Electroporated DNA Vaccine Clears Away Multifocal Mammary Carcinomas in Her-2/neu Transgenic Mice

Elena Quaglino,1 Manuela Iezzi,2 Cristina Mastini,1 Augusto Amici,3 Federica Pericle,1 Emma Di Carlo,2 Serenella M. Pupa,2 Carla De Giovanni,5 Michela Spadaro,1 Claudia Curcio,1 Pier Luigi Lollini,5 Piero Musiani,2 Guido Forni,1 and Federica Cavallo1

1Department of Clinical and Biological Sciences, University of Turin, Orbassano; 2Center of Excellence on Aging (CeSI), University of Chieti; 3Department of Molecular, Cellular and Animal Biology, University of Camerino, Camerino; 4Molecular Targeting Unit, National Cancer Institute, Milan; and 5Cancer Research Section, Department of Experimental Pathology, University of Bologna, Bologna, Italy

ABSTRACT

The transforming rat Her-2/neu oncogene embedded into the genome of virgin transgenic BALB/c mice (BALB-neuT) provokes the development of an invasive carcinoma in each of their 10 mammary glands. i.m. vaccination with DNA plasmids coding for the extracellular and transmembrane domains of the protein product of the Her-2/neu oncogene started when mice already display multifocal in situ carcinomas temporarily halts neoplastic progression, but all mice develop a tumor by week 43. By contrast, progressive clearance of neoplastic lesions and complete protection of all 1-year-old mice are achieved when the same plasmids are electroporated at 10-week intervals. Pathological findings, in vitro tests, and the results from the immunization of both IFN-γ and immunoglobulin gene knockout BALB-neuT mice, and of adoptive transfer experiments, all suggest that tumor clearance rests on the combination of antibodies and IFN-γ-releasing T cells. These findings show that an appropriate vaccine effectively inhibits the progression of multifocal preneoplastic lesions.

INTRODUCTION

The concept of the preventive efficacy of antitumor vaccines comes from a multitude of experiments showing that preimmunization efficiently inhibits subsequent lethal tumor challenges (1, 2). There is overwhelming evidence that eradication of fast growing transplantable tumors rests on swift high-affinity T-cell reactivity (3), whereas B cells may interfere with the efficiency of the reaction (4). By contrast, the question of whether a vaccine can inhibit the progression of carcinogenesis is rarely addressed (5). Although several theoretical considerations suggest that the chronic progression of early neoplastic lesions is an appropriate target for an immune control (6), the effective inhibitory potential of T cell and antibody-mediated reaction mechanisms elicited by a vaccine is still undetermined. This assessment is important because it may provide new information which may not coincide with that obtained from the study of fast growing transplantable tumors (7). Progression of a preneoplastic lesion is a lengthy process that may be hampered by mechanisms that are not efficacious when confronted with the unnatural speed of transplantable tumors.

Tumors which develop in transgenic mice as a consequence of a defined gene alteration form an experimental model for evaluation of the preventive efficacy of innate (8) and adaptive (7, 9) immunity. In these mice, tumors become evident after progressive stages of tumorigenesis, and the relationship between the tumor and surrounding tissues is preserved (10). Despite these important features, transgenic mouse models of cancer are not devoid of pitfalls. The transgene may not follow the same developmental expression pattern as the natural gene, because its promoter’s own peculiarities may not be shared by the mechanisms involved in the pathogenesis of natural tumors. The timing of transgene first expression during ontogenesis has a crucial importance because it shapes the features of mouse immune tolerance to the transgene protein product.

Thanks to the work of Leder and Muller (11), there are several lines of mice transgenic for the wild-type or transforming (T) rat (r) Her-2/neu oncogene under the control of mouse mammary tumor virus promoter. r185neu shares 94.8% sequence homology with its mouse counterpart (12). The females of the distinct transgenic lines overexpress r185neu at different periods of their life and develop multiple Her-2/neu mammary carcinomas after varying periods of latency (8). BALB/c female mice made transgenic for the Her-2/neuT display one of the most aggressive progressions of Her-2/neu carcinogenesis (8). r185neu is already overexpressed on the surface of the cells of the rudimentary mammary (10), salivary (13), and Harderian (data not shown) glands of 3-4-week-old mice. At 6 weeks, r185neu cells give rise to a widespread mammary atypical hyperplasia, which progresses to form an invasive and metastasizing carcinoma that becomes palpable in all 10 mammary glands between the 22nd and 27th week of age (8). No reactive lymphocyte infiltration nor antibody response is associated with carcinogenesis progression (7, 8).

