

A Better Immune Reaction to Erbb-2 Tumors Is Elicited in Mice by DNA Vaccines Encoding Rat/Human Chimeric Proteins

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Abstract

The Erbb-2 (neu in rat and Her-2 in humans) tyrosine kinase receptor is an oncoantigen (i.e., a tumor-associated molecule directly involved in cancer progression). Because oncoantigens are self-tolerated molecules, to trigger a response circumventing tolerance, we generated two plasmids (RHuT and HuRT) coding for chimeric neu-Her-2 extracellular and transmembrane proteins that are expressed on the cell membrane of the transfected cells and recognized by monoclonal antibodies reacting against neu and Her-2. RHuT encodes a protein in which the 410 NH₂-terminal residues are from the neu extracellular domain and the remaining residues from Her-2. Almost symmetrically, HuRT encodes for a protein in which the 390 NH₂-terminal residues are from Her-2 and the remainder from neu. The ability of RHuT and HuRT to elicit a protective response to neu and Her-2 in wild-type mice and in transgenic mice tolerant to neu and Her-2 proteins was compared with that of plasmids coding for the fully rat or fully human extracellular and transmembrane domains of the Erbb-2 receptor. In most cases, RHuT and HuRT elicited a stronger response, although this chimeric benefit is markedly modulated by the location of the heterologous moiety in the protein coded by the plasmid, the immune tolerance of the responding mouse, and the kind of Erbb-2 orthologue on the targeted tumor. *Cancer Res*; 70(7): 2604–12. ©2010 AACR.

Introduction

Erbb-2 is an ideal oncoantigen (i.e., a tumor-associated molecule with a causal role in cancer progression; ref. 1). It is overexpressed by several carcinomas with a more aggressive course, whereas its expression is low or absent in normal adult tissues (2, 3). Its expression on the cell membrane means it can be targeted by antibodies (3) and cell-mediated immunity (4, 5). By binding to it, antibodies directly inhibit the signaling pathway of an oncoantigen so as to impair the progression of transformed cells (6, 7). Indirect reactions, such as antibody-dependent cell and complement-mediated cytotoxicity, also have a major role in preventing the onset of a tumor and controlling its expansion (8, 9), whereas antibodies facilitate oncoantigen presentation by antigen-presenting cells (APC; ref. 10).

However, oncoantigens are self-tolerated molecules and triggering of a response to them has to circumvent central (11) and peripheral (12) tolerance mechanisms. Vaccination with altered forms of the antigen, xenogeneic protein that share a significant homology with the self-antigen and antigen mimicry by anti-idiotypic antibodies and peptides is an effective method of overcoming peripheral tolerance (13–16). B cells reacting with the self-epitopes endocytose the xenogeneic antigen, present its peptides on class II glycoproteins of the MHC, and activate helper T cells. These provide signals to B cells and trigger the production of high-affinity antibodies reacting with self-epitopes (17, 18). These antibodies are instrumental for the activation of a stronger T-cell reaction against self-oncoantigens (10), whereas the release of helper cytokines by T cells activated by not-self peptides could rescue bystander anergic T and B lymphocytes (19) and lead to the activation of dendritic cells (20). In addition, the foreign epitopes of an orthologue protein may lead to both heteroclitic CD8⁺ (21) and CD4⁺ (22) ligands with an enhanced ability to bind to MHC glycoproteins and effectively prime T cells able to react against the original nonmutated peptide.

Because the rat and the human extracellular and transmembrane domains of Erbb-2 protein (neu in the rat and Her-2 in humans) display 84% amino acid homology,⁷ we evaluated the immunogenicity of two plasmids (RHuT and

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-09-2548

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⁷ <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

HuRT) coding for chimeric neu and Her-2 extracellular and transmembrane domains. RHuT encodes a protein in which the 410 NH₂-terminal residues are from the neu extracellular domain and the remaining residues from Her-2. Almost symmetrically, HuRT encodes a protein in which 390 NH₂-terminal residues are from Her-2 and the remainder from neu. The ability of RHuT and HuRT to elicit a response to rat and human ErbB-2 orthologues in wild-type (wt) and in transgenic mice tolerant to neu and Her-2 proteins is compared with that of plasmids coding for the fully rat (RRT) or fully human (HuHuT) extracellular and transmembrane domains. In most cases, RHuT and HuRT elicited a stronger response than RRT or HuHuT, although this chimeric benefit is markedly modulated by the location of the heterologous moiety in the chimeric protein, the tolerance of the responding mouse, and the kind of ErbB-2 orthologue on the targeted tumor.

Materials and Methods

Plasmids. pVAX1 (Invitrogen) was the backbone for all the vaccines. The cDNA sequence for RRT was obtained as previously described (23), and that for HuHuT was obtained by digestion of pSVerbB2 (24) with *Hind*III and *Xba*I enzymes (Celbio) and insertion into pVAX (pVAX-Her-2). The intracellular domain of Her-2 was eliminated by digestion with *Acc*III and *Xba*I (Celbio); the TAA triplet was inserted using a synthetic double-stranded oligonucleotide sequence. The cDNA sequence for RHuT was obtained by digesting pVAX1-Her-2 plasmid with *Hind*III and *Bst*EII (Celbio), leading to the deletion of the leader sequence and that encoding the NH₂-410 amino acids (1–410 residues) of Her-2. The deleted portion was replaced with the neu cDNA fragment obtained by digesting RRT plasmid with *Hind*III and *Bst*EII. For HuRT, the cDNA encoding the COOH-299 residues (301–691 residues) of neu protein was obtained by PCR using RRT as template and the following primers: *Eco*RI, 5'-CATGGAATTCGCTCCGCTGAGGCTGAGCA-3' (forward); *Xba*I, 5'-GGCCTCTAGATTACATCGTATACTTCCGGATCTT-3' (reverse). The fragment obtained was cloned into pVAX1. The cDNA encoding the leader signal and the NH₂-390 amino acids (1–390 residues) of Her-2 was amplified by PCR using HuHuT as template and the following primers: *Eco*RI, 5'-CCGGGAATTCGGCAGTGTGGAGGCTGGGT-3' (reverse); T7, 5'-TAATACGACTCACTATAGGG-3'. To reconstitute the whole sequence of HuRT, the DNA obtained was inserted using *Hind*III and *Eco*RI enzymes into pVAX1 containing the sequence encoding the COOH-299 residues of neu. Two residues (Glu-Phe) belonging to *Eco*RI restriction site remained in the junction between Her-2 and neu sequence. All the sequences were verified by sequencing (BMR Genomics). Large-scale preparation of the plasmids was carried out with EndoFree Plasmid Giga kits (Qiagen, Inc.).

