

Specific immune response induced in mice by immunization with the human Epidermal Growth Factor Receptor extracellular domain

✉ Belinda Sánchez¹, Yeranddy Aguiar¹, Greta Garrido¹, Ailem Rabasa¹, Diana R Fernández¹, Rolando Pérez¹, Luis E Fernández¹

¹Vaccine Department, Center of Molecular Immunology, CIM
216 St and 15th Ave, Atabey Siboney, Playa, PO Box 16040, Havana, Cuba
E-mail: belinda@cim.sld.cu

RESEARCH

ABSTRACT

Epidermal Growth Factor Receptor (EGFR or HER1) is considered a tumor associated antigen. HER1 is overexpressed in many human epithelial tumors and involved in crucial cellular functions for tumor development. For this reason HER1 is considered a good target for cancer immunotherapy and some passive agents are being evaluated in clinical trials. However, HER1-based active immunotherapy has not been clinically explored. To develop an active immunotherapy approach based on HER1 for the treatment of HER1+ cancer patients, we cloned and expressed in mammalian cells the HER1 extracellular domain protein (HER1-ECD), which was specifically recognized by an anti-EGFR monoclonal antibody. HER1-ECD was purified by affinity chromatography and adjuvated in Very Small Size Proteoliposomes (VSSP) or Complete Freund adjuvant to immunize C57BL/6 mice. Immunization elicited specific humoral and cellular immune response. The polyclonal antibodies induced by immunization recognized the full length HER1 on the surface of a lung tumor cell line. These results suggest that HER1-ECD could be an appropriated antigen to induce specific immune response in patients with HER1+ tumors.

Keywords: HER1, immunization

Biotecnología Aplicada 2008;25:135-140

RESUMEN

Inducción de respuesta inmune específica contra el dominio extracelular del receptor del Factor de Crecimiento Epidérmico humano mediante inmunización en ratones. El Receptor del Factor del Crecimiento Epidérmico (EGFR o HER1) es considerado un antígeno asociado a los tumores. HER1 se encuentra sobreexpresado en muchos tumores humanos de origen epitelial y está implicado en funciones cruciales para el desarrollo de los mismos. Por estas razones HER1 es considerado un blanco apropiado para la inmunoterapia del cáncer y algunos agentes pasivos anti-HER1 están siendo evaluados en ensayos clínicos. Sin embargo, la inmunoterapia activa basada en el HER1 no ha sido evaluada en la clínica. Con el objetivo de desarrollar una terapia activa específica basada en el HER1, se clonó y expresó en células de mamíferos el dominio extracelular de HER1, que fue específicamente reconocido por un anticuerpos anti-EGFR. La proteína recombinante purificada por cromatografía de afinidad, se adyuvó en proteoliposomas de muy baja talla (VSSP) o Adyuvante Completo de Freund para inmunizar ratones C57BL/6. La inmunización indujo el desarrollo de una respuesta inmune específica de tipo celular y humoral. Los anticuerpos policlonales inducidos por la inmunización reconocieron específicamente al HER1 de talla completa en la superficie de una línea de carcinoma de pulmón humano HER1+. Estos resultados sugieren que el dominio extracelular del HER1 pudiera ser un antígeno adecuado para generar respuesta inmunes específicas en los pacientes portadores de tumores HER1+.

Palabras clave: HER1, inmunización

Introduction

Cancer cells have many distinctive properties in which the Epidermal Growth Factor Receptor (EGFR or HER1) is involved. These include the capacity of autocrine growth stimulation, apoptosis evasion, unlimited replication, angiogenesis promotion, and capacity for tissue invasion and metastasis [1]. HER1 is overexpressed in many human epithelial tumors, such as lung [2], breast [3], ovary [4], colon [5], head and neck [6], prostate [7, 8], bladder [9], and pancreatic [10] tumors. This overexpression has been associated in clinical studies with bad prognosis of the disease in bladder, cervical, ovarian and esophageal cancers, and with reduced survival rates in breast, gastric and colorectal cancers [11]. The apparent association of HER1 with poor patient outcome may also reflect its role in the promotion of tumor invasion and metastasis [12]. Besides, much evidence suggests that activation of

HER1 may promote resistance to the conventional cytotoxic therapies [13]. For these reasons, HER1 is considered a tumor associated antigen, and consequently, a good target for cancer immunotherapy.

