Inhibition of Mammary Carcinoma Development in HER-2/neu Transgenic Mice through Induction of Autoimmunity by Xenogeneic DNA Vaccination

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Abstract
Plasmid DNA vectors encoding the full-length (VR1012/HER-2-FL) or only the extracellular and transmembrane domains (VR1012/HER-2-ECD-TM) of human (h) HER-2/neu proto-oncogene were used to vaccinate HER-2/neu transgenic mice (N202) engineered to overexpress the rat (r) HER-2/neu oncogene product (r-p185neo). Both the full-length and the deleted vaccines were significantly (P = 0.0001 and P = 0.06, respectively) more active than the empty vector (VR1012/EV) in preventing and delaying HER-2/neu-driven mammary carcinogenesis. A low-level intratumoral infiltrate of dendritic cells, macrophages, CD8 T cells and polymorphonuclear granulocytes in association with low-level cytokine production was observed, which was not detected in tumors from control mice. Morphologic analyses showed that vaccination with VR1012/HER-2-FL or ECD-TM also efficiently hampered the development of terminal ductal lobular units (TDLU). Analyses of sera from vaccinated mice revealed high titers of antihuman HER-2/neu antibodies, which correlated with the delayed time of tumor onset (P = 0.002). These antibodies did not cross-react with r-p185neo. Nontransgenic mice treated with the vaccines produced autoreactive antibodies targeting mouse (m)-p185neo and showed impaired function of the lactating mammary gland and accelerated involution of the gland after weaning. Together, these data indicate that xenogeneic DNA immunization breaks tolerance against the endogenous m-p185neo, impairing the development of mammary TDLU in which m-p185neo expression is concentrated. The reduction in the number of TDLU decreases the number of glandular structures available for r-p185neo-dependent mammary carcinogenesis, resulting in a significant inhibition of mammary carcinoma development. (Cancer Res 2005; 65(3): 1071-8)

Introduction
The HER-2/neu oncogene is amplified and overexpressed in 20% of primary human breast cancers and is strongly associated with poor prognosis. Due to the tumor specificity of its overexpression, the HER-2/neu oncoprotein is considered one of the most attractive targets for immunotherapeutic intervention (1). We previously showed that HER-2/neu overexpression is associated with a better outcome when inflammatory infiltrates are present in the tumor (2–4), suggesting a role for HER-2/neu in tumor immunosurveillance. In addition, antibodies and T cells reactive to HER-2/neu occur naturally in tumor-bearing patients (5–8), confirming the in vivo immunogenicity and ability of HER-2/neu to break self-tolerance. Recently, active immunotherapy regimens have been devised to generate specific T-cell responses with or without antibody responses and are currently being tested in animal models or in clinical trials (9, 10). Transgenic mice overexpressing HER-2/neu oncoprotein in its inactivated or mutated forms (11) provide attractive models for testing immunoprevention of mammary carcinogenesis and for gathering preclinical data that might predict the response to active immunotherapy in a clinical setting (12, 13).

To date, the optimal vaccine formulation remains undefined and the mechanisms underlying the therapeutic and preventive effects mediated by such vaccines await elucidation (1, 14). Increasingly powerful methods to stimulate antitumor immune responses have been developed, including vaccination with "foreign" antigens through plasmid DNA xenoinmunization, and shown to break tolerance against self-antigen (15, 16). However, exploitation of overexpressed molecules requires that the immune system discriminate cells that express physiologic levels of the self-antigen (normal cells) from cells that overexpress the antigen (tumor cells). Nevertheless, vaccines that target self-antigens and destroy normal cells expressing these antigens might be a useful tool for cancers arising from cells of nonessential organs, such as melanocytes, breast, ovary, prostate, thyroid, and testis (14).

