

CASTELLANOS & CO.

A B O G A D O S

Calle 94 A No. 7 A - 09

Bogotá, D.C., COLOMBIA

Señora

Jefe de la División de Nuevas Creaciones

Superintendencia de Industria y Comercio

Ministerio de Desarrollo Económico

E.

S.

D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

**No. 03-048137- -00006-0000**

Fecha: 2008-05-21 14:36:46 Dep. 2020 NUECREACIONE
 Tra. 11 PATENTEIYII Eve: 379 FASENACIONALI
 Act. 444 RESPUESTAREQUE Folios: 21

Ref.: Expediente No. **03.048.137**

Solicitud de Patente de Invención para: **COMPOSICIONES FARMACEUTICAS PARA
 POTENCIAR LA INMUNOGENECIDAD DE ANTÍGENOS POCO INMUNOGÉNICOS**

Solicitante: **CENTRO DE INMUNOLOGÍA MOLECULAR****RESPUESTA AL EXAMEN DE FONDO**

MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, atentamente me permito presentar respuesta al oficio No. 2749 notificado por fijación en lista el 22 de febrero de 2008 correspondiente al examen de fondo, estando dentro del término legal en los siguientes términos:

Nivel inventivo

Presento a continuación los argumentos que demuestran el nivel inventivo de la presente invención en vista del documento D1.

El documento D1 enseña que la inserción hidrofóbica de una molécula anfipática (como pueden ser los gangliósidos) en la capa lipídica de proteoliposomas derivados de OMPC (Complejo de proteínas de la Membrana externa de Neisseria meningitidis) es suficiente para inducir la inmunogenicidad contra entidades tan poco inmunogénicas como son los gangliósidos. El problema técnico resuelto por el documento D1 es que hace inmunogénicas dos moléculas que son blancos importantes en la terapia del cáncer, NAcGM3 y NGcGM3.

D1 también enseña que para hacer inmunogénico cualquiera de estos antígenos, el antígeno debe ser modificado naturalmente o por síntesis de forma tal que contenga una región hidrofóbica y es justamente por esta región hidrofóbica que se insertará en la capa lipídica de la OMPC.

CASTELLANOS & CO.

A B O G A D O S

Calle 94 A No. 7 A - 09

Bogotá, D.C., COLOMBIA

Por el contrario, en la presente invención se reivindica una composición farmacéutica que tiene como característica técnica esencial un vehículo vacunal que consiste en nanopartículas de proteoliposomas (partículas mucho mas pequeñas que los proteoliposomas convencionales) derivadas del complejo de proteínas de la membrana externa de la bacteria *Neisseria meningitidis* denominada VSSP (Very Small Size Proteoliposomes) y gangliósidos previamente incorporados. Dicho vehículo es capaz de inducir la inmunogenicidad de antígenos polipeptídicos o proteicos poco inmunogénicos sin necesidad de que el antígeno u antígenos sean modificados de ninguna manera. La administración de la composición de la presente invención, capaz de inducir la inmunogenicidad de moléculas poco inmunogénicas, es lo que en el campo de la vacunología actualmente se conoce como "a bed-side" adjuvant". Cualquier experto medio reconocería fácilmente las ventajas de esta solución técnica sobre las descritas previamente.

Primero; que la estructura natural del antígeno no necesita ser modificada de ninguna forma y por tanto se mantendrá "antigénicamente virgen" para las células del sistema inmune. Esto hace que moléculas de diferente naturaleza (péptidos, polipéptidos, proteínas, secuencias de ADN, etc) puedan ser usadas en la composición vacunal de la presente invención.

Contrariamente, la tecnología de OMPC (proteoliposomas) requiere necesariamente la modificación química del antígeno. Y esto, como se sabe, da como resultado la obtención de los llamados "neo antígenos" los cual generalmente tienen cambios importantes en el sentido de la inmuno dominancia, básicamente para el repertorio de células TCD8 efectoras y por tanto limitando los antígenos que pueden usarse.

Por el contrario, D1 no aporta evidencias o sugerencias sobre el hecho de que los VSSP activen las células TCD8 efectoras, lo cual es aceptado por cualquier experto medio que son las más efectivas en la inmunoterapia del cáncer.

Esta inducción de la inmunogenicidad a expensa de las células TCD8 efectoras es la propiedad sorprendente de la presente invención, demostrando como los VSSP pueden promover el efecto anti-metastásico asociado a la respuesta inmune anti-HER1 y dependiente de la respuesta TCD8 efectora (ver adjuntos a este memorial)

Adicionalmente, podríamos mencionar dos características principales que distinguen la presente invención de los documentos previos citados en el estado del arte:

- Un aspecto innovador de la actual invención es el tamaño de las partículas en el VSSP. Se sabe muy bien en el estado del arte el uso del complejo de proteínas de la membrana externa de *Neisseria meningitidis* para obtener los inmunógenos pero los inventores de la presente invención encontraron que el uso de las partículas de tamaño muy pequeño aumentan la capacidad inmunogénica de este complejo de proteínas y la incorporación del gangliósido juega un papel importante en la estructura físico química de los VSSP.

CASTELLANOS & CO.
 ABOGADOS
 Calle 94 A No. 7 A - 09
 Bogotá, D.C., COLOMBIA

- El otro aspecto innovador es el uso del gangliósido. Debe observarse que en la presente invención no se reivindica cualquier gangliósido sino específicamente el GM3. Los inventores encontraron que el gangliósido tiene un papel funcional así como estructural (tamaño de partículas). Las composiciones farmacéuticas descritas en la actual invención tienen la capacidad de estimular la respuesta inmune TCD8 efectora, que es particularmente relevante para los individuos con un sistema inmune altamente deprimido, como por ejemplo pacientes con cáncer o SIDA. En estos individuos, cuando se usa solo el complejo de proteína de la membrana externa de *Neisseria meningitidis* no se induce la respuesta inmune perceptiblemente pero la inclusión del gangliósido provoca un aumento espectacular.

Por último y como evidencia del efecto inmunopotenciador de la composición farmacéutica de la presente invención (VSSP) el solicitante desea someter a consideración del señor examinador el reporte adjunto al presente memorial (*Torréns et.al.: "Immunotherapy with CTL peptide and VSSP eradicated established human papillomavirus (HPV) type 16 E7-expressing tumors". Vaccine 23 (2005) 5768- 5774*) el cual muestra otros resultados experimentales que sustentan el efecto sorprendente de la presente invención.

Finalmente, teniendo en cuenta que la composición reivindicada cumple con el requisito de nivel inventivo, solicito a su Despacho conceder el privilegio de patente a la presente invención.

Anexos

- *Artículo Torrén et.al.*
- *Artículo Sánchez Ramírez et al.*
- *Vaccination with mEGFR-ECD/VSSP has anti-metastatic effect*

De la Señora Jefe,


 Margarita Castellanos Abondano

C.C. No. 39.779.654 de Usaquén

T.P.A. No. 70819 del C. Sup. de la Judicatura

Calle 94 A No. 7 A - 09

Teléfonos: 610 74 20

Bogotá, D.C., 21 mayo 2008



Immunotherapy with CTL peptide and VSSP eradicated established human papillomavirus (HPV) type 16 E7-expressing tumors

Isis Torr  ns^{a,*}, Osmany Mendoza^a, Aileen Batte^a, Osvaldo Reyes^a,
Luis E. Fern  ndez^b, Circe Mesa^b, Gerardo Guill  n^a

^a Department of Cancer, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba

^b Department of Vaccines, Center of Molecular Immunology, 216 esq 15 Atabey, CP 16040, Havana, Cuba

Received 26 May 2005; accepted 22 July 2005

Available online 8 August 2005

Abstract

Peptide-based vaccines aimed at the induction of effective T-cell responses against established tumors have not been successful in clinic and require the use of new adjuvants. One of those is a new adjuvant in which gangliosides are incorporated into the outer membrane protein complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP). In a preclinical model of human papillomavirus HPV16-induced cervical cancer we show that vaccination with HPV 16 E7 derived minimal CTL peptide and VSSP protects mice against tumor challenge, induces regression of established tumors and produces E7-specific CD8⁺ T-cell responses.

  2005 Elsevier Ltd. All rights reserved.

Keywords: Immunotherapy; HPV; VSSP

1. Introduction

Cervical cancer is the second cause of women cancer mortality in the world [1]. Human papillomaviruses (HPV), particularly HPV-16, are associated with most cervical cancers [2]. More than 99% of cervical cancers and their precursor's lesions, squamous intraepithelial lesions (SILs), contain HPV DNA [3]. The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and are co-expressed in most HPV-containing cervical cancers [4]. Therefore, these oncogenic proteins represent ideal target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated tumors [5].

In the past, most HPV research focused on E7, and therefore an E7 immunodominant epitope. Vaccination with a synthetic MHC class-I H-2D^b-binding peptide, HPV16 E7_{49–57} (E7(p)), mixed with incomplete Freund's adjuvant (IFA) prevented tumor formation by HPV16-transformed B6 mouse

embryo cells and elicited CTL responses in C57BL/6 mice [6].

Over the years, various vaccines containing exactly fitting MHC class-I binding peptides have been tested for their therapeutic efficacy in patients with progressive disease associated to HPV [7–9]. Although moderate T-cell responses were observed, the clinical benefit was only modest. This may be due to failure of the vaccines to induce strong sustained immunity because of impaired immune system, which is commonly found in patients diagnosed with cancer.

Recently, it has been demonstrated that CD4⁺ T helper (Th) cells contribute to the development and efficacy of antitumor responses [10,11], probably due to their capacity to deliver essential activation signals to professional antigen presenting cells, such as dendritic cells (DC), needed for an optimal priming of tumor specific CTL [12–14]. Several studies show that effective CTL priming can be induced by: (a) the inclusion of Th epitopes in peptide vaccines [15,16]; (b) the use of long (32- to 35-aa) overlapping peptides including Th and CTL epitopes [17]; (c) the use of molecularly defined strong DC-activating adjuvants, such as monophosphoryl lipid A (MPL) [18,19], oligodeoxynucleotide (ODN)-CpG

* Corresponding author. Tel.: +53 7 2716022; fax: +53 7 336008/2714764.
E-mail address: isis.torrens@cigb.edu.cu (I. Torr  ns).

[20–22], anti-CD40 Ab [23], GM-CSF [24] and ISCOM [25].

A new adjuvant has been previously developed in which gangliosides are incorporated into the outer membrane protein complex (OMPC) of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP) [26]. A recent study reveals that VSSP have the ability to activate mouse and human DC, in vitro and in vivo, with the corresponding IL-12p40/p70, TNF- α and IL-6 production [27]. Therefore, VSSP is a potent adjuvant for DC activation and Th1 differentiation.

In the present study, we demonstrate that immunization of C57BL/6 mice with a vaccine based in adjuvancity of E7(p) + VSSP without oily component protects animals against challenge with the E7-expressing murine tumor cell line, TC-1 [28], and also protects them against re-challenge with higher doses of TC-1 cells. Similarly, immunization of tumor-bearing mice with E7(p) + VSSP leads to tumor regression and long-term survival and produces E7-specific CD8⁺ T-cell responses.

This is the first study to demonstrate that therapeutic immunization with a minimal CTL peptide and VSSP induces tumor regression and represents the first example of cancer immunotherapy based on the use of VSSP with a viral antigen.

2. Materials and methods

2.1. Mice

C57BL/6 mice (6–8 weeks old) were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). All animals were maintained under specific-pathogen-free conditions at the Center for Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). The experimental methods were approved by the Animal Care and Use Committee of the CIGB and were conducted in accordance with the Health Guide for the Care and Use of Laboratory Animals (HGCULA).

2.2. Cells

TC-1 cells were kindly provided by T.C. Wu (Johns Hopkins University, Baltimore). They were generated by cotransformations of primary C57BL/6 mouse lung epithelial cells with HPV-16 E6 and E7, and an activated *ras* oncogene as previously described [28]. The EL4 tumor cell line was obtained from American Culture Collection (TIB-39).

2.3. Peptides

All peptides were synthesized on solid phase and purified by reverse-phase high-performance liquid chromatography to >95% purity on an acetonitrile/H₂O-trifluoroacetic acid gradient and confirmed by ion-spray mass spectrometry

(Micromass, Manchester, United Kingdom). Lyophilized peptides were reconstituted in distilled H₂O for use in vitro and in vivo. The E7(p) (RAHYNIVTF) was selected because it was identified as an H2-D^b CTL epitope [6]. The H2-K^b-restricted Ova257–264 CTL peptide (SIINFEKL) was used as not related peptide (Ova(p)).

2.4. Adjuvants

VSSP [26] are produced and provided by the Center of Molecular Immunology (CIM, Havana, Cuba) and used at their suggested optimal working concentration of 160 μ g/mouse. LPS content in VSSP and OMPC is 30 μ g/mg protein and 20–60 μ g/mg protein, respectively. Quantification of LPS was performed by measurement of KDO according to the method of Karkhanis et al. [29]. IFA was obtained from Sigma, St. Louis, MO.

2.5. Immunizations

All vaccine formulations were prepared the day before injection, each formulation containing 50 μ g of E7(p) in 100 μ l was mixed with 160 μ g VSSP or emulsified in 100 μ l IFA. The total injected volume was 200 μ l/mouse. All immunizations were given subcutaneously in the flank, twice at 2-week intervals.

2.6. Implantation and measurement of TC-1 tumor

In preparation for implantation into mice, TC-1 cells were cultured until approximately 70% confluence and harvested with trypsin. TC-1 cells were injected subcutaneously in the leg at various doses, including 5×10^4 and 2×10^5 cells. Starting 7–10 days later and every 3–4 days thereafter, the inoculated area was observed and palpated for the presence of a tumor nodule. Tumor diameters were measured in two orthogonal dimensions using electronic digital callipers (Kell-Strom, Canada). Tumor volumes were calculated from these measurements according to: (length $\times$ width²)/2 [30]. Tumor-bearing mice were killed when moribund, as defined by the HGCULA.

2.7. Cytokine analysis

Spleen cells, harvested from three mice per group, were pooled and cultured in 96-well plates (5×10^5 cells/well) in culture medium containing 10% fetal bovine serum (FBS) and 10 U/ml human interleukin 2 (hIL-2). The spleen cells were sensitized either with or without 1 μ g/ml E7(p) for 72 h in triplicate conditions at 37 $^{\circ}$ C in humidified air containing 7% CO₂. Gamma interferon (IFN- γ) and interleukin 10 (IL-10) were quantified from cell supernatants using a specific ELISA according to the manufacturer's instructions (Quantikine[®] M murine, R&D Systems, MN, USA). Data were expressed as cytokine released in pg/ml $\pm$ S.D. of triplicate ELISA values.

2.8. Enzyme-linked immunospot (ELISPOT) assay

An ELISPOT assay was performed to detect HPV-16 E7-specific CD8⁺ T cells. A 96-well filtration plate (Millipore, Bedford, MA, USA) was coated with 5 µg/ml rat anti-mouse IFN-γ antibody (clone R4-6A2, Pharmingen, San Diego, CA) in 100 µl of PBS. After overnight incubation at 4 °C, the wells were washed with PBS and blocked with culture medium containing 10% fetal bovine serum (FBS) for 1 h. Mouse H-2^b target cells (EL4), previously pulsed with 10 µM of the E7(p) and suspended in RPMI 1640 supplemented with 10% of FBS plus hIL-2 (10 units/ml), were used as antigen presenting cells. Unloaded EL4 cells were also included for IFN-γ secreting cells background determination. Serial dilutions of fresh isolated splenocytes from each vaccinated group of mice (10⁶, 2 × 10⁵ and 4 × 10⁴ cells per well) and EL4 target cells (10⁵ cells per well) were, respectively, loaded to a final volume of 200 µl, and co-incubated by duplicate at 37 °C and 5% CO₂ for 17 h. Later, the plate was washed with PBS containing 0.2% Tween-20 (PBST) and then incubated with 5 µg/ml biotinylated anti-IFN-γ antibody (clone XMG1.2, Pharmingen) in 100 µl of PBS at room temperature for 2 h. After washing with PBST, spot development was performed using HRP-avidin complex (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at room temperature and AEC (Sigma, St. Louis, MO, USA). The spot forming cells (SFC), representing the number of IFN-γ producing cells were counted with a stereoscopic microscope. Values equal to or higher than double of negative control (EL4 without peptide) plus 10 SFC were considered as positive.

2.9. Statistical analysis

The statistical significance of the tumor protection and survival experiments was determined using log-rank test. Differences in tumor volumes among all treatment groups were

analyzed by Mann–Whitney *U*-test. For graphic representation of data, y-axis error bars indicate the S.D. for each point on the graph.

3. Results

3.1. Prophylactic immunization with E7(p) + VSSP confers protection against recurrent TC-1 tumor challenge

To examine the ability of E7(p) + VSSP immunization to confer protection against in vivo challenge with TC-1, an E7-expressing tumor cell line [28], female C57BL/6 mice were injected subcutaneously on days 7 and 14 with PBS, VSSP, E7(p) + IFA and E7(p) + VSSP. Seven days after the second immunization mice were challenged with 5 × 10⁴ TC-1 cells in the right leg. Fig. 1a shows that TC-1 cell implantation resulted in the appearance of palpable subcutaneous tumors in all mice (10/10) immunized with PBS or VSSP between 14 and 25 days; and all mice (10/10) immunized with E7(p) + IFA developed tumors between 19 and 30 days. In contrast, only 20% (2/10) of mice vaccinated with E7(p) + VSSP showed incidence of tumors at days 22 and 32, respectively, and no progress in the tumor size of these animals was observed. Compared with PBS, VSSP and E7(p) + IFA administrations, E7(p) + VSSP immunization induced statistically significant protection against TC-1 tumor challenge (*P* < 0.0001).