HER1 belongs to a type I tyrosine kinase family, named HER1 family, which includes four transmembrane growth factor receptors: HER1 [14], HER2/neu, HER3 and HER4 [15]. These receptors are structurally related, and each of them is composed of an extracellular domain (ECD) and a conserved cytoplasm signal transduction domain, separated by a hydrophobic membrane-spanning region. The ECD is the ligand binding domain, and is less conserved among the four receptors of the family, suggesting that they have different specificities in ligand binding. The more relevant HER1 ligands for tumor growth are the EGF [16] and the transforming growth factor α (TGF α) [17]. This ECD

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.
2. Hendler FJ, Ozanne BW. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J Clin Invest* 1984;74:647-51.
3. Perez R, Pascual M, Macias A, Lage A. Epidermal growth factor receptors in human breast cancer. *Breast Cancer Res Treat* 1984;4:189-93.
4. Gullick WJ, Marsden JJ, Whittle N, Ward B, Bobrow L, Waterfield MD. Expression of epidermal growth factor receptors on human cervical, ovarian, and vulvar carcinomas. *Cancer Res* 1986;46:285-92.
5. Lockhart C, Berlin JD. The epidermal growth factor receptor as a target for colorectal cancer therapy. *Semin Oncol* 2005;32:52-60.

region includes four subdomains. The subdomains I and III, contain the binding site, and subdomain II is responsible for receptor dimerization. Binding of the ligands to the HER1-ECD results in receptor homo- or hetero-dimerization, tyrosine kinase activation and initiation of signaling cascades [18, 19].

HER1 targeting immunotherapies have been limited to passive therapy with monoclonal antibodies (MAB) and small tyrosine kinase inhibitors (TKI). Some of these anti-HER1 agents have produced good clinical results. Cetuximab is a chimeric IgG1 MAB that has been approved by the US Food and Drug Administration (FDA), either as monotherapy or in combination with irinotecan, for the treatment of advanced colorectal carcinoma with detectable HER1 expression [20]. Another anti-HER1 MAB with relevant clinical results is the hR3/Nimotuzumab MAB (hR3), which has been approved combined with chemotherapy for the treatment of advanced head and neck tumors by the Cuban Center for State Control of Drug Quality (CECMED) [21]. These MABs recognize the ligand binding domain, and inhibit HER1 phosphorylation. In contrast to MABs, HER1-TKI exerts its activity intracellularly at the level of tyrosine kinase phosphorylation. The best characterized anti-HER1 TKI is gefitinib. This agent has entered phase II/III clinical testing in prostate, breast, bladder and renal cell carcinoma [22]. In contrast to passive therapy, anti-HER1 active immunotherapy has not been clinically tested, and only limited to preclinical studies [23, 24]. We have recently reported that vaccination of mice with the ECD of autologous EGFR overcomes the tolerance to the self EGFR and has an anti-metastatic effect on an EGFR+ tumor [25]. These results suggest that the immunization of cancer patients with the ECD of human HER1 could result in a good clinical outcome. This paper reports the cloning of the gene encoding HER1-ECD in a mammalian expression vector. The recombinant protein was expressed in HEK293 cells and purified by affinity chromatography. Mice were then immunized with HER1-ECD adjuvanted in Complete Freund Adjuvant (CFA) or in Very Small Size Proteoliposomes (VSSP). Immunization induced specific T-cell stimulation and antibodies which recognize the full length HER1 in a human tumor cell line.

Material and methods

HER1-ECD cloning and sequencing

DNA encoding HER1-ECD was amplified by PCR using the EGFRΔ533/pRK5 plasmid (kindly provided by Dr. Axel Ullrich, from Max-Planck Institute for Biochemistry, Germany) as template. The sense primer 5' GGGGTACCCTTCGGGGAGCAGCGATGC-GA-3' includes a KpnI excision site (underlined), the initiation codon ATG and 3 bp from the signal sequence of HER1. The anti-sense primer, 5'-GCTCTAGATC-AGGACGGGATCTTAGGCCCA-3' is complementary to bp 2103-2123 in the 3' region, and contains a stop codon (double underline) and an XbaI excision site (single underline). The PCR product, a 1.9-kb fragment, was cloned into KpnI/XbaI sites of the pcDNA 3-expression vector (Invitrogen, San Diego, USA), generating the HER1-ECD/pcDNA3 plasmid.

HER1-ECD sequences were confirmed by dideoxy nucleotide sequencing analysis to be identical to those previously reported [26, 27]. All enzymes were supplied by Boehringer-Mannheim, Germany.

Cell lines

Human embryonic kidney (HEK293, ATCC CRL-1573), human lung adenocarcinoma H125 [28], U1906 [29] and H661 [30] cell lines were grown in DMEM (Gibco, USA) supplemented with 10% fetal calf serum (FCS) (Hyclone, US), 2mM L-glutamine, 1mM sodium pyruvate, penicillin 100 U/mL / and streptomycin 100 mg/mL (Life Technologies, USA).

