Scientific Publications and Bibliographic Validation for

The Autologous Thermostable Hemoderivative-Cancer Vaccine (ATH-CV)

Per Review Publications About

a new cancer immunotherapy with anti-progressive tumor effect
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<td>Autologous Induction of tumor fibrogenesis [Inducción autóloga de</td>
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<tr>
<td>fibrogenesis tumoral] Lasalvia E, Cucchi S, DeStefani E, Deneo H,</td>
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<td>Fierro L, Mechoso B, Larrañaga J, Vázquez J. Neoplasia (Spain),</td>
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<td>1995(1): 5-10 Full text (Spanish) Abstract (English)</td>
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<td>Anti-metastatic effect of a blood fraction from cancer patients.</td>
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<td>Lasalvia E, Cucchi S, Carlevaro T, Vázquez J, Riotorto R, Fierro L.,</td>
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Erythrocytes as Autologous-Vaccine Carriers in Advanced Prostate Cancer. E. Lasalvia-Prisco, E. Garcia-Giralt, E. Lasalvia-Galante et al. ASCO 2008 J Clin Oncol 26: 2008 (May 20 suppl; Abs 14017)

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<td><strong>COMMENTS ABOUT OUR WORK</strong></td>
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<td>Leisha A. Emens MD. PhD, Assistant Professor of Oncology and Director of the Tumor Immunology and Immunotherapy Laboratory in Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, Maryland. Cancer Biology &amp; Therapy, March/April 2003, 2:2: 161-163</td>
<td>90</td>
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The Therapeutical Autologous Cancer Vaccine
In last 15 years, an innovative medical procedure for cancer immunotherapy has been described. Evidences of clinical and biological effects of this procedure have been reported in scientific peer review publications. Tested in hundred of patients with advanced malignant disease as a therapeutical cancer vaccine, this procedure has evidenced:

1. no relevant toxicity
2. favorable clinical and biological effects in cancer patients when other available effective treatments have been exhausted
3. favorable clinical and biological effects in cancer patients when the procedure was associated to other systemic treatments, mainly chemotherapy

It is important to remark that the main innovation in this description is not a new drug, molecule or antigen, but a medical procedure that uses known medical technologies according to a rationale proven in other experimental and clinical models.

Basically, the procedure is an active and specific immunotherapy through a therapeutic vaccine prepared from an autologous thermostable hemoderivative (ATH) used as vaccine immunogen, and an immunomodulatory adjuvant system (IAS) designed to condition the protective anti-tumor responses, switching the permissive tumor responses conditioned during carcinogenesis.

The Vaccine Immunogen
It is known that malignant tumor cells are submitted to mutations that are responsible of genetic or epigenetic modifications in the molecular structures synthesized by them. Therefore, these modified structures, known as Tumor Associated Antigens (TAA), can be recognized as non-self molecules by the patient’s immune system, and they can elicit a tumoral immune rejection. In some cases, the genetic and epigenetic modifications can produce over-expression of normal molecular components that also can induce immune rejection responses, unlike if they are in minimal levels; hence, they work also as TAA.

Malignant tumors deliver to the blood different molecules (hemotransference). This fact is well known today because some of them are used as tumor markers in clinical oncology (i.e. CEA, CA 15-3, CA125, PSA). Some tumor markers have been tested as cancer vaccine and have shown activity as immunogens, demonstrating that these hemotransferred molecules are TAA. Therefore, in the clinical cases of malignant tumors with high blood levels of tumor markers, a hemoderivative containing such tumor markers, and eventually other unknown TAAs, but also hemotransferred from the tumor, can be used as immunogen in an anti-tumoral vaccination. Quantitatively, this approach is also supported because the amount of some known tumor markers in blood samples, that can be used as cancer vaccines, are in the range of the amount of antigen present in several efficient preventive vaccines used against infectious diseases.

Favorable conditions of an autologous thermostable hemoderivative immunogen for cancer vaccines:

- Circulating in the blood the TAA hemotransferred from the tumor do not elicit anti-tumor immune responses. Several mechanisms are involved in such immune-tolerance or immune-ignorance.
  - One of these mechanisms is the change, spontaneous and/or treatment-induced, in the tumor antigen library. Each individual tumor has its own mutagenic history and antigens released, result from each individual and contemporary antigen library. An anti-tumoral vaccine prepared from autologous blood (hemoderivative) as immunogen, easily repeatable, is also an
individualized and contemporaneous vaccine, updated to the antigen library of the tumor at the moment of vaccination.

- Another mechanism involved in not eliciting anti-tumor immune responses by TAA released from tumors and circulating in the blood, is related to the own circulating TAA. These antigens, known or unknown, can be masked inside molecular conformations, linked to transporter molecules or complexed with chaperones. Also they can be mixed with immune-neutralizer molecules in the microenvironment of circulating blood. Historically, several technologies have been tested to process the blood sample, in order to release the immunogenicity of the potential antigens contained. One of the most used is the thermal fractionation, and several thermostable fractions have demonstrated immunogenic activity. In this vaccine, the TAA is an immunogen associated to the thermostable plasma proteome that in several models of immunization has been shown to be a microenvironment in which the ratio immune-rejection / immune-tolerance is increased.

b. Any escape mechanism is developed easily when the target of the immunotherapy is a unique molecule or antigenic site (monovalent). The hemoderivative used in this vaccine is polyvalent as other tissue or cell extracts used as immunogens. In cancer immunotherapy, the immune response politargeted is considered more efficient, and also is more difficult to be neutralized by the escape mechanisms.

Unfavorable conditions of an autologous thermostable hemoderivative immunogen for cancer vaccines:

In most vaccines using complex biological systems as immunogen (malignant cells, viruses, tissue extracts), the optimal standard protocol of vaccination is difficult to set up, due to poor antigen identification, polyvalent antigenicity and low antigen effective dose in the vaccine preparations. Therefore, the dosage of immunogen must be estimated through the volume of the biological source from which it is prepared, in this case the blood sample, and it is necessary to assess immunogenicity in each procedure. Efforts to improve antigen identification in this vaccination are being developed.

The vaccine's adjuvant

As it was mentioned above, the immune rejection failure in malignant tumor progression, with high blood levels of released TAA, is the evidence of mechanisms of tumoral escape from the immune rejection response. The poor results of most of cancer immunotherapy procedures are also an evidence of tumor escape mechanisms. As it was described, some of these mechanisms involve the antigen tumor library, the own TAA or the antigen monovalence. In addition, some escape mechanisms, related to the immune system of cancer patients, have been identified. The main of such mechanisms is the conditioning of the immune system of cancer patients to respond with immune tolerance (permissive immunity) when challenged by TAA. It is known as an active immune mechanism inducing effectors whose activity is to block the immune rejection response. The CD4+CD25+ cell population, or T-Regulatory cells, is one of these tolerogenic (tolerance-producing) or suppressor effectors. The permissive immunity is induced by malignant tumors early in carcinogenesis, probably through molecular messages from the cancer cells to the tumor sentinel lymph node. In cancer patients submitted to immunotherapy, two levels of such immune tolerance could be considered: a pre-existing tolerance induced in the tumor natural history that allows tumor persistence and progression, and the iatrogenic tolerance induced by immunotherapy.

The control of these two levels of tolerance is the main goal of the vaccine's adjuvant. At present, in order to control both levels, the Vaccine adjuvant system has the components described below. Optimization of this system is in course.
Components of the vaccine adjuvant system

Systemic Immunomodulation

Two technology options:

a. Cyclophosphamide, low dose in one injection, produces after 3 days a depletion of systemic T-Regulatory cells enhancing the immune protective responses

b. Metronomic chemotherapy, orally, after one month treatment, also induces depletion of systemic T-Regulatory cells, enhancing immune protective responses

Lymph node immunomodulation

An Immunomodulatory Adjuvant Site (IAS) is produced through inducing a transitory granuloma by S.C. injection of immunomodulatory agents in a skin site that drains in a sentinel lymph node of IAS. The immunomodulatory agents selected in the present configuration of the procedure are GM-CSF, Etoposide and a COX-2 inhibitor, all of them loaded in autologous erythrocytes, previously to be S.C. injected. The following mechanisms of action for the immunomodulatory activity of these agents have been demonstrated in other models:

a. Erythrocytes, as particles, induce a granuloma that elicits local and remote macrophage activation.

b. GM-CSF induces local and remote activation of macrophage and dendritic cells

c. Etoposide produces a depletion of T-Regulatory or suppressor cells

d. Cox-2 inhibition blocks the IDO (indoleamine 2,3-dioxygenase) expression, and therefore, the activity of T-Regulatory or suppressor cells.

Hence, the association of these agents produces changes in the local prevalence of activated antigen presenting cells and tolerogenic cells, conditioning protective immunity in the injection site. This protective immunity of the injection site is transferred to the sentinel lymph node of IAS because carrier erythrocytes are partially evacuated to this node. The sentinel lymph node of IAS, conditioned to produce protective immunity, induces in the systemic lymph nodes the same conditioning due to the decisional property of the sentinel lymph nodes.

Others immunomodulatory agents have been tested in the configuration of IAS: silicate, menadione, cyclophosphamide, active metabolites, 5-metyl tryptophan, etc

Pre-treatments

In certain clinical conditions, pre-treatments that increase the efficiency of the mechanisms involved in this immunotherapy can be associated

a. To increase the amount of TAA synthesized in tumor cells through mutagenic chemotherapy:
   • Standard Chemotherapy prolonged until reaching acquired resistance condition (a marker of mutagenic events)
   • Chemotherapy at mutagenic dose, selectively addressed to tumor cells (as IPT)

b. To increase hemotransference of TAA from tumor cells to the blood through cell destruction with technologies that allow TAA molecular preservation:
   • Chemotherapy induction of Apoptosis after stress of tumoral cells (an immunogenic tumoral cell death).
   • Induction of Autoschizis (a tumoral cell death with release to the blood of semi-conserved cell components).
Autologous induction of tumoral fibrogenesis

E. Lasalvia, S. Cucchi, E. Destefani, H. Deneo, L. Fierro, B. Mechoso, J. Larrañaga y J. Vazquez. (*)

(*) National Institute of Oncology. Uruguay

The procedure for obtaining a fraction inhibiting autologous tumor growth called the KIF (kinetic inhibitor fraction) from arterial blood is described within the framework of the systematic investigation of regulators of tumoral stromatic composition present in the circulating blood.

A total of 52 patients with metastatic cancer of different origins were included in a randomized prospective clinical trial to determine the effect of i.m. KIF administered over 18 months.

The results presented confirm the inhibitor effect of KIF on tumor growth associated with an improvement functional state and the absence of toxicity within the limits of this trial.

The histochemistry of the lesions which responded to KIF and the effect of this fraction in vitro are compatible with a mechanism of action promoting a stromatic reaction with functions of physical and/or biological barrier which block its progression and hamper its functional effects on the organisms without destroying the malignant cells.

This study should be included in the search for instruments which aid in the control of cancer progression by acting on fibrogenesis-fibrolysis.

Neoplasia 1995, 12,1:5-10
En el marco de la investigación sistemática de reguladores de la composición estromática tumoral presentes en la sangre circulante, se describe el procedimiento de obtención de una fracción obtenida de sangre arterial que inhibe el crecimiento tumoral autólogo y a la que se denomina KIF (kinetic inhibitor fraction).

Un total de 52 pacientes con cáncer metastático de diversos orígenes se incluyeron en un ensayo clínico prospectivo y aleatorizado para determinar el efecto de KIF administrado por vía intramuscular durante 18 meses.

Los resultados que se presentan confirman el efecto inhibitor de KIF sobre el crecimiento tumoral asociado a mejoría del estado funcional y ausencia de toxicidad en los límites de este ensayo.

La histoquímica de lesiones que respondieron a KIF y el efecto de esta fracción in vitro son compatibles con un mecanismo de acción que promueve una reacción estromática con funciones de barrera física y/o biológica que sin destruir las células malignas, bloquea su progresión y dificulta sus efectos funcionales sobre el organismo.

Este trabajo debe incluirse en la búsqueda de instrumentos que, actuando sobre la fibrogénesis-fibrolisis, ayuden a controlar la progresión cancerosa.

**Introducción**

Todo tumor maligno es un complejo de células cancerosas y estroma. La estroma tumoral constituye un entorno físico y biológico a las células malignas aisladas o agrupadas. Las características físicas de la estroma dependen fundamentalmente de los fibroblastos y sus productos de secreción: colágenos y otras macromoléculas extracelulares. Las propiedades biológicas de la estroma son mediadas a través de factores de crecimiento y otras citocinas que pueden inhibir o promover la proliferación celular maligna.

La composición de la estroma tumoral puede variar cualitativa y cuantitativamente en diferentes modelos experimentales o clínicos. Esta variación explica que algunos autores consideren a la estroma una estructura favorecedora de la progresión maligna, mientras otros autores describen circunstancias en las que ésta funciona como barrera de esta progresión.

Existen pocos estudios sobre los mecanismos que regulan la composición de la estroma tumoral. En un mismo paciente, la composición estromática puede variar cualitativamente y cuantitativamente en diferentes modelos experimentales o clínicos. Esta variación explica que algunos autores consideren a la estroma una estructura favorecedora de la progresión maligna, mientras otros autores describen circunstancias en las que ésta funciona como barrera de esta progresión.

**Materiales y métodos**

Para obtener la fracción kinetic inhibitor fraction (KIF), se utilizó un procedimiento descrito anteriormente. Se incluyeron 52 pacientes con cáncer metastático de diferentes orígenes en un ensayo clínico prospectivo y aleatorizado.

Los resultados presentados confirmaron el efecto inhibitor de KIF sobre el crecimiento tumoral asociado a mejoría del estado funcional y ausencia de toxicidad en los límites de este ensayo.

La histoquímica de lesiones que respondieron a KIF y el efecto de esta fracción in vitro son compatibles con un mecanismo de acción que promueve una reacción estromática con funciones de barrera física y/o biológica que sin destruir las células malignas, bloquea su progresión y dificulta sus efectos funcionales sobre el organismo.

Este trabajo debe incluirse en la búsqueda de instrumentos que, actuando sobre la fibrogénesis-fibrolisis, ayuden a controlar la progresión cancerosa.

**Discusión**

La inmunohistoquímica de lesiones que respondieron a KIF y el efecto de esta fracción in vitro son compatibles con un mecanismo de acción que promueve una reacción estromática con funciones de barrera física y/o biológica que sin destruir las células malignas, bloquea su progresión y dificulta sus efectos funcionales sobre el organismo.

Este estudio debería incluirse en la búsqueda de instrumentos que, actuando sobre la fibrogénesis-fibrolisis, ayuden a controlar la progresión cancerosa.

**Conclusiones**

El presente estudio ha confirmado la efectividad de la fracción KIF en la inhibición del crecimiento tumoral y mejoría del estado funcional de los pacientes. Los resultados apoyan la hipótesis de trabajo de que la fibrogénesis-fibrolisis puede ser una estrategia eficaz para controlar la progresión de los tumores metástaticos.

**Referencias**


**Correspondencia**: Prof. E. Lasalvia.

Recibido el 22 de julio de 1994.
Aceptado para su publicación el 29 de julio de 1994.
La presente comunicación refiere a una fracción que hemos aislado de sangre arterial en enfermos con cáncer diseminado y que demuestra propiedades compatibles con nuestra hipótesis de trabajo por lo cual ha sido denominada KIF (kinetic inhibitor fraction).

**PACIENTES Y MÉTODOS**

Preparación de la fracción hemoderivada KIF

a) Por punción de arteria femoral se obtienen 50 ml de sangre recogida en 5,000 unidades de heparina y se dejan sedimentar 60 minutos a 37 °C.

b) El plasma sobrenadante, que contiene células sanguíneas no sedimentadas y es rico en plaquetas, se lleva a hipotónia agregando 4 volúmenes de agua destilada y se deja estar durante 30 minutos.

c) El preparado se congela a -20 °C y se descongela a 4 °C a fin de favorecer la lisis celular.

d) La suspensión resultante se calienta a 100 °C durante 10 minutos, se enfria a temperatura ambiente y el precipitado resultante se descarta por filtración a través de filtro de lana de vidrio a fin de obtener una fracción termoestable.

e) Esta fracción se hace pasar a través de un filtro de acetato de celulosa, tamaño de poro 0,2 km, con los prefiltrados necesarios. Se obtiene así la fracción denominada KIF cuya riqueza proteica tiene valores entre 3 y 7 mg/ml, medida con el método de Lowry et al. La concentración proteica final se ajusta a 2,5 mg/ml.

f) Todo el procedimiento se realiza asegurando esterilidad y ética y se confirma por los tests microbiológicos correspondientes. La fracción KIF es mantenida entre 0 y 4 °C. Responde al diseño general de los ensayos terapéuticos fase 2 aleatorizados.

**Objetivo Tumoral Evaluable (OTE)** - En cada paciente considerado para ser incluido hasta su total utilización en un lapso no superior a 30 días

Ensayo clínico: en el ensayo clínico de la fracción KIF se definió un OTE, cuyo crecimiento fue seguido a fin de valorar la cinética clínica de, cada caso.

Los OTE fueron lesiones accesibles al examen clínico o imágenes obtenidas por exámenes adecuados (tomografías computarizadas, resonancia nuclear magnética, radiología convencional), asegurando en cada caso que fuera posible su medición seriada y comparativa a través del ensayo. En todas la evaluación de los OTE se midieron los diámetros mayor y menor, se expresaron en milímetros y se calculó su producto. Algunas lesiones accesibles, cuya localización y evolutividad fueron similares al OTE, se biopsiaron durante el transcurso del ensayo realizándose examen histoquímico de las mismas. Criterios para la inclusión de pacientes. Se consideraron para ser incluidos en el ensayo terapéutico de la fracción KIF, pacientes que cumplan los siguientes criterios: edad inferior a 75 años; diagnóstico histológico confirmado de cáncer; estadio de diseminación metastásica; estado funcional no superior a 2 en escala Zubrod/ECOG; no presentar patologías asociadas que requieran medicación permanente; resistencia conocida a tratamientos oncológicos de recibo; al menos una lesión cuyas características clínicas o de imagen permitieran su medición seriada en las condiciones descritas para los OTE, y consentimiento del paciente previa información adecuada.

En cada paciente considerado para ser incluido en el ensayo se iniciaron mediciones seriadas del OTE y sólo se incluyeron aquellos casos en que el producto de los diámetros incrementó su valor al menos 25 % en un periodo no mayor de 3 meses precediendo al inicio de dicho ensayo.

Aceleratorización: Los pacientes incluidos en el ensayo con el mismo tumor primario se aleatorizaron en 2 grupos: un grupo control sólo recibió tratamiento sintomático y un grupo tratado que, además, recibió tratamiento con KIF.

Protocolo de tratamiento. En cada paciente tratado, cada 30 días se practicó el procedimiento descrito para la obtención de KIF y a cada paciente se le administró este preparado, por vía intramuscular, a razón de 5 ml cada 48 horas durante todo el periodo de observación (18 meses). Evaluación. En cada paciente incluido en el ensayo, mensualmente se realizó una medición del OTE y una valoración clínica. La medición del OTE en cada caso consistió en asumir como valor inicial el valor del producto diámeto mayor por diámetro menor, al iniciar el ensayo expresando los valores subsiguientes como porcentaje del respectivo valor inicial. La valoración clínica incluyó el estado funcional aplicando la escala Zubrod/ECOG 18 y la toxicidad fue valorada como el más alto grado obtenido al aplicar los criterios de la OMS. También se registró la supervivencia durante todo el transcurso del ensayo.

**RESULTADOS**

Las tablas 1 y 2 muestran que en el ensayo se incluyeron varios tipos de tumores con alta prevalencia clínica. En general, el origen primario de los tumores así como la edad y sexo de los enfermos están equitativamente representados.

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<thead>
<tr>
<th>Tumor primario</th>
<th>Incluidos en el ensayo</th>
<th>12 meses</th>
<th>18 meses</th>
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<tr>
<td>Mama</td>
<td>6 (0-6) (38-65)</td>
<td>4 (0-4)</td>
<td>4 (0-4)</td>
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<tr>
<td>PulmNOC</td>
<td>4 (3-1) (52-68)</td>
<td>2 (1-1)</td>
<td>55-68</td>
</tr>
<tr>
<td>Melanoma</td>
<td>3 (2-1) (34-62)</td>
<td>2 (1-1)</td>
<td>34-62</td>
</tr>
<tr>
<td>Riñón</td>
<td>3 (3-0) (50-67)</td>
<td>3 (3-0)</td>
<td>50-67</td>
</tr>
<tr>
<td>Ovario</td>
<td>2 (0-2) (60-67)</td>
<td>2 (0-2)</td>
<td>60-67</td>
</tr>
<tr>
<td>Colon</td>
<td>2 (1-1) (65-68)</td>
<td>2 (0-2)</td>
<td>65-68</td>
</tr>
<tr>
<td>Utero-ovario</td>
<td>2 (0-2) (47-53)</td>
<td>2 (0-2)</td>
<td>47-53</td>
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<tr>
<td>Vejiga</td>
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<td>2 (2-0)</td>
<td>58-63</td>
</tr>
<tr>
<td>C. Oral</td>
<td>2 (2-0) (63-66)</td>
<td>2 (2-0)</td>
<td>63-66</td>
</tr>
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| Total          | 26 (13-13) (34-68)      | 9 (11)   | 34-68    | 20 (9-11)
**TABLA 2. Casuística en el grupo control**

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<tr>
<th>Tumor Incluidos</th>
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<td>primario en el ensayo</td>
<td>12 meses</td>
</tr>
<tr>
<td>Mama</td>
<td>6 (0-6) (40-68)</td>
</tr>
<tr>
<td>Pulm.</td>
<td>4 (3-1) (44-58)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>3 (2-1) (30-56)</td>
</tr>
<tr>
<td>Riñón</td>
<td>3 (3-0) (50-68)</td>
</tr>
<tr>
<td>Ovario</td>
<td>2 (0-2) (62-66)</td>
</tr>
<tr>
<td>Colon</td>
<td>2 (1-1) (62-64)</td>
</tr>
<tr>
<td>Ut. Cx</td>
<td>2 (0-2) (38-55)</td>
</tr>
<tr>
<td>Vejiga</td>
<td>2 (2-0) (55-68)</td>
</tr>
<tr>
<td>Cv. Oral</td>
<td>2 (1-1) (68-72)</td>
</tr>
<tr>
<td>Total</td>
<td>26 (12-14) (30-72)</td>
</tr>
</tbody>
</table>

Número de casos (varón-mujer) (edad mínima-edad máxima).

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**Fig. 1. Evolución de la media del tamaño tumoral en 16 pacientes del grupo control y 20 pacientes del grupo tratado con KIF, supervivientes durante 12 meses de los 52 enfermos inicialmente aleatorizados en ambos grupos. Ambas curvas pudieron ser ajustadas a una función de Gompertz: N= G (exp. ((Aola) (1-exp (-at))JJ, con diferentes parámetros: para el grupo tratado, Ao=0,54; a=0,91 y G=96; para el grupo control, Ao=0,001; a=0,001 y G=101, siendo G un parámetro de escala. La figura 1 muestra que en ambos grupos los OTE ajustaron la media mensual de su tamaño a una función gompertziana, pero KIF produjo en el grupo tratado una modificación de los parámetros de la misma que significa un alargamiento progresivo del tiempo de duplicación a partir del cuarto mes.**

**Fig. 2. Evolución del tamaño tumoral (media e intervalos de confianza) en 20 pacientes que pudieron ser observados durante 18 meses de tratamiento con KIF. La función a la que mejor ajustan estos valores es una coexponencial: A=AmIx [1- exp (kt)], donde AmIx=172,09 y k=0,5. La figura 2 permite demostrar que el efecto de KIF sobre la cinética de crecimiento de los OTE, representado como una función coexponencial, se mantiene por lo menos 18 meses.**

**Fig. 3. Supervivencia durante 18 meses de tratamiento con KIF en 26 pacientes inicialmente incluidos en el grupo.**
Fig. 4. Media del estado funcional y máxima toxicidad relacionadas a la evolución del tamaño tumoral en 20 pacientes tratados con KIF durante 18 meses.

