Breast Cancer Res Treat (2006) 100:149–160
DOI 10.1007/s10549-006-9235-7

CLINICAL TRIAL

Advanced breast cancer: anti-progressive immunotherapy using a thermostable autologous hemoderivative

Eduardo Lasalvia-Prisco · Emilio Garcia-Giralt · Silvia Cucchi · Jesús Vázquez · Eduardo Lasalvia-Galante · Wilson Golomar · Joshemaria Larrañaga

Received: 11 March 2006 / Accepted: 21 March 2006 / Published online: 4 July 2006

Abstract
Introduction Advanced breast cancer patients, acquired-chemotherapy resistant and in progression, are therapeutically terminal. We tested a recently described medical procedure using a thermostable autohemoderivative purported to inhibit tumor growth possibly through an immunological mechanism of action.
Patients and methods Metastatic breast cancer patients, chemotherapy-resistant, high CEA and CA 15-3 plasma levels of tumor markers, in progression, were 2-group randomized. Group 1 received the test procedure and Group 2 adequate measures to be comparable control. From 121 included patients, 108 could be evaluated. During 8-month follow-up period, tumor growth, number of cases attaining clinical non-progressive status and mortality were monthly assessed. Immunologic effect was assessed by delayed type hypersensitivity test and lymphocyte proliferation assay. Responding-tumors histopathologies were studied. The proteome of the autologous immunogen was characterized by 2-D electrophoresis.
Results and discussion In a significant number of cases, the test procedure promoted inhibition of tumor growth, non-progressive disease status, and lower cumulative mortality. These clinical results were associated with polyvalent immunization against several tested antigens: the hemoderivative used for treatment, the blood tumor markers and the derivative obtained from a regulatory lymphocyte population (CD4+CD25+). Interference with this regulatory activity could explain the selective autoimmunity suggested by the histopathology findings in responding tumors. The thermostability could be an essential property of the immunogen hemoderivative.
Conclusion The thermostable autohemoderivative tested is antigenically polyvalent and promoted a polytargeted immune response associated to a tumor anti-progressive effect, consequently, acting as an autohemoderivative cancer vaccine.

Keywords Autologous · Breast · Cancer · Hemoderivative · Vaccine

E. Lasalvia-Prisco (✉)
PharmaBlood Inc, Department of Research & Development, 2050 NE 163rd Street, # 202, North Miami Beach, Florida 33162, USA
e-mail: research@pharmablood.com

E. Lasalvia-Prisco · J. Vázquez · W. Golomar · J. Larrañaga
National Institute of Oncology, 8 de Octubre s/n, Montevideo, Uruguay (Initial Data)

E. Garcia-Giralt
Centre De Cancerologie Hartmann, 26, Boulevard Victor-Hugo, 92200 Neuilly Sur Seine, France

S. Cucchi · E. Lasalvia-Galante · J. Vázquez · W. Golomar · J. Larrañaga
Interdoctors, Department of Advanced Medical Treatments, 8 de Octubre s/n 2323, # 905, Montevideo, Uruguay

Introduction

Our team has described the clinical anti-progressive effect of a thermostable autologous hemoderivative (AHD) in a group of human malignant tumors [1, 2]. Some histopathological findings of tumors responding to this hemoderivative suggested an immuno-

logical mechanism of action, so we conducted further studies of this hemoderivative within the framework of an immunotherapy procedure [3]. In a series of chemotherapy-resistant, advanced cancer patients with a variety of primary sites, the progression of tumor growth was reduced by repeated injections of this hemoderivative associated with an immunomodulatory procedure which included low dose of cyclophosphamide as inhibitor of the immune-tolerance [4] and human recombinant granulocyte-macrophage stimulant factor (rhGM-CSF), a well known activator of dendritic cells [5]. A statistically significant correlation has been reported between this clinical anti-tumor-progressive effect and delayed-type hypersensitivity (DTH) to AHD, with a histological tumor response including CD3+/CD8+ lymphocyte infiltration and, above all, intense stromal fibrosis [3]. These findings are compatible with the hypothesis that, within the context of this procedure, AHD elicits an immune response that directly or indirectly modulates the components of the tumor stroma, making the environment less favorable for the survival, proliferation, and migration of malignant cells. These facts were recently confirmed in a more homogeneous patient population with advanced primary colon cancer [6]. In order to gain further insight regarding the anti-tumor effect of this procedure and the proposed immunologic mechanism of action, we designed a prospective randomized clinical trial in patients with advanced breast cancer.

Patients and methods

Patients

A prospective, randomized trial was carried out. Patient characteristics are summarized in Table 1. The study included 121 patients who met the following eligibility criteria: a histopathologically confirmed diagnosis of advanced metastatic breast adenocarcinoma, a tumor burden comprising 1–3 metastasis sites (brain excluded), conservation of organic functions (adequate bone marrow function: WBC $\geq 3000/mm^3$, ANC $\geq 1500/mm$, Hgb ≥ 9.0 g/dl, and platelets $\geq 100{,}000/mm^3$; adequate liver function: bilirubin ≤ 1.5 mg/dl, AST ≤ 2; adequate kidney function: creatinine ≤ 1.5 mg/dl); performance status ≤ 2 according to the Eastern Cooperative Oncology Group (ECOG) scale [7]; high levels of CEA and CA 15-3 (at least two times the upper limit of normality) and measurable progressive disease as assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) [8]. Eligible patients had to be HER-2 negative, resistant to hormone therapy and they had to meet strictly defined criteria for acquired chemotherapy resistance: recurrence after primary surgery followed by adjuvant chemotherapy and documented progressive disease after at least one favorable response to treatment including doxorubicin and taxanes for recurrence. The patients included had to have undergone the last cycle of chemotherapy more than 1 month earlier. The study was conducted in patients admitted to medical centers that submitted