When tumor-free animals from E7(p) + VSSP vaccinated group (five mice) were re-challenged with a larger dose of TC-1 cells (2 × 10⁵ cells) in the left leg, by the end of the 21-day observation period, a similar result was observed with 100% tumor protection (Fig. 1b). As expected, a new cohort of unimmunized mice verified the tumorigenicity of the TC-1 cells used for re-challenge.

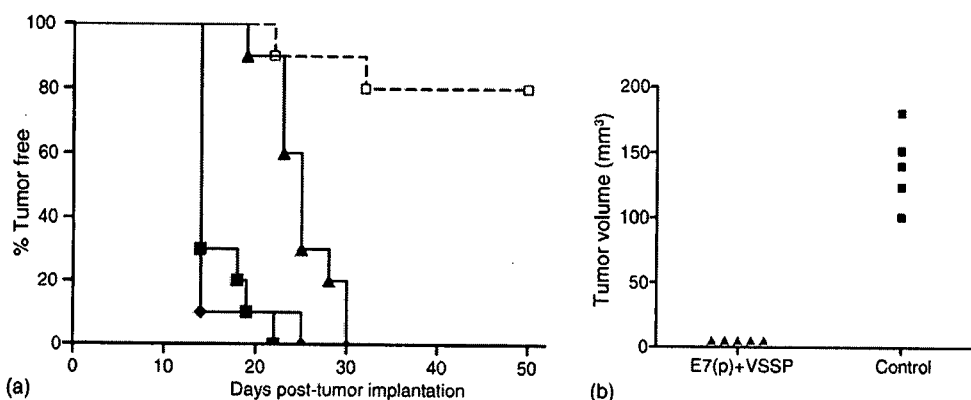


Fig. 1. Prophylactic immunization with E7(p) + VSSP protects against TC-1 tumor challenge and re-challenge. (a) C57BL/6 mice (10 per group) were immunized subcutaneously in the flank with PBS (■), VSSP (◆), E7(p) + IFA (▲), and E7(p) + VSSP (□) and then boosted 14 days later. Seven days after the last boost mice were challenged subcutaneously in the right leg with 5 × 10⁵ TC-1 cells (day 0) and observed for 50 days. Data are presented as percent of tumor incidence per group. (b) On day 50 from previous experiment, five tumor free mice immunized with E7(p) + VSSP were re-challenged with a larger dose of TC-1 cells (2 × 10⁵ cells) in the left flank and observed for additional 21 days. In addition, a new group of untreated mice (control) was challenged with tumor cells on day 50 to verify the tumorigenicity of the TC-1 inoculum. Data are presented as individual tumor volumes from mice.

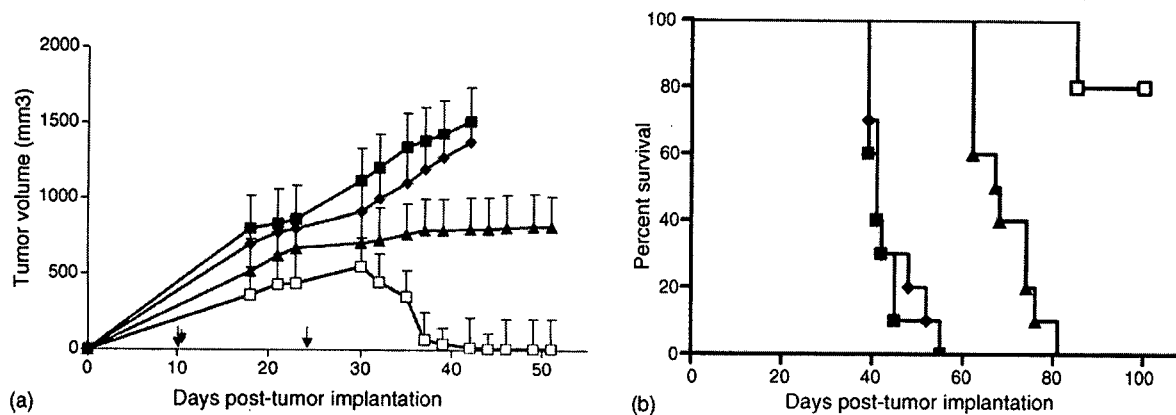


Fig. 2. Therapeutic immunization with E7(p) + VSSP induces TC-1 tumor regression and long-term survival. (a) C57BL/6 mice (10 per group) were injected subcutaneously with 2×10^5 TC-1 cells in the right leg (0). At 10 and 24 days post-implantation, mice were immunized with PBS (■), VSSP (◆), E7(p) + IFA (▲), and E7(p) + VSSP (□) in the flank (arrows). Tumor volumes of subcutaneous nodules were assessed for 50 days post-implantation (as described in Section 2). Data are presented as mean tumor volume (mm^3) $\pm$ S.D. in the different groups of mice. (b) Mice were monitored for survival over a 100-day period post-implantation. Mice that became moribund due to tumor burden were killed. Time to death is plotted on a Kaplan–Meier survival curve.

3.2. Therapeutic immunization with E7(p) + VSSP induces TC-1 tumor regression and promotes long-term survival

Given the ability of prophylactic E7(p) + VSSP immunization to confer protection against TC-1 tumor challenge, experiments were performed to determine whether or not E7(p) + VSSP immunization could induce regression of established TC-1 tumors. In these studies, therapy was initiated 10 days post-tumor implantation, when 100% of mice had sizeable subcutaneous tumors. Mice were injected subcutaneously with TC-1 cells (2×10^5 cells) on the right leg (Fig. 2a, day 0) and then 10 and 24 days later they were treated with PBS, VSSP, E7(p) + IFA and E7(p) + VSSP. By the end of the 50-day observation period, when tumor incidence in PBS, VSSP and E7(p) + IFA treated mice was 100%, E7(p) + VSSP therapy led to tumor regression in 100% of mice (Fig. 2a). Compared with PBS, VSSP and E7(p) + IFA treatments, E7(p) + VSSP immunization induced statistically significant regression of TC-1 tumors ($P < 0.0001$).

To determine the effects of E7(p) + VSSP therapy on long-term survival, these animals were followed over a period of 100 days. The Kaplan–Meier survival plot (Fig. 2b) shows that 100% (10/10) of PBS and VSSP-treated animals were moribund by day 55. For E7(p) + IFA-treated group, 100% (10/10) of animals were moribund by day 81. In contrast, survival in the E7(p) + VSSP-treated group was 80% (8/10) over this 100-day period, and it was statistically significant relative to PBS, VSSP and E7(p) + IFA-treated cohorts ($P < 0.0001$).

3.3. Th1 cytokine bias of splenocytes from animals immunized with E7(p) + VSSP

As an initial step in the identification of immune effectors mechanisms associated with tumor rejection, the

immune response induced by E7(p) + VSSP immunization was characterized. Mice were immunized with either VSSP, E7(p) + IFA and E7(p) + VSSP and boosted 14 days later. Seven days after the boost, splenocyte cultures were prepared and tested for their capacity to secrete IFN- γ or IL-10 upon re-stimulation with medium, E7(p) or the irrelevant antigen, Ova(p). As shown in Fig. 3, splenocyte cultures from E7(p) + VSSP-immunized mice released high levels of IFN- γ and low levels of IL-10 upon E7(p) restimulation. In contrast, splenocytes from E7(p) + IFA-immunized animals released moderate levels of IFN- γ . Cytokine release appeared to be specific for E7(p), as co-culturing with either medium or Ova(p) (added at the equimolar amount of E7(p)) resulted in release of minimal levels of IFN- γ and IL-10.

3.4. Enhancement of E7-specific CD8⁺ T cell-mediated immune response by immunization with E7(p) + VSSP with respect to E7(p) + IFA

To compare the E7-specific CD8⁺ T cell-mediated immunity in different vaccinated groups, the ELISPOT assay was performed. Mice were immunized with either VSSP, E7(p) + IFA and E7(p) + VSSP and boosted 14 days later. Seven days after the boost, splenocyte cultures were prepared. The ELISPOT assay determines the precursor frequencies of activated E7-specific CD8⁺ T cells [31]. As shown in Fig. 4, the highest ELISPOT numbers were found in mice receiving E7(p) + VSSP. The number of IFN- γ secreting splenocytes in mice immunized with E7(p) + VSSP was approximately nine-fold higher than that of naive animals, whereas in animals immunized with E7(p) + IFA the relation to naive animals was only approximately four-fold higher. The number of IFN- γ secreting splenocytes in mice immunized with E7(p) + VSSP was two-fold higher than that of animals immunized with E7(p) + IFA.

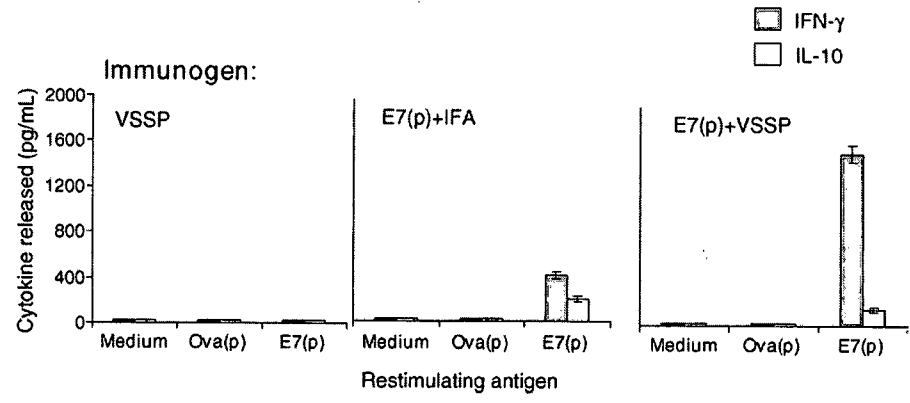


Fig. 3. Splenocytes from E7(p) + VSSP-immunized mice produce IFN-γ upon restimulation. C57BL/6 mice (three per group) were immunized subcutaneously in the flank with either 160 μg VSSP, 50 μg E7(p) + IFA or 50 μg E7(p) + 160 μg VSSP and then boosted 14 days later. Seven days after the boost, pooled splenocyte cultures were prepared and restimulated with medium, 1 μg/ml Ova(p) as irrelevant antigen or 1 μg/ml E7(p), as described in Section 2. IFN-γ (solid columns) and IL-10 (open columns) contents of 72 h culture supernatants were determined by ELISA and expressed as pg/ml ± S.D. of triplicate ELISA values.

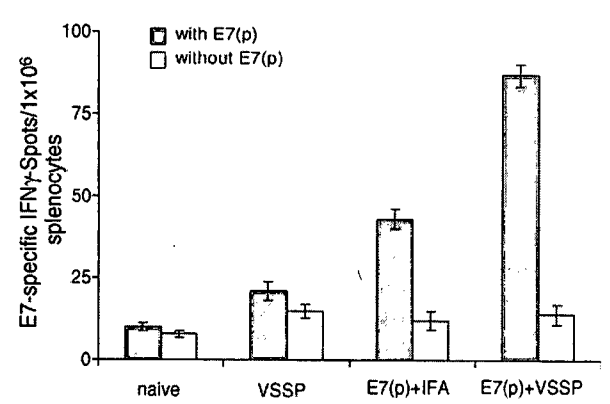


Fig. 4. Demonstration of E7-specific CD8+ T cell precursors in C57BL/6 mice immunized with various immunogens, using ELISPOT assays. Mice (three per group) were vaccinated with VSSP, E7(p) + IFA, E7(p) + VSSP, or were not vaccinated. The splenocytes were harvested 7 days after the last vaccination as described in Section 2. The number of IFN-γ-producing E7-specific T cells precursors was determined in the presence (solid columns) and absence (open columns) of the E7(p). Data are expressed as mean number of SFC per 1 × 10⁶ splenocytes ± S.D.

4. Discussion

The E7 protein of HPV 16 represents an interesting model of a tumor-specific antigen [32] because the expression of this oncogenic early protein is necessary for the maintenance of the transformed state of the HPV-infected cells and is conserved in HPV-induced preneoplastic lesions and cancer [4]. The identification and characterization of CTL epitopes for HPV has promoted the development of peptide vaccines against cervical cancer [6,8]. Several HPV-16 E7-specific CTL epitopes have been characterized for the HLA-A.2 haplotype [8] and have potential clinical applicability. Peptides relevant to other HPV types (HPV-18) [33] and other HLA backgrounds (HLA-B18) are also under investigation [34].

In human studies, CTL responses were observed in some HPV-associated cancer patients after vaccination with lipidate peptides derived from HPV-16 E7 [9] or HPV-18 E6 [35]. In one Phase I/II study, no adverse side effects of peptide-based HPV vaccine were observed in patients [36]. In another study, HPV-16- and HLA-A.2-positive patients with high-grade cervical or vulvar intraepithelial neoplasia were vaccinated with epitopes from E7 HPV16, 12–20 or 86–93; 10 out of 16 patients exhibited measurable enhancement in cytokine release and cytotoxicity mediated by CTLs derived from PBMCs and some patients had partial clearance of virus and regression of lesions [7]. Both clinical trials have been conducted using IFA [7] and Montanide ISA 51 adjuvant [36].

Many cancer vaccine approaches have managed to induce tumor-specific activation of both CD4 and CD8 T cells. However, these vaccines have generally not been effective enough to produce a real tumor rejection. Particularly, lack of immunogenicity constitutes one of the most significant challenges in peptide vaccine development. Current opinion suggests that the potency of HPV peptide-based vaccines can be further enhanced by the use of stronger-immunostimulatory adjuvants (for a review, see [37]), which may increase the immune response in immuno-compromised individuals. Several studies have demonstrated that peptide antigens must be prepared (formulated) with appropriate adjuvants to facilitate their delivery to DCs and at the same time to activate these antigen-presenting cells to trigger marked T-cell responses.

In this study, we have investigated the antitumor response ability of a vaccine based on the CTL peptide from E7 HPV16 formulated with CIM's proprietary adjuvant, VSSP [26]. This vaccine formulation lead to protection of mice against a lethal dose of HPV-transformed tumor cells, induced the regression of subcutaneous, pre-established E7-expressing TC-1 tumors [28] associated with the induction of an E7-specific, systemic, antitumor immune response, specifically, the release

of IFN- γ by the CD8⁺ T cells. In addition, in this study we have shown that the antitumor immune response and HPV16 E7-specific CD8⁺ T-cell response is far more vigorous after vaccination with a E7(p) + VSSP than following vaccination with a E7(p) + IFA.

VSSP belong to the new generation of adjuvants based on pathogen-related molecules identified as “danger” signals that are recognized by the innate immune system [38]. A number of adjuvants evoke strong immune responses, and are widely used in research, but they are unsuitable for human vaccines because of toxic side effects and serious adverse events they can provoke. However, phase I trials of VSSP in melanoma and breast cancer patients have already demonstrated the safety of these preparations [39,40]. Moreover, several trials against meningococcal disease using OMPC of *Neisseria meningitidis* preventive vaccines, an important component of VSSP, were undertaken in the 1980s in Cuba, Norway, Iceland, Brazil and Chile, showing the safety of these preparations in children [41–43].

It has been previously demonstrated that VSSP can stimulate the anti-OVA immune response without the use of oily component, which is associated to the toxicity of emulsified vaccine formulations [27]. Moreover, VSSP have shown the capability to exert an adjuvant effect without any covalent conjugation to the nominal antigen [27]. VSSP may induce potent adjuvant function through DC activation [27] and even though the precise mechanisms are not fully understood, the comparison of VSSP (characterized by a low level of LPS, 3% relative to the total protein) with LPS suggests that, perhaps a pattern recognition molecule in addition to Toll 4 is required for VSSP signalling [27].

Our data suggest that the combination of E7(p) with VSSP by a single mix have preferentially delivered the peptide to DC guaranteeing efficient presentation by MHC class-I molecules to naive T cells, to induce CTL responses and tumor rejection.

Our data are in accordance with data obtained by others in the TC-1 tumor model developed by Wu and colleagues [28], which has been validated for testing novel immunotherapeutics for the treatment of HPV-associated neoplasia. Our results also confirm the potential of the E7 antigen as a target for cancer immunotherapy, which has been previously validated. In contrast to other approaches, E7(p) + VSSP immunotherapy avoids the unwanted side effects of oil-based adjuvant, or the use of live recombinant viral/bacterial vaccines vectors and potentially oncogenic HPV DNA sequences. Moreover, using exact minimal CTL epitope length bypasses the need for antigen-presenting cells to correctly process a whole antigen or large peptides before presenting the T-cell epitopes to the immune system.

In summary, we have demonstrated that a vaccine composed of a minimal CTL peptide mixed in an VSSP adjuvant can generate strong E7-specific antitumor immunity and that this preparation might be successfully used to enhance the presentation of tumor-specific antigens, thereby increasing antigen-specific antitumor immune response. These results

suggest that VSSP markedly enhance the number of tumor-specific CTL.

