[CANCER RESEARCH 64, 2858–2864, April 15, 2004]

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

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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 adaptative (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. rp185^{neu} shares 94.8% sequence homology with its mouse counterpart (12). The females of the distinct transgenic lines overexpress rp185^{neu} 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 rHer-2/*neu*T display one of the most aggressive progressions of Her-2/*neu* carcinogenesis (8). rp185^{neu} 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, rp185^{neu} 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 rp185^{neu} (p185EC-TM plasmids) triggers a strong antibody and CTL response, protects all mice against a lethal challenge by a transplantable rp185^{neu+} carcinoma, and even leads to the eradication of clinically evident rp185^{neu+} tumors (7, 14). As a consequence of the early and diffuse tissue overexpression of endogenous rp185^{neu}, 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 rp185^{neu+} 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-2^{d}) 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

Received 9/19/03; revised 12/23/03; accepted 2/16/04.

Grant support: The Italian Association for Cancer Research, the Italian Ministries for the University and Health, the University of Torino, Compagnia di San Paolo, and the Center of Excellence on Aging, University of Chieti.

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Note: E. Quaglino and M. Iezzi contributed equally to this work.

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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 $\pm$ SE (8).

Cell Lines. rp185^{neu+} TUBO cells are from a carcinoma arising in a BALB-neuT mouse (7). N202.1A (rp185^{neu+}) and N202.1E (rp185^{neu-}) lines were taken randomly from a carcinoma of a FVB mouse (H-2^{q}) transgenic for the rat Her-2/*neu* protoncogene (16). Cells were cultured in DMEM (BioWhittaker Europe, Verviers, Belgium) with 20% FBS (Life Technologies, Inc., San Giuliano, Italy).

Injection of p185EC-TM Plasmids and Electric-Pulse Delivery. pcDNA3 vector coding the EC and TM domains of rp185^{neu} 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 p185EC-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 covered 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.[6] 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 (MZ6; Leica, Milano, Italy) with a 0.63 objective giving a total magnification of $\times$6.3. The resolution was 1600 $\times$ 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, $\geq$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 antidendritic cell antibodies (NLDC 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-Mac1 (anti- CD11b/CD18), anti-Mac3 (both from Boehringer Mannheim, Milan, Italy), antigranulocytes (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. Landolfo, Torino University, Torino, Italy). To evaluate the expression of rp185^{neu} and proliferating cell nuclear antigen, sections were incubated with polyclonal rabbit antineu antibody (C-18; Santa Cruz Biotechnology, Santa Cruz, CA) and antiproliferating cell nuclear antigen (Ylem, Roma, Italy) antibody, overlaid with biotinylated goat antirat, antihamster, and antirabbit or horse antigoat 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 30S11) 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 1×10^5 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 rounds 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, 1×10^7 Spc were stimulated for 6 days in a mixed lymphocyte tumor interaction (MLTI) with 5×10^5 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 [^{3}H]TdR release assay at E:T TUBO cells ratio from 50:1 to 6:1 in round bottomed 96-well microtiter plates in triplicate. The results were then expressed as $LU_{20}/10^7$ effector cells (17). To quantify IFN-γ production after MLTI, Spc (1×10^5) were cocultured in RPMI 1640 supplemented with 10% fetal bovine serum in the presence of 1×10^5 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 rp185^{neu+} N202.1A and rp185^{neu-} 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 2×10^5 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 antimouse IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 (Caltag Laboratories, Burlingame, CA), and then for 30 min with 5 μl of streptavidin-phycoeritrin (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 with test serum)(fluorescence mean)] $-$ [(% positive cells with control serum)(fluorescence mean)] $\times$ serum dilution (9). Viable cells (1×10^4) were analyzed in each evaluation.