In wild-type BALB/c mice, DNA vaccination with plasmids coding for the extracellular (EC) and transmembrane (TM) domains of r185neu (p185EC-TM plasmids) triggers a strong antibody and CTL response, protects all mice against a lethal challenge by a transplantable r185neu carcinoma, and even leads to the eradication of clinically evident r185neu tumors (7, 14). As a consequence of the early and diffuse tissue overexpression of endogenous r185neu, the same DNA plasmids fail to trigger in BALB-neuT mice the strong and rapid T-cell reactivity required for an efficient rejection of challenging r185neu carcinoma cells.

No CTL and a poor antibody response only is elicited (7). Induction of an immunity able to protect against Her-2/neu carcinogenesis is thus a daunting challenge (15). Nevertheless, in the present study, we show that the reactivity elicited through electroporation of p185EC-TM plasmids leads to a progressive and sustained clearance of already present multifocal preneoplastic lesions in all mammary glands and keeps all BALB-neuT mice free from palpable tumors at 1 year of age. No protection takes place in the absence of IFN-γ and antibody. Adoptive transfer experiments show that protection rests on both antibody and T cell-mediated reactivity, even if a CTL activity is not particularly evident.

MATERIALS AND METHODS

Mice. Virgin BALB-neuT female mice (H-2d) overexpressing the transforming rHer-2/neu oncogene under the control of the mouse mammary tumor virus (8) were bred by us. BALB-neuT mice knocked out for the IFN-γ gene (BALB-neuT/γKO) and those knocked out for the immunoglobulin μ chain
gene (BALB-neuT/µKO) were generated by crossing BALB-neuT mice with BALB/c mice KO for IFN-γ gene from The Jackson Laboratory (Bar Harbor, ME) and BALB/c mice KO for the immunoglobulin µ chain kindly provided by Dr. T. Blankenstein (Berlin, Germany; Ref. 4), respectively. Mice were treated according to the European Community guidelines. Female transgenic BALB-neuT mice of the same age were randomly assigned to the control and treatment groups, and all groups were specifically treated concurrently. As each experiment was repeated two to four times with similar results, data were cumulated and reported in the figures. Mammary glands were palpated at weekly intervals to note tumor appearance. Progressively growing masses > 1 mm mean diameter were regarded as tumors. Tumor multiplicity was calculated as the cumulative number of incident tumors per total number of mice, and it is reported as mean ± SE (8).

Cell Lines. p185<sup> mutants</sup> TUBO cells are from a carcinoma arising in a BALB-neuT mouse (7). N202.1A (p185<sup> mutants</sup>) and N202.1E (p185<sup> mutants</sup>) lines were taken randomly from a carcinoma of a FVB mouse (H-2<sup>b</sup>) transgenic for the rat Her-2/neu oncogene (16). Cells were cultured in DMEM (BioWhitaker Europe, Verviers, Belgium) with 20% FBS (Life Technologies, Inc., San Giuano, Italy).

Injection of p18SEC-TM Plasmids and Electric-Pulse Delivery. pcDNA3 vector coding the EC and TM domains of p185<sup> mutants</sup> was produced and used as described (7). Briefly, DNA was precipitated, suspended in sterile saline at 1 mg/ml, and stored in aliquots at −20°C for use in immunization protocols. A total of 100 µl of this solution (100 µg of DNA) was injected into the surgically exposed quadriceps of anesthetized mice at weeks 10 and 12. For DNA electroporation, 25 µg of p18SEC-TM plasmids in 20 µl of 0.9% of NaCl with 6 mg/ml polyglutamate were injected into the tibial muscle of anesthetized mice at weeks 10 and 12. Electric pulses were applied by two electrodes placed on the shaved skin covering with a conducting gel. Two square-wave 25 ms, 375 V/cm pulses were generated by a T820 electroporator (BTX, San Diego, CA). Each course of DNA i.m. vaccination or DNA electroporation consisted of two administrations with an interval of 14 days.

Whole Mount Image Analyses. Whole mounts of all mammary glands were performed as indicated on the Internet. Images were acquired by dividing each whole mount into 10 quadrants. Ten points were randomly chosen on the duct surface in each quadrant, and the corresponding lesions were measured. All lesions on a quadrant with a diameter > 150 µm were counted. Pictures were taken with a Nikon Coolpix 950 digital camera (Nital S.p.A., Torino, Italy) mounted on a stereoscopic microscope (M2Z6; Leica, Milano, Italy) with a 0.63 objective giving a total magnification of ×6.3. The resolution was 1600 × 1200 pixels. Images were acquired with an Adobe Photoshop v. 6.0 graphic software (Adobe Systems; San Jose, CA). Mammary glands that exceeded the size of a single imaging area were captured by photographing contiguous fields in a raster pattern. Each captured image was merged using the layer technique in Adobe Photoshop to form a single composite picture for analysis. Spatial calibration was determined by photographing a 1-mm stage using the same parameters as those for image capturing of whole mount preparations. The distance drawn on the 1-mm calibration image was divided by 1000 to find the number of pixels per micrometer. In each image, ≥ 100 discrete points were randomly chosen on the duct surface, the width in micrometers of any lesion in that point was measured perpendicular to the duct direction, and a value of zero was assigned if no lesion was present. Measurements for all lesions were recorded, and mean and SE were calculated for each treatment group. For each mammary gland, the number of lesions with a diameter > 150 µm was also recorded.