Table 1 Patient characteristics

Patient groups		Control	AHD-treated
Patient, number	Included/evaluable	61/54	60/54
Age, years	Mean (range)	57 (42–76)	59 (39–78)
Performance status: number cases in each grade (ECOG scale)	0	7	8
	1	22	19
	2	25	27
Receptor status	ER+	32	34
Menopause status	Pre/Post	18/36	17/37
Adjuvancy after surgery as primary tumor treatment: number of cases	None	7	8
	RGT	20	16
	RGT+CMF/RGT+FAC	14/13	16/14
Interval: surgery-to-recurrence	Months: mean (range)	7.2 (4.5–20)	7.4 (5–21)
Measurable tumor burden: number of cases by metastasis site	Lymph nodes	23	27
	Lung and pleura	12	9
	Liver	36	32
	Skin	9	10
Non-measurable tumor burden: number of cases by metastasis site	Osseous	34	32
Hormone therapy in recurrence	Refractory	28	24
	Resistance	26	30
Interval: initial response-to-progression	Months: mean (range)	7.3 (4–9)	6.8 (3–8)

medical data to the Cooperative Trials Center (CTC) of PharmaBlood, R&D Department, Florida, USA (PharmaBlood is a non-pharmaceutical concern company supporting scientific research in medical procedures using hemoderivatives). Written informed consent was obtained from all patients included in the study. The Institutional Review Board (IRB) approved the trial, which complied with the Declaration of Helsinki [9]. In a prospective, randomized, double blind, controlled trial, the treating physicians did not participate in the arm randomization for their patients that was performed remotely at the above referred CTC. The patients were randomized into 2 groups that received different treatments during 1 month: the AHD-treated group received the test immunotherapy procedure as previously reported [3], that is, subcutaneous injection of AHD (a heat-fractionated AHD) 3 times a week for 4 weeks plus an immunomodulatory procedure with low-dose cyclophosphamide (300 mg/m^2) 3 days before the first AHD injection, and rhGM-CSF (150 mcg/m^2) simultaneously with each AHD injection. The control group received the same protocol but AHD was substituted by a non heat-fractionated autologous hemoderivative (NHF-AHD). The ethical rationale for both groups was that eligible patients were therapeutically terminal; when these patients were included in the trial, all available efficient treatments, according to Physician Data Query (PDQ) of the National Cancer Institute [10], had been exhausted in both groups. The drugs administered to the control group (necessary for group comparability) were considered acceptable by the IRB; at the used dose both drugs have been repeatedly administered in oncological patients without relevant toxicity: cyclophosphamide at a low-dose of 300 mg/m^2 (only once) is not immunosuppressive [4] and rhGM-CSF is an adjuvant of hematological and immunological recovery [5]. Symptomatic treatment was allowed throughout the study in both groups. After 4 weeks of treatment (AHD or Control), all patients included in the study continued to receive only symptomatic treatment. According to previous reports, the maximal delay and the recovery of tumor growth in the observed AHD effect could be shown during an 8-month follow-up period [3, 6]. Therefore, the clinical parameters were assessed monthly for 8 months (primary endpoint). The secondary endpoint was the evaluation of immunologic responses 20 days after completing treatment (AHD and control). All assessments and analysis of this study were performed by two independent reviewers, using the imaging and/or measures sent by the clinical and laboratory performing teams.

Methods

AHD preparation and treatment procedure

AHD was obtained and administered as previously described [1–3, 6, 11]. Briefly, 20 ml of blood was drawn from the femoral artery into a syringe containing 5,000 IU of heparin and sedimented at 37°C for 1 h. Afterwards, cellular lysis was induced by exposing the supernatant of plasma and cells to hypotonic shock with 3 volumes of distilled water for 15 min, keeping the temperature at 4–8°C to control protease activity, and then freezing at −20°C. After 24 h, the preparation was thawed and incubated at 100°C for 10 min. After final filtration through a cellulose acetate membrane filter (0.22-μm pore diameter), the resulting AHD preparation was divided into 13 individual vials: one test vial with .5 ml and 12 vaccine vials, each containing an equal aliquot of the rest of the preparation. All vials were stored at −20°C until use. The procedure of NHF-AHD preparation is the same described for AHD except the step of submission to 100°C that was omitted. In both groups, the 12 vaccine vials were used for the vaccination procedure, one vial each time, three times a week. Each vaccination consisted of a mixture of the contents of the vaccine vial (AHD or NHF-AHD) and 150 mcg/m^2 of rhGM-CSF, a known activator of dendritic cells in the field of cancer vaccines [5], injected subcutaneous. No more than 2 ml were injected in each vaccination site at a time, so several subcutaneous injections were made on the abdominal surface in order to inject the entire contents of the vaccine vial. A single dose of cyclophosphamide 300 mg/m^2 was given 3 days before starting the vaccination procedure to inhibit tolerance immune responses, as described [4]. Aseptic technique was used in each phase of the procedure. The following assessments were made during the study:

1. *Tumor growth.* In each patient, tumor size was evaluated every 30 days according to the RECIST method (sum of the largest diameter of at least three preselected measurable tumor targets). This method of evaluating tumor size was validated by comparison with the results obtained by simultaneous assessment of tumor size through determination of tumor volume using VoluMeasure®, a volume-measurement application developed by Drs. Ge Wang, Jun Ni, and Simon Kao of the College of Medicine, University of Iowa. Tumor growth was calculated for each patient as the percent variation of tumor size in 30 days.
2. *Status of clinical response.* Clinical status, according to RECIST definitions (progressive disease (PD), stable disease (SD), partial remission (PR), or complete

remission (CR)) was recorded for each patient every 30 days. ST, PR, and CR were considered non-progressive disease (NPD) status. Under the conditions of this study, all patients with NPD were considered non-progressers. All patients included had met the eligibility criteria of PD (tumor growth increase > 20%) when starting the study. Any status was considered evaluable if it was maintained for at least 30 days.

3. *Immunologic response*. In vivo (a) and an in vitro (b) studies were made.

(a) DTH tests were performed in each patient using an intradermal injection of 0.1 ml of AHD or NHF-AHD (in treated or control group, respectively), lyophilized and reconstituted to 10× the original concentration. A test was performed before starting treatment (AHD and controls) and it was repeated 20 days after the last subcutaneous injection. The diameter of the skin induration elicited was measured at 48 h and it was considered positive if it was at least 5 mm.