Cell lines. NIH/3T3 mouse fibroblasts were from the American Type Culture Collection (ATCC). 3T3/KB cells express H-2K^d and B7.1, and 3T3/EKB or 3T3/NKB cells express additional Her-2 or neu (25). TUBO^{neu} carcinoma cells expressing H-2K^d and neu molecules are from a mammary carcinoma arisen in a BALB/c female transgenic mouse for the

activated *neu* (BALB-neuT mice; ref. 26). D2F2/E2^{Her-2} cells expressing Her-2 molecules were obtained by cotransfecting with pRSV/neo and Her-2 D2F2 mammary tumor cells from a BALB/c mouse (25). OVCAR-3 cells, a human ovary cancer cell line overexpressing Her-2, were from the ATCC. All cell lines were cultured in DMEM with Glutamax 1 (DMEM, Life Technologies) supplemented with 20% heat-inactivated fetal bovine serum (Invitrogen). 3T3/NKB and 3T3/EKB transfected cells were cultured with 0.6 mg/mL G418 (geneticin, Invitrogen) and 0.6 mg/mL zeocin (Invitrogen), 3T3/KB-transfected cells were cultured with 0.6 mg/mL G418 and 7.5 µg/mL puromycin (Invitrogen), and D2F2/E2^{Her-2} cells were cultured with 0.8 mg/mL G418.

Expression of plasmids following transfection. NIH/3T3 fibroblasts were transiently transfected with the plasmids using Lipofectamine 2000 Reagent (Invitrogen; ref. 27). Forty-eight hours later, they were fixed for 5 min with PBS–3% paraformaldehyde (Sigma-Aldrich), blocked with PBS–10% bovine serum albumin (Sigma-Aldrich) for 20 min, and stained (1 h at 4°C) with Ab4 (1:50 dilution; Oncogene), trastuzumab, and pertuzumab (1:50; Genentech) monoclonal antibodies (mAb). To detect Ab4 binding, an Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) was used; to detect trastuzumab and pertuzumab binding, a FITC-conjugated anti-human IgG (DakoCytomation) was used. Antibody staining was evaluated with Bio-Rad MRC 600 confocal microscope (Bio-Rad Laboratories).

Mice. Female BALB/c^{wt} (H-2^d), C57BL/6^{wt} (H-2^b), CB6F1^{wt} (H-2^{d/b}), and BALB-neuT mice were from Charles River Laboratory. CB6F1^{neu} mice transgenic for *neu* were generated by crossing BALB-neuT males (28) with C57BL/6^{wt} females. CB6F1^{Her-2} mice were obtained by crossing C57BL/6^{Her-2} males expressing *Her-2* gene (29) with BALB/c^{wt} females. Genotyped and individually tagged mice of the same age were treated according to the European Union guidelines.

Immunization and tumor growth. Anesthetized mice were vaccinated as described (30). The vaccination course consisted of two i.m. injections of 50 µg of plasmid followed by electroporation repeated with an interval of 14 d. When required, 1 wk after vaccination, mice were challenged in the mammary pad with a lethal dose of TUBO^{neu} (2×10^5 in CB6F1^{wt}; 1×10^5 in CB6F1^{neu} mice) or D2F2/E2^{Her-2} (5×10^5 in CB6F1^{wt}; 3.5×10^5 in CB6F1^{Her-2} mice) cells. In other cases, vaccination was started when TUBO^{neu} and D2F2/E2^{Her-2} tumors reached a mean diameter (hereafter diameter) of 2 or 4 mm. The mammary pad of challenged mice and CB6F1^{neu} mice was inspected weekly by palpation. Progressively growing masses >1 mm in diameter were regarded as tumors. Mice were sacrificed when one of the tumors exceeded 10-mm diameter. Differences in tumor incidence were analyzed by the log-rank (Mantel-Cox) test or Fisher's exact test.

Antibody response. Sera collected 2 wk after vaccination were diluted 1:200 in PBS and 100 µL were incubated for 30 min at 4°C with 3T3/NKB or OVCAR-3 cells pretreated with Fc receptor blocker (CD16/CD32; Pharmingen) for 15 min at 4°C. Total Ig binding was evaluated using a FITC-conjugated goat anti-mouse IgG Fc antibody (DakoCytomation). The Ab4 (Oncogene) and Ab5 (Calbiochem) mAbs were used as

positive controls for neu and Her-2 positivity, respectively. Flow cytometry was performed with a CyAn ADP (DakoCytometry). The results were expressed as mean fluorescence intensity (MFI) and analyzed with Summit 4.2 (DakoCytometry) software. Differences in MFI were analyzed by Student's *t* test.

Cytotoxic T-cell response. The percentage of specific killing *in vivo* was evaluated by labeling spleen cells (SPC) with different concentrations of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) as described (11). SPC labeled with 5 $\mu\text{mol/L}$ CFSE (CFSE^{high}) were pulsed with 15 $\mu\text{g/mL}$ of H-2K^d dominant neu (TYVPANASL) or Her-2 (TYLPTNASL) peptide (INBIOS). T cells recognizing the neu peptide do not effectively recognize the Her-2 peptide; thus, one was used as specificity control of the other.

