Advanced ovarian cancer: Vaccination site draining lymph node as target of immuno-modulative adjuvants in autologous cancer vaccine

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Abstract: Tumor as source of tumor associated antigens (TAA) and sentinel lymph node (SLN) configure the first interaction between the malignant disease and the patient's immune system. As consequence of this interaction, a local immune response is elicited inside the SLN. Tumor's cytokines reach the SLN conditioning its cellular microenvironment to produce local permissive immune responses. This local tolerogenic immunity is decisional because it starts a systemic also permissive immunity. The tumor progresses.

To counteract this mechanism, we have designed a medical procedure to create an immunotherapeutic site (ITS) that reproduces, distantly from the tumor, a TAA source and a draining lymph node but with a cellular microenvironment conditioned to promote local protective instead of permissive immune responses. Due to ITS decisional role, this local protective immunity starts a systemic anti-tumoral immune response.

In progressive ovarian cancer, we tested an ITS using the autologous thermostable hemoderivative-cancer vaccine as TAA source and granulocyte macrophage-colony stimulant factor plus etoposide, injected both at the vaccination site, as conditioner of the draining lymph node cellularity. The immunophenotyping of lymph node cell populations showed that ITS acquired a locally protective immune profile T-regulatory-cells/activated-antigen presenting-cells and systemically increased the antiprogressive effect of the tested vaccine.

Keywords: autologous vaccine, ovarian cancer, cancer vaccine, cancer immunotherapy, immunotherapy adjuvants

Introduction

In cancer disease, a locoregional immune microenvironment constituted by the tumor as source of tumor associated antigens (TAA) and the sentinel lymph node (SLN) as the first reactive draining site for TAA can be defined. During cancer development, a locoregional immune response is elicited at this microenvironment and it can potentially be permissive or protective, immunologically known as tolerogenic or immunogenic. This locoregional immune response is decisional because it starts a systemic immune response with the same configuration as its own: tolerogenic or immunogenic. In cancer patients, tumor invasion and dissemination evidence the predominance of tolerogenic over immunogenic immune responses, either local and/or systemic. Strong data supports that the tumor induces an immunomodulation of SLN, conditioning a tolerogenic locoregional immune response that allows lymph node metastatic invasion and starts a systemic immune response of tumor tolerance permitting tumor dissemination (Cochran et al 2006).

The therapeutic cancer vaccines can be considered as a medical procedure reproducing the above referred locoregional immune microenvironment of malignant tumors but without the tumor-induced immuno-modulative tolerogenic mechanism. In this

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case, the vaccination site is the source of TAA included in the vaccine and the SLN is the draining lymph node of the vaccination site known as sentinel immunized node (SIN). Like in tumor disease, the vaccine locoregional immune response can express tolerogenic and/or protective activity starting the corresponding systemic immune response (Disis et al 1996).

The goal of cancer vaccines is to elicit a protective systemic immune response. Therefore, the design of vaccine adjuvants addressed to switch the vaccine locoregional immune response from tolerogenic to protective must be investigated as a strategy for starting optimized systemic anti-tumoral vaccine effects. In tumors and cancer vaccines, several modulators of the locoregional immune microenvironment that condition the anti-tumoral immune responses have been identified. We have selected two of them to be studied taking in account their known mechanism of action and their safety proven in their extensive clinical use.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a potent immune stimulant when administered with different vaccines at the vaccination site. In addition, intratumoral GM-CSF induces local and remote antitumoral effects. The mechanism of action of GM-CSF resides in its ability to act locally, stimulating the proliferation and maturation of professional antigen-presenting cells (APCs) at the injection site. This mechanism is associated to the enrichment of activated dendritic cells within the regional draining lymph nodes (Leong et al 1999; Simons et al 1999; Wiseman et al 2001; Dranoff 2002; Yang et al 2003; Reali et al 2005). This agent has been used in clinical practice with different therapeutical objectives for a long time.

Etoposide (ETP) was the other agent selected. In experimental tumor models, administration of low dosages of certain cytostatic drugs at the site of antigenic stimulation (tumor or vaccine) facilitates the development of strong antitumoral T cell-immunity. T cell-suppressor depletion at the antigenic stimulation site and at the draining lymph nodes has been demonstrated as the locoregional mechanism of action for this immuno-potentiation. ETP, cisplatinum, and cyclophosphamide–active metabolites have been the main effective drugs in these local chemo-immunotherapeutic protocols (Scheper et al 1984; Tan et al 1986; Claessen et al 1989, 1991, 1992; Limpens, Garssen, Scheper et al 1990; Limpens, Garssen, Germeraad et al 1990; Limpens and Scheper 1991). ETP, one of these drugs, is active at the inoculation site, does not require liver activation and the dosage that can be safely inoculated locally is well known.