Here, we provide evidence that xenogeneic DNA vaccination with plasmid DNA vectors encoding the full-length HER-2/neu gene (VR1012/HER-2-FL) inhibits mammary carcinogenesis through an autoimmune reaction in HER-2/neu-overexpressing N202 mice by reducing the number of mammary epithelial cells at risk for transformation.

Materials and Methods
hHER-2/neu Plasmids and DNA Production. The plasmid vector VR1012/HER-2-FL was produced as described (17). The VR1012/HER-2-ECD-TM plasmid vector, encoding for a truncated molecule formed by extracellular (EC) and transmembrane (TM) domains, was generated by ligation of a BglI/BglI HER-2/neu cDNA fragment and a synthetic 50-bp BglI/XbaI DNA fragment into the SalI/XbaI-digested VR1012. The synthetic fragment, containing three in-frame stop codons, was generated by annealing two synthetic oligonucleotides.

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(5'-GTCCTGCGCTTGGAGGATGAGGTGACG-3' and 5'-CTGCTAGAGGATCACCAGGCTTACCTCCA-3') displaying an 11-bp complementary 3' end, followed by fill-in with DNA-polymerase I Klenow fragment (Roche, Milano, Italy). Sequence analysis verified the correct insertion of the resulting HER-2-ECD-TM fragment. Induction of protein expression by VR1012/HER-2-FL and VR1012/HER-2-ECD-TM was confirmed by indirect immunofluorescence and fluorescence-activated cell-sorting (FACS) analysis of human embryonic kidney (HEK 293) and murine neuroblastoma (Neuro2a) cells transiently transfected using the Fugene reagent (Roche). Plasmids were purified by anion-exchange chromatography using the Qiagen plasmid maxi kit (Valencia, CA) according to the manufacturer's instructions. Aliquots of purified plasmid (1 mg/mL in sterile PBS) were stored at −80°C.

Mice and Vaccination Schedules. N202 mice from FVB-neuT transgenic mice overexpressing the inactivated form of the rHER-2/neu proto-oncogene (18) were used. Animal care was in accordance with European Union institutional guidelines. Mammary glands of N202 mice were inspected weekly, and each tumor mass was measured with calipers in the two perpendicular diameters and calculated as described (17). Before manipulation, mice were anesthetized i.p. with 80 to 100 mg/kg of ketamine and 10 mg/kg of xylazine. Twelve-week-old virgin N202 mice were inoculated intradermally in the ear pinna using a 32-gauge needle once monthly for 10 months with 100 μg of plasmid DNA vectors (VR1012/HER-2-FL, -ECD-TM, and -EV) diluted in 50 μL of sterile PBS or left untreated and evaluated for spontaneous tumor incidence. BALB/c mice and mice purchased from Charles River (Calco, Lecco, Italy) were given injections (four times at 2-week intervals) of VR1012/HER-2-FL and VR1012-EVD plasmids (n = 5 in each group) before mating with BALB/c males. Immediately after delivery, two consecutive offspring were monitored daily for weight. For foster-nursing experiments, newly delivered pups from vaccinated and control BALB/c females were grouped in equal number (n = 5 in each group) for nursing by natural and foster-nursing mothers. Weight of pups was monitored daily in parallel. For cytofluorimetric analyses of mouse sera, blood was obtained from the retroorbital venous plexus of anesthetized mice. Sera from each group of mice were stored in aliquots at −30°C until tested.

Cells. The human breast carcinoma cell line SKBR-3 and the lung large cell carcinoma cell line H460, which overexpress or do not express, respectively, the hHER-2/neu oncoprotein, and the human embryonic kidney cell line HEK 293 were obtained from American Tissue Culture Collection (Rockville, MD). The murine mammary carcinoma lines N202.1A and N202.4 were described as described (19). All carcinoma cell lines were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated FCS (Life Technologies Inc., Paisley, United Kingdom) and 2 mMol/L l-glutamine at 37°C in a humidified atmosphere of 5% CO2 in air. The murine mammary normal gland epithelial cell line NMuMG (20) and the neuroblastoma Neuro2a were obtained from the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology and Research, Salisbury, United Kingdom) and maintained in DMEM (Sigma) supplemented with 10% FCS and 2 mmol/L L-glutamine and daily with 10 μg/mL of insulin (El Lilly Italia SpA, Sesto Fiorentino, Italy) only for NMuMG cells, at 37°C in a humidified atmosphere of 5% CO2 in air.