In this work, we have made a proof of concept in mouse using a single CTL peptide mixed with VSSP. In humans, a single epitope-based vaccine will however not likely be effective in all patients since the cellular response must match patient’s HLA. However, the use of multiple synthetic CTL epitopes restricted by different HLAs, mixed with VSSP could potentially overcome this restriction, covering ethnic backgrounds, and maintaining an adequate level of cellular immune response.

Since vaccine preparations types used in this study are easy to prepare and safe to administer to humans, our data provide important information for future clinical trials.

Acknowledgments

We thank Dr. T.C. Wu for providing the TC-1 cell line. We are also grateful to Dr. Silvio Perea for helpful discussions and to Dr. Ariana G. Ojalvo for her careful English revision.

References

- [1] Tomatis L. Cancer, cause occurrence and control. Lyon: World Health Organization, International Agency for Research on Cancer; 1990.
- [2] Bosch FX, Manos MM, Mu  oz N, Sherman M, Cansen AM, Peto J. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J Natl Cancer Inst* 1995;87:796–802.
- [3] Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999;189:12–9.
- [4] zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;5:342–50.
- [5] Melief CJ, Toes RE, Medema JP, van der Burg SH, Ossendorp F, Offringa R. Strategies for immunotherapy of cancer. *Adv Immunol* 2000;75:235–82.
- [6] Feltkamp MCW, Smits HL, Vierboom MPM, Minnaar RP, De Jongh BM, Drijfhout JW, et al. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol* 1993;23:2242–9.
- [7] Muderpach L, Wilczynski S, Roman L, Bade L, Felix J, Small LA. A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV16 positive. *Clin Cancer Res* 2000;6:3406–16.
- [8] Rensing ME, Stte A, Brandt RM, Ruppert J, Wentworth PA, Hartman M. Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*0201-binding peptides. *J Immunol* 1995;154:5934–43.
- [9] Steller MA, Gurski KJ, Murakami M, Daniel RW, Shah KV, Celis E. Cell-mediated immunological responses in cervical and vaginal cancer patients immunized with a lipidated epitope of human papillomavirus type 16 E7. *Clin Cancer Res* 1998;4:2103–9.
- [10] Ossendorp F, Mengede E, Camps M, Filius R, Melief CJ. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med* 1998;187:693–702.
- [11] Ossendorp F, Toes RE, Offringa R, van der Burg SH, Melief CJ. Importance of CD4⁺ T helper cell responses in tumor immunity. *Immunol Lett* 2000;74:75–9.

- [12] Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell response is mediated by CD40 signalling. *Nature* 1998;393:478–80.
- [13] Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4⁺ T-helper and T-killer cell. *Nature* 1998;393:474–8.
- [14] Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* 1998;393:480–3.
- [15] Hiranuma K, Tamaki S, Nishimura Y, Kusuki S, Isogawa M, Kim G, et al. Helper T cell determinant peptide contributes to induction of cellular immune responses by peptide vaccines against hepatitis C virus. *J Gen Virol* 1999;80:187–93.
- [16] Bristol JA, Orsini C, Lindinger P, Thalhamer J, Abrams SI. Identification of a *ras* oncogene peptide that contains both CD4⁺ and CD8⁺ T cell epitopes in a nested configuration and elicits both T cell subset responses by peptide or DNA immunization. *Cell Immunol* 2000;205:73–83.
- [17] Zwaveling S, Mota SCF, Nouta J, Johnson M, Lipford GB, Offringa R, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol* 2002;169:350–8.
- [18] Thompson HS, Davies ML, Watts MJ, Mann AE, Holding FP, O'Neill T, et al. Enhanced immunogenicity of a recombinant genital wart vaccine adjuvanted with monophosphoryl lipid A. *Vaccine* 1998;16:1993–9.
- [19] Ulrich JT, Myers KR. Monophosphoryl lipid A as an adjuvant: past experiences and new directions. *Pharm Biotechnol* 1995;6:495–524.
- [20] Sparwasser T, Koch ES, Vabulas RM, Heeg K, Grayson B, Lidford GB, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* 1998;28:2045–54.
- [21] Weiner GJ, Liu HM, Wooldridge JE, Dahle CE, Krieg AM. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc Natl Acad Sci USA* 1997;94:10833–7.
- [22] Vabulas RM, Pircher H, Lipford GB, Hacker H, Wagner H. CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J Immunol* 2000;164:2372–8.
- [23] Diehl L, den Boer AT, Schoenberger SP, van der Voort EIH, Schumacher TN, Melief CJM, et al. CD40 activation in vivo overcomes peptide-induced peripheral-cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat Med* 1999;5:774–9.
- [24] Disis ML, Bernhard H, Shiota FM, Hand SL, Gralow JR, Huseby ES, et al. Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood* 1996;88:202–10.
- [25] Fernando GJ, Stenzel DJ, Tindale RW, Merza MS, Morein B, Frazer IH. Peptide polymerisation facilitates incorporation into ISCOMs and increases antigen-specific IgG2a production. *Vaccine* 1995;13:1460–7.
- [26] Estevez F, Carr A, Solorzano L, Valiente O, Mesa C, Barroso O, et al. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). *Vaccine* 1999;18:190–7.
- [27] Mesa C, de Le  n J, Rigley K, Fern  ndez LE. Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for Th1 induction and dendritic cell activation. *Vaccine* 2004;22:3045–52.
- [28] Lin KY, Guarnieri FG, Staveley-O'Carroll K, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhance major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996;56:21–6.
- [29] Karkhanis YD, Zeltner JY, Jackson JJ, Carlo DJ. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative bacteria. *Anal Biochem* 1978;85:595–601.
- [30] Naito S, von Eschenbach AC, Giavazzi R, Fidler IJ. Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. *Cancer Res* 1986;46:4109–15.
- [31] Murali-Krishna K, Altman JD, Suresh M, Sourdive DJ, Zajac AJ, Miller JD, et al. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 1998;8:177–87.
- [32] Tindale RW. Human papillomavirus vaccines for cervical cancer. *Curr Opin Immunol* 1996;8:643–50.
- [33] Rudolf MP, Man S, Melief CJ, Sette A, Kast WM. Human T-cell responses to HLA-A-restricted high binding affinity peptides of human papillomavirus type 18 proteins E6 and E7. *Clin Cancer Res* 2001;7:788s–95s.
- [34] Bourgault Villada I, Beneton N, Bony C, Connan F, Monsonego J, Bianchi A, et al. Identifications in humans of HPV-16 E6 and E7 protein epitopes recognized by cytolytic T lymphocytes in association with HLA-B18 and determination of the HLA-B18-specific binding motif. *Eur J Immunol* 2000;30:2281–9.
- [35] Yoon H, Chung MK, Min SS, Lee HG, Yoo WD, Chung KT, et al. Synthetic peptides of human papillomavirus type 18 E6 harboring HLA-A2.1 motif can induce peptide-specific cytotoxic T-cells from peripheral blood mononuclear cells of healthy donors. *Virus Res* 1998;54:23–9.
- [36] Van Driel WJ, Rensing ME, Kenter GG, Brandt RM, Krul EJ, van Rossum AB, et al. Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a phase I–II trial. *Eur J Cancer* 1999;35:946–52.
- [37] Roden R, Wu TC. Preventive and therapeutic vaccines for cervical cancer. *Expert Rev Vaccines* 2003;2:495–516.
- [38] Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994;12:991–1045.
- [39] Carr A, Rodr  guez E, Arango M, Camacho R, Osorio M, Gabri M, et al. Immunotherapy of advanced breast cancer with a heterophilic ganglioside (NeuGcGM3) cancer vaccine. *J Clin Oncol* 2003;21:1015–21.
- [40] Guthmann MD, Bitton RJ, Carnero AJ, Gabri MR, Cinat G, Koliren L, et al. Active specific immunotherapy of melanoma with a GM3 ganglioside-based vaccine: a report on safety and immunogenicity. *J Immunother* 2004;27:442–51.
- [41] Tappero JW, Lagos R, Ballesteros AM, Plikaytis B, Williams D, Dykes J, et al. Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. *JAMA* 1999;281:1520–7.
- [42] de Kleijn ED, de Groot R, Labadie J, Laf  ber AB, van den Dobbelsteen G, van Alphen L, et al. Immunogenicity and safety of a hexavalent meningococcal outer-membrane-vesicle vaccine in children of 2–3 and 7–8 years of age. *Vaccine* 2000;18:1456–66.
- [43] Boslego J, Garcia J, Cruz C, Zollinger W, Brandt B, Ruiz S, et al. Efficacy, safety, and immunogenicity of a meningococcal group B (15, P1.3) outer membrane protein vaccine in Iquique Chile. Chilean National Committee for Meningococcal Disease. *Vaccine* 1995;13:821–9.

Active antimetastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant

Belinda Sánchez Ramírez^{1*}, Eduardo Suárez Pestana¹, Greta Garrido Hidalgo¹, Tays Hernández García¹, Rolando Pérez Rodríguez¹, Axel Ullrich² and Luis Enrique Fernández¹

¹Vaccine Department, Center of Molecular Immunology, Havana, Cuba

²Department of Molecular Biology, Max-Planck-Institute for Biochemistry, Martinsried, Munich, Germany

The epidermal growth factor receptor (EGFR) plays a central role in regulating neoplastic processes. The EGFR overexpression in many human epithelial tumors has been correlated with disease progression and bad prognosis. Passive EGFR-directed immunotherapy, but not active specific approaches, has already been introduced in medical oncology practice. Then we wonder if mice immunization with the extracellular domain of murine EGFR (mEGFR-ECD) in adjuvants can circumvent tolerance to self EGFR, by inducing an immune response with consequent antitumor effect. The present study demonstrated that despite mEGFR expression in thymus, strong DTH response was induced by inoculation of mice with the mEGFR-ECD. This self-immunization, using both CFA and very small sized proteoliposomes from *Neisseria meningitidis* (VSSP), promoted highly specific IgG titers, predominantly IgG2a and IgG2b. Sera from mice immunized with mEGFR-ECD/VSSP not only recognized EGFR+ tumor cell lines by FACS, but also inhibited their *in vitro* growth, even in the absence of complement. Noteworthy, vaccination of mice with mEGFR-ECD/VSSP stimulated a potent antimetastatic effect in the EGFR+ Lewis lung carcinoma model, while reproduction-associated side effects were absent. Curiously, mice immunized with the human EGFR-ECD (Her1-ECD) in VSSP though induced highly specific IgG antibodies with strong *in vitro* cytotoxic effect over EGFR+ human cell lines, showed low cross-reactivity with the mEGFR-ECD. These results further encouraged the development of the Her1-ECD/VSSP vaccine project for patients with EGFR+ tumors.

© 2006 Wiley-Liss, Inc.

Key words: EGFR; self-immunization; cancer therapy

The epidermal growth factor receptor (EGFR) belongs to the erbB family of 4 closely related cell membrane receptors, also known as the Type I receptor tyrosine kinase family: EGFR or HER1/erbB1, first to be molecularly cloned,¹ HER2/erbB2, HER3/erbB3 and HER4/erbB4. The 4 receptors consist of an extracellular ligand-binding domain (ECD), a transmembrane domain and an intracellular domain with tyrosine kinase activity for signal transduction. EGFR plays a central role in regulating both development and neoplastic processes. Binding of their specific ligands, such as epidermal growth factor (EGF) or transforming growth factor alpha (TGF- α) among others, induces receptor activation, modulation of cell proliferation and differentiation in normal tissues and tumors. Although expressed in nonmalignant cells, the EGFR can be found overexpressed or mutated in many human epithelial tumors such as breast,^{2,3} lung,⁴ prostate^{5,6} head and neck,⁷ colorectal,⁸ pancreatic,⁹ bladder,¹⁰ vulva and ovarian tumors.¹¹ This overexpression has been correlated with disease progression and poor prognosis.^{12,13} Activation of the EGFR signaling pathway in cancer cells has been shown to enhance cell proliferation, angiogenesis, tumor promotion and metastasis, and to decrease apoptosis. The potential of EGFR-targeted therapies for cancer treatment has increased the development of different passive agents. Passive immunotherapy with specific monoclonal antibodies (MAb)^{14,15} and treatment with tyrosine kinase inhibitor drugs such as Iressa^{16,17} and Tarceva¹⁸ are currently undergoing clinical trials with promising results or are commercially available. On the other hand, active immunotherapy strategies to block the EGF from binding to its receptor are being clinically tested by vaccinating patients with EGF coupled to P64k recombinant protein from *Neisseria meningitidis*.¹⁹

In addition, EGFR-based active specific immunotherapy may be an alternative and complementary approach for the treatment of epithelial tumors, provided that induction of an immune response against self EGFR is feasible. Preclinical studies of both a DNA vaccine based on xenogenic EGFR-ECD and dendritic cells pulsed with self EGFR-ECD have been recently published, demonstrating the validity of this active immunotherapy.^{20,21} Here an alternative, more simple, approach, based on vaccination with the mEGFR-ECD protein for exploring the possibility of circumventing tolerance to self EGFR, was proposed. We constructed DNA plasmids encoding the murine EGFR-ECD, which were stably transfected in mammalian cells, and the corresponding recombinant protein was used for vaccination protocols. Besides, we expressed the Her1-ECD to compare the relative efficacy of self and non-self-immunization and for evaluating the immune response specificity to EGFR+ human tumor cells. A strong DTH response and specific IgG titers with a TH1-associated subclass pattern were obtained by inoculation of mice with the self protein in very small sized proteoliposomes (VSSP) and complete Freund adjuvant. The corresponding immune sera showed *in vitro* antitumor effect, inhibiting EGFR+ tumor cells proliferation. mEGFR-ECD vaccination induced a potent antimetastatic effect in 3LL-D122 Lewis lung carcinoma. Antibodies obtained in mice immunized with Her1-ECD/VSSP evidenced a low cross-reaction with the parent mEGFR-ECD.

Material and methods

Construction of the expression vector encoding mEGFR-ECD and Her1-ECD

DNA encoding the extracellular domain of murine EGFR was amplified by PCR using total cDNA from mouse liver as template. The sense primer 5'-CGGAATTCCTCTCCCGGTCAGAGATGC-GAC-3' includes EcoRI excision site, the initiation codon and 4 bp from EGFR signal sequence. The antisense primer 5'-CGGGATCCTCAAGATGGTATCTTTGGCCCAGATG-3' is complementary to bp 1978–2000 in 3' region and contains a stop codon (double underlined) and a BamHI excision site (single underlined). The PCR product, a 1.9-kb fragment, was cloned into EcoRI/BamHI sites of the pBluescript KS⁺ vector. The fragment encoding for mEGFR-ECD was recovered using HindIII/BamHI enzymes and cloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA), generating the mEGFR-ECD/pcDNA3 plasmid.

DNA encoding the extracellular domain of human EGFR (Her1-ECD) was amplified by PCR using the Her1 Δ 533/pRK5 plasmid as template. The sense primer 5'-GGGGTACCCCTCGGG-GAGCAGCGATGCGA-3' includes a KpnI excision site (underlined), the initiation codon ATG and 3 bp from the signal sequence of Her1. The antisense primer 5'-GCTCTAGATCAGGACGGG-ATCTTAGGCCCA-3' is complementary to bp 2103–2123 in the

*Correspondence to: Center of Molecular Immunology (CIM), P.O. Box 16040, Havana 11600, Cuba. Fax: +53-7-272-0644, 53-7-273-3509. E-mail: belinda@cim.sld.cu

Received 14 October 2005; Accepted 8 March 2006

DOI 10.1002/ijc.22085

Published online 13 July 2006 in Wiley InterScience (www.interscience.wiley.com).



Publication of the International Union Against Cancer

3' region, and contains a stop codon (double underlined) and an XbaI excision site (single underlined). The PCR product, a 1.9-kb fragment, was cloned into KpnI/XbaI sites of the pcDNA 3-expression vector, generating the Her1-ECD/pcDNA3 plasmid.

mEGFR-ECD and Her1-ECD sequences were confirmed, by dideoxy nucleotide sequencing analysis, to be identical with those previously reported.^{22,23} All enzymes were supplied by Boehringer-Mannheim, Penzberg, Germany.

Cell lines

Ehrlich Ascites tumor (EAT, ECACC No. 87032503), 3LLD122, a metastatic variant of Lewis lung carcinoma,²⁴ the murine thymoma EL4 (ATCC TIB-39), human embryonic kidney (HEK293, ATCC CRL-1573), human epidermoid carcinoma A431 (ATCC CRL-1555) and human lung adenocarcinoma H125²⁵ cell lines were grown in DMEM (Gibco, NY, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone, Utah), 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin 100 U/ml and streptomycin 100 µg/ml (Life Technologies, Grand Island, NY). HEK293 transfectants were adapted to growth in HyQ PF 293 (protein-free medium from Hyclone, Utah).

Generation of HEK293 transfectants

HEK293 cells were grown in 6-well plates (1.75×10^5 cells/ml), and 8 hr later were transfected with 4 µg of mEGFR-ECD/pcDNA3 or Her1-ECD/pcDNA3 plasmids, using the calcium phosphate transfection system. Plates were incubated overnight at 3% CO₂, and then at 5% CO₂. Transfected cells were selected in medium containing 1,000 µg/ml of G418 (Geneticin, Sigma) starting 48 hr after transfection, for the generation of mEGFR-ECD/HEK293 and Her1-ECD/HEK293 stable cell lines. Mock transfection (with pcDNA3 vector) was used as a negative control.