Fig. 5. Incremento celular producido por KIF y suero fetal bovino (SFB) en 24 y 72 horas de cultivo en fase exponencial

Fig. 6. Microfotografías de metástasis biopsiadas en 4 pacientes diferentes tratados con KIF durante más de 6 meses cuando la inhibición del crecimiento tumoral había sido confirmada. Intensa reacción estromática con abundantes fibroblastos y fibras de colágeno. A), B) y D) Metástasis cutáneas de cáncer de mama. Metástasis peritoneal de cáncer de ovario. Columna izquierda: Tinción hematoxilina-eosina; columna derecha: tinción verde luz
que sólo adquiere un enlentecimiento gompertziano en las escasas observaciones (20 %) que incluyen etapas muy evolucionadas \(^{1,3,5}\). En algunas circunstancias el crecimiento maligno parece detenerse (cínica nula), sin que se conozcan las causas de este fenómeno.

En este trabajo el grupo control sigue el modelo más frecuente de historia natural. El grupo tratado con KIF se aparta significativamente de este comportamiento: sigue la evolución gompertziana pero con parámetros que implican un progresivo y permanente enlentecimiento, llegando en todos los casos evaluados a estados de aparente cínica nula.

El efecto de KIF no se detecta primariamente sobre las células cancerosas: la anatomía patológica las muestra indemnes y durante todo el ensayo no se comprobó la desaparición de ninguna lesión tumoral. La anatomía patológica sugiere que esta inhibición del crecimiento tumoral se asocia a la promoción por KIF de una diferenciación que esta barrera estromática parece imponer a la proliferación e invasión de las células malignas. Los componentes de la estroma tumoral interactuan por diferentes vías que pueden ligarse a heparina y factor de transformación beta que inhibe la proliferación de células malignas y produce fibrosis, también podrían participar directa o indirectamente en las propiedades de KIF. Sin embargo, hasta ahora todo intento de purificar KIF nos ha llevado a perder sus efectos, lo que podría indicar que varios principios activos interactuan en este hemoderivado.

Si bien algunos autores han observado efectos favorecedores de la proliferación maligna producidos por factores de crecimiento fibroblástico, la comparación con nuestros resultados debe tener en cuenta que existen múltiples variables conocidas para componentes del sistema en estudio: diferentes subtipos de fibroblastos \(^{2,3,16}\); efectos proliferativos o anti proliferativos de diferentes factores de crecimiento fibroblástico \(^{3,9,23}\); diferentes isoformas moleculares de los factores de crecimiento con diferente afinidad por los receptores \(^{37}\); diferente composición molecular de receptores \(^{38}\); modulación de la afinidad factor de crecimiento-receptor a través de citocinas liberadas en la respuesta biológica o inmunitaria frente al cáncer \(^{39,40}\); los factores de crecimiento fibroblástico circulan inactivos por su localización intracelular o su unión a proteínas transportadoras \(^{41,42}\), por lo cual su actividad en un hemoderivado depende del método de preparación del mismo.

El presente trabajo utiliza una fracción obtenida de sangre arterial y autóloga, empleando un procedimiento específico, lo cual conduce a un modelo donde los componentes variables han sido seleccionados en una forma que no permite fácilmente su comparación con modelos utilizados previamente. De nuestros resultados no puede desprenderse que las propiedades fibrogénicas de KIF tengan relevancia para su utilización terapéutica. Sin embargo, el desarrollo de subsiguientes estudios sobre la fibrogénesis y los agentes que la inducen podrían conducir a nuevos instrumentos para el control de la progresión cancerosa.

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**BIBLIOGRAFÍA**


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Anti-metastatic effect of a blood fraction from cancer patients

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Abstract: Growth factors are present in blood and urine from cancer patients and may be promoters or inhibitors of tumor growth, as I and other authors reported last year (Neoplasia 6, 1994; JNCI 5, 1994). We have tested the effect of one of these blood-derived fractions, known as tumor growth inhibitor, on the incidence of metastasis in cancer patients. Patients with different primary tumors and unsuccessful treatments were randomized in two groups: (I) treated with an autologous blood derivative, as we have previously described (Neoplasia 6, 1994), and (II) controls. The comparable (sex, age and primary tumor) two-year survivors in both groups were evaluated (I= 56 patients, and II = 51 patients). The total number of metastases diagnosed in the treated group was lower than that in the control group (I = 171, I I = 359), the mean number of metastases by patient was also lower in Group I, with p less than 0.01 in the chi square test, and more significant when only the late metastases were considered (1-2 years). No difference in metastasis localizations was observed between both groups. These results agree with the tumor growth inhibiting property observed in blood components of cancer patients as a consequence of their anti-angiogenic or fibroblastic growth factor activities.
ABSTRACT

Lately, the promising results obtained with autologous cancer vaccines are stimulating new research in the old field of cancer immunotherapy. This paper describes the development of a procedure previously reported by us that is used to obtain an autologous hemoderivative with antitumoral properties. The procedure has been tested in a phase I–II, randomized, controlled clinical trial of 28 cancer patients with different primary malignancies in metastatic and chemotherapy-resistant stages. The histology of the lesions that responded to this treatment was consistent with the characteristic histology observed in malignant lesions treated with a similar antitumoral hemoderivative: proliferation of stromal connective tissue, T-lymphocyte infiltration, and a reduction in the amount of tumor cells and blood vessels. We concluded that vaccination had elicited an immune response because a delayed-type hypersensitivity test made with the autologous hemoderivative produced a significantly more intense response in the responding treated patients. We propose that an immune mechanism acting on tumor cells and/or the regulatory system for stromal growth explains the histological results observed. The use of blood to obtain the immunogen allows vaccination to be repeated, so this method could avoid tumor escape responses due to mutations in the antigen library of the tumor. The results of our study justify further research to optimize the antitumoral effect of vaccination.

INTRODUCTION

Cancer vaccines have been a goal of medical research for many years. Lately, several studies have explored the autologous vaccination model in order to consider the antigenic individuality of malignant tumors. In this area of research, some experimental and clinical studies have obtained promising results. The results of melanoma treated with a vaccine manufactured with autologous hapten-modified tumor cells and adjuvant BCG1 and the results of renal-cell carcinoma, pancreatic cancer, and other solid tumors treated with a vaccine prepared from heat-shock proteins (HSP) from autologous tumors2-5 are examples. However, the favorable results reported were temporary in every case. Tumor escape mechanisms from immunological control include changes in malignant cell antigens due to a high rate of spontaneous mutations associated with their rapid proliferative rate, as well as environmental conditions including chemotherapy and the host biological response.6 Most autologous vaccines have been obtained from surgical tumor specimens. These vaccines, when successful, generated an immune response that recognized and controlled tumor cells with the antigen endowment present in the cells from which the vaccine was obtained. However, if the antigenic library of the tumor cells changes, immune mechanisms will be unable to recognize and control the malignant disease, thus resulting in clinical progression. It is not possible to update these vaccines for each and every new antigenic profile of tumor cells because it is not feasible to obtain new surgical specimens frequently. In order to obtain a series of autologous vaccines using frequently updated tumor antigens, we explored autologous blood as an immunogen source in cancer patients. In 1995, we reported the antitumoral effect of an autologous blood protein fraction that was inoculated repeatedly in cancer patients.7,8 This paper reports the procedure used to obtain an autologous blood fraction with immunogenic properties from cancer patients. The procedure can be performed repeatedly.

MATERIAL AND METHODS

Hemoderivative Preparation and Vaccination. The autologous hemoderivative was obtained by modifying a procedure that we developed previously.7 Twenty milliliters of blood was 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 vaccination cycle. Each vaccination consisted of a mixture of the vaccine-vial content and 300 µg of recombinant human granulocyte-macrophage colony stimulating factor (rH GM-CSF) and was given by subcutaneous injection. No more than 3 ml was injected in each subcutaneous vaccination site at a time, so several subcutaneous injections had to be made on the abdominal surface in order to inject the whole vaccine-vial. To prevent the development of immune-tolerance, cyclophosphamide 300 mg/m² was given 3 days before beginning the vaccination procedure. Ten days after the last vaccination, an intradermal test of delayed-type hypersensitivity (DTH) was made with the 0.1 ml of preparation conserved in the test-vial. Measures were taken to ensure sterility at each stage of the procedure (arterial blood extraction, vaccine preparation, and vaccination). The procedure was repeated every 45 days (counting from the first day of the previous cycle) until the diameter of the skin indurations elicited in response to the DTH test was at least 5 mm, or for 6 months if the diameter of the induration never reached 5 mm. The DTH test was not performed in the non-treated control group.

**Patients and Trial.** A phase I-II, randomized, controlled clinical trial was designed. The patient population studied was quite heterogeneous, as is common in phase I trials, but the objective of assessing an antitumoral effect in a controlled protocol is typical of phase II trials. As can be seen in Table 1, 28 cancer patients were included (11 breast, 7 colorectal, 4 ovarian, 4 pancreatic, 2 prostate) and distributed equally into two groups. The eligibility criteria were: adults up to 70 years old with a solid tumor and metastatic disease, at least one measurable mass, previous acquired resistance to chemotherapy, and an estimated survival of more than 6 months. Eligible patients also had abnormally high concentrations of the respective serum tumor markers released into blood by malignant cells: CA15-3, CEA, CA125, CA19-9 and PSA respectively in breast, colorectal, ovarian, pancreatic, and prostate cancer. The tumor burden in all cases was not minimal, it was evident in imaging studies and clinical assessment, but it did not affect the eligibility criteria in terms of performance status. Patients were registered centrally, stratified by primary malignant site, and then randomly distributed to one of the two study groups. In all cases, informed consent was obtained from patients or their guardians. An institutional review board approved the clinical trial.

- The first group underwent the vaccination procedure as described.
- The control group received cyclophosphamide 300 mg/m² three days before starting a series of subcutaneous injections of rH GM-CSF given on days 1, 14, 21, and 28. This procedure was repeated 4 times, beginning 45 days after the first day of the previous cycle (Total treatment time: 6 months). Vials were prepared with 300 µg of rH GM-CSF diluted in NaCl 0.9% to obtain the same final volume as the volume contained in the vaccine-vials. Vials were used to make control subcutaneous injections when vaccinations were scheduled. No more than 3 ml was injected per site at a time. The control group received no other antitumoral treatment, but did receive symptomatic treatment.
- Results were analyzed with the Student-t test.

**Assessment of the Antitumoral Effect.** The RECIST system was used every month to evaluate response in terms of tumor size. Performance status was also evaluated monthly. Follow-up lasted for 8 months from onset of vaccine treatment in all cases. The best RECIST-status maintained in 2 successive assessments (one month) was recorded as the response obtained in each patient. A histological study of the response was made in the treated patients; biopsies of accessible metastatic lesions were studied before and after treatment in 5 responders and in 4 non-responders.

Hematoxylin-eosin stain was complemented with more specific stains for connective tissue components: light green, Van Giesen (collagens), and Wides (reticular fibers). Immunocytochemical studies were made of the lymphocytes infiltrating the tumoral stroma to identify the activated populations. Monoclonal antibodies, CD3+ (Ventea) for T-lymphocytes, LS2 for B (CD20+) lymphocytes, PGM-1 for macrophages (DAKO), and CD8 (Novocastra) for cytotoxic suppressor T cells were used at a dilution of 1:50. Microvessels in the tumor tissue were detected by immunohistochemical staining with antifactor-VIII antigen-related polyclonal antibody (DAKO). The avidin-biotin complex staining method was used for all immunohistochemical stains, with dianaminobenzidine as a chromogen. Apoptotic tumor cells were assessed in representative microscopic fields by 3’-end labeling of apoptotic DNA using an ApoTag in situ apoptosis detection kit (Oncor).

**Immunization Assessment.** The DTH intradermal tests were read at 48 hours. In each case, we measured the largest and smallest diameters of the induration that appeared. Because the resulting induration had an almost circular shape, the distribution between positive and negative responses was the same regardless of whether the induration was assessed using the average of these two dimensions or the longest diameter. Therefore, positive DTH response was identified using only the longest diameter of the induration, expressed in millimeters.

**Protein Vaccine Assessment.** Samples of each final preparation were tested for total protein content using the Bradford assay. Chromatography by gel filtration (Sephadex G100 and G200, superfine, Pharmacia) was used to determine the molecular weight of protein fractions found in the vaccine.

The respective serum tumor marker was tested in each preparation, before and after thermal fractionation procedures. Because of reports that HSPs participate in autologous cancer immunotherapy, several HSP samples were tested by immunoblotting before and after thermal fractionation.

**Toxicity Assessment.** Toxicity was evaluated using the WHO (T) criteria and high levels of each detected toxicity type were recorded.

**Performance Status (PS) Assessment.** Performance status was evaluated in each patient before and after treatment and scored according to ECOG scale. The variation in PS was recorded for each patient. The mean and standard deviation were calculated for each group.

### RESULTS

As can be seen in Table 1, the two groups were comparable. Figure 1 shows the results of the RECIST status evaluation. At the end of the 8-month follow-up period, 1 patient from the non-treated group had died of progressive disease before completing follow-up, leaving 27 evaluable patients (14 treated and 13 non-treated). At the end of the follow-up period, we obtained the following findings. In Group 1, 5 patients (3 breast, 1 ovary, 1 pancreas) had progressive disease; 9 patients showed some type of...
favorable tumor response: 3 of 9 patients (1 breast, 1 colorectal, 1 ovary) reached levels of partial remission and 6 of 9 patients (2 breast, 2 colorectal, 1 pancreas, 1 prostate) had stable disease. In Group 2, 12 patients had progressive disease (5 breast, 4 colorectal, 2 ovary, 1 pancreas) and 1 patient had stable disease (1 prostate). Progressive disease was significantly more frequent in the non-treated group than in the treated group (p=0.01). The total number of favorable responses (partial remission + stable disease) was also significantly higher in the treated group (p<0.001). The median delay in response from the end of treatment to the first signs of a response was 12.7 weeks (range 5 to 19) in the 9 patients who had favorable responses. In treated patients (Group 1), the mean change in performance status in the 9 responders was -0.165 versus +1.750 in the 6 non-responders (p=0.02).

Figure 2 shows the results of the DTH test during the follow-up of Group 1: 7 of 9 patients with favorable responses and 1 of 5 patients with progressive disease had inductions of at least 5 mm.

Figures 3, 4, and 5 show histological preparations of tissue from accessible metastatic lesions after treatment in 5 responders in the treated group (4 skin, 1 peritoneal; 3 partial remissions, 2 stable disease). The histological findings were similar in all these lesions, which is consistent with a previously described character. The vaccine preparations were consistent with the characterization of an antitumoral autologous hemoderivative previously described. Briefly, the antitumoral effect was associated with a final vaccine whose composition included at least 5 protein fractions: a large homogeneous fraction (>50%), MW~50,000, and 4 non-homogeneous protein fractions. Unmodified structural HSPs and tumor markers were not identified in the final vaccines. Prior to the thermal fractionation process, among the many biomolecules present, increased serum concentrations of each patient’s respective tumor marker were identified, as well as several HSPs (Hsp70/72, Hsp90, Gp 96/94, and Hsp 47).

**DISCUSSION**

This phase I–II clinical trial had limitations due to the heterogeneity of the patient population and small number of cases. However, the results indicate the following innovative findings that merit further research in a trial of more advanced design.

**Antitumoral Effect.** The results categorically show that an autologous hemoderivative obtained by means of the procedure described had antitumoral activity. It inhibited tumor growth in a significant number of patients, according to assessment of tumor measures using the recommended RECIST system. It is known that assessment of tumor size by direct measurement of accessible tumors or measurement of CT-scan images cannot discern differences between viable tumor tissue and tumor fibrosis. Using tumor size alone to assess antitumoral effect in tumors that respond by producing fibrosis could be of questionable value. Vaccine-induced fibrosis can obscure the apparent antitumoral effect by increasing the size of tumors after treatment. Therefore, if fibrosis could be excluded from tumor measurements, the antitumoral effect might be even more significant. This is one reason why institutional references in oncology recommend the use of RECIST. This method is recommended to assess antitumoral effects, including those of therapeutic agents that induce tumor fibrosis. In addition, performance status, which is not recognized as a valid measure of response to antitumoral treatment, was significant in our results because it was linked to a reduction in targeted tumor mass growth. Three findings anticipate the potential clinical implications of this antitumoral effect:

a. the antitumoral effect becomes evident after a delay of several weeks from the beginning of treatment;

b. it is not specific to the primary tumor site in the tumors studied (breast, colon, ovary, prostate, and pancreas); and

c. it was observed in metastatic stages of the tumors studied.

**Characterization of the Vaccine.** We have shown that the vaccine is a preparation containing several species of proteins. It did not differ in composition from an autologous antitumoral hemoderivative previously described. According to filtration chromatography, it contained a pool of five protein fractions with a homogeneous main
fraction of molecular weight ~50,000. The vaccine lost its antitumoral properties with further purification of its poly-protein composition. The aim of this paper is to report the antitumoral effect of vaccinations of an autologous hemoderivative, so only a preliminary characterization of the vaccine preparation has been included. As in tumor vaccines using tumor cells as an immunogen, the chemical complexity of the hemoderivative vaccine described here, which contains several, mostly non-homogeneous, protein fractions, is difficult to define. In this study, the antitumoral effect elicited by an autologous hemoderivative, as demonstrated by immunological and histological evidence, is as appealing conceptually as the reported antitumoral effect of vaccines containing tumor cells, bacteria, viruses, biological adjuvants, and other immunogens that are as yet undefined chemically.

**Mechanism of Action.** Our results suggest two mechanisms that could be involved in the antitumoral effect shown:

**Immune Response.** The patients in the treated group who had DTH reactions also had a favorable antitumoral response more frequently. Therefore, even though the study design did not allow direct evidence of the involvement of antitumoral immunity to be obtained, we cannot disregard the possibility that such an involvement could link DTH results to the RECIST results. Our vaccine could elicit a direct or cross-immunization response against tumor-associated antigens (TAAs) released by malignant cells into the blood.

**Stromal Response.** Histopathological analysis was limited by the accessibility to biopsy of the tumor lesions before and after treatment. Therefore, in the treated group, the comparability of the histological results in responders and non-responders must be considered as just a preliminary finding. However, only patients with a favorable response to treatment showed an unusual histological image that was not found before treatment in any patient or in non-responders after treatment. Metastatic lesions in responders showed low T-lymphocyte infiltration. However, the most prominent histological finding was increased stromal fibrosis. This histological picture is not characteristic of a pure DTH immune response, and it suggests that our vaccine acts directly or indirectly on the growth of tumoral stroma components. Stromal fibrosis, changes in the blood vessel-to-tumor cell ratio, and the development of a more extensive barrier against tumor cell migration could reasonably have an antitumoral effect.

Immunological and stromal responses may both contribute to the antitumoral effect of this vaccine. The antitumoral effect was associated with DTH and also with stromal fibrosis. DTH to autologous hemoderivatives in the vaccine is not a direct sign of DTH against the tumor. This could have been tested if DTH testing had been performed with tumor cells or tumor extracts. However, such intradermal tests with tumor cells or tumor extracts were not feasible in the study design because repeated testing with extracts or cells from successive surgical tumor specimens cannot be used to control the effects of a sequence of vaccinations of a hemoderivative. However, it must be assumed that autologous blood can contain cells and molecules released by tumors. Several authors have identified cancer cells in the blood of cancer patients. Molecules synthesized in cancer

Figure 3. Skin metastasis of breast cancer six month after a partial remission (assessed by RECIST) in response to vaccination with an autologous hemoderivative. (A) hematoxylin-eosin, (B) light green, (C) Van Gieson, (D) Wilder. Increased tumor stroma with fibroblasts and lymphocytes is evident, as well as a reduction in blood vessels and tumor cells.

Figure 4. Peritoneal metastasis of ovarian cancer six months after partial remission (assessed by RECIST) in response to vaccination with an autologous hemoderivative. (A) hematoxylin-eosin, (B) light green, (C) Van Gieson, (D) Wilder. Increased tumor stroma with fibroblasts and lymphocytes is evident, as well as a reduction in blood vessels and tumor cells.
Cells, released into the blood, and recognized as tumor markers have also been found in the blood of cancer patients.\textsuperscript{11-13} All of the patients included in this study had abnormally high serum levels of tumor markers, which is evidence of disease with active release of malignant cell contents to blood. Under such circumstances, other molecules could be released by cancer cells. In this study, the respective tumor marker was present in the lysed blood supernatant from which the vaccine was obtained. As these molecules were not identified in the final vaccine, the only significance that we attributed to them is that they were markers of the presence of molecules released from tumor cells in the blood samples from which the vaccine was obtained. Additional studies must be made to determine if tumors release the molecules responsible for the antitumoral effect of these vaccines in the absence of elevation of serum tumor markers. It is known that the content of tumor cells released at cell death can be immunogenic for the host when this release is the result of the apoptosis of previously stressed tumor cells. A well-known cellular stressor is chemical stress induced by oncological chemotherapy. It is known that stress-induced immunogenesis in tumor cells is associated with HSP synthesis.\textsuperscript{14} All our patients had received previous chemotherapy. HSPs have been very well studied in cancer immunotherapy\textsuperscript{15-17} as antigens, adjuvants, or antigen chaperones.\textsuperscript{18-20} Hsp70/72, Hsp90, Gp 96/94, and Hsp 47 were demonstrated in vaccine preparations before thermal fractionation. This finding may be relevant to discussions of the DTH response and stromal fibrosis. Therefore, if thermal fractionation is applied to a lysed hemoderivative containing components of tumor cells, inductors of tumor-elicited immune response, and tumor stromal growth modifiers, this may produce molecular structural modifications, with the following consequences:

a. the procedure can be used to concentrate proteins with a molecular structure resistant to these conditions, thus resulting in a predominance of specific functional molecular forms of growth factors, as has been shown for platelet-derived growth factor;\textsuperscript{24} and

b. it maintains or increases the immunogenic properties of many proteins, with loss of biological properties, as described in toxicological models.\textsuperscript{25} Given the molecular complexity of blood from cancer patients, thermal antigenization can produce a vaccine with an antigenic polyvalence similar to that of autologous vaccines obtained from tumor cells. As a consequence, the immune response elicited by tumor-cell vaccines could be similar to that of vaccine obtained by the procedure described. Therefore, hemoderivative vaccines could complement tumor cell vaccines when tumor tissue is not available.

Antigen Empowerment. In order to explain how small amounts of antigens present in the blood sample can induce a clinical tumor response, it must be considered that the method for vaccine preparation described has some steps used to amplify the immune response elicited. These steps are the subcutaneous inoculation of immunogen, activation of dendritic cells with GM-CSF,\textsuperscript{26} and prevention of tolerance with cyclophosphamide.\textsuperscript{27} All these steps are already being used by other favorable cancer vaccine trials to enhance the antitumoral immune response. In addition, our procedure, as reported earlier, uses arterial blood instead of venous blood\textsuperscript{7,8} as a source of immunogen because most of the molecules delivered by tumor cells to the blood enter a deep vein, then travel through the right and left heart before reaching the arterial tree. Drawing blood from the arterial tree avoids the first-pass clearance in peripheral microcirculation and tissue that occurs in blood drawn from peripheral veins.

Figure 5. Lymphocyte tumor infiltration before and after vaccination treatment in a breast cancer (skin metastasis) that showed a favorable response in the antitumoral assessment. Histological sections from pre-treatment and post-treatment biopsy specimens were immunohistochemically stained for CD3. Immunohistochemical studies were made on formalin-fixed, paraffin-embedded sections using antibodies against the pan T-cell marker CD3 (Ventana). A minimal pre-treatment (A) and brisk post-treatment (B) T-lymphocyte (CD3+) infiltrate (x 400) is visible. The presence of B-lymphocytes (CD20+) in the infiltrate was minimal before and after treatment.
Finally, our vaccination procedure, because of the repeated preparation of the vaccine from autologous blood, updates the antigens released by tumors into the blood. Consequently, it could be used to avoid the tumor escape mechanism due to antigenic mutations or changes in the biological response or stroma regulators.

CONCLUSIONS

We conclude that with a procedure developed from an earlier one, it is possible to obtain an autologous hemoderivative that can be inoculated repeatedly to produce an antitumoral effect in patients with advanced cancer. A phase I-II clinical trial has shown that this antitumoral effect is statistically significant and can be elicited in advanced stages of different primary solid tumors. It takes several weeks of treatment for this antitumoral response to become evident. DTH to the hemoderivative was demonstrated by intradermal tests in most responding treated patients, suggesting a link between response to this vaccination and immune response. We discuss possible mechanisms of action of this antitumoral treatment, such as an immune response to modified proteins released into the blood by tumors, as well as stromal fibrosis associated with this immune response. Using autologous blood as the source material for the preparation of a poly-antigenic vaccine for cancer patients makes it feasible to make new vaccine preparations and continuously update the immunogen with the latest mutations of malignant cell antigens. The results of our study justify further research to optimize vaccine characterization, identify the primary molecular target of the immune and stromal responses elicited, and explore the clinical relevance of this therapeutic alternative.

