Vaccine 28 (2010) 162–170

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# HER-2/*neu* (657-665) represents an immunogenic epitope of HER-2/*neu* oncoprotein with potent antitumor properties

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ARTICLE INFO

*Article history:*
Received 22 July 2009
Received in revised form
10 September 2009
Accepted 22 September 2009
Available online 30 September 2009

*Keywords:*
Vaccination
Epitope
HLA-A2.1
Tumor immunity

ABSTRACT

The HER-2/*neu* oncoprotein is a promising cancer vaccine target. We describe herein a novel HLA-A2.1-restricted epitope, encompassing amino acids 657-665 (AVVGILLVV), which is naturally presented by human breast and ovarian cell lines. HER-2/*neu*(657-665), [HER-2($9_{657}$)], binds with high affinity to HLA-A2.1 molecules as revealed by a prediction algorithm (SYFPEITHI) and in functional assays. This peptide was found to be immunogenic in HLA-A2.1 transgenic (HHD) mice inducing peptide-specific CTL, which responded with increased IFNγ production, degranulation, and *in vitro* as well as *in vivo* cytotoxicity. Most important, HER-2($9_{657}$) functioned as a therapeutic vaccine by enabling HHD mice to reject established transplantable tumors. Cured mice resisted tumor growth when re-challenged with the same tumor, demonstrating the capacity of HER-2($9_{657}$) to generate tumor-specific memory immune response. Finally, this peptide was also found to be immunogenic in PBMCs from HLA-A2.1$^+$ patients with HER-2/*neu*$^+$ breast cancer. Our data encourage further exploitation of HER-2($9_{657}$) as a promising candidate for peptide-based cancer vaccines.



## 1. Introduction

Tumor cells express a range of antigens that can be recognized by CTLs. HER-2/*neu* oncoprotein is a promising target as it has been demonstrated to be immunogenic *in vivo* inducing specific CTL and IgG in a portion of patients with HER-2/*neu* positive breast cancer [1]. Moreover, tumor-reactive CTL and T helper responses have been induced *in vitro* using various MHC class I- and class II-binding peptides from the HER-2/*neu* sequence [1,2]. However, synthetic peptides representing HER-2/*neu* immunogenic epitopes have largely failed to function as therapeutic vaccines in phase I/II trials [1]. These disappointing results, which are not unique for HER-2/*neu* but have been observed also with peptide vaccines derived from a variety of other tumor antigens [3], should be attributed to the potent tolerizing conditions, generated by bulky tumors emerging during advanced stages of cancer, both in the periphery and within the tumor microenvironment [3–5], limiting immunotherapy. Therefore, efforts are now underway to circumvent this by combining vaccines with modalities aiming at reducing immunosuppression [6–8] and by vaccinating at the early stages of disease where tumor load is low enough to establish a strong immunosuppressive network [3,9,10]. However, in parallel with studies aiming at improving the effectiveness of vaccines, it is essential to identify novel immunogenic CTL epitopes in order to provide new tools for the design of more efficacious peptide-based treatment modalities.

We describe here an immunogenic nonamer encompassing amino acids 657 to 665 (AVVGILLVV) of HER-2/*neu*, and reveal several features of the immune response to this epitope both *in vitro* and *in vivo*. Our data clearly show capacity of HER-2($9_{657}$) to act as a potent cancer vaccine.

## 2. Materials and methods

### 2.1. Mice

HHD mice are $\beta2m^{-/-}$, $Db^{-/-}$, and express a HLA-A*0201 monochain composed of a chimeric H chain ($\alpha1$ and $\alpha2$ domains of HLA-A*0201 and the $\alpha3$ intracellular domain of $D^b$) linked by its $NH_2$ terminus to the COOH terminus of the human $\beta_2m$ [11]. HHD mice were provided by Prof. François Lemonnier (Unite d'Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France). All mice were maintained in pathogen-free conditions in the animal facilities of

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doi:10.1016/j.vaccine.2009.09.104

our center; all protocols were reviewed by the St. Savas Cancer Hospital competent authority for compliance with the Greek and European regulations on Animal Welfare and with Public Health Service recommendations.

### 2.2. Cell lines

The HER-2/*neu* overexpressing ovarian cancer cell line SKOV3 (donated by C. G. Ioannides, Department of Gynecologic Oncology and Immunology, University of Texas, Austin, TX and M.D. Anderson Cancer Center, Houston, TX) was maintained in culture in αMEM medium (Biochrom) supplemented with 10% FCS (Biochrom). The HER-2/*neu* overexpressing breast cancer cell line SKBR3 was purchased from the American Type Culture Collection and was also grown in αMEM medium. The murine ALC lymphoma cell line was provided by Prof. R. Kiessling (Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden). This cell line was grown *in vivo* as ascites by serial passages in C57BL/6 syngeneic mice. HLA-A2.1 transfectants of SKOV3 (SKOV3.A2), SKBR3 (SKBR3.A2) and ALC (ALC.A2.1), and human HER-2/*neu* transfectants of ALC (ALC.HER) and ALC.A2.1 (ALC.A2.1.HER) were produced as recently described [12].

### 2.3. Epitope prediction

This epitope was identified in Fridman et al. (manuscript in preparation) using a proprietary software [WO2006/124406] and confirmed by analysis with SYFPEITHI algorithm [13]. In brief, potential HLA-A*0201 ligands from the sequence of HER-2/*neu* were isolated using a matrix pattern suitable for calculations of peptides fitting to the HLA-A*0201 motif. Such motif predictions are available at www.syfpeithi.de.

