CASTELLANOS M. & CO
A B O G A D O S
Calle 94A No. 7A-09
Bogotá D.C.

Señora

JEFE DE LA DIVISIÓN DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

Ministerio de Desarrollo Económico

E. S. D.

Re.: Expediente No. **03.048.135**
Solicitud de Patente de Invención titulada: **"COMPOSICIÓN VACUNAL QUE COMPRENDE FACTOR DE CRECIMIENTO TRANSFORMANTE ALFA (TGF-ALPHA)"**
Solicitante: **CENTRO DE INMUNOLOGIA MOLECULAR**

RESPUESTA AL EXAMEN DE FONDO

Yo, **MARGARITA CASTELLANOS ABONDANO**, mayor de edad, identificada y domiciliada como aparece al pié de mi firma, atentamente me permito presentar respuesta al oficio **No. 12542** notificado por fijación en lista del 13 de noviembre de 2009, en los siguientes términos:

Claridad de las reivindicaciones

En el oficio No. 12542 se afirma que la definición del producto composicional que se reivindica en la vacuna no es clara porque se caracteriza la reivindicación por medio de su proceso de obtención y se caracteriza el adyuvante mediante un resultado a alcanzar y no por las características técnicas de dicho adyuvante. El señor examinador concluye que el capitulo reivindicatorio no cumple con el requisito de definición, claridad, concisión o sustento por la descripción.

Con el objetivo se dar mayor claridad a la invención y de superar la objeción hecha en el oficio No. 12543, el solicitante somete a consideración del señor examinador un nuevo juego de reivindicaciones donde se remarca el carácter inventivo del producto de la presente invención.

En la nueva reivindicación 1 se define la invención como "Una composición vacunal caracterizada porque comprende como principio activo una molécula compuesta por Factor de Crecimiento Transformante Alfa autólogo (TGFα) y otro péptido seleccionado del grupo consistente en, un péptido derivado del TGFα o un ligando del receptor del Factor de Crecimiento Epidérmico, y donde dicho conjugado está enlazado a una proteína transportadora."

Así entonces, en la nueva reivindicación, se reclama una composición vacunal por los componentes que comprende, estos son TGF combinado con otro péptido que puede ser cualquier otro ligando del receptor del EGF y una proteína transportadora. En la nueva reivindicación no se menciona el resultado que se espera alcanzar con dicha composición vacunal, que es provocar una respuesta inmune contra el TGFα, ni tampoco se menciona el proceso de obtención de la misma. Llamo la atención del señor examinador sobre el hecho que la nueva reivindicación 1 define una vacuna que comprende el TGF junto con otro péptido que puede ser un derivado del mismo u otro ligando del EGF-R.

Las modificaciones hechas al pliego reivindicatorio cumplen a cabalidad el requisito de claridad, concisión y sustento descriptivo de conformidad con el Artículo 30 de la Decisión 486.

Nivel Inventivo de la Solicitud

- En cuanto al nivel inventivo de la solicitud, el señor examinador señala que el hecho de usar TGFα no parece conceder un efecto técnico inesperado ya que tanto el TGFα como el EGF se encuentran íntimamente relacionados y se unen al mismo receptor.

El solicitante afirma que los inventores han caracterizado la respuesta inmune inducida por la composición de la presente invención y demuestran que los resultados no son extrapolables a los obtenidos con la vacuna que solamente contiene EGF. Adjunto a este memorial copia de los resultados publicados por los inventores que apoyan esta afirmación.

- El señor examinador plantea que la única diferencia entre la presente invención y el documento D1 es que la presente invención menciona al TGFα.

El solicitante respetuosamente discrepa con dicha afirmación, porque la presente invención según la nueva reivindicación 1 no se trata de la misma composición vacunal donde se usa uno u otro péptido indistintamente, pongo de presente que la nueva reivindicación 1 reclama una vacuna que comprende simultáneamente dos péptidos. En el artículo publicado por *Mulet et al.* en *Cancer Immunol Immunother (2006) 55: 628-638,* Pág. 632 capitulo de Resultados y luego Pág. 636 capitulo de discusión, la autora demuestra que inmunizando con diferentes formulaciones de la vacuna que comprende la proteína de fusión con TGFα, NO se obtienen células B que secreten anticuerpos contra el EGF, esto demuestra la especificidad de la respuesta obtenida. Por tanto, estos resultados demuestran que a pesar de que ambos factores (EGF y TGFα) reconocen el

mismo receptor, ellos son capaces de inducir respuestas de anticuerpo diferentes. La autora también discute (Pág. 636 del propio articulo) que sus resultados descartan que el efecto anti-tumoral de la preparación de con TGFα sea atribuido la eliminación del EGF circulante.

El examinador debe notar que la diferencia entre la vacuna de D1 y la vacuna de la presente invención es que con la vacuna que conjuga más de un péptido es posible inducir una respuesta inmune que funcione bloqueando simultáneamente diferentes mecanismos de regulación tanto autocrina como paracrina lo cual conlleva a un mayor efecto terapéutico sobre el control del crecimiento de los tumores. Dicho de otra manera, se ha demostrado que la respuesta inmune producida con la vacuna descrita en D1 no es idéntica a la respuesta inmune producida por la vacuna de la presente invención (ver *Mulet et al. Cancer Immunol Immunother (2006) 55: 628-638.*), entonces aquellos tumores cuyo crecimiento es sensible a la estimulación por diferentes vías, escaparán al bloqueo del crecimiento cuando se use la vacuna descrita en D1; sin embargo, cuando se usa la vacuna de la presente invención las diversas vías de estimulación serán bloqueadas por los anticuerpos levantados por esta composición vacunal.

Esto NO es obvio del estado del arte puesto que el mismo examinador considera que por el hecho de que ambas moléculas (TGFα y EGF) actúan a través de un receptor de membrana que es común a los dos (EGF-R), el efecto de la inmunización con ellos será el mismo.

Existe un consenso en el estado del arte de que el EGF ejerce su efecto de estimulación de la proliferación celular por medio de un mecanismo paracrino, esto quiere decir que las células que secretan EGF ejercen su efecto sobre las células del entorno y no sobre ellas mismas; sin embargo, el TGFα ejerce su función biológica de estimulación del crecimiento de algunos tumores a través de un mecanismo autocrino, es decir la célula tumoral produce el TGFα que actúa sobre ella misma. Estos conceptos se derivan de mediciones experimentales; es un dato verificado por diferentes autores que los niveles de EGF secretados por tumores que expresan el receptor (EGF-R) es muy bajo o nulo, sin embargo, muchos de estos tumores secretan cantidades medibles de TGFα.

Vale remarcar que aunque ambos factores ejercen su acción a través del mismo receptor, ellos tienen blancos diferentes de su acción de estimulación de la proliferación, además Mulet demostró (esto se discutirá en detalle más adelante) la inducción de anticuerpos anti- TGFα que no competían con el EGF, por tanto y basados en todos estos resultados, la composición de la presente invención pretende inducir diversos anticuerpos con el objetivo de bloquear simultáneamente diferentes mecanismos de estimulación y finalmente tener un efecto inhibidor sobre el crecimiento de los tumores.

Comentarios sobre la Respuesta a los argumentos del solicitante

El documento D2 en su primera página refiere que en carcinomas gástricos avanzados la expresión de TGFα es signo de mal pronóstico. También que en carcinomas escamosos de esófago, la expresión de este factor también se asocia a un pobre pronóstico. Sin

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embargo, seguidamente el propio autor refiere:"*In contrast, in preoperative colorectal carcinomas biopsies, Younes et al (1996) found that high expression of TGFα protein is a good prognostic indication*". Adicionalmente el examinador podrá encontrar en la literatura que NO hay un consenso sobre el papel de dicho factor en la transformación maligna, NO hay un consenso sobre la correlación directa entre la expresión de este factor y el pronóstico. Por tanto, y respetuosamente el solicitante considera que el examinador esta en un error cuando plantea que D2 desmiente la afirmación del solicitante. D2 es solo un caso en el que se encontró una buena correlación pero los tumores malignos son mucho más que los adenocarcinomas del esófago.

La presente invención proporciona una solución práctica al problema basada en el conocimiento anterior, solución técnica para aquellos tumores cuyo crecimiento se afecte al privarlos de estos factores de crecimiento de manera simultánea. El conocimiento no es nuevo, pero la solución práctica no ha sido anticipada por los documentos anteriores. Por otra parte, la solución práctica propuesta por la presente invención no es obvia porque, D'Errico, entre otros, han informado que el TGFα está relacionado con ciertos tipos de tumores (NO con cualquier tumor), por lo tanto, no está claro cómo los anticuerpos circulantes en contra de este factor de crecimiento podrían funcionar, es decir, no es posible prever el efecto que sobre el crecimiento tumoral tendrá la privación del TGFα circulante.

Los anteriores argumentos aclaran las ventajas técnicas de la presente invención sobre el arte previo y por ende sustentan el nivel inventivo de la solicitud. Respetuosamente solicito a ese Despacho que se conceda el privilegio de patente a la presente solicitud toda vez que la misma cumple con las disposiciones de los artículos 30 y 18 de la Decisión 486.