In this study, we have explored GM-CSF and ETP as isolated or associated local adjuvants of a cancer vaccine, assuming that these agents can be complementary in the locoregional immunomodulation to enhance the systemic antitumoral protective immunity. Advanced ovarian cancer patients were the patient population studied. As cancer vaccine we have tested the autologous thermostable hemoderivative cancer vaccine (ATH-CV), a procedure with well-documented systemic effects of sensitization against vaccine antigens and antiprogressive activity upon tumor growth including ovarian cancer (Lasalvia, Cucchi, Carlevaro, et al 1995; Lasalvia, Cucchi, DeStefani et al 1995; Lasalvia et al 2003; Garcia-Giralt et al 2006; Lasalvia-Prisco et al 2006a, 2006b).

Patients and methods

Patients

The study was conducted in patients admitted to medical centers that submitted medical data to the Cooperative Trials Center (CTC) of PharmaBlood, R and D Department, Florida, USA (PharmaBlood is a non-pharmaceutical concerned company supporting scientific research in medical procedures using hemoderivatives).

Eligibility criteria

Patients who were enrolled in this open, randomized phase II study had to meet the following criteria: to have histologically or cytologically proven persistent or recurrent (relapse being observed within 1 year of the last platinum-based chemotherapy regimen) stage III or IV ovarian adenocarcinoma, excluding borderline tumor, low-potential tumors, squamous cell carcinoma, and granulosa-theca cell tumors (International Federation of Gynecology and Obstetrics); in demonstrated progression according to RECIST (Therasse et al 2000) criteria with serum CA-125 level also progressing; and to have received at least one chemotherapeutic regimen, with the last regimen featuring platinum compounds at therapeutically adequate and potentially active doses. Patients must have at least one bidimensionally measurable lesion by computed tomography scan or magnetic resonance imaging, with at least one diameter greater than or equal to 2 cm. Patients had to be 18 years or older, they must have had a performance status of $\leqslant 2$ on the Eastern Cooperative Oncology Group scale (Oken et al 1982) or $\geqslant 60\%$ on the Karnofsky scale (Schag et al 1984) with an expected survival of at least 6 months. Baseline blood laboratory assessment of organic functions was as follows: adequate bone marrow function: WBC $\geqslant 3000/mm^3$, ANC $\geqslant 1500/mm$, Hgb $\geqslant 9.0$ g/dl, and

platelets ⩾100,000/ mm^3; adequate liver function: bilirubin ⩽ 1.5 mg/dl, AST ⩽ 2; adequate kidney function: creatinine ⩽ 1.5 mg/dl.

Written informed consent was obtained from each patient.

Exclusion criteria

Exclusion criteria included the following: brain or leptomeningeal metastasis; previous or concurrent malignancies at other sites including abdominal adenocarcinoma of unknown origin and symptomatic peripheral neuropathy; patients with documented anaphylactic reaction to any drug, recognized immunodeficiency disease or active autoimmune disease and those who have had previously immunotherapy of any type within the past 6 weeks or who were receiving treatment with immunosuppressive therapy. In addition, significant cardiovascular abnormalities, active infection causing fever or other medical condition requiring specific treatment were within the exclusion criteria.

Clinical trial

The Institutional Review Board (IRB) approved the trial, which complied with the Declaration of Helsinki (World Medical Association, 2004, website at http://www.wma.net/e/policy/b3.htm). It was a prospective, randomized, controlled trial; the treating physicians did not participate in the arm randomization for their patients that was performed remotely at the above-mentioned CTC. The patients were randomized into 4 Groups that received different treatments. The trial duration for each patient was 6 months (180 trial days): 1 month of pre-treatment background, 1 month of different treatments for each Group accomplishing the predetermined treatment schedule and 4 months of post-treatment follow-up after finishing treatments.

Treatments schedule

Patients assigned to Group 1 received the ATH-CV, as it was previously described (Lasalvia-Prisco et al 2003). Briefly, five days before the beginning of the treatment period (26th trial day), twenty milliliters of blood were drawn from the femoral artery in a tube containing 5000 IU heparin. The blood was allowed to sediment at 37 °C for 1 hour. Then, cellular lysis was produced by exposing the supernatant of plasma and cells to hypotonic shock with 3 volumes of distilled water for 15 min, and followed by freezing at –20 °C. After 24 hours, 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 preparation was divided into 5 vials: 1 test-vial containing 0.5 ml and 4 vaccine-vials containing equal aliquots of the remaining preparation. All vials were stored at –20 °C until use. The 4 vaccine-vials were used on days 1, 14, 21, and 28 of the treatment period (one month: 31st to 60th trial day).