Immunohistochemistry, Whole-Mount Analysis, and Histology. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 minutes with antibodies that specifically detect the following: dendritic cells (NLD 145, Cederlane, Ontario, Canada), Mac1 (anti-CD11b/CD18) and Mac3 (both from Boehringer Mannheim, Milan, Italy), polymorphonuclear granulocytes (RB-6-8C5, provided by Dr. R.L. Coffman, DNAX Research Institute of Molecular and Cellular Biology Inc, Palo Alto, CA), CD8 and CD4 T cells (both from Sera-Lab, Crawley Down, Sussex, United Kingdom), asialo-GM1 (Wako Chemicals GmbH, Dusseldorf, Germany), interleukin (IL)-1β (Genzyme, Cambridge, MA), tumor necrosis factor α (TNF-α; Immuno Kontakt, Frankfurt, Germany), IFN-γ (provided by Dr. S. Landolfo, University of Turin, Turin, Italy), macrophage chemotactant protein (MCP)-1 (PharMingen, San Diego, CA), macrophage inflammatory protein (MIP)-2 (Valter Occhiena s.r.l., Turin, Italy), Rantes (PeproTech Inc., Rocky Hill, NJ), inducible nitric oxide (iNOS; macrophage inducible; Transduction Laboratories, Lexington, KY). To evaluate the expression of r-p185neu, paraffin-embedded sections were tested with anti-neu antibody (C-18, Santa Cruz Biotechnology, Santa Cruz, CA). After washing, sections were overlaid with biotinylated goat antirat, antihamster, and antirabbit or horse anti-goat immunoglobulin (Vector Laboratories, Burlingame, CA) for 30 minutes. Unbound antibody was removed by washing and the slides were incubated with avidin-biotin complex/alkaline phosphatase (Dako). Quantitative studies of immunohistochemically stained sections (Table 1) were done independently by three pathologists in a blind fashion. From mice with multiple tumors, tumor growth area and 10 randomly chosen fields were evaluated for each sample. Positive cells were counted under a microscope (×40 objective and ×10 ocular lens; 0.180 mm2 per field). Cytokine expression was defined as absent (−), scarce (±), and moderate (+) in cryostat sections tested with the corresponding antibody.

Whole-mount preparations of mammary glands were done as indicated in http://ccm.ucdavis.edu/tgmouse/HistoLab/wholmt1.htm and stained with ferric hematoxylin. Images of whole-mount preparations were taken with a Nikon Coolpix 990 digital camera (Nital Spa, Torino, Italy) mounted on a stereoscopic MZ 6 microscope (Leica Microsystems, Milano, Italy). After whole-mount image acquisition, two thoracic mammary glands for each animal were embedded in paraffin, sectioned at 4 μm, and stained with H&E for histologic evaluation.

Indirect Immunofluorescence and FACS Analysis. The rHER-2/neu transgene product was detected using mouse monoclonal antibody (mAb) Ab4 (2.5 μg/mL; Oncogene Research Products, Boston, MA). Products of the h- and mHER-2/neu oncoprotein were revealed using

