Vaccination with Human HER-2/neu (435-443) CTL Peptide Induces Effective Antitumor Immunity against HER-2/neu-Expressing Tumor Cells In vivo

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Abstract
HER-2/neu is a self-antigen expressed by tumors and nonmalignant epithelial tissues. The possibility of self-tolerance to HER-2/neu-derived epitopes has raised questions concerning their utility in antitumor immunotherapy. Altered HER-2/neu peptide ligands capable of eliciting enhanced immunity to tumor-associated HER-2/neu epitopes may circumvent this problem. The human CTL peptide HER-2/neu (435-443) [hHER-2(9435)] represents a xenogeneic altered peptide ligand of its mouse homologue, differing by one amino acid residue at position 4. In contrast to mHER-2(9435), vaccination of HLA-A*0201 transgenic (HHD) mice with hHER-2(9435) significantly increased the frequency of mHER-2(9435)-specific CTL and also induced strong protective and therapeutic immunity against the transplantable ALC tumor cell line transfected to coexpress HLA-A*0201 and hHER-2/neu or rHER-2/neu. Similar results were also obtained with wild-type C57BL/6 mice inoculated with HER-2/neu transfectants of ALC. Adoptive transfer of CD8+ CTL from mice immunized with hHER-2(9435) efficiently protected naive syngeneic mice inoculated with ALC tumors. In conclusion, our results show that HER-2(9435) serves as a tumor rejection molecule. They also propose a novel approach for generating enhanced immunity against a self-HER-2/neu CTL epitope by vaccinating with xenogeneic altered peptide ligands and provide useful insights for the design of improved peptide-based vaccines for the treatment of patients with HER-2/neu-overexpressing tumors. (Cancer Res 2006; 66(10): 5452-60)

Introduction
CTLs recognize antigenic peptides, 8 to 12 amino acids long, presented to them by antigen-presenting cells in the context of MHC class I molecules (1). CTL peptides have been identified in several proteins, including those expressed by tumor cells (2). CTL sensitized in vitro against tumor-associated peptides have been shown to lyse cancer cell lines expressing both the appropriate MHC class I allele and the relevant peptide (3, 4).

HER-2/neu is a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity that has a similarity in structure and sequence to the epidermal growth factor receptor (5). It is reportedly overexpressed, mostly via gene amplification, in a large proportion of aggressive cancers (6, 7). The HER-2/neu protein seems to be an ideal tumor-associated antigen for immunotherapy, because CTL responses specific for MHC class I epitopes have been observed in some cancer patients (3, 8). Moreover, tumor-reactive CTL and helper T-cell responses have been induced in vitro using various MHC class I and II binding synthetic peptides derived from the HER-2/neu sequence (9–17).

The HER-2/neu epitope spanning amino acids 435 to 443 [HER-2(9435)] has been shown by us (17) and others (12) to bind with high affinity to HLA-A2.1 molecules and to elicit CTLs from tumor-associated lymphocytes of patients with ovarian cancer. We were also able to show increased frequencies of CTL precursors specific for HER-2(9435) among peripheral blood mononuclear cells (PBMC) from patients with HER-2/neu+ tumors (13). PBMC from those patients showed specific lysis of autologous tumor cells, suggesting that HER-2(9435) is naturally processed and expressed by HER-2/neu+ carcinomas. Thus far, no HER-2(9435)-based vaccination studies have been reported in preclinical or clinical models.

