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Human ErbB-2 (Her-2) Transgenic Mice: A Model System for Testing Her-2 Based Vaccines

Marie P. Piechocki,*† Ye-Shih Ho,§ Shari Pilon,*† and Wei-Zen Wei†‡

Her-2 transgenic (Tg) mice were generated with wild-type human c-ErbB-2 (Her-2) under the whey acidic protein promoter. They are tolerant to Her-2 and appropriate for testing Her-2 vaccines. The expression of transmembrane ErbB-2 from the whey acidic protein-Her-2 cassette and its up-regulation by insulin and hydrocortisone was verified by in vitro transfection. The transgene cassette was microinjected into fertilized eggs from B6C3 (C3H × C57BL/6) females mated with B6C3 males. Transgene-positive mice were backcrossed onto C57BL/6 mice. Human ErbB-2 was expressed in the secretory mammary epithelia during pregnancy and lactation and expressed constitutively in the Bergman glia cells within the molecular layer of the cerebellum. Overt, neoplastic transformation was not detected in any tissue examined. Tolerance to Her-2 was demonstrated by inoculating mice with a syngenic tumor expressing high levels of human ErbB-2. Tumors grew exclusively in Her-2 Tg mice without inducing an Ab response, while the nontransgenic littermates remained tumor free for 10 mo and mounted a robust anti-ErbB-2 Ab response. When immunized with a DNA sequence comprising the promoter-enhancer region of the MMTV-long terminal repeat and a constitutively activated allele of the human ErbB-2 proteins (14). When immunized with human ErbB-2 DNA, FVB-NeuN females developed less tumors, but neither humoral nor cellular immunity to rat neu was detected and there remained uncertainty whether tolerance to rat neu was overcome (15). These results indicated that rat neu Tg mice may be somewhat inadequate for testing human ErbB-2 vaccines.

The development of human ErbB-2 Tg mice was previously attempted with a DNA sequence comprising the promoter-enhancer activated allele of the MMTV-long terminal repeat and a constitutively activated allele of the human ErbB-2 (16). Expression of the transgene was observed in kidney, lung, mammary gland, salivary gland, and in the male reproductive track. All Tg mice expressing ErbB-2 died by 4 mo of age, probably due to kidney and lung failure following the development of preneoplastic lesions. Mammary glands in parous females were underdeveloped and some gave rise to tumors. Tg males were sterile.

To generate a new model of human ErbB-2 Tg mice, we have chosen the whey acidic protein (WAP) promoter. WAP is a major whey protein secreted in rodent’s milk and WAP promoter has been used for targeting heterologous genes to the mammary gland. Although typically described as being under strict hormonal and lactational control, the expression of WAP transgenes in normal mice has also been observed in other organs, especially in the brain (17). Here we report the generation of Her-2 Tg mice using a WAP promoter regulated c-ErbB-2 transgene and the immune tolerance in these mice.

Materials and Methods

Construction of pWAP-human ErbB-2 (pWAP-Her-2)

The full-length human c-ErbB-2 cDNA was isolated from plasmid pCMV-ErbB-2 (9) as a 4.4-kb EcoRI restriction fragment and cloned into the KpnI site downstream of the 2.5-kb WAP promoter in pBSK kindly provided by

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Dr. B. Groner (Chemotherapeutisches Forschungsinstitut, Frankfurt, Germany). Escherichia coli DH5a (Life Technologies, Gaithersburg, MD) or Top10 (Invitrogen, Carlsbad, CA) was used to propagate pWAP-Her-2, using Terrific Broth (Life Technologies) containing 100 µg/ml ampicillin. Plasmid was purified with QIAfilter Giga kit (Qiagen, Valencia, CA). The 6.9-kb WAP-Her-2 expression cassette was liberated with HindIII and used for transfection and microinjection.