Antibody-Dependent Cellular Cytotoxicity. [^{3}H]TdR TUBO cells (5×10^3) 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. CD90$^+$ Spc were isolated by magnetic cell sorting with an autoMACS (Miltenyi Biotech). Briefly, Spc were labeled with a PE-conjugated anti-CD90 mAb (clone 53–2.1; PharMingen), and then anti-PE microbeads (Miltenyi Biotech) were used to isolate CD90$^+$ cells by two rounds

[6] http://ccm.ucdavis.edu/tgmouse/HistoLab/wholmt1.html.

on a magnetic separator (MS^+ MACS; Miltenyi Biotech). $CD90^+$ Spc (1×10^7, 90–92% positive) were administered i.v. in 0.2 ml of PBS to recipient BALB-neuT mice at weeks 10, 12, and 13. Other mice received 0.5 ml of pooled sera from 15-week-old electroporated donors. Lastly, one group received both spleen T cells and sera administered 4 h apart. Recipient BALB-neuT mice were sacrificed at week 17, and the whole mounts of all 10 mammary glands were evaluated. These results were compared with the staging of the fourth right and left mammary glands.

Statistics. Differences in tumor incidence were evaluated with the Mantel-Haenszel Log-rank test; differences in tumor multiplicity, mammary lesions, LU_{20}, cytokine production, and antibody titer were evaluated with Student's *t* test.

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 10^{th} and 12^{th} 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. 1*A*). 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. 1*C*). Tumor multiplicity, too, was significantly reduced (Fig. 1*C*). 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. 2*C*) but markedly reduced by week 21 (Fig. 2*D*). 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. 3*C*). The poor expression of