Patients assigned to Group 2 received the same protocol than Group 1 but GM-CSF 300 micrograms were injected in the vaccination sites simultaneously with each vaccine following the chronology Vaccine-Adjuvant selected for GM-CSF in previous reports (Disis et al 1996).

Patients assigned to Group 3 received the same protocol than Group 1 plus ETP 10 mg/day injected at vaccination sites 1 to 4 days after each vaccine, following the chronology Vaccine-Adjuvant selected for ETP in previous reports (Limpens, Garssen, Germeraad et al 1990).

Patients assigned to Group 4 received the same protocol than Group 1 but GM-CSF 300 micrograms were injected in the vaccination sites simultaneously with each vaccine and ETP 10 mg/day was injected at vaccination sites 1 to 4 days after each vaccine.

Evaluations

The vaccine effect was assessed in each Group comparing the post-treatment versus the pre-treatment evaluations of parameters measuring the vaccine effects, in other words, each Group was its own control. Afterwards, the vaccine effects assessed in the different Groups including adjuvants (Groups 2, 3 and 4) were compared with the Group without the tested adjuvants (Group 1) in order to validate the adjuvant activity.

Pre-treatment background evaluations

The following evaluations were performed before starting treatments:

1. Tumor growth assessed in a measurable lesion image 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 measured tumor size at 1st and 30th trial days (same image technology for same lesion localization allowed comparative assessment).
2. Thirty-day variation of CA-125, measured between the 1st and 30th trial days.
3. Delayed type hypersensitivity (DTH) test was performed at the 28th trial day in each patient with an aliquot of the autologous thermostable hemoderivative (ATH) lyophilized and recovered in 1/10 of initial volume (ATH × 10). DTH test

was read 48 hours after the intradermal inoculation (30th trial day) and it will be considered positive if it produced an induration ⩾5 mm.

Post-treatment follow-up evaluation

The post-treatment assessments were:

1. At day 30th after finishing treatments (90th trial day), DTH elicited by ATH × 10 was performed and the positive (induration ⩾5 mm) or negative (induration < 5 mm) was registered 48 hours after the test.
2. At day 120th after finishing treatments (180th trial day), the previous 30-day increments of CA-125 and tumor growth were registered, as in the pre-treatment background evaluation, taking into account that in previous reports optimal tumor anti-progressive effect was demonstrated 120 days after ATH vaccination (Lasalvia-Prisco et al 2003, 2006a, 2006b).
3. At day 120th after finishing treatments (180th trial day), in each patient a SIN localization by technetium scintigraphy was performed as it was described (Cochran et al 2000) and mainly used for melanoma SLN (Mariani et al 2002). Afterwards, SIN was surgically removed and immunophenotyping of cell populations was processed by flow cytometry and immunocytochemistry, expanding the antibodies set as it was practiced to explore all relevant lymph node cell populations (Bryan et al 1993; Vuylsteke et al 2002). Briefly, 0.4 ml of Technetium-99 m of unfiltered sulfur colloid (containing 37 MBq/ml, or 1 mCi/ml) was injected intradermally in the four quadrants (0.1 ml each) around the circumference of the vaccination site 24 hours before the SIN removal. Dynamic sequences and static views with a gamma camera allowed the radiologist to mark on the skin the SIN localization. Ten to fifteen minutes before the surgery, 5 ml of 1% isosulfan blue dye were injected around the vaccination site. Local anesthesia was used for inguinal SIN removal and it was performed in the outpatient clinic. The incision was routinely 1 inch long. The surgeon using a sterile hand-held gamma probe confirmed location of the SIN that was marked on the skin by the radiologist, detecting the blue dye stained node. Patients consented to this procedure as part of the initial informed consent process for the trial. Immediately after removal, SINs were collected in sterile ice-cold complete medium, comprising IMDM supplemented with 25 mmol/L Hepes buffer with 10% fetal calf serum, 50 IU/ml penicillin-streptomycin, 1.6 mmol/L L-glutamine, and 0.05 mmol/L ß-mercaptoethanol Viable cells were isolated and analyzed using a scraping method. In short, SIN was bisected crosswise with a surgical scalpel and the cutting surface of the SIN was scraped 10 times with a surgical blade. SIN cells were rinsed from the blade with medium containing 0.1% DNase I, 0.14% collagenase A, and 5% fetal calf serum, incubated for 45 minutes at 37 °C, and subsequently in phosphate-buffered saline with 5 mmol/L ethylenediamine tetraacetic acid for 10 minutes on ice. Finally, the SIN cells were washed twice in complete medium, counted, and directly stained with antibodies labeled with either phycoerythrin or fluorescein isothiocyanate, and analyzed by flow cytometry at 10,000 or 100,000 events per measurement. In each case, the amount of antibody used was based on the manufacturer's suggestion or titration experiments to optimize the signal/noise ratio. Immunocytochemistry was performed in cytospin preparations acetone-fixed and stained immunocytochemically with different monoclonal antibodies. In preliminary studies, we have assessed a wide spectra of cell markers by flow cytometry and immunocytochemistry in order to identify the mobilized cell populations in immunized lymph nodes: CD1a, CD83 and CD86 for dendritic cells ; CD9, CD10, CD14, CD19, CD20, CD21, CD24, CD38, CD40, CD45 and the surface Immunoglobulins IgG, IgM, IgA, IgD, kappa, lambda for B lymphocytes, CD3, CD4, CD5, CD8, and CD25 for T-lymphocytes (results not shown). According to these preliminary observations, mature dendritic cells ($CD1a^+/CD83^+$) and T-regulatory cells ($CD4^+/CD25^+$) were the marker sets defined in the frame of this study as the highly modified cell populations in the examined lymph nodes of the four Groups of patients studied. Therefore, these cell populations were the target of the performed assessments (CD86 marker had the same variations as CD83+).
4. During the 150 days after pre-treatment, toxicities were registered according to the Common Terminology Criteria for Adverse Events v3.0 or CTCAE (National Cancer Institute website at http://ctep.cancer.gov/forms/CTCAEv3.pdf).
5. As it was incorporated in the informed consent, independent and blinded reviewers performed assessment of each one of the CA-125, tumor growth, DTH tests and SIN immunophenotyping.