Transfection

HEK293 cells were grown in 6-well plates (1.75×10^5 cell/mL) and 8 hours later they were transfected with 4 mg of the HER1-ECD/pcDNA3 plasmid using the calcium phosphate transfection procedure. Plates were incubated overnight at 3% CO₂, and transferred to 5% CO₂, 16 hours later. Transfected cells were grown in a selective medium (containing 1 mg/mL of G418; Geneticin, Sigma, USA) starting 48 hours after transfection for the generation of HER1-ECD/HEK293 and HER1-ECD/HEK293 stable cell lines. The mock transfection with the pcDNA3 vector was used as a negative control.

HEK293 transfectants were grown in a SFM4-CHO serum-free medium (Hyclone, USA) supplemented with 4 mM glutamine, 1 g/L Pluronic F68, and 26 mM of NaHCO₃.

Immunoprecipitation

The supernatant from the HER1-ECD/HEK293 transfectant cultures (2 mL) was mixed with 1 mg of hR3 MAB (specific for HER1-extracellular domain, CIM, Cuba) and 20 ml of Protein A-Sepharose (Amersham-Pharmacia Biotech, Uppsala, Sweden). Samples were gently shaken overnight at 4°C and then centrifuged 1 min at 11 000 g. The precipitated recombinant protein was separated on 7.5% SDS-PAGE and visualized by silver staining.

Purification

Recombinant HER1-ECD was purified from confluent cultures of the transfectant agent by affinity chromatography using EAH-Sepharose 4B (Amersham Pharmacia Biotech, USA) covalently coupled to hR3 MAB. Equilibration and washing steps were performed with PBS/NaCl 1M pH 7.0 and protein elution with Glycine 0.2 M pH 2.8. Purity was assessed by densitometry, using a personal densitometer SI (Amersham Pharmacia Biotech, USA) and Imag Quant Software. Protein concentration was assayed by Lowry's method.

Western blot

Purified HER1-ECD protein (15 µg) was applied into 7.5% SDS-PAGE gels and transferred to the Hybond-ECL Nitrocellulose membrane (Amersham Pharmacia Biotech, USA, UK). Membranes were blocked with NEG buffer (0.15 M NaCl, 5 mM EDTA, 500 mM Tris-HCl (pH 7.5), 0.02% Tween 20, 0.04% Gelatin) and incubated with 10 µg/mL of hR3 MAB. The pro-

6. Dassonville O, Formento JL, Francoual M, Ramaoli 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.

7. Zhou 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.

8. 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.

9. 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.

10. 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.

11. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer* 2001;37(4):S9-15.

12. Wells A. Tumor invasion: role of growth factor-induced cell motility. *Adv Cancer Res* 2000;78:31-101.

13. Holbro T, Civenni G, Hynes NE. The ErbB receptors and their role in cancer progression. *Exp Cell Res* 2003;284:99-110.

14. Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, *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.

15. Normanno N, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: an overview. *Curr Drug Targets* 2005;6:243-57.

16. Gregory H. Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature* 1975;257:325-7.

17. Salomon DS, Kim N, Saeki T, Ciardiello F. Transforming growth factor-α: an oncogene/developmental growth factor. *Cancer Cells* 1990;2:389-97.

18. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW, Ward CW. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol Cell* 2003;11:495-505.

19. Boerner JL, Danielsen A, Mähle NJ. Ligand-independent oncogenic signaling by the epidermal growth factor receptor: v-ErbB as a paradigm. *Exp Cell Res* 2003;284:111-21.

20. Systems-ImClone, Erbitux (Cetuximab). US Prescribing Information. ImClone System, 2004.

tein content was visualized using horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch laboratories Inc., USA) followed by Chemiluminescent Substrate (Amersham Pharmacia Biotech, USA).

Enzyme immunoassay for testing hR3 MAb recognition

Microtiter plates (High binding, Costar, USA) were coated with 5 µg or 10 µg of 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 hR3 MAb at different concentrations added into the plate and incubated 1 hour at 37°C. Alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma, USA) was added and incubated 1 hour at 37°C. After the addition of P nitrophenylphosphate (1 mg/mL) (Sigma, USA) the Optical Density (OD) was measured at 405 nm using a micro well system reader (Organon Teknica Inc., Salzburg, Austria). All washes were made with PBS/Tween-20. ELISA test background was two times the OD at 405 nm for PBS.

Mice and immunization protocols

Female C57BL/6 mice, aged 8-12 weeks old, were purchased from the National Center for Laboratory Animals Production (CENPALAB, 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, Havana, Cuba).