**Mice and cell lines**

Male and female C57BL/6 (H-2Kb) mice (6–8 wk of age) were obtained from Charles River Laboratory (Frederick, MD) or the Jackson Laboratory (Bar Harbor, ME). Mouse mammary tumor line D2F2 was derived from a spontaneous mammary tumor which arose in a BALB/c hyperplastic alveolar cystic tumor line (13). The cell line was maintained in vitro DMEM supplemented with 5% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO) and 5% Cosmic Calf Serum (HyClone, Logan, UT), 10% NCTC 109 medium (Sigma-Aldrich), 2.5 mM Na₂-HEPES, 0.5 mM sodium pyruvate, 2 mM l-glutamate, 1.0 mM MEM nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. D8/E2 is a C57BL/6 ovarian cancer cell line (19) transfected with pCMV-ErbB-2. EL-4/E2 is a C57BL/6 thymoma cell line transfected with pCMV-ErbB-2. All transfected cells were cloned twice by limiting dilution to isolate clones of stable expression. Transfected cell lines were maintained in medium containing 0.8 mg/ml G418 (Genetech, Cambridge, MA) and 0.45% v/v IGEPAL CA-630 (Sigma-Aldrich) supplemented with 10% NCTC 109 medium (Sigma-Aldrich), 2.5 mM Na₂-HEPES, 0.5 mM sodium pyruvate, 2 mM l-glutamate, 1.0 mM MEM nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**Expression of WAP-Her-2 cassette in D2F2 cells**

D2F2 cells were cotransfected with the 6.9-kb HindIII WAP-Her-2 expression cassette and linearized pRSV/neo, at a 10:1 ratio, using LipofectAMINE Plus reagent purchased from Life Technologies. Individual colonies were expanded and expression of the recombinant protein was analyzed by flow cytometry, or immunoprecipitation and Western blot. Some of the transfected D2F2 clones were cultured in the presence of 10 µg/ml insulin and 10 µM hydrocortisone to enhance transcription from WAP promoter.

**Flow cytometric analysis**

mAbs TA-1 (AB-5) and 3B5 (AB-3) which recognize the extracellular and cytoplasmic domains of ErbB-2, respectively, were purchased from Oncogene Research Products (Cambridge, MA). FITC conjugated goat anti-mouse-IgG was the secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Normal mouse Ig or isotype-matched mAb were the negative controls. Flow cytometric analysis was performed with a FACS-Calibur (BD Biosciences, Mountain View, CA).

**Identification of Her-2 Tg mice by Southern blotting and PCR**

For initial identification of Her-2 Tg mice, genomic DNA was isolated from the tail tissues of 3- to 4-wk-old mice using Qiagen genomic DNA isolation kit (Qiagen) and digested to completion with EcoRI (Life Technologies), transferred to charged nylon membranes in 20× SSC, cross-linked to the membrane in a UV cross-linker and blocked overnight at 56°C in blocking solution (5× SSC, 0.5% SDS, 5× Denhardt’s reagent, 1 µg/ml BSA, and supplemented with 10 µg/ml sheared salmon sperm DNA) in a Stratagene rotary hybridization oven.

Southern blot DNA probe was isolated from human ErbB-2 cDNA by EcoRI restriction (Fig. 1A). A 1.6-kb DNA fragment encoding ErbB-2 at 1440–3000 was isolated and random primer labeled using the Klenow fragment of DNA polymerase to incorporate radioactive [α-32P]dCTP (DuPont NEN, Boston, MA, 3000 Ci/mmol). The reaction was stopped by the addition of EDTA and the product was purified by passage through a sephadex G-50 column which was packed and blocked with salmon sperm DNA (5 Prime–3 Prime, Boulder, CO). Purified probe was denatured by boiling for 5 min and snap cooled on ice. Denatured probe was added to hybridization buffer to achieve a final concentration of 2×10⁶ cpm/mg DNA. Specific activity of the probe was typically >10⁸ cpm/µg DNA. Membranes were hybridized overnight at 56°C and then washed to ultimate stringency (0.1× SSC, 0.1% SDS, 65°C). Membranes were sealed in plastic wrap and exposed to Kodak MR-1 film with an intensifying screen for 2–3 days at +80°C.