Statistical methods

The primary end-point of the study was SIN cell immunophenotyping treatment-induced variations. Secondary efficacy end-points included treatment-induced variations

of tumor growth, increments of CA-125 serum level and number of cases acquiring systemic sensitization to vaccine antigens (DTH).

The assessment of SIN cell immunophenotyping targeted cell populations: mature dendritic cells ($CD1a^+/CD83^+$) and T-reg cells ($CD4^+/CD25^+$) in Groups 2, 3 and 4 were compared versus the respectively evaluations in Group 1 using the unpaired two-tailed Student t-test.

The assessment of tumor growth, CA-125 serum level increment and number of patients with positive DTH response was performed comparing the follow up evaluations in each Group versus the respectively pre-treatment evaluations using the paired two-tailed Student t-test. The assessment of the differences between the post-treatment evaluations among the studied Groups was performed using the unpaired two-tailed Student t-test.

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

Figures 1 and 2 show mean and standard deviation of pre-treatment and post-treatment evaluations of tumor growth and CA-125 serum level increment, respectively in each one of the 4 different treated Groups. In all groups, tumor growth decreased with statistical significance after treatment: only vaccination (ATH-CV) (Figure 1, Group 1, $p < 0.05$), vaccination (ATH-CV) plus GM-CSF as singular adjuvant (Figure 1, Group 2, $p < 0.02$), vaccination (ATH-CV) plus ETP as singular adjuvant (Figure 1, Group 3, $p < 0.02$) and vaccination (ATH-CV) plus both GM-CSF and ETP as double