Mice (n = 10) were immunized four times biweekly with 50 µg of HER1-ECD in Freund Adjuvant (FA; complete for the first immunization and incomplete for the rest) (HER1-ECD/FA) or in VSSP adjuvant [31, 32], in water/oil (Montanide ISA 51, Seppic, France) emulsion (HER1-ECD/VSSP). The routes of vaccine administration were subcutaneous (sc) or intramuscular (im) respectively. Sera samples were obtained on days 0, 21, 35, and 56.

T-cell proliferation assay

Inguinal lymph nodes (LN) were harvested from mice immunized with 50 mg of HER1 ECD adjuvanted in VSSP or FA. Single cell suspensions were plated at 5 x 10⁵ cells/well in flat-bottomed 96-well microculture plates (TPP, Switzerland), using DMEM plus 4% FCS. Cells were cultured in triplicate with 50, 75 and 100 µg of HER1-ECD, at 37°C in a 5% CO₂ incubator. On the fourth day of the culture, wells were pulsed with 1mCi [³H]thymidine for 18 hr. Plates were harvested and the incorporated [³H]thymidine was measured using a scintillation counter. Results were expressed as stimulation index (S.I).

DTH test

Mice were immunized three times with 50 µg of HER1-ECD adjuvanted in VSSP or FA as described above. Seven days after the last immunization mice were sensitized by intradermal injection with 50 µg of HER1-ECD in 50 µL of PBS in the right hind foot pad and the same volume of PBS in the left foot pad. After 48 hours foot swelling was measured using a plethysmometer (Ugo Basile, Italy). Mice inoculated

with PBS in VSSP and sensitized with HER1-ECD in PBS were used as negative control. Differences in DTH between treated and control groups were translated into differences in displaced liquid volume and were statistically validated by Kruskal Wallis and Dunn's Multiple Comparison test.

Enzyme immunoassay for testing biological activity

Microtiter plates (High binding, Costar, USA) were coated with 10 mg/mL of 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 pre-immune sera (as negative control) were added and incubated 1 hour at 37°C. Alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma, USA) was added and incubated 1 hour at 37°C. After the addition of P nitrophenylphosphate (1 mg/mL) (Sigma, US) the Optical Density (OD) was measured at 405 nm using a micro well system reader (Organon Teknica Inc., 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 kinetics. ELISA test background was two times the OD at 405 nm of pre-immune sera.

RT-PCR

Total RNA from H125, U1906 and H661 cell lines was extracted using the RNAsy minikit (QIAGEN, Germany) and one microgram of RNA was reverse transcribed using QIAGEN reverse transcription kit (QIAGEN, Germany) in accordance with the manufacturer's protocols. To amplify cDNAs, 1 µg of the reverse-transcribed cDNA was subjected to PCR in 50 µL of PCR buffer [10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, and 2.5 mM MgCl₂] containing 0.8 mM deoxynucleotide triphosphates, 0.5 mM of each set of primers, and 2.5 units of Taq DNA polymerase (QIAGEN, Germany). The specific primers for HER1 gene amplification were: sense 5'-CTGAGGAC-3' and antisense 5'-C GACCCTTA-3'. The specific primers for HER2 and β-actin gene amplification (used as control of the experiment) were designed from published sequences [33]. After PCR amplification, 10 µL of the RT-PCR products were separated by electrophoresis on 1.5% agarose gels and visualized with Ethidium bromide.

FACS Analysis

Cells were stained with sera from immunized mice (1/200 dilution) or 5 µg/mL of hR3 MAb (Center of Molecular Immunology, Cuba), or 5 µg/mL of anti HER2 MAb (Oncogene, US), followed by FITC-goat anti-mouse IgG (Jackson, Immunoresearch laboratories Inc, USA). Up to 10 000 cells were acquired using a FACScan flow cytometer and analyzed using the CellQuest software (Beckton Dickinson, San Jose, CA, US).

Results

HER1-ECD cloning and expression

PCR amplified DNA encoding HER1-ECD was cloned into pcDNA3 expression vector, and HEK293 cells

21. Crombet T, Osorio M, Cruz T, Roca C, del Castillo R, Mon R, Iznaga-Escobar N, Figueredo R, Koropatnick J, Rengifo E, Fernandez E, Alvarez D, *et al.* Use of the humanized anti-epidermal growth factor receptor monoclonal antibody h-R3 in combination with radiotherapy in the treatment of locally advanced head and neck cancer patients. *J Clin Oncol* 2004; 22:1646-54.

22. Ferry D, Hammond L, Rason M, *et al.* Phase Intermittent oral ZD1839 (Iressa), a novel epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), shows evidence of good tolerability and activity: Final results from a phase I study. *Proc Am Soc Clin Oncol* 2000;19.