Immunohistochemical Analysis. Groups of three mice were sacrificed at the indicated times, and mammary tissue was processed for morphological analysis (10). For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with H&E. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with antidiendritic cell antibodies (NLDIC 145; Cederlane Laboratories, Ontario, Canada), anti-CD11c (Chemicon International, Inc., Temecula, CA), anti-CD4, anti-CD8a (both from Sera-Lab, Crawley Down, Sussex, United Kingdom), anti-Mac-1 (anti-CD11b/CD18), anti-Mac3 (both from Boehringer Mannheim, Milan, Italy), anti-granulocytes (RB6-8C5; provided by Dr. R. L. Coffman, DNAX, Inc., Palo Alto, CA), anti-interleukin1β (Genzyme, Cambridge, MA), and anti-IFN-γ mAb (provided by Dr. S. Landolfi, Torino University, Torino, Italy). To evaluate the expression of p185<sup> mutants</sup> and proliferating cell nuclear antigen, sections were incubated with polyclonal rabbit antiniu antibody (C-18; Santa Cruz Biotechnology, Santa Cruz, CA) and antiproliferating cell nuclear antigen (Ylem, Roma, Italy) antibody, overlaid with biotinylated goat antirat, antianthamser, and antirabbit or horse antiogul immunoglobulin (Vector Laboratories, Burlingame, CA) for 30 min and incubated with avidin-biotin complex/AP (DAKO, Glostrup, Denmark). At 52 weeks of age, the heart, kidney, and liver of each mouse were examined. Morphological studies were conducted by three pathologists in a blind fashion.

In Vitro Assays. In all tests, spleen cells (SpC) obtained from mice in each treatment group were depleted of erythrocytes by hypotonic lysis and cultured in RPMI 1640 supplemented with 10% fetal bovine serum. To evaluate IFN-γ production by fresh T cells, SpC were stimulated with anti-CD28 and -CD3 (1 µg/ml final concentration; Pharmingen, San Diego, CA) for 8 h. IFN-γ-producing cells were identified using the mouse IFN-γ cell enrichment and detection kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). Activated SpC were labeled with an anti-IFN-γ (clone R4–6A2) conjugated with an anti-CD45 (clone 50S11) monoclonal antibody (mAb; Miltenyi Biotec) for 5 min on ice, then incubated for 45 min at 37°. Cross-staining was avoided by keeping the cell density at 1 × 10<sup>6</sup> cells/ml. IFN-γ bound to the capture matrix was stained with phycoerythrin (PE)-conjugated mAb against IFN-γ (clone AN.18.17.24; Miltenyi Biotec). Anti-PE microbeads were used to enrich PE (IFN-γ)-stained cells with two magnets on a magnetic separator (MS "MACS"; Miltenyi Biotec).

The cells were counterstained with mAb against CD8-FITC (Pharmingen) and analyzed by flow cytometry. To evaluate CTL activity, 1 × 10<sup>5</sup> SpC were stimulated for 6 days in a mixed lymphocyte tumor interaction (MLTI) with 5 × 10<sup>5</sup> mitomycin-C (Sigma, St. Louis, MO)-treated TUBO cells in the presence of 10 units/ml interleukin-2 (Eurocetus, Milan, Italy). CTL activity was then assayed in a 48-h [3H]Tdr release assay at E:T TUBO cells ratios from 50:1 to 6:1 in round bottomed 96-well microtiter plates in triplicate. The results were then expressed as LU<sub>50</sub>/10<sup>7</sup> effector cells (17). To quantify IFN-γ production after MLTI, SpC (1 × 10<sup>5</sup>) were cocultured in RPMI 1640 supplemented with 10% fetal bovine serum in the presence of 1 × 10<sup>5</sup> TUBO cells. Supernatants were collected after 24 h and tested with a mouse IFN-γ ELISA kit (Pharmingen) according to the manufacturer’s protocol.

Flow Cytometry. SpC were stained immediately or after MLTI restimulation with mAb against CD4 or CD8a (Cedarlane, Hornby, Ontario, Canada). Intracellular cytokine assay was performed after polyclonal activation with the Leukocytes Activation Cocktail (BD Pharmingen) and stained using the Intracellular Cytokine Staining Starter kit-mouse (BD Pharmingen) according to the manufacturer’s recommendations. Samples were analyzed with a FACScan (Becton Dickinson, Mountain View, CA). Data elaborated through CellQuest (Becton Dickinson) software were displayed as a percentage of positivity.