(b) A laboratory antigen-induced lymphocyte proliferation assay with different autoantigens presumably present in AHD and NHF-AHD was made before and after AHD or control treatment. Before cyclophosphamide was administered, a first sample of peripheral blood mononuclear cells (PBMC) was collected in each patient to study in vitro response before AHD or control treatment. Twenty days after the last subcutaneous injection, before the DTH intradermal test, a second PBMC sample was collected from each patient to study the in vitro response after AHD or control treatment. Preliminary assays to identify in vitro autoimmunity elicited by AHD treatment had revealed some level of lymphocyte response against the autologous CD4+ cell population (results not shown); therefore, we tested two immunologically relevant subpopulations of autologous CD4+ cells as a challenge for AHD and control PBMC. Two aliquots were obtained from each of the two PBMC samples collected: one to be used as responder cells in the proliferation assay and the other to obtain four challengers in the same assay. The four challengers were: two autologous PBMC derivatives in each patient, one from the CD4+CD25+ cell population and the other from the CD4+CD25− cell population; the other two challengers were the intact CD4+CD25+ and CD4+CD25− cells. Both cell populations (CD4+CD25+ and CD4+CD25−) were obtained from the second PBMC aliquot following the procedure described by Wan Fal Ng et al. [12]. To obtain the hemoderivative challengers, an aliquot of each cell population was subjected to the same procedure used to prepare AHD or NHF-AHD as described above. To use intact cells as challenger, an aliquot of each cell population was mixed with non-responder cells at a ratio of 1 challenger cell by 1 responder cell. Briefly, autologous CD4+CD25+ and CD4+CD25− cells and their thermostable fractioned derivatives from each patient were tested as an antigen inductor of in vitro proliferation of responder cells. Simultaneously, tumor markers (CEA and CA 15-3) and appropriate controls were tested as challenger of lymphocyte proliferation.

Proliferation assays were made immediately after obtaining PBMCs by incubating 10^5 PBMCs from the first aliquot, obtained as mentioned above, added to 100 μl of RPMI 1640 with 10% human AB serum and deposited in round-bottomed wells on a 96-well plate. Several immunologic challenges and controls were tested in triplicate: the medium control of 12 wells in the top row contained 10^5 PBMCs in 100 μl of working RPMI 1640 medium plus an additional 100 μl of working RPMI 1640 medium. One hundred microliters of 4 dilutions (1:10; 1:100; 1:1,000 and 1:10,000) of the two PBMC hemoderivatives, CD4+CD25+ and CD4+CD25− (equivalent to 1×10^6 cells), the two aliquots of intact CD4+CD25+ and CD4+CD25− cells (3×10^5 cells), AHD or NHF-AHD (in treated or control group, respectively, concentrated by lyophilization and reconstituted to 10× treatment dilution), CA 15-3 (1,000 U), CEA (2 μg) and 1/100 dilution of control autologous plasma were added in triplicate to the wells of the second through sixth rows of 96-well plates. One hundred microliters of positive control consisting of a serial dilution of stock phytohemaglutinin (PHA, 0.5 mg/ml) in RPMI 1640 (1:10, 1:100 and 1:500) was placed in triplicate wells of the first 9 wells of the seventh row. One hundred microliters of negative control (1:100 dilution of healthy male plasma in RPMI 1640 medium) was added to each of last 3 wells of the seventh row. PHA and RPMI 1640 were obtained from Sigma. Human CEA, CA 15-3 and CA 125 were from Fitzgerald Industries International, MA. Plates were incubated in a 5% CO_2 incubator at 37°C for 5 days. One microcurie of tritiated thymidine was then added to each well in a volume of 20 μl and plates were again incubated, at 37°C for 16 h. The contents of each well were harvested and counted in a liquid scintillation beta-counter. The mean of the three determinations per point was registered. The cpm of the PHA dilution with the highest cpm was divided by the average cpm of the media control. If this ratio was greater than 2.00, then the positive control was accepted. The cpm of the negative control was divided by the average cpm of the media control. If this ratio was less than 2.00, then the negative control was accepted.

The results of the lymphocyte proliferation assay were expressed as Net Counts or cpm (cpm experimental—cpm background unstimulated). The effect of the treatment (AHD or control) was assessed as the ratio of the final cpm (20 days after the last injection) to the initial cpm (before injections) and recorded in each patient as the lymphocyte proliferation response (LPR). A LPR higher than 4 was considered a positive response to treatment.

4. *Circulating cancer cells*. In order to better define the source of the immunogen, an assessment of cancer cells in blood of included patients was performed according to the method described by Gauthier et al. [13].

5. *AHD Proteomics*. Samples of AHD, NHF-AHD and autologous plasma of 20 patients of each group were concentrated by lyophilization and subjected to Two Dimensional Electrophoresis (2-DE). Two-dimensional electrophoresis was performed essentially as reported [14]. Samples of 750 μg were applied on immobilized pH 3–7 or pH 3–10 non-linear IPG strips. Focusing started at 200 V and the voltage was gradually increased to 5,000 V at 3 V/min and kept constant for further 6 h. The second-dimensional separation was performed in 125 SDS-polyacrylamide gels. The gels ($180 \times 200 \times 1.5$ mm) were run at 50 mA per gel, in an ETTAN DALT II apparatus (Amersham Biosciences). After protein fixation with 50% methanol, containing 5% phosphoric acid for 2 h, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 16 h. Excess of dye was washed out from the gels with H_2O and the gels were scanned in an Agfa DUOSCAN densitometer (resolution 400). Protein spots were quantified using the Imga Master 2-D Elite software (Amersham Biosciences). The percentage of the volume of the spots representing a certain protein was determined in comparison with the total proteins present in the 2-D gel. The same procedure was performed with previous albumin + immunoglobulins depletion (ProteoExtract™ Albumin/IgG Removal Kit from Calbiochem).

6. *Histopathology*. The tumor histopathology of several responding patients (DTH-positive) was studied to confirm that the immune response observed with AHD was a response against tumor TAAs shared with the hemoderivative. In DTH positive patients, biopsies of accessible lesions and normal surrounding tissue before and after AHD treatment were available. Specimens were stained with hematoxylin-eosin (Fisher Scientific, Pittsburgh, PA). Immunohistochemical staining was performed using DAKO EnVision Systems. The following antibodies were used: CD8, 1:50 for C8+ lymphocytes; CD4, 1:100 for C4+ lymphocytes; and CD20, 1:1000 for B-lymphocytes (DAKO Corp).