Lectin- or antibody-mediated precipitations of the recombinant proteins

Supernatants from mEGFR-ECD/HEK293 or Her1-ECD/HEK293 cultures (2 ml) were mixed with 10 µl of lectin-agarose (a lectin from *Triticum vulgaris*, Sigma, St. Louis) or 1 µg of R3 MAb (a MAb specific for human EGFR extracellular domain; CIM, Havana, Cuba) plus 20 µl of Protein A-Sepharose (Amershan-Pharmacia Biotech, Uppsala, Sweden), respectively. Samples were gently shaken overnight at 4°C, and afterwards centrifuged for 1 min at 11,000g. The precipitated recombinant proteins were separated on SDS-PAGE 7.5% and visualized by silver staining.

Purification and immunoblotting of mEGFR-ECD and Her1-ECD

Recombinant proteins were purified from confluent cultures of the respective transfectants by affinity chromatography. EAH-Sepharose 4B (Amershan-Pharmacia Biotech) was covalently coupled to human recombinant EGF (hrEGF) (Center of Genetic Engineering and Biotechnology, CIGB, Cuba) or to R3 MAb for mEGFR-ECD and Her1-ECD purification, respectively. Equilibration and washing steps were performed with PBS/NaCl (1 M, pH 7.0) and protein elution with glycine (0.2 M, pH 2.8). Purity was assessed by densitometry, using a personal densitometer SI (Amershan-Pharmacia Biotech) and Imag Quant Software. Protein concentrations were assayed by Lowry's method.²⁶

Purified proteins' identity was established by immunoblotting. Recombinant proteins (30 µg) were applied to 7.5% SDS-PAGE gels and transferred to PVDF membranes (Gelman, Ann Arbor, MI). After blocking with NEG buffer (0.15 M NaCl, 5 mM EDTA, 500 mM Tris-HCl (pH 7.5), 0.02% Tween 20, 0.04% gelatin), membranes were incubated with R3 (for Her1-ECD, data not shown) or 7A7 (for mEGFR-ECD) MAbs and proteins were visualized using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Perkin Elmer Life Sciences).

RT-PCR

Total RNA was isolated from Balb/c or C57BL/6 mice thymus using Trizol reagent (Life Technologies) according to the manufacturer's instructions. The reverse transcription and polymerase

chain reaction (RT-PCR) was performed using the SUPER-SCRIPTTM one-step RT-PCR system. Used EGFR and β -actin primers were designed from the published sequences.²⁷ After PCR amplification, 10 µl of the RT-PCR products were separated by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide. Total RNAs from murine thymoma EL4 was used as negative control.

Mice and immunization protocols

Female C57BL/6 mice, aged 8–12 weeks, were purchased from the National Center for Laboratory Animals Production (CEN-PALAB, Havana, Cuba). All mice were kept under pathogen-free conditions. Animal experiments were approved by the Center of Molecular Immunology's Institutional Animal Care and Use Committee (CIM).

Mice ($n = 5$ or $n = 10$ for DTH and humoral response studies, respectively) were immunized with 50 µg of either mEGFR-ECD or Her1-ECD in FA adjuvant, complete for the first immunization and incomplete for the rest (Sigma) or in VSSP adjuvant, obtained from the combination of the outer membrane proteins of *Neisseria meningitidis* with GM3 ganglioside, in water/oil (Montanide ISA 51, Seppic, Paris, France) emulsion.²⁸ Immunizations were made, subcutaneously (sc) for FA-adjuvanted or intramuscularly (im) for VSSP-adjuvanted preparations, on days 0, 14 (for the DTH study) and 0, 14, 28 and 42 (for humoral response studies). Sera were extracted on days 0, 21, 35 and 56. Control groups received PBS/FA or PBS/VSSP.

DTH test

Seven days after the last immunization, mice were sensitized by intradermal injection with 50 µg of mEGFR-ECD in 50 µl of PBS in the right hind foot pad and by the same volume of PBS in the left foot pad. After 48 hr, mice foot swellings were measured using a plethysmometer (Ugo Basile, VA, Italy). Mice immunized with 100 µg of Keyhole Limphet Hemocyanin (KLH), (Sigma, Aldrich) in FA and sensitized with KLH in PBS were used as positive controls, while those injected with PBS/FA and sensitized with mEGFR-ECD were considered as negative controls. Differences in DTH between treatment groups were statistically validated by Kruskal Wallis and Dunn's multiple comparison test.

Enzyme immunoassay

Microtiter plates (High binding, Costar) were coated with 10 µg/ml of mEGFR-ECD or Her1-ECD in carbonate buffer (0.1 M, pH 9.6) and incubated overnight at 4°C. Plates were blocked with 5% calf serum in PBS/Tween-20, and sera dilutions in duplicate, from immunized mice ($n = 10$), or preimmune sera (as negative control) were incubated for 1 hr at 37°C. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma) was added and incubated for 1 hr at 37°C. After addition of *p*-nitrophenylphosphate (1 mg/ml) (Sigma), the optical density (OD) was measured at 405 nm using a microwell system reader (Organon Teknica, Salzburg, Austria). All washes were made with PBS/Tween-20. The Mann Whitney U test was used to assess statistical differences between individual time points in the humoral response kinetic. ELISA test background was 2 times the OD at 405 nm of preimmune sera, which coincides with the OD value for PBS.

For determination of serum IgG subclasses, secondary isotype-specific biotinylated rat anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibodies were used (PharMingen). Optimal secondary reagent dilutions were established by ELISA with 14F7 (IgG1-specific for NGcGM3), T3 (IgG2a-specific for CD3) and T4 (IgG2b-specific for CD4) MAbs (CIM), while R24 MAb (IgG3-specific for GD3) (kindly provided by Dr. Philip O. Livingston, Memorial Sloan Kettering Cancer Center, NY). Unpaired *t*-test was used to check statistically significant differences between sera dilutions.

ELISPOT

Specific inguinal lymph (LN) nodes spot forming cells (SFC) were obtained from mice immunized with Her1-ECD/VSSP and

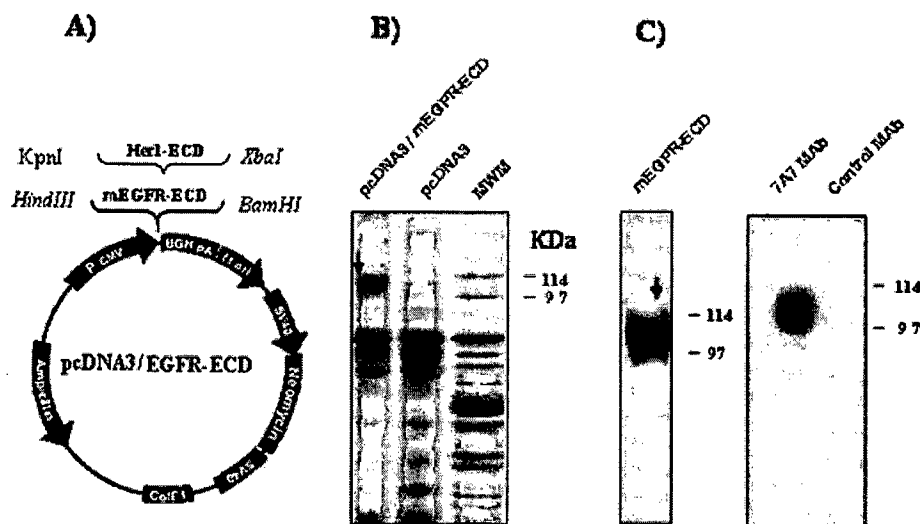


FIGURE 1 – Construction and functionality of the expression vector and purification of the mEGFR-ECD. (a) DNAs encoding the mEGFR-ECD or the Her1-ECD were inserted into the pcDNA3 expression vector. (b) mEGFR-ECD/pcDNA3 construct was verified by sequencing, and protein expression was checked by precipitation with lectin-agarose from supernatants of the HEK293 transfectant and visualized in 7.5% SDS-PAGE gels with silver staining. Mock transfection (pcDNA3) was used as control. (c) mEGFR-ECD purification was afforded by affinity chromatography with hEGF-EAH Sepharose (left) and protein identity confirmation by Western blotting (right) using 7A7 Mab. An isotype control Mab was used as negative control.

tested by enzyme-linked immunospot (ELISPOT) as previously described,²⁹ with some modifications. Maxisorp 96-well plates (Nunc) were coated with 10 µg/ml of Her1-ECD or mEGFR-ECD in 50 µl carbonate buffer (pH 9.8) at 4°C overnight. After blocking with 5% BSA in PBS, different dilutions of pooled LN cells were incubated in triplicate for 6 hr at 37°C in a 5% CO₂ incubator. Antibodies secreted by individual cells were revealed as spots by the stepwise addition of 1.5 µg/ml of alkaline phosphatase-conjugated goat anti-mouse IgG (Fcγ) or IgM (Fcμ) antibodies (Jackson Immunoresearch laboratories, West Grove, PA) and the addition of 1 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Sigma, San Louis, MO) in 0.1 M AMP buffer (pH 10.5) containing 0.6% agarose. Plates were incubated overnight at 4°C and the results were scored the next day by counting the number of specific SFC in a stereoscopic microscope. LN cells from mice injected with PBS/FA were used as negative control.

FACS analysis for EGFR recognition

Cells were stained with sera from immunized mice (1/200 dilution) followed by FITC-goat anti-mouse IgG (Jackson Immunoresearch laboratories). Up to 10,000 cells were acquired using a FACSscan flow cytometer and analyzed using the CellQuest software (Beckton Dickinson, San Jose, CA). PCR and 7A7 Mab,³⁰ which is specific for mEGFR-ECD, were used to confirm EGFR expression in murine cells. Human EGFR expression in the corresponding cells was confirmed with R3 Mab. EL4 murine cell line and human lymphocytes were used as negative control cells.

Growth assay

Flat-bottomed 96-well microculture plates were seeded with 10⁴ cells in 100 µl/well and grown in DMEM supplemented with 1% FCS in the presence of sera dilutions. After 48 hr of incubation at 5% of CO₂, cells' viability was measured by the modified colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay.^{31,32} Media were replaced by 100 µl/well of MTT (1 mg/ml) and plates were incubated under culture conditions for 4 hr. Formazan crystals were dissolved by addition of 100 µl/well of dimethyl sulfoxide followed by 30 min incubation at 37°C. Absorbance (OD) was measured at 540 nm using a microplate spectrophotometer and the reference wavelength (620 nm) OD subtracted. Background control contained only culture me-

dium without cells. Cells without treatment were included as a maximum cell growth point. R3 and 7A7 MABs were used as positive controls for human and murine cells lines, respectively. Statistical differences in the *in vitro* viability assay were evaluated by the unpaired *t*-test.

Cytotoxicity assay

Flat-bottomed 12-well microculture plates were seeded with 5 × 10⁵ cells in 100 µl/well and grown in DMEM supplemented with 1% FCS in the presence of diluted sera (1/10). Sera from nonimmunized mice were used as control for unspecific complement-mediated cytotoxicity. To measure specific complement-independent cytotoxicity, immune sera were heated for 30 min at 56°C, and after 24 hr of incubation at 5% of CO₂, death cells were counted by FACS using propidium iodide. Cells without treatment were included as control for minimum death cell. R3 and 7A7 MABs were used as positive controls for human and murine cells lines, respectively.

Tumor challenge assay

C57/BL6 mice (*n* = 10) were immunized, intramuscularly, 3 times biweekly with 100 µg of mEGFR-ECD/VSSP or PBS/VSSP. One day before the second immunization, mice were challenged with 2 × 10⁵ tumor cells, subcutaneously, in the foot pad. Three weeks later, tumors reached 0.8 cm and were surgically removed. Twenty-one days after surgery, mice were sacrificed and spontaneous lung metastases quantified by weighing the lungs. Statistical differences between groups were determined by unpaired *t*-test.

Reproductive side effects studies

Female Balb/c mice (*n* = 10) were immunized with mEGFR-ECD/VSSP or PBS/VSSP (control group) as previously described. After checking the induction of specific antibodies against mEGFR-ECD, mice were mated with nonimmunized male animals. Fertility (number of mice completing pregnancy), number of pups, pups' birth weights and certain postnatal developmental features such as eyes opening, hair growth and incisor eruption were observed. The Mann Whitney U test was used to test statistically significant variations in the reproduction parameters between treated and control animals.

Statistical analyses

Variance homogeneity and data normal distribution were analyzed by Bartlett's and Bonferroni tests, respectively, using the

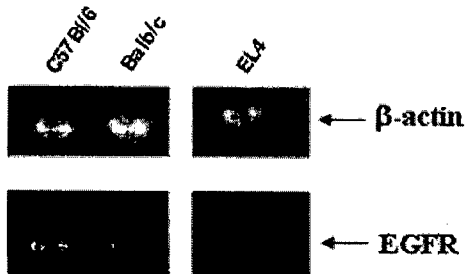


FIGURE 2 – EGFR is expressed in mice thymus tissue. Thymuses from C57Bl/6 and Balb/c mice were analyzed by RT-PCR for EGFR expression. Total RNA was extracted from thymuses of C57Bl/6 or Balb/c mice and from EL4 tumor (negative control) using TRIZOL reagent. RNA (1 μ g) was reverse-transcribed to cDNA and the specific fragments amplified by PCR. β -actin mRNA served as an internal control.

SPSS version 10.0 software. All statistical tests (Kruskal Wallis and unpaired *t*-tests) were 2-sided and conducted using the Graph Pad Prism version 4.00 software. A probability value of $p < 0.05$ was considered as statistically significant.

Results

mEGFR-ECD and Her1-ECD expression and purification

cDNAs encoding mEGFR-ECD and Her1-ECD were successfully cloned into the pcDNA3 expression vector (Fig. 1a) and transfected in HEK293 cells as previous description. Expression of the soluble recombinant proteins by stable HEK293 transfectants was checked by lectin-agarose precipitation (mEGFR-ECD) or immune-precipitation with R3 MAb (Her1-ECD). In each case, as expected, a 105-kDa protein band was displayed but not for mock transfection, as determined by SDS-PAGE. Figure 1b shows the corresponding results for the mEGFR-ECD. mEGFR-ECD identity was further confirmed by affinity chromatography (with hrEGF-EAH Sepharose) purification combined with SDS-PAGE and western blotting, showing again the 105-kDa protein band (Fig. 1c). Achieved protein purity was 98% after only 1 purification step as determined by densitometry (data not shown).

mEGFR is expressed in thymus

As has been previously reported from studies with rat cell lines³³ and human thymus,³⁴ the EGFR presence in mice thymus was demonstrated by RT-PCR. The corresponding EGFR cDNA band (Fig. 2) became apparent when thymuses from C57BL/6 and Balb/c mice were analyzed, but not from murine thymoma EL4, used as a negative control.

mEGFR-ECD immunization induces DTH response

Generation of specific DTH responses was considered as a primary endpoint for the mEGFR-ECD vaccine (emulsified in FA) ability in immunizing mice. Mice were subcutaneously injected with mEGFR-ECD/FA and then sensitized with 50 μ g of the nominal antigen. After 48 hr, mice foot swellings were measured, and inflammation scores in the vaccinated group animals were higher than those of the negative control group ($p < 0.05$, Dunn's multiple comparison test). Noteworthy, DTH responses induced in mice vaccinated with either mEGFR-ECD or KLH, a strongly immunogenic protein, were similar ($p > 0.05$) (Fig. 3).

mEGFR-ECD immunization induces a strong and long-lasting specific humoral response

Vaccine-related humoral responses were explored by immunizing C57BL/6 mice 4 times biweekly with 50 μ g of mEGFR-ECD in 2 different adjuvants: FA, the reference adjuvant, and VSSP, a new product already clinically tested in humans. Inoculated mice developed high serum IgG antibody levels against the immunizing

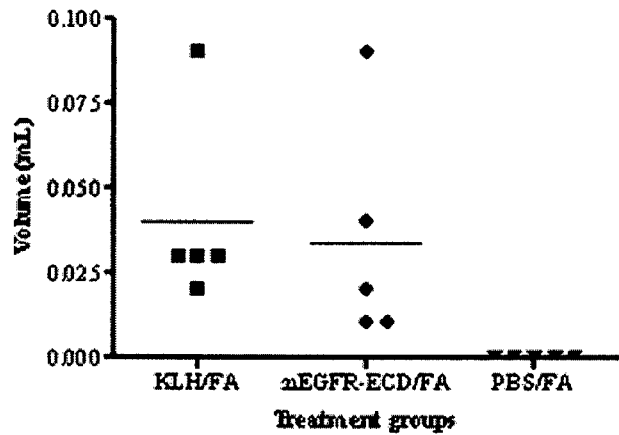


FIGURE 3 – mEGFR-ECD specific DTH response. Vaccine-induced DTH response was assayed by immunizing C57Bl/6 mice 2 times with 50 μ g of mEGFR-ECD/FA. KLH/FA (50 μ g) was used as positive control and PBS/FA as negative control. Mice included in the first and third groups were sensitized 7 days later with the mEGFR-ECD in PBS, while animals belonging to the second group were sensitized with KLH. mEGFR-ECD/FA-treated mice showed higher foot pad inflammations (Dunn's multiple comparison test, $p < 0.05$) than those of PBS control mice, but similar (Dunn's multiple comparison test, $p > 0.05$) to those in KLH group. A representative experiment from 2 independent ones is shown.