Anexos

Capitulo reivindicatorio

Formato de modificaciones

Recibo de pago por modificaciones

Señora Jefe,

Margarita Castellanos Abondano
C.C. No. **39.779.654** de Usaquén
T.P.A. No. **70819** del Cons. Sup. de la Judicatura
Carrera 9ª. No. 93-09 - Bogotá, D.C.
Teléfono: 7560770

Bogotá D.C. 10 FEB. 2010

PATENTES/P0968/MEMORIAL FONDO III

Industria y Comercio
SUPERINTENDENCIA

Espacio reservado para el adhesivo de radicación

FORMULARIO ÚNICO DE CORRECCIONES Y MODIFICACIONES

(1) SOLICITUD DE:

☐ **Corrección de Errror Material** ☒ **Modificación a una solicitud**

(2) SOLICITANTE

Nombre: **CENTRO DE INMUNOLOGIA MOLECULAR**

Dirección: **Calle 216 Esq. 15, Atabey, Playa, C. Habana 12100, CUBA**

Nacionalidad o Domicilio: **La Habana, Cuba**

Lugar de Constitución: **Cuba**

Teléfono: Fax:
E-mail:

IDENTIFICACIÓN

C.C. ☐ NIT ☐
C.E. ☐ Otro ☐
Cual ____
Número ____

(3) REPRESENTANTE O APODERADO

Nombre: **MARGARITA CASTELLANOS A.**

Dirección: **CALLE 94A No. 7A-09**

Teléfono: **756 07 70** Fax: **7 55 01 58**

E-mail: info@castellanosyco.com

IDENTIFICACIÓN

C.C. ☒ NIT ☐
C.E. ☐ Otro ☐
Cual ____
Número **39'779.654** TPA **70.819**

(4) Número de Expediente: **03.048.135**

(5) Descripción de la corrección o modificación solicitada:

Presento nuevo pliego reivindicatorio al cual solicito se conceda el privilegio de patente.

(6) Comprobante de pago No. **10 - 6,190** **Fecha** **27/01/2010**

(7) Anexos:

☒ Comprobante de pago de la tasa.
☐ Poderes si fuere el caso, junto con la sustitución de los mismos a mi favor.
☐ Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas
☒ Nuevo capítulo reivindicatorio

(8) **NOMBRE** **MARGARITA CASTELLANOS A.** **FIRMA** Margarita Castellanos

CC 39'779.654 Usaquén - TPA 70.819 CSJ

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página - MCA/zca-P0968

10 FEB. 2010

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 444 RESPUESTAREQUE Folios: 18

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
ALVARO CASTELLANOS	CONSIGNACION	BANCO DE BOGOTA	062754387	175511696	27/01/2010	3,133,000.00

***** C O N C E P T O *****

CANT.	RENTISTICO	CONCEPTO		TOTAL CONCEPTO
1	50005-01-03 CAMBIO DE MODALIDAD Y MODIFICA	12 MARCAS Y LEMAS COMERCIALES		114,000.00
		TOTAL :	$	114,000.00

CON: CIENTO CATORCE MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

REIVINDICACIONES MODIFICADAS EN RESPUESTA AL OFICIO No. 12542

1. Una composición vacunal caracterizada porque comprende como principio activo una molécula compuesta por Factor de Crecimiento Transformante Alfa humano recombinante (TGFα) y otro péptido seleccionado del grupo consistente en, un péptido derivado del TGF-alfa o un ligando del receptor del Factor de Crecimiento Epidérmico, y donde dicho conjugado está enlazado a una proteína transportadora, adicionalmente la composición vacunal comprende un adyuvante.

2. La composición vacunal según la reivindicación 1, caracterizada porque la proteína transportadora es la proteína P64K de Neisseria meningitis.

3. La composición vacunal según la reivindicación 1, caracterizada porque el adyuvante es Adyuvante Incompleto de Freund.

4. La composición vacunal según la reivindicación 1, caracterizada porque el adyuvante es Al(OH)3.

5. La composición vacunal según la reivindicación 1, caracterizada porque comprende como principio activo un conjugado químico entre el TGFα, EGF y la proteína transportadora P64K de Neisseria meningitidis.

6. La composición vacunal según la reivindicación 1, caracterizada porque comprende como principio activo una proteína de fusión recombinante entre el TGFα, el EGF y la proteína transportadora P64K de Neisseria meningitidis.

7. La composición vacunal según la reivindicación 6, caracterizada porque comprende una proteína de fusión recombinante entre el TGFα y la P64K de Neisseria meningitidis, la cual presenta una cola de seis histidinas en el extremo N-terminal de dicha proteína P64K.

8. La composición vacunal según la reivindicación 7 caracterizada porque la proteína de fusión comprende una molécula de EGF unida al TGFα y a la proteína transportadora la P64K.

Cancer Immunol Immunother (2006) 55: 628–638
DOI 10.1007/s00262-005-0030-9

ORIGINAL ARTICLE

Aillette Mulet · Greta Garrido · Anabel Álvarez
Tamara Menéndez · Frank-D Böhmer · Rolando Pérez
Luis Enrique Fernández

The enlargement of the hormone immune deprivation concept to the blocking of TGFα-autocrine loop: EGFR signaling inhibition

Received: 25 August 2004 / Accepted: 12 May 2005 / Published online: 18 November 2005

Abstract Transforming growth factor alpha (TGFα) is a potent ligand of the epidermal growth factor receptor (EGFR). EGFR is frequently over-expressed in epithelial tumors and endogenous ligands, mostly TGFα, are frequently co-expressed with EGFR, potentially resulting in autocrine stimulation of tumor cell growth. Therefore, different therapeutic approaches aim for the inactivation of TGFα/EGF/EGFR signaling system, but no approach is based on TGFα as a target. The principal goal of this work was to assess the potential of an active specific immunotherapy approach to block the TGFα/EGFR autocrine loop. For the proof of the concept, a fusion protein between human TGFα (hTGFα) and P64k protein from *Neisseria meningitidis* was generated, and its immunogenicity characterized in a mouse model using different adjuvants. All immunogens were effective for the generation of specific humoral responses against hTGFα. The inmunodominant epitope of hTGFα when immunizing mice with the fusion protein involved the C-loop/C-terminal region. This region includes key residues for hTGFα binding to EGFR. The anti-hTGFα immune mice sera recognized the natural hTGFα precursor in A431 cells and hTGFα-transfected 3T3 fibroblasts as revealed by flow cytometry analysis and immunoblotting. They inhibited the binding of ^{125}I-TGFα to the EGFR, EGFR-autophosphorylation, and downstream activation of MAP kinases as well as proliferation of two EGFR-expressing human carcinoma cell lines. These data suggest that EGFR signaling activation by the hTGFα autocrine loop may be inhibited in vivo by induction of specifically blocking antibodies. The fusion protein reported in this paper could be a potential immunogen for the development of a new cancer vaccine.

Keywords hTGFα · Active specific immunotherapy · Growth factor immunodeprivation · Cancer vaccine

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A. Mulet (✉) · G. Garrido · R. Pérez · L. E. Fernández
Center of Molecular Immunology (CIM),
P.C.Box 16040, Havana 11600, Cuba
E-mail: aillette@ict.cim.sld.cu
Tel.: +53-7-2716810
Fax: +53-7-2720644

A. Álvarez · T. Menéndez
Center of Genetical Engineer and Biotechnology,
P.O.Box 6162, Havana 10600, Cuba

Frank-D Böhmer
Molecular Cell Biology, Jena University Hospital,
07747 Jena, Germany

Introduction

The epidermal growth factor receptor (EGFR) family and the corresponding peptide ligands are involved in normal and neoplastic development, and many of them are over-expressed in human carcinomas as compared with their normal counterpart [1]. The simultaneous presence of the EGFR and its ligand transforming growth factor alpha (TGFα) in human tumor tissues suggests that the TGFα autocrine loop drives tumor growth. In fact, TGFα/EGFR co-expression is considered to be an indicator of bad prognosis for many epithelial tumors [2–9]. Besides, TGFα is a more potent angiogenic factor than EGF [10] and cooperates with other oncogenes (e.g., c-myc and ras) or chemical carcinogens to promote tumorigenesis [11]. In this context, TGFα might represent a suitable target for novel therapeutic approaches in cancer.

Passive immunotherapy with specific monoclonal antibodies (MAbs) against EGFR (e.g., Imclone C225 and Theracim hR3), combined or not with chemotherapy or radiotherapy [1, 12, 13], and receptor tyrosine kinase inhibitor drugs [14] are currently in clinical trials. Following an active specific immunotherapy approach, our group has developed a cancer vaccine based on EGF-immune deprivation, aiming to stop EGF-dependent

tumor growth. This vaccine has revealed promising preclinical [15, 16] and clinical results [17, 18]. Obviously, an EGF vaccine can only block the action of EGF but will have no effect on TGFα. Therefore, many epithelial tumors which depend in growth on EGFR signaling would escape from EGF-immunodeprivation therapy by up-regulation of the TGFα autocrine loop. Thus, an active specific immunotherapy approach targeting TGFα seems highly warranted. To our best knowledge, such an approach has not yet been attempted.