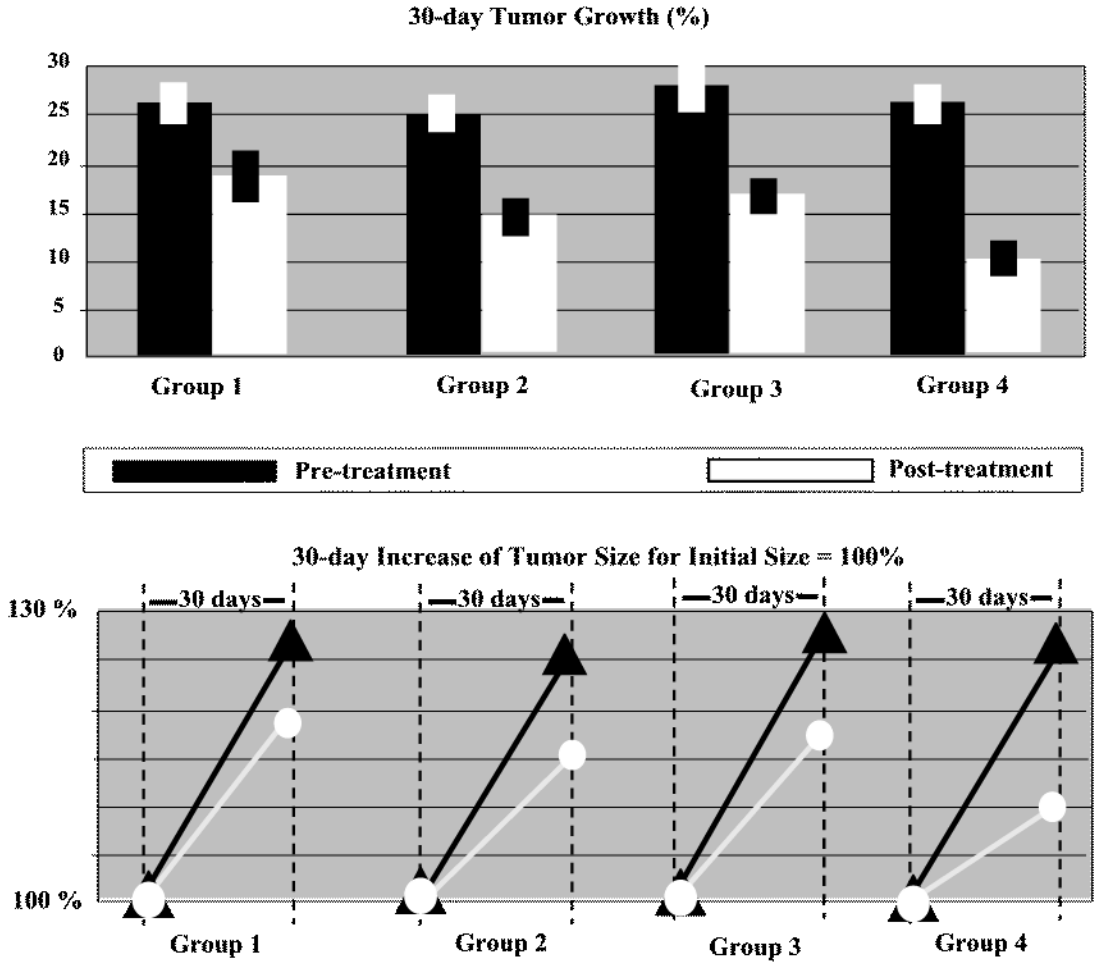

Figure 1 Pre-treatment and post-treatment tumor growth: mean (± standard deviation) in the four 30-day differently treated groups. Upper Row – Pre-treatment: tumor size growth (RECIST) % in the 30 days previous to treatment start. Post-treatment: tumor size growth (RECIST) % in the 30 days between 120th and 150th day after finishing treatment: Group 1: ATH-CV. Pre-treatment 27.1 (±5.1); post-treatment 18.2 (±6.0); Group 2: ATH-CV + GM-CSF. Pre-treatment 24.7 (±4.3); post-treatment 14.9 (±4.6); Group 3: ATH-CV + ETP treated. Pre-treatment 28.2 (±6.1); post-treatment 16.7 (±2.7) and Group 4: ATH-CV + GM-CSF + ETP treated. Pre-treatment 25.6 (±4.4); post-treatment 10.0 (±3.1). Lower Row – Linear increase in tumor size in all groups and in pre and post-treatment. The treatments slowed the tumor growth but did not stop it.