| Table 1. Immunohistochemical analysis of mammary tumors in vaccinated N202 mice |
|-------------------|-------------------|-------------------|
|                   | VR1012/HER-2-FL   | VR1012/HER-2-ECD-TM |
| Reactive cells*   |                   |                   |
| Dendritic cells   | 4.1 ± 1.2         | 5.8 ± 1.5         |
| Macrophages       | 4.2 ± 1.3         | 6.4 ± 1.2         |
| Polymorphonuclear granulocytes | 3.3 ± 1.4   | 13.1 ± 3.5 |
| CD8               | 5.3 ± 2.2         | 11.6 ± 3.0        |
| CD4               | 3.0 ± 1.2         | 3.0 ± 1.5         |
| Natural killer cells | 3.0 ± 1.1   | 3.2 ± 1.0        |
| Cytokines†        |                   |                   |
| IL-1β             | ±                 | ±                 |
| TNF-α             | ±                 | ±                 |
| IFN-γ             | ±                 | ±                 |
| MCP-1             | ±                 | ±                 |
| Chemokines        |                   |                   |
| MIP-2             | ±                 | ±                 |
| Rantes            | ±                 | ±                 |
| iNOS              | ±                 | ±                 |

NOTE: Mammary tissues of 38-week-old mice were analyzed.
*Cell counts were determined at ×400 magnification in 10 randomly chosen fields, each 0.180 mm2. Results are expressed as mean ± SD positive cells per 0.180-mm2 field.
†Values significantly different (P < 0.005) from the corresponding values in untreated and empty vector–vaccinated mice.
‡Expression of cytokines was defined as absent (−), scarce (±), moderate (+) in cryostat sections tested with the corresponding antibody.

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mouse mAb MGR-2 (2.5 μg/mL) produced in our laboratory (21) and the rabbit polyclonal antibody C-18 (2 μg/mL), respectively. Cells were stained for 1 h at 0°C with the primary antibodies listed above or sera from mice (diluted 1:20) followed by a fluorescein-conjugated anti-mouse and anti-rabbit immunoglobulin G (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:100 for 1 h at 0°C. After final washings, cells were resuspended in PBS containing propidium iodide (1 μg/mL) to identify dead cells and evaluated in a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Viable cells (1 × 10^6) were analyzed in each evaluation. Data acquisition and analysis were done using CellQuest software.

**Radioimmunoprecipitation.** SKBR-3 and N202.1A live cells in suspension (10 × 10^6) were membrane-labeled by lactoperoxidase-catalyzed radiodiobination (22) and immunoprecipitation was done as described (23). The eluted and denatured immune complexes were resolved on 7.5% polyacrylamide gels and the separated proteins autoradiographed at −80°C.

**Results**

**Incidence of Mammary Carcinomas in N202 Mice after Xenogeneic DNA Vaccination.** Analysis of N202 mice vaccinated with plasmid vectors VR1012/HER-2-FL, VR1012/HER-2-ECT-TM, or VR1012/EV revealed significantly longer tumor-free survival in mice given injections of VR1012/HER-2-FL compared with animals given injections of VR1012/EV (P < 0.0001) or left untreated (P < 0.0001; Fig. 1). The tumor-preventive effect of the VR1012/HER-2-ECT-TM construct, which lacks most of the catalytic and autophosphorylation sites of hHER-2/neu, showed borderline significance when compared with controls (P = 0.06).

**Histologic and Immunohistochemical Features of Mammary Tissue in Vaccinated Transgenic Mice.** By 39 weeks of age, untreated and VR1012/EV-treated N202 mice displayed one or more mammary glands with a lobuloalveolar carcinoma formed by neoplastic cells that overexpressed the rHER-2/neu oncogene product, r-p185neu, on their surface (data not shown). Mouse treated with VR1012/HER-2-FL or VR1012/HER-2-ECT-TM plasmids also showed mammary carcinomas widely and homogeneously expressing r-p185neu on the neoplastic cell membrane, but the tumors were smaller (data not shown).