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

24. 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.

25. Ramirez BS, Pestana ES, Hidalgo GG, Garcia TH, Rodriguez RP, Ullrich A, Fernandez LE. Active antitumor immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant. *Int J Cancer* 2006;119:2190-9.

26. Downward J, Yarden Y, Mayes E, Scarse 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.

27. 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 isolation of human and mouse alternative EGFR transcripts encoding truncated receptor isoforms. *Genomics* 2001; 71:1-20.

28. 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.

29. Bergh J, Nilsson K, Ekman R, Giovanna B. Establishment and characterization of cell lines from human small cell and large cell carcinomas of the lung. *Acta Pathol Microbiol Immunol Scand [A]* 1985;93:133-47.

30. Katabi MM, Chan HL, Karp SE, Batist G. Hexokinase type II: a novel tumor-specific promoter for gene-targeted therapy differentially expressed and regulated in human cancer cells. *Hum Gene Ther* 1999;10:155-64.

were transfected with the resulting plasmid. Protein expression by stable HEK293 transfectants was evaluated by immunoprecipitation with the hR3 MAb, which is specific for HER1. Mock transfection was used as a negative control. A protein band of approximately 105 kDa corresponding with the expected size of HER1-ECD was visualized in SDS PAGE (Figure 1).

HER1-ECD purification

HEK293 transfectant was grown in an SFM4-CHO serum free medium, and the supernatant was used to purify the soluble HER1-ECD recombinant protein. The purification was made by affinity chromatography using EAH sepharose covalently coupled to hR3 MAb. The purity after one step of purification was 98% (Figure 2a), determined by densitometry.

HER1-ECD identity was determined by western blot by using hR3 MAb, as shown in figure 2b. Besides, we confirmed the specificity of that recognition by ELISA, using different concentrations of hR3 MAb (Figure 2c).

Immune response induced by immunization with HER1-ECD

C57BL/6 mice were immunized four times biweekly with the HER1-ECD in FA or the VSSP adjuvant, which has been tested in humans. To evaluate the immunogenicity of HER1-ECD adjuvanted in VSSP, the stimulation of T cells was measured *in vitro*. For this purpose, LN cells from immunized mice were isolated and stimulated *in vitro* with different concentrations of HER1-ECD purified protein. T cells from immunized mice with HER1-ECD/VSSP had a significant stimulation index (S.I) compared with control mice, reaching up to 9.53 S.I (Mann Whitney U test, $p < 0.05$) (Figure 3a). The specificity of the lymphocyte stimulation was verified using EGF as an irrelevant stimulation antigen (Figure 3b).

The activation *in vivo* of a cellular immune response was measured by the DTH test. Mice immunized with HER1 ECD in VSSP or FA were sensitized at the foot pad with the HER1-ECD protein, and swelling was

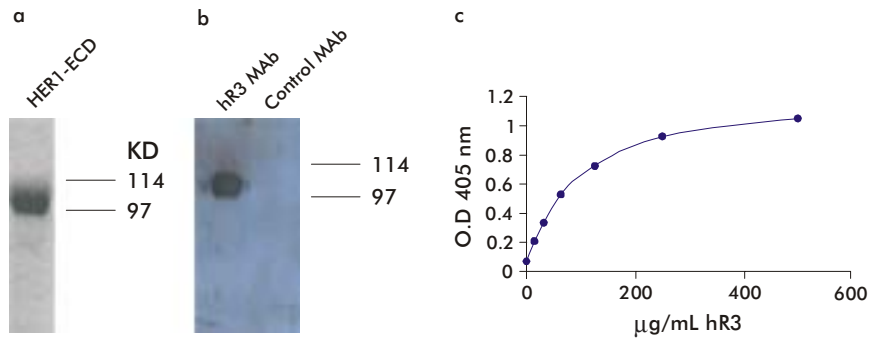


Figure 2. Purification and identity of HER1-ECD. HER1-ECD recombinant protein was purified by affinity chromatography using hR3 MAb covalently coupled to EAH sepharose. The purity was determined by SDS-PAGE (a). The identity of HER1 ECD was verified by Western Blot with the hR3 MAb. The negative control used was an irrelevant IgG1 MAb (b). The specificity of HER1-ECD recognition by the hR3 MAb was tested by ELISA, using different concentrations of hR3 MAb (c).