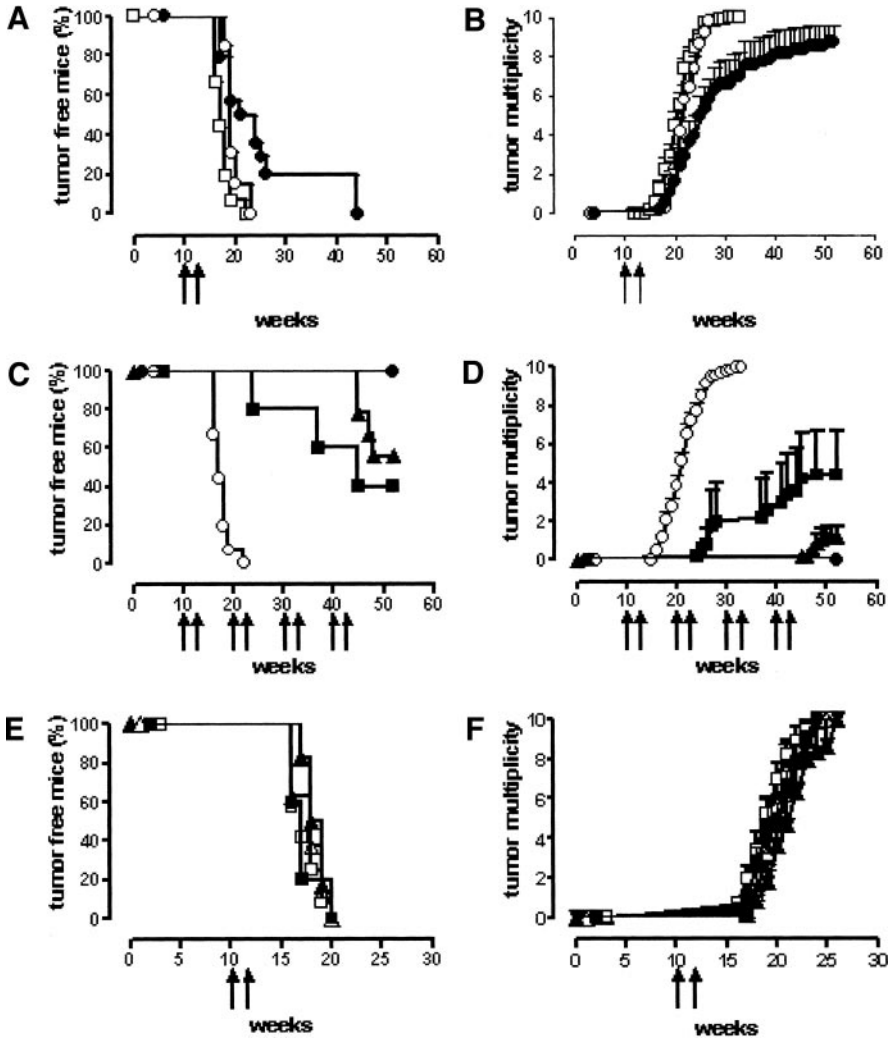

Fig. 1. Incidence and multiplicity of carcinomas in i.m. DNA-vaccinated and DNA-electroporated BALB-neuT, BALB-neuT/μKO, and BALB-neuT/γKO mice. BALB-neuT mice of the same age were randomly assigned to the concurrently treated control and experimental groups. Each experiment was repeated two to four times, and the results were cumulated. Mice with at least one progressively growing mammary tumor $>$ 1 mm mean diameter were classed as tumor-bearing mice. *A* and *B*, i. m. vaccinated BALB-neuT mice. Mice untreated (□, 27 mice) and injected i.m. at weeks 10–12 with pcDNA3 empty plasmids (○, 13 mice) and p185EC-TM plasmids (●, 14 mice). *B*, tumor multiplicity is significantly lower in p185EC-TM-vaccinated mice as compared with all of the other groups from week 22 to 33 ($P = 0.04$). *C* and *D*, electroporated BALB-neuT mice. Mice electroporated with pcDNA3 empty plasmids, weeks 10–12 (○, 10 mice); p185EC-TM plasmids, weeks 10–12 (■, 19 mice); p185EC-TM plasmids, weeks 10–12, 20–22, and 30–32 (▲, 8 mice); with p185EC-TM plasmids, weeks 10–12, 20–22, 30–32, and 40–42 (●, 6 mice). In *C*, tumor incidence is significantly different in p185EC-TM-electroporated mice as compared with controls electroporated with empty vector. Moreover, tumor incidence in p185EC-TM-electroporated mice is significantly different ($P < 0.0001$) as compared with all other groups. *E* and *F*, BALB-neuT/μKO and BALB-neuT/γKO mice electroporated at weeks 10–12 with pcDNA3 empty plasmid (□, 9 BALB-neuT/μKO mice; △, 12 BALB-neuT/γKO mice) or p185EC-TM plasmids (■, 6 BALB-neuT/μKO mice; ▲, 5 BALB-neuT/γKO mice). Vertical bars, SE.

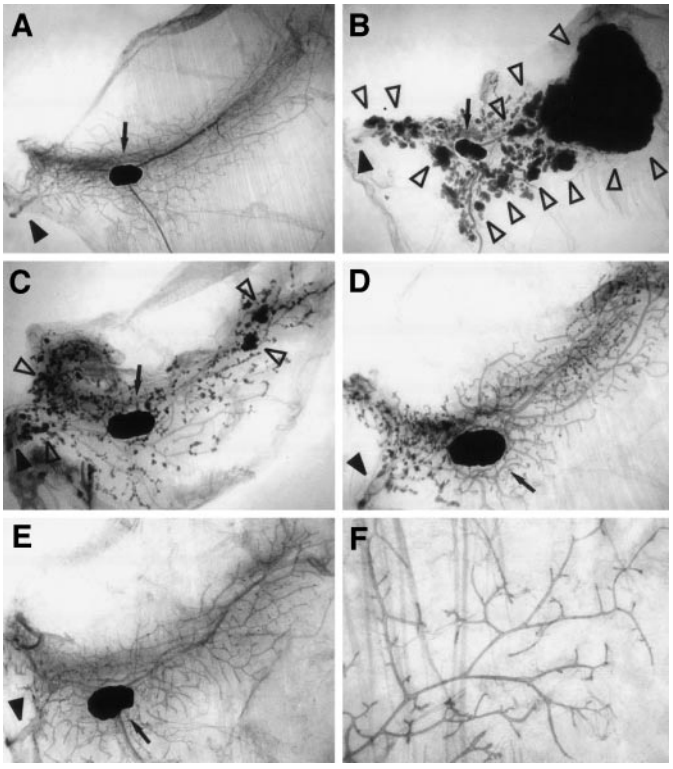

Fig. 2. Whole mount of the mammary glands from wild-type BALB/c (*A*) and BALB-neuT mice untreated (*B*) and DNA electroporated (*C–F*). The mammary gland of a wild-type 21-week-old BALB/c mouse is a tree-like duct structure originating from the nipple (*A, black arrowhead*) and extending into the fat 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.