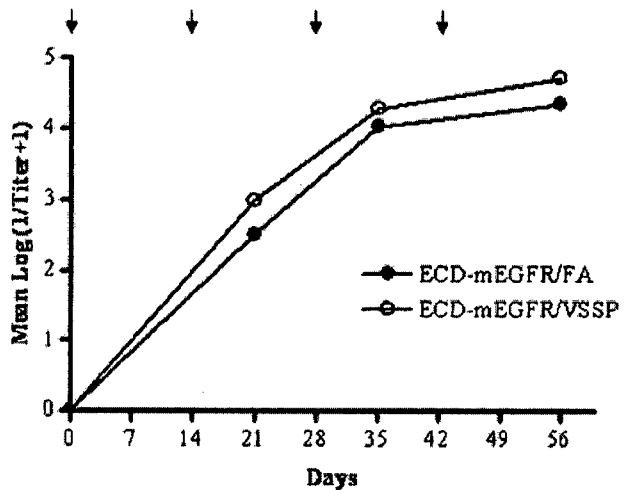


FIGURE 4 – Kinetics of the anti-mEGFR-ECD humoral response. Mice were immunized 4 times with 50 μ g of the mEGFR-ECD in VSSP or FA biweekly. Antibody titers were quantified by ELISA in sera collected on days 0, 21, 35 and 56. Data was log transformed ($1 + 1/\text{titer}$) for graphic representation. While absent before the first vaccine administration (day 0), an increased antibody presence was detected after immunizations, preferentially when VSSP was used as adjuvant (Mann Whitney U test, $p < 0.05$). Immunization days are represented by arrows. A representative experiment from 3 independent ones is shown.

protein, which increased with successive immunizations, for both adjuvant formulations (Fig. 4). Indeed, the mEGFR-ECD/VSSP vaccine induced higher antibody titers than the FA one in each sera collection day (Mann Whitney U test, $p < 0.05$). Eight of 10 mice (80%) immunized with mEGFR-ECD/VSSP showed specific antibody titers above 1/40,000 by day 56, even reaching values up to 1/160,000, whilst only 2 of 10 mice (20%) rose titers above 1/40,000 in the mEGFR-ECD/FA immunized group (Table I). One year after having finished the immunization schedule and

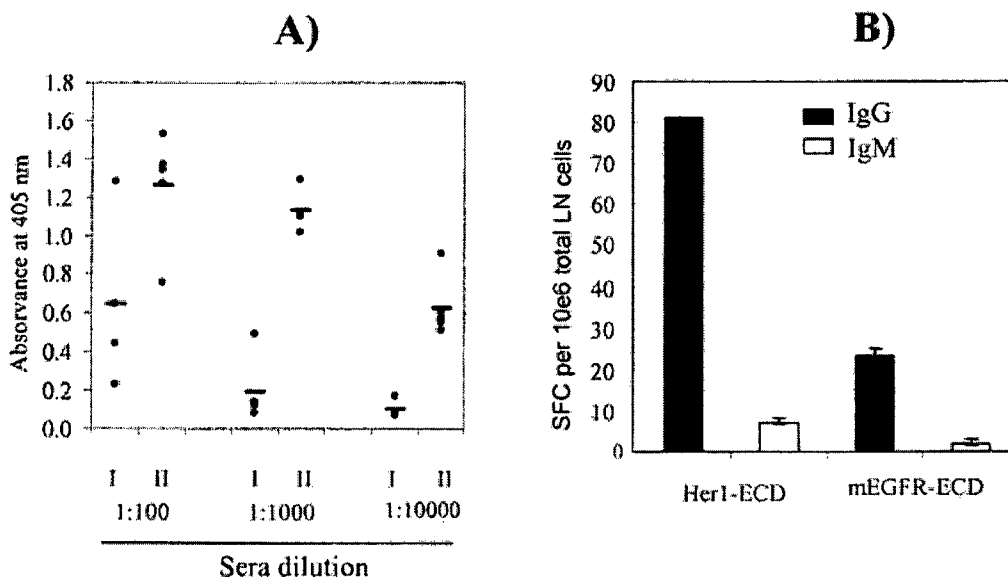


FIGURE 6 – Immunization with Her1-ECD/VSSP mobilizes B cell responses. Mice were immunized 2 times with 50 μ g of Her1-ECD/VSSP biweekly. (a) Specific IgG antibodies against the Her1-ECD (II) and their cross-reaction with mEGFR-ECD (I) were assayed by ELISA employing sera collected on day 21. (b) Specific SFC, secreting IgG and IgM antibodies against the Her1-ECD and their cross-reaction with the mEGFR-ECD were measured by ELISPOT. A representative experiment from 2 independent ones is shown.

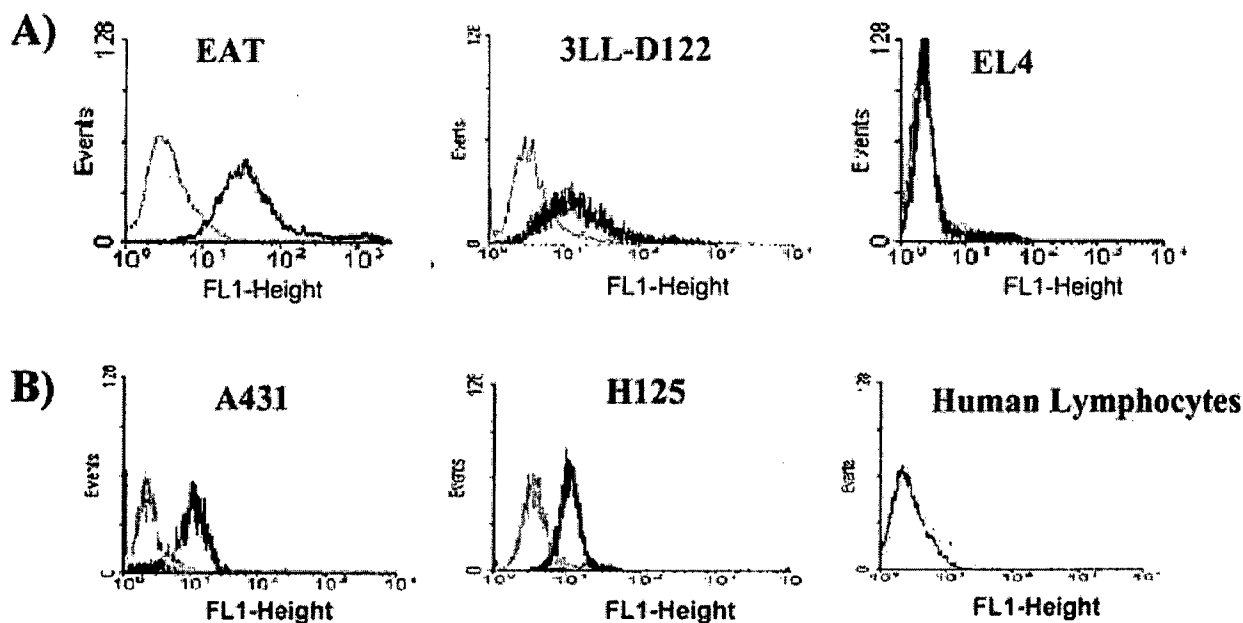


FIGURE 7 – Immune sera recognize EGFR+ tumor cells. (a) 1/200 sera dilutions, obtained from mice immunized with mEGFR-ECD/VSSP, reacted with 3LL-122 and EAT cell lines (black line) but not with EL4 cells. (b) 1/200 sera dilutions, obtained from mice immunized with Her1-ECD/VSSP, reacted with A431 and H125 cell lines (black line) but not with human lymphocytes. Sera from mice immunized with PBS/VSSP (gray line) were used as negative control.

Absence of reproductive side effects in mice immunized with mEGFR-ECD/VSSP

The potential side effects of “self” immunization in humans were stressed by examining the appearance of possible toxic symptoms in female mice immunized with the mEGFR-ECD in VSSP. After the vaccine, inoculation animals were mated and their progeny studied. Pregnancies rates were 5 out of 10 in the immunized group, while 3 out of 10 in control mice. The median

number of pups per litter in both groups was 6 (range 5–7). Features like newborns’ weights, hair growth, eyes opening and incisor appearance did not comparatively differ (Mann Whitney U test, $p > 0.05$) (Table II). On the other hand, a group of mice were observed for 1 year after fulfilling the immunization protocol, and the vitality, temperature and food intake were completely normal, without changes in functional hepatic parameters when compared with those of nonimmunized mice (data not shown).

TABLE I - RESPONSE FREQUENCY AND IgG TITERS¹ IN INDIVIDUAL ANIMALS BY DAY 56 AFTER IMMUNIZATION WITH THE mEGFR-ECD, USING FA OR VSSP AS ADJUVANTS

Treatment groups	Response frequency	1/IgG titer						
		2,500	5,000	10,000	20,000	40,000	80,000	160,000
mEGFR-ECD in FA	10/10	1	1	3	3	1	1	
mEGFR-ECD in VSSP	10/10		1		1	2	4	2

¹ Assayed by ELISA.

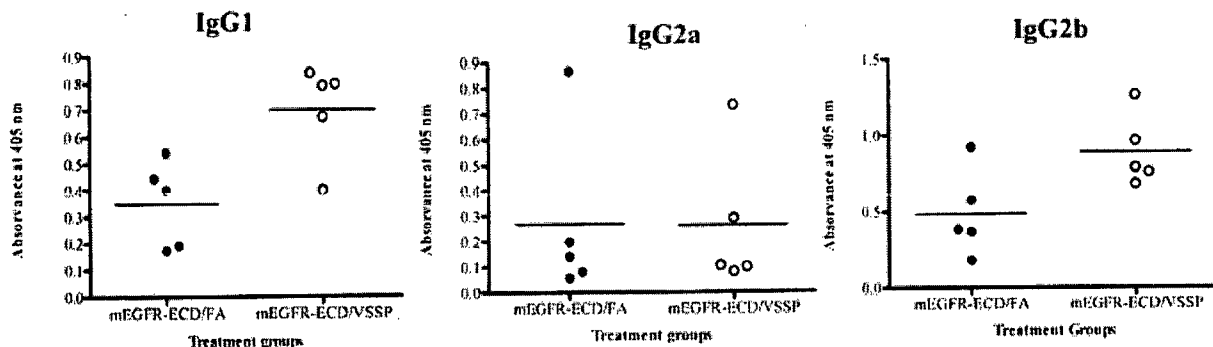


FIGURE 5 - IgG subclasses induced by immunization. Serum IgG subclasses pattern in C57Bl/6 mice after 2 inoculations with 50 μ g of the mEGFR-ECD in VSSP or CFA biweekly, was measured by ELISA with samples collected on day 21, and diluted 1/10,000. Each point represents the mean absorbance value of duplicate samples in individual mice ($n = 5$). IgG2b levels were higher (unpaired t -test, $p < 0.05$) in the group immunized with mEGFR-ECD/VSSP, while no differences were found for IgG2a. A representative experiment from 3 independent ones is shown.

without antigen recall, the sera-specific IgG levels decreased in both groups of mice, although an appreciable response was still detected in 100% of animals (1/1,000 and 1/100 sera dilutions) (data not shown).

Administration of mEGFR-ECD in FA or VSSP to Balb/c mice produced elevated IgG titers in 100% of animals, similarly to what was observed in the C57Bl/6 case (data not shown).

Vaccination with mEGFR-ECD polarizes systemic immunity to a TH1 pattern

Consistent with previous results demonstrating that complete FA preferentially promotes a TH1 type response to the accompanying antigen,³⁵ elevated levels of IgG2a, IgG2b, and IgG1 were detected in day 21 sera corresponding to mice immunized with mEGFR-ECD/FA. As shown in Figure 5, while no differences in IgG2a levels were found in sera of mice vaccinated with FA or VSSP formulations (unpaired t test, $p > 0.05$), the use of VSSP promoted the induction of higher levels of IgG2b ($p < 0.05$).

Her1-ECD/VSSP immunization generates specific B cell clones with low cross-reactivity to the mEGFR-ECD

We wonder if immunization with the xenogenic EGFR-ECD could induce antibodies reacting with the self EGFR. C57Bl/6 mice were immunized with 50 μ g of Her1-ECD in FA or VSSP. All immunized mice, independent of the used adjuvant, developed high IgG antibody titers against the human protein (1/320,000) by day 56 (data not shown). Alternatively, ELISA experiments reflected an evident but low cross-reactivity with the murine protein by day 21 (Fig. 6a). This low cross-reactivity against the mEGFR-ECD was confirmed by ELISPOT assay. Sixty-two IgG and 6 IgM secreting specific SFC in 10^6 LN cells were found in LN of Her1-ECD-immunized mice, while only 21 IgG secreting SFC/ 10^6 LN cells cross-reacted with the mEGFR-ECD (Fig. 6b).

Immune sera recognizes full length EGFR by FACS

To check whether immunizations with a truncated EGFR affected the recognition of the full length EGFR in its native conformation on the cell surface, EGFR+ cells were analyzed by FACS. EAT³⁶

and 3LL-D122 murine cell lines were positively stained by sera from mice immunized with mEGFR-ECD/VSSP (Fig. 7a). Besides, sera from mice immunized with Her1-ECD/VSSP readily reacted with A431 and H125 cell lines (Fig. 7b). Sera from control mice, immunized with PBS/VSSP, neither recognized murine nor human EGFR+ cells.

Immune sera inhibit EGFR+ tumor cells growth and possess cytotoxic effect

To determine whether immunization with mEGFR-ECD/VSSP or Her1-ECD/VSSP can generate serum antibodies affecting murine or human tumor cells growth *in vitro*, the MTT viability assay was performed. Incubation of 3LL-D122 or H125 cells with sera obtained from mice immunized with mEGFR-ECD/VSSP or Her1-ECD/VSSP, respectively, decreased the number of viable cells if compared with preimmune sera after 48 hr, and this effect was sera-dilution-dependent (unpaired t -test, $p < 0.05$) (Fig. 8). In addition, the immune sera *in vitro* cytotoxicity over EGFR+ cells was evaluated after treating cells for 24 hr with the corresponding complement inactivated samples, following propidium iodide staining. FACS analysis showed that treatment of 3LL-D122 cells with inactivated sera produced 55.83% of death cells, suggesting that a complement-independent cytotoxicity mechanism is operating (Fig. 9a). In parallel, H125 cells were treated with sera from mice immunized with Her1-ECD/VSSP, and the effects over the cells evaluated. Incubation with immune sera manifested cytotoxic effects (FACS) (Fig. 9b).

Antimetastatic effect of mEGFR-ECD/VSSP vaccination

To investigate whether the autologous vaccination can protect individuals from metastatic widespread, mice were immunized with the mEGFR-ECD in VSSP and, 1 day before the second immunization, challenged with 2×10^5 tumor cells in the foot pad. Three weeks after malignant cells inoculation, tumors were surgically removed. Twenty-one days after surgery, mice were sacrificed and the spontaneous lung metastases quantified by weighing the lungs. As shown in Figure 10, vaccination of mice significantly reduced lung metastasis ($p < 0.01$), compared with animals in the control group.

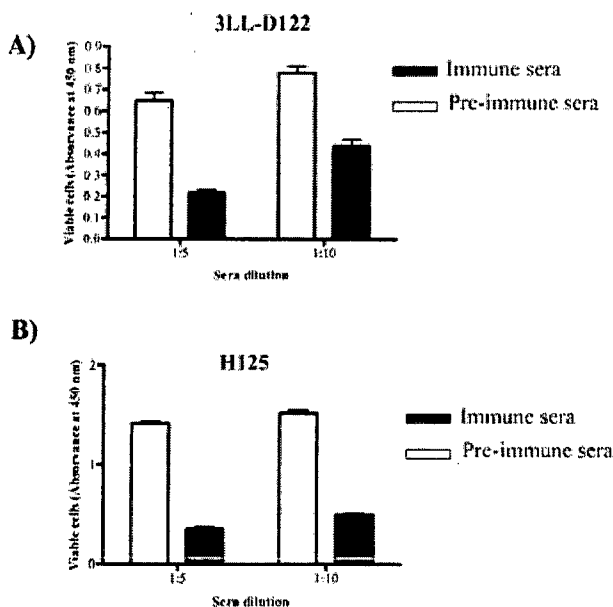


FIGURE 8 – Immune sera inhibit EGFR+ tumor cells growth. (a) 3LL-D122 cells or (b) H125 cells were grown in the presence of sera obtained from mice immunized with the mEGFR-ECD or the Her1-ECD, respectively. Preimmune sera were used as negative controls. After 48 hr, cells' viability was measured by the MTT colorimetric assay. Both kinds of hyperimmune sera were able to significantly decrease viable cells number (unpaired *t*-test, $p < 0.05$). Each bar represents the mean absorbance $\pm$ SD of 2 independent experiments.

Discussion

Despite the EGFR wide expression in the organism, it can be considered as a tumor-associated antigen (TAA) because of its overexpression in many epithelial tumors,³⁷ its implication in tumor growth and correlation with bad prognosis.¹² For that reason, the EGFR has become an attractive target for cancer therapy, and many attempts are currently ongoing, using this molecule as target for passive therapy with tyrosine kinase inhibitors and MAb.³⁸⁻⁴⁰ Our results demonstrated that the murine EGFR extracellular domain, in an appropriate adjuvant, is enough immunogenic in mice to promote a strong antitumorigenic effect in a relevant EGFR+ tumor model, highlighting this particular approach as an attractive new strategy for cancer treatment.