Analyses to determine whether antitumor immunologic events in the mammary glands mediate the tumor growth inhibition observed after vaccine administration revealed a significant infiltration (P < 0.005) of dendritic cells, macrophages, polymorphonuclear granulocytes, and CD8+ lymphocytes in association with low to moderate production of IL-1β, TNF-α, IFN-γ, and MCP-1 in the mammary tumor stroma after VR1012/HER-2-FL or VR1012/HER-2/ECT-TM administration as compared with tumors grown in VR1012/EV-treated or in untreated mice (Table 1; Fig. 2A, a-f). No expression of chemokines such as MIP-2, or Rantes was detected, and no induction of macrophagic iNOS, an activation marker of these cells, was observed after such vaccinations (Table 1).

**Mammary Gland Morphology in Vaccinated N202 Mice.** Whole-mount analysis of mammary glands from N202 mice vaccinated with VR1012/HER-2-FL, VR1012/HER-2-ECT-TM, or VR1012/EV or left untreated was done at 29, 39, and 52 weeks of age. At 29 weeks, an average of two mammary glands per mouse in all the experimental groups revealed small tumor foci. The tumor lesions found in VR1012/HER-2-FL-vaccinated or untreated mice were larger compared with those in the other two groups. The glands from VR1012/HER-2-FL–vaccinated mice showed an impaired lobulization with fewer and less developed terminal ductal lobular units (TDLU) (data not shown). At 39 weeks, multiple tumor foci of different sizes were present in all of the mice. In VR1012/HER-2-FL–immunized mice, fewer glands were affected by tumors and the average number of tumor foci per mammary gland was lower compared with VR1012/HER-2–treated mice (mean ± SD; 0.6 ± 0.58 versus 1.5 ± 0.50; P < 0.005). At this age, the group of VR1012/HER-2-FL–vaccinated mice also showed an impairment in the number and the development of TDLUs (Fig. 2B).

The pathologic evaluation of 52-week-old mice showed that more than 90% of VR1012/HER-2-treated or untreated mice had one or more mammary glands with palpable tumors (diameters > 3 × 3 mm), and multiple tumor foci were present in the other glands macroscopically free of tumors. About 70% of mice vaccinated with VR1012/HER-2-FL were free of macroscopic tumors, and whole-mount analysis showed that several mammary glands were also free of microscopic tumor foci, whereas the others presented only a small number of tumor foci.

**Immunologic Events Associated with Tumor Prevention.** The antibody response present in the sera from the different transgenic mice groups was tested by FACS analysis using the hHER-2/neu-overexpressing breast carcinoma cell line SKBR-3 (H in Fig. 3A) and the rHER-2/neu-overexpressing syngeneic mammary carcinoma cell line N202.1A (R in Fig. 3A). Sera from DNA-vaccinated mice revealed high titers of anti-hHER-2/neu immunoglobulin G antibodies, whereas no reactivity on rHER-2/neu was observed. The specificity of the antibody was confirmed by biochemical analysis of 125I-labeled SKBR-3 cells (Fig. 3B), in which only antibodies present in the pool of SKBR-3–immunoreactive sera recognized the 185-kDa molecule (lane 4) identified by MGR-2 mAb as hHER-2/neu (lane 1), whereas the pools of control sera (lanes 2 and 3, respectively) were negative. Similar analysis using radiolabeled N202.1A cell extract and the same serum samples revealed no reactivity at the 185-kDa position (data not shown).

The level of anti-hHER-2/neu antibodies present in the sera of vaccinated mice, even after seven DNA vaccinations, was heterogeneous (Fig. 3C), but the level of antibody response nevertheless correlated significantly with the time of tumor onset (P = 0.002) in mice vaccinated with VR1012/HER-2-FL (Fig. 3C), indicating that vaccination efficiency is associated with a stronger preventive
effect. In four VR1012/HER-2-FL–vaccinated mice protected from tumor development the mean (± SE) immunofluorescence value for anti-hHER-2/neu antibody levels measured at 52 weeks of age was 61.5 ± 9.3 as compared with 30.6 ± 3.3 determined for DNA-vaccinated mice at tumor onset. The mean (± SE) immunofluorescence of seronegative sera was 18.4 ± 0.88.