7. *Toxicity assessment*. Toxicity was evaluated using the common terminology criteria for adverse events, version 3.0 (CTCAE), of the US National Cancer Institute [15], the highest levels of each toxicity type detected were recorded.

8. *Monthly number of patients' deaths* associated to cancer progression was recorded.

9. *Statistical analysis*. Tumor growth, number of patients persisting in PD and NPD, number of patients with positive DTH response and in vitro proliferation responses in the AHD-treated group were compared versus the control group using the unpaired two-tailed Student t-test. The overall survival was compared using Kaplan-Meier curve and Log-rank (Peto) / Wilcoxon (Peto-Prentice) analysis. Additionally, in the group of all AHD-treated patients, the results of two stratified subgroups were examined: patients attaining the clinical status of stable disease (non-progressors) were compared with patients persisting in progressive disease status (progressors). Immunologic responses (DTH test and cpm in proliferation assay) in both subgroups were compared using the two-tailed, unpaired Student *t*-test. LPR, as a ratio, was assessed considering as positive the values higher than 4. In all statistical assessments, P values ≤ 0.05 were considered significant. Sample size was assessed to determine if it was sufficient to attain a power of 80% with a significance of 0.05.

Results

The AHD-treated and control groups were comparable with respect to the parameters recognized as influencing tumor growth (Table 1). Table 1 also shows the evaluable patients in each group at end-of-study: six patients (out of 60) in the AHD-treated group and seven patients (out of 61) in the control group were not evaluable. In the AHD-treated group, two patients died of intercurrent cardiovascular complications and four patients refused to continue the programmed treatments. In the control group, one patient died of intercurrent cardiovascular complications, four patients did not complete follow up and two patients refused to continue the programmed treatment.

Tumor growth was significantly lower in the group treated with AHD than in the control group (Table 2). Mean tumor growth with 95% confidence intervals in both groups is also shown in Table 2. The sample size was sufficient to satisfy the criterion of 80% predictive power with a level of significance of 0.05. In terms of clinical tumor progression status, all patients were in

Table 2 Patients assessment

Month	Control group		AHD-treated group		*P*
Tumor growth % increase of tumor size in 30 days					
[−1]	24.7	23.8–25.4	24.7	23.8–25.6	0.95
1	24.8	24.1–25.9	25.3	24.5–26.1	0.74
2	24.2	23.1–25.3	24.6	23.3–25.5	0.58
3	24.6	23.5–25.6	20.5	19.3–21.8	0.018
4	25.2	24.1–26.1	18.4	17.8–19.1	0.0001
5	24.7	23.5–26.9	18.2	17.5–19.7	0.0001
6	24.9	23.0–26.1	18.6	17.3–20.1	0.0004
7	24.7	23.6–25.9	22.0	20.7–23.1	0.0011
8	24.8	23.4–25.7	24.3	23.1–26.1	0.073

Month	Control group						AHD-treated group					
Disease progression monthly number of patients in each RESIST status												
	D	PD	SD	PR	CR	NPD	D	PD	SD	PR	CR	NPD
[−1]	0	54	0	0	0	0	0	54	0	0	0	0
1	0	54	0	0	0	0	0	54	0	0	0	0
2	0	54	0	0	0	0	0	52	2	0	0	2
3	0	54	0	0	0	0	0	49	5	0	0	5
4	1	53	0	0	0	0	0	34	20	0	0	20
5	3	50	0	0	0	0	0	26	28	0	0	28
6	4	46	0	0	0	0	0	36	18	0	0	18
7	4	42	0	0	0	0	2	40	12	0	0	12
8	5	37	0	0	0	0	3	45	4	0	0	4

	t-Test: Control group vs. AHD-treated group
All months	
PD	$P = 0.0057$
NPD	$P = 0.0074$
Months 4–6 (*)	
PD	$P = 0.0020$
NPD	$P = 0.0020$

(*) Months of maximal significance		
	Control group	AHD group
Maximal toxicity [CTECAE]		
Systemic	1	1
Local	2	2

D: Monthly number of patients' deaths due to disease progression

PD: Progressive disease, SD: Stable disease, PR: Partial remission, CR: Complete remission (RESIST)

NPD: Non-progressive disease

progression (PD) according to RECIST when they entered the study. At end-of-study, the number of patients in PD was significantly lower in the AHD-treated group than in the control group and the number of NPD patients was higher in the AHD-treated group than in the control group (Table 2). Twenty-eight patients reached SD status in the AHD-treated group (non-progressors). SD was not reached in the control group. Table 2 also shows that the number of the monthly patients' deaths due to disease progression (D) was lower in the AHD-treated group than in control group. Survival curves and statistical analysis shown in Fig. 1 confirmed these findings. As it is shown in Table 2, no systemic toxicities higher than 1 (CTCAE) were recorded, only mild fever (37.5–38.5°C) was observed in both groups and it was considered a side effect of rhGM-CSF. No evidence of any autoimmune phenomenon was evident. Local reactions recorded in both groups at the injection sites consisted of toxicity grade 1–2 (pain or pain + inflammation) in all cases. No patient had to discontinue or modify treatment due to toxicity or side effects.