For routine screening of Her-2 Tg mice, a 2-mm ear punch or tail tissue was collected from 3- to 4-wk-old pups and used for PCR analysis. The tissue was digested in 200 µl of sterile lysis buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.2 mg/ml gelatin (Sigma-Aldrich) and 0.45% v/v IGEPAL CA-630 (Sigma-Aldrich) supplemented with 100 µg/ml proteinase K (Sigma-Aldrich P20308). The tissue was digested overnight at 56°C. Proteinase K was heat inactivated at 95°C for 30 min. The upper primer, 5′-CCC CCA CCC CAC CCC CAA AGT C-3′, anneals to the WAP promoter at position –22 relative to the ATG start codon. The lower primer, 5′-CGG GGG CCA AGA GGG CGA GGA G-3′, anneals to the human ErbB-2 cDNA at aa 18 downstream of the signal peptide. Amplification of the transgene results in a 352 bp PCR product. Briefly, 2–5 µl of genomic DNA was amplified in 1× Qiagen reaction buffer, 200 µM dNTPs, 1 µM of each primer, and 1× Q buffer in a total volume of 50 µl with 1 U of Taq polymerase. After an initial denaturation at 94°C for 3 min, samples were amplified for 30–35 cycles, consisting of denaturing at 94°C for 1 min, annealing at 58°C for 1 min, followed by extension at 72°C for 1 min. After the last cycle, samples were incubated for 5 min at 72°C and resolved in 1.5% TAE-agarose gels.

**Immunoprecipitation and Western blot analysis**

Lysates from fresh tissues were prepared by mincing tissues (3 mm³) in prechilled 1.5 ml microcentrifuge tubes on ice in tissue lysis buffer (50 mM HEPES (pH 8.0), 10% glycerol, and 1% Triton X-100) supplemented with protease and phosphatase inhibitor mixtures (Oncogene Sciences, Cambridge, MA). Human ErbB-2 protein was immunoprecipitated from the tissue lysates by incubation with an anti-ErbB-2 mAb 4D5, or Herceptin (Genentech, South San Francisco, CA) or mAb 9G10.6 (Neomarkers, Fremont, CA) for 2–4 h. Immune complexes were recovered by incubation with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 16–18 h. The agarose beads were subjected to centrifugation and washed twice with lysis buffer. Proteins were eluted in 1× sample buffer and boiled for 3 min before fractionation in 6% SDS-PAGE. Proteins were electrotransferred to Immobilon-P (Millipore, Bedford, MA) polyvinylidene difluoride membranes. Membranes were fixed with methanol, rehy- drated, and blocked overnight at 4°C in TBST buffer (10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1% Tween 20) with 1% BSA. ErbB-2 protein was detected by immunoblotting with mAb 3B5 or polyclonal C-18 (Santa Cruz Biotechnology). Phosphotyrosine was detected with mAb PY20 (Transduction Laboratories, Lexington, KY). Blots were developed with ECL reagents (Amersham, Arlington Heights, IL) and Kodak MR film.

**Immunohistochemical analysis**

Tissues were removed from mice and placed immediately in phosphate buffered formalin. Paraffin sections were prepared at 4–5 µm thickness and stained with H&E. For immunohistochemical analysis, endogenous peroxidase was blocked by incubation in 3% H₂O₂ in methanol or Peroxoblock (Neomarkers). Mild treatment with ficin was used for epitope retrieval. Tissues were processed using the HISTOMOUSE SP kit from Zymed Laboratories (South San Francisco, CA) designed to stain mouse tissues with mouse mAbs. The primary Ab TAB250 (Zymed Laboratories) which recognizes an epitope in the extracellular domain of the human ErbB-2 protein was used according to the recommended procedure. Alternatively, paraffin sections were subjected to HIER (citrate pH 6.0) and stained with Z4881 specific for the intracellular domain of human ErbB-2 (Zymed Laboratories) followed by detection with anti-rabbit poly-HRP (Chemicon). Immunostaining was developed using DAB as the chromagen and nuclei were counterstained with hematoxylin. Sections were viewed under a Zeiss microscope equipped with a Sony 970 CCD camera and MCID5+ software interface for data acquisition and image analysis with the 25× objective (100× total magnification).

**Testing tolerance and immunization of Her-2 Tg mice**

For plasmid DNA immunization, mice from the eighth generation back-cross were injected intramuscularly (i.m.) with 100 µl of saline containing 100 µg of each component plasmid at 2-wk intervals for a total of five vaccinations. Plasmid DNA expressing full-length and truncated variants of ErbB-2 gene have been described and characterized (9–11). The plasmid pEBos-G-CSF encoding murine GM-CSF was provided by Dr. Nishikawa (Osaka University, Osaka, Japan). At 2 wk after the final DNA vaccination, mice were challenged s.c. with 2×10⁶ EL-4/E2. Tumors were measured weekly with calipers and animals were sacrificed when any dimension of the tumor exceeded 15 mm. The percentage of tumor-free mice was analyzed by Kaplan-Meier method and statistical significance was determined by the log-rank test.