Because CD8+ cells significantly infiltrated the tumor growth area in VR1012/HER-2-FL–vaccinated mice, we further analyzed the possible involvement of a CTL response in this model. No induction of CTL activity against the syngeneic N202.1A mammary tumor was observed in splenocytes from vaccinated mice upon in vitro mixed lymphocyte-tumor culture restimulation (data not shown), consistent with our previous findings (17).

Effects of Xenogeneic DNA Vaccination on Normal Mammary Tissue Development and Function. Based on our analyses revealing that vaccination with either VR1012/HER-2-FL or VR1012/HER-2-ECD-TM (a) elicited only a moderate inflammatory reaction in the neoplastic mammary tissue without altering r-p185<sup>++</sup> tumor cell expression and (b) inhibited the development of mammary TDLU, we tested whether the preventive antitumor effect was due largely to autoreactivity against normal mammary gland structures. Indeed, the sequence alignment of the r-, m- and hHER-2/neu proteins showed several regions, which are more conserved between h- and m- than in rECD domain, and may therefore represent the source of potential epitopes common to h- and mECD domain (Fig. 3D).

To test the ability of xenogeneic DNA vaccination to induce an immunologic response against mHER-2/neu protein that affects lobular structures development (24), we repeated the vaccinations in BALB/c mice characterized by mammary lobular development only during gestation and lactation, and evaluated their lactating capabilities. The mean weight of BALB/c pups from VR1012/HER-2-FL–vaccinated mice, similar at birth to the mean of pups from control mice, was significantly lower after 7 days than that from VR1012/EV-vaccinated control females in two consecutive offspring (<i>P</i> < 0.001, <i>r</i><sup>2</sup> > 0.9; Fig. 4A and B). Foster-nursing experiments to verify the role of lactation in this pup weight decrease showed that pups from VR1012/HER-2-FL–vaccinated mothers and fostered by control mothers (column c) displayed increased weights compared with pups fostered by their vaccinated mothers (column d; <i>P</i> = 0.0004). By contrast, pups from control mothers fostered by DNA-vaccinated mothers (column b) showed decreased weight compared with pups nursed by the control mothers (column a; <i>P</i> = 0.0014; Fig. 4C). In keeping, morphologic analysis of mammary tissue from DNA-vaccinated BALB/c mothers evaluated 5 days after weaning (Fig. 4D) showed that postlactational involution was much more advanced compared with that of postlactating controls evaluated at the same time (Fig. 4E).

To investigate whether antibodies cross-reactive with mHER-2/neu were produced after anti-hHER-2/neu vaccinations, the serum reactivity of control and vaccinated BALB/c (Fig. 5A) and N202...
(Fig. 5B) mice was tested against the normal murine mammary epithelial cell line NMuMG, which expresses mHER-2/neu protein. Different pools of sera including sera with high (pool c) or low (pool b) reactivity on SKBR-3 cells were tested. Cytofluorimetric analysis revealed higher levels of autoreactive antibodies in the two pools c from vaccinated BALB/c and N202 mice than in the two pools b (histograms c and b, respectively). The pools of control sera (pools a) were negative (histograms a) on murine NMuMG cells, as the internal controls of the assay. Histograms d show the binding of anti-mHER-2/neu C18 antibody (positive control) on NMuMG cells.

**Discussion**

We previously reported that xenogeneic DNA strategy with VR1012/HER-2-FL construct induced tumor protection only when immunization was started in 3-month-old but not in 6-month-old N202 mice and that the preventive effect achieved by our immunologic approach seemed not to be based on anti-rHER-2/neu specific B- and T-cell immune attacks (17). In addition, immunity to the rHER-2/neu molecule does not seem to be involved in tumor prevention because vaccination with VR1012/HER-2-FL did not exert any therapeutic benefits on syngeneic implanted mammary adenocarcinoma N202.1A cells, as we recently reported (1).