The main goal of this work was to assess the potential of an active specific immunotherapy approach to block the TGFα/EGFR autocrine loop. For the proof of the concept a fusion protein between human TGFα (hTGFα) and a highly immunogenic carrier protein was designed and expressed in *Escherichia coli*. The immunogenicity of this recombinant protein was characterized in a mouse model using different adjuvants and the induction of TGFα-blocking antibodies was analyzed. Sera from immunized mice inhibited in vitro TGFα-mediated EGFR signaling activation.

Materials and methods

Cell culture

A431 human epidermoid carcinoma, H125 human lung adenocarcinoma, and Caco-2 colon cancer cell lines were obtained from the ATCC (USA). Balb-3T3 murine fibroblast cell line and Balb-3T3 cells transfected with hTGFα cDNA were kindly provided by Dr. Diana del Barco (CIGB, Havana, Cuba). All cell lines were maintained in DMEM medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (FCS, Hyclone).

Design of pMhisTGFα expression vector

The gene coding for hTGFα (150 bp long) was amplified by PCR using the PSK-TGFα plasmid (kindly provided by CIGB, Havana, Cuba) coding for the complete hTGFα gene (500 bp long) as template and oligonucleotides 4292 (sense, 5′ GCT CTA GAA GTG GTG TCC CAT TTT AAT GAC 3′) and 5353 (antisense, 5′ C GGA ATT CGC CAG CAG GTC CGC ATG CTC AC 3′) as primers. The resulting DNA fragment was chloroform extracted from 2% agarose gel before a digestion with *Xba*I/*Eco*RI and ligated into the pM229 vector. This plasmid contains the *lpdA* gene, coding for P64k protein of *Neisseria meningitidis* (strain B385) with a six His tag at the N-terminal region under the control of *E. coli* tryptophan operon promoter (ptrp) and phage T4 transcriptional terminator (tT4) [19]. The resulting pMhisTGFα plasmid codes for a $(His)_6$ fusion protein containing the mature hTGFα sequence inserted between amino acids 45/46 of P64k (hTGFα–P64k). This plasmid was verified by restriction analysis, DNA sequencing, and expressed in *E. coli* strain MM294 (ATCC 39607).

Fusion protein purification

Escherichia coli expressing the recombinant protein hTGFα–P64k was grown in an LB medium supplemented with 50 μg/ml ampicillin for 10 h at 37°C. After collecting cells, all steps were performed at 4°C. Bacterial disruption was achieved in a French press at 1,500 kg/cm^2, and the insoluble fraction was removed by high-speed centrifugation for 30 min at 11,000×*g*. As an initial purification step, a 40% ammonium sulfate precipitation was done. The obtained pellet by centrifugation at 4°C for 30 min at 11,000×*g* was diluted in 20 mM Tris buffer, pH 6, containing 0.5 M of NaCl and 50 mM of Imidazole and fractionated by chelating metal affinity chromatography (Sepharose fast flow charged with Cu^{2+}, Pharmacia, Uppsala, Sweden) with an increasing gradient of imidazole from 50 mM to 200 mM. Finally the salt excess was eliminated by dialyzing against PBS at 4°C overnight. Protein concentration was determined as described by Lowry et al. [20]. The hTGFα–P64k solutions were filtered by 0.2 μM and kept at −20°C until used. The purity of the isolated fusion protein was determined by SDS-PAGE using the Molecular Analyst software (Bio-Rad, CA, USA).

Mice and immunization protocols

Balb/c female mice, 8–12 weeks of age, purchased from CENPALAB (Havana, Cuba) were used.

All immunizations were done by subcutaneous injection of 232 μg of fusion protein (20 μg of hTGFα) on days 0, 14, 28, and 32. Immunogens were prepared either by mixing the fusion protein with 2 mg of aluminum hydroxide (alum) per dose (Superfos Biosector, Frederikssund, Denmark) or by emulsifying equal volumes of fusion protein solution and oily adjuvants: complete (CFA, Sigma) and incomplete (Montanide ISA 51, SEPPIC, France) Freund adjuvants. On days 0, 21, 35, 56, blood was drawn to determine antibody titers.

For ELISPOT or Cytokine ELISA techniques, mice were re-immunized subcutaneously in the tail base and sacrificed 3 days later by cervical dislocation and the inguinal lymph nodes (LNs) were extracted. Animal studies were performed with approval from CENPALAB's and CIM's Institutional Animal Care and Use Committees according to international guidelines.

Obtaining purified polyclonal anti-hTGFα antibodies

Balb/c female mice were immunized with hTGFα-fusion protein emulsified in Montanide ISA 51 as described above and inoculated with 1×10^6 cells of X63 murine

myeloma cells in PBS intraperitonieally. After 7 days, the ascites containing the antibodies was obtained and the IgG polyclonal antibodies were obtained by affinity chromatography with Protein A-Sepharose according to the protocol of the manufacturer (Pharmacia, Uppsala, Sweden). The control IgG was obtained from mice immunized with the adjuvant only.

ELISA

Microtiter plates (High binding; Costar, Cambridge, MA, USA) were coated with 50 ng/well hTGFα (R&D System, Minneapolis, MN, USA) in a coating buffer at 4°C overnight. Afterward, plates were blocked with 5% of FCS in PBS-0.05%Tween 20. Serum sample dilution and AP-labeled goat anti-mouse antiserum (Sigma) were diluted in blocking buffer and stepwise incubated on the plates at 37°C for 1 h with extensive washing with PBS-0.05%Tween 20 between both steps. *p*-Nitrophenyl phospate (Sigma) 50 ng/well in dietanolamine buffer pH 9.8 was used as substrate and absorbance (*A*) was measured at 405 nm.

Assays were performed in triplicate for each sample and the SD was less than 10%. Background values of *A* were less than 0.1. Titer was defined as the highest serum dilution giving *A* values equal to or greater than two times the value of a same dilution of pre-immune serum. The geometric mean of antibody titer was used to compare the different treatment groups.

To determine the epitopes recognized by the sera of immunized mice, four synthetic peptides that represent N-terminal, A-loop, B-loop, and C-loop/C-terminal region of hTGFα (Table 1) were synthesized. The murine counterpart of the C-loop/C-terminal region was also synthesized. These cyclic (A-loop and B-loop) and semi-cyclic (C-loop/C-terminal) peptides reproduced disulfide bonds present in the original molecule. The serum titers against those peptides were determined using plates coated either with each peptide or TGFα at 0.5 mM. To determine to which extent anti-peptide responses abrogated the total response against the whole hTGFα, mouse serum dilutions were pre-incubated with different concentrations of immunodominant or unrelated peptides at 4°C overnight and then incubated in a plate coated with hTGFα (50 ng/well) following the same protocol described above.

IgG isotype profiling was performed using secondary biotinylated anti-mouse IgG1, IgG2a, IgG2b and IgG3 rat MAbs (Pharmingen, San Diego, CA, USA). An ELISA system as described previously [21] was used to establish optimal secondary antibody dilutions. Then, streptavidine-phosphatase conjugate (Sigma) was added. Enzymatic reaction was developed and measured at 405 nm.

Cytokine response

IL-4 and IFN-γ were determined using a commercial ELISA kit (Pharmingen) according to the manufacturer protocol. Briefly, 200,000 LN cells from the immunized or control (mice immunized with saline) mice were plated on 96 U bottom culture plates and incubated with 100 μg of hTGFα-P64k protein at 37°C for 5 days, 5% of CO_2. At day 4, 5 ng/ml of PMA (Sigma) and 200 ng/ml of Ionomycin (Sigma) were added to each well. Twenty-four hours later, 100 μl of supernatant was harvested and tested.

ELISPOT

Microtiter plates (High binding; Costar) were coated with 125 ng/well of hTGFα (R&D System), hEGF (CIGB, Cuba), mEGF (R&D System) or P64k (CIGB, Havana, Cuba) in coating buffer at 4°C overnight. After blocking with 5% BSA in PBS at 37°C for 30 min, serial dilutions of LN cells in RPMI medium were incubated for 6 h at 37°C, 5% CO_2. Specific secreting B-cells were revealed as spots forming cells (SFC) using AP-labeled goat anti-mouse IgG (Fcγ specific) or IgM (Jackson, West Grove, PA, USA) antibodies in blocking buffer at 4°C overnight. After extended washes with PBS-Tween 2.5% solution and tap water, BCIP (Sigma) in AMP buffer containing 0.6% agarose was used as the substrate for the enzyme. The plates were incubated at 4°C overnight and the spots were counted under a microscope. Lymph node cells from naive mice were used as a negative control.