As most tumor-associated antigens are self-antigens, the antigen-specific CTL repertoires may be significantly reduced during the processes of negative selection, leaving a T-cell repertoire that is poorly effective at mounting a productive antitumor response. Efforts to use unmodified forms of peptide immunogens to elicit antitumor responses in vivo have been largely unsuccessful, leading investigators to modify naturally occurring tumor antigens. Indeed, several peptide analogues capable of eliciting enhanced immunity to tumor-associated epitopes have been developed (18–20). In the present study, we have tested the capacity of hHER-2(9435), which differs from its murine homologue by one amino acid substitution at position 4, to function both as a protective and as a therapeutic vaccine in HHD mice inoculated with transplantable ALC tumor cells. We have cotransfected ALC with HLA-A2.1 cDNA and hHER-2/neu cDNA (ALC.A2.1.hHER) to enable expression of hHER-2(9435) epitope. Cotransfection of ALC was also done with a rHER-2/neu cDNA (ALC.A2.1.rHER) because (a) rHER-2/neu differs from mHER-2/neu in <6% of the amino residues (21) and (b) rHER-2(9435) and mHER-2(9435) nonamers have identical sequences (http://ncbi.nlm.nih.gov/BLAST). hHER-2(9435) and mHER-2(9435) are predicted to bind to HLA-A2.1 or H-2Kb molecules with equivalent efficacy [see ref. 22 and SYFPEITHI (http://www.uni-tuebingen.de/uni.kki/database.html)]. Therefore, we also tested the potency of these two peptide vaccines in C57BL/6 mice inoculated with HER-2/neu (human or rat) single transfectants of ALC (ALC.rHER and ALC.hHER, respectively). We found that both HHD and C57BL/6 mice developed significant antitumor responses when prophylactically immunized with hHER-2(9435). Moreover, treatment with hHER-2(9435) of established syngeneic ALC.A2.1.rHER tumors showed an impressive therapeutic effect. Finally, CD8+ T cells from mice immunized...
with hHER-2(9 435) when adoptively transferred in naive syngeneic recipients highly protected them against the growth of ALC transfectants. Our data provide the basis for clinical use of xenogeneic HER-2/neu CTL epitopes in vaccination protocols.

Materials and Methods

Animals. HHD mice are \( \beta_{2m}{^-}{^{-}} \cdot D^{b}{^-}{^-} \) and express a HLA-A*0201 monochain composed of a chimeric heavy chain (\( a1 \) and \( a2 \) domains of HLA-A*0201 and the \( a3 \) intracellular domain of \( D^{b} \) linked by its NH\(_2\) terminus to the COOH terminus of the human \( \beta_{2m} \) (23). These mice were generated by crossing HHD transgenic C57BL/6sxSJL mice with H-2b^-/-\( \beta_{2m}{^-}{^-} \cdot C57BL/6 \) double-knockout mice. HHD mice were kindly provided by Prof. Francois Lemmonnier (Unite d’Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France).

All mice were maintained in pathogen-free conditions in the animal facilities of 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.

Synthetic peptides. The mHER-2(9 435) (ILHDGAYSL) and hHER-2(9 435) (ILHNGAYSL) were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer and analyzed by mass spectrometry. The same procedure was also applied for the human gp100-derived peptide hgp(9 154), (ILHNGAYSL) were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer and analyzed by mass spectrometry. The same synthetic peptides.

Adoptive cellular therapy model. HHD or C57BL/6 mice were immunized with peptides as described above. One day after the end of the immunization protocol, CD8\(^+\) T cells were isolated from total immune splenocytes by negative selection using the CD8\(^+\) -cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Freshly isolated CD8\(^+\) HHD splenocytes were expanded in vitro, as described below, tested for specificity in cytotoxicity assays, and then given i.p. (4 \( \times \) 10\(^5\) cells in 0.5 mL PBS) in syngeneic mice carrying palpable ALC tumors transfected with rHER-2/neu. Tumor growth was monitored as described above. Each group consisted of five mice and experiments were done twice.

CTL expansion procedures. For adoptive transfer experiments, CTLs from immunized animals were expanded in cultures following a method described by Riddell et al. (24) with some modifications. A total of 1 \( \times \) 10\(^5\) CD8\(^+\) cells were resuspended in 25 mL complete medium with 25 \( \times \) 10\(^5\) peptide-pulsed, syngeneic, irradiated (3,000 rads) splenocytes. One day after initiating the cultures, 100 \( \mu\)L recombinant interleukin (IL)-2 (20 U/mL; Chiron BV, Amsterdam, the Netherlands) was added to the medium. Usually on days 10 to 12, cultures were split because T-cell concentration reached numbers >1.5 \( \times \) 10\(^5\)/mL. After this time point, cultures were restimulated with IL-2 and 30 ng/mL anti-CD3 mAb (CD3\( \varepsilon \)-chain, clone 145-2C11; BD Pharmingen, San Diego, CA) to achieve higher expansion. On average, \( -4 \times 10^5 \) to \( 6 \times 10^5 \) CTL were obtained by days 15 to 18. Before transferring into syngeneic HHD mice, CTLs were tested for specificity against various appropriate tumor targets or peptide-pulsed T2 targets.