IFN- γ enzyme-linked immunospot assay. Two weeks after vaccination, 0.5×10^6 or 1×10^6 SPC were added to the wells of 96-well HTS IP plates (Millipore) precoated with 5 $\mu\text{g/mL}$ of rat anti-mouse IFN- γ (clone R4-6A2, BD Biosciences). SPC were stimulated with 15 $\mu\text{g/mL}$ of neu TYVPANASL or Her-2 TYLPTNASL peptides for 16 h or incubated for 48 h with 3T3/KB, 3T3/NKB, or 3T3/EKB at an APC-to-lymphocyte ratio of 1:10. IFN- γ spots were enumerated as previously described (31). Data were analyzed by the Student's *t* test.

Results

The proteins encoded by RHuT and HuRT are recognized by anti-human and anti-rat mAb. To assess whether the protein coded by the various plasmids (Fig. 1A) is recognized by mAb selectively binding neu or Her-2, NIH/3T3 fibroblasts were transfected with each plasmid. The Ab4 mAb (recognizing a rat epitope in the II domain of neu) binds fibroblasts transfected with RRT and RHuT. Pertuzumab (recognizing a Her-2 epitope in the II extracellular domain, residues 195–320; ref. 32) binds fibroblasts transfected with HuHuT and HuRT. Trastuzumab (recognizing a Her-2 epitope in the IV domain, residues 489–560; ref. 33) binds fibroblasts transfected with HuHuT and RHuT (Fig. 1B).

RHuT and HuRT elicit a stronger immunity than RRT and HuHuT in CB6F1^{wt} mice. When CB6F1^{wt} mice were vaccinated, RHuT elicited the highest antibody response to neu (Fig. 2A, left). Both RRT and RHuT induced a strong cytotoxic response to TYVPANASL, the H-2^d dominant neu peptide (Fig. 2B, left), whereas RHuT triggered a higher number of IFN- γ -producing cells following peptide (Fig. 2C, left) and neu-transfected 3T3/NKB (Fig. 2D, left) restimulation.

When the anti-Her-2 response was evaluated, mice vaccinated with HuRT displayed the highest titer of antibodies (Fig. 2A, right). Mice vaccinated with both HuRT and HuHuT displayed a strong cytotoxic response to Her-2 TYLPTNASL peptide (Fig. 2B, right). Following Her-2 TYLPTNASL peptide restimulation, mice immunized with HuRT displayed the greatest number of IFN- γ -producing cells (Fig. 2C, right), whereas, following Her-2-transfected 3T3/EKB restimulation, HuHuT-vaccinated mice displayed more IFN- γ -producing T cells (Fig. 2D, right).

The sera from RHuT and HuRT immunized mice better down regulated from cell membrane and confine in the cytoplasm RRT and HuHuT protein, respectively (Supplementary Fig. S1).

In CB6F1^{wt} mice, the immunity elicited by RHuT and HuRT better inhibits tumors driven by neu or expressing Her-2. First, CB6F1^{wt} mice were challenged with TUBO^{neu} cells, whose ability to give rise to a tumor depends on neu receptor-transduced signals (23, 34). When mice were challenged 1 week after vaccination, a growing tumor was displayed by all mice vaccinated with the pVAX, whereas the challenge was rejected by all those vaccinated with RRT, RHuT, and HuRT. Slightly less protection was provided by HuHuT (Fig. 3A). When mice were already bearing a 4-mm TUBO^{neu} carcinoma invading the s.c. tissue (35), a significantly better protection was afforded by vaccination with RHuT (95%, 19 of 20 tumor-free mice) compared with RRT (65%, 11 of 17; $P = 0.03$, Fisher's exact test; Fig. 3B).

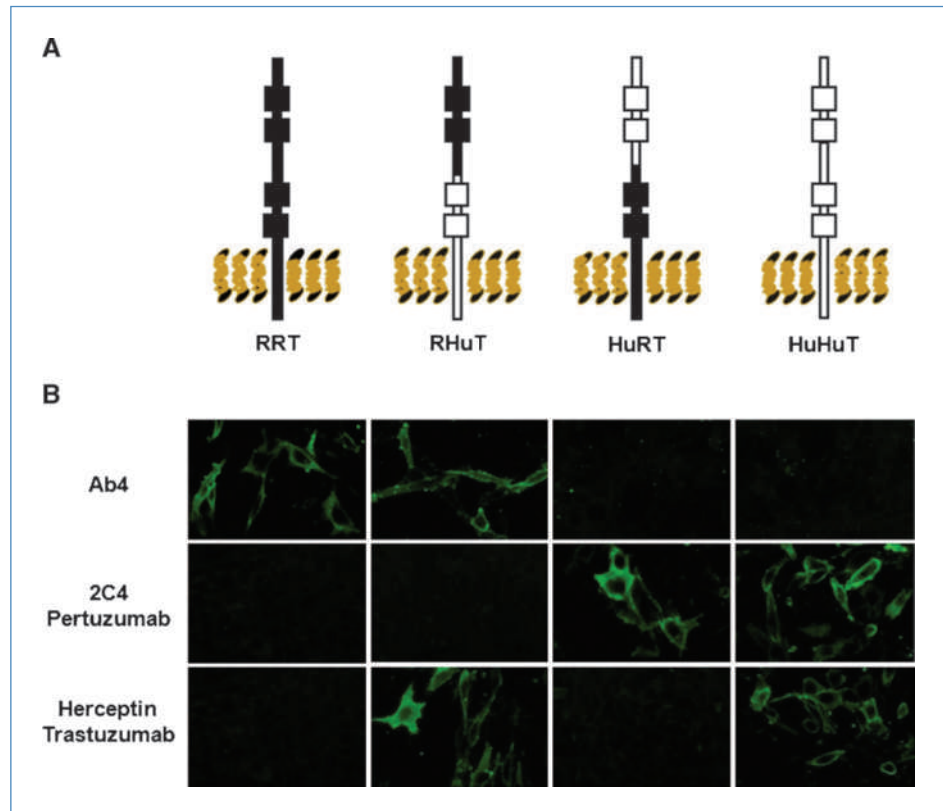
A different scenario was evident when mice were challenged with Her-2-transfected D2F2/E2^{Her-2} cells. Whereas Her-2 protein is overexpressed in a way comparable with the neu on TUBO^{neu} cells, in D2F2/E2^{Her-2} cells it is solely a surrogate tumor-associated antigen (36). A growing tumor was displayed by all mice vaccinated with pVAX, whereas the challenge was rejected by all those vaccinated with RRT, RHuT, HuRT, and HuHuT (Fig. 3C). By contrast, vaccination did not cure established 4-mm D2F2/E2^{Her-2} carcinomas but only delayed their growth (Fig. 3D). The mean time required for a carcinoma to exceed a 6-mm threshold was 31 ± 2 days in mice vaccinated with HuRT and progressively shorter in those vaccinated with HuHuT (29 ± 3 days), RHuT (25 ± 2 days), and RRT (13 ± 1 days). Here, too, the protection afforded by RHuT is significantly better of that of RRT ($P = 0.002$).