TAA tolerance represents a significant challenge for effective immunotherapy of human cancer, and in any successful vaccine strategy, this issue should be conveniently addressed in advance. The experimental demonstration that breaking self tolerance is possible has been recently published and is generally accepted under the principle that self recognition is a physiological phenomenon.⁴¹ The fact that the EGFR is widely distributed in normal epithelial tissues hardly suggests that this molecule could be a very poor immunogen, introducing additional difficulties to any vaccine design. In our vaccine approach, the construction of chimerical molecules was avoided, introducing instead potent TH1 adjuvants. Emulsifying the recombinant protein either in FA or VSSP (a product already clinically tested in humans) renders a rather simple formulation. As the primary endpoint for the vaccine induced mEGFR-ECD immunogenicity, the induction of DTH was considered. Surprisingly, mEGFR-ECD/FA vaccination promoted severe inflammations in mice foot pads after the specific sensitization, similar to those mice previously immunized with KLH/FA and sensitized with this "foreign" protein.

Immunization of mice with the mEGFR-ECD in FA or VSSP also stimulated the specific humoral immunity, characterized by ele-

vated IgG antibody titers, successively incremented with reimmunizations, an indicative of a mature response. Both adjuvants influenced IgG subclasses distribution in favor of IgG2a and IgG2b, an indirect indication of TH1 differentiation. Particularly, higher levels of IgG2b were associated with VSSP formulations. An indicative that the immune reaction against this kind of self proteins is limited came from other member of the EGFR family, Her2. Monkeys were successfully immunized only after 6 immunizations with the Her2-ECD, formulated in the powerful adjuvant Detox, and specific IgG titers never reached 1/10,000,⁴² a value lower than that induced in mice with the mEGFR-ECD in FA or VSSP. Besides, Disis *et al.*⁴³ have shown that a Her2 neu-peptide-based vaccine, but not a whole-protein vaccine, can elicit humoral and cellular responses in rats. The EGFR is a tolerated self-antigen for which a specific T cells thymic deletion mechanism could be operating, requiring the presence of the auto-antigen in the thymus.^{44,45} The EGFR thymic expression, although earlier reported in humans³⁴ and rats,³³ was confirmed in our lab for Balb/c and C57Bl/6 mice by RT-PCR. Similar to what has been reported in normal people, a total absence of anti-EGFR natural auto-antibodies was also observed in mice sera by ELISA and FACS. Nevertheless, evidences coming from cancer patients indicated that the presence of discrete serum anti-EGFR natural antibodies can be detected with certain frequency.⁴⁶ The same observations have been reported for breast cancer patients in which anti-Her2 antibodies and CTL could be measured,⁴⁷ lacking enough efficacy in preventing tumor progression. This "natural" immunity to Her2, present only in a minority of patients overexpressing the receptor, is of low magnitude.⁴⁸ The low, natural immune responses to Her1 and Her2 in cancer patients means that any associated target-directed therapeutic vaccine must efficaciously stimulate naïve B and T lymphocytes. In fact, Her2 vaccines, constructed with synthetic peptides mixed with granulocyte-macrophage colony stimulating factor as adjuvant, have been clinically tested in breast, ovarian and lung cancer patients overexpressing Her2 and 68% of them developed T-cell immunity against the self Her2 protein.⁴⁹

More likely explanations for the unusually strong immunogenicity of the autologous Her1 protein observed in this study might be the full length EGFR truncation, the adjuvant conditioning of the antigen presentation context or both. EGFR truncation could modify the T cells repertoire immunodominance, favoring the presentation of cryptic determinants.⁵⁰ In this case, protein truncation did not affect the full length EGFR recognition in its natural conformation in the cells by the vaccine-induced serum antibodies, as determined in FACS experiments. This result suggests certain differences with the Her2 model, in which specific antibodies were undetectable in sera obtained from rats immunized with the rat Her2 intracellular domain (ICD), while CTL and antibodies with degenerated specificities for the human and rat Her2/neu were produced when the inoculated immunogen was the highly homologous foreign human ICD.⁵¹

The use of potent TH1-type adjuvants in combination with poorly immunogenic self proteins to promote a proinflammatory context for loosing the regulatory cells circuit was an attractive, tested idea. In this sense and as usual in experimental vaccine approaches complete FA was selected as reference TH1 adjuvant.³⁵ While complete FA cannot be used in human vaccines, a new, already clinically tested adjuvant (VSSP) with peculiar immune-modulatory properties was introduced in the EGFR-ECD vaccines. VSSP monotherapy in mice induced elevated IgG levels with a TH1-related pattern against GM3, a poorly immunogenic ganglioside,⁵² and dendritic cell maturation with IL-12 production,⁵² which in turn is pivotal for proinflammatory responses.^{53,54}

Although the amino acid sequence homology between human and murine EGFR-ECDs is about 87%, sera obtained from mice immunized with the Her1-ECD formulation were unable to appropriately react with the murine protein, while only 1/3 of the vaccine-stimulated B cells, secreting specific IgG antibodies, cross-reacted with the mEGFR-ECD. The finding that serum-specific antibodies, induced in mice by immunization with the corresponding EGFR-ECDs in VSSP, caused the slow growing of EGFR+

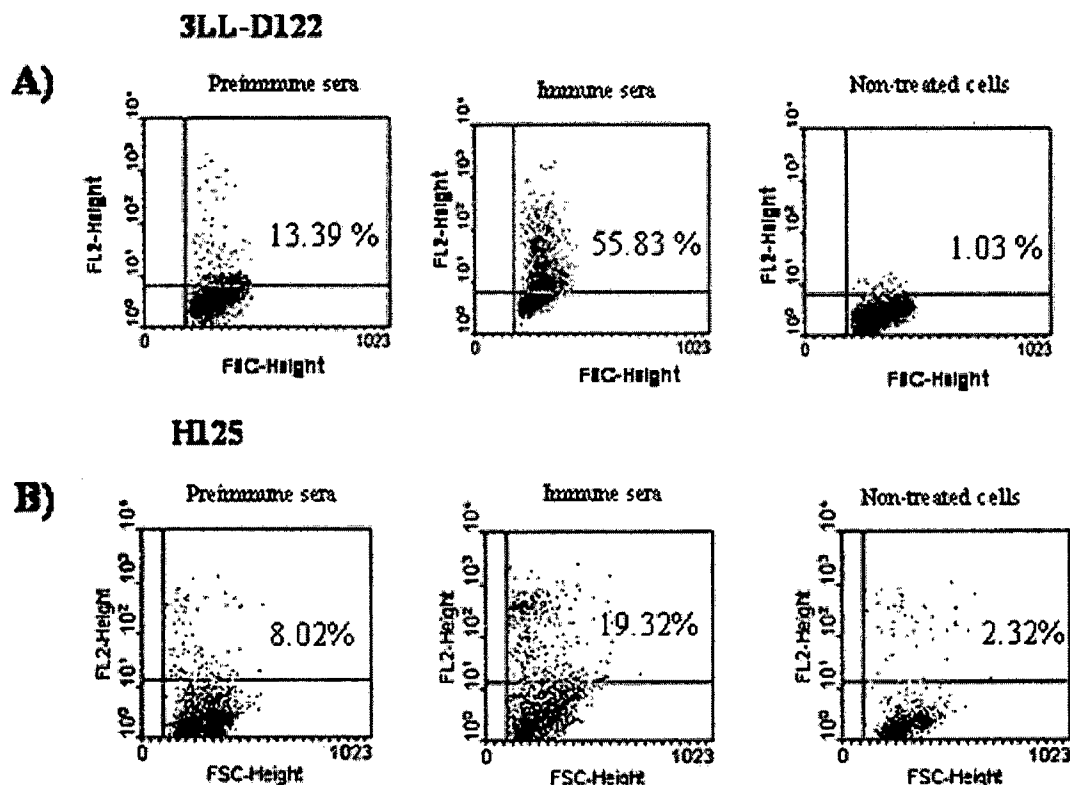


FIGURE 9 – Immune sera cytotoxic effect over EGFR+ cells. (a) 3LL-D122 and (b) H125 cells were incubated for 24 hr with inactivated complement sera from mice immunized with mEGFR-ECD/VSSP or Her1-ECD/VSSP, respectively. The immune sera cytotoxic effect was determined by FACS analysis. 7A7 (50% of cytotoxicity) and R3 (25% of cytotoxicity) MABs were used as positive controls for 3LL-D122 and H125 cells, respectively. This experiment is representative of 2 independent ones.

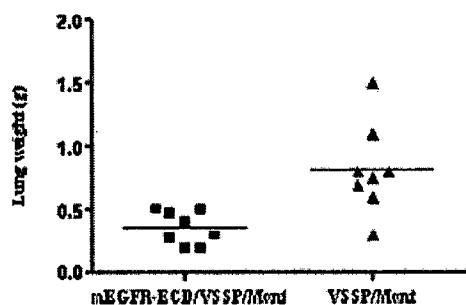


FIGURE 10 – Antimetastatic effect of mEGFR-ECD/VSSP vaccination. Mice were immunized 3 times with 100 µg of mEGFR-ECD/VSSP biweekly. One day before the second immunization, mice were challenged with 2×10^5 3LL-D122 tumor cells in the foot pad. Three weeks after malignant cells inoculation, tumors were surgically removed, and 21 days later, mice were sacrificed and the spontaneous lung metastases quantified by weighing the lungs. In vaccinated animals lung weights were significantly reduced ($p < 0.01$, unpaired *t*-test) compared with that of the corresponding control group. This experiment is representative of 2 independent ones.

tumor cells and also promoted a strong target-directed complement-independent cytotoxic effect emphasize the quality of the vaccine-induced immune response.

Further results associated with the *in vivo* mEGFR-ECD/VSSP antimetastatic effect in the EGFR+ 3LL-D122 Lewis lung carcinoma model are encouraging if the intermediate character of the selected experimental setting (rather therapeutic than prophylactic) was considered. Even though in this case tumor cells were inocu-

lated into mice foot pads just after vaccine priming, supplying afterwards the 2 booster injections, a significant decrease in lung metastases number, after primary tumor surgical removal, was evident.

One week after the third vaccine administration, and coincident with the surgical removal of primary tumors, specific IgG titers in mice sera were elevated as noticed from the humoral response kinetics, probably suggesting that the induced antibodies might have a role in avoiding tumor cells' dissemination or in turn the growth of malignant cells already lodged in the lungs, or both. Future studies stressing the relative contribution of humoral and cellular immunity in the vaccine-induced antimetastatic effect, through hyperimmune serum transfer and the appropriate lymphocyte subsets depletion experiments are currently ongoing. The "spontaneous metastasis" 3LL Lewis lung carcinoma model, employed in this study, is rather significant because of the most likely resemblance to the real clinical situation in which surgeons frequently remove patients' primary tumors successfully, but unfortunately and more commonly, disease spreading will follow. Indeed, avoiding distant metastatic dissemination could be the most appropriate task for effective cancer vaccines. Interestingly, other 2 active immunotherapy approaches targeting the EGFR have shown recently *in vivo* efficacy in the Lewis lung carcinoma model but in a primary tumor scenario. A DNA vaccine,²⁰ based on the xenogenic EGFR-ECD gene and a self EGFR-ECD protein pulsed dendritic cells vaccine²¹ were able to keep alive 60% of mice preventatively injected and afterwards challenged with LL/2c tumor. Although together all these results strongly suggest that EGFR could be a significant target not only for passive but also for active immunotherapy, another crucial remaining question is the better vaccine approach to follow up in the upcoming future clinical trials. A relevant learning from the present work is that

TABLE II – PREGNANCY AND NEWBORN REPRODUCTIVE PARAMETERS MEASURED AFTER mEGFR-ECD/VSSP IMMUNIZATION

Group	Fertility	Number of pups per litter	Weight of 1-day-old pups (g) (mean ± SD)	Hair growth (d.a.b.) ¹	Eyes opening (d.a.b.)	Incisor eruption (d.a.b.)
Treated	5/10	5–7	1.298 ± 0.03	5–7	13–15	10–13
Control	3/10	5–7	1.33 ± 0.07	5–7	13–16	10–13

¹d.a.b. = days after birth.

just the use of the recombinant self protein in a potent adjuvant, like VSSP, could be appropriate for establishing an antitumor immunity in patients with EGFR+ tumors, indicating the existence of a new opportunity in this particular target, different from sophisticated vaccine technologies like the dendritic cells approach, or up to now ineffective vaccine formulations in humans like naked DNA.

EGFR-targeted therapies are expected to produce side effects related to the induction of autoimmunity. In fact, some side effects as skin rash have been reported for some related drugs,⁵⁵ but important toxic effects have not been found for the majority of the different approaches tested in clinical trials. As an example THERACIM, an anti-EGFR MAb (humanized R3, CIM), has been clinically tested (Phase II trials), in combination with radiotherapy, in head and neck cancer patients, raising up to 600 mg/cycle, without the detection of skin rash symptoms. Although it has been reported that EGFR gene expression inhibition is critical for cancerous cell growth but not for normal cells,⁵⁶ active immunization, providing a long lasting specific immune response, should be carefully monitored for possible side effects. The conducted immunization experiments with mEGFR-ECD/VSSP showed that while

1 year after the last vaccine booster anti-mEGFR-ECD IgG low levels were detectable in most animals, signs of toxicity were absent and functional hepatic parameters behave as in naïve mice.

Induction of EGF deficiency in rats affects the development of fetal but not adult tissues.⁵⁷ Considering the role of EGF in the epigenetic regulation of fetal and neonatal development, we studied the effect of anti-EGFR immunity in female mice fertility and their progeny. Marked side effects in these animals as a consequence of vaccination were not detected.

We conclude that self EGFR-ECD is rather immunogenic in FA or VSSP contexts. Taken together, the immunization approach described here may be an attractive and novel strategy for EGFR+ cancer active immunotherapy.

Acknowledgements

We thank Armando López (CIM, Cuba) for his technical assistance in animal's experiments, and also Dr. Donald Morton (John Wayne Cancer Institute, USA) and Dr. Agustín Lage (CIM, Cuba) for their critical revision of the manuscript and useful suggestions.

References

1. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schelessinger J, Downward J, Mayes ELV, et al. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 1984;309:418–25.

2. Perez R, Pascual M, Macias A, Lage A. Epidermal growth factor receptors in human breast cancer. *Breast Cancer Res Treat* 1984;4:189–93.

3. Klijn JG, Look MP, Portengen H, Alexieva-Figusch J, van Putten WL, Foekens JA. The prognostic value of epidermal growth factor receptor (EGF-R) in primary breast cancer: results of a 10 year follow-up study. *Breast Cancer Res Treat* 1994;29:73–83.

4. Hendler FJ, Ozanne BW. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J Clin Invest* 1984;74: 647–51.

5. Zhau HY, Chang SM, Chen BQ, Wang Y, Zhang H, Kao C, Sang QA, Pathak SJ, Chung LW. Androgen-repressed phenotype in human prostate cancer. *Proc Natl Acad Sci USA* 1996;93:15152–7.

6. Liu XH, Wiley HS, Meikle AW. Androgens regulate proliferation of human prostate cancer cells in culture by increasing transforming growth factor- α (TGF- α) and epidermal growth factor (EGF)/TGF- α receptor. *J Clin Endocrinol Metab* 1993;77:1472–8.

7. Dassonville O, Formento JL, Francoual M, Ramaioli A, Santini J, Schneider M, Demard F, Milano G. Expression of epidermal growth factor receptor and survival in upper aerodigestive tract cancer. *J Clin Oncol* 1993;11:1873–8.

8. Lockhart C, Berlin JD. The epidermal growth factor receptor as a target for colorectal cancer therapy. *Semin Oncol* 2005;32:52–60.

9. Tan X, Egami H, Ishikawa S, Nakagawa M, Ishiko T, Kamohara H, Hirota M, Ogawa M. Relationship between activation of epidermal growth factor receptor and cell dissociation in pancreatic cancer. *Int J Oncol* 2004;25:1303–9.

10. Neal DE, Marsh C, Bennett MK, Abel PD, Hall RR, Sainsbury JR, Harris AL. Epidermal-growth-factor receptors in human bladder cancer: comparison of invasive and superficial tumours. *Lancet* 1985;1: 366–8.

11. Gullick WJ, Marsden JJ, Whittle N, Ward B, Bobrow L, Waterfield MD. Expression of epidermal growth factor receptors on human cervical, ovarian, and vulval carcinomas. *Cancer Res* 1986;46:285–92.

12. Khademi B, Shirazi FM, Vasei M, Doroudchi M, Gandomi B, Modjtahedi H, Pezeshki AM, Ghaderi A. The expression of p53, c-erbB-1 and c-erbB-2 molecules and their correlation with prognostic markers in patients with head and neck tumors. *Cancer Lett* 2002;184:223–30.

13. Brabender J, Danenberg KD, Metzger R, Schneider PM, Park J, Salonga D, Holscher AH, Danenberg PV. Epidermal growth factor receptor and HER2-neu mRNA expression in non-small cell lung cancer is correlated with survival. *Clin Cancer Res* 2001;7:1850–5.