Cytotoxicity assay. The cytotoxicity assay was done as described previously (25). Briefly, effector CTLs (1 \( \times \) 10\(^5\)/mL) in 100 mL medium were placed in 96-well V-bottomed plates (Costar, Cambridge, MA). As targets, tumor cell lines were labeled with sodium chromate (Radiochemical Centre, Amersham, the Netherlands; 100-200 \( \mu\)Ci isotopes per 1 \( \times \) 10\(^5\) to 2 \( \times \) 10\(^5\) target cells) and added to effectors at the indicated E:T ratios. For peptide recognition, T2 cells were incubated overnight at 26°C together with 20 \( \mu\)g/mL peptide, washed, and then labeled. Incubation was done for 6 hours in CO\(_2\) incubators. Supernatants (100 mL) were collected from each well (all determinations done in triplicates) and radioactivity was measured in a gamma counter (Packard, Downers Grove, IL). The percentage of cytotoxicity was calculated according to the formula: % lysis = 100 \( \times \) (test 51Cr release – spontaneous 51Cr release) / (maximum 51Cr release – spontaneous 51Cr release). Cytotoxicity values were considered to indicate significant recognition of a target when the differences between mean values (from triplicate analyses) for % lysis of the particular target (e.g., pulsed T2 cells or transfected tumor cell lines) and unloaded T2 cells (or nontransfected tumor cell lines) were \( >10\% \) at an E:T ratio of 20:1 and statistically significant (\( p < 0.05 \)). Wherever indicated, mAb were added throughout the 6 hours incubation period (at 10 \( \mu\)g/mL final concentration).
T2 binding assay. Each peptide was tested for concentration-dependent binding to T2 cells in a HLA-A*0201 stabilization assay (26). T2 (TAP-)deficient cells, which have been incubated at room temperature the previous night to increase cell-surface MHC class I molecule expression, were then incubated overnight without or with each peptide over a range of peptide 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 anti-HLA-A2 mAb and phycoerythrin (PE)–conjugated anti-mouse immunoglobulins (Dako). The HLA-A*0201 strongly binding Flu peptide matrix (amino acids S8–66; EZBioLab, Inc. Westfield, NJ) was used as a positive control. Results are presented as fold of increase of mean fluorescence intensities (MFI) in the presence of peptides relative to MFI without peptides.

Measurement of peptide/HLA-A*0201 complex stability. This was done as described (27). Briefly, T2 cells were incubated overnight at 37°C without peptides or with 1 μmol/L of each peptide in serum-free RPMI 1640 supplemented with 3% -microglobulin at 100 ng/mL. Next, cells were incubated with brefeldin A (10 μg/mL; Sigma-Aldrich) for 1 hour to block cell-surface expression of newly synthesized HLA-A*0201 molecules, washed, and further incubated for 0, 2, 4, or 6 hours. Subsequently, cells were stained with mAb BB7.2 followed by anti-mouse PE. MFI measured at 0 hours was considered as 100%. MFI at all other time points are expressed relative to MFI at 0 hours and calculated as follows: [MFI (0 hour) – MFI (2, 4, or 6 hours)] / MFI (0 hour)] × 100.

ELISPOT assay. Splenocytes from every single immunized mouse were plated at 500,000 per well in quadruplicates in 96-well flat-bottomed plates. Irradiated (3,000 rads) HHD splenocytes pulsed with 500 nmol/mL mHER-2(9435) were added to the syngeneic responder splenocytes at a cell ratio of 1:1 in a total volume of 200 μL/well RPMI 1640 supplemented with 10% FBS, murine recombinant IL-7 (10 ng/mL), and murine recombinant IL-12 (100 pg/mL; both purchased from R&D Systems, Abingdon, United Kingdom). Control cultures were stimulated with mitogenic, irradiated splenocytes pulsed with 500 nmol/L hgp(9 154). Cultures were incubated at 37°C in a CO2 incubator. On day 3, 100 μL culture supernatant was replenished with fresh medium supplemented with 20 ng/mL IL-7 and 200 pg/mL IL-12. Growing microcultures were restimulated on day 7 with T2 cells pulsed with 500 nmol/L of either mHER-2(9435) or hgp(9 154) in control cultures. Twenty-four hours later, IFN-γ production was estimated using the IFNγ ELISPOT Assay kit (BD Pharmingen) according to the manufacturer's protocol. Spots were counted under a stereomicroscope (Zeiss, Jena, Germany) using the Image ProPlus software (Media Cybernetics, San Diego, CA). Specific spots were calculated by subtracting the mean number of spots obtained from the control cultures.