Antibody Response. Sera were collected from mice from each treatment group and diluted 1:100. Their binding to p185<sup> mutants</sup> N202.1A and p185<sup> mutants</sup>-N202.1E cells was assayed by flow cytometry (9). Isotype determinations were carried out by an indirect immunofluorescence procedure. Dilutions (1:20) of sera in PBS-azide-BSA were incubated with 2 × 10<sup>5</sup> N202.1A or N202.1E cells for 45 min at 4°C. After washing, the cells were incubated for 30 min with rat biotin-conjugated antibodies antinuse IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 (Caltag Laboratories, Burlingame, CA), and then for 30 min with 5 µl of streptavidin-phycocerytin (DAKO), and resuspended in PBS-azide-BSA containing 1 mg/ml propidium iodide and evaluated in a FACSCan. The specific N202.1A-binding potential was calculated as follows: [(% positive cells in test serum)/fluorescence mean] - [(% positive cells with control serum)/fluorescence mean] × serum dilution (9). Viable cells (1 × 10<sup>5</sup>) were analyzed in each evaluation.

Antibody-Dependent Cellular Cytotoxicity. [3H]Tdr TUBO cells (5 × 10<sup>4</sup>) were incubated for 2 h at 4°C with progressive dilutions (1:10 to 1:100) in HBSS of serum and washed. SpC from untreated mice were then added at 50:1 E:T ratio to each well in triplicate, and lysis was determined as described (18).

Adoptive Transfer. CD8<sup>+</sup> SpC were isolated by magnetic cell sorting with an autoMACS (Miltenyi Biotech). Briefly, SpC were labeled with a PE-conjugated anti-CD90 mAb (clone 53–2; Pharmingen), and then anti-PE microbeads (Miltenyi Biotech) were used to isolate CD8<sup>+</sup> cells by two rounds

RESULTS

DNA Electroporation Inhibits Carcinogenesis. i.m. DNA vaccination with p185EC-TM plasmids begun when mammary glands display atypical hyperplasia (week 6) impaired carcinogenic progression (7, 9). To test whether it was also able to inhibit the progression of more advanced forms, mice received a course of two p185EC-TM plasmids injected i.m. (DNA vaccination course), or electroporated (DNA electroporation course), at the 10th and 12th week of age, when multiple in situ carcinomas were present. A delayed occurrence of the first palpable tumor was observed in a few vaccinated mice, although by week 43, all mice displayed a palpable tumor (Fig. 1A). The protection was not significantly improved by simply repeating the vaccination courses at weeks 20–22 in those few mice in which tumors were not yet palpable (data not shown). By contrast, 47% of DNA-electroporated mice remained tumor free at 1 year of age when the experiment was ended (Fig. 1C). Two additional DNA electroporation courses at weeks 20–22 and 30–32 extended the tumor-free survival, and no tumor was palpable until week 45. Although the final number of mice without tumor was only marginally extended, tumor multiplicity was significantly lower. Finally, all mice that received four DNA electroporation courses at weeks 10–12, 20–22, 30–32, and 40–42 were tumor free at 1 year of age. Their disease-free survival was more than doubled compared with mice electroporated with the empty vector. The aggressiveness and ineluctability of the progression of the multifocal carcinoma in situ of BALB-neuT mice make this complete and persistent inhibition of advanced preneoplastic lesions a significant finding.

Clearance of in Situ Carcinomas. In DNA-electroporated mice, the carcinoma in situ lesions present at the time of vaccination (9) were almost unchanged at week 15 (Fig. 2C) but markedly reduced by week 21 (Fig. 2D). At week 52, neoplastic side buds had completely vanished in mice receiving the four electroporation courses (Fig. 2, E and F). This progressive clearance of in situ carcinomas in DNA-electroporated mice is associated with a marked decrease of membrane expression of rp185 neu. At week 21, rp185 neu was only expressed in the cytoplasm, but even there, its expression was fainter than in untreated BALB-neuT mice (Fig. 3C). The poor expression of
ranging from atypical hyperplasia foci to mammary gland of BALB-neuT mice is almost totally formed by neoplastic lesions and week-old mice electroporated at weeks 10 and 12 still display diffuse neoplastic side buds BALB-neuT mice vaccinated by p185EC-TM electroporation. Mammary glands of 15–C–F show progressive clearance of preneoplastic lesions in whole mounts (A, black arrowhead) and extending into the fat pad. At 21 weeks of age, the mammary gland of a wild-type 21-week-old BALB/c mouse is a tree-like duct structure originating from the nipple (A, black arrowhead) and extending into the fat pad. At 21 weeks of age, the mammary gland of BALB-neuT mice is almost totally formed by neoplastic lesions ranging from atypical hyperplasia foci to in situ and large invasive carcinomas (B, empty arrowheads). Whole mounts (C–F) show progressive clearance of preneoplastic lesions in BALB-neuT mice vaccinated by p185EC-TM electroporation. Mammary glands of 15-week-old mice electroporated at weeks 10 and 12 still display diffuse neoplastic side buds and in situ carcinoma foci (C). Although in situ carcinomas are missing, side buds are still present, but markedly reduced, at week 21 (D) and have completely vanished at week 52 in mice receiving the four electroporation courses (E and F). The central oval black areas in a–e (arrows) are mammary lymph nodes. Magnification: A–E, ×6; 3; F, ×12.