14. Milas L, Mason K, Hunter N, Petersen S, Yamakawa M, Ang K, Mendelsohn J, Fan Z. In vivo enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clin Cancer Res* 2000;6:701–8.

15. Crombet T, Torres L, Neninger E, Catala M, Solano ME, Perera A, Torres O, Iznaga N, Torres F, Perez R, Lage A. Pharmacological evaluation of humanized anti-epidermal growth factor receptor, monoclonal antibody h-R3, in patients with advanced epithelial-derived cancer. *J Immunother* 2003;26:139–48.

16. Blackledge G, Averbuch S. Gefitinib ('Iressa', ZD1839) and new epidermal growth factor receptor inhibitors. *Br J Cancer* 2004;90:566–72.

17. Sirotak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000;6:4885–92.

18. Herbst RS, Johnson DH, Mininberg E, Carbone DP, Henderson T, Kim ES, Blumenschein G, Jr, Lee JJ, Liu DD, Truong MT, Hong WK, Tran H, et al. Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. *J Clin Oncol* 2005;23:2544–55.

19. Gonzalez G, Crombet T, Torres F, Catala M, Alfonso L, Osorio M, Neninger E, Garcia B, Mulet A, Perez R, Lage R. Epidermal growth factor-based cancer vaccine for non-small-cell lung cancer therapy. *Ann Oncol* 2003;14:461–6.

20. Lu Y, Wei YQ, Tian L, Zhao X, Yang L, Hu B, Kan B, Wen YJ, Liu F, Deng HX, Li J, Mao Y et al. Immunogene therapy of tumors with vaccine based on xenogeneic epidermal growth factor receptor. *J Immunol* 2003;170:3162–70.

21. Hu B, Wei Y, Tian L, Zhao X, Lu Y, Wu Y, Yao B, Liu J, Niu T, Wen Y, He Q, Su J et al. Active antitumor immunity elicited by vaccine based on recombinant form of epidermal growth factor receptor. *J Immunother* 2005;28:236–44.

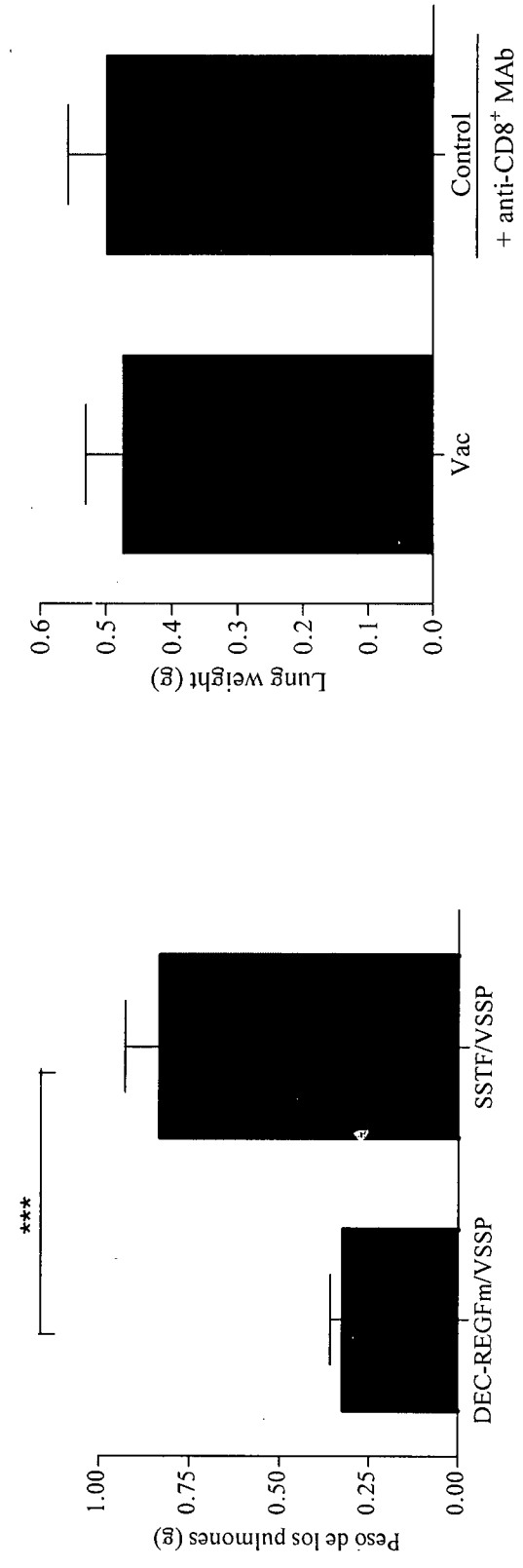
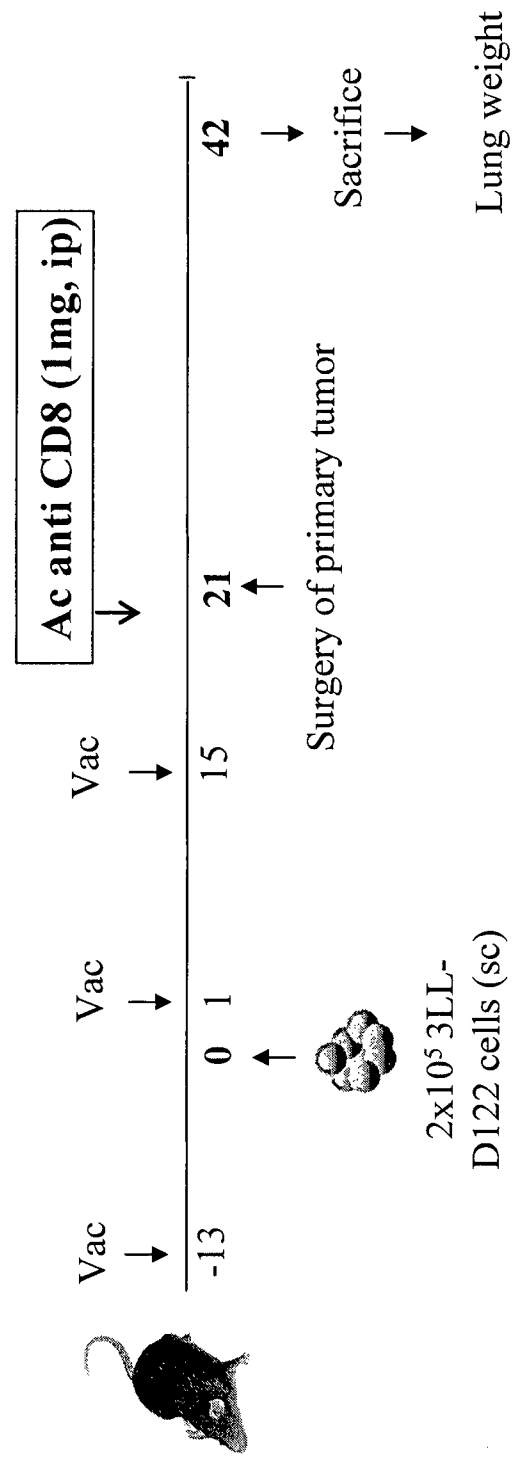
22. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature* 1984;307:521–7.

23. Reiter JL, Threadgill DW, Eley GD, Strunk KE, Danielsen AJ, Sinclair CS, Pearsall RS, Green PJ, Yee D, Lampland AL, Balasubramaniam S, Crossley TD et al. Comparative genomic sequence analysis and isola-

- tion of human and mouse alternative EGFR transcripts encoding truncated receptor isoforms. *Genomics* 2001;71:1-20.
24. Eisenbach L, Hollander N, Greenfield L, Yakor H, Segal S, Feldman M. The differential expression of H-2K versus H-2D antigens, distinguishing high-metastatic from low-metastatic clones, is correlated with the immunogenic properties of the tumor cells. *Int J Cancer* 1984;34:567-73.
 25. Carney DN, Gazdar AF, Bepler G, Guccion JG, Marangos PJ, Moody TW, Zweig MH, Minna JD. Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res* 1985;45:2913-23.
 26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
 27. Honda J, Oomizu S, Kiuchi Y, Komatsu N, Takeuchi S, Takahashi S. Identification of epidermal growth factor mRNA-expressing cells in the mouse anterior pituitary. *Neuroendocrinology* 2000;71:155-62.
 28. Estevez F, Carr A, Solorzano L, Valiente O, Mesa C, Barroso O, Sierra GV, Fernandez LE. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). *Vaccine* 1999;18:190-7.
 29. Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods* 1983;65:109-21.
 30. Garrido G, Sanchez B, Rodriguez HM, Lorenzano P, Alonso D, Fernandez LE. 7A7 MAb: a new tool for the pre-clinical evaluation of EGFR-based therapies. *Hybrid Hybridomics* 2004;23:168-75.
 31. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
 32. Brezicka T, Einbeigi Z. Supra-additive cytotoxic effects of a combination of cytostatic drugs and antibody-induced complement activation on tumor cells in vitro. *Tumour Biol* 2001;22:97-103.
 33. Sakai Y, Wajjwalku W, Takahashi M, Masuda A, Utsumi KR, Matsuyama M. Expression of proto-oncogenes and tumor suppressor genes in in vitro cell lines derived from a thymus, thymoma, and malignant thymoma of rats. *Nagoya J Med Sci* 1993;55:125-30.
 34. Pescarmona E, Pisacane A, Pignatelli E, Baroni CD. Expression of epidermal and nerve growth factor receptors in human thymus and thymomas. *Histopathology* 1993;23:39-44.
 35. Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. *Science* 1996;271:1728-30.
 36. Gonzalez GSB, Suarez E, Beausoleil I, Perez R, Lastre M, Lage A. Induction of immune recognition of self epidermal growth factor (EGF): effect on EGF-biodistribution and tumor growth. *Vaccine Res* 1996;5:233-44.
 37. Urban JL, Schreiber H. Tumor antigens. *Annu Rev Immunol* 1992;10:617-4.
 38. Crombet T, Torres O, Rodriguez V, Menendez A, Stevenson A, Ramos M, Torres F, Figueredo R, Veitia I, Iznaga N, Perez R, Lage A. Phase I clinical evaluation of a neutralizing monoclonal antibody against epidermal growth factor receptor in advanced brain tumor patients: preliminary study. *Hybridoma* 2001;20:131-6.
 39. Saltz LB, Meropol NJ, Loehrer PJ, Sr, Needle MN, Kopit J, Mayer RJ. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 2004;22:1201-8.
 40. Janne PA, Gurubhagavatula S, Yeap BY, Lucca J, Ostler P, Skarin AT, Fidias P, Lynch TJ, Johnson BE. Outcomes of patients with advanced non-small cell lung cancer treated with gefitinib (ZD1839, "Iressa") on an expanded access study. *Lung Cancer* 2004;44:221-30.
 41. Coutinho A, Hori S, Carvalho T, Caramalho I, Demengeot J. Regulatory T cells: the physiology of autoreactivity in dominant tolerance and "quality control" of immune responses. *Immunol Rev* 2001;182:89-98.
 42. Fendly BM, Kotts C, Wong WLT, Figari I, Harel W, Staib L, Carver ME, Vetterlein D, Mitchell MS, Shepard M. Successful immunization of Rhesus monkeys with the extracellular domain of p185HER2: a potential approach to human breast cancer. *Vaccine Res* 1993;2:129-39.
 43. Disis ML, Gralow JR, Bernhard H, Hand SL, Rubin WD, Cheever MA. Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self-protein. *J Immunol* 1996;156:3151-8.
 44. Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat Genet* 1997;15:289-92.
 45. Akkaraju S, Ho WY, Leong D, Cnaan K, Davis MM, Goodnow CC. A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulinitis. *Immunity* 1997;7:255-71.
 46. Bei R, Masuelli L, Moriconi E, Visco V, Moretti A, Kraus MH, Muraro R. Immune responses to all ErbB family receptors detectable in serum of cancer patients. *Oncogene* 1999;18:1267-75.
 47. Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB, Moe R, Cheever MA. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res* 1994;54:16-20.
 48. Bernhard H, Salazar L, Schiffman K, Smorlesi A, Schmidt B, Knutson KL, Disis ML. Vaccination against the HER-2/neu oncogenic protein. *Endocr Relat Cancer* 2002;9:33-44.
 49. Disis ML, Gooley TA, Rinn K, Davis D, Piepkorn M, Cheever MA, Knutson KL, Schiffman K. Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. *J Clin Oncol* 2002;20:2624-32.
 50. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 1992;358:155-7.
 51. Disis ML, Shiota FM, Cheever MA. Human HER-2/neu protein immunization circumvents tolerance to rat neu: a vaccine strategy for 'self' tumour antigens. *Immunology* 1998;93:192-9.
 52. Mesa C, De Leon J, Rigley K, Fernandez LE. Very small size proteoliposomes derived from *Neisseria meningitidis*: an effective adjuvant for TH1 induction and dendritic cell activation. *Vaccine* 2004;22:3045-52.
 53. Trinchieri G. Interleukin-12 and its role in the generation of TH1 cells. *Immunol Today* 1993;14:335-8.
 54. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med* 1996;184:747-52.
 55. Lee MW, Seo CW, Kim SW, Yang HJ, Lee HW, Choi JH, Moon KC, Koh JK. Cutaneous side effects in non-small cell lung cancer patients treated with Iressa (ZD1839), an inhibitor of epidermal growth factor. *Acta Derm Venereol* 2004;84:23-6.
 56. Rubin Grandis J, Chakraborty A, Melhem MF, Zeng Q, Tweardy DJ. Inhibition of epidermal growth factor receptor gene expression and function decreases proliferation of head and neck squamous carcinoma but not normal mucosal epithelial cells. *Oncogene* 1997;15:409-16.
 57. Raaberg L, Nexø E, Jorgensen PE, Poulsen SS, Jakab M. Fetal effects of epidermal growth factor deficiency induced in rats by autoantibodies against epidermal growth factor. *Pediatr Res* 1995;37:175-81.

Vaccination with mEGFR-ECD/VSSP has anti-metastatic effect

Spontaneous metastasis/3LL-D122 model



Student *t* test; $p < 0,0001$

Student *t* test; $p > 0,05$



Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP)

Francisco Estevez^{a,*}, Adriana Carr^b, Leobaldo Solorzano^a, Oscar Valiente^b,
Circe Mesa^b, Osquel Barroso^b, Gustavo Victoriano Sierra^a, Luis Enrique Fernandez^b

^aFinlay Institute, Ave. 27 No. 19805, La Lisa, P.O. Box 16 017, C. de La Habana, Cuba

^bCenter of Molecular Immunology, Calle 216 esq. a 15, Atabey, Playa, P.O. Box 16 040, C. de La Habana, Cuba

Received 25 November 1998; received in revised form 19 April 1999; accepted 26 April 1999

Abstract

Certain gangliosides are tumor-associated antigens that constitute potential targets for cancer immunotherapy. A major drawback in the design of ganglioside-based cancer vaccines, however, is the poor immunogenicity of these glycolipids. Here we report the immunological and physicochemical properties of very small size proteoliposomes (VSSP) obtained by using anionic detergents to incorporate gangliosides into the outer membrane protein complex (OMPC) of *N. meningitidis*. VSSP of three different gangliosides, GM3, NGcGM3 and GD3, were tested. These gangliosides differ in level of expression in normal tissues and in immunogenicity in different animal species. We show that the immunization with VSSP in an oil adjuvant consistently induced both IgM and IgG anti-ganglioside antibodies. In the mouse, the anti-ganglioside IgG fraction was not restricted to the typical T-independent isotype IgG3. Unexpectedly, significant levels of the T-dependent IgG1, IgG2a and particularly IgG2b were also found. VSSP-mediated enhancement of the immunogenicity was not restricted to the relatively immunogenic ganglioside GD3, satisfactory immune responses against highly tolerated GM3 and NGcGM3 were also obtained. Similar results were achieved in chickens and monkeys. No reactogenicity was observed even when self-gangliosides were used for immunization. VSSP overcame natural tolerance to gangliosides in an adjuvant dependent fashion. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Cancer vaccines; Proteosomes; Gangliosides; Proteoliposomes

1. Introduction

Gangliosides are sialylated glycosphingolipids present on the plasma membrane of mammalian cells. In tumors, however, they are overexpressed or display abnormal glycosylation patterns [1,2]. Sometimes, free gangliosides are also shed into the blood stream of patients suffering certain malignancies [3,4].

It has been observed that cancer patients who

develop antibodies against certain gangliosides have better prognosis and prolonged survival compared to those that are antibody-negative [5]. Similarly, antibodies against different gangliosides have been shown to mediate protection in animal models against challenge with ganglioside-expressing tumors [6,7]. These findings, among others, point to gangliosides as promising targets for the active specific immunotherapy of cancer [8]. However, a major drawback of ganglioside vaccines is the poor immunogenicity of these glycolipids. Gangliosides are generally autoantigens tolerated immunologically to a greater or lesser degree. Furthermore, they are categorized as T-cell-independent antigens, i.e. they are not recognized by T lymphocytes, which therefore do not provide help

* Corresponding author. Present address: Center of Molecular Immunology, Calle 216 esq. a 15, Atabey, Playa, A. Postal 16040, C. de La Habana, Cuba. Tel.: +53-7-335049; fax: +53-7-333509.