There were significant variations in immunological parameters between AHD-treated and NHF-AHD-treated group (control). In addition, significant variations in immunological parameters were observed within the AHD-treated group among the patients that attained non-progressive disease status (non-progressors) and those who kept their progressive disease status (progressors). DTH tested by the intradermal AHD became positive after AHD treatment in 24 of 54 evaluable patients (treated group) and it remained negative when it was tested with NHF-AHD in all 54 evaluable patients of the control group ($P < 0.001$). In

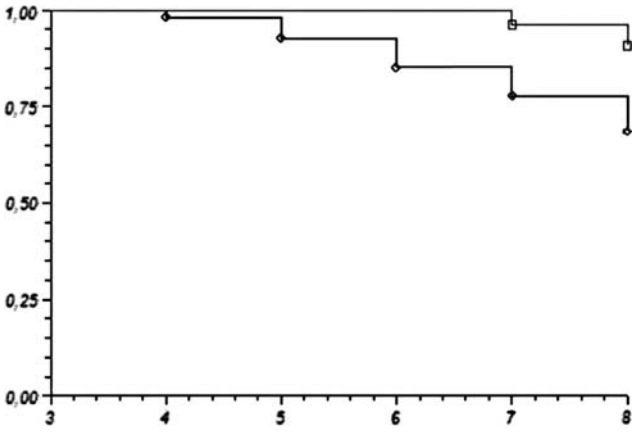

Fig. 1 Overall Survival for AHD (Thermostable Autologous Hemoderivative)-treated patients and control patients NHF-AHD (Non-Heat Fractionated Autologous Hemoderivative)-treated. Kaplan–Meier Survival Plot (PL estimates). Analysis by Log-rank and Wilcoxon tests

the AHD-treated group, the number of patients with a positive (≥ 5 mm) response to the DTH test with AHD was significantly greater ($P < 0.001$) in the non-progressor patients than in the progressors (Table 3). The lymphocyte proliferation assay was evaluable (positive and negative controls accepted) in 46 and 48 patients in the AHD group and the control group respectively. Significant increases (cpm: $P < 0.001$, LPR > 4) in the responses of lymphocyte proliferation were evident after AHD treatment when responder cells were challenged with CEA, CA 15-3 (Table 4), the AHD hemoderivative obtained from the autologous CD4+CD25+ cell population or with the intact CD4+CD25+ cells (Table 5). The selectivity of this immunologic response was evident because AHD treatment failed to modify the negative response of lymphocytes to the autologous plasma (Table 4), the hemoderivative from CD4+CD25− cells or the intact CD4+CD25− cells (Table 5). In the AHD-treated group, the lymphocyte proliferation responses to CEA, CEA 15-3 (Table 4), AHD, CD4+CD25+ derivative and CD4+CD25+ cells (Table 5) were significantly greater ($P < 0.001$) in the non-progressor patients than in the progressors. In the control group, NHF-AHD and all other tested challengers failed to elicit proliferation responses.

Table 3 Delayed type hypersensitivity elicited by a thermostable AHD

	DTH: Initial (+)	DTH: Final (+)
All patients		
AHD-treated group (n = 54)	0	24
Control group (n = 54)	0	0
t-Test		P = 0.0001
AHD-treated group		
Progressors (n = 26)	0	20
Non-progressors (n = 28)	0	4
t-Test		P = 0.001

Delayed type hypersensitivity test (DTH) performed with an autologous hemoderivative (AHD or NHF-AHD in treated and control group, respectively); before (initial) and after (final) treatment: control or AHD. Progressors or Non-progressors to AHD treatment are stratified. AHD group: patients treated with AHD; Control group: appropriate controls with NHF-AHD. Progressors: patients maintaining progressive disease status; Non-progressors: patients achieving stable disease status. (RECIST criteria)

n: number of patients; +: diameter ≥5 mm

AHD: heat fractionated autologous hemoderivative; NHF-AHD: non-heat fractionated autologous hemoderivative

Within the limitations of the assessment method employed, no relation was established between the presence of circulating cancer cells and the efficiency of the hemoderivative to induce in vitro or in vivo immune responses. Circulating cancer cells were identified, respectively, in 30%, 28% and 31% of the patients in the control group, progressors in AHD treated group and non-progressors in AHD treated group.

Figure 2 shows an example of the main histological changes found in biopsies obtained before and after AHD treatment from patients who responded with a positive DTH test (≥5 mm) and significant anti-progressive effect on tumor growth. The example shown is from biopsies of cancer and control biopsies from the surrounding breast tissue (not compromised by cancer). Samples obtained before and after treatment (AHD and control) are shown. After AHD treatment the results reproduced the histopathology previously reported in AHD-treated and non-progressors: tumor stromal fibrosis with lymphocyte infiltration and a decrease in tumor cells and vascularity [1, 3, 6]. The histochemistry of the infiltrating lymphocytes showed a high predominance of CD8+ and CD20+ cells (B cells), with minimal presence of CD4+ cells. In this non-progressor patient, after AHD treatment the control biopsies obtained from normal breast did not show any of these findings. Before AHD treatment the biopsies in tumor and normal breast were both negative for stromal and infiltrative responses. Having the sample-accessibility conditions, 19 cases of non-progressor patients could be studied and their histopathology conclusions were in all cases exemplified in Fig. 2 (No similar change was seen in 14 progressor cases with the same sample-accessibility conditions).

Figure 3 shows an example of the 2-DE of AHD compared with NHF-AHD and plasma. These results allowed identifying a proteome range between 447 and 536 spots in the different tested samples of AHD that

Table 4 Immunonologic response to thermostable AHD: Lymphocyte proliferation assay stimulated with CEA and CA 15-3

	CEA		CA 15-3		AP	
	cpm before treatment cpm after treatment Mean [95% CI]	LPR Mean[95% CI]	cpm before treatment cpm after treatment Mean [95% CI]	LPR Mean [95% CI]	cpm before treatment cpm after treatment Mean [95% CI]	LPR Mean [95% CI]
All patients						
AHD group (n/v = 54/46)	142 [130–154] 714 [698–730] (*)	5.03 [4.16–5.90]	134 [120–148] 616 [598–634] (*)	4.59 [4.05–5.13]	131 [120–142] 133 [106–150] (*)	1.01 [0.92–1.10]
Control group (*n*/*v* = 54/48)	136 [118–154] 141 [120–162] (*)	1.03 [0.78–1.28]	128 [117–139] 136 [120–152] (*)	1.06 [0.84–1.28]	132 [122–142] 126 [118–134] (*)	0.95 [0.88–1.02]
t-test	*P* < *0.001*		*P* < 0.001		*P* = 0.18	
AHD-treated patients						
Progressors (*n*/*v* = 26/23)	158 [144–172] 1206 [1010–1402] (*)	7.63[6.43–8.83]	130[118–148] 922[888–956] (*)	7.05[6.42–7.52]	128 [114–142] 124 [114–134] (*)	0.97 [0.69–1.25]
Non-progressors (*n*/*v* = 28/23)	158 [148–168] 172 [156–188] (*)	1.09[0.76–1.42]	134[114–154] 146[130–162] (*)	1.08[0.92–1.24]	136 [122–150] 140 [116–164] (*)	1.07[0.85–1.29]
t-Test [Comparing (*)]	*P* < 0.001		*P* < 0.001		*P* = 0.30	