Statistics. Tumor sizes among the respective groups were compared with Wilcoxon's rank-sum test. Kaplan-Meier curves were plotted for survival analyses. All P values were two tailed and considered significant at P < 0.05. Significance of differences among groups in the cytotoxicity and ELISPOT assays was assessed with the two-tailed Student's t test. Statistically significant differences were considered at P < 0.05.

Results

ALC tumor cells transfected to coexpress HLA-A*0201 and hHER-2/neo or rHER-2/neo, process, and present HLA-A*0201-restricted HER-2/neo peptides. The capacity of hHER-2(9435) and mHER-2(9435) to bind to HLA-A*0201 and to form stable peptide/HLA-A*0201 complexes was tested in standard T2 binding and MHC peptide stability assays, respectively. As shown in Fig. 1A and B, both peptides exhibited a comparable strong binding and stabilization capacity.

To evaluate the ability of HLA-A*0201-restricted, HER-2/neo–derived epitopes to control tumor growth, we first cotransfected the ALC leukemic cell line (C57BL/6 origin) with the genes for the MHC class I HLA-A2.1 molecule and the full-length hHER-2/neo or rHER-2/neo. Flow cytometric evaluation of HER-2/neo and HLA-A2.1 expression on a stable double transfected (called ALC.A2.1.hHER or ALC.A2.1.rHER) maintained under neomycin selection is shown in Fig. 1C and D. HLA-A2.1 and HER-2/neo expression by the transfected ALC remained constant after 8 to 9 weeks in culture without neomycin selection (data not shown), the anticipated duration of in vivo tumor growth in naive animals.

We next examined whether the ALC expressing both HLA-A2.1 and HER-2/neo could function as a target recognized by HER-2/neo peptide-specific effectors. To address this, we used a CTL clone, which was elicited previously from an ovarian patient's T lymphocytes with pooled peptide extracts from HLA-A2.1, HER-2/neo–overexpressing autologous tumor cells (17). This particular clone was shown to specifically lyse T2 cells pulsed with peptide hHER-2(9435) as well as the HLA-A2.1 transfectant of the HER-2/neo–overexpressing SKOV3 human ovarian cell line (SKOV3.A2), which naturally expresses this epitope (17, 28).

In addition, as shown in Fig. 1E, ALC.A2.1.hHER targets, but not the wild-type ALC or the ALC.A2.1 and ALC.hHER single transfectants, were lysed by our CTL clone. Furthermore, the growth kinetics of double-transfected ALC in naive C57BL/6 and HHD mice (Fig. 2A) were similar to the HHD mice with GM-CSF, the peptide-induced antitumor effect observed in T2 loaded with hHER(9435) and ALC.A2.1.hHER, respectively. Such differences could be attributed to the one amino acid difference at position 4 between hHER-2(9435) (ILHNGAYSL) and m,HER-2(9435) (ILHDGAYSL), which may cause less effective recognition of the respective targets by our CTL clone. These data suggested that our ALC double transfectants stably expressed the HLA-A2.1-restricted human or rat CTL epitope HER-2(9435), making these cell lines suitable for evaluating the antitumor efficacy of immune responses to this antigen.

To evaluate the effect of HLA-A2.1 and hHER-2/neo or rHER-2/neo expression in ALC on its immunogenicity and tumorigenicity, we compared the growth of wild-type or single-transfected ALC and double-transfected ALC in naive C57BL/6 and HHD mice. ALC.A2.1.hHER and ALC.A2.1.rHER as well as ALC.A2.1 were rejected by normal C57BL/6 mice as a xenogeneic target consistent with the expression of HLA-A2.1 (Fig. 2A). In contrast, ALC.hHER and ALC.rHER as well as the wild-type ALC [Fig. 2A; or a mock transfectant of ALC (data not shown)] grew and killed all C57BL/6 animals. Apparently, the expression of hHER-2/neo or rHER-2/neo, both showing great homology with mHER-2/neo, was insufficient to induce any antitumor responses. Moreover, the growth kinetics of double-transfected ALC in HHD mice were identical with those of wild-type ALC (or ALC-mock; data not shown) growing in either C57BL/6 or HHD mice (Fig. 2A and B). These results showed that neither HLA-A2.1 nor HER-2/neo (or HER-2/neo peptide antigens presented by HLA-A2.1) function as effective rejection antigens in immunized HHD mice. Therefore, changes in kinetics of tumor progression on immunization with a HLA-A*0201-restricted HER-2/neo peptide should be interpreted as an antitumor response directed toward this particular peptide presented on ALC in the context of HLA-A2.1 molecules.