**Measurement of anti-ErbB-2 Ab**

Serum samples were diluted 1:20 and the presence of anti-ErbB2 Ab was determined by flow cytometry using SKBR-3 cells, a human breast carcinoma cell line with amplified ErbB-2. FITC conjugated goat anti-mouse Ab specific for mouse IgG Fcγ (Jackson ImmunoResearch Laboratories) was used to detect bound primary Ab. Normal mouse serum or isotype-matched mAb was the negative control. The mAb TA-1 (Oncogene Research Products), which recognizes an extracellular domain of ErbB-2, was

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used as a positive control for detection of ErbB-2 expression on SKBR-3 cells (Oncogene Research Products). Serial dilutions of TA-1 were used to generate a standard curve to determine the concentration (micrograms per milliliter) of anti-ErbB-2 Ab in serum. Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences). Results are presented as concentration, or mean channel fluorescence. Statistical analysis was performed with Student’s t test.

Results

Construction and expression of WAP-Her-2

WAP-Her-2 transgene was constructed by fusing the 2.6-kb mouse WAP promoter with the 4.4-kb human ErbB-2 cDNA as described in Materials and Methods (Fig. 1A). Expression of WAP-Her-2 cassette was initially tested by transfecting the 6939 bp fragment into mouse mammary tumor D2F2 cells. Human ErbB-2 protein was detected on the surface of transected cells by flow cytometry (Fig. 1B). Treatment of the transfected cells with insulin and hydrocortisone resulted in nearly a 4-fold increase in ErbB-2 expression, indicating responsiveness of WAP promoter to the hormone. Immunoprecipitation and Western blot analysis verified the presence of the 185 kDa, phosphorylated human ErbB-2 (data not shown).

Purified WAP-Her-2 expression cassette was microinjected into fertilized eggs from B6C3 (C57BL/6 × C3H)F1 females mated with B6C3 males following the procedure described by Hogan et al. (20). The embryos were implanted into pseudopregnant CD-1 surrogate mothers. From 97 live births, 7 positive mice were identified by Southern blot analysis using tail tissue lysates. These Tg founders were bred with C57BL/6 mice. A total of 60 pups were produced. Seven pups from 4 founders, carried the transgene as detected by Southern blot. These 7 pups were regarded as the F1 founders and were mated with C57BL/6 mice to produce the first generation of backcross (B1) mice. Transgene-positive B1 male mice were backcrossed with female C57BL/6 mice and transgene distribution in subsequent offspring followed Mendalian rule.

FIGURE 1. Construction of Her-2 transgene and genotypic identification of Her-2 Tg mice. A, A schematic representation of WAP regulated Human ErbB-2 (Her-2) transgene. The transgene is a 6939 bp linear HindIII fragment consisting of the 2.6-kb mouse WAP promoter fused with downstream 4.4 kb human ErbB-2 cDNA. Polyadenylation sequence at the 3’ end of the gene are from the bovine growth hormone gene. The shaded arrows indicated the location of PCR primers used in genotyping. Probe for Southern blotting and the relevant EcoRI restriction sites are indicated. B, Expression of the human ErbB-2 transgene. D2F2 mouse mammary tumor cells were cotransfected with the WAP-Her-2 cassette and pRSV-neo. Stable clones expressing human ErbB-2 were selected and cultured in the absence (left panel) or presence (right panel) of 10 μM hydrocortisone and 10 μg/ml insulin for 48 h. Cell surface expression of human ErbB-2 was evaluated by flow cytometry with mAb TA-1 and detected with FITC conjugated goat anti-mouse secondary Ab (shaded histogram). The clear histogram represents binding of an isotype control Ab. C, Genotype analysis of Her-2 Tg mice. Positive identification of Tg animals is indicated by hybridization of the probe to a 1.6-kb EcoRI fragment (top) and by the amplification of a 352-bp PCR product (bottom). Southern blot and PCR analyses were performed on genomic DNA from the same 10 pups and the results demonstrated perfect concordance.
Tg mouse line which had the highest and most consistent Tg expression in the early generations was chosen to establish our colony. All studies described here were performed on the progeny of this line.

For routine screening of transgene expression, PCR primers were designed so that the upper primer annealed to the WAP promoter and the lower primer annealed to ErbB-2 cDNA. A 351-bp product was diagnostic of WAP-Her-2 gene. Using ear punch tissue from 10 pups of the first backcross (B1) generation, there was 100% concordance between Southern blotting and PCR analysis (Fig. 1C). Therefore, routine screening was performed with PCR.