References

Breast cancer: Autologous immunogenicity elicited by chemotherapy

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The study was conducted in 30 breast cancer patients admitted in Medical Centers that referred medical data to the Cooperative Trials Center of PharmaBlood, Florida, USA. The eligibility criteria were: women, age ≤ 74 y.o., carcinoma of the breast histologically confirmed, non-surgery of primary lesion, M1 disease with more than 2 measurable lesions as targets for the Response Evaluation Criteria In Solid Tumors (RECIST) assessment, adequate hematologic, renal and hepatic function, performance status according to the scale of the Eastern Cooperative Oncology Group (ECOG) ≤ 2, life expectancy > 6 months, no previous chemotherapy or hormonotherapy and programmed to receive Cyclophosphamide-Methotrexate-5Fluorouracil (CMF) chemotherapy. Written informed consent was obtained from each patient and the trial was approved by the institutional ethical committee. Pre-CMF treatment and Post-CMF treatment (one month after last course), an intra-dermal test was performed with an autologous hemodervative protein fraction previously reported as immunogenic in advanced cancer patients and the Delayed Type Hypersensitivity (DTH) responses were registered. After 6 courses of CMF treatment, the tumor responses were assessed in each patient according to the RECIST system. Twenty-eight patients were evaluated. Initial characteristics of the patients were comparable in the groups of RECIST response and in the groups of DTH response. DTH was negative in all patients pre-CMF treatment. Post-CMF treatment, the distribution of DTH positive (more than 5 mm) in the different RECIST tumor responses was: in 1 of 12 patient with Progressive Disease, in 3 of 6 patients with Stable Disease and in 8 of 10 patients with Partial Remission.

There is a statistically significant relationship between the variables (P < 0.01). These results are consistent with the release of Tumor Associated Antigens by the chemotherapy induced death of tumor cells and/or the rise of TAAs in malignant cells associated to chemotherapy induced genetic or epigenetic modifications in protein structures. The significance of this mechanism in order to perform an internal vaccination through chemotherapy will be investigated.-
Introduction

A high dose of ascorbic acid has an antitumoral effect in some individual cases of human solid tumors (1), but the statistical significance of these results has been controversial (2). Menadione has also inhibited the growth of human solid tumors in preclinical studies (3–5) and it was also reported that it has a clinical antitumoral effect acting as a radiosensitizer (6). When technological advances in plasma determination of menadione were available (7), the difficulty of achieving in patients the plasma concentration/time of in vitro cell exposure to menadione in order to reproduce the effect of this drug as a single antitumoral agent at the clinical level was evidenced (8).

Serum Markers Variation Consistent with Autoschizis Induced by Ascorbic Acid–Menadione in Patients with Prostate Cancer

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Abstract

In vitro exposure of malignant prostate cell lines to ascorbic acid–menadione showed that tumor cells were killed through a mechanism named autoschizis. Experimental animal studies showed that autoschizis is also evident when ascorbic acid–menadione is administered to nude mice with implanted human prostate tumors. Prostate-specific antigen (PSA) is a known serum marker of prostate tumor cells specific activity. Recently, total serum homocysteine has been identified as a marker of tumor cell numbers with sensitivity for early detection of tumor cell death induced by treatments. A clinical trial with prostate cancer patients submitted to the association of ascorbic acid–menadione was performed and PSA/homocysteine was assessed in the follow-up. The early response in serum PSA and homocysteine levels was reported. The results showed that ascorbic acid–menadione produced an immediate drop in tumor cell numbers as assessed by homocysteine levels. Serum PSA levels showed an early rise and later dropped. These results suggest that autoschizis can also be induced by this pharmacological association at the clinical level in prostate cancer patients. Further studies are being performed in order to research if these results can be found with other primary tumors as it was shown in in vitro and experimental models. Ascorbic acid–menadione could be emerging as a new antitumoral chemotherapy.

Key Words: Menadione; ascorbic acid; chemotherapy; tumor cell death; prostate cancer.

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Tests in models of human tumor cell lines (9–11), animal transplantable tumors (12,13), and human tumor implants in nude mice (14,15) have shown that the association of ascorbic acid and menadione in a ratio of 100/1 induces antitumoral effects in a concentration 10–50 times lower than each agent acting alone. In the same conditions, an antitumoral synergist effect with radiotherapy and chemotherapy was also demonstrated (12,13). Scanning and transmission electron microscopies were employed to characterize these cytotoxic effects of ascorbic acid–menadione association. Following 1-h treatment of a human bladder carcinoma cell line (T24 line), the cells display membrane and mitochondrial defects as well as excision of cytoplasm fragments that contain no organelles. These continuous self-excisions reduce the cell size. Concomitantly, nuclear changes, chromatin disassembly, nucleolar condensation and fragmentation, and decreased nuclear volume lead to cell death via a process similar to karyorrhexis and karyolysis. Because this cell death is achieved through a progressive loss of cytoplasm because of self-morcellation, the authors reporting this study named this mode of cell death autoschizis, from the Greek autos, self, and schizein, to split (16,17). Flow-cytometric studies have also identified autoschizis as an ultrastructural perturbation with the break of organoids (18).

Among the different primary human solid tumors submitted to ascorbic acid–menadione in the referred preclinical studies, prostate cancer cell lines and human prostate tumor implants have been well documented (11,14,15). It has been reported that in nude mice implanted with human prostate tumor, a week of oral ascorbic acid–menadione treatment induced a more significant antitumoral effect than the same drugs administered by injection (15). Therefore, clinical trials to assess the oral association ascorbic acid–menadione as antitumoral treatment in patients with prostate cancer have started.

As autoschizis is a mechanism of tumor cell death, the study of the ascorbic acid–menadione effect upon tumor cells in vivo in patients with prostate cancer required a marker of tumor cell death induced by antitumoral treatments. Serum level of the prostate-specific antigen (PSA) has been considered as a serum marker of most prostate cancers. However, the correlation with a live malignant cell mass can be difficult because the serum level can be modified, among other factors, by the synthetic activity of tumor cells and the content release from cells to serum during and after the tumor cell death. In this framework, it has been important to report that serum total homocysteine (HCY) is a good marker of the number of live tumor cells, including human prostate tumor cell lines, and that it falls immediately after the death of tumor cell induced by antitumoral treatments. Therefore, in prostate cancer, serum measure of both markers, PSA and HCY, has been proposed as a very efficient procedure to obtain early information about the tumor cell death induced by treatments (19).

**Patients and Methods**

**Treatment**

Two 7-d courses of menadione at 50 mg/m²/d and ascorbic acid at 5 g/m²/d were administered orally. The 7-d courses of treatment began d 1 and d 22 of the study. Only two treatment courses were tested in this study because the purpose was to identify the immediate effect of this drug’s association in a clinical model reproducing the previously proven immediate autoschizis elicited by them in preclinical models.

**Assay for Homocysteine**

The measure of total HCY in serum was performed weekly, with a previously reported high-performance liquid chromatographic (HPLC) procedure (17). The first measure was taken prior to starting the first course of treatment (d 1) and the last one on the d 42 of the study.

**Assay for PSA**

Total PSA was measured in the same blood specimens where homocysteine was measured, weekly, d 1 through d 42.

**Patients**

The study was conducted in 20 prostate cancer patients admitted in medical centers that referred medical data to the Cooperative Trials Center (CTC) of PharmaBlood, R&D Department (Florida, USA). A prospective and randomized trial was performed. Written informed consent was obtained from all
patients. The Institutional Ethical Committee approved the trial.

All patients fulfilled the following eligibility criteria: pathologically proven prostate cancer, advanced stages (M1), osseous metastasis; resistant to hormonotherapy, performance status (PS) according to the Eastern Cooperative Oncology Group (ECOG) ≤ 2; age ≤ 74 yr old; adequate hematological function (WBC count ≥ 4000/µL, neutrophils count ≥ 2000/µL, platelet count ≥ 10 × 10^4/µL); renal function (serum creatinine ≤ 1.5 mg/dL, 24-h creatinine clearance ≤ 60 mL/min), hepatic function (total bilirubin ≤ 2.0 mg/dL, serum transaminases ≤ 2.0 × upper limit of normal range) and pulmonary function (PaO_2 ≥ 60 torr). The patients were randomly distributed in four groups of five patients each. Group 1 was submitted to the described two courses of ascorbic acid–menadione treatment. In group 2, ascorbic acid was omitted. In group 3, menadione was omitted. A placebo was administered to patients of group 4. The homocysteine and PSA assays were performed in the four groups as described.

**Statistics**

Three-way analysis of variance (ANOVA) was performed using Statsdirect statistical software. The two-way interactions were tested at the 0.005 level of significance.

**Results**

Table 1 shows that the four groups of patients were comparable in most relevant parameters. Figure 1 shows the results registered in the 42-d follow-up of this clinical trial. The two courses of treatment were administered the first and fourth week of the study. Serum homocysteine and PSA were measured the first day of each week (d 1, 8, 15, 22, 29, 36, and 42 of the study). In group 1, treated with ascorbic acid–menadione, the pretreatment serum level of HCY (d 1) fell in all posttreatment samples (d 8, 15, 22, 29, 36, and 42). Also in the group 1, the PSA serum levels rose in the two initial weeks (d 8 and 15) and, afterward, it fell at d 36 and 42. Variations that were not relevant were observed in homocysteine and PSA serum levels in groups 2 and 3, which were treated with a single agent—menadione and ascorbic acid, respectively.

Table 2 shows the statistical analysis of weekly measures in the three drug-treated groups comparative with the placebo (control) group (group 4). Group 1 showed a fall of HCY associated to ascorbic acid–menadione treatment, with high statistical significance (p < 0.01), in all weekly measures after starting the first series (d 8–42). Nonsignificant variation of HCY was evidenced for group 2 (menadione, single agent) and group 3 (acid ascorbic, single agent) comparative to group 4 (control placebo).
Fig. 1. **Left:** Variation of PSA serum level (µg/mL) (y-axis); **right:** Variation of HCY serum level (µM/mL) (y-axis).  
**x-axis:** weeks of the study, marking the days of PSA and HCY measures. ■, group 1; ●, group 2; ▲, group 3; and ◆, group 4. Assuming that the initial value (pretreatment measure) is 0, the PSA and HCY were measured each week during the 42 trial days. At d 1 and d 22, each one of the two series of 7-d treatment (—) included in this study was started (two-course treatment). In each one of these courses, group 1 was treated with ascorbic acid–menadione association; group 2 was treated only with menadione; group 3 was treated only with ascorbic acid; and group 4 received an appropriate placebo. The figure shows the mean PSA values and HCY values obtained from the five patients of each group, weekly.
### Table 2

Variation of Serum HCY after two treatment courses (d 1–8 and 22–28)

<table>
<thead>
<tr>
<th>Assay day</th>
<th>Group 1 (mean ± SD) (95% CI)</th>
<th>Group 2 (mean ± SD) (95% CI)</th>
<th>Group 3 (mean ± SD) (95% CI)</th>
<th>Group 4 (mean ± SD) (95% CI)</th>
<th>Group 1 vs Group 4</th>
<th>Group 2 vs Group 4</th>
<th>Group 3 vs Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>-1.2 ± 0.568 (−1.904/−0.496)</td>
<td>0.468 ± 0.362 (0.018/−0.918)</td>
<td>0.59 ± 0.508 (−0.040/1.220)</td>
<td>0.45 ± 0.335 (0.034/0.866)</td>
<td>p = 0.0029</td>
<td>p = 0.9462</td>
<td>p = 0.5608</td>
</tr>
<tr>
<td>15</td>
<td>-3 ± 1.712 (−5.126/−0.874)</td>
<td>0.59 ± 0.227 (−3.010/0.874)</td>
<td>0.74 ± 0.349 (−0.304/1.172)</td>
<td>0.64 ± 0.221 (−0.365/0.915)</td>
<td>p = 0.0057</td>
<td>p = 0.7936</td>
<td>p = 0.6608</td>
</tr>
<tr>
<td>22</td>
<td>-3.6 ± 1.437 (−5.385/−1.815)</td>
<td>0.68 ± 0.210 (−0.649/0.941)</td>
<td>1.24 ± 0.448 (−0.684/1.796)</td>
<td>0.88 ± 0.316 (−0.487/1.273)</td>
<td>p = 0.0012</td>
<td>p = 0.2133</td>
<td>p = 0.2703</td>
</tr>
<tr>
<td>29</td>
<td>-5.0 ± 1.595 (−6.980/−3.020)</td>
<td>0.76 ± 0.341 (−0.337/1.183)</td>
<td>1.45 ± 0.498 (−0.832/2.068)</td>
<td>0.86 ± 0.298 (−0.490/1.230)</td>
<td>p = 0.0016</td>
<td>p = 0.4260</td>
<td>p = 0.0519</td>
</tr>
<tr>
<td>36</td>
<td>-6.9 ± 1.799 (−9.134/−4.466)</td>
<td>1.12 ± 0.400 (−0.624/1.616)</td>
<td>1.32 ± 0.448 (−0.763/1.877)</td>
<td>1.12 ± 0.273 (−0.781/1.459)</td>
<td>p = 0.0009</td>
<td>p &gt; 0.9999</td>
<td>p = 0.1634</td>
</tr>
<tr>
<td>42</td>
<td>-7.3 ± 1.394 (−9.030/−5.570)</td>
<td>1.3 ± 0.432 (−0.763/1.837)</td>
<td>1.34 ± 0.486 (−0.736/1.944)</td>
<td>1.24 ± 0.365 (−0.786/1.694)</td>
<td>p = 0.0001</td>
<td>p = 0.8405</td>
<td>p = 0.6909</td>
</tr>
</tbody>
</table>

**Note:** Twenty prostate cancer patients, all of them resistant to hormonotherapy, with bone metastases were randomized in four groups of five patients each. In group 1, the treatment course was ascorbic acid–menadione; in group 2, it was menadione; and in group 3, it was ascorbic acid. Group 4 received a placebo. Assuming the initial value (pretreatment measure) is 0, HCY variation was measured each week during the 42-d trial period (assay days). The mean ± standard deviation and 95% confidence interval (95% CI) of each group is shown. The statistical significance of mean variation in treated groups comparative with the control group (p-value) for each assay day, calculated by ANOVA, of is also shown.
### Table 3
Variation of serum PSA after two treatment courses (d 1–8 and 22–28)

<table>
<thead>
<tr>
<th>Assay day</th>
<th>Group 1 (mean ± SD, 95% CI)</th>
<th>Group 2 (mean ± SD, 95% CI)</th>
<th>Group 3 (mean ± SD, 95% CI)</th>
<th>Group 4 (mean ± SD, 95% CI)</th>
<th>Group 1 vs group 4</th>
<th>Group 2 vs group 4</th>
<th>Group 3 vs group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.54 ± 0.286 (0.185/0.895)</td>
<td>0.12 ± 0.275 (–0.462/0.223)</td>
<td>0.21 ± 0.244 (–0.093/0.513)</td>
<td>0.16 ± 0.157 (–0.035/0.355)</td>
<td>p = 0.0799</td>
<td>p = 0.0200</td>
<td>p = 0.7629</td>
</tr>
<tr>
<td>8</td>
<td>0.94 ± 0.266 (0.610/1.270)</td>
<td>0.32 ± 0.131 (0.157/0.483)</td>
<td>0.38 ± 0.191 (0.143/0.617)</td>
<td>0.21 ± 0.223 (–0.067/0.487)</td>
<td>p = 0.0024</td>
<td>p = 0.3893</td>
<td>p = 0.1923</td>
</tr>
<tr>
<td>15</td>
<td>0.07 ± 0.193 (–0.170/0.310)</td>
<td>0.52 ± 0.290 (0.160/0.880)</td>
<td>0.48 ± 0.186 (0.249/0.711)</td>
<td>0.42 ± 0.199 (0.173/0.667)</td>
<td>p = 0.0022</td>
<td>p = 0.6075</td>
<td>p = 0.5466</td>
</tr>
<tr>
<td>22</td>
<td>–0.24 ± 0.237 (–0.534/0.054)</td>
<td>0.66 ± 0.369 (0.201/1.119)</td>
<td>0.66 ± 0.241 (0.361/0.959)</td>
<td>0.81 ± 0.260 (0.488/1.132)</td>
<td>p &lt; 0.0001</td>
<td>p = 0.4438</td>
<td>p = 0.4931</td>
</tr>
<tr>
<td>29</td>
<td>–1.8 ± 0.380 (–2.272/–1.328)</td>
<td>0.82 ± 0.301 (0.446/1.194)</td>
<td>0.58 ± 0.258 (0.260/0.890)</td>
<td>0.78 ± 0.311 (0.394/1.166)</td>
<td>p &lt; 0.0001</td>
<td>p = 0.8063</td>
<td>p = 0.3268</td>
</tr>
<tr>
<td>36</td>
<td>–2.6 ± 0.570 (–3.307/–1.893)</td>
<td>1.08 ± 0.235 (0.789/1.371)</td>
<td>0.96 ± 0.223 (0.683/1.237)</td>
<td>1.12 ± 0.360 (0.674/1.566)</td>
<td>p = 0.0002</td>
<td>p = 0.8309</td>
<td>p = 0.4050</td>
</tr>
</tbody>
</table>

**Note:** Twenty prostate cancer patients, all of them resistant to hormonotherapy, with bone metastases were randomized in four groups of five patients each. In group 1, the treatment courses was ascorbic acid-menadione; in Group 2, it was menadione; and in group 3, it was ascorbic acid. Group 4 received an appropriate placebo. Assuming that the initial value (pretreatment measure) is 0, the PSA was measured each week during a 42-d trial period (assay days). The mean ± standard deviation and 95% confidence interval (95% CI) of each group is shown. The statistical significance of mean variation in treated groups comparative with control group (p-value) for each assay day, calculated by ANOVA, is also shown.
Table 3 shows the statistical analysis of PSA serum levels. For group 1, the rise of PSA at d 15 and the fall of PSA at d 22, 29, 36, and 42 were significantly different \((p < 0.01)\) compared to the control group (group 4). A nonsignificant difference was observed between group 2 or group 3 and group 4.

**Discussion**

This article reported a study about the variation of two serum tumor markers in prostate cancer patients who were treated with two 7-d courses of oral ascorbic acid–menadione. The total homocysteine was one of the assessed serum markers. It was previously identified as a marker of tumor proliferative mass and its rise was linked to tumor cell folate depletion. This depletion of folate inactivates the remethylation reaction catalyzed by the methionine synthetase, and as consequence, HCY cannot be converted to methionine and increase. High levels of HCY have been shown in cancer patients treated with antifolate and nonantifolate drugs; therefore, it was concluded that folate depletion is not dependent on antifolate treatments. It is caused by the high proliferation rate and, obviously, by the high number of cells. The fall of this high serum HCY level in cancer patients, induced by treatments, could be a marker of the deacceleration of the tumor cell proliferation rate and/or a marker of the death of tumor cells with the decrease in proliferating tumor cell numbers. Briefly, the serum HCY level has been previously identified as an indicator of the number of live and proliferative tumor cells with early response (fall) to death and/or slowing the growth of tumor cells induced by treatments. Serum is not recommended for HCY determination, but when it is carefully collected, a nonsignificant difference was found between the serum and the plasma measures \((19)\). The results reported in this article show that the HCY serum level drops in the first day after the treatment series in group 1. This decrease in tumor cell number induced by ascorbic acid–menadione association persists the following 15 d in the interseries of this short treatment. No similar HCY variation was evident in groups 2 and 3, which were treated with each drug separately. It was reported that the association of ascorbic acid–menadione produces a potentiation of the antitumoral activity of each isolated agent by a factor of 50–100. Therefore, it is possible that any antitumoral effect of each isolated tested agent in groups 2 and 3 did not reach the level of minimum sensitivity of our marker. PSA was the other marker assessed. It is known that serum PSA is a specific indicator of tumor cell activity in most prostate tumors. It is a glycoprotein with protease activity synthesized by epithelial prostate cells, including prostate tumor cells. The permeation of PSA from inside the tumor cells to extracellular media is responsible of the high serum level of PSA in most prostate cancer patients. The main mechanism through which the treatments kill prostate tumor cells is apoptosis. The serum PSA falls as a consequence of the decrease of tumor cells mass; the total source of PSA synthesis decreases and the total PSA permeated from tumor cells to serum also decreases. However, this fall in serum PSA is frequently evident with a delay of days or weeks after tumor treatment. Apoptosis time, changes in the level of remnant tumor cell activity, new conditions of PSA permeation, and the efficiency in the clearance of serum PSA are parameters used to explain the delay in the response of PSA serum level to prostate cancer treatments. In vitro studies have shown that dead prostate tumor cells can release their PSA within hours or days \((19)\). An early increase of PSA after the induction of tumor cells death by treatments can be observed as a consequence of the release of cell contents into the blood. As a consequence, PSA is not recommendable as a measure of early tumor cell death induced by treatments. In this trial, PSA increases in the initial serum specimens taken immediately after treatment with ascorbic acid–menadione and it drops slowly afterward. In the frame of this short treatment, PSA reaches a significant drop at the end of the follow-up. The variations of serum homocysteine and PSA suggest that malignant cell death is induced in prostate cancer patients by the administration of ascorbic acid–menadione. Preclinical studies have shown that this drug association induces prostate tumor cell death by autoschizis in vitro and in experimental animal models \((11,14,15)\). Our results suggest that ascorbic acid–menadione also produces autoschizis in vivo in human prostate cancer patients. The membrane and cell injury produced by oxidative radicals gen-
erated by H\textsubscript{2}O\textsubscript{2} has been proposed as the mechanism of action involved in the autoschizis induced by ascorbic acid–menadione. It is well known that the ascorbic acid–menadione association generates H\textsubscript{2}O\textsubscript{2}. It is also known that most malignant cells are often catalase deficient. This mechanism of action seems to be confirmed because autoschizis is inhibited by exogenous catalase (9,11). Cytoskeletal structures and ATP pool have been considered as the target of this peroxidative mechanism of action (5,11). Activation of DNAses with decrease of cell DNA is also a major fact in autoschizis (14). These oxidative mechanisms of action and these targets can be involved in other primary tumors submitted to the same treatment as suggested by the pioneer preclinical studies (9). Further studies are necessary to investigate the optimal antitumoral conditions and clinical relevance of ascorbic acid–menadione, which could make this association a new antitumoral chemotherapy.

References

Breast Cancer: Updated Vaccination With An Autologous Hemoderivative in Changing Tumor Antigen Library

Short Title: Updated Breast Cancer Vaccine

E. Garcia-Giralt, E. Lasalvia-Prisco, S. Cucchi, J. Vazquez; Centre De Cancerologie Hartmann, Neuilly, Sur Seine, France; PharmaBlood Inc Dept Research &Development, North Miami Beach, FL

Background. We have previously described a vaccination procedure with an autologous Hemoderivative (AH) that in solid tumors, including breast cancer, inhibited the progressive disease condition, elicited Delayed Type Hypersensibility (DTH) and induced a tumor stroma modification in responder patients (E. Lasalvia-Prisco et al, Cancer Biology & Therapy, 2003; ASCO 2003). The present study describes the disease and DTH evolution in responder vaccinated patients.

Methods. Thirty metastatic (1-3 sites), chemotherapy resistant (3-lines), breast cancer patients (Ages: 38-72 y.o.) in progressive disease (RECIST assessment) were included. Written informed consents and institutional ethical committee approval were accomplished. The included patients were vaccinated with AH with the procedure above referred. After 6 months, 11 patients were responders (Stable disease + Partial Remission) and DTH-positive response elicited by AH (= 5mm). When progressive disease reappeared, a second vaccination with a second AH was performed. Follow up of these 11 responder patients was analyzed (RECIST assessment and intradermal DTH-test elicited with the first and second AH).

Results. Progressive Disease reappeared in 10 responder patients 7-14 months after vaccination. In these 10 patients, a second AH was freshly prepared and a second vaccination was performed. Previously to the second vaccination, DTH response elicited by intradermal test with the first AH persisted positive in 9, and it was negative in all 10 when it was performed with the second AH. Six month after the second vaccination with the new AH, a second Tumor-Growth response (Stable disease + Partial Remission) was evident in 8 patients and the DTH response elicited with the second AH was positive in 7 of them.

Conclusions Results are consistent with a tumor immunoescape by changes in the neo-antigen library and the feasibility of re-vaccination with an updated AH containing neo-antigens delivered from the tumors to the blood.