Active immunization with hHER-2(9435) in the presence of GM-CSF delays tumor outgrowth. We next sought to investigate whether active immunization against these HLA-A2.1-restricted peptides could augment protection against the growth of ALC double transfectants in HHD mice. No protection against ALC.A2.1.hHER was observed when HHD mice were immunized thrice with mHER-2(9435) in IFA alone. In contrast, immunization with hHER-2(9435) induced a low, although significant, effect (mice survived up to day 95 versus day 65 in the other groups; P < 0.05; Fig. 3A). By coinjecting HHD mice with GM-CSF, the peptide-induced antitumor effect...
was greatly enhanced: 50% of mice injected with hHER-2(9435) survived up to day 140, whereas in total from three independently done experiments there were 10 of 30 (33%) long-term survivors (Fig. 3B). The latter resisted rechallenge with ALC.A2.1.hHER but not with wild-type ALC or single-transfected ALC (ALC.A2.1.HHER or ALC.A2.1.rHER) after selection with neomycin. HER-2/neu expression on ALC mock transfectants (mock). HER-2/neu-specific CTL recognition of ALC.A2.1.hHER (E) or ALC.A2.1.rHER (F) was assessed using a CTL clone established from an ovarian patient’s T lymphocytes recognizing hHER-2(9435) in the context of HLA-A2.1 (17). The SKOV3.A2 cell line, naturally processing and expressing hHER-2(9435), and T2 cells, pulsed with hHER(9435) (E) or mHER(9435) (F), were used as positive controls. All other cell targets served as negative controls.

To further substantiate the observed difference in the potency of the two vaccines (i.e., hHER-2(9435) versus mHER-2(9435)), we compared ALC growth in C57BL/6 mice actively immunized with either of the peptides. Also in this model, hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from the two vaccines [i.e., hHER-2(9435) versus mHER-2(9435)], we compared ALC growth in C57BL/6 mice actively immunized with either of the peptides. Also in this model, hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435). The results from this type of experiments, which were repeated twice, showed that vaccination with hHER-2(9435) induced significantly higher protection compared with mHER-2(9435).
inoculated HHD mice with ALC.A2.1.rHER tumor cells, and once tumors were palpable (≈22-26 days after administration), peptides were injected with rat GM-CSF. As shown in Fig. 4C, when hHER-2(9,435) was given, three mice were totally cured, and in the remainders (n = 7), tumor growth was greatly delayed. A delay in tumor growth was also observed when immunizations were done with mHER-2(9,435) (Fig. 4B), which however was significantly less pronounced (P < 0.01). Nontreated mice (data not shown) or mice injected with control peptide hgp(9,154) (Fig. 4A) were sacrificed by euthanasia already by days 60 to 65, at which time point their tumors had already grown >200 mm² mean diameter.

Therapeutic immunity induced by peptide immunization is mediated by CD8⁺ CTL. To confirm that CD8⁺ CTLs were responsible for in vivo therapeutic protection against the ALC transfectants tumors, we studied the ability of adoptively transferred CD8⁺ T lymphocytes from immunized mice to cause tumor regression in syngeneic, naive hosts, carrying established tumors.

Figure 2. Expression of HLA-A2.1 and hHER-2/neu or rHER-2/neu in ALC does not induce any changes of tumor progression in unimmunized HHD mice. All animals were inoculated s.c. in the right flank with 2 × 10⁴ ALC cells either nontransfected or transfected. Tumor growth was observed regularly every 4 to 5 days. Representative of two experiments. Five mice per group were included in every experiment.