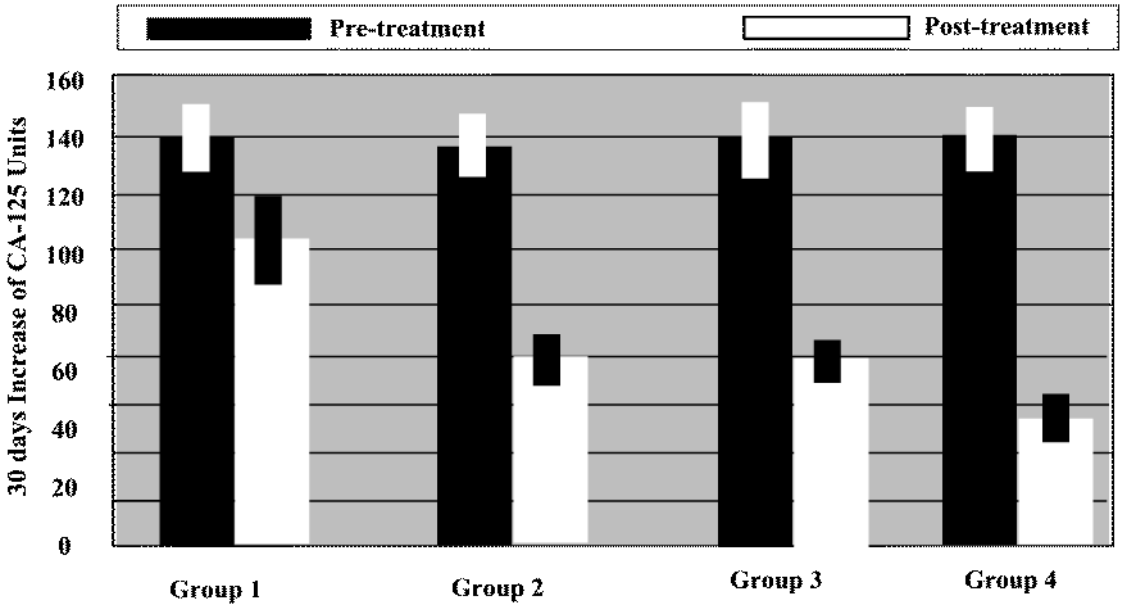

Figure 2 Pre-treatment and post-treatment CA-125 serum level increase. Mean (± standard deviation) in the four 30-day differently treated groups. Pre-treatment: Increase of CA-125 in the 30 days previous to treatment start. Post-treatment: Increase of CA-125 in the 30 days between 120th and 150th day after finishing treatment. Treatments and results: Group 1: ATH-CV. Pre-treatment 138.6 (±6.1); post-treatment 103.9 (±4.6). Group 2: ATH-CV + GM-CSF. Pre-treatment 136.3 (±5.8); post-treatment 78.3 (±5.2). Group 3: ATH-CV + ETP treated. Pre-treatment 140.5 (±7.1); post-treatment 80.1 (±6.7). Group 4: ATH-CV + GM-CSF + ETP treated. Pre-treatment 139.6 (±5.9); post-treatment 54.2 (±4.1).

adjuvants (Figure 1, Group 4, $p < 0.005$). The increase of CA-125 serum level was also lower in the post-treatment of the four Groups: only vaccination (ATH-CV) (Figure 2, Group 1, $p < 0.04$), vaccination (ATH-CV) plus GM-CSF as singular adjuvant (Figure 2, Group 2, $p < 0.01$), vaccination (ATH-CV) plus ETP as singular adjuvant (Figure 2, Group 3, $p < 0.01$) and vaccination (ATH-CV) plus both GM-CSF and ETP as double adjuvants (Figure 2, Group 4, $p < 0.001$).

Figure 3 shows DTH test against the vaccine immunogen ATH (negative before treatment). The number of positive–tests elicited by ATH-CV (Group 1) was significantly increased by GM-CSF (Group 2 vs Group 1: $p < 0.04$), by ETP (Group 3 vs Group 1: $p < 0.04$) and with higher significance by both GM-CSF and ETP (Group 4 vs Group 1: $p < 0.001$).

Table 1 shows the significant differences that were identified in the immunophenotyped cell populations recovered from the vaccination site draining lymph nodes when the four Groups were compared: the mature dendritic cells ($C1a^+CD83^+$) and the T-regulatory cells ($CD4^+CD25^+$). For the mature dendritic cells, a marker of activated antigen presenting cells, the number identified at the end of post-treatment follow-up was significant higher in Group 4 and Group 2 than in Group 1 and Group 3. For the T-Regulatory cells (T-Reg), a marker of SIN tolerogenic activity, the cell population in lymph nodes was significant lower in Group 4 and Group 3 than in Group 1 and Group 2.

In all four groups studied, no significant toxicity (CTCAE higher than 2) was registered. No patients refused the SIN biopsy. There were no infections or complications at these surgical sites. In Table 1, the other cell-populations' immunophenotypes evaluated in preliminary studies were also indicated. For these cell populations, any statistic difference among the four Groups was evidenced in the frame of this study.

Discussion

When patients were treated with ATH-CV (Group 1), the post-treatment observations were a slow down of tumor growth (Figure 1), a decrease of CA-125 serum level increment (Figure 2) and an increase of the number of cases sensitized to the ATH-CV immunogen (Figure 3). These facts confirmed the previously reported antiprogressive and immunogenic effects of this vaccine (Lasalvia-Prisco et al 2006a, 2006b) supporting the development of the ATH-CV procedure as immunotherapy in advanced ovarian cancer.