Fig. 3. Immunohistochemical analysis of mammary glands from 21-week-old wild-type BALB/c (A and D) and BALB-neuT mice untreated (B, E, and G) and DNA electroporated (C, F, H, and I). Wild-type BALB/c mammary samples display a duct lined by monostratified epithelial cells which do not express rp185\(^{\text{neu}}\) (A) and display a low proliferating activity as assessed by antiproliferating cell nuclear antigen staining (Fig. 3F). On the other hand, although numerous dendritic cells (NLDC 145\(^+\), CD11c\(^−\)), macrophages (Mac1\(^+\), Mac3\(^−\)), and CD4 (data not shown) were present in the stroma surrounding the hyperplasia foci, several CD8 (Fig. 3H) neutrophils (RB6–8C5\(^−\); data not shown) overcame the basal membrane and were briskly intermingled with the residual neoplastic cells present in DNA-electroporated mice. Anti-IFN-γ (Fig. 3I) widely stained the cytoplasm of most infiltrating T cells, whereas anti-interleukin-1β antibody stained areas rich in infiltrating macrophages (data not shown). At week 52, rp185\(^{\text{neu}}\) cells had vanished in the mice that received the four electroporation courses, and little or no reactive cell infiltrate was still evident. In only a few mammary glands, residual and constrained foci of hyperplasia associated with a distinct reactive cell infiltrate were present close to the nipple (data not shown).

**Immunological Events Associated with Carcinogenesis Inhibition.** A compendium of the immunological reactivity found in the Spc from vaccinated BALB-neuT mice is displayed in Table 1. Two weeks after the first vaccination or electroporation course, fresh Spc displayed no detectable CTL activity against rp185\(^{\text{neu}}\) targets (data not shown), whereas a low CTL activity only was displayed by Spc from p185EC-TM plasmid-vaccinated and -electroporated mice cultured for 6 days in MLTI with rp185\(^{\text{neu}}\) tumor cells (Table 1). By contrast, after anti-CD3 and -CD28 stimulation, a significant number of both CD8\(^+\) and CD8\(^−\) cells produced IFN-γ as assessed with the Miltenyi Biotec cell enrichment and detection kit. No higher CTL activity was observed by repeating these experiments after the four courses of immunization by electroporation (data not shown).

Two weeks after the first vaccination course, the titer of anti-rp185\(^{\text{neu}}\) antibodies was significantly higher in the sera from the electroporated compared with vaccinated mice (Fig. 4A; P < 0.009). After both vaccination and electroporation, anti-rp185\(^{\text{neu}}\) antibodies were mainly of the IgG subclasses, with IgG2a as the most and IgG1 as the least represented (Fig. 4B). Sera from electroporated mice show a significantly higher titer of IgG2a (P < 0.0001), IgG2b

rp185\(^{\text{neu}}\) goes along with a marked reduction of epithelial cell proliferation as assessed by antiproliferating cell nuclear antigen staining (Fig. 3F). On the other hand, although numerous dendritic cells (NLDC 145\(^+\), CD11c\(^−\)), macrophages (Mac1\(^+\), Mac3\(^−\)), and CD4 (data not shown) were present in the stroma surrounding the hyperplasia foci, several CD8 (Fig. 3H) neutrophils (RB6–8C5\(^−\); data not shown) overcame the basal membrane and were briskly intermingled with the residual neoplastic cells present in DNA-electroporated mice. Anti-IFN-γ (Fig. 3I) widely stained the cytoplasm of most infiltrating T cells, whereas anti-interleukin-1β antibody stained areas rich in infiltrating macrophages (data not shown). At week 52, rp185\(^{\text{neu}}\) cells had vanished in the mice that received the four electroporation courses, and little or no reactive cell infiltrate was still evident. In only a few mammary glands, residual and constrained foci of hyperplasia associated with a distinct reactive cell infiltrate were present close to the nipple (data not shown).