RHuT affords the best protection against neu-driven tumors in CB6F1^{neu} mice. The ability of chimeric proteins to elicit a response was assessed in CB6F1^{neu} mice that express neu protein in the newborn thymus (Supplementary Fig. S2A) and display an aggressive mammary carcinogenesis with 100% penetrance (37; Supplementary Fig. S2B).

When 10-week-old CB6F1^{neu} mice were vaccinated before a challenge with TUBO^{neu} cells, the tumor was rejected by all those vaccinated with RHuT, 67% of those vaccinated with RRT, and only 12% and 11% of those vaccinated with HuRT and HuHuT, respectively (Fig. 4A). All the plasmids failed to cure CB6F1^{neu} mice bearing 4-mm TUBO^{neu} tumors (data not shown). However, by starting the vaccinations when mice bear a 2-mm tumor, RHuT significantly delayed tumor onset, whereas RRT was ineffective (Fig. 4B).

Because all CB6F1^{neu} mice develop neu-driven mammary carcinomas (Supplementary Fig. S2C; ref. 37), the potential of the vaccines against progressive stages of carcinogenesis was evaluated. Vaccination with RHuT started when the mammary glands displaying atypical hyperplasia (week 10 of age) kept all mice tumor-free until week 43, at which time 46% of RRT- and all HuRT- and HuHuT-vaccinated mice displayed at least one palpable tumor (Fig. 4C, left). RHuT and RRT vaccinations started when mice harboring multiple mammary lesions similar to *in situ* carcinoma (week 14) were

Figure 1. Characterization of the chimeric proteins encoded by RHuT and HuRT. A, drawing of the proteins encoded by RRT, RHuT, HuRT, and HuHuT. Black regions, rat moieties; white regions, human moieties. B, recognition by Ab4, trastuzumab, and pertuzumab mAb of the proteins expressed on NIH/3T3 fibroblasts following transfection with the various plasmids. Confocal microscope analysis performed 48 h after transfection. Results are representative of one of three independent experiments.



still able to extend the tumor-free survival, RHuT being more protective (Fig. 4C, middle). When vaccinations were delayed until mice harbored microscopic invasive carcinomas (week 18), RHuT alone was still able to significantly extend the tumor-free survival (Fig. 4C, right).

As RHuT provides the best protection, we evaluated whether repeated vaccinations could prolong this protection to nearly the natural murine life span. Median survival of $CB6F1^{neu}$ mice vaccinated every 10 weeks with RHuT was extended to week 95 (Supplementary Fig. S3), and 45% of 104-week-old mice were still tumor free. No pathologic evidence of autoimmunity was found (data not shown).

These rejection patterns correlate with a high titer of anti-neu antibodies. Antibody titers of mice vaccinated with RRT and HuRT were lower than those of mice vaccinated with RHuT, whereas those of mice vaccinated with HuHuT were similar to those of mice electroporated with pVAX (Fig. 4D, left). The titer of anti-neu antibodies (Fig. 4D, middle and right) and the efficacy of the antitumor protection (Fig. 4C, middle and right) were progressively lower when vaccination was started at the later stages of carcinogenesis.

The cytotoxic response against cells pulsed with the dominant neu TYVPANASL peptide and the ability of SPC to produce $IFN-\gamma$ following TYVPANASL restimulation were nil (data not shown). This is not surprising because $CB6F1^{neu}$ mice, like parental BALB-neuT mice, express neu in the newborn thymus (Supplementary Fig. S2A) and display central tolerance with deletion of $CD8^+$ T cells reacting with the

dominant neu TYVPANASL with high affinity (11). However, $IFN-\gamma$ -secreting T cells were observed when SPC from mice immunized with RRT and RHuT were restimulated by neu-expressing 3T3/NKB cells. This suggests the recognition of subdominant neu epitopes. As expected, a marked response was found when SPC from mice immunized with HuHuT and HuRT were restimulated by Her-2-expressing 3T3/EKB cells (Supplementary Fig. S4).

HuRT induces the best response to Her-2⁺ tumors in $CB6F1^{Her-2}$ mice. $CB6F1^{Her-2}$ mice do not develop Her-2-driven mammary tumors (29). When these mice were challenged with D2F2/E2^{Her-2} cells after vaccination, the challenge was rejected by 80% and 70% of mice vaccinated with HuRT and HuHuT, respectively. RHuT vaccination protected 40% of mice, whereas that with RRT was ineffective (Fig. 5A). All plasmids induced a significant anti-Her-2 antibody response. HuRT and HuHuT elicited the highest titer (Fig. 5B, left).

A cytotoxic response against cells pulsed with the dominant Her-2 TYLPTNASL peptide and $IFN-\gamma$ release following TYLPTNASL restimulation was evident in mice vaccinated with HuRT and HuHuT only (Fig. 5B, middle and right).

Discussion

The electroporation of RHuT and HuRT chimeric rat-human plasmids in $CB6F1$ mice elicits an immune reaction that is (a) enhanced by the presence of the heterologous moiety,

(b) critically dependent on the location of this moiety on the molecule, and (c) markedly modulated by the different degree of tolerance of the responding mouse to the Erbb-2 orthologues.

In CB6F1^{wt} mice, not tolerant to rat or human Erbb-2 epitopes, preimmunization with RHuT, HuRT, the fully rat RRT, and the fully human HuHuT leads to the almost total rejection of a lethal challenge of both TUBO^{neu} cells and D2F2/E2^{Her-2} cells, although a response sufficient to cure 4-mm TUBO^{neu} tumors was only triggered by RHuT and (to a lesser degree) RRT.