**Tissue distribution of human ErbB-2 in Her-2 Tg mice**

Human ErbB-2 expression in the mammary gland was examined in several Her-2 Tg females of the F1 (founder × C57BL/6) generation (Fig. 2) at days 1–2 of lactation.

The no. 4 mammary glands were removed from Her-2 Tg females at 1–3 days after they delivered the first or second litter. Tissue lysates were prepared and immunoprecipitated with mAb Herceptin which recognized an epitope in the extracellular domain of human ErbB-2 and blotted with mAb 3B5 which recognized a carboxyl-terminal epitope. In Her-2 Tg mice from the F1 (lanes 4–5) generation, strong expression of p185 was detected. The same dominant band was detected in tissue lysate without immunoprecipitation (lane 3). Furthermore, this female produced two female pups with abundant human ErbB-2 expression in the mammary gland (lanes 6–7). No expression was detectable in the non-transgenic lactating littersates (lane 8).

Tg mice were crossed with C57BL/6 mice for 12 generations to establish Her-2 Tg mice in the C57BL/6 background. Mammary glands from Her-2 Tg females exhibit the HER2/Neu “signature” phenotype of branching mammary structures with extension beyond the normal fat pad and lobules arrayed in parallel along the milk line and the main artery that supplies all of the mammary glands.

Expression of human ErbB-2 was sustained in mice backcrossed with C57BL/6. In Fig. 3A, immunoprecipitation (IP) and Western blot was used to detect Human ErbB-2 gene product in the mammary tissue and cerebellum of B11 females on days 17–19 of pregnancy or, postvaginal plug release. Tissue lysates were immunoprecipitated with mAbs: 9G6.10, (Fig. 3A, lanes 2 and 4) or Herceptin (lanes 3 and 5) which recognized different epitopes of human ErbB-2. Immunoprecipitated proteins were detected by Western blot using pAb C-18 (Santa Cruz Biotechnology). Similar to F1 mice, p185 was detected in both mammary (lanes 2 and 3) and cerebellum (lanes 4 and 5) tissue lysates. Lane 1 is the cerebellum tissue lysate used for immunoprecipitation analysis in lanes 4 and 5. In Fig. 3B, we defined the localization of human ErbB-2 in the mammary gland (Fig 3B, a and b) and cerebellum (Fig. 3B, c and d) using immunohistochemistry. Secretory mammary epithelia lining mature ductules and cross-sections of terminal endbud clusters exhibit intensely positive membrane staining by anti-human ErbB-2 Ab (arrows) while supporting stromal cells and fibroblasts are clearly negative (asterisks). In the cerebellum, intense ErbB-2 was detected in the molecular layer of the cerebellum (Fig. 3B, c and d). Distribution of the protein was prominent along the Bergman glia fibers (arrows, see figure legend) in the molecular layer (ML) and to a lesser extent in the membranes of the purkinje cells (PCL), but not detectable in the granular layer (GL). Transgene expression in the brain was also documented in WAP-hGH Tg mice (21).

ErbB-2 protein expression in Her-2 Tg males was also characterized. As in the females (Fig. 3B, c and d), intense human ErbB-2-specific immunoreactivity was observed in the molecular layer of the cerebellum in Her-2 Tg males (Fig. 3B, e and f). Human ErbB-2 protein was detected in the cerebellum by immunoprecipitation and Western Blotting (Fig. 3C, lane 3). Human ErbB-2 protein was not detected in the parotid (lane 1), other salivary glands (lane 2), or cerebrum (lane 4). The same tissues taken from a transgene-negative littermate were unequivocally negative (lanes 5–8). We further demonstrated that Tg ErbB-2 in the cerebellum was indeed phosphorylated on tyrosine residues using an HRP-conjugated mAb against phosphotyrosine (Fig. 3D) and verified the specificity using mAb P2NA that exclusively detects human ErbB-2 phosphorylated at tyrosine residue 1248 (not shown).

In males and females, human ErbB-2 protein distribution in the cerebellum was prominent in the Bergman glia cells, along their fibrous extensions and on the membranes of the purkinje cells which interface the molecular layer and the granular layer where the transgene is not expressed. This localization may indicate an association with the endogenous ErbB-3, which is expressed in the Bergman glia fibers and purkinje cells in adult cerebellum (22, 23). Other WAP-transgenes have been expressed in the brain, under hormonal regulation (24) or present in other regions of the brain (17). Constitutive ErbB-2 expression in the cerebellum appears unique to our Tg strain.