Figure 3. Active immunization with hHER-2(9,435) and GM-CSF induces strong antitumor immunity in mice. Animals (n = 10 per group) were injected as described in Materials and Methods either with peptides in IFA (A) or peptides in IFA + GM-CSF (B-E). Peptides were applied s.c. on days 0, 5, and 10, whereas GM-CSF was applied i.p. on days 2, 4, 6, 8, and 10. One day after the last injection, HHD mice were inoculated s.c. with 2 × 10⁴ ALC.A2.1.hHER (A and B) or 2 × 10⁴ ALC.A2.1.rHER (C) tumor cells. C57BL/6 mice were inoculated s.c. with 2 × 10⁴ ALC:hHER (D) or 2 × 10⁴ ALC:rHER (E) tumor cells. Representative of a total of three experiments.
CD8+ T cells were first expanded with IL-2 and anti-CD3-mAb and then tested for cytotoxicity against the transfected ALC and SKOV3 targets before adoptive transfer. Lysis of ALC.A2.lHER and SKOV3.A2 was more profound with CD8+ CTL from HHD mice immunized with hHER-2(9435) (HHDCD8-hHER435) as opposed to killing with HHDCD8-mHER435 (P < 0.001; Fig. 5A). Similarly, CD8 CTL from C57BL/6 immunized with hHER-2(9435) (C57BL/6CD8-hHER435) mice exhibited significantly increased lytic activity against ALCrHER targets compared with syngeneic C57BL/6CD8-mHER435 (P < 0.001; Fig. 5B). Cytotoxicity against the ALC.A2.lHER or SKOV3.A2 targets was abolished in the presence of anti-H-2Kb mAb, whereas lysis of ALCrHER was abolished by anti-H-2Kb mAb (data not shown).

In response to hHER-2(9435), HHD-derived CTL with profoundly higher functional activity compared with mHER-2(9435)-primed HHD mice were induced in all five HHD mice tested. Thus, HHDCD8-hHER435 lysed T2 targets coated with 10 nmol/L mHER-2(9435) at 12.4 ± 5.22%, which is not statistically different compared with 11.5 ± 5.0% lysis of T2 pulsed with 100 nmol/L of this peptide by HHDCD8-mHER435 effectors (Fig. 5C and D). The increased avidity of HHDCD8-hHER435 versus HHDCD8-mHER435 for mHER-2(9435) was evident at each peptide concentration used for coating T2 targets: 18 ± 2.96% versus 11.5 ± 5.0% (P < 0.05), 12.4 ± 5.22% versus 5.8 ± 3.9% (P < 0.005), and 1.33 ± 0.57% versus 0.46 ± 0.11% (P < 0.05) at 100, 10, and 1 nmol/L, respectively [pooled data (n = 5) from the individual results shown in Fig. 5C and D].

Pulsing of T2 cells with doses of peptide higher than 100 nmol/L (i.e., 1.000 nmol/L) was inappropriate to uncover differences in the avidity between these two CTL effectors (comparable levels of lysis were achieved; Fig. 5C and D).

When adoptively transferred, HHDCD8-hHER435 cells efficiently protected all syngeneic mice with established ALC.A2.1.rHER tumors. This therapeutic model is described in detail in Materials and Methods. Immunizations with the indicated peptides started at the time points when tumors were palpable (i.e., 22-26 days after administration). Tumor growth thereafter was observed regularly every 4 days (starting by days 32-34). Curves, individual mice.

In the present study, we report the development of a preclinical model based on human HLA-A2.1 transgenic HHD mice and a transplantable ALC tumor cell line transfected to coexpress HLA-A*0201 and hHER-2/neu or rHER-2/neu molecules. This model can be used to evaluate candidate antigens to control tumor outgrowth and immunization strategies for human use. We used this model to estimate the significance of a HLA-A2.1-restricted HER-2/neu CTL peptide epitope in active immunization studies. hHER-2(9435) is important not only for its potential use in the immunotherapy of hHER-2/neu-overexpressing tumors but also because HER-2/neu is a self-protein and thus is subjected to self-tolerance. hHER-2(9435) has been recently shown by us (13, 17, 24) and others (12) to represent a naturally processed and presented CTL peptide epitope in active immunization studies.