FACS analysis

A431, Balb-3T3 and Balb-3T3-TGFα cells (5×10^5) were stained in PBS containing 0.1% NaN_3 and 1% BSA for 30 min at 4°C. Different dilutions of pre-immune (negative control) or sera from immunized mice were added

Table 1 hTGFα synthetic peptide sequences

Peptide sequence	TGFα region (amino acids encoded[a])
VVSHFNDCPDSHTQF	N-terminal (1–15)
CPDSHTQFI[b] FHGTC cyclic peptide (C_8–C_{21})	A-loop (8–21)
CFHGTS[c] LVQEDKPACV semi-cyclic peptide (C_{16}–C_{32})	B-loop (16–33)
CHSGYVGARCEHADLLA semi-cyclic peptide (C_{34}–C_{43})	C-loop and C-terminal (34–50)

[a]Numbering is relative to the mature hTGFα
[b]C_{16} is substituted for I
[c]C_{21} is substituted for S

and incubated for 30 min at 4°C. After washing, a goat anti-mouse conjugate with FITC was added (Jakson). Acquisition was performed on a FACScalibur (Becton Dickinson, San José, CA, USA), using forward- and side-scatter characteristic to exclude dead cells. Data were analyzed using Cell Quest (Becton Dickinson).

Radio receptor assay

A431 cells (10^4 per well) were seeded in 96 wells culture plates using DMEM medium (Hyclone) with 5% FCS (Hyclone) and kept overnight with 5% CO_2 at 37°C. The next day the cells were washed three times with PBS and incubated with 100,000 cpm of ^{125}I-hTGFα (110 μCi/μg) in varying dilutions of immune sera for 1 h at room temperature. Binding inhibition by an excess of nonradioactive TGFα was used as the positive control. After three washes, 50 μl of a solution of NaOH 2 M was added to each well and the total ^{125}I-hTGFα bound to cell membranes was measured in an automatic gamma counter (Wallac, Turku, Finland). The hTGFα was labeled by Chloramine T method [22].

Cell lysates and Western blotting

Caco-2 or A431 cells were serum starved for 24 h and incubated with control or anti-hTGFα polyclonal antibodies for 12 h. Incubation with 1 μM tyrphostin AG 1478 for 1 h was used as the positive control. Cell lysates were prepared using 50 mM Hepes pH 7.4, 0.15 M NaCl, 1% Triton X-100 buffer containing 1 mM EDTA, 1 mM EGTA, 2 μg/ml Leupeptin, 1% Aprotinin, 2 μg/ml Pepstatin, 1 mM PMSF and 1 mM Na_3VO_4 and clarified by centrifugation. The protein concentration of the lysates was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were resolved on SDS-PAGE, transferred to polyvinyldifluoride nitrocellulose (Gelmar, Ann Arbor, MI, USA) followed by blocking with NEGT buffer (0,15 M NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 7.5, 0.02% Tween 20 and 0.04% Gelatine) overnight at 4°C. Then the membranes were incubated with specific anti-p44/42 MAP kinase antibody or anti-phosphotyrosine (PY) antibody (Cell Signaling Technology, Beverly, MA, USA) at room temperature for 2 h. After washing with NEGT buffer, the membranes were incubated with secondary antibody (anti-mouse or anti-rabbit antibodies conjugated with horseradish peroxidase) for 30 min at room temperature. The signal was visualized by enhanced chemiluminescence according to manufacturer's instruction (PerkinElmer Life Sciences, Foster City, CA, USA) and band intensity was quantitated using a personal densitometer SI (Pharmacia) and ImagQuant Software. Where indicated, the membranes were stripped and reproved with anti-MAP kinase (PAN ERK, transduction laboratories, Lexington, KY, USA) or anti-EGFR (Santa Cruz Biotechnology) antibodies for 1 h at room temperature to normalize for protein loading on the gel.

To assay the polyclonal anti-hTGFα antibodies for recognition of hTGFα precursor in immunoblots, A431, H661, Balb-3T3 and Balb-3T3-TGFα cells were grown in DMEM-FCS 10% until confluency and lysed. Lysates were processed for immunoblotting as described above. The polyclonal antibodies' anti-hTGFα at 1/500 was used as a primary antibody and anti-mouse antibodies conjugated with horseradish peroxidase were used as a secondary antibody.

MTT assay

Growth inhibition/cytotoxicity determination of immune sera in human cell lines was performed according to the ATCC MTT cell proliferation assay instructions. Briefly, 10^4 cells were plated on flat-bottomed 96-well plates in RPMI medium with 1% of FCS for 24 h at 37°C and 5% CO_2. Control or anti-hTGFα polyclonal antibodies in different dilutions were added to the plates for another 24 h. Then, 10 μl of MTT (Sigma) solution (5 mg/ml) was added to each well and incubated for 2 h at 37°C. The intracellular purple formazan crystal formed in living cells was solubilized using 100 μl of solubilization buffer (10% SDS in 0.01 M HCl). The A was measured at 570 nm with subtraction of the A at a reference wavelength of 690 nm. All tests were performed in duplicate. The percentage of viable cells in test wells relative to the A of maximum reference wells was calculated using the following formula:

$$\text{Viable cells (\%)} = \frac{T-B}{M-B} \times 100\%,$$

where $T = A$ of test well, $B =$ mean A of background wells (wells with culture medium only), $M =$ mean A of maximum reference wells.

Statistical analysis

ANOVA with the multiple comparison parametric test of Bonferroni was performed. Wilcoxon–Mann-Whitney test was used when a non-parametric test was necessary. $P < 0.05$ was accepted as significant.

Results

Fusion protein characterization

To obtain an immunogenic fusion protein of hTGFα, we chose the P64k protein from *N. meningitidis* as a fusion partner. P64k has previously been successfully used for the generation of immunogenic hEGF-chemical conjugates and revealed no side-effects in clinical trials [17, 18]. A plasmid encoding the mature hTGFα sequence, inserted between the amino acids 45 and 46 of

P64k protein and additionally including a segment of six His at the N-terminal region, was obtained as described in Materials and methods and used for expression in *E. coli*. The soluble, 69 kDa fusion protein (hTGFα–P64k) was obtained from the *E. coli* cytoplasm, with at least 90% purity using chelating-metal affinity chromatography (data not shown). The identity of expressed hTGFα–P64k was verified by immunoblotting. Specific antibodies recognized both hTGFα and P64k in the fusion protein (data not shown). The recombinant protein was emulsified in oily adjuvants either CFA or IFA (Montanide ISA 51) or adsorbed in alum to prepare different vaccine formulations.

Immunization with hTGFα–P64k induced a serological response versus hTGFα

Mice immunized with different formulations containing hTGFα-fusion protein developed antibodies against hTGFα, predominantly of the IgG class. The kinetics of the serum anti-hTGFα antibody response was followed for 56 days after the initiation of the immunization protocol and were similar, independent of the adjuvant employed in the vaccine formulation ($P=0.594$, ANOVA). Mean antibody titers reached a peak value by day 35 (Fig. 1a) and remained almost constant up to the end of the observation period (7 months). It is noteworthy that, when mice were re-immunized, antibody titers increased up to 50,000 in all animals (data not shown).

To compare the specific humoral responses obtained with the different adjuvants, inguinal LNs of mice immunized with hTGFα–P64k in oily adjuvants were examined for the presence of antibody-secreting B cells. Mice immunized with the fusion protein in oily adjuvant showed a higher number of anti-hTGFα-specific antibody secreting B cells, compared to mice immunized with the antigen adsorbed in alum (ANOVA $P<0.005$, Fig. 1b). B cells secreting antibodies against the carrier protein P64k predominated in LNs of all mice as expected. No B cells secreting cross-reactive antibodies against human or murine EGF were found (data not shown), indicating the specificity of the antibody response obtained.

With respect to the quality of the antibody response, whereas similar levels of IgG1 were observed in sera of immunized mice irrespective of the adjuvant, an increase in IgG2a and IgG2b was associated to CFA or IFA (Fig. 2). Moreover, not only was the (IgG2a + IgG2b)/IgG1 ratio similar for the groups immunized with oily adjuvants ($P=0.622$, Bonferroni test) but also the ratio was in both cases different, compared to the alum group ($P<0.05$). As expected, no IgG3 antibodies were generated with any vaccine formulation.