Compared with other organs tested, (including salivary gland, kidney, thymus, esophagus, adrenals, ovaries, and testes), ErbB-2 expression in the brain has been most consistent and at the highest level in Her-2 Tg mice from F1 to B12 generation independent of sex, lactational status, and parity (not shown). None of the tissues expressing human ErbB-2 demonstrated obvious abnormality. The mice were healthy and have a normal life span.

**Immune tolerance to ErbB-2**

To determine whether Her-2 Tg mice were tolerant to ErbB-2, growth of an immunogenic ErbB-2-bearing tumor was tested (Fig. 4). In normal C57BL/6 mice, injection of 5 × 106 ID8 ovarian cancer cells resulted in tumor growth after ~2 mo (19). Transfection of ID8 with ErbB-2 (ID8/E2) increased the immunogenicity of the tumor and C57BL/6 mice rejected ID8/E2 tumor cells. Her-2 Tg mice injected with 5 × 106 ID8/E2 cells began developing tumors after 5 mo and all mice were tumor positive by 9 mo (Fig. 4). Transgene-negative littersates did not develop ID8/E2 tumors (p < 0.01 when compared with Her-2 Tg mice). Flow cytometric
In a pilot study, we tested the induction of humoral immunity with vaccination. Anti-tumor immunity induced in Her-2 Tg mice by DNA. Tolerance was not broken by the growth of an ErbB-2 overexpression in the mammary tissue and cerebellum at lactation day (17–19). Tissues were minced and proteins were immunoprecipitated with mAbs specific for different epitopes of the human Erb-B2 protein; 9G6.10, (lanes 2 and 4) or Herceptin (lanes 3 and 5) in mammary (lanes 2 and 3) or cerebellum (lanes 4 and 5) tissue lysates. Proteins were detected using the polyclonal Ab C-18 (Santa Cruz Biotechnology). Lane 1 is a positive control cerebellum tissue lysate. The arrow points to the 185-kDa human Erb-B2. B) Immunohistochemical localization of human Erb-B2 in the mammary gland (a and b) and cerebellum (c and d) of a lactating (days 17–19) Her2-Tg female from the 11th backcross onto the C57BL/6 background. Secretory mammary epithelia lining mature ductules and cross-sections of terminal endbud clusters exhibit intensely positive membrane staining (arrows) while supporting stromal cells and fibroblasts are clearly negative (+). These mature lobulo-alveolar structures are characterized by single layers of secretory epithelial cells with few adipocytes. In addition, the nonsecretory immature, undifferentiated mammary gland cells failed to demonstrate robust membrane Erb-B2 staining. Expression of human Erb-B2 in the cerebellum of Her-2 Tg female (c and d) and male (e and f) mice. Intense immunoreactivity using the TAB250 human-Erb-B2 specific mAb was observed in the molecular layer (ML) of the cerebellum (d and f). Distribution of the protein was prominent alongside the Bergman glial fibers (arrows) and to a lesser extent in the membranes of the Purkinje cells (PCL), but not evident in the granular layer (GL). C) Immunoprecipitation and Western blot analysis of human Erb-B2 gene product in salivary gland and brain tissues of male Her-2 Tg mice. Human Erb-B2 protein is exclusively expressed in the cerebellum of males from the 10th backcross (lane 3). No human Erb-B2 protein was detected in the cerebrum (lane 4) parotid (lane 1), other salivary glands (lane 2) nor in any of these corresponding tissues in transgene-negative littermates (lanes 5–8). (D) The immunoprecipitated human ErbB-2 in the cerebellum is phosphorylated on tyrosine residues as detected by the anti-phosphotyrosine mAb, PY20-HRP.