**Figure 4.** Therapeutic effect induced on active immunization with HER-2/neu peptides in HHD mice carrying established syngeneic ALC.A2.1.rHER tumors. This therapeutic model is described in detail in Materials and Methods. Immunizations with the indicated peptides started at the time points when tumors were palpable (i.e., 22-26 days after administration). Tumor growth thereafter was observed regularly every 4 days (starting by days 32-34). Curves, individual mice.
epitope on the surface of primary tumor cells or tumor cell lines constitutively expressing HER-2/neu, such as SKOV3.A2 and SW-626.

As a basis for studies of antitumor immunization efficacy, we created an ALC (H-2b) double transfectant. These cells express HER-2/neu and HLA-A2.1 and are efficiently lysed by a CTL clone specifically recognizing HER-2(9435) in the context of HLA-A2.1 (17, 28). The growth of ALC transfectants in HHD mice is identical with that of parental ALC in HHD or in C57BL/6 wild-type mice. These data indicate that the expression of both HLA-A2.1 and HER-2/neu (human or rat) molecules in ALC does not create significantly immunogenic antigens, which is in keeping with its generally poor immunogenicity. The advantage of this is that it allows evaluation of immune responses to specific antigens in the absence of such responses to the parental tumor. Additionally, considering the fact that (a) rHER-2/neu shows a great homology (>94%) to mHER-2/neu (21) and (b) mHER-2(9435) is identical to rHER-2(9435) in amino acid composition and sequence, it is fair to conclude that the rHER-2/neu transfectants of ALC would behave similarly to ALC transfected with a mHER-2/neu plasmid. We would thus anticipate that any results obtained on active immunization with hHER-2(9435) or mHER-2(9435) against ALC.A2.1.rHER should also be valid for ALC cells cotransfected with HLA-A2.1 and mHER-2/neu. The fast growth kinetics of ALC (inoculation of as few as 2 × 10^4 cells create palpable tumors by days 22-26) render complete control of this tumor a difficult task. In spite of this, we observed complete cures in ~30% of the mice inoculated with ALC tumor cells. Besides the percentages of long-term survivors, we interpret shifts in tumor outgrowth as indicators of successful immune-mediated intervention.

Figure 5. In vitro CTL activity of CD8+ T lymphocytes isolated from spleens of HHD (n = 5) or C57BL/6 (n = 5) mice immunized with HER-2/neu peptides and GM-CSF. Immunizations were done as described in Materials and Methods. Pooled CD8+ T cells from HHD (A) or C57BL/6 (B) mice immunized either with hHER-2(9435) or mHER-2(9435) were used as CTL effectors against ALC or SKOV3 targets at the indicated E:T ratios. C and D, efficiency of peptide vaccination by CD8+ T cells derived from HHD mice immunized with either hHER-2(9435) or mHER-2(9435). These CTL effectors were tested against T2 targets (E:T ratio of 20:1) pulsed with mHER-2(9435) at the indicated concentrations. C and D, each curve represents one individual mouse tested. A to D, mean of triplicate cultures. The SD was negligible (always <10% of the mean values) and thus omitted. E to H, in vivo antitumor activity of the indicated CTL adoptively transferred in syngeneic animals carrying established ALC tumors. E and F, HHD mice were inoculated with ALC.A2.1.rHER. G and H, C57BL/6 mice were inoculated with ALC:HER. Curves, individual mice.
In this study, we show that vaccination with mHER-2(9335) generates T cells with low avidity for this self-peptide resulting in weak immunity against ALC tumor cells expressing hHER-2/ neu or rHER-2/neu. In contrast, the human variant of this peptide is a more potent immunogen acting as a better protective and therapeutic vaccine. hHER-2(9335)-induced CTL from all HHD mice cross-recognized the mHER-2(9335) self-peptide and T2 cells pulsed with mHER-2(9335) were lysed more efficiently by HHDCD8-hHER-235 effectors compared with HHDDB-mHER-235 effectors. In functional assays, we have found that both peptides bind with similar affinities to HLA-A2.1 molecules, an observation that is in agreement with SYPPEITHI [calculates binding scores 28 and 27 for mHER-2(9335) and hHER-2(9335), respectively]. These data suggest that immunization with the hHER-2(9335) peptide plus GM-CSF generates CTL lines that recognize mHER-2(9335) with higher avidity than their mHER-2(9335) plus GM-CSF-induced counterparts. To this end, we showed that CD8+ CTL from HHD or C57BL/6 mice immunized with hHER-2(9335) plus GM-CSF induce significantly higher levels of cytotoxicity against ALC.A2.1.rHER or ALC.rHER targets, respectively, compared with mHER-2(9335)-induced CTL effectors. In agreement with the superior in vitro recognition of the transfected ALC tumors, the hHER-2(9335)-specific CTL on adoptive transfer effectively controlled tumor growth in mice. Importantly, HHD mice which rejected HLA-A2.1-rHER ALC tumors on vaccination with hHER-2(9435) became resistant to a second challenge with the same tumor cells but not to wild-type ALC or HLA-A2.1 single transfecants of ALC lacking the expression of HER-2/neu showing the capacity of the vaccine to induce HER-2/neu peptide-specific memory T cells.