Both, the GM-CSF (Group 2) and ETP (Group 3) administered locally as singular ATH-CV adjuvant enhanced the vaccine immunotherapy activity measured as: slow down of pre-treatment tumor growth (Figure 1), decrease of CA-125 serum level increment (Figure 2) or increase of the number of positive DTH tests (Figure 3). Therefore, GM-CSF and ETP can be defined as local vaccine adjuvants for ATH-CV in advanced ovarian cancer. The use of both adjuvants,

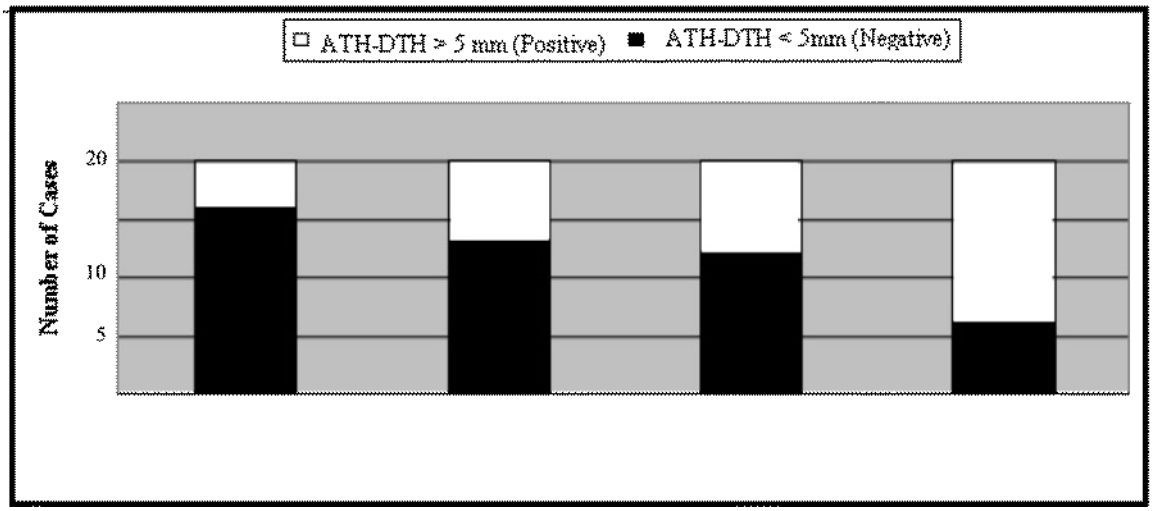

Figure 3 Delayed type hypersensitivity (DTH) positive (>5 mm) and negative (<5 mm) elicited by the vaccine autologous thermostable hemoderivative cancer vaccine (ATH-CV) after vaccination with: ATH-CV (Group 1); ATH-CV plus different vaccine adjuvants: GM-CSF (Group 2), ETP (Group 3) and GM-CSF + ETP (Group 4).

GM-CSF and ETP, jointly administered with ATH-CV (Group 4) produced stronger effects than the use of each one separately (Figures 1, 2, and 3), evidencing the cumulative adjuvant effect of these two agents for this model. Immunophenotyping studies of draining lymph nodes (Table 1) suggest as mechanism of action for the tested vaccine adjuvants their known effects upon the immunity committed cell populations at SIN level: GM-CSF recruits and activates the antigen presenting cells increasing the locoregional presence of mature dendritic cells (Molenkamp et al 2005) and ETP produces a locoregional depletion of suppressor cells (Limpens and Scheper 1991) today specifically identified as the tolerogenic lymphocyte population of T-reg cells or $CD4^+CD25^+$ (Battaglia et al 2003; Viguier et al 2004). In melanoma patients, it was reported that mature dendritic cells ($CD1a^+CD83^+$) induce T-cell activation under pro-inflammatory conditions. Mature Dendritic cells also increase significantly their frequency in the SLN after intradermal

Table 1 Among the cell populations recovered from draining lymph nodes of vaccination sites and immunophenotyped, this table shows the cell populations with statistical significant differences when the four differently treated Groups were compared. Group 1 (ATH-CV): Autologous Thermostable Hemoderivative-Cancer Vaccine; Group 2 (ATH-CV + GM-CSF): Granulocyte Macrophage-Colony Stimulant Factor as local adjuvant of ATH-CV; Group 3 (ATH-CV + ETP): Etoposide as local adjuvant of ATH-CV; Group 4 (ATH-CV + GM-CSF + ETP) Granulocyte Macrophage-Colony Stimulant Factor plus Etoposide as local adjuvants of ATH-CV.