**Immunological Events Associated with Carcinogenesis Inhibition.** A compendium of the immunological reactivity found in the Spc from vaccinated BALB-neuT mice is displayed in Table 1. Two weeks after the first vaccination or electroporation course, fresh Spc displayed no detectable CTL activity against rp185\(^{\text{neu}}\) targets (data not shown), whereas a low CTL activity only was displayed by Spc from p185EC-TM plasmid-vaccinated and -electroporated mice cultured for 6 days in MLTI with rp185\(^{\text{neu}}\) tumor cells (Table 1). By contrast, after anti-CD3 and -CD28 stimulation, a significant number of both CD8\(^+\) and CD8\(^−\) cells produced IFN-γ as assessed with the Miltenyi Biotec cell enrichment and detection kit. No higher CTL activity was observed by repeating these experiments after the four courses of immunization by electroporation (data not shown).

Two weeks after the first vaccination course, the titer of anti-rp185\(^{\text{neu}}\) antibodies was significantly higher in the sera from the electroporated compared with vaccinated mice (Fig. 4A; P < 0.009). After both vaccination and electroporation, anti-rp185\(^{\text{neu}}\) antibodies were mainly of the IgG subclasses, with IgG2a as the most and IgG1 as the least represented (Fig. 4B). Sera from electroporated mice show a significantly higher titer of IgG2a (P < 0.0001), IgG2b
PREVENTION OF HER-2/NEU MAMMARY CARCINOMAS

Table 1 Immune reactivity elicited in BALB-neuT mice by i.m. DNA vaccination and electroporation

<table>
<thead>
<tr>
<th>Mice i.m. DNA vaccinated with</th>
<th>Mice DNA electroporated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty pcDNA3 plasmids</td>
<td>p185EC-TM plasmids</td>
</tr>
<tr>
<td>Cytotoxicity (LU80)%</td>
<td>4.9 ± 2.5</td>
</tr>
<tr>
<td>IFN-γ producing CD8+ cells%</td>
<td>N.D.</td>
</tr>
<tr>
<td>IFN-γ producing CD8+ cells%</td>
<td>N.D.</td>
</tr>
<tr>
<td>ADCC%</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*pBALB-neuT mice were i.m. DNA vaccinated or electroporated at weeks 10 and 12. Two weeks later, the mice were sacrificed, and spleen and sera were collected.

†Mean ± SE of LU80 against TUBO cells generated in the spleen cells from three mice from each treatment group after a 6-day mixed lymphocyte tumor interaction (pcDNA3 vs. p185EC-TM plasmids electroporated). As a rough calibration of these results, the percentage of lysis displayed by spleen cells from DNA electroporated mice at 50:1 E:T ratio ranged from 16 to 20%.

(See Table 1 for details.)

Considering that sequential courses of DNA electroporation are required to restrain the progression of the neoplastic lesions, the kinetics of antibody titer were evaluated. Eight weeks after each electroporation course, the titer of anti-p185 neu antibodies decreased, whereas it was boosted (P < 0.0001) by the following course (Fig. 4C). An increase in CD8+ cells was also evident in the Spc of mice that received the four electroporation courses (Table 2). After a MLTI, the amount of IFN-γ released by Spc from mice that received the four electroporation courses was significantly higher than that released after one. In agreement with the immunohistochemistry findings (Fig. 3H), the majority (63–68%) of cells that produced IFN-γ was CD8+ lymphocytes. By contrast, no higher CTL activity was observed after the four courses of immunization by electroporation (data not shown).

Table 2 Percentage of CD4+ and CD8+ cells and IFN-γ production in the spleen of BALB-neuT mice after one and four DNA electroporation courses

<table>
<thead>
<tr>
<th>Splenocytes from BALB-neuT mice</th>
<th>CD4+ (%)</th>
<th>CD8+ (%)</th>
<th>IFN-γ productionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-week-old untreated</td>
<td>22 ± 2</td>
<td>9 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>14-week-old, one DNA</td>
<td>24 ± 2</td>
<td>9 ± 4</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>electroporation course</td>
<td>33 ± 3</td>
<td>12 ± 3</td>
<td>176 ± 3</td>
</tr>
</tbody>
</table>

bPercentage of positive spleen cells as evaluated by cytotoxicity.

DNA Electroporation Does Not Protect BALB-neuT/γKO and BALB-neuT/μKO Mice. The production of antibodies to p185 neu and the secretion of IFN-γ were the two prominent immune activities associated with the clearance of HER-2/neu lesions. Because selective depletion of immune functions through antibody administration is awkward in experiments lasting 1 year, BALB-neuT/μKO mice (4) that do not produce immunoglobulin in response to p185EC-TM plasmid electroporation (data not shown), and BALB-neuT/γKO that do not produce IFN-γ (16), were bred to weigh the in vivo importance of these immune mechanisms. Although the kinetics of HER-2/neu tumor onset in untreated BALB-neuT/μKO and BALB-neuT/γKO were similar to that of BALB-neuT mice, electroporation did not significantly protect either strain (Fig. 1, E and F). No anti-p185 neu antibodies were detectable in the sera of any BALB-neuT/μKO mice 2 weeks after the electroporation course (data not shown).