PBMCs were incubated with the following challenger: CEA, CA 15-3 or Autologous plasma (AP); cpm = net counts (challenged—control in the same experiment); LPR = Lymphocyte proliferation response = ratio cpm after/cpm before treatment, mean [95% CI]; AHD group: patients treated with AHD; Control group: appropriate controls with NHF-AHD; Progressors: patients maintaining progressive disease status; Non-progressors: patients achieving stable disease status. *n* = number of patients; *v* = number of evaluable patients' tests (controls accepted)

AHD: heat-fractionated autologous hemoderivative; NHF-AHD: non-heat fractionated autologous hemoderivative

Table 5 Immunologic response: lymphocyte proliferation assay stimulated with the AHD (Immunogen), regulatory cells and their derivatives

	AHD or NHF-AHD (AHD or Control groups)		[CD4+CD25−] HD		[CD4+CD25+] HD	
	cpm before treatment cpm after treatment Mean [95% CI]	LPR Mean [95% CI]	cpm before treatment cpm after treatment Mean [95% CI]	LPR Mean [95% CI]	cpm before treatment cpm after treatment Mean [95% CI]	LPR Mean [95% CI]
All patients						
AHD group (n/v = 54/46)	124 [108–140] 810 [732–888] (*)	6.53 [4.90–8.16]	133 [112–154] 127 [107–147] (*)	0.95 [0.82–1.08]	148 [132–150] 746 [824–898] (*)	5.04 [4.88–5.20]
Control group (n/v = 54/48)	132 [106–148] 145 [111–179] (*)	1.09 [0.80–1.38]	144 [126–162] 145 [122–178] (*)	1.01 [0.87–1.15]	124 [103–145] 132 [120–144] (*)	1.06 [0.94–1.18]
t-Test [Comparing (*)]	$P < 0.001$		$P = 0.30$		$P < 0.001$	
AHD-treated patients						
Progressors (n = 26/23)	130 [126–154] 1010 [890–1062] (*)	7.77 [5.60–9.94]	120 [106–146] 124 [110–158] (*)	1.03 [0.92–1.14]	126 [109–127] 816 [498–726] (*)	6.48 [5.01–7.95]
Non-progressors (n = 28/23)	134 [132–168] 126 [144–164] (*)	0.94 [0.79–1.09]	142 [130–158] 155 [123–167] (*)	1.09 [0.90–1.28]	130 [116–132] 138 [114–142] (*)	1.06 [0.86–1.46]
t-Test [Comparing (*)]	$P < 0.002$		$P = 0.76$		$P < 0.001$	
All patients						
AHD group (n/v = 54/46)	124 [148–164] 740 [701–745] (*)	5.97 [4.45–7.49]	116 [130–162] 122 [122–172] (*)	1.05 [0.82–1.28]	122 [132–150] 828 [824–898] (*)	6.79 [5.29–8.29]
Control group (n/v = 54/48)	138 [150–186] 145 [161–189] (*)	1.05 [0.78–1.32]	122 [130–158] 116 [116–174] (*)	0.95 [0.80–1.10]	122 [142–166] 126 [111–201] (*)	1.03 [0.90–1.16]
t-Test [Comparing (*)]	$P < 0.001$		$P = 0.28$		$P < 0.001$	
AHD-treated patients						
Progressors (n = 26/23)	126 [126–154] 966 [890–1062] (*)	7.67 [6.62–8.72]	120 [106–146] 124 [110–158] (*)	1.03 [0.91–1.15]	124 [109–127] 749 [498–726] (*)	6.04 [5.24–6.84]
Non-progressors (n = 28/23)	121 [132–168] 117 [144–164] (*)	0.97 [0.90–1.04]	122 [130–158] 126 [123–1.67] (*)	1.03 [0.94–1.12]	132 [116–132] 126 [114–142] (*)	0.95 [0.84–1.06]
t-Test [Comparing (*)]	$P < 0.003$		$P = 0.90$		$P < 0.0001$	

PBMCs were incubated with the following challenger: autologous hemoderivative used as immunogen (AHD); autologous derivative (AD) from (CD4+CD25−) and (CD4+CD25+) cells; intact (CD4+CD25−) or (CD4+CD25+) cells; cpm = net counts (challenged—control in the same experiment); LPR = Lymphocyte proliferation response = ratio cpm after/cpm before treatment, mean [95% CI]

AHD group: patients treated with AHD; Control group: appropriate controls with NHF-AHD; Progressors: patients maintaining progressive disease status; Non-progressors: patients achieving stable disease status. n = number of patients; v = number of evaluable patients' tests (controls accepted); AHD: heat-fractionated autologous hemoderivative; NHF-AHD: non-heat fractionated AHD

concurs with the 400 spots from 83 gene-products for plasma-proteome previously reported with the same technology [16]. Obviously, this profile was increased to a range of 605–710 spots in samples of NHF-AHD because the preparation procedure of NHF-AHD included proteins from blood cells contained in the supernatant of sedimented blood used as source of the immunogen hemoderivative. In the tested samples, AHD proteome lost 158–174 spots compared with NHF-AHD that must be considered the thermolabile fraction in these experimental conditions. However, AHD proteome showed that the thermostable fraction still contained multiple proteins that were distributed in the first dimensional electrophoresis, in sites with alpha, beta, and gamma electrophoretic mobility compared with the classical electrophoresis of plasma proteins. In the second dimensional electrophoresis, the molecular weight of the AHD proteome ranged from 24.000 to 190.000 kD. The AHD proteome also contained site-spots non-identified in NHF-AHD suggesting molecular modifications induced by heat treatment. As a whole, under the used experimental heat conditions, these results confirmed the thermostability of several proteins with well known high biological significance, i.e. immunoglobulins, tumor markers (CEA), growth factor components.