Keyword: Breast cancer ; Vaccines .
Background. – In cancer patients, including colorectal cancer, we have previously reported that vaccination with an Autologous Hemoderivative (AH) elicits Delayed Type Hypersensibility (DTH) associated with an antitumoral effect. (E. Lasalvia-Prisco et al, Cancer Biology & Therapy, 2003; ASCO 2003). It was also shown that 5-fluorouracil (5-FU) can upregulate the expression of malignant neo-antigens and MHC molecules in colon cancer cell lines, enhancing their sensitivity to the Cytotoxic T-lymphocytes, suggesting that chemotherapy (5FU) could increase the antigenicity of colorectal malignant cells and it suggests that pre-treatment with this chemotherapy agent could enhance the efficacy of malignant cell derivatives as autologous cancer vaccines.

Results. - DTH positive responses (= 5 mm) were 1 of 10, 2 of 10, 4 of 10, 5 of 10 and 7 of 10 in the groups pre-treated respectively with 2, 4, 6, 8 and 10, 5FU series. There is a statistically significant relationship between the variables: 5FU series and DTH response (P<0.01). Conclusions. - These clinical results are consistent with the reported in vitro results that 5FU enhances the antigenicity of colorectal malignant cells and it suggests that pre-treatment with this chemotherapy agent could enhance the efficacy of malignant cell derivatives as autologous cancer vaccines.
Eduardo Lasalvia-Prisco · Silvia Cucchi · Jesús Vázquez · Eduardo Lasalvia-Galante · Wilson Golomar · William Gordon

Insulin-induced enhancement of antitumoral response to methotrexate in breast cancer patients

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Abstract Purpose: It has been reported that insulin increases the cytotoxic effect in vitro of methotrexate by as much as 10,000-fold. The purpose of this study was to explore the clinical value of insulin as a potentiator of methotrexate. Patients and methods: Included in this prospective, randomized clinical trial were 30 women with metastatic breast cancer resistant to fluorouracil + Adriamycin + cyclophosphamide and also resistant to hormone therapy with measurable lesions. Three groups each of ten patients received two 21-day courses of the following treatments: insulin + methotrexate, methotrexate, and insulin, respectively. In each patient, the size of the target tumor was measured before and after treatment according to the Response Evaluation Criteria In Solid Tumors. The changes in the size of the target tumor in the three groups were compared statistically. Results: Under the trial conditions, the methotrexate-treated group and the insulin-treated group responded most frequently with progressive disease. The group treated with insulin + methotrexate responded most frequently with stable disease. The median increase in tumor size was significantly lower with insulin + methotrexate than with each drug used separately. Discussion: Our results confirmed in vivo the results of previous in vitro studies showing clinical evidence that insulin potentiates methotrexate under conditions where insulin alone does not promote an increase in tumor growth. Therefore, the chemotherapy antitumoral activity must have been enhanced by the biochemical events elicited in tumor cells by insulin. Conclusions: In multidrug-resistant metastatic breast cancer, methotrexate + insulin produced a significant antitumoral response that was not seen with either methotrexate or insulin used separately.

Keywords Breast Cancer · Chemotherapy · Insulin · Methotrexate · Tumor growth

Introduction

It is known that slowly growing cancers have tumor cell populations with a low-growth fraction and are less sensitive to chemotherapy than rapidly growing tumors with high-growth fractions [11]. Slowly growing malignancies have relatively more cells in a noncycling status and fewer cells in a cycling status than rapidly growing malignancies. It has been demonstrated that insulin as a pharmacological agent induces the switch from a noncycling to a cycling status in tumor cells [5]. In MCF-7 human breast cancer cells, insulin has been shown to increase the cytotoxic effect of methotrexate up to 10,000-fold in vitro [1]. Ellipticine uptake is also increased by insulin [9]. It has been suggested that insulin is effective in potentiating most chemotherapy drugs. This insulin-induced potentiation has been
proposed as a strategy for breast cancer treatment, but confirmatory clinical trials are still lacking [2]. This study was carried out to confirm insulin-induced clinical potentiation of the antitumoral effect of methotrexate as suggested by preclinical studies and to establish a mechanism of action for this antitumoral effect.

**Patients and methods**

**Patients**

The study was conducted in 30 patients with breast cancer admitted to medical centers that reported medical data to the Cooperative Trials Center (CTC) of PharmaBlood, R&D Department, Florida. A prospective, randomized trial was carried out. All patients met the following eligibility criteria: histologically confirmed breast carcinoma, metastatic stage (M1); Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 2; age ≤ 74 years; and adequate hematological function (WBC count ≥4000/µl, neutrophil count ≥2000/µl, hemoglobin level ≥9.0 g/dl, platelet count ≥100000/µl, serum creatinine ≤1.5 mg/dl, 24-h creatinine clearance ≤ 60 ml/min), liver function (total bilirubin ≤ 2.0 mg/dl, serum transaminases not more than twice the upper limit of the normal range), and respiratory function (PaO2 ≥60 Torr). The patients included had measurable lesions, as required by the Response Evaluation Criteria In Solid Tumors (RECIST) system of tumor assessment [13], and if they had a positive estrogen receptor status, they had been treated with and become resistant to hormone therapy.

All patients included in the study had progressive disease (RECIST criteria) after chemotherapy with at least four series of fluorouracil + Adriamycin + cyclophosphamide (FAC) and had not been treated with any other chemotherapy. They were randomly allocated to three groups of ten patients each: group 1 was treated with insulin + methotrexate as described below, group 2 was treated with methotrexate without insulin, and group 3 was treated with insulin without methotrexate. Written informed consent, including detailed information about risks and benefits, was approved and signed by all the patients included in the study. Central computerized remote randomization was performed, with patients being allocated to one of the groups through random sequence generation by the permuted block method. An assessment of the results of 30 patients had completed the trial showed that this sample size was enough. The patients were recruited from two oncological medical centers in Montevideo, Uruguay (first at the National Cancer Institute and then at Interdoctors Medical Center), both of which participated with their data in the network operated and sponsored by the Cooperative Trials Center (CTC) of PharmaBlood R&D Department.

The institutional ethics committee of PharmaBlood and the institutional review boards of the participating medical centers approved the trial. The ethical reviewers considered that an 8-week delay before starting second-line chemotherapy after FAC had failed in all the patients included in the trial was acceptable. This determination was consistent with the standard of care in this clinical situation which has been recently well summarized [3];

Despite almost 30 years of clinical cancer research, the true impact of second and subsequent lines of chemotherapy on the outcome of metastatic breast cancer patients, especially on the duration of survival, is still unknown. In the virtually incurable metastatic setting, issues like quality of life and patients' preferences gain particular relevance.

The accepted protocol was resubmitted to the committee for review in order to obtain approval for treatment of patients with insulin alone considering the potentially harmful effect through the activation of receptors for insulin/insulin-like growth factors. The committee confirmed the approval on the basis of reports of no harmful effect of this treatment [6, 7]. The results of the study confirmed the committee's criteria because no significant differences were found in tumor growth either between the insulin-alone group and the methotrexate-alone group or between before and after treatment in the insulin-alone group.

**Treatment**

All the patients included in the study received two 21-day courses of treatment separated by a 7-day interval without treatment between courses. In group 1, the treatment course was intravenous human recombinant insulin (0.3 U/kg body weight every other day) followed 20 min later by a 15-min intravenous infusion of methotrexate (2.5 mg/m² in 50 ml 30% glucose). If symptomatic hypoglycemia was observed, the 30% glucose solution containing methotrexate was infused immediately. An oral glucose supplement was also prescribed to prevent delayed hypoglycemic symptoms. In group 2, insulin was omitted and methotrexate was administered intravenously at the same dose and in the same solution (2.5 mg/m² in 50 ml 30% glucose) as in group 1. In group 3, methotrexate was omitted, insulin was administered at the same dose as in group 1, and 30% glucose solution was also administered intravenously 20 min after insulin or sooner if hypoglycemic symptoms were evident.

**Tumor growth assessment**

After 8 weeks (two 3-week courses plus 1 week interval after each course), the response to treatment was assessed in each patient using RECIST criteria [13]. The sum of the longest diameter of measurable target lesions and the number of non-target lesions were recorded immediately before and after this 8-week period. Skin nodules and palpable lymph nodes were measured using calipers. Lung and liver target lesions were measured by a CAT scan. Responses were confirmed by repeating the assessment 4 weeks after status assignment. Three independent reviewers performed all image measures (Telemedicinal Organization, North Miami Beach, FL).

The distribution of RECIST status (progressive disease, stable disease, or remission) in each group was recorded. This distribution was dependent on treatments that showed statistical significance according to the Chi-squared test. The data from the RECIST measurements of the change in tumor size of the patients in each treatment group, expressed as a percentage of pretreatment measurements, were compared using Student’s t-test. Additionally, increases in tumor size were expressed as a proportion of the initial value and analyzed by the two-proportion test comparing pairs of groups: group 3 vs group 1, and group 2 vs group 1. The sample size was assessed after analysis of the results when the trial was finished for the 30 patients allocated to the three groups. The above pairs of groups were analyzed for the proportion of progressive disease in each. Ten patients in each group was the required sample size for an 80% chance of rejecting the hypothesis of equal proportions at the 0.05 level of significance when the true proportions were those shown by the study. Statistical analysis was performed using StatsDirect software and an independent expert was consulted.

**Results**

The characteristics of the patients included are shown in Table 1. The three groups were comparable in the most relevant prognostic parameters for the clinical condition studied. Previous treatments were also comparable. The similar range of sizes of target lesions measured before treatment was especially significant, allowing the change in size to be measured as a percentage of initial size.
Figure 1 shows the RECIST status assessed under the study conditions. Progressive disease was the most frequent response in two of the three groups: in group 2 (treated with methotrexate alone) there were seven progressive disease and three stable disease, and in group 3 (treated with insulin alone) there were eight progressive disease and two stable disease. In group 1 (treated with insulin + methotrexate), stable disease was the most frequent response (nine stable disease, one progressive disease). The distribution of RECIST type responses (stable disease and progressive disease) was dependent on the treatments tested, and was statistically significant ($P<0.01$, Chi-squared test).

Figure 2 shows the means and 95% confidence intervals (CI) of the percentage increase in tumor size after treatment in the three groups. Increases in tumor size were significantly lower in patients treated with insulin + methotrexate than in those treated with insulin alone and significantly lower than in those treated with methotrexate alone.

From the same set of measurements, Figs. 1 and 2 show the clinical and biological effects of the treatments, respectively. Figure 1 indicates that the decrease in tumor growth induced by insulin + methotrexate reached the level of a clinically confirmed antitumoral response because more patients in this group achieved stable disease. Figure 2 shows that insulin + methotrexate treatment reduced tumor growth. All patients completed the study. Hypoglycemia was induced in all patients receiving insulin as part of their protocol. Eight patients in group 1 and nine patients in group 3 showed no hypoglycemic symptoms during the 20 min after insulin injection; they showed a mean blood glucose level of 456 mg/dl (range 376–520 mg/dl). Two patients in group

Table 1 Clinical characteristics of the 30 women with metastatic breast cancer (M1) included in the three treatment groups

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (insulin + methotrexate)</th>
<th>Group 2 (methotrexate)</th>
<th>Group 3 (insulin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>42–64</td>
<td>44–68</td>
<td>39–69</td>
</tr>
<tr>
<td>&lt; 50 years</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Estrogen receptor-positive</td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Progesterone receptor-positive</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Measurable M1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Liver</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Skin</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Range of initial (pretreatment) RECIST sum of target measures (mm)</td>
<td>57–65</td>
<td>59–64</td>
<td>56–66</td>
</tr>
</tbody>
</table>
Table 2 Maximum recorded WHO toxicity grade in the patients included in the trial comparing insulin + methotrexate (group 1), methotrexate (group 2) and insulin (group 3). The numbers of patients with each toxicity grade (0 to 4) in the three groups are shown. No other toxicities referred to in the WHO criteria were recorded.

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Grade</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td></td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
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<td>2</td>
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<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leukocytes</td>
<td></td>
<td>10</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0</td>
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<td>3</td>
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<tr>
<td></td>
<td>4</td>
<td>0</td>
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</tr>
<tr>
<td>Platelets</td>
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<td>10</td>
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<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mucositis</td>
<td></td>
<td>8</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
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</tr>
</tbody>
</table>

1 and one patient in group 3 showed hypoglycemic symptoms within 20 min of insulin injection (13, 16 and 19 min), but recovered immediately after starting the glucose infusion. There was no evidence of any harmful sequelae attributable to the hypoglycemia induced.

Table 2 shows the toxicities associated with antitumoral chemotherapy (according to WHO criteria) recorded in this study.

Discussion

The methotrexate dose used in this study was chosen because a similar dose of methotrexate had been used previously in patients receiving low-dose combined chemotherapy potentiated with insulin [2]. In addition, the cumulative monthly dose was no higher than the monthly dose used in the well-known standard protocol of methotrexate + fluorouracil + cyclophosphamide (CMF). Indeed, each individual methotrexate injection (2.5 mg/m²) was less than the dose usually considered optimal in non-potentiated protocols but is within the presumed range of effective dose for a potentiation similar to the one observed in vitro. The results of this study confirmed the expected safety of the selected methotrexate dose. The toxicities in the methotrexate-alone group were not relevant (WHO grades 1/2) and they were even lower when methotrexate was associated with insulin, only producing a grade 1 mucositis. In this study, methotrexate at this safe low dose did not have an antitumoral effect when used alone (group 2), but it did produce a significant antitumoral effect when administered after insulin (group 1). The term antitumoral is used here as a description of the clinical effect of a reduction in the proportion of patients showing progressive disease.

Therefore, as reported previously, our results support the hypothesis that insulin can potentiate the antitumoral effect of methotrexate [2] and confirm in vivo previously reported in vitro results [10]. Our results also show insulin potentiation of methotrexate in this condition, where insulin alone did not promote an increase in tumor growth (group 3). This effect is in agreement with previous results from in vitro models where insulin enhancement of cytotoxicity was not a direct consequence of an insulin-dependent increase in the growth rate of tumor cells [1, 10]. The same in vitro models do not allow an explanation of the insulin potentiation of methotrexate in terms of the known effects of insulin treatment upon the specific metabolism of methotrexate which include a decrease in intracellular pH induced by glucose metabolism and tight binding of the drug to its target, dihydrofolate reductase. Insulin potentiation of other antitumoral drugs has been reported [9].

If we discount the promotion of tumor cell growth and the interaction with the specific target as the mechanism of potentiation of methotrexate by insulin, we can hypothesize that this mechanism could involve another general insulin-dependent biochemical pathway as has been previously suggested to explain the in vitro potentiation of methotrexate by insulin [1]: protein synthesis in tumor cells is one of the biochemical pathways activated by insulin [8]. Most chemotherapy drugs that have been tested using insulin to increase cytotoxicity are known modifiers of protein structure that act at the genetic or epigenetic level [12]. High levels of mutated or epigenetically modified proteins could be responsible for the cytotoxic mechanism elicited by the insulin-dependent increase in protein synthesis associated with chemotherapy drugs. The relative selectivity of this mechanism of action for insulin + methotrexate in malignant cells is attributed to the agonism of insulin and insulin-like receptors in tumor cells. Certainly, the response to insulin is more intense in most tested cancer cells than in most normal cells. This is probably because cancer cells are richer in receptors for insulin-like growth factors that are cross-stimulated by insulin [4].

Conclusion

The in vitro potentiation of methotrexate cytotoxicity by insulin in human breast cancer cell lines was previously known. We report the results of a randomized, controlled trial that confirmed, at the clinical level, the potentiation by insulin of the antitumoral effect of methotrexate in women with advanced breast cancer. The term antitumoral is used as a description of the clinical effect of a reduction in the proportion of patients with progressive disease. Under the conditions of this study, the dose of insulin used did not increase tumor growth. Therefore, we suggest that, as has been reported
in vitro, methotrexate potentiation by insulin was not a direct consequence of the expansion of the tumor cycling cell population but a consequence of some of the biochemical events that are simultaneously activated. The enhancement of methotrexate uptake by tumor cells and/or the promotion of protein synthesis in a mutagenic intracellular environment are hypothesized to be mechanisms of potentiation. It is known that both events are promoted by insulin acting as a cross-agonist of the highly expressed receptors for insulin-like growth factors in breast cancer cells.

These mechanisms, which are shared with other primary tumor cells and with other chemotherapeutic agents suggest that it would be worthwhile to pursue further study of these phenomena in other tumors and with other chemotherapeutic agents.

References


Author(s): E. Garcia-Giralt, E. Lasalvia-Prisco, S. Cucchi, J. Vázquez, E. Lasalvia-Galante, W. Golomar

Abstract: **Background:** In advanced cancer from different primary tumors, our team (E. Lasalvia-Prisco et al, Cancer Biology & Therapy, 2003*; ASCO 2003, ASCO 2004) has reported anti-tumoral effect of an immunotherapy procedure using as immunogen an autologous hemoderivative (AHD). We explored in a breast cancer population the immunological mechanisms involved. **Methods:** Patients.- Metastatic breast cancer patients (n= 121), hormone/chemotherapy resistant, high CEA/CA 15-3 plasma levels, PS<3 and documented progressive disease were 2-group randomized: AHD-vaccinated as reported* and controls. Assessment.- **Clinical response**: monthly assessed according to RECIST as progressive disease (PD) or stable disease (SD). **Immune response:** pre and 20-day post treatment, Lymphocyte Proliferation Assay measured as thymidine-H3 incorporation (LPA) using peripheral blood mononuclear cells (PBMC) as responder cells and, as challengers: CEA, CA 15-3, AHD and 2 other autologous hemoderivatives (AH) obtained as AHD but from 2 lymphocyte populations (Wan Fal Ng preparation method): [CD4+CD25+] and [CD4+CD25-]. **Results:** After 8-month follow-up, 101 patients were evaluated (50/51 control/treated). Groups were comparable. In the treated group, 25/51 patients reached SD status, significantly higher (p<0.001) than in the controls (1/50 pts), and the responders (reaching SD) were 25 patients and nonresponders (persisting PD) were 26. Post treatment, the responders have LPA positive (challenged > 4 x control) when their PBMCs were challenged with AHD, CEA, CA 15-3 (p<0.001) and AH from [CD4+CD25+] cells (p<002). LPA was negative before treatment and after treatment when challenger was AH from [CD4+CD25-] cells (p>0.5). **Conclusions:** AHD induced anti-progressive effect upon the tumor growth in 25/51 patients. This effect was associated with an immune response poli-targeted against molecules released from malignant cells to the blood (CEA/CA 15-3) and also against regulatory (tolerance) cells [CD4+CD25+] but not against [CD4+CD25-] cells. These facts are compatible with an anti-tumoral mechanism of autoimmunity elicited by AHD vaccine.
Colorectal Cancer: Comparative effects of an Autologous Hemoderivative Vaccine with a CEA vaccine and/or an autologous [CD4+CD25+] vaccine.

Lasalvia-Prisco, E. Garcia-Giralt, S. Cucchi, J. Larrañaga

Abstract: **Background:** In advanced cancer from different primary tumors, our team (E. Lasalvia-Prisco et al, Cancer Biology & Therapy, 2003*; ASCO 2003; ASCO 2004) has reported anti-tumoral effect of an immunotherapy procedure using as immunogen a hemoderivative prepared from autologous blood (AHD). Further studies have suggested that AHD, as polyvalent immunogen, elicits an immune response against tumor-associated antigens released by tumor cells to the blood (i.e. CEA) and an autoimmune response against regulatory or tolerance cells [CD4+CD25+]. **Methods:** Patients.- Metastatic colorectal adenocarcinoma patients (n= 66), chemotherapy-resistant, high CEA plasma levels, PS<3 were 2 group randomized. Group 1 (n=33), AHD vaccination as has been described*. Group 2 (n=33), 3 successive vaccination procedures: (2a) first 2 months, same procedure than group 1 but CEA instead of AHD as immunogen; (2b) following 2 months, CEA was substituted by an autologous hemoderivative prepared as AHD but from autologous [CD4+CD25+] cells (Wan Fal Ng preparation method) and (2c) last 2 months, regular AHD as immunogen. **Assessment.-** After each 2-month vaccination, lymphocyte proliferation assays measured as thymidine-H3 incorporation (LPAs) were performed in all patients. Peripheral blood mononuclear cells (PBMC) were used as responder cells and every used immunogen was tested as challenger. **Results:** When challenged with CEA, LPAs were positive (challenged > 4 x control) in 16/33 pts of group 1, 0/33 of 2a, 0/33 of 2b and 12/33 of 2c. When challenged with AH [CD4+CD25+], LPAs were positive in 15 /33 pts of group 1, 0/33 of 2a, 2/33 of 2b and 10/33 of 2c. When challenged with AHD, LPAs were positive in 17 /33 pts of group 1, 0/33 of 2a, 3/33 of 2b and 16/33 of 2c. All Specificity Controls with [CD4+CD25+] were negative. Group 1 and 2c positive responses were significant (p<0.001). **Conclusions:** AHD induced a sensitization, at least, to blood-released CEA tumor marker and the autologous regulatory cell population [CD4+CD25+]. The blockage of the tolerance system of regulatory cells could explain the efficacy of the anti-tumoral immune response elicited by the tumor-associated antigens released to the blood.
Advanced Colon Cancer

Antiprogressive Immunotherapy Using an Autologous Hemoderivative

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Abstract

Introduction. Advanced colon cancer patients, acquired-chemotherapy resistant and in progression, are therapeutically terminal. We tested a recently described medical procedure using a thermostable autologous hemoderivative purported to inhibit tumor growth possibly through an immunological mechanism of action.

Patients and Methods. Metastatic colon cancer patients chemotherapeutic-resistant, high CEA plasma levels, in progression, were 2-group randomized. Group 1 received the test procedure and Group 2 adequate control measures. During an 8-mo follow-up period (n = 101), tumor growth, number of cases attaining clinical non-progressive status, and mortality were assessed monthly. Immunological effect was assessed by delayed-type hypersensitivity test and lymphocyte proliferation assay. Responding-tumors histopathologies were studied.

Results and Discussion. In a significant number of cases, the test procedure promoted inhibition of tumor growth, nonprogressive 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 regulatory lymphocyte population (CD4+CD25+). Interference with this regulatory activity could explain the selective autoimmunity suggested by the histopathology findings in responding tumors.

Conclusion. The autologous hemoderivative tested is antigenically polyvalent and promotes a polytargeted immune response associated with a tumor antiprogressive effect, consequently, acting as an autologous hemoderivative cancer vaccine.

Key Words: Autologous hemoderivative; colon cancer; vaccine.

Introduction

Our team has described the clinical antiprogressive effect of an autologous hemoderivative (AHD) in a group of human malignant tumors (1–3). Some histopathology findings of tumors responding to this hemoderivative suggest an immunological mechanism of action, so we conducted further studies of this hemoderivative within the framework of an immunotherapy procedure. 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 a 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 antitumor-progressive effect and delayed-type hypersensitivity (DTH) to AHD, with a histologic 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. In order to confirm the antiprogressive effect of this procedure and the proposed immunological mechanism of action, we designed the present study of a patient population (advanced colon cancer) more homogeneous than the populations included in the studies referenced above (1–3) and included laboratory tests to characterize the hemoderivative antigenic activity.

Patients and Methods

Patients

A prospective, randomized trial was carried out. Patient characteristics are summarized in Table 1. The study included 120 patients who met the following eligibility criteria: a histopathologically confirmed diagnosis of advanced metastatic colon adenocarcinoma; a tumor burden comprising one to three metastasis sites (brain excluded); conservation of organic functions (adequate bone marrow function: WBC ≥ 3000/mm³, ANC ≥ 1500/mm, Hgb ≥ 9.0 g/dL, and platelets ≥ 100,000/mm³; 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 (6); high levels of CEA (at least two times the upper limit of normality), and measurable progressive disease as assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) (7). Eligible patients 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 5-fluorouracil/leucovorin and irinotecan for recurrence. The patients included had to have undergone the last cycle of chemotherapy more than 1 mo earlier. The study was conducted in patients admitted to medical centers that submitted medical data to the Cooperative Trials Center (CTC) of PharmaBlood, R&D Department, Florida, USA. 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 (8).

