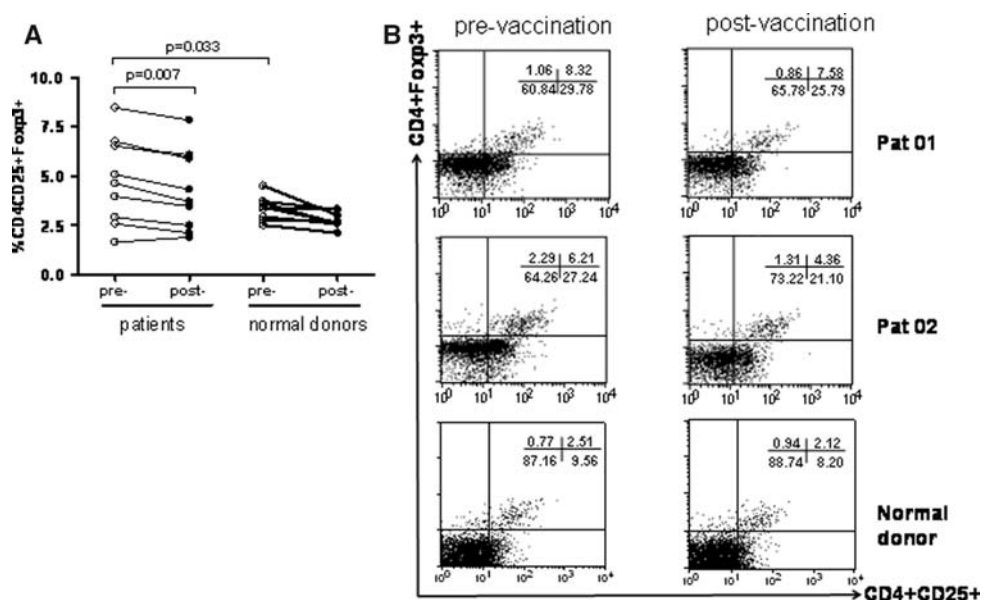
and T cell responses in patients with advanced breast cancer. The current protocol foresaw vaccination in patients with metastatic breast cancer without overexpression of the Her-2/neu protein (+ and ++ upon immunohistochemistry, FISH negative), which was due to ethical reasons in order not to withhold standard anti-Her-2/neu immunotherapy with trastuzumab from patients with Her-2/neu-driven disease.

According to the current trial conduct, this phase I study had safety as primary endpoint. Additionally, immunogenicity of the vaccine was chosen as secondary endpoint, since the specificity of the evoked immune responses toward Her-2/neu is of utmost importance as previously demonstrated in our preclinical studies. Characterization of the B- and T cell compartment was particularly interesting, as immune responsiveness has been repeatedly shown to be perturbed in cancer patients [15–17].

Unlike many peptide vaccines against cancers which have been designed to induce cytotoxic T cell responses using class I restricted peptides [18–23], we used putative B cell epitopes from the extracellular domain of the Her-2/neu protein being HLA type independent. In order to induce CD4+ T helper cell responses, important for the induction of antibody isotype switching and memory responses [24], these peptides were coupled to immunopotentiating reconstituted influenza virosomes (IRIV), an adjuvant system currently used in two licensed vaccines, a hepatitis A vaccine and an influenza vaccine, which proved to cause minimal side effects while being very immunogenic [11, 25].

Indeed, the results from the primary study endpoint on vaccine safety showed that only 4 of 10 vaccinees displayed minimal local reactions of grade 1, consisting of mild redness and swelling or pain at the injection site, while no fever or other systemic side effects (grade 4/5) were reported. The deaths of two study participants, 1 and 6 months after vaccination, respectively, were not vaccine-related but due to disease progression. On these occasions an independent DSMB arrived at the same conclusion as the investigators. Half of the patient collective (50%) presented with stable disease up to 12 months after vaccination. Six months after initiation of vaccination one patient showed a partial remission according to RECIST and three developed tumor progression (Table 2). As all patients received hormone treatment and did not show Her-2/neu overexpression it is more probable to attribute any clinical effects to the applied endocrine medication rather than to the Her-2/neu vaccine.

The second endpoint of the study concentrated on the immunogenicity of the vaccine. The majority of patients, 8 of 10, developed a significant increase in anti-peptide titers to all three peptides (Fig. 1a), while only two participants did not respond with peptide-specific titers. As all patients received endocrine therapy by aromatase inhibitors during this vaccination trial for ethical reasons, the question whether aromatase inhibition might have influenced the magnitude of the antibody responses remains unanswered to date. This appears to be unlikely though, because recent studies with other adjuvant therapies have shown not to impact on immune response levels [26].