When immunization began when mice displayed a 4-mm D2F2/E2^{Her-2} tumor, HuRT, RHuT, and HuHuT were able to delay its growth, HuRT and HuHuT being the most effective.

A different scenario emerges when the same plasmids are used to immunize neu-tolerant and Her-2-tolerant mice. CB6F1^{neu} mice are transgenic for the *neu* oncogene, express the neu protein in their thymus, display the deletion of T-cell clones reacting with neu protein at high affinity (11), and develop lethal neu⁺ mammary carcinomas (37). The immunity elicited by RHuT confers full protection against a TUBO^{neu}

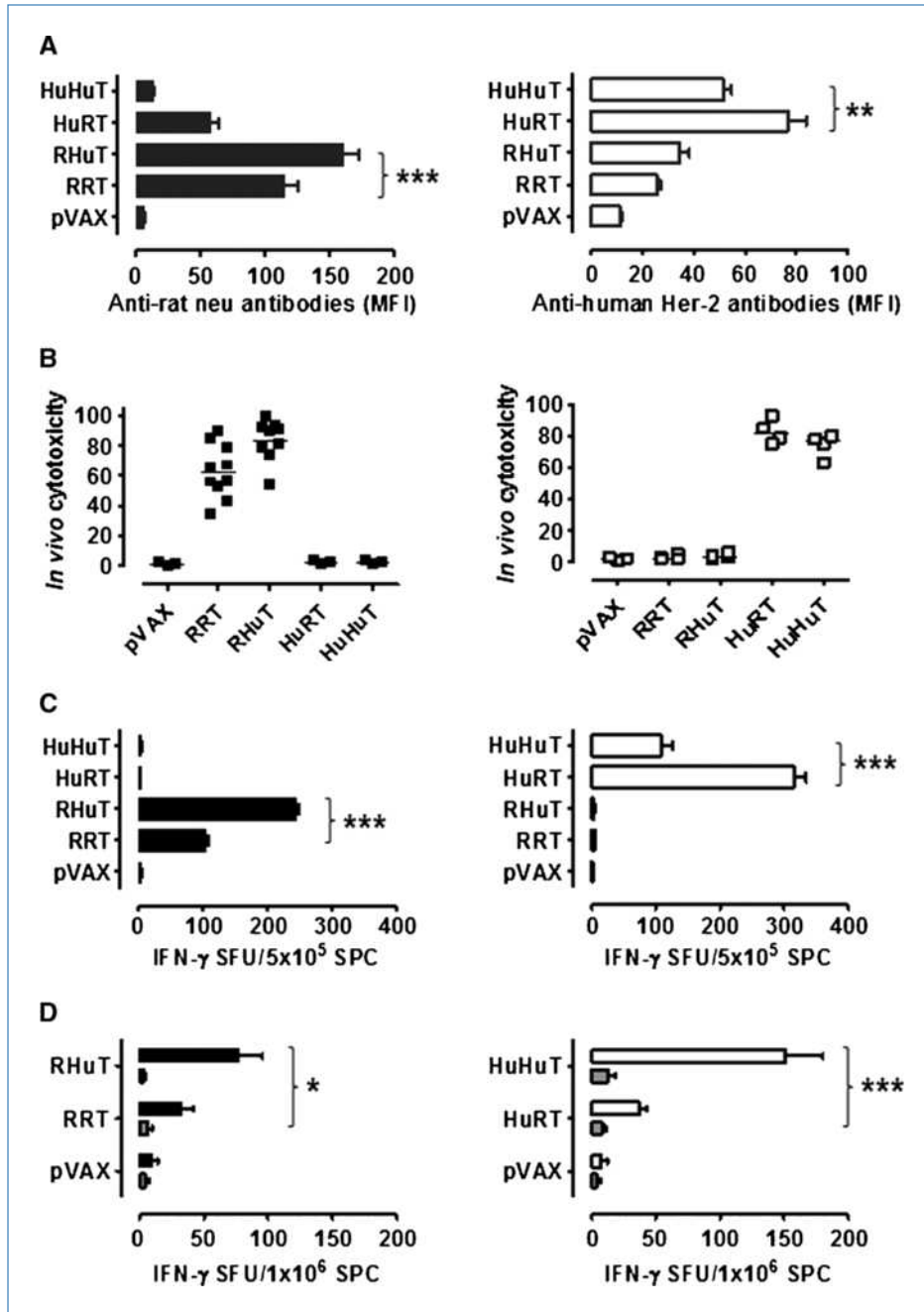


Figure 2. Antibody and cellular response against neu (left) and Her-2 (right) 2 wk after vaccination of CB6F1^{wt} mice with RHuT, HuRT, RRT, and HuHuT. A, anti-ErbB-2 antibody titer (MFI ± SE). B, *in vivo* cytotoxicity against the H-2^d dominant peptide. Each square, % of lysis displayed by each mouse. C and D, IFN-γ-producing cells, expressed as spot-forming unit (SFU), after restimulation with dominant H-2^d peptide (C) or cells (D) evaluated by enzyme-linked immunospot assay. 3T3/KB (gray columns), 3T3/NKB (black columns), and 3T3/EKB (white columns). *, *P* = 0.05; **, *P* = 0.002; ***, *P* < 0.0009, Student's *t* test.