The trial design included central randomization of patients into two groups that received different treatments during 1 mo: an AHD-treated group and a control group. The AHD-treated group received the test immunotherapy procedure as previously reported (3), that is, subcutaneous injection of AHD three times a week for 4 wk plus an immunomodulatory procedure with low-dose cyclophosphamide (300 mg/m²) 3 d before the first AHD injection and rhGM-CSF (150 µg/m²) simultaneously with each AHD injection. The control group received only the immunomodulatory procedure, cyclophosphamide (300 mg/m²) 3 d before starting rhGM-CSF (150 mcg/m²) sc three times a week for 4 wk. The ethical rationale for these 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 (9), 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 dose used both drugs have been repeatedly administered in oncological patients without relevant toxicity: cyclophosphamide at a low dose of 300 mg/m² (only once) is not immunosuppressive (4) and rhGM-CSF is an adjuvant of hematological and immunological recovery (10). Symptomatic treatment was allowed throughout the study in both groups. According to previous reports, the maximal delay and the recovery of tumor growth in the observed AHD effect could be shown during an 8-mo follow up period (3). Therefore, the clinical para-
AHD Preparation and Treatment Procedure

AHD was obtained and administered as previously described (1–3,11). Briefly, 20 mL of blood was drawn from the femoral artery into a syringe containing 5000 IU of heparin and sediments at 37°C for 1 h. Afterwards, cellular lysis was induced by exposing the supernatant of plasma and cells to hypotonic shock with 3 vol 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 five individual vials: one test vial with 0.5 mL and four vaccine vials, each containing an equal aliquot of the rest of the preparation. All vials were stored at −20°C until use. In the AHD-treated group, the four vaccine vials were used for the vaccination procedure, one vial each successive week. Each vaccination consisted of a mixture of the contents of the vaccine vial and 150 µg/m² 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 300 mg/m² cyclophosphamide was given 3 d before starting the vaccination procedure to inhibit tolerance immune responses, as described (4). Aseptic technique was used in each phase of the procedure. In the control group, AHD was prepared to perform the laboratory test, but only 150 µg/m² of rhGM-CSF was injected subcutaneously three times a wk, for 4 weeks, and 300 mg/m²
cyclophosphamide was given 3 d before the first injection.

The following assessments were made during the study:

1. Tumor growth: In each patient, tumor size was evaluated every 30 d 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. An independent reviewer (Telemedical Organization) in a blind procedure confirmed the measurements. Tumor growth was calculated for each patient as the percentage variation of tumor size in 30 d.

2. Status of clinical response in each patient was recorded every 30 d, according to RECIST definitions, as progressive disease (PD), stable disease (SD), partial remission (PR), or complete remission (CR). SD, PR, and CR were considered non-progressive disease (NPD) status. Under the conditions of this study, all patients with NPD had SD (tumor growth increased by < 20% or decreased by < 30%). 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 d.

3. Immunological response. An in vivo and an in vitro study were made.

(a) Delayed-type hypersensitivity (DTH) tests were performed in each patient using an intradermal injection of 0.1 mL of AHD lyophilized and reconstituted to 10X the original concentration. A test was performed before starting treatment (AHD and control) and it was repeated 20 d 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 was made before and after AHD 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 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 treatment. The previous assay to identify in vitro autoimmunity elicited by AHD treatment 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. 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 Fai 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 as described above. To use intact cells as challenger, an aliquot of each cell population was mixed with responder cells at a ratio of 1 challenger cell to 1 responder cell. Briefly, autologous CD4+ CD25+ and CD4+CD25- cells and their thermal fractioned derivatives from each patient were tested as an antigen inducer of in vitro proliferation of responder cells.

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 immunological challenges and controls were tested in triplicate: the
medium control of 12 wells in the top row contained $10^3$ PBMCs in 100 $\mu$L of working RPMI 1640 medium plus an additional 100 $\mu$L of working RPMI 1640 medium. One hundred microliters of four dilutions (1:10, 1:100, 1:1,000, and 1:10,000) of the two PBMC hemoderivatives, CD4+CD25+ and CD4+CD25− (equivalent to $1 \times 10^6$ cells), the two aliquots of intact CD4+CD25+ and CD4+CD25− cells (10^5 cells), AHD (concentrated by lyophilization and reconstituted to 10X treatment dilution), CEA (2 $\mu$g), and 1/100 dilution of autologous plasma were added in triplicate to the wells of the second through the 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 nine wells of the seventh row. One hundred microliters of negative control (1:100 dilution of healthy male plasma in RPMI 1640 medium) were added to each of last three wells of the seventh row. PHA and RPMI 1640 were obtained from Sigma. Human CEA was from Fitzgerald Industries International, MA.

Plates were incubated in a 5% CO_2 incubator at 37°C for 5 d. One microcurie of tritiated thymidine was then added to each well in a volume of 20 $\mu$L 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 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 d 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. 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 11 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).

5. 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)

6. Toxicity assessment. Toxicity was evaluated using the common terminology criteria for adverse events, version 3.0 (CTCAE), of the US National Cancer Institute (14), the highest levels of each toxicity type detected were recorded.

7. Cumulative number of patient deaths associated to cancer progression was recorded monthly.

8. Statistical analysis. Tumor growth, number of patients persisting in PD, 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’s t-test. The cumulative number of deaths in each group was compared with the Fisher exact test. 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 (responders) were compared with patients persisting in progressive disease status (nonresponders). Immunological responses (DTH test and proliferation assay) in both subgroups were compared using the two-tailed, unpaired Student’s 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. An independent statistical review was made (Telemedical Organization).

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: 10 patients (out of 60) in the AHD-treated group and 9 patients (out of 60) in the control group were not evaluable. In the AHD-treated group, two patients refused to continue the programmed treatment and eight patients did not complete follow up. In the control group, two patients died of intercurrent cardiovascular complications, three patients did not complete follow up, and four patients refused to continue the programmed treatment.

Tumor growth was significantly lower in the group treated with A HD than in the untreated controls (Table 2A). Mean tumor growth with 95% confidence intervals in both groups is also shown in Table 2A. 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 pro-
gression status, all patients were in progression (PD) according to RECIST when they entered the study. The number of patients in PD at end of study was significantly lower in the AHD-treated group than in the untreated group (Table 2A). Twenty-six patients reach SD status in the AHD-treated group (responders). SD was not reached in control group. Table 2B shows that the mortality throughout the study was significantly lower in the AHD-treated group than in the control group. No clinically relevant toxicities, whether systemic or local, were reported in the AHD-treated group. No patient had to discontinue or modify treatment due to toxicity or side effects. As it is shown in Table 2B, 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 AHD-treated patients at the injection sites consisted of toxicity grade 1–2 (pain or pain + inflammation) in all cases.

There were significant variations in immunological parameters between AHD-treated and non-AHD-treated groups. In addition, significant variations in immunological parameters were observed within the AHD-treated group between the patients that attained non-progressive disease status (responders) and those who kept their progressive disease status (non-responders). DTH elicited by the intradermal AHD test became positive after AHD treatment in 24 of 50 evaluable patients and it remained negative in all 51 evaluable non-AHD-treated patients ($p < 0.001$). In the AHD-treated group, the number of patients with a positive ($\geq 5$ mm) response to the delayed-type hypersensitivity (DTH) test with AHD was significantly greater ($p < 0.001$) in the responder
patients than in the non-responders (Table 3A). 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 ($p < 0.001$) in the responses of lymphocyte proliferation were evident after AHD treatment when responder cells were challenged with CEA (Table 3B), the AHD hemoderivative obtained from the autologous CD4+CD25+ cell population or with the intact CD4+CD25+ cells (Table 3C). The selectivity of this immunologocal response was evident because AHD treatment failed to modify the negative response of lymphocytes to the autologous plasma (Table 3B), the hemoderivative from the autologous CD4+CD25+ cell population or with the intact CD4+CD25+ cells (Table 3C). In the AHD-treated group, the lymphocyte proliferation responses to CEA (Table 3B), AHD, CD4+CD25+ derivative and CD4+CD25+ cells (Table 3C) were significantly greater ($p < 0.001$) in the responder patients than in the non-responders.

Within the limitations of the assessment method employed, no relation was established between the circulating cancer cells and the efficiency of the hemoderivative to induce in vitro or in vivo immune responses.

Table 3A

<table>
<thead>
<tr>
<th>All Patients</th>
<th>DTH: initial (+)</th>
<th>DTH: final (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group ($n = 51$)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AHD Group ($n = 50$)</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>t-Test</td>
<td></td>
<td>$p = 0.0001$</td>
</tr>
<tr>
<td>AHD-Treated Group</td>
<td>DTH: initial (+)</td>
<td>DTH: final (+)</td>
</tr>
<tr>
<td>Responders ($n = 26$)</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Non-responders ($n = 24$)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>t-Test</td>
<td></td>
<td>$p = 0.001$</td>
</tr>
</tbody>
</table>

* Delayed type hypersensitivity test (DTH) performed with an autologous hemoderivative (AHD) before (INITIAL) and after (FINAL) treatment: control or AHD.

Responders or Non-Responders to AHD treatment are stratified.

AHD Group: patients treated with AHD; Control group: appropriate controls without AHD

Responders: patients achieving stable disease status; Non-responders: patients maintaining progressive disease status.

$n$: number of patients

+: diameter $\geq 5$ mm

Figure 1 show an example of the main histologic changes found in biopsies obtained before and after AHD treatment from patients who responded with a positive DTH test ($\geq 5$ mm) and significant antiprogressive effect on tumor growth. The example shown is from biopsies of colon cancer and control biopsies from the surrounding colon mucosa tissue (not compromised by cancer). Samples obtained before and after treatment (AHD and control) are shown. After AHD treatment, the results reproduce the histopathology previously reported in AHD responders: tumor stromal fibrosis with lymphocyte infiltration and a decrease in tumor cells and vascularity (1,3). The histology of the infiltrating lymphocytes showed a high predominance of CD8+ and CD20+ cells (B cells), with minimal presence of CD4+ cells. In this responder patient, after AHD treatment the control biopsies obtained from normal colon mucosa did not show any of these findings. Before AHD treatment the biopsies in tumor and normal colon mucosa were both negative for stroma and infiltration responses. Having the sample-accessibility conditions, 15 cases of responder patients could be studied and their histopathology conclusions were in all cases exemplified in Fig. 1. (No similar change was seen in 10 non-responder cases.)
Discussion

Antiprogresive Effect

Spontaneous tumor growth in this population of patients with advanced colon cancer was slowed down by AHD treatment in a statistically significant way. The sample size provided acceptable predictive power and significance. The advanced stage of the selected patients explains the high level of non-evaluable patients at the end of the follow up in both groups (risk factors for concurrent disease and protocol desertions are highly prevalent). It could be discussed if an antitumoral effect without disease remission (limited to disease stabilization) can be considered clinically relevant. However, these results are clinically auspicious because the study population was composed of patients with recurrent metastatic colon cancer, in progression and therapeutically terminal (resistant to available chemotherapy). Under these circumstances, the significant reduction in the number of cases in progression (PD) in AHD-treated patients is a favorable antiprogresive response that is an unexpected event in the spontaneous evolution of the disease. It may offer a new opportunity to these patients and opens a field of research on ways to optimize this result.

Immunological Mechanism

AHD treatment elicited statistically significant immunological responses in vivo (DTH) and in vitro (proliferation assay). The immunogen activity of AHD suggested in previous reports was confirmed in this study. The control group, which underwent the same immunomodulatory procedure (cyclophosphamide and rh-GM CSF) but did not receive AHD, showed no immunological response to AHD.

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Table 3B

<table>
<thead>
<tr>
<th></th>
<th>CEA</th>
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<th>AP</th>
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<tbody>
<tr>
<td></td>
<td>cpm</td>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td>Before treatment</td>
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<tr>
<td>After treatment</td>
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<tr>
<td>Mean [95% CI]</td>
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<td></td>
<td></td>
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<tr>
<td>Control Group</td>
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<tr>
<td>(n/v = 51/48)</td>
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<tr>
<td>181 [171–191]</td>
<td></td>
<td>1.10 [0.84–1.36] (*)</td>
<td></td>
</tr>
<tr>
<td>187 [174–200] (*)</td>
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<tr>
<td>AHD Group</td>
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<td></td>
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<td>(n/v = 50/46)</td>
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<td></td>
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</tr>
<tr>
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<tr>
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<tr>
<td></td>
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<tr>
<td>All Patients</td>
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</tr>
<tr>
<td>AHD-Treated Patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
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<td>(n/v = 26/24)</td>
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<td>6.86 [4.21–8.18] (*)</td>
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<td>174 [164–184]</td>
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<tr>
<td>1.121 [904–1.338] (*)</td>
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<td>Non responders</td>
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<tr>
<td>(n/v = 24/22)</td>
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<td>1.36 [0.56–2.14] (*)</td>
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<tr>
<td>176 [168–184]</td>
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<td></td>
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<tr>
<td>192 [168–216] (*)</td>
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<tr>
<td>t-test [Comparing (*)]</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.44</td>
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<tr>
<td></td>
<td>170 [160–180]</td>
<td>1.00 [0.97–1.03] (*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>168 [154–182] (*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>176 [162–190]</td>
<td>1.04 [0.96–1.12] (*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>180 [166–196] (*)</td>
<td></td>
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</tr>
</tbody>
</table>

AHD Group: patients treated with autologous hemoderivative (AHD); Control Group: appropriate controls without AHD; Responders: patients achieving stable disease status; Non-Responders: patients maintaining progressive disease status.

n = number of patients; v = number of evaluable patients’ tests (controls accepted).
Table 3C

Immunological Response: Lymphocyte Proliferation Assay Stimulated with the Autologous Hemoderivative (Immunogen), Regulatory Cells, and Their Derivatives

<table>
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<tr>
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<th>AHD</th>
<th>[CD4+CD25-] AD</th>
<th>[CD4+CD25+] AD</th>
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<tr>
<td></td>
<td>cpm Before</td>
<td>cpm Before</td>
<td>cpm Before</td>
</tr>
<tr>
<td></td>
<td>treatment</td>
<td>treatment</td>
<td>treatment</td>
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<tr>
<td></td>
<td>After treatment</td>
<td>After treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td></td>
<td>Mean [95% CI]</td>
<td>Mean [95% CI]</td>
<td>Mean [95% CI]</td>
</tr>
<tr>
<td>All Patients</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>p = 0.0001</td>
<td></td>
<td>p = 0.26</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>t-test p &lt; 0.0001</td>
<td></td>
<td></td>
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<tr>
<td>AHD-Treated Patients</td>
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<td></td>
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<tr>
<td>p = 0.002</td>
<td></td>
<td>p = 0.82</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>t-test p &lt; 0.002</td>
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<td></td>
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<tr>
<td>AHD</td>
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<tr>
<td>p = 0.0001</td>
<td></td>
<td>p = 0.26</td>
<td>p &lt; 0.001</td>
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<tr>
<td>t-test p &lt; 0.0001</td>
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<tr>
<td>AHD-Treated Patients</td>
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</tr>
<tr>
<td>p = 0.002</td>
<td></td>
<td>p = 0.82</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>t-test p &lt; 0.002</td>
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</tbody>
</table>

*PBMCs were incubated with the following challenge: autologous hemoderivative used as immunogen (AHD), autologous derivative (AD) from (CD4+CD25-) and (CD4+CD25+) cells, intact (CD4+CD25-) or (CD4+CD25+) cells.

AHD Group: patients treated with autologous hemoderivative (AHD); Control Group: appropriate controls without AHD; Responders: patients achieving stable disease status; Non-responders: patients maintaining progressive disease status.

n = number of patients; v = number of evaluable patients’ tests (controls accepted).
Comparing AHD-treated patients and the control group, laboratory assays were confirmatory of AHD intradermal tests. In a statistically significant number of cases, AHD elicited a positive skin DTH (> 5 mm) and positive proliferation of responder cells (PBMC) measured by thymidine incorporation (more than a fourfold increase).

Immunogen Characterization

As all cancer vaccines using other cells or tissue extracts as polyvalent immunogen, the cancer vaccination with an antigenically polyvalent blood extract allows better immunological characterization than exhaustive chemical characterization of its biological complexity. The present study, advancing from previous reports, reaches that level of immunogen characterization for AHD. Certainly, it was previously reported that AHD obtained from healthy individuals did not induce autologous immunization, that AHD obtained from arterial blood was a more efficient immunogen in cancer patients than AHD obtained from venous blood, and that the preliminary chemical characterization of AHD showed at least five protein fractions and several peptide components (1–3). As vaccines using cells as immunogens, i.e., tumor cell vaccines, AHD is a polymolecular and complex biological system, so it is potentially a polyvalent antigenic immunogen. In fact, this study showed that AHD works as a polyvalent antigen preparation because it induced a lymphocyte proliferative response addressed against at least one tumor marker (CEA), which is a tumor-associated antigen (TAA) present in the blood of the patients included in this study in compliance with

**Fig. 1.** Biopsies of colon mucosa from a patient with favorable response to AHD. Upper row: biopsy from tumor site. Lower row: biopsy from a non-tumor site. Columns 1 and 2: Before treatment. Columns 3 and 4: After 6 mo of treatment. Columns 1 and 3: Hematoxylin-eosin (HE). Columns 2 and 4: Immunohistochemistry. From top to bottom: Lymphocytes (HE), CD4+, CD8+, and B (CD20+) cells. (Histochemistry with the respective antibodies.)
the eligibility criteria. It is possible that other molecules transferred from malignant cells to the blood and not yet characterized could also be components of the antigenic polyvalence of AHD. The eligibility criteria of acquired resistance to chemotherapy and high serum tumor-marker levels define a patient population with tumors experiencing mutational events manifested by TAA expression and release to the blood. In addition to the tumor marker mentioned, among the antigenic activities identified in AHD by lymphocyte proliferation assay, this study showed that lymphocytes from patients with an antiprogressive response to AHD treatment were sensitized against components of a derivative that was prepared from autologous CD4+CD25+ cells and not to the same derivative prepared from other autologous CD4+ cell populations (CD25-). The results showed that intact CD4+CD25+ cells were also targets of this induced immune response and selectively in addition, because CD4+CD25- cells did not induce responder proliferation. AHD was prepared from blood supernatant containing plasma, platelets, and blood cells (mainly white blood cells) subjected to cytolysis by hypoosmosis and freezing. Therefore, molecules from plasma, such as tumor markers, and molecules from lysed platelets and blood cells, including the molecular components of different lymphocyte populations, were recovered in AHD. The presence of circulating tumor cells, evidenced by the employed method, is not a requirement for the autologous blood as source of the hemodervative immunogen (38% and 36%, respectively, in responders and non-responders), suggesting that the foundation of the hemodervative antigenicity is the molecular hemotransference from the tumor and not the cell migration to the blood.

**Autologous Antigenicity**

The polyvalent autoantigenicity of AHD must be the result of AHD preparation and the vaccination procedures. Molecular-selective denaturation is a known mechanism for enhancing or expressing antigenic activity. During AHD preparation, blood components are subjected to thermal fractionation, which has been used as a selective denaturation method to prepare immunogens from snake venoms (15). Reject immune response and breaking of tolerance have been reported repeatedly when different models of viral, autoimmune and malignant diseases were treated with a serum thermostable fraction (16-18). Inhibition of tolerance by pre-treatment with a low dose of cyclophosphamide (4) and activation of antigen-presenting cells (5) are also known mechanisms for improving immune responses elicited by low-power antigens or self-derivative antigens. During the vaccination procedure, AHD (a thermostable blood fraction) was repeatedly inoculated by the immunogenic subcutaneous route; tolerance was inhibited by cyclophosphamide, and human recombinant rhGM-CSF was used to activate antigen-presenting cells. The TAA s, which are nonspecific molecules generated specifically in malignancy or self-molecules overexpressed in malignancy, can elicit autologous immune responses (autoimmunity) when their antigenicity and immune presentation are empowered and the protecting tolerance is inhibited. Whichever was the mechanism involved; this study demonstrated that AHD, prepared and administered as described, effectively elicited an autologous immune response in vivo and in vitro.

**Tumor-Targeted Response**

In order to stratify the AHD-treated group into responders and nonresponders, patients attaining a clinical stable disease status according to the RECIST definition (between –30% and +20% of tumor growth variation) were considered responders. All patients were in progressive disease (more than 20% of tumor growth increase) when they were included in the study (eligibility criteria). The results showed that an autologous immune response in vivo (DTH) and in vitro (lymphocyte proliferation) was associated with antiprogressive clinical effect (stabilization disease) with very high statistical significance. The association and statistical significance was maintained for all antigens that induced in vitro lymphocyte proliferation.

In this study, the histopathology of biopsies obtained before and after AHD treatment in patients with an antiprogressive response confirms the reported effects of AHD in the pathology of autologous tumors of different primary sites: lymphocyte infiltration and stroma proliferation (1,3). It is shown that tumor tissue was the target of a host response, with mainly CD8+ lymphocyte and CD20+ B-lymphocyte infiltration with minimal presence of CD4+.
This host response was selective for tumor sites. Simultaneous biopsies of the same organ in sites not compromised by tumor did not show lymphocyte infiltration. These histologic findings in AHD-treated and responding patients are compatible with the fact that the immune response against AHD was also addressed against the tumor expressing TAA. However, the confirmation of these histopathologic findings also suggests direct or indirect activation of other intermediary mechanisms of action that originate the intense fibrosis observed. The predominant modification of tumor stroma composition is a novel and unexpected consequence of the tested AHD treatment. It could explain the AHD effect, which is mainly antiprogressive (disease stabilization) rather than tumoricidal (remissions), and it recalls connective tissue affection in autoimmune disease.

**Regulatory Cell-Targeted Response**

In recent years, it has been shown that a population of CD4+ T cells constitutively expressing the interleukin-2-alpha chain receptor or CD25+ has a prominent role in the maintenance of self-tolerance in humans, as was already known in rodents. These cells reach 10–15% of T4+ cells and are recognized as a regulatory population of T cells that is capable of suppressing the responses elicited in CD4+CD25– cells. Autoimmunity would be associated with quantitative or qualitative dysfunction of regulatory cells (12). More recently, it has been reported that the prevalence of regulatory T cells is increased in the peripheral blood and tumor microenvironment of patients with pancreas and breast adenocarcinoma (19). According to the results of the present study, the sensitization induced by AHD treatment was elicited by a CD4+CD25+ derivative but not by a similar derivative obtained from CD4+CD25– cells. The intact CD4+CD25+ cells were also a target of this autoimmune response, and this fact supports the hypothesis that AHD treatment enhances the autologous immune reject response against tumor antigens through down-regulation of CD4+CD25+ regulatory activity. As an immunogen, AHD is antigenically polyvalent and the response elicited by AHD is polytargeted, at least against autologous tumors and autologous regulatory cells. The advantage of simultaneous vaccination against CEA and CD4+CD25+ cells in colon cancer has recently been reported (11).

As has previously been reported, the toxicity of the AHD procedure was negligible, and no clinical autoimmunity or immunosuppressive phenomena were observed under the specific conditions of this study. The profile of the immunological response elicited by AHD could be defined as selective autoimmunity. The absence of autoimmune and immunosuppressive secondary effects suggests that the targets of this vaccination procedure include regulatory cells or regulatory cell components involved in the immune response elicited by AHD but do not include either pre-existing regulatory cells responsible of self-tolerance (i.e., native CD25+) or cells of the protective immune system (i.e., native CD4+). Within the frame of this study, the failure of AHD vaccination to elicit an immune response against autologous CD4+CD25– cells supports these suggestions.

**Conclusion**

It is confirmed that in recurrent metastatic colon cancer in progression with acquired resistance to available efficient chemotherapy, a previously described thermostable autologous hemoderivative can elicit a DTH immune response that is associated with an antiprogressive effect on tumor growth, disease stabilization, and a modification in tumor histopathology compatible with an immunological response and intense stromal fibrosis. These effects are associated to evidence of induction by AHD of lymphocyte sensitization against at least one tumor marker released by tumor cells (CEA) as well as a derivative obtained from autologous regulatory CD4+CD25+ cells. The absence of relevant toxicity in this study allows stating that the thermostable autologous hemoderivative described is a tool for developing a colon cancer vaccine that is antigenically polyvalent, cytologically polytargeted with tumor-antiprogressive effect and could be considered as a therapeutic option when all efficient chemotherapy treatments have been exhausted.