In concordance with the immunoglobulin subclass pattern obtained with the oily adjuvants, the cytokine profile secreted by LN cells from mice immunized with the TGFα-fusion protein in IFA was characteristic of a Th1 pattern and similar to the profile obtained when

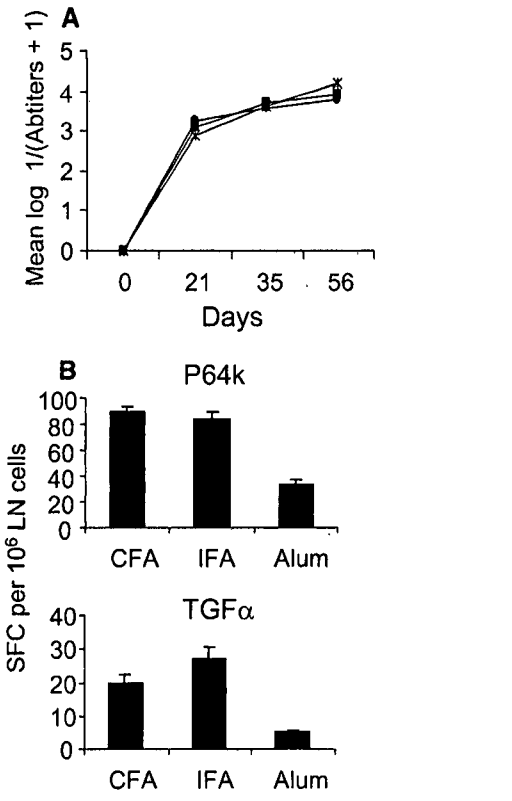

Fig. 1 Anti-hTGFα humoral response. **a** Kinetics of antibody response. Mice were immunized with 116 μg of fusion protein (10 μg equivalent of hTGFα), four doses biweekly, in CFA (*Asterisk*), IFA (*filled square*) and adsorbed in alum (*filled circle*). Antibody titers (logarithm of 1/(Ab titer + 1)) were measured by ELISA in the sera of mice at days 0, 21, 35, and 56 and are plotted for the different days of sera collection. **b** Clonal expansion of specific IgG producing B cells after hTGFα–P64k immunization, measured by ELISPOT. Lymph node cells from naïve mice or mice immunized with the fusion protein in CFA, IFA or alum were plated on wells coated with P64k or hTGFα (**a**). Results are expressed as the mean of SFC per 10^6 LN cells ± SD

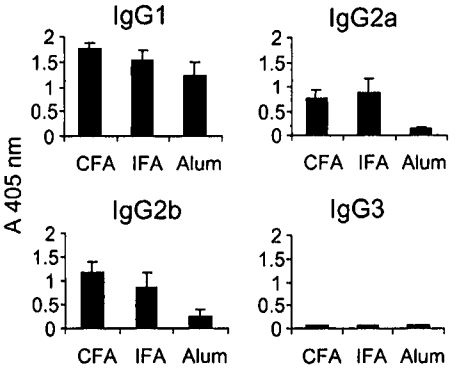

Fig. 2 IgG isotype profile in immunized mice. Sera from immunized mice were diluted 1/500 and the levels of each IgG subclass was determined by ELISA. Data represent average of absorbance (*A*) ± SD from 5 to 10 mice

mice were immunized with this fusion protein in combination with a very small size proteoliposome (VSSP) obtained from *N. meningitidis*, a potent stimulator of the

Th1 cytokine pattern [23]. As shown in Fig. 3, the stimulated specific T cells from mice immunized with the fusion protein in oily adjuvant secreted only IFN-γ and not IL-4 when stimulated either with the entire fusion protein or the hTGFα peptides.

Predominantly the C-loop and the C-terminal region of hTGFα were recognized by sera from immunized mice

To study the immunodominance of the antibody response induced by hTGFα–P64k, four sequential peptides covering the whole hTGFα molecule were synthesized (see Materials and methods, Table 1). These peptides represent the different loops of the hTGFα molecule. The immune sera mainly recognized the semi-cyclic peptide corresponding to the C-loop and the C-terminal region (Fig. 4) independently of the adjuvant used for the immunization. Noteworthy, immune sera also recognized the murine counterpart of the immunodominant peptide (C-loop/C-terminal) with antibody titers up to 1/1,000 (data not shown). Further evidence for the dominant role of the C-loop/C-terminal peptide in eliciting the serological response was obtained by

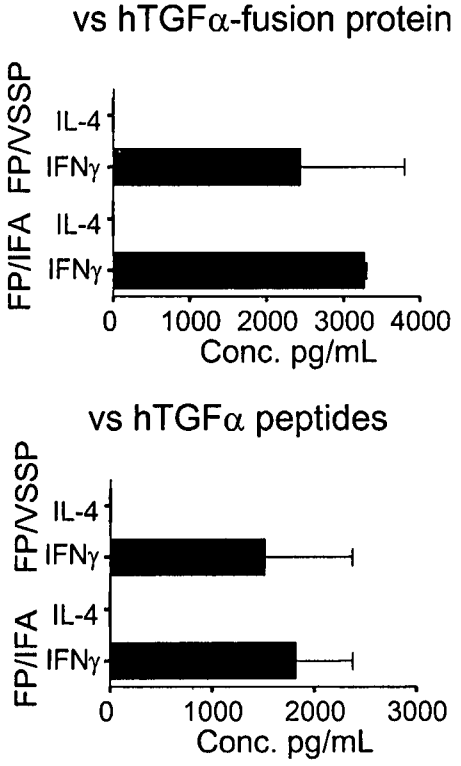

Fig. 3 Profile of cytokines secreted by Th specific cells induced by vaccination. Mice were immunized with the TGFα-fusion protein (FP) in Montanide ISA 51 alone (FP/IFA) or with VSSP (FP/VSSP) and LN cells wAS isolated and incubated with 20 μg of TGFα-FP (*Top*) or hTGFα peptides for 5 days. After an overnight stimulation with PMA (5 ng/ml) and Ionomycin (200 ng/ml),100 μl of supernatant were harvested and tested for IFN-γ or IL-4 as indicated on the graph. The results are expressed as mean with error bars representing ±SD (three animals per group)

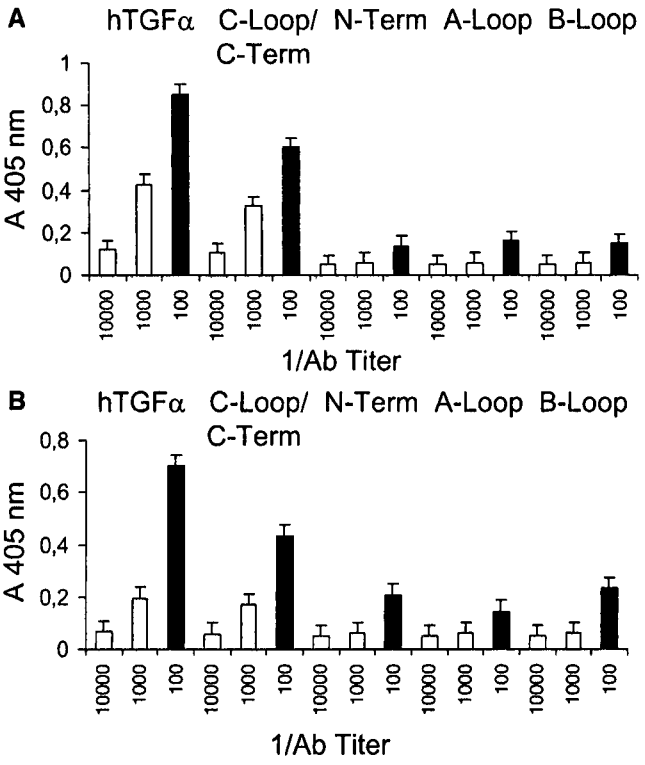

Fig. 4 Immunodominancy of anti-TGFα antibody response. Four peptides covering the whole hTGFα sequence were synthesized (see Table 1 for peptides sequences). Serial dilutions of sera from mice immunized with hTGFα–P64k emulsified in CFA (**a**) or adsorbed in alum (**b**) were added to microtiter ELISA plates coated with 0.5 mM of each peptide representing N-terminal, A-loop, B-loop and C-loop/C-terminal region of hTGFα or the whole molecule as is indicated above each graph. *Y*-axis represents the mean of *A* (405 nm) ± SD obtained from six mice of each group at different dilutions that are represented by each bar. *X*-axis indicates the sera dilutions

inhibition experiments. Incubation of hyperimmune sera with different concentrations of the immunodominant peptide inhibited the majority but not the total anti-hTGFα response (data not shown). We also explored whether the hTGFα–P64k fusion protein itself had any activity as an EGFR ligand. However, no activation of EGFR in A431 cells was detectable when exposing the cells to the fusion protein (data not shown). Thus, despite maintaining an antigenic structure, fusion of the TGFα sequence to the P64k protein leads to abrogation of its biological activity.