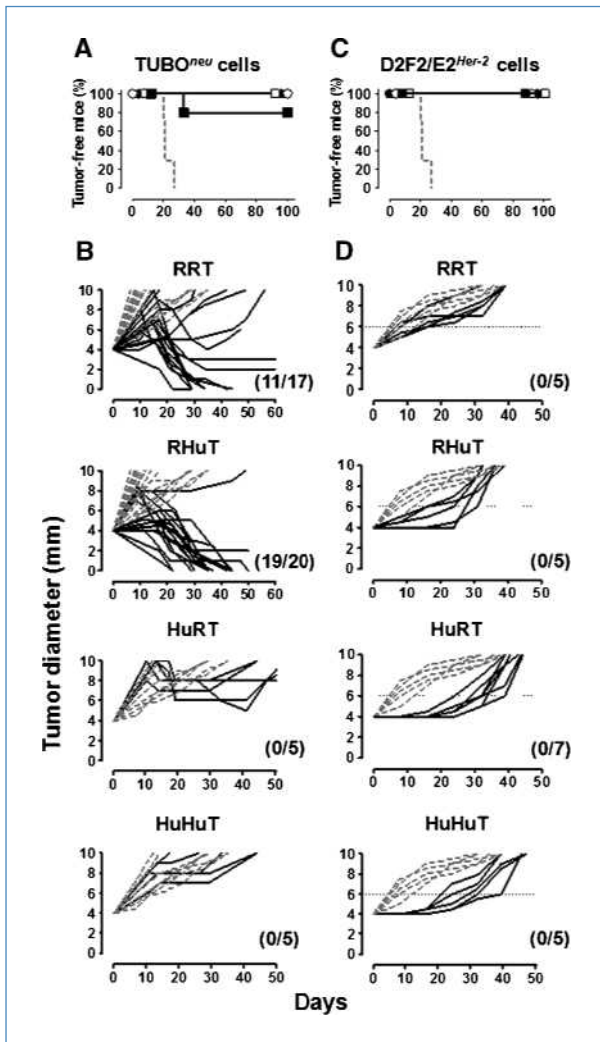


Figure 3. Antitumor response elicited in CB6F1^{wt} mice by RHuT, HuRT, RRT, and HuHuT. The protection was evaluated against neu⁺ (TUBO^{neu} cells, left) and Her-2⁺ (D2F2/E2^{Her-2} cells, right). A and C, groups of 10 mice vaccinated with RRT (●), RHuT (○), HuRT (□), HuHuT (■), and pVAX (dotted gray lines) challenged 1 wk after vaccination with a lethal dose of tumor cells. B and D, ability of vaccination to cure established 4-mm tumors. Each line refers to an individual tumor; dotted gray lines show tumor growth in mice vaccinated with the empty pVAX. Brackets, number of mice that rejected the tumor/total mice.

challenge, and a modest, but significant, ability to cure 4-mm TUBO^{neu} tumors, as well as the best protection when these mice were vaccinated at progressive stages of autochthonous tumor progression. Moreover, repeated RHuT vaccinations kept 45% of mice free from palpable tumors until week 104.

CB6F1^{Her-2} mice are transgenic for the *Her-2* oncogene and tolerant to the Her-2 epitopes (29). Their preimmunization with HuRT confers the best protection against a challenge of a Her-2⁺ tumor. However, an only slightly inferior protection is afforded by HuHuT, whereas RHuT is less protective and the protection afforded by RRT is almost negligible. The lesser protection afforded by vaccination against the

D2F2/E2^{Her-2} tumor cells is partially due to the fact that their Her-2 protein is simply a transfected surrogate antigen not directly involved in their growth (25), unlike TUBO^{neu} cells whose progression rests on the signals transduced by the neu receptor (7, 30). Whereas TUBO^{neu} cells are sensitive to the direct action of antibodies and T cells (7, 34), D2F2/E2^{Her-2} cell expansion can only be inhibited by T cells (4).

All these studies were performed in mice with a CB6F1 genetic background. However, because transgenic CB6F1^{Her-2} mice do not develop Her-2⁺ tumors, the ability of chimeric plasmids to inhibit the onset of autochthonous tumors was tested in FVB^{Her-2} mice that differ from CB6F1 mice at both the H-2 (H-2^q versus H-2^{d/b}) and background genes (38). In these mice, immunizations with both RHuT and HuRT repeated at 10-week intervals afforded significant protection (Supplementary Fig. S5A), HuRT being apparently more effective. Unfortunately, the recent establishment of our FVB^{Her-2} colony and this slow tumor progression precluded direct comparison with the protection afforded by RRT and HuHuT, and this result is only proof of their potential in hampering *Her-2*-driven autochthonous carcinogenesis in genetically different mice.

To move toward a mechanistic explanation of the chimeric benefit observed *in vivo*, we mainly focused on comparison of the reactivity induced by RHuT and RRT against neu because the chimeric benefit of HuRT is less evident. Germane with the stronger protection against neu⁺ tumors, CB6F1^{wt} mice vaccinated with RHuT displayed the highest number of IFN- γ -producing CD8⁺ T cells and a significant cytotoxicity against cells pulsed with the dominant neu peptide. Nevertheless, in the Winn-type assay performed in nonobese diabetic-severe combined immunodeficient mice, these CD8⁺ T cells were unable to impair the progression of TUBO^{neu} cells (Supplementary Fig. S6A, left), probably because of TUBO^{neu} defects in the antigen-presenting machinery. By contrast, CD4⁺ T cells from immunized mice significantly delayed TUBO^{neu} tumor onset (Supplementary Fig. S6A, right). When the assay was performed in immunocompetent CB6F1^{wt} mice, CD4⁺ T cells from RHuT-vaccinated, but not from RRT-vaccinated, mice induced regression of TUBO^{neu} tumors in four of seven mice (Supplementary Fig. S6B). This tumor inhibition was associated with the recruitment of massive leukocyte infiltration (Supplementary Fig. S7).

This major role of the CD4⁺ T cells goes along with the stronger antibody response observed in RHuT immunized CB6F1^{wt} and CB6F1^{neu} mice. The more efficient downregulation and cytoplasmic confinement of ErbB-2 surface receptors observed with the sera from RHuT and HuRT immunized mice suggests that the amino acid sequence and the structural conformation of the proteins encoded by these chimeric plasmids (Supplementary Fig. S8) trigger new immune responses to subdominant as well as to new epitopes cross-reacting with the neu.

Our combined *in vivo* and *in vitro* findings further expand the notion that vaccination with an altered form of the antigen is an effective way of generating a robust B-cell and T-cell response to a self-antigen and overcoming tolerance (14).