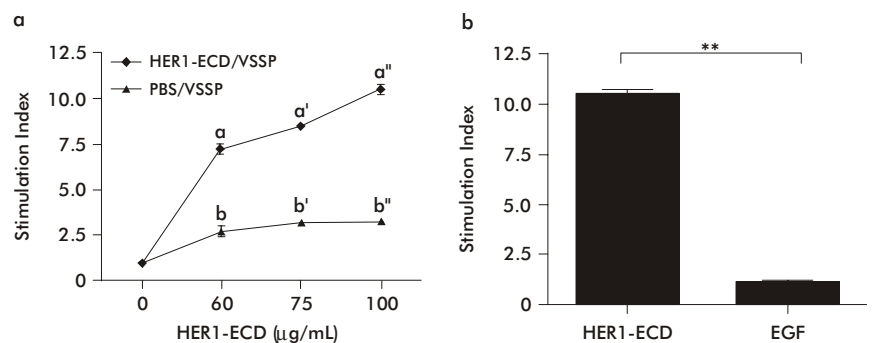


Figure 3. *In vitro* proliferation of HER1-ECD stimulated lymphocytes. C57BL/6 mice were immunized four times biweekly with 50 µg of HER1-ECD adjuvanted in VSSP. Lymph-nodes were extracted and stimulated *in vitro* for four days with the indicated concentrations of HER1-ECD. Each value represents the mean ± SD. Different letters indicate statistical significance using the Mann Whitney U test ($p < 0,05$) (a). Lymphocytes proliferation specificity was evaluated by stimulation with human EGF as the irrelevant antigen. (**) indicates statistical significance using the Mann Whitney U test ($p < 0.001$) (b). Proliferation was measured by [3H]-Thymidin incorporation. Similar results were obtained in two independent experiments.

measured after 48 hours. Animals immunized with HER1-ECD in VSSP and FA showed inflammation 30 times higher than the negative control group ($p < 0.05$, Dunn's multiple comparison test) (Figure 4).

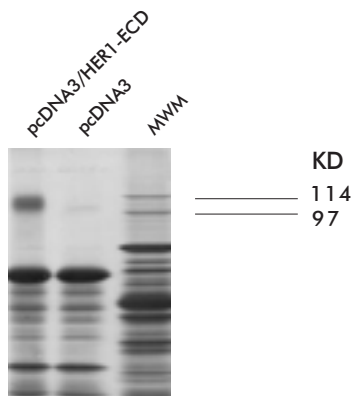


Figure 1. Expression of HER1-ECD by HEK293 transfectant. The expression of HER1-ECD in the HEK293 transfectant was verified by immunoprecipitation with hR3 MAb, from transfectant culture supernatants. The arrow indicates the band corresponding to HER1-ECD. A mock transfectant culture supernatant was used as the negative control.

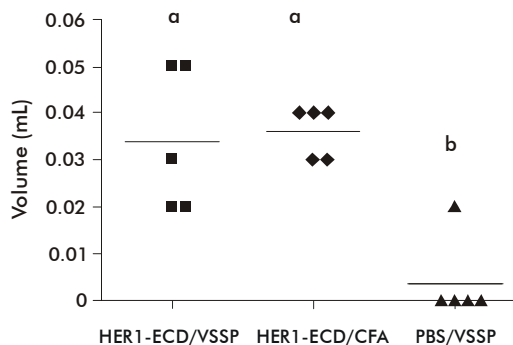


Figure 4. Specific DTH induced by immunization with HER1-ECD. DTH response induced by immunization with HER1-ECD was assayed by immunizing C57BL/6 mice three times with 50 mg of HER1-ECD adjuvanted in VSSP or FA. Mice were sensitized on the foot pad seven days later with the HER1-ECD in PBS. Different letters indicate statistical significance using Dunn's Multiple Comparison test, $p < 0.05$. A representative experiment of the two independent studies is shown.

To evaluate the humoral induced immune response, sera from mice immunized four times biweekly were tested on day 56. All immunized mice developed high serum IgG polyclonal antibody (PAb) levels against HER1-ECD using both VSSP and the FA adjuvant, as determined by ELISA (Table 1).

Specific polyclonal antibodies recognize full length HER1 in cancer cells, but not HER2

To determine whether polyclonal antibodies (PAb) induced by immunization with a truncated HER1 could bind a full length receptor on the cell surface, three lung carcinoma cell lines were characterized in terms of HER1 and HER2 (homologous members of the EGFR family) expression. All tested cell lines (H125, U1906 and H661) expressed HER1 and HER2 at the mRNA level (Figure 5a). However, while all cells showed HER2 expression by FACS, only H125 cells expressed HER1 on the membrane (Figure 5b).