In this study, we provide evidence that early immunization of N202 mice with the xenogeneic DNA vaccine VR1012/HER-2-FL induces protection from and delayed onset of tumors through induction of autoantibodies that target the endogenous mHER-2/neu protein, leading to a reduced number of normal mammary epithelial cells at risk for transformation.

We also tested the VR1012/HER-2-ECD-TM plasmidic vector because vaccination with a construct lacking the tyrosine kinase activity of the immunogen should be preferred in patients. Unexpectedly, differently from the active immunization reported using the rECD-TM construct in a different transgenic mouse model.
(25–27), our human plasmidic vector is less efficient. The poor effectiveness of the VR1012/HER-2-ECD-TM construct in tumor prevention indicates that relevant helper epitopes might be present in the intracellular domain region (9). In agreement, previous preclinical studies showed that the inclusion of the HER-2/neu intracellular domain subunit in active vaccination protocols elicited protective antitumor immunity (28). Other explanations for the low efficacy of the truncated construct might rest in its low in vivo transfection capability, as suggested by in vitro transient transfections and immunofluorescence assays with both the Neuro2a and HEK 293 cell lines, showing that the surface expression of the hHER-2/neu was about 50% (as mean fluorescence intensity) lower when induced by the VR1012/HER-2-ECD-TM compared with the VR1012/HER-2-FL plasmid (data not shown).

On the other hand, the lack of anti tumor activity mediated by the injection of the empty vector indicates that CpG motifs present in the VR1012 vector backbone do not induce a detectable antitumor response (29).

Xenogeneic hHER-2/neu DNA codes not only for peptides that are identical or peptides that are completely different from those of mouse and rat but also for peptides with minor alterations that function as "heteroclitic" peptides, with increased binding affinity

Figure 4. A and B, effect of xenogeneic DNA vaccination on the growth of pups from five vaccinated (▲, VR1012/HER-2-FL) and control (●, empty vector) BALB/c females. A, first offspring. The mean weight of 36 newborns in the vaccinated group differed significantly (P < 0.001) from that of 24 newborns in the control group. B, second offspring. The mean weight of 34 newborns in the vaccinated group differed significantly (P < 0.001) from that of 39 newborns in the control group. C, weight of foster-nursed pups at 7 days from birth (n = 5 in each group). Column a, control pups nursed by control (empty vector vaccinated) mother (mean weight ± SEM, 4.38 ± 0.09); column b, control pups foster-nursed by VR1012/HER-2-FL–vaccinated mother (3.90 ± 0.15); column c, pups from VR1012/HER-2-FL–vaccinated mother foster-nursed by control mother (3.94 ± 0.08); column d, pups from VR1012/HER-2-FL–vaccinated mother nursed by VR1012/HER-2-FL–vaccinated mother (3.44 ± 0.06). Differences between columns: a and d, P < 0.0001; a and b, P = 0.014; a and c, P = 0.008; c and d, P = 0.0004 were analyzed by unpaired t tests. D and E, morphology of mammary tissue from VR1012/HER-2-FL–treated BALB/c mice examined 5 days after weaning. The mammary gland of VR1012/HER-2-FL–vaccinated mice showed an advanced grade of involution, with disappearance of secretory lobules and ducts actively remodeling to the prepregnancy status, occupying 8% of the mammary fat pad. Mammary glands from VR1012-EV–treated mice remained composed of collapsing secretory lobules, with infiltrating inflammatory cells (open arrows). Droplets of secretum (arrowheads) are visible in collecting ducts, and vascular (arrows) and stromal component are still well represented. Glandular ducts occupy 29% of the mammary fat pad. Magnification ×200.