Sera from immunized mice recognized natural hTGFα and inhibited TGFα-dependent EGFR signaling and cell growth

Antibodies generated against the fusion protein specifically bound to the transmembrane form of hTGFα (pro-TGFα) present in cell lines expressing this growth factor. As revealed by FACS analysis (Fig. 5a, b), immune sera reacted with Balb3T3 cells transfected with hTGFα

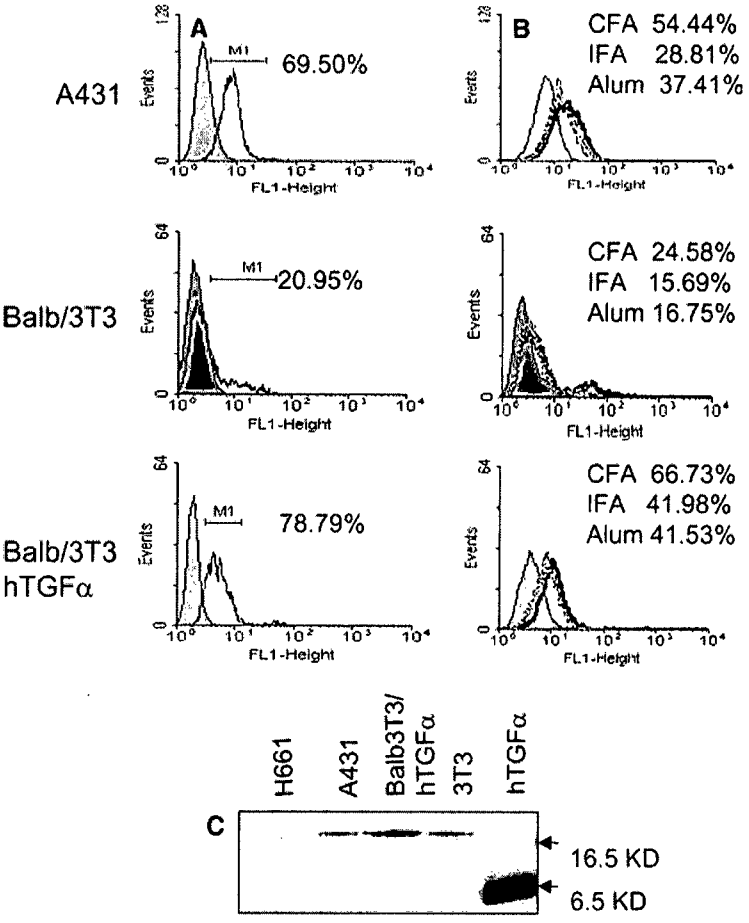

Fig. 5 Anti-TGFα immune sera recognize specifically the membrane form of TGFα (pro-TGFα). **a** Binding of an anti-hTGFα MAb (cross-reactive with mTGFα) to A431, Balb-3T3 and Balb-3T3/hTGFα cell lines. **b** Pre-immune sera (*solid histogram*) and immune sera from mice immunized with the fusion protein in CFA (*solid line*), IFA (*broken line*) or alum (*dotted line*) adjuvant, diluted 1/20, were incubated with those cell lines. Then FITC-conjugated anti-mouse secondary antibody was added to determine the specific binding. Results are shown as histograms and the percentage of positively recognized cells is indicated in each graph. **c** Recognition of TGFα precursor by immunoblotting. One hundred micrograms of total protein from A431, H661, Balb/3T3-hTGFα and 3T3 cells were separated on 10% SDS-PAGE gel, followed by transfer to PVDF membrane. Total protein from H661 cells was used as negative control (line 1). Immunodetection was performed using polyclonal anti-hTGFα antibodies obtained from immunized mice

cDNA (Balb3T3-hTGFα), but not with the parental cell line, and also recognized human pro-TGFα present in A431 cells. Recognition of the cellular TGFα precursor of about 17 kDa by the anti-hTGFα antibodies could also be demonstrated by immunoblotting using cell lysates of these cell lines (Fig. 5c). In this case, the reactivity was strongest with pro-TGFα in Balb3T3-hTGFα cells and less pronounced with A431 cells. Importantly, nearly no reactivity was detectable in H661 NSCLC cells, previously reported to not express TGFα [24]. Some material was also detected in the non-transfected 3T3 cells, indicating some cross-reactivity against murine pro-TGFα. In summary, both types of analysis revealed TGFα recognition by the generated antibodies. Some quantitative differences in results with both techniques may have been caused by different antigen accessibility in denatured lysates and on intact cells. To know the blocking capacity of immune sera, preimmune and immune sera were incubated with ^{125}I-radiolabeled hTGFα and binding to EGFR was tested using the A431 cell line. As shown in Fig. 6, the antibodies clearly inhibited binding of TGFα to EGFR in a dose-dependent fashion.

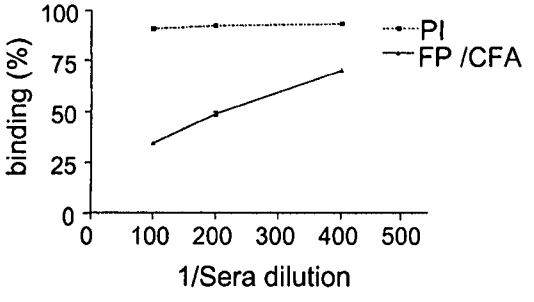

Fig. 6 TGFα/EGFR binding inhibition capacity of serum from mice immunized with TGFα-fusion protein in CFA. A preimmune serum was used as non-specific inhibition control and unlabled TGFα (5 ng/ml) was used as positive control. The ordinate represents the percent of binding of different sera dilutions. The total ^{125}I-hTGFα binding in absence of any competitor was set 100%. Each point is the mean ± SD of duplicate measurements

We also explored the obtained antibodies with respect to their capacity for functional interference with TGFα-induced EGFR signaling. For this purpose, we employed cellular systems where a role of endogenous TGFα for activation of resident EGFRs, i.e., an autocrine mechanism, had previously been shown [25–28].

A constitutive basal phosphorylation of EGFR in the A431 cell line has been described [25]. This activation could be due to the autocrine loop between endogenous TGFα and the EGFR. As shown in Fig. 7, polyclonal antibodies from mice immunized with hTGFα–P64k

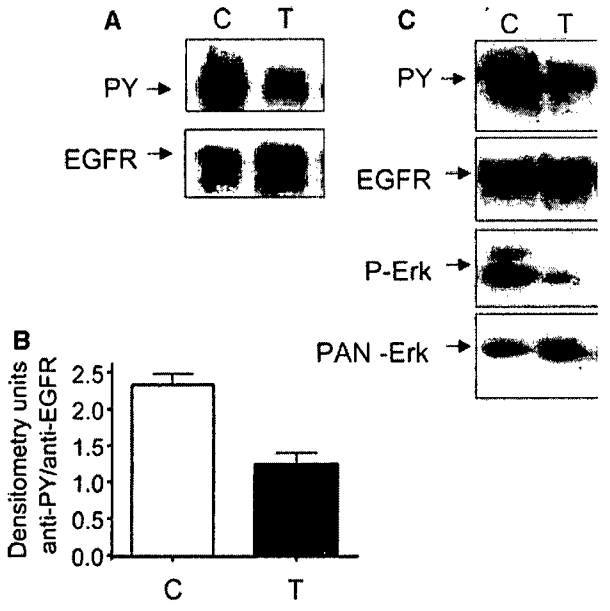

Fig. 7 Inhibition of basal EGFR signaling activity by anti-TGFα specific antibodies in A431 cells. After serum starvation, A431 cells were incubated with negative control (C) or anti-hTGFα (T) polyclonal antibodies (1/100 dilution) for 12 h and were subsequently lysed. Proteins were loaded onto a SDS-PAGE gel and transferred to a PVDF-membrane for immunoblotting. **a** Representative immunoblot showing the phosphorylation levels of EGFR and Erk1/2 kinase in cells incubated with control or anti-hTGFα antibodies and total EGFR or Erk1/2 proteins level. **b** Graphs represent the relation between the densitometry units obtained with the anti-PY or anti-pErk antibody and the same blot reprobed with anti-EGFR or PAN Erk antibody