The location of the heterologous and identical moieties on the chimeric molecule is not a neutral factor. The data showing

that RHuT and RRT elicit the best responses against neu, and HuRT and HuHuT the best against Her-2, suggest that the optimal response is elicited when the NH₂-terminal portion of the chimeric protein and the corresponding portion on the targeted ErbB-2 orthologue are identical. This identity is an almost absolute requirement, whose importance goes beyond the chimeric benefit. Plasmids coding for a protein differing from the targeted ErbB-2 orthologue at the NH₂-terminal elicit a poor cross-reactive antibody response and an almost nil T-cell response. Therefore, the location of the heterologous moieties may determine both the presence of the chimeric benefit and even the ability to elicit a response.

An additional variable is the genetic makeup and the central immune tolerance to the ErbB-2 orthologues of the

recipient mouse. Whereas both RHuT and HuRT elicit a protection toward transplantable and autochthonous Her-2⁺ tumors in CB6F1^{Her-2} and FVB^{Her-2} mice, surprisingly RHuT elicits much less antibodies to Her-2 in FVB^{Her-2} mice, suggesting that the protection rests mainly on cell-mediated immunity. In addition, as expected, in transgenic CB6F1^{neu}, CB6F1^{Her-2}, and FVB^{Her-2} mice, the response elicited by HuRT and RHuT is lower and less efficacious than that elicited in wt mice. Nevertheless, the rank of the efficacy of the response triggered by the plasmids against neu and Her-2 in CB6F1^{neu} and CB6F1^{Her-2} mice, respectively, remains the same as in CB6F1^{wt} mice. Vaccination may be supposed to overcome, at least in part, tolerance related to the transgene overexpression (39).