Importantly, in all but one of the peptide-responsive vaccinees, the antibody levels were also directed against the native Her-2/neu antigen. The Her-2/neu antibody responses were measured by quantitative ELISA and verified by Western blot analysis. The discrepancy in the detected number of Her-2/neu-specific IgG between the two methods might be explained by the fact that only linear epitopes can be recognized by Western blot analysis, while also conformational epitopes can be measured by ELISA. In contrast to other studies [27], the results from our trial indicate that the B cell-derived peptide vaccine was potent enough to overcome tolerance to the self-antigen Her-2/neu (Fig. 1b, c). Whether the quality of the induced antibodies is related to the kappa or lambda light chain IgG is impossible to estimate at this stage. Studies on the antibody avidity, as described by Goldblatt et al. [28], could give further indications on the biological activity of the induced antibodies. It must be stressed, however, that this phase I study does not allow any evaluation of antibody/vaccine efficacy, since the selection of patients with only weak Her-2/neu expression, due to ethical reasons in order not to withhold trastuzumab treatment when indicated, does not represent the actual target population in which the anti-tumor effects of the induced antibodies can be properly evaluated. Along with further assessment of safety, evaluation of efficacy of the present vaccine would be the focus of a subsequent phase II trial.

It has been proposed that successful vaccination to generate Her-2/neu immunity is not only dependent on the appropriate selection of epitopes but also on the use of a potent adjuvant system. In this respect, we chose IRIV as conjugation partner for two reasons: firstly, the presence of the influenza hemagglutinin antigen from the influenza A Singapore strain on the virosome surface to which the majority of the population has previously been exposed allows the induction of booster responses to this antigen. Accordingly, the majority of the vaccinees displayed a significant increase in the influenza neutralizing antibody titers (Table 3). The two mentioned Her-2/neu non-responder vaccinees also failed to develop influenza-specific antibody levels indicative for immunological incompetence. Secondly, the virosome system was used to generate bystander CD4+ T cell responses [24] necessary to induce antibody isotype switching to IgG as well as memory responses to the B cell peptides. The production of IL-2 in particular, but also of TNF-α as well as IFN-γ

markedly increased in cultures of virosome-stimulated PBMCs after completion of the vaccination series. These results are in line with our preclinical data showing that the selected B cell peptides from the ECD in conjunction with a strong adjuvant system were able to induce both, humoral and cellular responses. Noteworthy, the vaccine was immunogenic (except for two) in patients across all HLA types in stage IV disease. Thus, our study indicates that patients with advanced metastatic breast cancer are immunocompetent and fully susceptible to vaccination. This is further supported by characterization of the lymphocyte population based on CD surface marker expression in comparison to age- and gender-matched healthy controls vaccinated with a hepatitis A vaccine [13] (Table 4). Except for the number of CD3+ T cells, no significant differences in the distribution of lymphocyte subpopulations (CD4, CD45RO, CD8, CD19) were detected between the tumor patients and the healthy controls before and after vaccination. Also the percentage of CD4 T cells expressing CD62L, a homing receptor on naïve T cells, did not significantly differ between the two groups and declined after vaccination, most probably as a result of immune responsiveness to the vaccine antigens, as also described for other vaccines in healthy adults [13, 29].

Several studies have shown that T regulatory cells (Tregs) are increased in patients with a variety of malignancies and that higher numbers of Tregs are associated with tumor progression [30–34]. Recently, it was shown that tumor infiltration by Tregs is associated with a reduced survival of breast cancer patients [35]. Treg cells may also be responsible for the failure of an anticancer immune response by hindering the generation and activity of anti-tumor reactive T cells thereby maintaining self-tolerance to the tumor antigens. Along these lines, also our data showed that the patients had a significantly higher number of circulating Treg cells, defined as CD4+CD25+Foxp3+ T cells, than the healthy matched control group prior to vaccination. Of notice was the fact that the number of Tregs significantly decreased after vaccination with the multi-peptide vaccine (Fig. 3). The fact that a decline in Treg cells was also observed in healthy donors after vaccination with a hepatitis A vaccine (Fig. 3) raises the question whether each vaccination has the potential to interfere with this cell population in order to guarantee optimal immune responses. In tumor patients this suppressive effect on Tregs might be particularly crucial for the efficacy of a vaccine. Similar results have very recently been described in breast cancer patients treated with trastuzumab [30] as well as with an aromatase inhibitor [35], thus suggesting that overcoming Treg cell reconstitution might not only be seen in the present context, but perhaps also as an important contributor to success in a wider range of anti-tumor treatment strategies.

Taken together, the results from our phase I trial show that the described Her-2/neu vaccine was safe and immunogenic in 8 of 10 patients. As a next step it is of interest whether an increased vaccine dose might lead to an ameliorated seroconversion rate. Current studies on the impact of higher peptide vaccine doses on anti-tumor immunity show controversial results [36, 37]. Thus, a second part of this phase I study is on the way to be able to answer this question. In future settings, a series of different options for further phase II studies exist, such as the combination of the current vaccine with passive antibody administration or with other treatment modalities including Treg cell inhibitors.

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