Adoptive Transfer of Protection. Because the whole mount provides a picture of the stage of carcinogenesis, we exploited this as an intermediate end point to assess the protection afforded by the transfer of antibody and T lymphocytes from DNA-electroporated mice in otherwise untreated BALB-neuT recipients. Spleen T cells and pooled sera from three groups of 15-week-old, DNA-electroporated BALB-neuT mice were transferred to untreated BALB-neuT mice at weeks 10, 12, and 13. Four weeks after the last transfer, the stage of carcinogenic progression was evaluated through a computer-aided image analysis of the mammary gland whole mounts (Table 3). Transfer of either T cells or sera provided significant inhibition, and their combination produced a marked additive effect. No inhibition followed the transfer of serum and T lymphocytes from age-matched BALB-neuT mice electroporated with the empty vector.

Table 3 Mammary carcinogenesis in recipient BALB-neuT mice to which T cells and sera from DNA-electroporated BALB-neuT mice were adoptively transferred

<table>
<thead>
<tr>
<th>Mean lesion size (μm²) ± SE</th>
<th>Number of lesions &gt; 150 μm² ± SE</th>
<th>Tumor index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors electroporated with empty pcDNA3 plasmidsa</td>
<td>Sera</td>
<td>392.4 ± 43.3</td>
</tr>
<tr>
<td>CD3 T lymphocytesa</td>
<td>348.7 ± 63.6</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Sera + CD3 T lymphocytes</td>
<td>361.8 ± 60.1</td>
<td>2.78 ± 1.0</td>
</tr>
<tr>
<td>Donors electroporated with the p185EC-TM plasmids</td>
<td>Sera</td>
<td>84.0 ± 15.4</td>
</tr>
<tr>
<td>CD3 T lymphocytes</td>
<td>121.2 ± 27.8</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Sera plus CD3 T lymphocytes</td>
<td>30.0 ± 13.5</td>
<td>0.1 ± 0.01</td>
</tr>
</tbody>
</table>

aValues significantly different (P ≤ 0.01; Student’s t test) (a) from those of mice receiving sera and CD3 lymphocytes from p185EC-TM plasmid-electroporated mice, (c) from those of mice receiving sera and CD3 T lymphocytes from p185EC-TM plasmid-electroporated mice. Values assigned to points without lesions. The tumor index is the product of the mean lesion size and number.

DISCUSSION

BALB-neuT mice are genetically predestined to develop lethal invasive carcinomas in their mammary glands by week 22. The early and diffuse overexpression of p185 neu that occurs in suckling BALB-neuT mice (10) hinders the induction of a protective immunity against a challenge of p185 neu + transplantable tumors (7). Nevertheless, repeated DNA electroporations of p185EC-TM plasmids begun when multiple in situ carcinomas were present slowly cleared the neoplastic lesions and maintained all 1-year-old BALB-neuT mice free from detectable disease.
As the transforming oncogene is embedded in the genome of these mice, a unique dynamic relationship between the oncogenic signals and inhibitory immune reactions is taking place. In BALB-neuT mice, i.m. DNA vaccination triggers a reaction that inhibits the progression of neoplastic lesions but is unable to elicit their regression. As immunity fades, lesions progress, and none of the 1-year-old mice is tumor free. As expected (19, 20), transfection of muscle cells and expression of the plasmid-encoded proteins are greatly increased by electroporation. As compared with simple i.m. injection, vaccination by electroporation provides a greater and more persistent mass of the plasmid-coded antigen (21).

In the absence of the continuous offsetting by neoplastic stem cells, the clearance of neoplastic lesions that follows a single DNA electroporation course would coincide with a definitive cure. To keep all 1-year-old BALB-neuT mice free of tumors, repeated courses of DNA electroporation are required. About 3 months after each course, the immune response declines, and carcinogenesis is no longer controlled.

By contrast with transplantable tumors, clearance of in situ carcinoma and inhibition of carcinogenesis are unaccompanied by marked CTL reactivity, and both the lack of protection in BALB-neuT/μKO mice and data from the adoptive experiments suggest that anti-rp185neu antibody plays a significant protective role, presumably because rp185neu is both the target tumor antigen and a receptor regulating cell growth (22). By down-regulating rp185neu expression (23), and impeding the formation of homo or heterodimers that transduce proliferative signals (24), the antibody impedes neoplastic proliferation. The membrane down-modulation of rp185neu, its intracytoplasmic confinement, and the morphological features of inhibited proliferation associated with diminished nuclear positivity of proliferating cell nuclear antigen in the mammary glands of DNA-electroporated mice endorse this noncytotoxic role of the anti-rp185neu antibody, which may also activate complement-mediated lysis as suggested by their isotype. The ability of anti-rp185neu antibody to mediate antibody-dependent cellular cytotoxicity is another mechanism for cooperation between antibody and intratumor delayed type hypersensitivity (22).