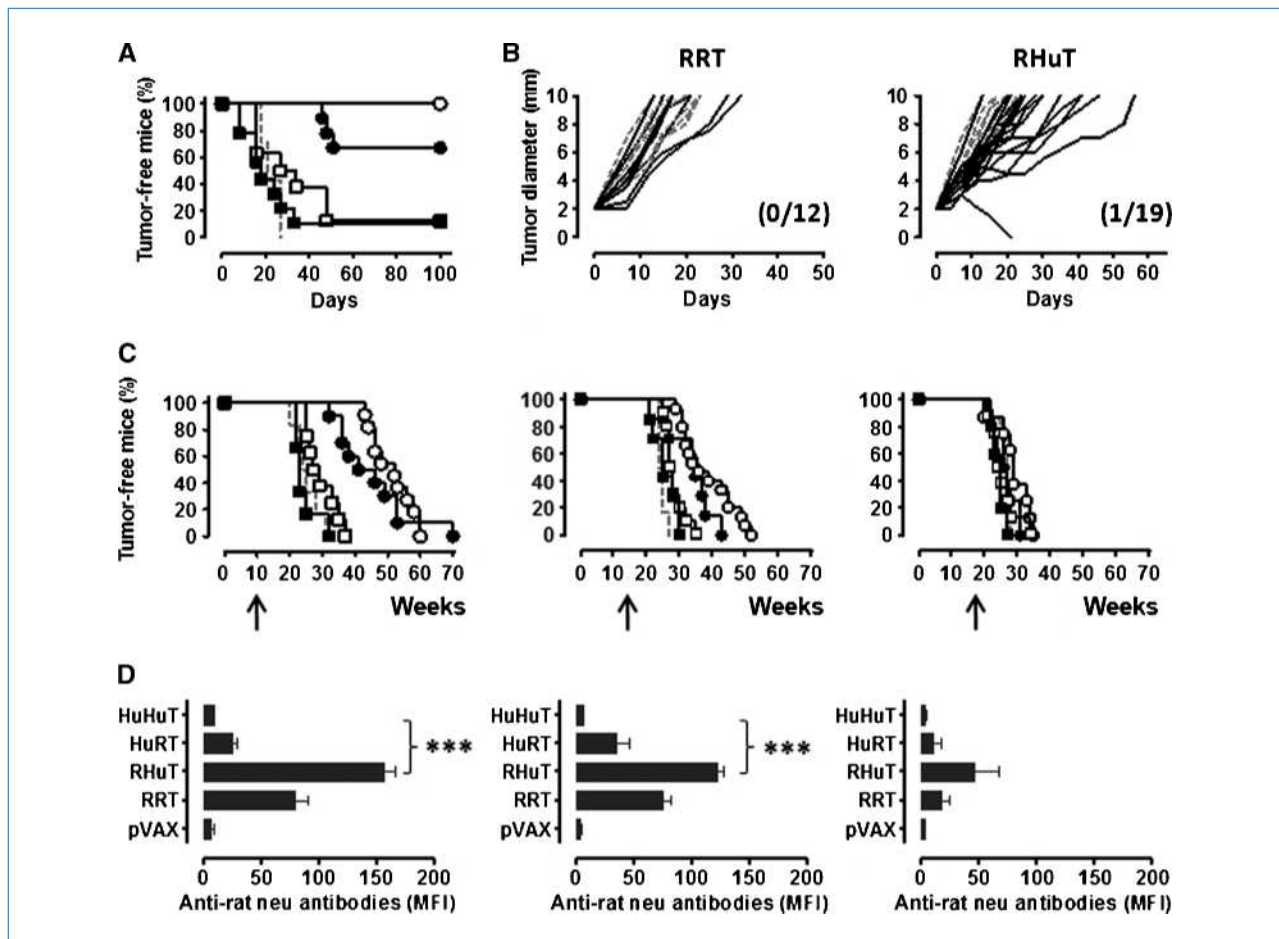


Figure 4. Immune response against neu elicited in CB6F1^{neu} mice by RHuT, HuRT, RRT, and HuHuT. A and C, groups of at least five mice vaccinated with RRT (●), RHuT (○), HuRT (□), HuHuT (■) and pVAX (dotted gray lines). A, mice were challenged 1 wk after vaccination with a lethal dose of TUBO^{neu} tumor cells. B, ability of vaccination to cure established 2-mm tumors. Each line refers to an individual tumor; dotted gray lines show tumor growth in mice vaccinated with the empty pVAX plasmid. Brackets, number of mice that rejected the tumor/total mice. In RHuT-vaccinated mice, tumors grew to 10-mm mean diameter more slowly than in RRT-vaccinated mice (18.7 ± 1.8 versus 30.0 ± 2.5; *P* = 0.002, Student's *t* test). C, protection against autochthonous neu⁺ mammary tumors provided by vaccination started when mice display mammary atypical hyperplasia (week 10, left panel), *in situ* carcinoma (week 14, middle panel), and invasive microscopic cancer (week 18, right panel). When vaccination is started at weeks 10 and 14, tumor incidence in RRT and RHuT mice is significantly different (*P* < 0.0001) compared with pVAX-vaccinated mice, whereas when it began at week 18 only RHuT-vaccinated animals showed a statistically different tumor incidence (*P* = 0.03). D, anti-neu antibodies in mice vaccinated starting at 10 wk (left), 14 wk (middle), and 18 wk (right) of age and tested 2 wk after vaccination. Results are expressed as MFI ± SE. ***, *P* < 0.0006, Student's *t* test.

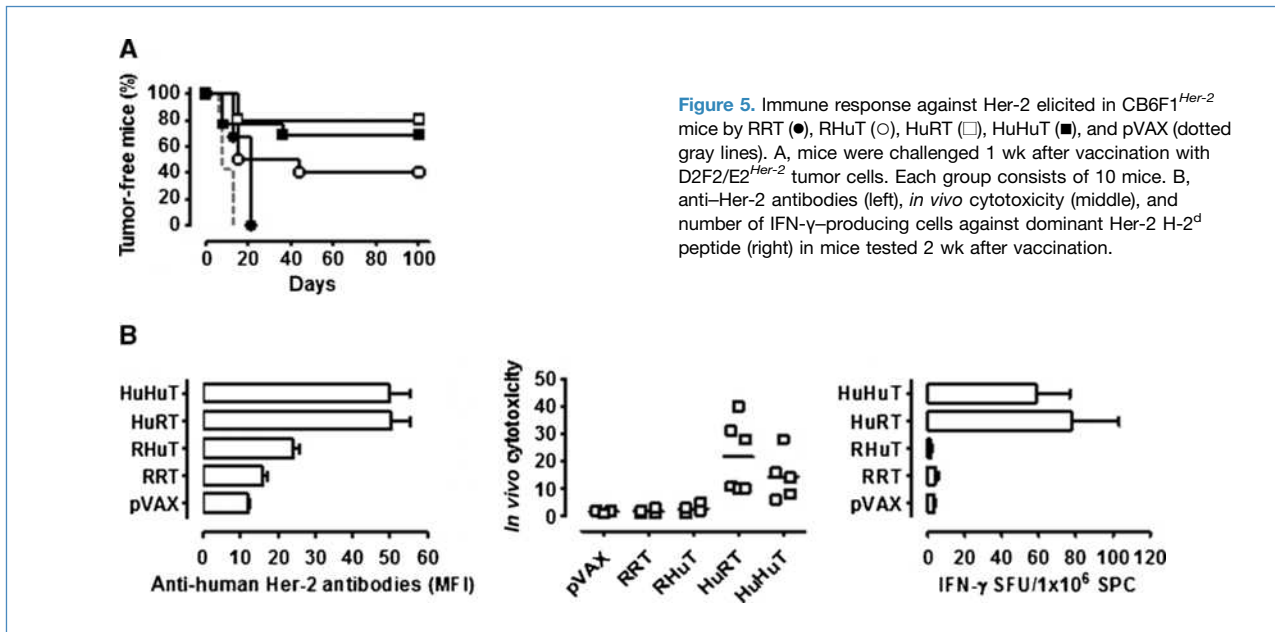


Figure 5. Immune response against Her-2 elicited in CB6F1^{Her-2} mice by RRT (●), RHuT (○), HuRT (□), HuHuT (■), and pVAX (dotted gray lines). A, mice were challenged 1 wk after vaccination with D2F2/E2^{Her-2} tumor cells. Each group consists of 10 mice. B, anti-Her-2 antibodies (left), *in vivo* cytotoxicity (middle), and number of IFN- γ -producing cells against dominant Her-2 H-2^d peptide (right) in mice tested 2 wk after vaccination.

All these responses were elicited and had to sneak through the natural tolerance of mice to mouse Erbb-2 orthologues. The ability of a plasmid to elicit a murine response to both rat and human Erbb-2 also rests on its ability to overcome tolerance to the amino acid sequences that are identical in the mouse and rat and mouse and human Erbb-2 orthologues. Vaccination may primarily induce a response to epitopes that are different from mouse to rat, or mouse to human, and hence are not tolerated. This major interference imposed by the natural tolerance to mouse Erbb-2 is often ignored in experimental vaccination studies in the mouse. However, it has to be carefully taken into account because it may markedly sway the results obtained. Although RHuT and HuRT proved to be more immunogenic in many mouse-tumor combinations, the different genetic makeup and the different state of tolerance of patients to Her-2 preclude ranking their immunogenicity in patients. The differences between the rat moieties coded by both HuRT and RHuT with Her-2 will be sufficient to warrant a major chimeric benefit. Thus, their availability holds the promise of an interesting clinical perspective.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Wei-Zen Wei for helpful discussions, cells, and mice and Dr. Federico Gabrielli for molecular modeling.

Grant Support

Associazione Italiana per la Ricerca sul Cancro, Ministero dell'Università e della Ricerca, University of Torino, Compagnia di San Paolo, Fondazione Denegri, Fondazione Internazionale di Ricerca in Medicina Sperimentale, Fondazione CRT Progetto Alfiere, Regione Piemonte, and Nordic Centre of Excellence for the Development of Anti-Tumor Vaccine Concepts. This study was funded under the auspices of EU Consortium for Anticancer Antibody Development (EUCAAD) 200755. The project EUCAAD has received research funding from the EU Community's Seventh Framework Programme.

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Received 07/09/2009; revised 12/15/2009; accepted 01/12/2010; published OnlineFirst 03/23/2010.

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