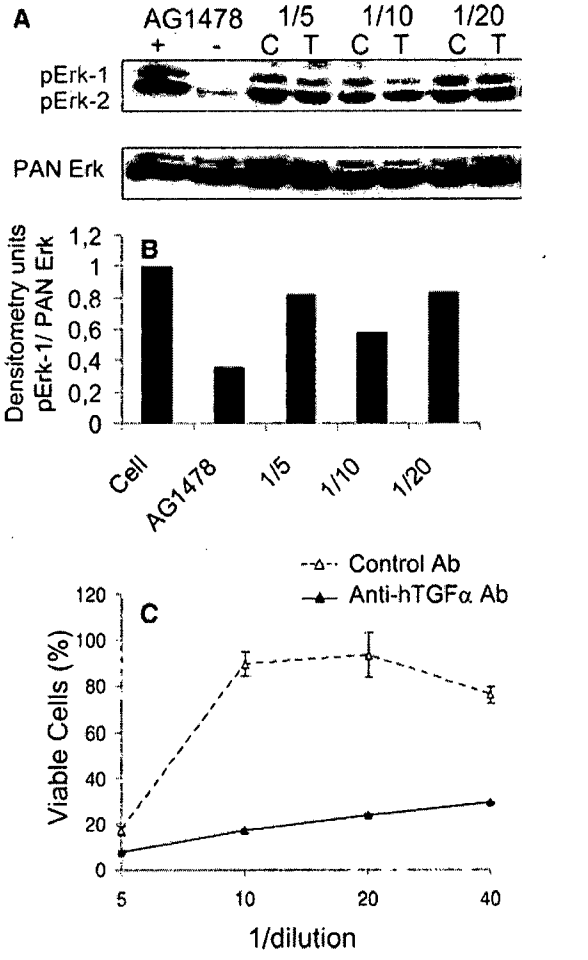

Fig. 8 Neutralizing antibodies generated by TGFα-FP vaccination inhibit the Erk signaling and growth of Caco-2 colon carcinoma cells. Serum starved Caco-2 cells were incubated with negative control (C) or anti-hTGFα (T) polyclonal antibodies (at different dilutions) for 12 h and subsequently lysed with lysis buffer. Proteins were loaded onto a SDS-PAGE gel and transferred to a PVDF-membrane for immunoblotting. **a** Representative immunoblot showing the phosphorylation levels of Erk1/2 kinases (*top*) in cells incubated with control or anti-hTGFα antibodies and total Erk protein level (*bottom*). **b** Relative phosphorylation levels of Erk1 in cells incubated with control or anti-hTGFα antibodies. The data represent the mean ± SD. The ordinate represents the ratio between the densitometry analysis of pErk1 and PAN Erk immunoblot, in case of antibody (T) treatment normalized to the signal with control (C) antibody at the same dilution. **c** Caco-2 cells were incubated in RPMI-FCS 1% for 24 h, negative control or anti-hTGFα polyclonal antibodies were added at different dilutions, and incubation was continued for another 24 h. Then, cell amounts were assessed by MTT assay. The ordinate represents the amount of cells relative to the amount of cells in cultures without treatment (100%). The EGFR phosphorylation inhibitor AG1478 was used as a positive control

fusion protein inhibited the basal EGFR phosphorylation as compared with control polyclonal antibodies. Also an inhibition of constitutive MAPK activity was detected when the cells were incubated with the specific anti-TGFα polyclonal antibodies. In Caco-2 colon cancer cells, the existence of an autocrine TGFα/EGFR loop, leading to activation of MAPK signaling, has been reported [26]. While we were unable to detect sufficient EGFR phosphorylation to use this as readout for treatment with the anti-hTGFα-specific antibodies, a robust constitutive MAPK phosphorylation was detectable. Polyclonal antibodies obtained from mice immunized with hTGFα–P64k fusion protein moderately inhibited the basal Erk1 phosphorylation in this cell line (Fig. 8). The incubation of cells with AG1478 was used as positive control and control polyclonal antibodies obtained from mice immunized only with the adjuvant as negative control. The inhibition was most pronounced at intermediate antibody dilutions, suggesting that at high antibody concentrations some component in the preparation may interfere with this assay. Consistent with the inhibition of MAPK activity, the anti-hTGFα polyclonal antibodies also blocked the growth of Caco-2 cells, as detected by MTT assays (Fig. 8c). The levels of growth inhibition were, however, more pronounced as inhibition of MAPK activity and similar to those obtained with the positive control, AG1478, an inhibitor of EGFR phosphorylation (data not shown). At high concentrations, control IgG led to a non-specific growth inhibition. Also, a tendency to inhibit the growth of the human non-small cell lung carcinoma cell line H125 by the polyclonal anti-TGFα antibodies was detectable (Fig. 9). For this cell line the effect of the specific antibodies generated by the TGFα

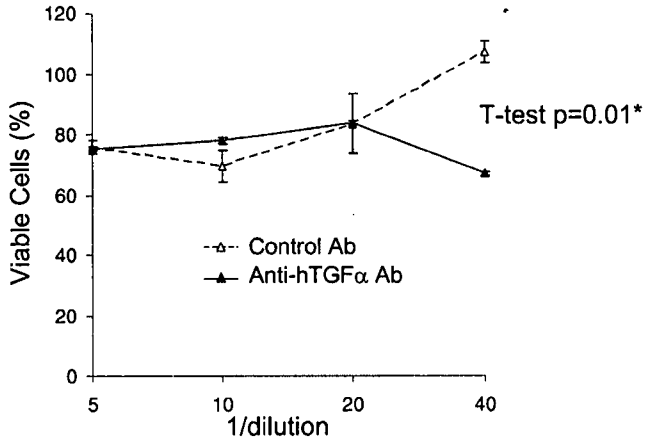

Fig. 9 Neutralizing antibodies generated by TGFα-FP vaccination inhibit the growth of H125 non-small cell lung cancer cells. H125 cells were incubated in RPMI-FCS 1% for 24 h, then negative control (C) or anti-hTGFα (T) polyclonal antibodies were added at different dilutions and incubation was continued for another 24 h. Then, cell amounts were assessed by MTT assay. The ordinate represents the amount of cells relative to the amount of cells in cultures without treatment (100%). The EGFR phosphorylation inhibitor AG1478 was used as positive control

vaccine was achieved only when applied in higher dilution compared with the control antibody ($P=0.01$, t test). These results suggest again that there may be some factor in the purified antibodies that could stimulate cell growth, covering up the effect of TGFα neutralization.

Discussion

Several approaches have been assessed for their ability to interfere with the function of the erbB receptors and ligands family, a validated target for cancer therapy, which might be useful for therapeutic intervention. These approaches include the use of MAbs that block ligand binding (e.g., Imclone 225 and Theracim hR3). Other alternatives are based on the specific removal of EGFR ligands by antibodies generated by active specific immunotherapy (ASI). The eventual ligand vaccines might be less toxic than receptor antagonists and potentially more efficacious for long-term treatments.

Our present study addressed the question of whether a fusion protein containing hTGFα could raise a specific immune response in a mouse model. This animal model is relevant for this purpose due to the high homology between human and mouse TGFα (93% identity in amino acid sequence) and the fact that this molecule is not species-specific in its biological effects [2]. Because TGFα is a self-protein, a carrier protein is required to increase its immunogenicity. P64k, a recombinant protein originally isolated from *N. meningitides*, was selected as the carrier protein according to previous results [15–18], to build up a fusion protein. It is not obvious that the use of a recombinant fusion protein could be useful in order to obtain a neutralizing TGFα-specific antibody response. For this purpose the mature hTGFα cDNA was inserted into the N-terminal region of the gene coding for P64k protein. Preliminary structural studies suggested that P64k N-terminal region is quite flexible [29], providing a suitable framework for the native folding of the inserted growth factor polypeptide. hTGFα–P64k immunogen, combined with different adjuvants, was rather effective for the generation of anti-hTGFα-specific humoral responses. Montanide ISA 51 and Freund's complete adjuvant were superior to alum in terms of anti-TGFα serum antibody titers and LN-specific IgG secreting B cells from immunized mice. The same adjuvant dependence was reported for an EGF-P64k vaccine with respect to anti-EGF antibody titers [16]. Additionally anti-hTGFα vaccine-induced serum antibody levels were similar (~1/20,000) to those obtained against mEGF (60% identity in amino acid sequence with hEGF) when vaccinating mice with a preparation containing a hEGF-P64k fusion protein (unpublished observations), evidencing the efficacy of this strategy of vaccine design for poorly immunogenic growth factor peptides. Moreover a potential cross-reactive response against EGF, induced by immunization with the TGFα vaccine was discarded by ELISPOT, excluding that any possible antitumoral effect of this preparation could be attributed to EGF immune deprivation. TGFα in the described fusion protein context was immunogenic not only at an antibody response level but also TGFα-specific IFNγ-secreting Th cells were obtained with this immunogen. This result suggests that it will be possible to induce an effective autoimmunity against tumors which overexpress this growth factor using this vaccine.

Despite the widespread expression of TGFα in adult rodent tissues [2] and the vaccine-induced autoimmunity versus TGFα (serum anti-ligand antibodies, specific antibodies secreting B cells and specific IFNγ-secreting Th cells) no visible toxic effects were observed in treated mice. Besides, experiments in vaccinated pregnant mice showed no toxicity in their progeny (data not shown). This result corresponded with the reported behavior of TGFα −/− knockout mice, which were viable, generally healthy, and fertile animals [30, 31].

According to the reactivity pattern against the different hTGFα peptides obtained with all of the vaccine formulations, the most immunogenic part seems to be the C-loop–C-terminal region. This result might indicate that the disulfide bond between Cys_{34} and Cys_{43}, which forms the TGFα C-loop in the original molecule, is conserved in the fusion protein. This region contains the six key residues for the TGFα-binding to EGFR [2]. In fact, we demonstrated that the specific immune response generated by our vaccine is able to block the binding between hTGFα and its receptor in an in vitro assay using a A431 human carcinoma cell line.

Moreover, it was important to know if the specific antibodies generated by the vaccination could block the downstream signaling from the autocrine TGFα/EGFR loop. The first evidence was the decrease in the EGFR basal signaling activation in the A431 epidermoid cancer

cell and Caco-2 colon cancer cell line. This last result was in correspondence with the result obtained by the group of Tarnawski in the demonstration of the EGFR transactivation by prostaglandin E_2. In this report, the inhibition of Erk phophorylation induced by prostaglandin E_2 by the addition of TGFα neutralizing antibodies in the Caco-2 cell line was demonstrated [26]. Taken together, our results suggest that our vaccination would be effective in the generation of anti-TGFα neutralizing antibodies that could block the EGFR signaling cascade. In concordance with this result, the incubation of Caco-2 cells with anti-hTGFα-specific antibodies resulted in a remarkable decrease in the growth. Also, some inhibitory effects were seen in the human non-small cell lung carcinoma cell line H125 which also depends on the TGFα/EGFR autocrine loop for growth [28, 29]. Further experiments are required analyze the effects of the anti-hTGFα antibodies in these human tumor cell lines in more detail.