**References**


9. PDQ (Physician Data Query) National Cancer Institute, comprehensive cancer database.


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

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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. Immuno-

logic 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

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 ≥3000/mm³, ANC ≥1500/mm³, Hgb ≥9.0 g/dl, and platelets ≥100,000/mm³; 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>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Control</th>
<th>AHD-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Included/evaluable</td>
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<td>60/54</td>
</tr>
<tr>
<td>Age, years</td>
<td>57 (42–76)</td>
<td>59 (39–78)</td>
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<tr>
<td>Performance status: number cases in each grade (ECOG scale)</td>
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<tr>
<td>0</td>
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<tr>
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<td>Menopause status</td>
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<tr>
<td>Pre/Post</td>
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<td>17/37</td>
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<tr>
<td>Adjuvancy after surgery as primary tumor treatment: number of cases</td>
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<tr>
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<tr>
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<tr>
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<td>Measurable tumor burden: number of cases by metastasis site</td>
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<tr>
<td>Lymph nodes</td>
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<tr>
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<td>Non-measurable tumor burden: number of cases by metastasis site</td>
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<tr>
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<td>Resistance</td>
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<td>Interval: initial response-to-progression</td>
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<tr>
<td>Months: mean (range)</td>
<td>7.3 (4–9)</td>
<td>6.8 (3–8)</td>
</tr>
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</table>
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/m²) 3 days before the first AHD injection, and rhGM-CSF (150 mcg/m²) 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 and rhGM-CSF is an adjuvant of hematological and immunological recovery [5]. Symptomatic treatment was allowed throughout the study 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 and rhGM-CSF is an adjuvant of hematological and immunological recovery [5]. Symptomatic treatment was allowed throughout the study in both groups. A 4 weeks of treatment (AHD or Control), all patients included in the study continued to receive only symptomatic treatment. A 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. A after 24 h, the preparation was thawed and incubated at 100°C for 10 min. A after final filtration through a cellulose acetate membrane filter (0.22-µm pore diameter), the resulting A HD 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 A HD 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/m² 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/m² was given 3 days before starting the vaccination procedure to inhibit tolerance immune responses, as described [4]. A septic 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 VoluMasure®, 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-progressors. 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 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 CD 4+ cell population (results not shown); therefore, we tested two immunologically relevant subpopulations of autologous CD 4+ 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 CD 4+CD 25+ cell population and the other from the CD 4+CD 25− cell population; the other two challengers were the intact CD 4+CD 25+ and CD 4+CD 25− cells. Both cell populations (CD 4+CD 25+ and CD 4+CD 25−) 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 CD 4+CD 25+ and CD 4+CD 25− cells and their thermostable fractioned derivatives from each patient were tested as an antigen inducer 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 10⁵ PBMCs from the first aliquot, obtained as mentioned above, added to 100 µl of RPMI 1640 with 10% human A B 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 10⁵ 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, CD 4+CD 25+ and CD 4+CD 25− (equivalent to 1 × 10⁵ cells), the two aliquots of intact CD 4+CD 25+ and CD 4+CD 25− cells (3 × 10⁵ 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 phytohemagglutinin (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% CO₂ 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-D E ). 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 × 200 × 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 apparatus (Amersham Biosciences). After protein fixation with 50% methanol, containing 5% phosphoric acid excess of dye was washed out from the gels with H2O 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™ A lbumin/IgG R emoval K it 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 (CTCA E ), 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 treatment. 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
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).

### Table 2 Patients assessment

<table>
<thead>
<tr>
<th>Month</th>
<th>Control group</th>
<th>AHD-treated group</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Tumor growth % increase of tumor size in 30 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[-1] 24.7 23.8-25.4 24.7 23.8-25.6 0.95</td>
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<tr>
<td></td>
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<td>1 24.8 24.1-25.9 25.3 24.5-26.1 0.74</td>
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<td>2 24.2 23.1-25.3 24.6 23.3-25.5 0.58</td>
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<tr>
<th>Month</th>
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<tr>
<td></td>
<td>Disease progression monthly number of patients in each RESIST status</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>5</td>
<td>37</td>
</tr>
</tbody>
</table>

*-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

<table>
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<tr>
<th>Maximal toxicity [CTCAE]</th>
<th>Control group</th>
<th>AHD group</th>
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<tbody>
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<td>Systemic</td>
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<tr>
<td>Local</td>
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</table>

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
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. 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 3** Delayed type hypersensitivity elicited by a thermostable AHD

<table>
<thead>
<tr>
<th></th>
<th>DTH: Initial (+)</th>
<th>DTH: Final (+)</th>
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<tbody>
<tr>
<td>All patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHD-treated group ($n = 54$)</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Control group ($n = 54$)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$t$-Test</td>
<td></td>
<td>$P = 0.0001$</td>
</tr>
<tr>
<td>AHD-treated group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progressors ($n = 26$)</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Non-progressors ($n = 28$)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>$t$-Test</td>
<td></td>
<td>$P = 0.001$</td>
</tr>
</tbody>
</table>

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

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**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.
<table>
<thead>
<tr>
<th></th>
<th>CEA</th>
<th>CA 15-3</th>
<th>A P</th>
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<tr>
<td></td>
<td>cpm before treatment</td>
<td>LPR</td>
<td>cpm before treatment</td>
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<tr>
<td></td>
<td>Mean [95% CI]</td>
<td></td>
<td>Mean [95% CI]</td>
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<tr>
<td>All patients</td>
<td></td>
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<tr>
<td>Control group (n/v = 54/48)</td>
<td>136 [118–154]</td>
<td>1.03 [0.78–1.28]</td>
<td>128 [117–139]</td>
</tr>
<tr>
<td>* t-test</td>
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<tr>
<td>P &lt; 0.001</td>
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<tr>
<td>AHD-treated patients</td>
<td></td>
<td></td>
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<tr>
<td>* t-test [Comparing (*)]</td>
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<td></td>
<td></td>
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<tr>
<td>P &lt; 0.001</td>
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</table>

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

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<tr>
<td></td>
<td>cpm before treatment</td>
<td>cpm after treatment</td>
<td>cpm after treatment</td>
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<tr>
<td>Mean [95% CI]</td>
<td>Mean [95% CI]</td>
<td>Mean [95% CI]</td>
<td>Mean [95% CI]</td>
</tr>
<tr>
<td>All patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHD group (n/v = 54/46)</td>
<td>124 [108–140]</td>
<td>6.53 [4.90–8.16]</td>
<td>0.95 [0.62–1.08]</td>
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<td></td>
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<td></td>
<td>746 [824–898]</td>
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<tr>
<td>Control group (n/v = 54/48)</td>
<td>132 [106–148]</td>
<td>1.09 [0.80–1.38]</td>
<td>1.01 [0.87–1.15]</td>
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<td></td>
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<td></td>
<td>132 [120–144]</td>
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<tr>
<td>t-Test [Comparing (*)]</td>
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<tr>
<td>AHD-treated patients</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Progressors (n = 26/23)</td>
<td>130 [126–154]</td>
<td>7.77 [5.60–9.94]</td>
<td>1.03 [0.92–1.14]</td>
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<tr>
<td></td>
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<td></td>
<td>816 [498–726]</td>
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<tr>
<td>Non-progressors (n = 28/23)</td>
<td>134 [132–168]</td>
<td>0.94 [0.79–1.09]</td>
<td>1.09 [0.90–1.28]</td>
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<td></td>
<td>126 [144–164]</td>
<td>(*)</td>
<td>130 [116–132]</td>
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<td></td>
<td>138 [114–142]</td>
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<tr>
<td>t-Test [Comparing (*)]</td>
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</tr>
<tr>
<td>AHD group (n/v = 54/46)</td>
<td>124 [148–164]</td>
<td>5.97 [4.45–7.49]</td>
<td>1.05 [0.82–1.28]</td>
</tr>
<tr>
<td></td>
<td>740 [701–745]</td>
<td>(*)</td>
<td>122 [132–150]</td>
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<td></td>
<td></td>
<td></td>
<td>828 [824–898]</td>
</tr>
<tr>
<td>Control group (n/v = 54/48)</td>
<td>138 [150–186]</td>
<td>1.05 [0.78–1.32]</td>
<td>0.95 [0.80–1.10]</td>
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<tr>
<td></td>
<td>145 [161–189]</td>
<td>(*)</td>
<td>122 [142–166]</td>
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<td></td>
<td>126 [111–201]</td>
</tr>
<tr>
<td>t-Test [Comparing (*)]</td>
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<tr>
<td>AHD-treated patients</td>
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</tr>
<tr>
<td>Progressors (n = 26/23)</td>
<td>126 [126–154]</td>
<td>7.69 [6.62–8.72]</td>
<td>1.03 [0.91–1.15]</td>
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<td>749 [498–726]</td>
</tr>
<tr>
<td>Non-progressors (n = 28/23)</td>
<td>121 [132–168]</td>
<td>0.97 [0.90–1.04]</td>
<td>1.03 [0.94–1.12]</td>
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<td>126 [114–142]</td>
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<tr>
<td>t-Test [Comparing (*)]</td>
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</tbody>
</table>

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
concur 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, A HD proteome lost 158–174 spots compared with NHF-AHD that must be considered the thermolabile fraction in these experimental conditions. However, A HD 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 A HD proteome ranged from 24.000 to 190.000 kD. The A HD 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 A HD versus those treated with control. Of 54 patients, 28 (52%) met RECIST criteria for NPD. Therefore, these results provide evidence that treatment with A HD may be an effective anti-breast cancer strategy.

Immunological mechanism

A HD treatment elicited statistically significant autologous immunologic responses in vivo (DTH ≥ 5 mm) and in vitro (LPR > 4). A HD elicited in vivo DTH against A HD and in vitro immune responses against several tested challengers including the immunogen A HD and the TAAs (CEA and CA 15-3). These in vivo and in vitro immunologic responses were statistically associated not only to A HD treatment but also to the non-progressor condition of A HD treated patients. In addition, among the antigenic activities identified in A HD by lymphocyte proliferation assay, this study showed that lymphocytes from patients with an anti-progressive response to A HD treatment were sensitized against components of an autologous CD 4+CD 25+ cells derivative and not against the same derivative prepared from other autologous CD 4+ cell populations (CD 25–).

Immunogen characterization

In the present study, it was shown that after heat treatment, A HD 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 A HD. 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, CD 4+, CD 8+ and B (CD 20+) cells.
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, A HD is a polymolecular biological system; therefore it is potentially a polyvalent antigenic immunogen.

Autologous antigenicity

At least some of the antigenic activities demonstrated in A HD-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 T A A s 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 A HD 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 A HD procedure was negligible and no clinical autoimmunity or immunosuppressive phenomena were observed under the specific conditions of this study.

Anti-cancer activity of A HD 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 A HD 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


10. PDQ (Physician Data Query) National Cancer Institute, comprehensive cancer database


15. National Cancer Institute, US: Common terminology criteria for adverse events v3.0 (CTCAE)


Prostate cancer: autologous immunotherapy optimized by indoleamine-2,3-dioxygenase (IDO)-inhibitor as immune-tolerance breaker

E. Lasalvia-Prisco, E. Garcia-Giralt, S. Cucchi, J. Larrañaga

Background- In several peer review publications (Cancer Biol Ther 2003) our team has reported a procedure of cancer immunotherapy using an autologous thermostable hemoderivative vaccine (ATHV) with anti-progressive tumor effect in metastatic malignant disease from different primary tumors including prostate cancer. Last year (ASCO Meeting 2005) we have reported that the tolerance break for tumor associated antigens through the interference with CD4+CD25+ regulatory cells is a component of the mechanism of action of the ATHV antitumoral effect. Therefore, we have intended to optimize this autologous immunotherapy adding to the procedure different steps of immune-tolerance blockage selected due to their proven efficacy in pre-clinical models and their feasibility in the frame of ATHV technology. In this study we report the results in prostate cancer when the adjuvant step added was the translational knowledge the tolerance blockage by IDO-inhibition through 1-Methyl-Tryptophane or 1-MT (Munn DH et al J Exp Med 1999).

Methods- Thirty metastasic prostate cancer patients, hormone and chemotherapy resistant, Performance Status ≤ 2 and PSA progressing serum level, were included in this institutional-IRB approved phase I/II trial. The patients were randomized in 3 groups submitted to 3 different treatments: I, only sympthomatic; II, the previously reported ATHV and III, ATHV + simultaneous s.c. 1-MT. Tumor size increase (tumor growth) measured according RECIST was registered in each case. Mean difference in the 3 groups was statistically assessed (Student’s t-test). Tryptophane to Kynurenine conversion was tested to assess IDO inhibition.

Results- Tumor growth was significantly slower in Group II and III than in Group I (p<0.01 and p<0.005). Tumor growth was also significantly slower in Group III than in Group II (p<0.02). IDO-inhibition was confirmed only in Group III. No relevant toxicities were detected.

Conclusions- These results support that additional tolerance break by IDO-inhibition optimizes the tumor growth inhibition through immunotherapy with an autologous thermostable hemoderivative vaccine.

Topic Category: Developmental therapeutics: immunotherapy
Keyword: vaccines ; colon cancer ; autologous hemoderivative
Abstract ID: 12515

**Ovarian cancer: autologous immunotherapy optimized by remote adjuvancy of a silicate-induced granuloma**

E. Garcia-Giralt, E. Lasalvia-Prisco, S. Cucchi, E. Lasalvia-Galante, J. Vazquez, W. Golomar, J. P. Vincent

**Background-** Advanced ovarian cancer has been included in several studies about an immunotherapy procedure using an Autologous Thermostable Hemoderivative Vaccine (ATHV) with anti-progressive tumor effect in metastatic malignant disease from different primary tumors (*Cancer Biol Ther* 2003). Like in most cancer vaccines, the net antitumoral effect of ATHV has low intensity despite the addition of several adjuvant steps: immune response enhancement by GM-CSF, immune tolerance break by cyclophosphamide or interference with CD4+CD25+ regulatory cells (*E. Lasalvia-Prisco et al. ASCO 2003-2005*). The inclusion of another adjuvant immunomodulatory step to ATHV in patients with advanced ovarian cancer was explored in this study. The tested adjuvancy was the local and remote immune-stimulation elicited by a subcutaneous silicate-granuloma (SSG), a phenomenon well proven in preclinical models (*RM Fauve et al. Immune Lett 1987; E Fontan et al Proc Nat Acad of Sci 1983*).

**Methods-** Twenty four metastasic ovarian cancer patients, chemotherapy resistant, performance status $\leq 2$ and CA-125 progressing serum level, were included in this institutional-IRB approved phase I/II trial. The patients were 3-group randomized, submitted to 3 different treatments: I only symptomatic; II the previously reported ATHV and III ATHV + SSG. Tumor Growth was assessed in each case by the tumor size increase (RECIST measured). Mean difference in the 3 groups was statistically assessed (Student’s test).

**Results-** Tumor Growth was slower in Group II and III than in Group I (p<0.02, p<0.005). Tumor growth was slower in Group III than in Group II (p<0.02). No relevant toxicities were detected.

**Conclusions-** The results support that in advanced ovarian cancer an additional subcutaneous silicate granuloma as adjuvant agent optimizes the tumor growth inhibition through immunotherapy with an autologous thermostable hemoderivative vaccine. The bypass of the immune-ignorance could be discussed as its mechanism of action.

**Topic:** Developmental therapeutics: immunotherapy

**Keyword:** vaccines ; ovarian cancer ; autolgous immunotherapy
Breast cancer: draining lymph node of vaccination site targeted by adjuvant GM-CSF in an autologous vaccine

E. Garcia-Giralt, E. Lasalvia-Prisco, S. Cucchi, M. Aghazarian, J. Larrañaga, M. Brent, D. Walton

Background:
We have reported the sensitization against Tumor Associated Antigens (TAA) and the tumor antiproliferative effect of Autologous Thermostable Hemoderivative-Cancer Vaccine (ATH-CV) in breast cancer patients (E. Garcia-Giralt et al ASCO, 2006). Systemic immune response elicited by TAA is started by a locoregional immune response at the microenvironment constituted by the antigens source and the draining lymph node. The antigens source can be a tumor or a vaccination site and respectively, the draining lymph node is the sentinel lymph node (SLN) or the Sentinel Immunized Node (SIN). Tolerogenic or protective locoregional immune response is decisional because it starts a corresponding systemic immune response, tolerogenic or protective. GM-CSF is a conditioner of SLN and SIN, switching the locoregional immune responses from tolerogenic to protective (ML Disis et al Blood, 1996; AJ Cochran et al Nat Rev Immunology, 2006). Therefore, to optimize the ATH-CV antitumoral effect, we have explored GM-CSF as a locoregional adjuvant.

Methods:
Thirty six M1 breast cancer patients, ER+,HER 2-, hormone and chemotherapy resistant, performance status ≤ 2, and CA 15-3 rising level, were included in this prospective, IRB-approved phase I/II trial. Patients were 3-group (G) randomized (12 each), submitted to different treatments: G1, no additional oncology treatment; G2, ATH-CV; G3, ATH-CV plus GM-CSF at vaccination site 150 µg/day, 5 days.

Statistic assessment (Student’s t-test) was performed at the 30-day end point: ATH sensitization by Delayed Type Hypersensibility test (DTH) and Lymphocyte Proliferative Assay (LPA); CA 15-3 serum level and immunophenotyping of lymph node cells in biopsies of SIN scintigraphy-localized.

Results:
Significant differences (p<0.05):
DTH test > 5 mm: G1, 0/12; G2, 3/12 and G3, 6/12.
LPA > 2.0: G1, 0/12; G2, 4/12 and G3, 7/12.
30 days CA 15-3 increase: G1>G2>G3.
No relevant toxicities were evidenced.

Conclusions:
In advanced breast cancer, ATH-CV sensitization and tumor antiproliferative effect were potentiated by GM-CSF as local adjuvant. Increase of SIN mature dendritic cells is suggested as mechanism of action.
Prostate cancer: vaccine Sentinel Immunized Node (SIN) target for adjuvant locoregional chemotherapy in autologous vaccine

E. Lasalvia-Prisco, E. Garcia-Giralt, S. Cucchi, J. Vázquez, M. Brent, D. Walton

Background
In cancer patients, the systemic immune response elicited by Tumor Associated Antigens is started by a locoregional immune response at the microenvironment constituted by the antigen source (the tumor) and the draining sentinel lymph node (SLN). The locoregional immune response is decisional because it starts a systemic immune response configured as the SLN response: tolerogenic or protective (AJ Cochran, Nat Rev Immunol, 2006).

Cancer vaccines reproduce this locoregional immune response: the vaccination site as the tumor antigen source and the draining SIN as the SLN (ML Disis, Blood, 1996). In experimental models, several chemotherapy drugs, especially Etoposide, injected in the vaccination site switch the locoregional immune response from tolerogenic to protective (RJ Scheper, Invest New Drugs, 1984). This study explores Etoposide as a locoregional adjuvant of the previously described Autologous Thermostable Hemoderivative-Cancer Vaccine ATH-CV (E Lasalvia-Prisco, ASCO, 2006).

Methods
Thirty three M1 prostate cancer patients, hormone and chemotherapy resistant, performance status \(\leq 2\), and PSA rising level, were included in this prospective, IRB-approved phase I/II trial. Patients were 3-group (G) randomized (11 each), submitted to different treatments: G1, no additional oncology treatment; G2, ATH-CV; G3, ATH-CV plus Etoposide 200 µg at vaccination site days 1-4 after vaccine.

Statistic assessment (Student’s t-test) was performed at the 30-day end point: ATH sensitization by Delayed Type Hypersensibility test (DTH) and Lymphocyte Proliferative Assay (LPA); PSA serum level and cells immunophenotyping in biopsies of SIN scintigraphy-localized.

Results
Statistical significant (p<0.05): 
DTH test > 5 mm: G1, 0/11; G2, 4/11 and G3, 8/11. 
LPA > 2.0: G1, 0/11; G2, 5/11 and G3, 9/11. 
30 days PSA increase: G1>G2>G3. 
No relevant toxicity was evidenced.

Conclusions
In advanced prostate cancer, when all other treatments are exhausted, the sensitization and tumor antiprogressive effect of ATH-CV were potentiated by Etoposide as local adjuvant. SIN depletion of tolerogenic cells is suggested as mechanism of action.
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IMMUNOTHERAPEUTIC SITE IN BREAST CANCER: A NEW METHODOLOGY FOR THE AUTOLOGOUS THERMOSTABLE HEMODERIVATIVE CANCER VACCINE (ATH-CV)

Garcia-Giralt Emilio1, Lasalvia-Prisco Eduardo2,3,4, Cucchi Silvia3, Robinson Leonard2, Dalton John2
1Centre De Cancérologie Hartmann, Neuilly Sur Seine, France, 2PharmaBlood Inc, Department of Research & Development, Florida, USA, 3Interdoctors, Department of Advanced Medical Treatments, Montevideo, Uruguay, 4National Institute of Oncology, Montevideo, Uruguay

Tumor as source of Tumor Associated Antigens (TAA) and Sentinel Lymph Node (SLN) have been recognized as the scenario for the first immune 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. In early stages of cancer, tumor's cytokines reach the SLN conditioning its cellular microenvironment to produce local permissive (tolerogenic) immune responses. This local tolerogenic immunity is decisional because it starts a systemic also permissive immunity. The tumor grows and disseminates.

To counteract this malignant mechanism of immune escape, we have designed a medical procedure to create an Immunotherapeutic Site (IT) that reproduces distant from the tumor, a TAA source and a draining lymph node but with a cellular microenvironment conditioned to promote local protective immune responses instead of permissive immune responses.

In this study, we tested an IT using the ATH-CV as TAA source and Granulocyte Macrophage-Colony Stimulant Factor (GM-CSF) plus Etoposide (ETP), injected both in the vaccination site, as conditioner agents to promote the protective (immunogenic) immune responses. The immunophenotyping of lymph node cell populations showed that IT reverted the ratio between T-Regulatory (suppressor) cells and Activated Antigen Presenting (Mature Dendritic) cells inside the lymph nodes, in contrary sensu to the ratio induced by the tumor. The results also showed that the lymph node conditioning was first limited to the vaccination-site draining lymph node or Sentinel Immunized Node (SIN) and later reached the systematic lymph nodes. In conclusion, the tested procedure, IT with ATH-CV, GM-CSF and ETP, conditions a cancer vaccination inducing a better cellular frame to elicit a protective systemic anti-tumoral immune response.
Conditioning of vaccine sentinel lymph node as adjuvant of Autologous Hemoderivative Breast Cancer Vaccine


Background.— Tumor and Sentinel Lymph Node (SLN) configure the first interaction between malignant disease and patient's immune system. As a consequence of this interaction, Tumor Associated Antigens (TAA) elicit a local immune response 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 and 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, the local interaction tumor-immune system but producing tumor antiprogressive immunity. In this design, TAA source is a cancer vaccine and its draining lymph node is the Vaccine Sentinel Lymph Node (VSLN). Immuno-modulative adjuvants (IMAs) condition ITS cellular microenvironment, promoting local protective instead of permissive immune responses. Due to ITS decisional role, this local protective immunity starts a systemic anti-tumoral immune response.

Material and Methods.— Forty breast cancer patients (T3N1M0), 4-group randomized, were submitted to Autologous Thermostable Hemoderivative-Cancer Vaccine (ATH-CV) according Lasalvia-Prisco et al (1995-2006). In Groups 2 to 4, ATH-CV was associated with 1 to 3 IMAs: Magnesium Silicate (Si), Granulocyte Macrophage-Colony Stimulant Factor (GM-CSF) and Etoposide (ETP). Thirty days after vaccination, the groups were compared exploring the VSLN immunophenotyping (IP) and the systemic immunogenic and immunotherapy vaccine effects (IG & IT). IP was evaluated as % of mean values reported in lymph nodes of cadaver samples. IG was assessed by percent of positive cases (>5mm) in Delayed Type Hypersensitivity (DTH) tested with ATH-CV. IT was measured as percent of 30 days tumor growth (RECIST). Statistic assessment was performed by Student's t-test.

Results
Groups (n=10), alla treated with ATH-CV
1: No adjuvant :
VSLN (IP) : Activated APC + 15 ; T-Reg Cells + 5
IG & IT Effects: DTH+ (%) 30; Tumor Growth – 26

2: Si Adjuvant
VSLN (IP) : Activated APC + 24 ; T-Reg Cells + 2
IG & IT Effects: DTH+ (%) 33; Tumor Growth – 34

3: Si + GM-CSF adjuvant :
VSLN (IP) : Activated APC + 44 ; T-Reg Cells + 3
IG & IT Effects: DTH+ (%) 43; Tumor Growth – 42

4: Si + GM-CSF + ETP adjuvant :
VSLN (IP) : Activated APC + 45 ; T-Reg Cells - 20
IG & IT Effects: DTH+ (%) 55; Tumor Growth - 52

Conclusions.— ITS, including ATH-CV, Si, GM-CSF and ETP, enhances the effects of ATH-CV only, immunogenicity and slowing tumor growth. These ITS effects are associated with a switch of VSLN cellular microenvironment, conditioning a local and systemic protective immunity.
Conditioning of Vaccine Sentinel Lymph Node as adjuvant of Autologous Hemoderivative Breast Cancer Vaccine

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Tumor and Sentinel Lymph Node (SLN) configure the first interaction between malignant disease and patient’s immune system. As consequence of this interaction, Tumor Associated Antigens (TAA) elicit a local immune response 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 and permissive immunity. The tumor progresses (1).