### 2.4. Peptide synthesis

The nonamer HER-2/*neu* (657-665) (AVVGILLVV) [HER-2($9_{657}$)] and control peptides HER-2/*neu* (85-94) (LIAHNQVRQV) [HER-2($10_{85}$)], HER-2(369-377) (KIFGSLAFL) [HER-2($9_{369}$)] and gp100-derived peptide gp(154-162) (KTWGQYWQV) [gp($9_{154}$)] were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer using Fmoc-chemistry and analyzed by mass spectrometry. Peptides were >95% pure as analyzed by reverse-phase HPLC. The influenza flu matrix peptide (aa 58–66; flu (58–66)) was purchased from EZBiolab.

### 2.5. T2 binding assay

HER-2($9_{657}$) was tested for dose-dependent binding to T2 cells in a HLA-A*0201 stabilization assay, as described recently [13]. In brief, T2 cells were incubated overnight in serum-free RPMI 1640 (Biochrom) in the presence of 100 ng/ml β2-microglobulin (Sigma–Aldrich) without (background immunofluorescence) or with peptide over a range of concentrations from 1 nmol/L to 10 μmol/L. Stability of HLA-A*0201 was assayed by flow cytometry after staining the cells with the BB7.2 mAb and FITC-conjugated anti-mouse IgG (DakoCytomation). Results are expressed as fold of increase of mean fluorescence intensities (MFI) in the presence of peptide relative to MFI without peptide.

### 2.6. Measurement of peptide/HLA-A*0201 complex stability

The method for this has been reported recently [13]. In brief, T2 cells were incubated overnight at 37 °C without peptide or with 1 μmol/L of peptide in serum-free RPMI 1640 (Biochrom) supplemented with β2-microglobulin at 100 ng/ml. Next, cells were incubated with brefeldin A (10 μg/ml; Sigma–Aldrich) for 1 h, washed, and further incubated for 0 h, 2 h, 4 h, and 6 h. Subsequently, cells were stained with BB7.2 mAb followed by staining with a FITC-conjugated anti-mouse IgG (DakoCytomation). MFI measured at 0 h was considered as 100%. MFI measured at all other time-points are expressed relative to MFI at 0 h and calculated as follows: [MFI (0 h) – MFI (2 h, 4 h, or 6 h)/MFI (0 h)] × 100.

### 2.7. Preparation of dendritic cells

Dendritic cells (DCs) were generated from murine bone marrow (BM) cells as previously described [14] with some modifications. In brief, BM cells were flushed from the long bones of HHD mice and depleted of red cells with ammonium chloride. BM cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, $5 \times 10^{-5}$ M 2-ME and 10 mM HEPES (thereafter referred to as complete medium (CM)). 20 ng/ml of recombinant murine (rm) GM-CSF and 20 ng/ml of rmIL-4 (R&D Systems) were added to CM. On day 0, $3 \times 10^6$ cells were seeded per well in 6-well plates (Costar) in 3 ml CM/well and incubated at 37 °C in 5% $CO_2$. On days 2 and 4, floating cells were gently removed, and fresh CM supplemented with rmGM-CSF and rmIL-4 was added to the cells. On day 6, culture supernatants were aspirated and replaced with fresh CM supplemented with 100 U/ml TNFα (Sigma Chemicals). Purity of $CD11c^+$ cells was >90%, as detected with PE-conjugated anti-CD11c (HL3) mAb (Pharmingen) by direct immunofluorescence.

### 2.8. In vivo generation of HER-2($9_{657}$)-specific CTL

*In vivo* generation of HER-2($9_{657}$)-specific CTL was tested in HHD mice by applying a protocol recently published by us [12,13]. Briefly, mice were injected thrice with HER-2($9_{657}$) peptide (100 μg per injection emulsified in 200 μl incomplete Freund's adjuvant s.c. at the basis of the tail) every 5 days (i.e., days 0, 5 and 10). During this period CpG (5'-TCCATGACGTTCCTGACGTT-3', PTO backbone, MWG, Ebersberg, Germany), at 50 μg in 300 μl PBS, was given i.p. five times every other day. In parallel performed experiments, CpG was proven to act similarly with GM-CSF (which was used in our recent reports [12,13]) as an adjuvant for the generation of vaccine-specific CTL *in vivo* (data not shown). One day after the last injection splenic $CD8^+$ T cells from the immunized mice were used as effectors/responders in assays.

### 2.9. In vitro generation of HER-2($9_{657}$)-specific CTL

This was performed as recently described [13]. Briefly, $CD4^+CD25^+T_{reg}$ cell-depleted PBMC from HER-2/$neu^+$, HLA-$A^*0201^+$ breast cancer patients were used for the generation of HER-2($9_{657}$)-specific CTL. $CD4^+CD25^+$ $T_{reg}$ cells were depleted from PBMC before cell culture in a two-step procedure using anti-CD25-PE mAb (clone 2A3) (BD Biosciences) and goat-anti-mouse IgG microbeads (Miltenyi Biotec).The $T_{reg}$ cell-depleted PBMC were cultured, in parallel with autologous total PBMC, in the presence of 10 μg/ml of HER-2($9_{657}$) in RPMI 1640 culture medium supplemented with 10% FCS, antibiotics, 10 IU/ml IL 2 (IL-2; Chiron), and 5 ng/ml of IL-7 (Immunotools). Cells were plated at $2 \times 10^6$ cells/ml in 96-well U-bottom plates (Costar/Corning) and placed in an incubator with 5% $CO_2$ and humidified atmosphere. Weekly restimulations were performed with irradiated autologous PBMC pulsed with HER-2($9_{657}$) at a ratio of 5:1 CTL to autologous irradiated (3000 rads) PBMC. Every other day, fresh IL-2 was added at 10 IU/ml. At 5–7 days after the last restimulation, cells were harvested, washed and used as effectors in cytotoxicity assays.