Figure 5. Evidence of anti-mHER-2/neu autoreactive antibodies. Cytofluorimetric analysis of murine NMuMG cells tested with sera from BALB/c (A) and N202 (B) mice. Antibody C-18 recognizing mHER-2/neu (histograms d); pools c, from VR1012/HER-2-FL–vaccinated BALB/c (n = 3) and N202 (n = 2) mice containing antibodies with high reactivity on SKBR-3 cells (histograms c); pools b, from VR1012/HER-2-FL–vaccinated BALB/c (n = 3) and N202 (n = 2) mice containing antibodies with low reactivity on SKBR-3 cells (histograms b); pools a, from control BALB/c mothers (n = 4) and untreated N202 (n = 5) mice (histograms a).
for MHC molecules (30). The use of such heteroclitic peptides allowed generation of specific T cells not only against the heteroclitic but also against the native peptide (31). The presence of heteroclitic peptides may thus induce a cross-reactive immune responses against a self-antigen. Indeed, we found that vaccinations with xenogeneic DNA constructs gave rise to auto-antibodies against HER-2/neu self-antigen and hampered the development of TDLU in normal mice, which in turn reduced the incidence of tumors derived from these structures. The reasons why xenogeneic vaccination does not break tolerance against the rat transgene might rest in the sequences highly conserved between mouse and human and not present in the rat structure. Accordingly, immunizations with DNA vaccines coding for the rHER-2/neu was able to break tolerance against the transgene with production of antibodies against r-p185neo which cross-reacted with mHER-2/neu (27).

The absence of obvious autoimmune lesions may be mainly attributable to the very poor expression of mHER-2/neu by the tissues of adult mice. However, in the mammary gland the cells expressing mHER-2/neu are inhibited by the autoreactive antibodies. Possibly, the reactive cell infiltrate observed in the stroma of tumors from both VR1012/HER-2-FL and VR1012/HER-2-ECD-TM plasmid–vaccinated mice mirrors an attempt of antibody-dependent cellular cytotoxicity toward the m-p185neo expressed by TDLU from which the r-p185neo driven mammary cancer takes place. HER-2/neu and the other receptors of the HER family play a role in different stages of mammary gland development as shown by impairment of mammary development in mice with some defects in these receptors (32). HER-2/neu also plays a fundamental role in development of other organs such as heart and central nervous system. However, its role is confined to embryonic differentiation, unlike the mammary gland, which continues to develop even in adult life after hormone stimulation. Thus, an immunologic response induced against HER-2/neu antigen is expected to perturb mammary gland development but not heart or nervous system. Accordingly, no systemic pathologic damage was evidenced in either vaccinated adult female mice or their offspring, which were healthy after birth and developed normally except for a lower weight during mother nursing. However, in view of a future clinical trial based on vaccination against HER-2/neu, detailed analyses of a possible cardiac dysfunction mediated by induction of anti-HER-2/neu “trustuzumb-like” antibodies will be required (33).

Previous studies have revealed the existence of B-cell epitopes shared by the h- and rHER-2/neu molecules in the ECD domain (34, 35). Our present findings reveal that other promiscuous B-cell epitopes are shared by the h- and the m- molecules, but not by the r- suggesting the use of the mHER-2/neu construct for immunization of patients.

The generation of tissue-specific autoimmune responses represents an approach to cancer immunotherapy that is gaining momentum, and the ability to induce tissue-specific autoreactivity will allow treatment of cancers arising from nonessential organs, such as melanocytes, breast, prostate, thyroid, and testis (14). This issue is of particular practical importance for young women at hereditary risk of breast cancers, especially those with mutations in the BRCA1 or BRCA2 gene, for whom an “immunologic mastectomy” would represent a great improvement over double-surgical prophylactic mastectomy.

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References


Inhibition of Mammary Carcinoma Development in HER-2/neu Transgenic Mice through Induction of Autoimmunity by Xenogeneic DNA Vaccination

Serenella M. Pupa, Manuela Iezzi, Emma Di Carlo, et al.


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