Recently, in order to counteract this mechanism, we have designed a medical procedure to create an Immunotherapeutic Site (ITS) that reproduces, distant from the tumor, the local interaction tumor-immune system but producing antiprogressive tumor immunity (2-5). In this design, TAA source is a cancer vaccine previously described, the Autologous Thermostable Hemoderivative-Cancer Vaccine or ATH-CV (6) and its draining lymph node is the Vaccine Sentinel Lymph Node (VSLN). ITS also includes the local injection of immunomodulative adjuvants (IMAs) that condition its cellular microenvironment, promoting local protective instead of permissive immune responses. Due to ITS decisional role, this local protective immunity starts a systemic anti-tumor immune response. The IMAs included in ITS are the known immunomodulative agents: Magnesium Silicate subcutaneous granuloma or Si (7), GM-Colony Stimulating Factor or GM-CSF (8) and Etoposide or ETP (9). ITS enhances the immunogenicity and slowing tumor growth effects repeatedly reported using ATH-CV exclusively. These ITS effects are associated with a switch to cellular VSLN local and systemic protective immunity.

Methods

Forty breast cancer patients (T3N1M0), 4-group randomized, were submitted to Autologous Thermostable Hemoderivative-Cancer Vaccine (ATH-CV) as it was described (6). In Groups 2 to 4, ATH-CV was associated with 1 to 3 IMAs: Magnesium Silicate (Si), Granulocyte Macrophage-Colony Stimulant Factor (GM-CSF) and Etoposide (ETP). Thirty days after vaccination, the groups were compared exploring:

- the VSLN immunophenotyping (IP)
- the systemic immunogenic vaccine effect (IG)
- the immunotherapy vaccine effects (IT)

IP was evaluated as % of mean values reported in lymph nodes of cadaver samples (10).

IG was assessed by percent of positive cases (>5mm) in Delayed Type Hypersensitivity (DTH) tested with ATH-CV.

IT was measured as percent of pre-treatment 30 day tumor growth (RECIST).

Statistic assessment was performed by Student’s t-test.

Results

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ATH-CV Adjuvant(s)</th>
<th>VSLN (IP)</th>
<th>IG &amp; IT Effects</th>
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</tr>
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<td>+3</td>
</tr>
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<td>4</td>
<td>10</td>
<td>Si + GM-CSF + ETP</td>
<td>+45</td>
<td>-20</td>
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</table>

Conclusions

Bibliographic references showed that Immunotherapeutic Sites (ITS) are a new medical procedure to improve the anti-tumor immune responses in cancer patients. To perform ITS, a cancer vaccine and three immunomodulative agents are injected in one or several subcutaneous sites draining to a non-tumor-draining lymph node. Each of these ITS works as an engine switching the systemic tumor-conditioned-tolerogenic immunity to anti-tumor systemic immunity. We are reporting the Vaccine Sentinel Lymph Node and systemic anti-tumor immunomodulative effects obtained in advanced breast cancer patients using an Autologous Thermostable Hemoderivative as Cancer Vaccine and Magnesium Silicate Granuloma, GM-CSF and Etoposide as the 3 immunomodulative agents. These immunological effects were associated with slowing of tumor growth.

References


The Objective of the authors team:
to develop new and better medical procedures
Advanced ovarian cancer: Vaccination site draining lymph node as target of immuno-modulative adjuvants in autologous cancer vaccine

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
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 antitumoral 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 (Schepers et al 1984; Tan et al 1986; Claessen et al 1989, 1991, 1992; Limpens, Garssen, Schepers et al 1990. Limpens. Garssen, Gemertaad et al 1990; Limpens and Schepers 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 ≤2 on the Eastern Cooperative Oncology Group scale (Oken et al 1982) or ≥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 ≥ 3000/mm³, ANC ≥ 1500/mm³, Hgb ≥ 9.0 g/dl, and
platelets ≥100,000/ mm³; 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 ru 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 until use. 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 marked on the skin by the radiologist, detecting the blue dye stained node. Patients consented to this procedure 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 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.

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 30-day Tumor Growth (%)

- **Group 1**
  - Pre-treatment: 27.1 (±5.1)
  - Post-treatment: 18.2 (±4.9)

- **Group 2**
  - Pre-treatment: 24.7 (±4.3)
  - Post-treatment: 14.9 (±3.1)

- **Group 3**
  - Pre-treatment: 28.2 (±5.1)
  - Post-treatment: 16.7 (±2.7)

- **Group 4**
  - Pre-treatment: 25.6 (±4.3)
  - Post-treatment: 10.0 (±3.1)

**Figures**

**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 (±4.9). Group 2: ATH-CV + GM-CSF. Pre-treatment: 24.7 (±4.3); post-treatment: 14.9 (±3.1). Group 3: ATH-CV + ETP. Pre-treatment: 28.2 (±5.1); post-treatment: 16.7 (±2.7). Group 4: ATH-CV + GM-CSF + ETP. Pre-treatment: 25.6 (±4.9); post-treatment: 10.0 (±3.1). Lower Row – Lineal increase in tumor size in all groups and in pre and post-treatment. The treatments slowed the tumor growth but did not stop it.
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 (CD1a^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,
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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>S.D.</th>
<th>Mean</th>
<th>S.D.</th>
<th>p</th>
<th>Mean</th>
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<th>p</th>
<th>Mean</th>
<th>S.D.</th>
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<td>7.5</td>
<td>1.6</td>
<td>&gt;0.9</td>
<td>11.8</td>
<td>2.0</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Group 2 (**)</td>
<td>9.2</td>
<td>1.1</td>
<td>9.0</td>
<td>1.2</td>
<td>&gt;0.9</td>
<td>3.4</td>
<td>1.1</td>
<td>&lt;0.03</td>
<td>3.2</td>
<td>0.8</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

*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; CD11c; 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.

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).
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.

**References**


Erythrocytes as autologous-vaccine carriers in advanced prostate cancer.

**Abstract No:** 14017  
**Citation:** *J Clin Oncol* 26: 2008 (May 20 suppl; abstr 14017)  
**Author(s):** E. M. Lasalvia-Prisco, E. Garcia-Giralt, E. M. Lasalvia-Galante, J. Vázquez, S. Cucchi, G. Spera, J. P. Valentine  

**Background:** Malignant tumors condition sentinel lymph nodes to produce permissive immune responses. This local-regional conditioning is decisional because it starts also permissive systemic immunity (Cochran 2006). In some cancer vaccine platforms, immunomodulatory adjuvants counteract this mechanism of tumor progression switching the cell population profile of vaccine sentinel lymph node (VSLN) from permissive to protective (Disis 1996). GM-CSF and etoposide (ETP) showed this activity in the VSLN of a subcutaneous immunotherapeutic site (ITS) where autologous thermostable hemoderivative cancer vaccine (ATH-CV) was also injected (Lasalvia-Prisco 2007). In advanced prostate cancer, we explored to optimize the delivery of ATH-CV to the conditioned VSLN using autologous erythrocytes (AE) as carriers emulating other demonstrated models (Chiarantini 1997).

**Methods:** Thirty M1 prostate cancer patients (Pts), hormone and chemotherapy resistant, performance status <2, PSA rising level, were included in this Helsinki Declaration compliant phase I/II trial and 3-group randomized: G1, no additional treatment; G2, ITS with (ATH-CV)+(GM-CSF)+ETP; G3, ITS with ATH-CV encapsulated in AE as it was described (Ihler 1973)+(GM-CSF)+ETP. The procedures were repeated each 30 days, 4 times; 180-day follow-up; end point 180th day. Assessments were performed at 1st and 180th day: (a) delayed hypersensitivity test (DHT) and lymphocyte proliferation assay (LPA) elicited with pre-treatment ATH-CV, (b) 30-day PSA increase and tumor growth. Groups were statistically compared (Student t-test). CTCAE toxicity was assessed. **Results:** Post-treatment Increase of cases DHT>5mm (G1=0, G2=4, G3=6) and LPA>2.00 (G1=1, G2=5, G3=8) were significantly higher in G2 vs G1 (p<.02, p<.03), G3 vs G2 (p<.05, p<.03). Post-treatment variation (%) of 30-day PSA increase (+8,-16,-34) and tumor growth (+6,-22,-38) were significantly higher in G2 vs G1 (p<.01, p<.01, G3 vs G2 (p<.03, p<.02). No significant toxicity (>2) was registered. **Conclusions:** In advanced prostate cancer, all other treatments exhausted, the sensitization and tumor anti-progressive effect of ITS with GM-CSF, ETP and ATH-CV were safely potentiated if ATH-CV is carried by autologous erythrocytes.

**Abstract Disclosures**

http://www.abstract.asco.org/AbstView_55_30995.html
Erythrocytes as autologous-vaccine carriers targeted in breast cancer sentinel lymph node.

Sub-category: Other: developmental therapeutics: immunotherapy
Category: Developmental Therapeutics: Immunotherapy
Meeting: 2008 ASCO Annual Meeting

Abstract No: 14018
Citation: J Clin Oncol 26: 2008 (May 20 suppl; abstr 14018)
Author(s): E. Garcia-Giralt, E. M. Lasalvia-Prisco, E. M. Lasalvia-Galante, J. Vázquez, S. Cucchi, G. Spera, J. P. Valentine

Abstract: In the development of an autologous thermostable hemoderivative cancer vaccine (ATH-CV), an immunotherapeutic site (ITS) was described as the subcutaneous access to the vaccine sentinel lymph node (VSLN). Through ITS, VSLN is reached first by immunomodulatory agents and later by the immunogen ATH-CV. It was demonstrated that immunomodulatory agents GM-CSF and etoposide (ETP) condition VSLN, switching permissive to protective immunity (Disis, 1996; Lasalvia-Prisco, 2007). Continuing the development of this platform, we explored to optimize vaccine immunogen delivery from ITS to the conditioned protective responder VSLN using autologous erythrocytes (AE) as carriers following other demonstrated models (Chiarantini, 1997). In this study we report the results in breast cancer patients.

**Methods:** Immediately after diagnosis, 30 breast cancer patients, stage I-II, with surgery and sentinel lymph node (SLN) staging scheduled, were included in this prospective, Helsinki Declaration compliant, phase I/II trial, 3-group randomized and submitted to an ITS in tumor periphery: G1, Saline; G2, GM-CSF+ETP+(ATH-CV); G3, GM-CSF+ETP+AE (ATH-CV) loaded. At surgery, 26 to 28 days afterwards, SLN was localized by technetium-99, removed and examined: half, pathology and half, flow cytometry immunophenotyping (CD1a+CD83+) and (CD4+CD25+) cells. After surgery, ATH-CV sensitization was tested: intradermal DTH and lab lymphocyte proliferation assay (LPA). Groups were statistically compared by Student t-test (St).

**Results:**
- VSLN pathology was negative for cancer cells in all specimens.
- CD1a+CD83+ cells in VSLN (by 600 CD3+T cells): G1=4.2; G2=11.4; G3=15.4. St: G2 vs G1, p<0.01; G3 vs G2, p<0.05.
- CD4+CD25+ cells in VSLN (% of CD4+ T cells): G1=14.3; G2=4.1; G3=4.3. St: G2 vs G1, p<0.01; G3 vs G2, p>0.05.
- DTH>5mm (% cases): G1=0; G2=40; G3=80.
- LPA; (CPM Test / CPM Control); mean (SD): G1=0.98 (0.34); G2=3.66 (0.68); G3=5.17 (0.76).

**Conclusions:** Autologous erythrocytes enhanced the systemic immune responses elicited by ATH-CV acting as carrier of vaccine immunogen from ITS to the immunological protective conditioned VSLN.

**Abstract Disclosures**
Randomized phase II clinical trial of chemo-immunotherapy in advanced nonsmall cell lung cancer

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Emilio García-Giralt2
Jesús Vázquez2,4
Marta Aghazarian4
Eduardo Lasalvia-Galante3,4
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Abstract: The purpose of this study was to compare chemotherapy-naive patients with stage IV nonsmall cell lung cancer patients treated with chemotherapy or chemoimmunotherapy. We tested doxetacel plus cisplatinum as chemotherapy protocol. An immunomodulatory adjuvant system was added as chemoimmunotherapy to the previously mentioned protocol. This system contains three well-known and complementary conditioners of protective immune-responses: cyclophosphamide low-dose, granulocyte macrophage-colony stimulant factor and magnesium silicate granuloma. Eighty-eight patients were randomly assigned to receive every 3-weeks one of the treatments under comparison. Patients received four cycles of treatment unless disease progression or unacceptable toxicity was documented. The maximum follow-up was one year. In each arm, tumor response (rate,duration), median survival time, 1-year overall survival, safety, and immunity modifications were assessed. Immunity was evaluated by submitting peripheral blood mononuclear cells to laboratory tests for nonspecific immunity: a) phytohemaglutinin-induced lymphocyte proliferation, b) prevalence of T-Regulatory (CD4+CD25+) cells and for specific immunity: a) lymphocyte proliferation induced by tumor-associated antigens (TAA) contained in a previously described autologous thermostable hemoderivative. The difference (chemotherapy vs. chemoimmunotherapy) in response rate induced by the two treatments (39.0% and 35.0%) was not statistically significant. However, the response duration (22 and 31 weeks), the median survival time (32 and 44 weeks) and 1-year survival (33.3% and 39.1%) were statistically higher with chemoimmunotherapy. No difference in toxicity between both arms was demonstrated. A switch in the laboratory immunity profile, nonspecific and specific, was associated with the chemoimmunotherapy treatment: increase of proliferative lymphocyte response, decrease of tolerogenic T-regulatory cells and eliciting TAA-sensitization.

Keywords: lung cancer chemotherapy, lung cancer chemoimmunotherapy, cancer vaccine, immunomodulatory cancer treatment, immunotherapy adjuvants, cancer therapy

Introduction

Despite aggressive treatment with surgery, radiation and chemotherapy, lung cancer is the leading cause of cancer mortality, resulting in more than 160,000 deaths per year in the United States and 1.2 million worldwide. Nonsmall cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers (ALA 2005).

The treatment of advanced stages of lung cancer with systemic chemotherapy has obtained modest results (Socinski et al 2003). Immunotherapy, another systemic treatment, also has been disappointing since the historic intents with BCG (bacille Calmette Guérin; Hadžiev et al 1982), Corynebacterium parvum (Issell et al 1978), and thymosin (Chretien et al 1979) up to the recent trials with humanized antibodies targeting specific tumor-associated antigens (Lynch et al 2004). However, in last years, new platforms for chemotherapy as tumor antigens releaser and immunotherapy as immunomodulatory conditioner have introduced a rational for the association of these platforms in a
chemoimmunotherapy protocol in order to improve the results of advanced NSCLC treatment.

In fact, chemotherapy is a tumor antigens releaser. In malignant disease spontaneously progressing, without treatment, the content of dying tumor cells is transferred to the interstice, to the lymph and to the blood. Some components of that content are tumor-associated antigens (TAA). This malignant tumor content, released from dying cells, meets the immune system in the interstice, in the lymph, and also in the circulating blood. This mechanism can work as a spontaneous endogenous vaccination. However, it is evident the failure of tumor immune control by this spontaneous endogenous vaccination in progressive cancer. This failure could be result of the low antigenicity of TAA released from spontaneous apoptotic tumor cells death (Melcher et al 1998) and the conditioning of tolerogenic or permissive immune response induced during the carcinogenesis (Cochran et al 2006). Apoptotic tumor cell death increases when malignant disease is submitted to oncological treatments, mainly chemotherapy. Cell death through apoptosis is less immunogenic than cell death through necrosis (Melcher et al 1999). However, the immunogenicity is maintained when apoptosis follows a cellular stress (Feng et al 2002) as it is produced by chemotherapy (Tiligada 2006). Therefore, chemotherapy works, at least partially, as an endogenous vaccination enhancer with high efficacy as releaser of TAA antigens, one of the components in the configuration of cancer vaccines (Raez 2005).

Immunomodulatory conditioning is a new platform of immunotherapy. The goal is to switch the tolerogenic (permissive) tumor-induced conditioning of the immune responses to immunogenic (protective) immune responses to immunogenic (protective) immune responses (Pinedo et al 2000; Rini et al 2005). A tool to accomplish this goal is the described systemic depletion of tolerogenic T-regulatory cells population (CD4+CD25+) by cyclophosphamide at low dose, injected a short period (three days) before antigen stimulation (Berd et al 1982; Ghiringhelli et al 2004). Another tool is the recently described immunomodulatory site (ITS), where different immunomodulatory agents are injected in order to induce in the draining lymph node an increase of activated antigen-presenting cells and a depletion of tolerogenic T-regulatory cells. This locoregional immunomodulation is decisional because it elicits a systemic protective lymph nodes conditioning. Among other agents (erythrocytes or other local inflammatory agents) to create an ITS, magnesium silicate that produces a subcutaneous granuloma (MSG) was reported as a strong inducer of remote macrophage activation with enhancement of protective antitumor immune responses, when it is performed during the 4 days previous to the antigen (Fauve and Hevin 1977; Fontan et al 1983, 1992; Fauve et al 1987). It was also reported that granulocyte macrophage-colony stimulating factor (GM-CSF), injected subcutaneously, simultaneously or around the time of antigen stimulation, is a recruiter and activator of antigen presenting cells, mainly dendritic cells (Disis et al 1996; Dranoff 2002).

Briefly, the association of systemic low-dose cyclophosphamide and a locoregional lymph node immunomodulation by MSG and GM-CSF is a safe immunomodulatory adjuvant system (IAS) of cancer vaccines supported by the proven properties of their components and evidenced in previous clinical trials (Garcia-Giralt et al 2006, 2007; Lasalvia-Prisco et al 2007a, 2007b, 2007c). This study compared in advanced NSCLC, the antitumoral and immunological effects of the standard chemotherapy and chemoimmunotherapy designed using the same chemotherapy in its platform of tumor antigens releaser and the platform of immunomodulatory conditioning immunotherapy through and IAS with cyclophosphamide, MSG, and GM-CSF.

Patients and methods

Patients and trial

Patients were submitted to a randomized phase II study. The trial accomplished the flow diagram and checklist of the CONSORT statement in conjunction with the CONSORT explanation and elaboration document (Altman et al 2001; Moher et al 2001).

The patient’s characteristics are summarized in Table I. Eighty-eight patients who met the following eligibility criteria

<table>
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<th>Table 1. Patients’ characteristics</th>
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<tr>
<td>Adenocarcinoma</td>
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Notes: Stage IV NSCLC randomized in two arms: CHT, treated with chemotherapy (doxetacel + cisplatinum) and CHIMT. Performance status ≤2. No prior chemotherapy, radiotherapy or surgery. No brain metastasis. Abbreviations: CHT, treated with chemotherapy (doxetacel + cisplatinum); CHIMT, treated with same chemotherapy + an immunomodulatory adjuvant system (IAS).
were included. The criteria were: histopathologically confirmed diagnosis of inoperable NSCLC (stage IV); age ≤ 75 years; Eastern Cooperative Oncology Group performance status (Oken et al 1982) ≤ 2; no prior malignancy, chemotherapy, surgery, or radiotherapy; no central nervous system metastases and at least one measurable lesion; tumor burden comprising no more than 3 metastasis sites; no associated acute disease. Conservation of organic functions was confirmed (adequate bone marrow function: WBC 3000/mm³, ANC 1500/mm, Hgb 9.0 g/dl, and platelets 100,000/mm³; adequate liver function: bilirubin ≤ 1.5 mg/dl, aspartate aminotransferase ≤ 40 IU/L; adequate kidney function: creatinine ≤ 1.5 mg/dl). The study was conducted in patients admitted to medical centers that submitted medical data to the Cooperative Trials Center (CTC) of Interdoctors Medical Procedures, Florida, USA (Interdoctors Medical Procedures is a nonpharmaceutical concern group supporting scientific research in medical procedures). 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 (WMA 1997). In a prospective, randomized, controlled trial, the treating physicians did not participate in the arm randomization for their patients. The patients were randomized into 2 groups that received different treatments: chemotherapy (CHT) or chemoimmunotherapy (CHIMT). Basically, CHIMT is CHT with the IAS associated to each chemotherapy series.

**Chemotherapy**

CHT protocol was one of the therapeutic options recommended in the PDQ (Physician Data Query) database of the National Cancer Institute for Stage IV, NSCLS: doxetacel plus cisplatinum (Georgoulias et al 2004). Briefly, doxetacel 100 mg/m² on day 1, and cisplatinum 80 mg/m² on day 2. The series were repeated every 3 weeks.

**Immunomodulative adjuvant system**

IAS was added to the study design (CHIMT arm) in each chemotherapy series, considering day 2 as the start day of tumor cell affectation by chemotherapy. Cyclophosphamide 300 mg/m² was administered on day-1 of each series; GM-CSF 300 µg SC was administrated days 2 to 5, daily, of each series and a subcutaneous granuloma was induced with 500 mg magnesium silicate on day-2 of each series.

A maximum of 4 series of CHT or CHIMT were programmed.

**Assessment**

In all patients, data was collected at baseline and followed until death, loss of follow-up, or until a maximum of one year.

Overall tumor response and safety were documented 8 weeks after completion of treatment. The overall response rate was expressed as the proportion of patients demonstrating CR (complete remission) or PR (partial remission) based on all patients randomly assigned to receive treatment and according to the response evaluation criteria in solid tumors (RECIST). All responses required confirmation at least four weeks after they were first observed (Therasse et al 2000).

Response duration (RD) was calculated from the first date of a 50% reduction in the tumor was registered to the last date that tumor reduction was documented.

At the end of the follow-up (1 year), survival parameters were analyzed for each group using the Kaplan-Meier method from the first day of treatment to death or the date of the last follow-up visit for patients who were still alive. Median time survival (MTS) and one year overall survival (1-OS) were estimated. Assessment of safety was based on reports of adverse events, laboratory-test results, and vital-sign measurements. Adverse events were categorized according to the common toxicity criteria of the National Cancer Institute (CTCAE), version 2 (NCI 2006).

**Laboratory tests**

The laboratory tests were designed taking in account preliminary assays in patients treated with each component of IAS, showing accumulative increase of peripheral blood monomolecular cells (PBMC) proliferation responses to phytohemaglutinin (PHA) and decrease of T-regulatory cells (results not shown) supporting the use of the associated three IAS components in this study.

In a sample of PBMC, lymphocyte proliferation assay induced with PHA and T-regulatory Cells (CD4+CD25+) prevalence (T-Reg) were assessed as nonspecific immune-reactivity parameters. It was previously demonstrated that an autologous thermostable hemoderivative (ATH) contained TAA (Lasalvia-Prisco et al 2003, 2006a, 2006b); therefore, lymphocytes proliferation assay induced with this ATH was evaluated as specific assay for immunity against circulant TAA.

**Isolation of PBMC**

Heparinized blood was diluted 1/1 v/v with phosphate buffer solution (PBS) before Ficoll density centrifugation. The buffy coat containing PBMC was harvested, contaminating
red blood cells lysed by incubating in ACK-lysing buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4), and washed twice in cold PBS. A PBMC sample in each patient was prepared at days-3, 3 and 20 of the first treatment series.

**PHA and ATH lymphocyte proliferation assay**

PHA was obtained from Sigma (St. Louis, MO). For each test, a fresh ATH was prepared as it was described (Lasalvia-Prisco et al 2003).

PHA and ATH lymphocyte proliferation assays were made immediately after obtaining each sample of PBMCs, days-3, 3 and 20 by incubating 10⁷ PBMCs, from an aliquot of PBMC sample 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. Two immunologic challenges and appropriate controls were tested in triplicate:

- a) Medium control in the top row, an additional 100 μl of working RPMI 1640 medium;
- b) PHA, 100 μl of a serial dilution of stock 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 second row;
- c) ATH, 100 μl of a serial dilution of ATH (10x concentrate from 20 mL blood) in RPMI 1640 (1:10, 1:100 and 1:500) was placed in triplicate wells of the first 9 wells of the second row;
- d) Negative control, 100 μl of 1:100 dilution of healthy male plasma in RPMI 1640 medium was added to each of first 3 wells of the forth row. RPMI 1640 were also obtained from Sigma. Plates were incubated in a 5% CO₂ incubator at 37 °C for 5 days. One microliter 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 content of each well was harvested and counted in a liquid scintillation beta-counter. The mean of three determinations per point was registered as mean-cpm of each challenger. The mean-cpm of the negative control was divided by the mean-cpm of the media control. If this ratio was less than 2.00, then the negative control was accepted. For each test in each patient, the mean-cpm of the PHA dilution with the highest cpm was divided by the mean of the media control, expressed as % of the value calculated for the day -3 and defined as PHA lymphocyte proliferation response (PHA-LPR). Also for each test in each patient, the mean-cpm of the ATH dilution with the highest cpm was divided by the mean-cpm of the media control expressed as a percentage of the value calculated for the day -3 and defined as ATH lymphocyte proliferation response (ATH-LPR). The results were statistically compared in both treatment arms.