The inability of DNA electroporation to protect BALB-neuT/γKO mice points to a critical role of IFN-γ. IgG2a is by far the most predominant isotype of the anti-rp185neu antibody elicited, and T cell-released IFN-γ is the main IgG2a switch factor (25). Moreover, clearance of in situ carcinomas is associated with a massive infiltration of IFN-γ-releasing T cells that penetrate the basement membrane and interact with tumor cells. Intratumor IFN-γ activation of many proinflammatory and antitumor cell activities leads to both tumor rejection and induction of an efficient immune memory (25), even in the absence of a significant CTL response (26). After anti-rp185neu and -CD28 stimulation, a significant number of both CD8+ and CD8− cells produced IFN-γ. In a similar way, both CD4+ and CD8+ T cell-depleted Spc populations specifically released IFN-γ after expansion in the presence of mitomycin-C-treated p185neu+ cells, as assessed in ELISPOT (data not shown). Intratumor IFN-γ also directly inhibits the proliferation of p185neu+ tumor cells and their production of proangiogenic factors (27).

As numerous peptides potentially fitting in the H-2d glycoproteins are already present in the protein encoded by p185EC-TM plasmid (28), plasmids coding for the full-length rp185neu were not used. This rules out the possibility that cells which take up plasmid DNA receive positive growth signals. Moreover, concerns over the use of the full-length Her-2/neu oncogene as a DNA vaccine also include the risk of induction of autoimmunity against the intracellular domain that is highly conserved by members of the epidermal growth factor receptor family (28).

The combination between cellular and humoral reactivity improves inhibition of carcinogenesis in BALB-neuT mice receiving the adoptive transfer of sera and T lymphocytes. The collaboration of both humoral and cellular immune response in the rejection of Her-2/neu tumors in transgenic mice has been clearly documented (29). This combination was also required for the eradication of rp185neu+ tumors transplanted in wild-type BALB/c mice, for which rp185neu is a xenogenic nontolerated model tumor antigen (14). Although vaccination cured all wild-type BALB/c mice, no cure was observed in the absence of antibody, in FcyRII/III KO mice and both mice with a deficient CD8 lymphocyte effector function and those with IFN-γ KO gene.

DNA-electroporated BALB-neuT mice necropsied at week 52 were free from overt signs of autoimmune lesions in the heart, kidney, and liver (data not shown), even if the induced anti-rp185neu antibodies cross-reacted with mouse p185neu. As autoimmunity cannot easily be dissociated from effective tumor immunity (30), the absence of obvious autoimmune lesions may be attributable to the combination of the very poor expression of p185neu by the tissues of adult mice and the inability of BALB-neuT mice to generate a high-affinity immune response to the early and diffusely expressed transgenic rp185neu protein. An efficient immune response rests on high-avidity reaction mechanisms (3). Even so, a low-avidity response may be effective in tumor rejection and discriminate between quantitative differences in the expression of the target antigen (31). The response to rp185neu in BALB-neuT mice generated by DNA electroporation appears to successfully control the slow but devastating progression of multiple preneoplastic lesions and even induce their long-lasting regression without causing evident autoimmune aggression of the normal tissues where mouse p185neu or rp185neu is expressed at a much lower level.

A similar vaccination could be considered in the management of early lesions expressing one of the many deregulated oncogenic protein kinase membrane receptors directly involved in cell carcino-
11. Muller WJ, Sinn E, Wallace R, Pattengale PK, Leder P. Single-step induction of vaccination, the positive results obtained in large animals (35), along provides a relatively simple method for inducing a strong protection.\(^7\) DNA electroporation \(^8\) provides a relatively simple method for inducing a strong protection. The lower amount of DNA required as compared with i.m. DNA vaccination, the positive results obtained in large animals (35), along with the availability of devices for electroporation in humans,\(^7\) could make this translation not too unlikely. Enhancement of the protection elicited by DNA electroporation through the concurrence of various accessory signals is being investigated.

REFERENCES


Electroporated DNA Vaccine Clears Away Multifocal Mammary Carcinomas in Her-2/ \textit{neu} Transgenic Mice

Elena Quaglino, Manuela Iezzi, Cristina Mastini, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/8/2858

Cited articles
This article cites 34 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/8/2858.full.html#ref-list-1

Citing articles
This article has been cited by 22 HighWire-hosted articles. Access the articles at:
/content/64/8/2858.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.