Discussion

In this study, we observed a statistically significantly superior progression-free and overall survival in patients who received AHD versus those treated with control. Of 54 patients, 28 (52%) met RECIST criteria for NPD. Therefore, these results provide evidence that treatment with AHD may be an effective anti-breast cancer strategy.

Immunological mechanism

AHD treatment elicited statistically significant autologous immunologic responses in vivo (DTH $\geq$ 5 mm) and in vitro (LPR > 4). AHD elicited in vivo DTH against AHD and in vitro immune responses against several tested challengers including the immunogen AHD and the TAAs (CEA and CA 15-3). These in vivo and in vitro immunologic responses were statistically associated not only to AHD treatment but also to the non-progressor condition of AHD treated patients. In addition, among the antigenic activities identified in AHD by lymphocyte proliferation assay, this study showed that lymphocytes from patients with an anti-progressive response to AHD treatment were sensitized against components of an autologous CD4+CD25+ cells derivative and not against the same derivative prepared from other autologous CD4+ cell populations (CD25−).

Immunogen characterization

In the present study, it was shown that after heat treatment, AHD retained multiple molecular species of proteins configuring a thermostable proteome obtained from the supernatant of sedimented blood that is a thermostable plasma proteome plus a cytolysed-blood-cells

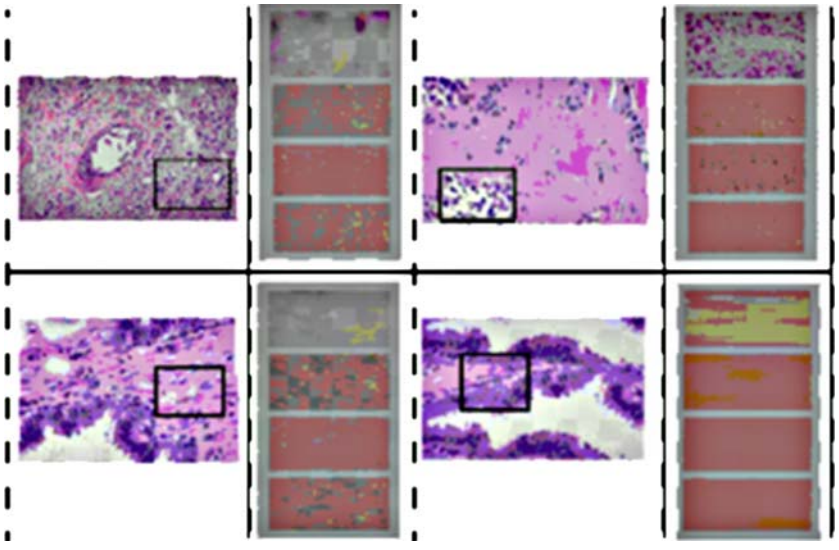

Fig. 2 Breast biopsies from a patient with favorable response to AHD. Upper row: biopsy from tumor site. Lower row: biopsy from a non-tumor site: Column 1 and 2: before treatment; Column 3 and 4: after 6 months of treatment; Columns 1 and 3: hematoxylin-eosin; Columns 2 and 4: immunohistochemistry. From top to bottom: Lymphocytes, CD4+, CD8+ and B (CD20+) cells

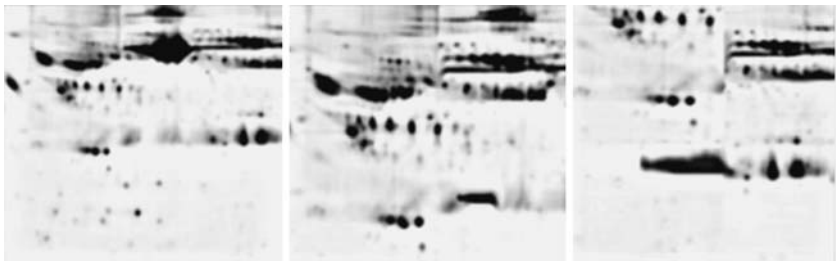

Fig. 3 2-D Electrophoresis of the thermostable Autologous Hemoderivative used as immunogen. A number of spots between 447 and 536 to be compared with the 400 spots (83 gene products) of the plasma proteome identified with the same methodology and the 605–710 spots of the Non-heated Autologous Hemoderivative which lost 158–174 thermolabile spots

proteome. Compared with NHF-AHD, this thermostable proteome has some new 2-DE spots and it has lost some molecular components, but in summary, like other cancer vaccines using cells as immunogens, i.e. tumor cell vaccines, AHD is a polymolecular biological system; therefore it is potentially a polyvalent antigenic immunogen.

Autologous antigenicity

At least some of the antigenic activities demonstrated in AHD-treated patients had as targets molecular components that were present in the patients' blood and in the immunogen-control NHF-AHD. This is the case of TAAs leaked from tumor cells to the blood and blood cells' components including CD4+CD25+ cells. These molecular targets were indicative of an immune response targeted to the cells containing these molecules: tumor cells (as shown in the pathology) and intact CD4+CD25+ cells (as shown in LPR tests). These facts evoke a selective auto-immunity. The histopathology of treated non-progressor tumors also confirmed the reported effects of AHD in the different primary sites: an intense stroma proliferation was associated with the lymphocyte infiltration [1, 3].

Safety

As it has been previously reported [1–3, 6], the toxicity of the AHD procedure was negligible and no clinical autoimmunity or immunosuppressive phenomena were observed under the specific conditions of this study.

Anti-cancer activity of AHD has been observed in several different types of cancers. In that regard, a variety of mechanisms might be invoked to explain the superior outcomes with AHD treatment. These include interference with CD4+/CD25+ activity and acquired autologous antigenicity. Several correlative studies have provided evidence for these two mechanisms of action [12, 17–25].

In conclusion, this study suggests that tumor molecular components transferred to the blood from malignant cells or biological responder cells can elicit an autologous immune response in cancer patients, producing a clinically beneficial effect. This approach has little toxicity and is associated with a DTH immune response and modification in tumor histopathology compatible with an immunological response. The association of these effects with an immune response bypassing the pre-existing tolerance against components of regulatory and tumor cells could be the basis for an immunotherapeutic procedure in further studies. Future trials with this agent will include optimizing adjuvant treatment and/or repeating the treatment over several months.