**T-regulatory (CD4+CD25+) prevalence**

An aliquot of each PBMC sample, days-3, 3 and 20, isolated from peripheral blood, were used for two and three color cell surface labeling using Abs against CD4 and CD25. We analyzed the cell samples by flow cytometry after cell surface labeling for co-expression of CD4 and CD25 molecules. The prevalence of CD4+CD25+ cells as a percentage of total CD4+ population was determined by standard determination of quadrant statistics and registered as T-regulatory cells value (T-reg ). For each test, days-3, 3 and 20, in each patient, T-reg values were expressed as percent of the day -3 mean values and defined as T-reg response (T-regr). The results in both treatment arms were statistically compared.

**Statistical analysis**

Response rate to treatment (RR), response duration (RD), median survival time (MST), and 1-OS were the primary end-point. Secondary end-points included safety and laboratory tests.

Survival rates at 95% confidence intervals (CIs) were estimated and the comparisons between the two treatment procedures were carried out using the two-tailed log-rank test. The sample size for survival (log-rank) was assessed using the approach of Schoenfeld and Richter. RR in both groups was compared using Fisher’s exact test. RD difference was evaluated using the generalized Wilcoxon test. Results of the laboratory tests (PHA LPR, ATH LPR, and T-regr) in the two arms were statistically compared by unpaired Student’s t-test. Statistical analysis was performed with WINKS Statistical Data Analysis (SDA) software (TexasSoft, Cedar Hill, TX).

**Results**

Eighty-eight patients who met the eligibility criteria were accrued into the study; 44 patients were randomly assigned to each treatment arm, CHT and CHIMT. Eighty-one patients were assessable, 41 in CHT arm and 40 in CHIMT arm. Seven patients were nonassessable because of unrelated intercurrent diseases (4 patients), refused further treatment after one chemotherapy series (2 patients), and nonaccomplishing follow-up (1 patient).

Patient characteristics are listed in Table 1.

The treatment arms, CHT and CHIMT, were balanced with respect to the clinical prognosis parameter, respectively. The median age was 56 and 58 (range 40 to 73 and 33 to 74 years); at least 5% weight loss (48.8% and 55.0%); cell type was squamous cell carcinoma in 15 and 16 patients (36.6% and 40.0%), large cell in 10 and 9 patients (24.4% and 22.5%), adenocarcinoma in 12 and 10 patients (29.3%...
Chemo-immunotherapy in lung cancer

Table 2 shows the analysis of tumor response in both arms.

RD was 39.0% (1 patient with CR and 15 patients with PR) in CHT arm and 35.0% (14 patients with PR) in CHIMT arm. The difference in response was not statistically significant (p = 0.12).

RD was 22 weeks in CHT arm and 31 weeks in CHIMT arm. This difference was significant (p < 0.05).

Figure 1 shows the survival parameters in the two treatment arms.

The values of MST and 1-OS: in CHT and CHIMT arms, MSTs were 32 and 44 weeks, respectively; 1-OS were 33.3% and 39.1%, respectively. Figure 1 also shows overall survival curves for the two treatment arms. The log rank test showed a statistical significant difference between the survival curves favorable to CHIMT versus CHT (p = 0.02). The sample size was small but reached a power of 80% for an alpha value of 0.05%.

No different toxicity according CTCAE (NCI 2006) between CHT and CHIMT was evident. The most significant (grade 3/4) toxicities found included, respectively: nausea (7 and 5 cases), hypertension (4 and 3 cases), diarrhea (1 and 2 cases), dyspnea (1 and 0 cases), neurosensory toxicity (3 and 5 cases), hypomagnesaeemia (1 and 1 cases), and neutropenia (7 and 5 cases).

Table 3 shows the PBMC tests results.

The mean ± SD of PHA-LPR was significant higher in CHIMT posttreatment samples, day 20, than in CHIMT pretreatment samples, day -3. No significant difference was in evidence in PHA-LPR day 3 and day -3 of CHIMT treatment. No significant difference found in CHT tests.

The mean ± SD of T-regr prevalence was a mirror image of PHA-LPR. It was lower in CHIMT posttreatment samples, day 20, than in CHIMT pretreatment samples, day -3. No significant difference in T-reg day -3 and day 3 of CHIMT treatment was in evidence. No significant difference found in CHT tests.

The mean ± SD of ATH-LPR was also higher in CHIMT posttreatment latest samples, day 20, than in CHIMT pretreatment samples, day -3. However, there was a small significant difference of higher values in early posttreatments tests, day 3, versus pretreatment tests, day -3 in both, CHT and CHIMT arms. No significant variation was found in late CHT posttreatments tests, day 20.

Discussion
This study of an innovative therapeutic platform reports exhaustively from previous own and independent references supporting the rational of this procedure.

The patients in the two arms were comparable in the considered prognostic parameters (Table 1). The sample size was acceptable according to the approach of Schoenfeld and Richter and it also accomplished the criteria recently reported for the two-arm design of phase II clinical trials (Taylor et al 2006). The RRs were statistically nondifferent between CHM and CHIMT and the values are in the range of RR reported in similar medical conditions treated only with

<table>
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<td>Response (CR + PR)</td>
</tr>
<tr>
<td>% Response</td>
</tr>
<tr>
<td>Response duration (weeks)</td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; PR, partial response.
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Table 3. Immune assessment of peripheral blood mononuclear cells

<table>
<thead>
<tr>
<th>Test</th>
<th>PHA-LPR</th>
<th>T-Reg</th>
<th>ATH-LPR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Series day</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>CHT</td>
<td>100</td>
<td>100</td>
<td>103(\pm) 4.2</td>
</tr>
<tr>
<td>3 vs. -3</td>
<td>p &gt; 0.1</td>
<td>p &gt; 0.1</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>20 vs. -3</td>
<td>p &gt; 0.1</td>
<td>p &gt; 0.1</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>CHIMT</td>
<td>100</td>
<td>103 (\pm) 3.6</td>
<td>144 (\pm) 6.2</td>
</tr>
<tr>
<td>3 vs. -3</td>
<td>p &gt; 0.1</td>
<td>p &gt; 0.1</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>20 vs. -3</td>
<td>P = 0.01</td>
<td>P = 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Notes: Lymphocyte proliferation assays responses (PHA-LPR and ATH-LPR) were calculated in each test dividing the highest cpm in the respective challenger/dilution by the mean of the media control and expressed as % of the value calculated for the day -3. The table shows the mean \(\pm\) SD of these percents obtained for the patients in each arm at each sample-day. The T-regulatory cells response (T-reg) were calculated in each test as the % of CD4\(^+\) cells marking CD4\(^+\)CD25\(^+\) and expressed as % of the value calculated for the day -3. The table shows the mean \(\pm\) SD of these percents obtained for the patients in each arm at each sample-day. Series day: Ordinal day referred to the first day of chemotherapy. Considering day 1 as the first day of chemotherapy.

Abbreviations: CHT, Chemotherapy arm (n = 41); CHIMT, Chemotherapy + Immuno-adjuvant arm (n = 40).

comparable chemotherapy (Socinski et al 2003). We infer that the tested immunomodulatory adjuvant had no influence upon the initial response to chemotherapy. Differently, the duration of response and the survival parameters were all statistically increased in CHIMT compared with CHT. This increment is also evident if we compare CHIMT results with the previously reported same parameters in trials of comparable patients treated with comparable chemotherapy (Socinski et al 2003). We deduce that the tested immunomodulatory adjuvant elicits a mechanism that maintains the response to chemotherapy. The statistically significant increase of PHA-LPR and the significant decrease of T-reg are compatible with a switch of the immune system, conditioning the immune responses from the permissive (tolerogenic) to the protective (immunogenic). These facts concur with the reported immunomodulatory activity of the IAS components: Cyclophosphamide, low dose, 3 days before antigen stimulation, decreases the CD4\(^+\)CD25\(^+\) (T-reg) cell population (Berd et al 1982; Ghiringhelli et al 2004). The magnesium silicate granuloma has been demonstrated as a remote enhancer of systemic macrophage activity committed to the protective responses (Fauve and Hevin 1977; Fontan et al 1983, 1992; Fauve et al 1987). GM-CSF, subcutaneously, administered around the antigenic stimulation, recruits and activates the antigen presenting cells, mainly dendritic cells, allowing a stronger immune response (Disis et al 1996; Dranoff 2002).

The influence of this nonspecific immunity modulation upon the specific antitumor immunity is suggested by the results of ATH-LPR. The early increase of ATH-LPR at day 3, in both arms, CHT and CHIMT, is compatible with higher release of TAA induced by chemotherapy and recovered in the contemporary ATH. The most important increase of ATH-LPR, evidenced at day 20, is exclusive of CHIMT and is associated with the immunity nonspecific modulation. We interpret that chemotherapy-induced apoptosis produced an immunogenic TAA release from tumor cells, impacting upon an immune system that could be conditioned by IAS to elicit protective responses, resulting in an immunotherapy mechanism added to the chemotherapy antitumor effect. The antitumor response duration and the survival parameters are higher in CHIMT arm than in CHT arm. This result is compatible with a protracted antitumor effect of chemotherapy plus immunotherapy, which delays the recovery of tumor growth after remission induced by cytotoxic drugs.

Conclusion

CHT and CHIMT resulted in a not-different RR but CHIMT maintained the cancer control safely for a longer period, improving RD, MST, and 1-OS compared with CHT. The immunity-associated changes are compatible with a potentiation of the time-effective chemotherapy-antitumor-effect through an internal vaccination by TAA (released from chemotherapy-induced tumor cells apoptosis) impacting upon a modulated proprotective/antipermissive immune system. Despite the small number of cases of this phase II study, the statistical significance evidenced warrants further investigations in order to assess the clinical relevance of this chemo-immunotherapy.

Disclosure

The authors report no conflicts of interest.

References


[NCI] National Cancer Institute, US. 2006: Common terminology criteria or adverse events V3.0 (CTCAE).


Chemo-immunotherapy in lung cancer
Background: From Classic Autohemotherapy to Autologous Hemoderivative Cancer Vaccine

From Classic Autohemotherapy to Autologous Hemoderivative Cancer Vaccine through a drug and drug-carrier immunomodulatory Adjuvant System. Evidence of safety and efficiency in immunomodotherapy of Non Small Cell Lung Cancer

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1898 to 1940

The interest in classic autohemotherapy was dimmed after the antibiotics and the chemotherapy emerged. But in the field of oncology, in 1965, the first serum tumor marker (CEA) was identified, followed by the knowledge of other serum tumor markers between 1979 and 1985 (PSA, CA19-9; CA-125, CA15-3). In 1996, solid evidences of the possibility to design a vaccine with CEA as immunogen began to be reported. These facts confirmed a paradigm of classic cancer autohemotherapy: the presence in the patient’s blood of potential tumor antigens, released from the tumor to the blood. In addition, the blood was known as a property of several oncoproteins/merit necessary to tumor biology. These oncoproteins molecules, as certain growth factors and chaperones, could be from the tumor associated antigens released to the blood. In time, two mechanisms of immunomodulatory conditioning the immune responses were evinced: the heat-enhancement of the activity as protective immune adjutants of the blood-components and the access to the sentinel lymph nodes of immunomodulatory agents, through subcutaneous spaces, to induce protective immune responses in that node and, by its decisional property, in the systemic lymph nodes. These facts confirmed other paradigm of classic cancer autohemotherapy: the immunogenicity of the extra-vascular re-injected heat-processed blood.

1950-1995

Following this definition of ITS, and taking into account the recent reports that showed that ITS potentiates chemotherapy when it was intercalated with chemotherapy, we hypothesize a new strategy for cancer treatment using ITS with AT-CV previous to chemotherapy. The cell death through chemotherapy-induced cell death, followed by apoptosis, is a known immunogenic cell death. Therefore, the rational for the strategy of successive immunomodulatory-adjuvancy and chemotherapy is to elicit anti-tumor responses by the tumor associated antigens present in the chemotherapy-released tumor-components, impacting in the previously appropriately-conditioned immune system. It would be an immunotherapeutical site (ITS) configured with subcutaneous GM-CSF.

1995-2007

In the field of oncology, in 1965, the first serum tumor marker (CEA) was identified, followed by the knowledge of other serum tumor markers between 1979 and 1985 (PSA, CA19-9; CA-125, CA15-3). In 1996, solid evidences of the possibility to design a vaccine with CEA as immunogen began to be reported. These facts confirmed a paradigm of classic cancer autohemotherapy: the presence in the patient’s blood of potential tumor antigens, released from the tumor to the blood. In addition, the blood was known as a property of several oncoproteins/merit necessary to tumor biology. These oncoproteins molecules, as certain growth factors and chaperones, could be from the tumor associated antigens released to the blood. In time, two mechanisms of immunomodulatory conditioning the immune responses were evinced: the heat-enhancement of the activity as protective immune adjutants of the blood-components and the access to the sentinel lymph nodes of immunomodulatory agents, through subcutaneous spaces, to induce protective immune responses in that node and, by its decisional property, in the systemic lymph nodes. These facts confirmed other paradigm of classic cancer autohemotherapy: the immunogenicity of the extra-vascular re-injected heat-processed blood.

2008

Conclusions. Injecting intravenous cyclophosphamide, followed by an immunotherapeutical site (ITS) configured with subcutaneous GM-CSF, etoposide and an autologous thermostable hemoderivative, conditioned a potentiated anti-tumor effect of successive chemotherapy in advanced Non-Small Cell Lung Cancer. The results are compatible with a chemotherapy-induced release of tumor associated antigens challenging a protective conditioned immune system as mechanism of action.

Methods

Patients. - 120 patients. Stage IV NSCLC. 2-arm randomized (90 each), and assigned to either chemotherapy or ITS with AT-CV followed by chemotherapy

Trial Design. - The study was a prospective, controlled, randomized, phase III study. The study was conducted in accordance with the Declaration of Helsinki.

Arm 1 was treated with chemotherapy (CHEMO): docetaxel 100 mg/m² on series-day 1, and cisplatinum 80 mg/m² on series-day 2. The series were started at trial-day 31 and it was repeated every 3 weeks. CHEMO was continued until uncontrollable intolerance or until a maximum of six series

Arm 2 was treated starting ITS with AT-CV at trial-day 1 during 30 days, followed by the same chemotherapy as arm 1 (CHEMO) beginning at trial-day 31. Best supportive care (BSC) was prescribed to all patients in both arms, at the investigator’s discretion. The follow-up was of two years in order to calculate the assessments later described.

Assessments. - Thirty-day tumor growth

Survival curves. Median Time Survival. One-year survival

For each patient, the survival was calculated as the time in days from randomization to the date of death or the end of the study.

Results of the Immunity tests and TG in both arms were statistically compared by log-rank test. For survival (log-rank) was assessed using the approach of Schoenfeld and Richter. 95% confidence intervals (95% CI) were estimated and the comparisons between arms were done using the approach of Schoenfeld and Richter. In the hemoderivative, the number of patients with a survival of more than 1 year was noted.

Secondary end-points included immunity assessments and safety. Survival rates at 95% confidence intervals (95% CI) were estimated and the comparisons between the two arms were carried out using the log-rank test. The sample size for survival (log-rank) was assessed using the approach of Schoenfeld and Richter. Results of the immunity tests and TG in both arms were statistically compared by unpaired Student’s t-test.

Selected References:


Conclusions. Injecting intravenous cyclophosphamide, followed by an immunotherapeutical site (ITS) configured with subcutaneous GM-CSF, etoposide and an autologous thermostable hemoderivative, conditioned a potentiated anti-tumor effect of successive chemotherapy in advanced Non-Small Cell Lung Cancer. The results are compatible with a chemotherapy-induced release of tumor associated antigens challenging a protective conditioned immune system as mechanism of action.
A New Twist on Autologous Cancer Vaccines

Physicians have had a long-standing interest in marshalling the cancer patient's own immune system to effect tumor rejection. The use of cancer vaccines to activate an endogenous antitumor immune response has the advantages of exquisite tumor specificity, low toxicity, and potential durability due to the phenomenon of immunologic memory. Moreover, cancer vaccines are an attractive complement to the standard cancer treatment modalities of surgery, radiation therapy, and chemotherapy, offering a non-toxic treatment strategy that is likely to be non-cross resistant. Even with these advantages, the use of therapeutic cancer vaccines also poses significant challenges. Their efficacy is hampered by the extent of the tumor burden, relatively well entrenched mechanisms of tumor-specific immune tolerance, and the potential plasticity of the tumor cells themselves. From a practical point of view, the development of tumor vaccines is further limited by the technical limitations posed by the nature of the vaccination platform itself. In this issue of Cancer Biology & Therapy, Lasalvia-Prisco and colleagues report the results of the first clinical trial testing a novel vaccine formulation utilizing an autologous hemoderivative for the treatment of advanced solid malignancies. While clearly preliminary, their approach is intriguing because it circumvents many of the practical obstacles to the development of effective vaccines for cancer therapy.

Tumor vaccine formulations can be broadly divided into those that are well defined, and those that are not. Well-defined tumor vaccines contain known tumor antigens; examples include peptide-based, protein-based, and plasmid DNA vaccines. These vaccines offer the advantages of relative ease of manufacture, clear targets for the monitoring of immune responses to vaccination, and a good safety record to date. One disadvantage to the use of precisely targeted tumor vaccines is that many tumor antigens seem to activate antigen-specific immunity that is incapable of mediating a tumor rejection response. This concept is supported by the results of studies characterizing the natural and vaccine-induced immune responses in melanoma patients who continue to have disease progression despite the presence of antigen-specific tumor immunity. A second disadvantage to highly targeted cancer immunotherapies is that they favor the selection of antigen loss variants, ultimately resulting in the outgrowth of a subpopulation of antigen-negative tumor cells that are by definition resistant to therapy.

Tumor vaccines that are less well defined are generally formulated either directly from tumor cells themselves to make a cellular vaccine, or are derived from tumor cells as a crude preparation of viral lysate or heat shock protein (HSP)-peptide complexes. There are two primary advantages to cancer vaccine formulations that are less well defined. First, these vaccine platforms deliver a variety of tumor antigens. By definition, they are capable of directing the immune response simultaneously to multiple antigens, greatly decreasing the probability of immune-mediated selection of antigen loss variants. Second, the menu of antigens delivered can include both known and as yet unknown tumor antigens. The sheer number of antigens delivered thus increases the likelihood of activating the immune system to recognize an antigen that can mediate tumor rejection. One disadvantage to the use of undefined antigen vaccines relates to the primary antigen source. The use of autologous tumor cells is often preferred due to the possibility that critical targets for immune-mediated tumor rejection are unique to each tumor. However, sufficient numbers of autologous tumor cells are frequently not available to support full vaccination regimens, leading some to investigate the use of allogeneic tumor cells to deliver tumor antigens common to a given histology. A second disadvantage of using relatively undefined vaccine platforms is that there is often no clear target for monitoring vaccine-induced immune responses. Some investigators have used the development of delayed type hypersensitivity (DTH) to autologous tumor as an informative measure of vaccine-induced antitumor immunity. Again, this is possible only when autologous tumor is available for processing, and this is frequently not the case for advanced solid tumors. A third drawback to less defined vaccine formulations is that the quality of the manufacturing process may be difficult to ensure.
In particular, the antigenic content of autologous tumor cell-based formulations will be unique. Appropriate measures of manufacturing consistency and potency may thus be more difficult to define for cell-based as opposed to highly targeted cancer vaccines.

Lasalvía-Prisco and colleagues describe a novel vaccine formulation derived from the arterial blood of advanced solid tumor patients. They develop a procedure for manufacturing and partially characterizing an autologous hemoderivative, and then test it as a cancer vaccine in a clinical trial involving patients with a variety of advanced solid tumors. The processing of the vaccine itself is simple. It is derived from 20 milliliters of femoral arterial blood. After sedimentation at 37°C, the supernatant of plasma and cells is subjected to hypotonic shock, followed by freezing. Twenty-four hours later the preparation is thawed, exposed to 100°C for 10 minutes, and filtered over cellulose acetate. A crude analysis of the vaccine preparation showed it to consist of a minimum of five protein fractions, with a major homogeneous protein component of approximately 50,000 kD. Although they were present prior to processing, heat shock proteins and known tumor markers were not detected in the final product.

Although it remains relatively uncharacterized, this vaccine platform in principle offers multiple advantages. First, the small quantity of arterial blood required is quickly and safely accessible by femoral arterial puncture. Second, the manufacturing process is relatively simple and cost-effective, requiring minimal manipulation and no direct chemical or genetic modification of the cellular component prior to processing. Finally, and most importantly, the patient himself is a renewable source of therapeutic material. At the time of tumor recurrence, an updated vaccine product that accurately reflects that antigenic profile of the tumor at that point in time could be easily obtained and prepared.

The investigators designed the clinical trial to overcome some other limitations to the efficacy of therapeutic cancer vaccines. They include GM-CSF, a cytokine that is well known for its immune-stimulating properties, as a local vaccine adjuvant. They also incorporate a low dose of intravenous Cyclophosphamide three days prior to vaccination. Although chemotherapeutic immunomodulation is not widely used in cancer vaccine trials, there is substantial preclinical and clinical evidence to suggest that Cyclophosphamide can augment the induction of antigen-specific immunity.\(^2\) Importantly, the trial also included a control group that received Cyclophosphamide and GM-CSF but no autologous hemoderivative. The vaccinated group had a higher frequency of stable (SD) or responding (PR) disease than did the control group (p < 0.001). Also, the vaccinated patients demonstrated a correlation between clinical response (SD + PR) and the development of DTH to the autologous hemoderivative of at least 5 mm in diameter (p < 0.02). Moreover, histologic analysis of responding metastatic lesions were characterized by stromal fibrosis and CD^3^+ T cell infiltration not characteristic of pre-treatment biopsies.

The mechanism underlying the reported bioactivity of this treatment approach remains an open question. Although no HSPs are found in the final vaccine product, the overall vaccine preparation is reminiscent of HSP cancer vaccines.\(^2\) It is possible that a stress-related protein present in the vaccine preparation delivers tumor-derived antigens present in the arterial blood in a form capable of activating T cell-dependent immunity. This is supported by the development of DTH to the homodervative, and by the presence of T cells infiltrating the responding tumors. It would be more strongly supported by additional measures of antitumor immunity, such as DTH to autologous tumor where available (rather than the autologous hemodervative), or cellular immune responses to a defined antigen known to be present in the patients tumor (by ELISPOT).

Interestingly, the manufacturing process includes a step of hypotonic shock. Hypotonic shock is known to activate monocytes and macrophages, enhancing phagocytosis and the secretion of cytokines such as interleukin 1 and interleukin 6.\(^2\) The contribution of this step to the bioactivity of the vaccine is unclear, but it could provide a vaccine adjuvant that is an integral component of the vaccine itself.

The most striking histopathologic feature of responding lesions is the presence of marked fibrosis. The extent of this fibrotic response suggests an additional mechanism distinct from that mediated by T cell against the tumor cells themselves. The observed histology strongly argues for a therapeutic effect that shifts the balance of interactions between the tumor cells and the supporting stroma to favor tumor regression. Carefully elucidating the regulatory pathways underlying this aspect of the hemoderivative's bioactivity should facilitate the development of informative surrogate measures of clinical response for use in future clinical trials.

In summary, Lasalvía-Prisco and colleagues have described a novel cancer vaccine platform consisting of an autologous hemoderivative, with a suggestion of clinical response. These results are preliminary, and require confirmation in larger trials and by other investigators. Further characterization of the critical parameters of vaccine formulation and the mechanism of bioactivity will facilitate the development of more informative clinical trials. If these results are confirmed and extended, this vaccine platform represents an exciting development in the field of cancer immunotherapy. The ability to re-derive a potent vaccine in response to the changing antigenic profile of an evolving metastatic tumor is a powerful and unique feature of this vaccine platform. Clearly, an active, individualized cancer vaccine that is cost-effective and simple to manufacture would be a welcome addition to the treatment armamentarium for metastatic solid tumors.

References