**FIGURE 3.** Expression of Erb-B2 protein in Her-2 Tg mice fully backcrossed (11 generations) onto the C57BL/6 background. (A) Immunoprecipitation and Western blot analysis of human Erb-B2 gene product in the mammary tissue and cerebellum at lactation day (17–19). (B) Immunohistochemical localization of human Erb-B2 in the mammary gland (a and b) and cerebellum (c and d) of a lactating (days 17–19) Her2-Tg female from the 11th backcross onto the C57BL/6 background. Secretory mammary epithelia lining mature ductules and cross-sections of terminal endbud clusters exhibit intensely positive membrane staining (arrows) while supporting stromal cells and fibroblasts are clearly negative (+). These mature lobulo-alveolar structures are characterized by single layers of secretory epithelial cells with few adipocytes. In addition, the nonsecretory immature, undifferentiated mammary gland cells failed to demonstrate robust membrane Erb-B2 staining. Expression of human Erb-B2 in the cerebellum of Her-2 Tg female (c and d) and male (e and f) mice. Intense immunoreactivity using the TAB250 human-Erb-B2 specific mAb was observed in the molecular layer (ML) of the cerebellum (d and f). Distribution of the protein was prominent alongside the Bergman glial fibers (arrows) and to a lesser extent in the membranes of the Purkinje cells (PCL), but not evident in the granular layer (GL). C) Immunoprecipitation and Western blot analysis of human Erb-B2 gene product in salivary gland and brain tissues of male Her-2 Tg mice. Human Erb-B2 protein is exclusively expressed in the cerebellum of males from the 10th backcross (lane 3). No human Erb-B2 protein was detected in the cerebrum (lane 4) parotid (lane 1), other salivary glands (lane 2) nor in any of these corresponding tissues in transgene-negative littermates (lanes 5–8). (D) The immunoprecipitated human ErbB-2 in the cerebellum is phosphorylated on tyrosine residues as detected by the anti-phosphotyrosine mAb, PY20-HRP.

To examine the induction of anti-Erb-B2 Abs in Her-2 Tg mice, serum was collected 3 mo after ID8/E2 tumor cell injection. Anti-Erb-B2 Abs were not detected in Her-2 Tg mice while a significant induction of anti-Erb-B2 IgG Abs was detected in transgene-negative littermates (p < 0.05) (Fig. 5). Abs against the parental ID8 tumor were not detected in either group (Fig. 4). These results indicated that Her-2 Tg mice were tolerant to Erb-B2 and this tolerance was not broken by the growth of an Erb-B2 overexpressing tumor.

**Anti-tumor immunity induced in Her-2 Tg mice by DNA vaccination**

In a pilot study, we tested the induction of humoral immunity with pCMV-Erb-B2. After four i.m. injections with 100 μg DNA, 2 wk apart, anti-Erb-B2 IgG was detected in 2 of 10 Her-2 Tg mice, whereas 3 of 3 transgene-negative littermates generated high levels of anti-Erb-B2 specific Abs (not shown). To overcome tolerance in Her-2 Tg mice, we subjected Her-2 Tg mice and their transgene-negative littermates to a more robust DNA vaccination regimen. Her-2 Tg mice were covaccinated four times with pCMV secE2 encoding a secreted Erb-B2 extracellular domain and DNA encoding GM-CSF. Sera was collected one week after the fourth DNA vaccination and anti-Erb-B2 IgG Abs were measured as an indicator of anti-Erb-B2 immune response. Transgene-negative mice had significant anti-Erb-B2 IgG in their serum with an average of 32 ± 14 μg/ml (Fig. 6). Low level anti-Erb-B2 Abs were detected in 8 of 9 Her-2 Tg mice. One Her-2 Tg mouse had 43 μg/ml anti-Erb-B2 IgG indicating that tolerance to Erb-B2 was clearly overcome in this mouse. Anti-Erb-B2 Abs were not detected in any unvaccinated Her-2 Tg mice.

To test the effect of vaccination on tumor growth, vaccinated mice were boosted once more and challenged 2 wk later with EL-4/E2 cells. All transgene-negative littermates were protected against EL-4/E2 challenge (Fig. 7). In contrast, only 33% of Her-2 Tg mice were protected. Using log rank test, the protection of
vaccinated Her-2 Tg mice was less than that of vaccinated transgene-negative littermates (p < 0.05), but greater than that of control vector-injected Her-2 Tg mice (p < 0.05). Therefore, five courses of i.m. injection with DNA encoding human ErbB-2 partially overcame humoral and possibly cellular tolerance to protect mice from tumor growth.