References

1. Lasalvia E, Cucchi S, DeStefani E et al (1995) Autologous induction of tumoral fibrogenesis. Neoplasia 12(1):5–10
2. Lasalvia E, Cucchi S, Carlevaro T et al (1995) Antimetastatic effect of a blood fraction from cancer patients. (Meeting abstract). Proceedings 31st Annual Meeting of the American Society of Clinical Oncology, Chicago, IL, USA, Abstract No: 730
3. Lasalvia-Prisco E, Cucchi S, Vázquez J et al (2003) Antitumoral effect of a vaccination procedure with an autologous hemoderivative. Cancer Biol Ther March/April 2(2):155–160
4. Berd D, Mastrangelo MJ, Engstrong P et al (1982) Augmentation of the human immune response by cyclophosphamide. Cancer Res 42:4862–4866
5. Leong SP, Enders-Zohr P, Zhou YM et al (1999) Recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) and autologous melanoma vaccine mediate tumor regression in patients with metastatic melanoma. J Immunother 22(2):166–174
6. Lasalvia-Prisco E, Garcia-Giralt E, Cucchi S et al (2006) Advanced colon cancer: antiprogressive immunotherapy using an autologous hemoderivative. Med Oncol 23(1):91–104
7. Oken MM, Creech RH, Tormey DC et al (1982) Toxicity and response criteria of the Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649–655
8. Therasse P, Arbuck SG, Einsenhauer EA et al (2000) New guideline to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment

Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Nat Cancer Inst 92:205–216
9. World Medical Association Declaration of Helsinki: Recommendations guiding medical doctors in biomedical research involving human subjects. Adopted by the 18th World Medical Assembly, Helsinki, Finland, 1964 and as revised in Tokyo, 1975, in Venice, 1983, in Hong Kong, 1989. Version with changes of 1997
10. PDQ (Physician Data Query) National Cancer Institute, comprehensive cancer database
11. Garcia-Giralt E, Lasalvia-Prisco E, Cucchi S et al (2005) Breast cancer: role of tumor associated antigens and regulatory cells [CD4+CD25+] as targets of the immune response elicited by an anti-progressive autologous hemoderivative vaccine (Meeting abstract). Proceedings 41st Annual Meeting of the American Society of Clinical Oncology, Orlando, FL, USA, Abstract No. 2589
12. Wan Fai Ng, Dugan Phillip J, Ponchel FE et al (2001). Human CD4+CD25+ cells: a naturally occurring population of regulatory T cells. Blood 98(9):2736–2744
13. Gauthier LR, Granotier C, Soria JC et al (2001) Detection of circulating carcinoma cells by telomerase activity. Br J Cancer 84(5):631–635
14. Langen H, Roeder D, Juranville JF et al (1997) Effect of the protein application mode and the acrylamide concentration on the resolution of protein spots separated by two-dimensional gel electrophoresis. Electrophoresis 18:2085–2090
15. National Cancer Institute, US: Common terminology criteria for adverse events v3.0 (CTCAE)
16. Fountoulakis M, Juranville JF, Jiang L et al (2004) Depletion of the high-abundance plasma proteins. Amino Acids 27:249–259
17. Jones E, Dahm-Vicker M, Simon AK et al (2002) Depletion of CD25+ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. Cancer Immun. 2:1–12
18. Liyanage UK, Moore TT, Hong-Gu Joo et al (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 169:2756–2761
19. Josic D, Buchacher A, Kannicht C et al (1999) Degradation products of factor VIII which can lead to increased immunogenicity. Vox Sang 77:90–99
20. Robinson WE Jr, Montefiori DC, Mitchell WM (1988) Antibody-dependent enhancement of human immunodeficiency virus type 1 infection. Lancet 1:790–794
21. Abeyounis CJ, Milgrom F (1976) A thermostable antigen characteristic for carcinogen-induced rat intestinal tumors. J Immunol 116(1):30–34
22. Lomakin MS, Larin AS, Maiskii IN (1978) Thermostable antigens of malignant tumors and normal tissues of experimental animals [Termostabil'nye antigeny zlokachestvennykh tkanei eksperimental'nykh zhivotnykh]. Biull Eksp Biol Med 85(6):726–728
23. Lomakin MS, Larin AS, Maiskii IN (1980) Thermo-stable tumor-associated antigens in the serum of tumor-bearing animals and in tissue cultures of malignant tumors [Termostabil'nye opukhole-assotsiirovannye antigeny v syvorotke krovi zhivotnykh-opukholenositelei i v kul'ture tkani zlokachestvennykh opukholei]. Biull Eksp Biol Med 89(4):452–454
24. Hampton R, Walker M, Marshall J et al (2002). Differential expression of carcinoembryonic antigen (CEA) splice variants in whole blood of colon cancer patients and healthy volunteers: implication for the detection of circulating colon cancer cells. Oncogene 21(51):7817–7823
25. Burton RM, Hope NJ, Lubbers (1976) A thermostable antigen associated with ovarian cancer. Am J Obstet Gynecol 125(4):472–477
26. Conry RM, Khazaeli MB, Saleh MN et al (1999) Phase I trial of a recombinant vaccinia virus encoding carcinoembryonic antigen in metastatic adenocarcinoma: comparison of intradermal versus subcutaneous administration. Clin Cancer Res 5(9):2330–2337
27. Saetang T, Suttijitpaisal P, Ratanabanangkoon K (1998). Preparations of toxic components from *Naja kaouthia* venom by selective heat denaturation. J Nat Toxins 7(1):37–44
28. Petrunin DD, Lopukin Iu M, Molodenkov MN et al (1982). Immunochemical identification of thermostable alpha-glycoprotein in blood sera in various immune system diseases. Biull Eksp Biol Med 93(4):66–68
29. Bruster HTH, Kuntz BME, Scheja JW (1988) Autovaccination plus heat-inactivated autologous plasma in AIDS patients. Lancet 4:1284–1285
30. Snegotska O (1965) Autovaccines for the treatment of cancer. Folia Clin Int (Barc Spain) 5(6):303–308
31. Karcher D, Lowenthal A, Stoppie P et al (1984) A serum protein affecting the regulation of the immune system. Neurochem Res 9(10): 1423–1430