PAb induced in mice by the immunization with HER1-ECD adjuvanted in VSSP were evaluated by FACS for their capacity to bind the above mentioned cell lines. As shown in figure 6, induced PAb recognized the HER1+ H125 cell lines but did not bind to U1906 which was selected as negative control of HER1 expression.

Discussion

HER1 is a well-characterized tumor associated antigen. It has been widely explored as a target for cancer immunotherapy, but these studies have been limited to a passive immunotherapy with MABs [21] and TKI [22, 34]. Active immunotherapy based on HER1 would need to circumvent the tolerance to the self protein, but previous studies where mice were immunized with murine EGFR-ECD and generated an immune response with antimetastatic effect over an EGFR+ tumor [25], pointed out the potential effectiveness of a vaccine based on autologous HER1.

The present study was conducted to further explore the efficacy of active immunotherapy based on human HER1-ECD in the adequate adjuvant to generate a specific immune response in mice. DNA encoding HER1 ECD was cloned into a pcDNA3 mammalian expression vector and HEK293 human cells were successfully transfected. The HER1-ECD recombinant protein expression was verified by immunoprecipitation using hR3 MAB and was then purified from the culture supernatant by affinity chromatography. The high purity protein identity was checked by using hR3 MAB.

The HER1-ECD protein was mixed with the VSSP adjuvant and then emulsified in Montanide ISA 51. VSSP was the selected adjuvant due to its capacity to promote a pro-inflammatory context and avoid the tolerance to poorly immunogenic proteins. VSSP/Montanide ISA 51 has been clinically tested [35] without showing high toxicity. The HER1-ECD/VSSP vaccine preparation elicited specific cellular and humoral immune response in immunized mice. Lymph node cells from immunized mice were stimulated *in vitro* in a concentration depending manner with HER1-ECD. CD4+ T cell proliferation could be induced by the inflammatory context provided by VSSP in the formulation. The in-

Table 1. Frequency of responder animals and IgG titers* of C57BL/6 animals on day 56 after 4 immunizations with HER1-ECD/VSSP or HER1-ECD/FA

Treatment groups	Frequency	1: IgG titer*						
		5000	10 000	20 000	40 000	80 000	160 000	320 000
HER1-ECD in FA	10/10		1	1	2	3	2	1
HER1-ECD in vssp	10/10	1		2	3	1	1	2

*Measured by ELISA.

duction of specific cellular immune response was confirmed by the DTH test. Mice immunized with HER1-ECD/VSSP and then sensitized with the same antigen developed inflammation, indicating that T CD4+ cells were activated.

The induction of specific humoral immune response was also evaluated in C57BL/6 mice. FA, even when it can not be used in humans due its high toxicity, was included in our studies as a reference TH1 adjuvant. Both HER1-ECD/VSSP and HER1 ECD/FA vaccine formulations elicited specific IgG antibodies in immunized mice, with titers reaching 1/320 000 after four inoculations. The induction of high antibody titers were expected from our previous studies immunizing mice with the murine EGFR-ECD in VSSP which generated very high specific IgG response [25]. Varying results have been published by other authors when HER2 has been used as the immunization antigen. Vaccination of monkeys with HER2-ECD in Detox [36], a potent adjuvant [37], induced no more than 1/

31. 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.

32. Mesa C, De Leon J, Ringley 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.

33. Thomas DG, Giordano TJ, Sanders D, Biermann JS, Baker L. Absence of HER2/neu gene expression in osteosarcoma and skeletal Ewing's sarcoma. *Clin Cancer Res* 2002;8:788-93.