TGFα is biosynthesized as a membrane-bound precursor protein (pro-TFGα) that undergoes sequential endoproteolytic cleavage to release a soluble form of the factor [2]. The transmembrane form is biologically active and can interact with extracellular proteins through a juxtacrine pathway and can function as a cell–cell adhesion molecule that regulates migration and colonization of specific organs during metastasis [32, 33]. Also, a more aggressive truncated form of this transmembrane factor (TGFα precursor which underwent only the first cleavage at the N-terminus) has been detected in membranes of tumor cell lines grown to high cell density, including A431 [34]. This tethered pro-TGFα precursor showed an enhanced capacity to activate EGFR and it has been associated with the growth advantage of some tumor cells [35]. In this regard we show that the specific antibodies generated by our vaccine recognize the TGFα tethered precursor and could potentially also block the action of this molecule in tumor growth. The vaccine formulations containing our TGFα-fusion protein and oily adjuvant are able to raise a Th1 response. This was observed at the level of IgG isotype pattern and by analyzing the cytokine response. These results were expected for alum and CFA adjuvants (classic Th2 inductor [15] and Th1 adjuvant [36], respectively), but unexpected for IFA, which for protein antigens rather promotes a more Th2-like immune response [37, 38]. One possible explanation for this fact could be that the P64k protein (obtained from *N. meningitidis*) would be recognized by some receptor of the innate immune system. Other proteins from microorganisms such as P40 of Klebsiella *pneumoniae* have been used to elicit specific CTL response to antigenic peptides [38]. Specific T cells to p64K and hTGFα, generated by vaccine formulations containing oily adjuvant, secreted IFN-γ and not IL-4, similar to the cytokine profile obtained with VSSP as adjuvant. VSSP has been reported as a potent stimulator of dendritic cells to polarize the immune response to a Th1 pattern [22]. Although a Th1-like response is not strictly necessary for an anti-tumoral mechanism based on antibody-mediated TGFα deprivation, it could be beneficial for any possible cytotoxic effect. Besides, other antitumoral effects of IFN-γ produced by either CD4+ or CD8+ T cells have been described by Blankenstein and Qin [39]. This cytokine acts on non-hematopoietic tumor stroma cells and, either directly or indirectly, induces angiostasis. This effect prevents rapid tumor burden outgrowth and allows residual tumor cells to be eliminated. In some models, IFN-γ also contributes to the destruction of existing tumor blood vessels.

The induction of a hormone-specific immune castration by autoantibodies generated by vaccination has been used at the clinical setting for chorionic gonadotropin to control fertility [40] and also in the treatment of hormone-dependent cancers such as colorectal [41] and prostate [42] carcinomas. This concept has been extended to EGF in patients with NSCLC with encouraging results [17, 18]. Hitherto, TGFα has only been used to target a toxin to a tumor site in clinical trials [43].

EGF and TGFα have distinct influence in tumor biology [4]. Accordingly, it makes sense to develop a cancer vaccine strategy targeting TGFα, which eventually could be effective against tumors that develop resistance to the EGF vaccine. Moreover, even a better approach could be to combine both EGFR ligand vaccines. Pre-clinical experiments to address these questions are currently ongoing in our lab.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

Radicación No. 03-48135
Clasificación Internacional (IPC): A61K 39/00, 38/18, C12P 21/02, C12N 1/12.
Concepto Técnico No. 987

Patente de Invención	■	Patente de Modelo de Utilidad	☐	
Vía Nacional	☐			
Vía PCT (fase nacional)	■	Cap.I	☐	Cap. II ■

No. Sol. Internacional PCT7CU01700011
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

Descripción:	Folios: 9 a 56	como se radicó inicialmente
Reivindicaciones:	Folios: 75	como se radicó inicialmente
	Folio: 150	como se radicó el 05/10/09
	Folio: 162	como se radicó el 10/02/2010
Dibujos:	Folios: 87 a 91	como se radicó inicialmente
Prioridad:	Folios: 1 Fecha: 12 de Junio de 2000 País: CU Número: 286/2000	
Respuesta del solicitante:	Folios: 156 a 159	como se radicó el 10/02/2010
Otros:	Folios: 163 a 173	como se radicó el 10/02/2010

2. Modificaciones (artículo 34 D.486 y art. 22 Tratado PCT)

Se aceptan las modificaciones realizadas por el solicitante toda vez que no amplían el objeto inicial de la solicitud.

3. Objeto de la invención

- Título de la solicitud (folio 1): "Composición vacunal que comprende factor de crecimiento transformante alfa".

- La presente solicitud se refiere a una composición vacunal capaz de provocar una respuesta contra TGFα autólogo, caracterizada porque comprende como principio activo una proteína de fusión entre el TGFα autologo y cualquier péptido derivado del mismo y la proteína transportadora P64K de Neisseria meningitidis, en combinación con un adyuvante.

- El problema planteado en esta solicitud es la necesidad de composiciones antigénicas para la inmunoterapia activa de cáncer.

- Para solucionar este problema técnico la solicitud en estudio proporciona un preparado vacunal que contiene TGFα humano o cualquier derivado del mismo, o una combinación de éste con otro ligando del DGF-R, EGF.

- La Clasificación Internacional de Patentes (IPC) es A61K 39/00, A61K 38/18, C12P 21/02, C12N 1/12, A61K 38/18.

4. De la solicitud de Patente

4.1. Reivindicaciones *(artículo 30 D 486)*

Habiéndose reconocido la claridad y el efecto de la composición en el anterior punto, se encuentra que el capítulo reivindicatorio define, es claro y conciso y además está lo suficientemente sustentado por la descripción. Por lo anterior, las reivindicaciones sí cumplen con el art. 30 de la Decisión 486.

5. Determinación del Estado de la Técnica

- La fecha que se ha tenido en cuenta para determinar el estado de la técnica es: 12 de Junio de 2000

- Consultadas las bases de datos con que cuenta la oficina y las otras fuentes utilizadas para determinar el estado de la técnica y habiéndose subsanado las objeciones realizadas en el anterior concepto técnico 3548, no se encontró documento alguno que afecte el objeto de la solicitud.

6. Análisis de patentabilidad (artículo 14 D486)

6.1. Novedad (artículo 16 D. 486)

- La reivindicación 1 y sus dependientes divulgan una composición una composición vacunal caracterizada porque comprende como principio activo una molécula compuesta por factor de crecimiento transformante Alfa humano recombinante (TGFα) y otro péptido seleccionado del grupo consistente en, un péptido derivado del TGF-α o un ligando del receptor del factor de crecimiento epidérmico y donde dicho conjugado está enlazado a una proteína transportadora, adicionalmente la composición vacunal comprende un adyuvante

Según el numeral 5, no se encontró dentro del estado de la técnica, documento alguno que evidenciara la existencia de la invención para el momento de la presentación prioritaria.

Por tal razón las reivindicaciones 1 a 8 folio(s) 162 son novedosas según lo estipulado en el artículo 16 de la Decisión 486.

6.2. Nivel Inventivo (artículo 18 D486)

Las reivindicaciones estudiadas corresponden del numeral 1 a 8 contenidas el folio 162.

Habiéndose superado las objeciones al capítulo reivindicatorio, habiéndose definido correctamente la composición de la vacuna, ésta comprende el TGFα, otro péptido seleccionado del grupo consistente en, un péptido derivado del TGF-α o un ligando del receptor del factor de crecimiento epidérmico y dicho conjugado está enlazado a una proteína transportadora que adicionalmente comprende un adyuvante. Se reconoce entonces que no presentaba obviedad para un técnico medio versado en la materia el que dicha combinación pudiera estar presente en una misma vacuna, y que tuviera la relevancia y el efecto que demuestra en la presente solicitud.

Por consiguiente se concluye que la presente solicitud tiene nivel inventivo de acuerdo a lo estipulado en el art. 18 de la Decisión 486 de la Comisión de la Comunidad Andina.

6.3. Aplicación industrial *(artículo19 D 486)*

Las reivindicaciones estudiadas corresponden de la número 1 a 8 contenidas en el folio 162.

La presente invención sí tiene Aplicación Industrial ya que su objeto puede ser aplicado en la industria de farmacéuticos

7. Conclusión

En consecuencia y por todo lo expuesto anteriormente, se recomienda **Conceder** las reivindicaciones: 1 a 8 (folios: 162) con el título **"Composición vacunal que comprende factor de crecimiento transformante alfa".**

Bogotá, D.C.,

CONCEPTO TECNICO No. 987	**EXAMINADOR TECNICO** Código No. 202089