	Group 1 (**)		Group 2 (**)		Group 2 vs Group 1	Group 3 (**)		Group 3 vs Group 1	Group 4 (**)		Group 4 vs Group 1
	Mean	S.D.	Mean	S.D.	p	Mean	S.D.	p	Mean	S.D.	p
$CD1a^+$ $CD83^+$ (*)											
Number by 600 CD3 +T cells	7.4	1.8	11.6	2.1	<0.02	7.5	1.6	>0.9	11.8	2.0	<0.02
$CD4^+$ $CD25^+$ (*)											
% of $CD4^+$ T cells	9.2	1.1	9.0	1.2	>0.9	3.4	1.1	<0.03	3.2	0.8	<0.02

*Immunophenotyping
CD1a+ CD83+ : Mature dendritic cells
$CD4^+$ $CD25^+$: T-Regulatory cells
**Treatments
Group 1: ATH-CV
Group 2: ATH-CV + GM-CSF
Group 3: ATH-CV + ETP
Group 4: ATH-CV + GM-CSF + ETP
The following immunophenotypes were assessed. CD1; CD2; CD3; CD4; CD5; CD8; CD9; CD10; CD13; CD14; CD19; CD20; CD21; CD22; CD24; CD33; CD38; CD43; CD45; CD56; CD71; CD83; bcl-2; CD1a; CD25. No other set than $CD1a^+$ $CD83^+$ cells and $CD4^+$ $CD25^+$ showed any statistical difference among the tested Groups. At the dose used, Etoposide did not produce the known cytotoxic effects upon other lymphocyte as $CD3^+$ or $CD8^+$. The dendritic marker $CD86^+$ considering its variation similar to CD83+ in this model, was not tested.

administration of GM-CSF at the melanoma excision site, as indicative of increased DC migration (Molenkamp et al 2005).

Therefore, the role of GM-CSF as adjuvant of protective cancer vaccine effects at the local level could be an increase of T-cell activation at SIN level as result of the increased number of mature dendritic cells.

T-reg cells are well identified as tolerogenic circulant cells in cancer patients and it was confirmed that $CD4^+CD25^+$ cells are involved in the control of the local immune response in human lymph node metastatic melanoma. Their frequency is clearly increased in metastatic lymph nodes (SLN) compared with tumor-free satellite lymph nodes. The $CD4^+CD25^+$, lymph node occupants, inhibit in vitro the proliferation and cytokine production of autologous tumor infiltrating $CD4^+CD25^-$ as well as $CD8^+$ T cells in a cell-contact-dependent manner (Viguier et al 2004). Therefore, decreasing the number of $CD4^+CD25^+$ in SIN is a contribution of the Etoposide Adjuvancy to enhance the local protective immune responses in vaccinated lymph nodes. The expression of FOXP3 and the functional analysis of the in vitro or in vivo immune-inhibitory activity of these $CD4^+CD25^+$ cells could confirm the T-Reg phenotype of these cells but the found variation of the CD^+CD25^+ population is compatible with the immune conditioning proposed.

This double and complementary immunomodulation of SIN cell populations elicits a SIN microenvironment conditioned to produce a locoregional immune response protective or immunogenic, minimizing the option of permissive or tolerogenic immune responses.

As it was mentioned, this locoregional immune response against the vaccine TAA configured at vaccination site draining lymph node or SIN is decisional because it starts a systemic immune response with the same configuration as its own. Therefore, immune effectors cells with predominantly protective or anti-tumoral activity will be found as circulant cells, lymph organ cells and tissue infiltrative cells. The remote effects upon tumor growth and DTH test are evidence of this systemic immune effector activity.

The GM-CSF and ETP, as complementary local adjuvants of the tested cancer vaccine, promote the vaccination site and SIN as a biological start-engine of a systemic protective-immune-response that could be denominated immunotherapeutic site (ITS). As consequence, the vaccination could counteract the biological start-engine of a permissive tolerogenic microenvironment configured by the SLN-conditioned by tumor cytokines during the natural history of malignancy (Cochran et al 2006).

This counteraction between the permissive tumor immunomodulation and the vaccine plus adjuvant protective immunomodulation must be optimized through future research of more powerful adjuvant systems, maintaining the safety of the double adjuvancy GM-CSF + ETP that was evidenced in this study, where no relevant clinical toxicity or side effects were registered.

Conclusions

In advanced ovarian cancer, the vaccination site in ATH-CV can be used to access SIN safely with vaccine adjuvants as GM-CSF and ETP in order to elicit an immunomodulation of the locoregional immunity and to start a systemic immune response predominantly protective or antitumoral. In the tested conditions and during the observation period, all tumors continued growing but a slower rate of tumor growth was observed. To optimize this antitumoral effect will be the goal of future studies. At the basic level, further research of cell functionality of SIN is warranted. At the clinical level, if other cancer vaccines could obtain benefits from the association of these adjuvants, if other adjuvant designs could optimize these results and if the same results could be reproduced using this immunotherapy procedure in other malignancies also must be investigated.

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