Based on transgenic mouse models, it is now clear that tolerance is capable of deleting or inactivating high-avidity T cells against the transgene, thereby leading to self-tolerance (29). A typical example for this is tolerance induced to HER-2/neu in mice where the rat neu proto-oncogene is expressed in the mammary tissue under the control of the mouse mammary tumor virus promoter (30, 31). However, low-avidity self-specific T cells can be isolated from tolerant hosts (32–35) and there are reports that such cells can be activated, expanded, and involved in antitumor responses (32, 33). To this end, Lustgarten et al. (36) showed that multiple injections of dendritic cells pulsed with HER-2(9335) or HER-2(9773) in neu/2A.1 double transgenic mice stimulated low-avidity peptide-specific CD8+ CTL, which were capable of mediating antitumor activity in vivo. In agreement with this, we vaccinated our mice thrice with the HER-2/neu peptides to achieve tumor retardation. In other experimental models (37, 38), tolerance to self-melanocyte differentiation proteins tyrosinase and gp100 was successfully broken by immunization with dendritic cells pulsed with tyrosinase or gp100 peptide ligands containing conservative substitutions that were cross-reactive with the original antigens. Altered peptide ligands have been used to improve the reactivity of T cells specific for self/tumor antigens in mouse and human (18–21, 39–44). For melanocyte differentiation antigens, H-2D^d-restricted CD8+ T cells capable of recognizing B16 melanoma could only be elicited when the altered peptide was used (42). Recently, hgp100(925) that represents an altered peptide ligand form of mgp100(935) has been successfully used to induce in vivo tumor regression in combination with adoptively transferred hgp100(925)-specific T cells and IL-2 (43). The enhanced antitumor activity, induced on immunization with the hHER-2(9335) vaccine in our model, is consistent with the generation of higher numbers of murine CTLs with increased avidity for mHER-2(9335). Whether vaccination of mice with this hHER-2/neu peptide breaks tolerance to self-HER-2/neu will be definitively tested in HER-2/neu transgenic mice, which is currently under investigation in our laboratory.

An important issue for improvement of cancer immunotherapy is the identification of immunogenic tumor-associated peptide epitopes. HHD mice and the ALC.A2.1.HER tumor cell line constitute useful tools for rapid evaluation and optimization of such immunogenic epitopes in a context that would have relevance to therapy of HER-2/neu-overexpressing human cancers. In the present study, we used this preclinical model to show that repeated injections with mHER-2(9335) and GM-CSF delayed outgrowth of ALC double transfecants expressing hHER-2/neu or rHER-2/neu. Furthermore, when the same protocol is applied but using the xenogenic hHER-2(9335), which contains a single amino acid substitution at position 4, as an altered peptide ligand, we could show a considerably enhanced antitumor effect. These data suggest that vaccination of cancer patients with similar, xenogeneic tumor peptides may be considerably more effective compared with self-tumor peptides.

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References


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Control of Tumor Growth with a HER-2/neu CTL Epitope

Figure 6. mHER-2(9335)-specific CTL precursors in HHD mice nonimmunized or immunized as indicated and described in Materials and Methods. CTL precursors were measured as IFN-γ ELISpot responses of total splenocytes to T2 cells pulsed with 500 nmol/L mHER-2(9335). Closed circle, one single mouse tested (total spots from quadruplicates); solid line, mean CTL precursor frequency.


Vaccination with Human HER-2/neu (435-443) CTL Peptide Induces Effective Antitumor Immunity against HER-2/neu-Expressing Tumor Cells In vivo

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