E-mail address: frank@ict.cim.sld.cu (F. Estevez)

fuged to obtain 500 g (wet weight) of biomass. This biomass was resuspended in 25 l of buffer containing 0.09% sodium deoxycholate (DOC), 50 mM Tris-HCl, pH 8.5, and 2 mM EDTA. The extraction process was carried out at 4°C for 2.5 h. During this period, 10 treatments of 30 s each were given in an ultrasonic bath (Ultraware, Radleys, UK), alternating with magnetic stirring at 250 rpm.

Cell debris was separated by centrifugation at 10,000g and the supernatant treated with DNase and RNase (5 mg/l each) at 37°C. The extract was centrifuged at 100,000g for 2 h and the pellet resuspended in 200 ml 5% sodium deoxycholate/(50 mM) Tris-EDTA buffer, pH 9. Gel filtration on Sephacryl S-300 HR (Pharmacia Biotech, Sweden) was carried out using 1% sodium deoxycholate as eluant. The first eluted peak was the basic material containing the vesicles as revealed by electron microscopy following negative staining with uranyl acetate. The OMPC was precipitated with ethanol, washed with the same solvent, and stored at -80°C in 40 mg portions.

To incorporate gangliosides into the OMPC (VSSP preparation procedure), 40 mg aliquots of OMPC were dissolved to a final concentration of 1 mg/ml in 0.01 M Tris-HCl buffer, pH 8.5, containing 12 mM DOC and 1 mM sodium dodecylsulfate (SDS). Forty mg ganglioside was then added and the mixture stirred. The resultant solution was dialyzed for 14 d against 0.01 M Tris-HCl buffer, pH 8.5, using a 3.5 KDa cut-off membrane (Spectra/Por, Spectrum Medical Industries, Houston, Texas) to remove detergents. The dialyzed preparation was centrifuged for 1 h at 100,000g, pellets were discarded and supernatants assayed for protein and ganglioside contents. Finally, the VSSP solution obtained was filter-sterilized (0.2 µm cellulose acetate membranes; Sartorius, Germany) and stored at 4°C.

Vaccine lots NGcGM3/VSSP, GM3/VSSP and GD3/VSSP were prepared with the respective ganglioside and the OMPC. The lot designated OMPC was prepared in the same way but without the addition of any ganglioside, and the lot designated GM3 was prepared with the ganglioside alone.

2.4. Physical and chemical characterization of vaccines

Protein assay was performed by a modification of the method of Lowry [21]. Gangliosides were identified by HPTLC as described below and their concentration determined by the resorcinol method [22].

For the electron microscopy study, samples were adsorbed 5 min onto carbon-coated Formvar grids and negatively stained with 1% phosphotungstic acid, pH 7.0, or 2% uranyl acetate. Grids were examined at 20–80,000-fold magnification at 60–80 kV.

Gel filtration chromatography on Sepharose CL-4B (Pharmacia Biotech, Sweden) was carried out in a C10/40 column equilibrated with 0.01 M Tris-HCl buffer, pH 8.5, containing 0.15 M NaCl. Fractions of 1 ml were collected and assayed for protein and ganglioside concentration. The partition coefficient K_{av} was done as described by Sofer and Nystrom [23]. Standard proteins were used as molecular weight markers in the same conditions as the samples. A calibration curve, K_{av} vs. MW, was established.

CsCl equilibrium density gradient analysis of the vaccine was performed with an initial one-step gradient with a density of 1.525 g/ml in the lower half and 1.005 g/ml in the upper half. Tris-HCl buffer (0.01 M, pH 8.5) was present throughout the gradient, and the vaccine was initially uniformly distributed in the upper solution. Centrifugation was performed in a Kontron TFT 70.13 angle head rotor at 55,000 rpm (211,000g) and 10°C for 48 h. Fractions were collected, and density, sialic acid content and protein concentration determined for each fraction.

2.5. ELISA

PolySorp 96-well plates (Nunc, Denmark) were coated with 0.16 nmol/well of ganglioside dissolved in methanol and dried at 37°C for 1.5 h. To reduce background, plates were washed with PBS containing Tween 20 (0.05%, v/v) plus 0.2 M extra of NaCl and then blocked with skimmed milk (2%, w/v) in PBS. Serum samples, biotinylated goat anti-IgM or anti-IgG antibodies specific for each animal species and streptavidin-conjugated alkaline phosphatase (Jackson, West Grove, PA) were diluted in blocking buffer and sequentially incubated on the plates for 2 h at room temperature with extensive washing between each incubation. The enzymatic reaction was visualized with *p*-nitrophenyl phosphate (PNPP), 1 mg/ml, dissolved in 1M diethanolamine buffer, pH 9.6, plus 1 mM $MgCl_2$.

To eliminate the effect of non-specific recognition, sera were also tested on wells to which no gangliosides had been added. The absorbance at each serum dilution obtained on these wells was subtracted from that of the ganglioside-coated wells. Serological titer was defined as the inverse of the highest dilution yielding a final absorbance value higher than 0.1.

Determination of IgG isotype profile was performed using secondary isotype-specific biotinylated rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies (PharMingen, San Diego, CA). Optimal secondary antibody dilutions were established by ELISA with the MAbs 14F7 (IgG1), T3 (IgG2a), T4 (IgG2b) (Center of Molecular Immunology, Havana, Cuba) and R24 (IgG3) (kindly provided by Dr. Philip O.

arations of the outer membrane proteins of *N. meningitidis* [27,28]. The physicochemical properties of VSSP, however, differ from other proteosome-based preparations described before [29]. Ganglioside incorporation into the OMPC leads to a dramatic reduction in the size of the complex that, unlike other proteosome-based preparations, is not visible by electron microscopy. On the other hand, the relatively high density of VSSP (1.265 g/ml) reveals that VSSP are non-vesicular proteo-lipidic bodies from which most water has been excluded.

The basis for this compact structure might be explained if we consider the molecular properties of gangliosides. These glycosphingolipids have very large oligosaccharide moieties relative to their hydrocarbon portions. For reasons of molecular geometry, these structures are not thermodynamically compatible with the formation of bilayer vesicles but with micelles [30]. The presence of high concentrations of gangliosides in a solution of membrane proteins, highly dissociated by the presence of anionic detergents, leads to the formation of mixed micelles from which most water is excluded. After detergent removal, the system should reach a new equilibrium in which the ganglioside properties predominate.

Our study on the immunogenicity of VSSP confirms and extends a previous report [12], and shows that not only IgM but also high-level IgG anti-ganglioside antibodies can be induced in the mice. However, in addition to GD3 ganglioside, which is considered the most immunogenic in this animal model [13], highly expressed gangliosides such as GM3 and NGcGM3 were also highly immunogenic when used as VSSP. Similar results were obtained in the other two animal species studied. Unexpectedly, the anti-ganglioside IgG fraction in mice was not restricted to the typical T-independent isotype IgG3. High levels of the Th1-related IgG isotypes IgG1, IgG2a and particularly IgG2b were also found. These phenomena, however, were strongly dependent on the use of an oil-in-water adjuvant. VSSP overcame natural tolerance to ganglioside in an adjuvant-dependent fashion.

The mechanisms involved in VSSP immuno-enhancing properties are not yet understood. The observed effect is not inherent to the adjuvant itself since in previous experiences GM3/VLDL and NGcGM3/VLDL conjugates administered either in CFA or in Montanide ISA 51 were not immunogenic in mice. It is known that serotype proteins, which are the main components of the OMPC, induce proliferation and activation of lymphocytes and lead to secretion of IL-2 by a mechanism different to that of polyclonal activators such as LPS [31]. On the other hand, the adjuvant activity of oil-in-water emulsions has been suggested to be linked to their interaction with dendritic cells [32] which, at the same time, have been implicated in the

presentation of non-classical antigens that are very closely related to gangliosides [33]. The xenogenic environment created by the bacterial proteins and some residual lipids that, like LPS, are natural activators of the immune system, together with the very small size of the complexes, might facilitate ganglioside uptake by this subset of antigen-presenting cells that triggers the maturation of an incipient immune response.

No reactogenicity was observed even when VSSP of self-gangliosides such as GM3 were administered in Montanide ISA in any of the three animal species used. These gangliosides, as has been suggested before [34], should remain 'cryptic' in normal tissues, out of reach of circulating antibodies.

Based on these observations and on our recent finding that threshold inocula of syngeneic melanoma B16 tumor cells in mice immunized with GM3-containing VSSP leads to a very high tumor rejection rate and increased survival (submitted for publication), we have started two clinical trials, following international guidelines [35]. Patients suffering metastatic breast cancer have been immunized with GM3/VSSP and NGcGM3/VSSP vaccines in Montanide ISA 51 to evaluate the immunogenicity and the therapeutic potential of these preparations.

Acknowledgements

The authors thank Miss Marilyn Clavell and Mr. Dariel Morales for expert technical assistance, Ms. Amada Valdez for laboratory assistance, Dr. Ester M. Fajardo for help with the ELISA technique and Dr. Philip O. Livingston for his kindness in providing us with the R24 MAb and his words of encouragement.

References

- [1] Hakomori SI. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res* 1996;56:5309–18.
- [2] Morton DL, Ravindranath MH, Irie RF. Tumor gangliosides as targets for active specific immunotherapy of melanoma in man. In: Svennerholm L, Asbury AK, Reisfeld RA, Sandhoff K, Suzuki K, Tettamanti G, Toffano G, editors. *Progress in Brain Research*. Amsterdam: Elsevier Science, 1994. p. 251.
- [3] Portoukalian J, David MJ, Gain P, Richard M. Shedding of GD2 ganglioside in patients with neuroblastoma. *Int J Cancer* 1993;53:948–51.
- [4] Takahashi K, Ono K, Hirabayashi Y, Taniguchi M. Escape mechanisms of melanoma from immune system by soluble melanoma antigens. *J Immunol* 1988;140:3244–8.
- [5] Portoukalian J, Carrel S, Dore JF, Rumke P. Humoral immune response in disease-free advanced melanoma patients after vaccination with melanoma-associated gangliosides. *Int J Cancer* 1991;49:893–9.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES

Aboqado

Margarita Castellanos Abondano

ASUNTO	Radicación	03-48137
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	
	Folios	007

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☒ Cap. I ☐ Cap. II ☐

No. Sol. Internacional PCT/CU01/00010

Fecha de Presentación Internal. 06/12/2001

Como resultado del examen definitivo, realizado con fundamento en el artículo 48 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

- 1.1. Descripción: Folios: 42 a 79 como se radicó inicialmente.
- 1.2. Reivindicaciones: Folios: 109 a 110 según modificación de 28/12/2007.
- 1.3. Figuras: Folios: 84 a 86 como se radicó inicialmente.
- 1.4. Prioridad: 06/12/2000, Cuba, CU285/2000
12/07/2001, Cuba, CU167/2001
- 1.5. Respuesta del solicitante: Folios: 105 a 110.
Folios 115 a 117

2. Objeto de la invención

Título de la solicitud: "Composiciones farmacéuticas para potenciar la inmunogenicidad de antígenos pocos inmunogénicos".

El objeto de la presente solicitud de patente de invención consiste en composiciones inmunoestimuladoras capaces de generar respuestas inmunes antígeno-específicas.

Reivindicaciones 1– 13: Una composición farmacéutica para potenciar la inmunogenicidad de antígenos poco inmunogénicos, que contiene:

- (A) uno o más antígenos poco inmunogénicos;
(B) un vehículo vacunal que consiste en proteoliposomas derivados del complejo de proteínas de la membrana de *Neisseria meningitidis* los cuales contienen

gangliósidos incorporados; y
(C) eventualmente uno o más adyuvantes.

El problema planteado en esta solicitud es la baja inmunogenicidad de antígenos importantes en enfermedades infecciosas, autoinmunes y el cáncer y la consecuente necesidad de composiciones farmacéuticas adyuvantes que superen o contrarresten este hecho.

Para solucionar este problema técnico la solicitud en estudio proporciona formulaciones que hacen inmunogénicas moléculas poco antigénicas mediante su asociación con proteoliposomas de talla muy pequeña, que contienen incorporados gangliósidos.

El objeto de la solicitud definida anteriormente se clasificó en la IPC A61K39/385 .

3. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria 06/12/2000

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

Nº	Documento	Título	Fecha de publicación
D1	Estevez F. <i>et.al.</i>	"Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP)".	20/08/1999

4. Análisis de patentabilidad (según requisito) (artículo 14 D486)

4.1. Nivel inventivo (artículo18 D486)

La solicitud en estudio reclama una composición farmacéutica para potenciar la inmunogenicidad de antígenos poco inmunogénicos, que contiene:
(A) uno o más antígenos poco inmunogénicos;
(B) un vehículo vacunal que consiste en proteoliposomas derivados del complejo de proteínas de la membrana de *Neisseria meningitidis* los cuales contienen gangliósidos incorporados; y
(C) eventualmente uno o más adyuvantes.

El documento de Estevez *et.al.*(D1) divulga el uso de proteoliposomas de tamaño muy pequeño derivados del complejo de proteínas de membrana de *Neisseria meningitidis*, denominados VSSP, a los que se les incorpora gangliósidos (pag 196, columna izq, párrafo 1), estas partículas logran potenciar antígenos poco inmunógenos mediante una composición que incluye adyuvantes tipo aceite en agua (pagina 196, columna izq, 3er párrafo). En D1 hay una clara indicación sobre el efecto inmunopotenciador de esta asociación VSSP-adyuvante respecto de la respuesta inmune general (no restringida a respuesta específica anti-gangliosídica) que se produce luego de su administración (ver Resumen), pero menciona la mayor potenciación de respuesta inmune cuando el gangliósido utilizado es el GD3 (pág 196, columna izq, 3er párrafo).

La única diferencia entre la solicitud en estudio y D1, es que en la solicitud incluye un segundo antígeno en la partícula VSSP, de manera opcional. Sin embargo, los datos presentados evidencian un resultado equivalente al de los productos existentes en el estado de la técnica, antes que un efecto técnico sorprendente que pueda considerarse como un aporte cualitativo al estado de la técnica.

De esta manera, según el estado de la técnica, siendo el problema técnico persistente, sin evidencia concluyente de un efecto técnico extraordinario, el desarrollo reclamado se considera carente de actividad inventiva y por lo tanto no cumple con el requisito de nivel inventivo establecido en el artículo 18 de la Decisión 486.

5. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

N°	Documento N°	Reivindicaciones afectadas	Requisito que afecta
D1	Estevez F. et. al.	1-13	Nivel Inventivo

6. Respuesta a los argumentos del solicitante:

En relación a los argumentos del solicitante se hacen las siguientes consideraciones:

1. El solicitante argumenta que la solicitud reivindica una composición farmacéutica que tiene como característica técnica esencial un vehículo vacunal que consiste en nanopartículas de proteoliposomas derivadas del complejo de proteínas de la membrana externa de la bacteria *Neisseria meningitidis* denominada VSSP. Este mismo tipo de partículas son divulgadas en D1, incluso en este documento se refieren a estas partículas con el mismo nombre, se explica que son nanopartículas no visibles al microscopio electrónico y derivadas del complejo de proteínas de la membrana externa de la bacteria *Neisseria meningitidis* (pág 196, columna izq, párrafo 1). Por tanto, se trata del mismo tipo de partículas usadas como vehículo vacunal.
2. El solicitante argumenta que el vehículo vacunal de la solicitud es capaz de inducir la inmunogenicidad de antígenos poco inmunógenos sin necesidad de que el antígeno u antígenos sean modificados de alguna manera lo cual hace que moléculas de diferente naturaleza (péptidos, polipéptidos, proteínas, secuencias de ADN, etc) puedan ser usadas en la composición vacunal. Sin embargo, al revisar el ejemplo 2 de la solicitud (folio 61, líneas 7 a 13), se observa que para la preparación de los VSSP estos fueron dispersos en una solución de 0.5% deoxicolato de sodio y 0.1% dodecil sulfato sodio, que son soluciones detergentes, y que se adicionó además 10 mg de NAcGM3, que es el gangliósido.

D1 también realiza la inclusión del gangliósido en la moléculas VSSP a partir de la utilización de detergentes (pág 192, columna izq, 3er párrafo). De lo anterior se concluye que la modificación del antígeno principal de la molécula que es el gangliósido, es igual en los dos estudios.
3. El solicitante menciona que la inducción de la inmunogenicidad a expensas de las células TCD8 efectoras es la propiedad sorprendente de la invención. Sin embargo, esta propiedad es un resultado esperado por el uso de este tipo de partículas VSSP cuyos antígenos estas incluidos de la forma mencionada en el

numeral anterior, por tanto esta propiedad de respuesta inmune de células TCD8 esta implícita al tipo de partícula en sí y no es un efecto técnico aportado por la solicitud.

4. El solicitante menciona como aspecto innovador de la invención el uso del gangliósido específico GM3, porque este gangliósido específicamente tiene un efecto tanto funcional como estructural en la partícula VSSP. Sin embargo, esta mención también se encuentra en D1, en el cual se divulga que el gangliósido GM3 es considerado el más inmunógeno de los ensayados (pág 196, columna izq, 3er párrafo). Por tanto, el efecto de GM3 no puede considerarse como un aspecto innovador de la invención.

Por las anteriores consideraciones y en vista de la información que había sido suministrada por D1, se recomienda **Negar** el privilegio de patente sobre las reivindicaciones 1 a 13 (folios 109-110) de la solicitud.

CONCEPTO TÉCNICO No. 3514	EXAMINADOR TÉCNICO Código No.202052
-------------------------------------	---