34. Sirotnak 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.

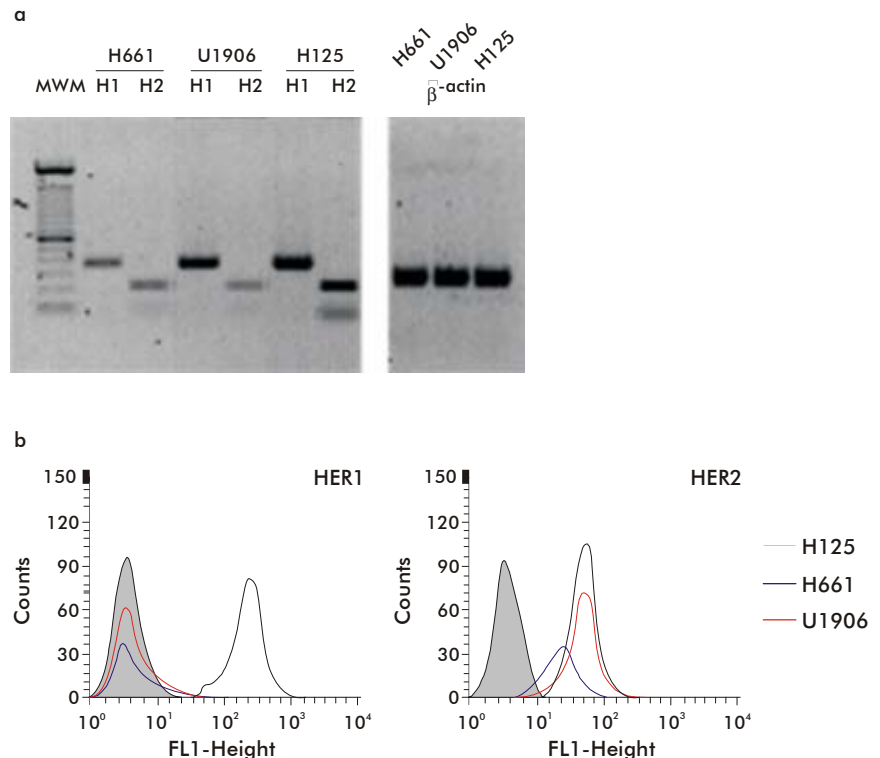


Figure 5. HER1 and HER2 expression in lung carcinoma cell lines. The expression of HER1 and HER2 at the mRNA level was assayed by RT-PCR using specific oligonucleotides and visualized in agarose gels. The positive control measured was the β-actine expression (a). The expressions of HER1 and HER2 proteins in tumor cells were assayed by FACS using the hR3 MAB (for HER1 expression) and the anti-HER2 MAB (for HER2 expression) (b).

10 000 specific IgG titers, indicating that HER2 could be less immunogenic than HER1.

The immunization with the truncated HER1-ECD protein did not affect the recognition of the PAb of the full length protein on the tumor cell surface. Specificity for HER1 was also demonstrated as shown in the FACS experiments with human tumor cell lines. Using FACS the anti-HER1 PAb only recognized the H125 cell line, which is HER1+/HER2+, but did not recognize the U1906 cell line, which is HER1-/HER2+.

In summary, the present study demonstrates that a vaccine composition containing HER1-ECD adjuvanted in VSSP/Montanide ISA 51 is able to induce specific cellular and humoral response in mice. These results, together with the previously demonstrated anti-metastatic effect of autologous EGFR-ECD vaccination in mice, suggest that this novel therapeutic approach could be used to target EGFR+ tumors in patients.

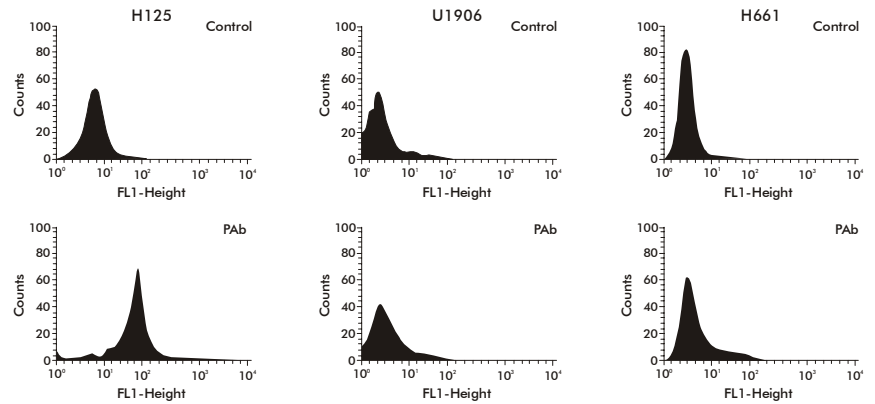


Figure 6. Polyclonal antibodies induced by HER1-ECD immunization recognize full length HER1 but not HER2. Polyclonal antibodies from mice immunized with HER1-ECD adjuvanted in VSSP, were assayed by FACS for the recognition of full length HER1 in the HER1+/HER2+ H125 cell line and HER1-/HER2+ U1906 and H661 cell lines.

35. Guthmann MD, Bitton RJ, Carnero AJ, Gabri MR, Cinat G, Koliren L, Lewi D, Fernandez LE, Alonso DF, Gomez DE, Fainboim L. Active specific immunotherapy of melanoma with a GM3 ganglioside-based vaccine: a report on safety and immunogenicity. *J Immunother* 2004;27:442-51.

36. Fendly BM, KC, 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 Research* 1993;2:129-39.

37. Schultz N, Oratz R, Chen D, Zeleniuch-Jacquotte A, Abeles G, Bystryn JC. Effect of DETOX as an adjuvant for melanoma vaccine. *Vaccine* 1995;13:503-8.

Received in September, 2007. Accepted for publication in June, 2008.