Discussion

A human ErbB-2 Tg mouse strain (Her-2 Tg) syngeneic to C57BL/6 background has been established. Strong expression of human ErbB-2 was detected in lactationally active mammary epithelium and a high level of expression was detected constitutively in the brain of both male and female mice. Several other organs demonstrated variable expression. Her-2 Tg mice were tolerant to human ErbB-2 and permissive to the out-growth of tumors expressing human ErbB-2 without generating an Ab response. DNA vaccination in Her-2 Tg mice produced a modest but detectable anti-ErbB-2 Ab response and 33% of the animals were protected from tumor growth although 100% of the transgene-negative mice were protected. Induction of anti-ErbB-2 Ab was a sensitive indicator of immune response to ErbB-2 in the otherwise tolerant hosts, but cellular immunity may contribute significantly to tumor rejection as Ab level did not correlate directly with tumor rejection.

These results are comparable to those found in neu Tg mouse models. Tg mice expressing either normal or activated rat neu demonstrated tolerance to neu Ag. In FVB Neu-N Tg mice expressing MMTV-neu, immunization with irradiated whole cell or recombinant vaccinia virus induced very weak cellular and humoral response when compared with FVB mice (25). In BALB NeuT mice expressing MMTV-NeuT, neu specific Ab and antitumor immunity was induced by vaccination with Neu DNA, although the level of response is much reduced when compared with transgene-negative littermates (26). Our findings demonstrate that tolerance in Her-2 Tg mice was partially overcome by DNA vaccination. This model enables us to test human ErbB-2 based vaccination strategies in a realistic, tolerant host.

Previous report demonstrated that MMTV-Her-2 Tg mice died within 4 mo of age, probably due to kidney and lung failure following the development of preneoplastic lesion (16). A viable
Sprague-Dawley rat Tg for the wild-type human ErbB-2 gene has been described (27). In this model, expression of the Tg mRNA (under the control of the MMTV-long terminal repeat) was detectable in mid-pregnant but not virgin mammary tissues. After repeated cycles of pregnancy and lactation, pathological changes were produced in the mammary glands.

Using WAP promoter to express c-ErbB-2, Her-2 Tg mice developed normally without detectable lesions in the lung or kidney. ErbB-2 expression in the mammary gland was significant. The consistent and high level expression in the brain has been reported before with WAP promoter regulated transgenes. Human growth hormone driven by WAP promoter was highly expressed in the brain of both male and female Tg mice (21). Mice carrying WAP promoter regulated human urokinase-type plasminogen activator also demonstrated consistent transgene expression in the brain extract (28). Expression of human ErbB-2 in the brain did not have detectable pathological consequence in Her-2 Tg mice.

In normal human brain, ErbB-2 was detected consistently in oligodendrocytes, astrocytes and microglial cells and the level was elevated in patients with multiple sclerosis (29). ErbB-2 expressed in hypothalamic astrocytes mediates neuroendocrine functions. Luteinizing hormone-releasing hormone is released following stimulation of ErbB-2/4 complex on astrocytes by neuregulin, resulting in the development of female reproductive capacity (30). ErbB-2 is expressed in 86% of medulloblastoma, although it is not detected at any stage of cerebellar development, suggesting deregulation of ErbB-2 during medulloblastoma tumorigenesis (22). Cranial ganglia defects were detected in ErbB-2 knockout mice (31). ErbB-2 appears to play critical roles in normal and diseased brain. Further analysis of ErbB-2 in Her-2 Tg mouse brain may provide useful information. Vaccination of Her-2 Tg mice did not result in detectable autoimmune symptoms. Mice lived and reproduced normally. When Her-2 DNA vaccination efficacy is enhanced by increasing DNA uptake or modulating regulatory T cell, autoimmunity may be manifested and this should be closely monitored.

FVB NeuN mice crossed with C57BL/6 mice exhibit attenuated mammary tumor formation and increased tumor latency (32) and female mice develop tumors only after they experienced two to four pregnancies (33). The influence of strain background on mammary gland lesion incidence and phenotypes in WAP-TGFα TgS has recently been described by Rose-Hellekant et al. (34). Here, we show that expression of WAP-ErbB-2 in C57BL/6 background resulted in a Her-2 tolerant mouse strain without spontaneous tumor growth. This strain will be valuable for testing ErbB-2 based vaccination against different tumor types. They can also be bred with mice expressing human HLA transgene to test human ErbB-2 vaccination in different HLA background. If spontaneous tumors are desired, Her-2 Tg mice can be bred with various oncogene Tg or tumor suppressor gene knockout mice to produce tumors of different nature.

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References

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