rp185^{neu} goes along with a marked reduction of epithelial cell proliferation as assessed by antiproliferating cell nuclear antigen staining (Fig. 3*F*). 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. 3*H*) 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. 3*I*) 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^{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^{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^{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^{neu} antibodies was significantly higher in the sera from the electroporated compared with vaccinated mice (Fig. 4*A*; $P < 0.009$). After both vaccination and electroporation, anti-rp185^{neu} antibodies were mainly of the IgG subclasses, with IgG2a as the most and IgG1 as the least represented (Fig. 4*B*). Sera from electroporated mice show a significantly higher titer of IgG2a ($P < 0.0001$), IgG2b

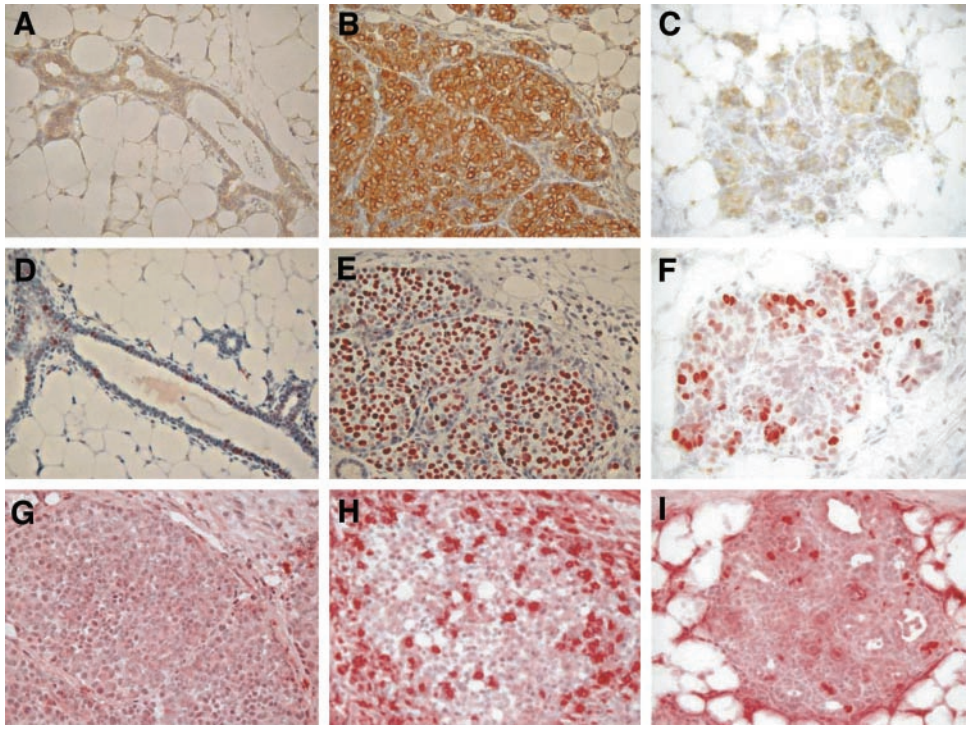

Fig. 3. Immunohistochemical analysis of mammary glands from 21-week-old wild-type BALB/c (*A* and *D*) and BALB-neuT mice untreated (*B, E,* and *G*) and DNA electroporated (*C, F, H,* and *I*). Wild-type BALB/c mammary samples display a duct lined by monostratified epithelial cells which do not express rp185^{neu} (*A*) and display a low proliferating activity as assessed by antiproliferating cell nuclear antigen (*D*). Large solid tumoral masses in untreated BALB-neuT mice show a strong membrane rp185^{neu} (*B*) and diffuse proliferating cell nuclear antigen (*E*) positivity. The residual mammary preneoplastic and early neoplastic lesions in electroporated BALB-neuT mice are associated to scarce or absent membrane and faint cytoplasmic expression of rp185^{neu} (*C*) and scanty nuclear expression of proliferating cell nuclear antigen (*F*). CD8$^+$ lymphocytes, scarcely present around the tumor developed in untreated BALB-neuT mice (*G*), briskly infiltrate the neoplastic side buds after overcoming the basement membrane in electroporated BALB-neuT mice (*H*). Their cytoplasm frequently expresses IFN-γ (*I*). Magnification: *A–I*, ×400.

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

	Mice i.m. DNA vaccinated with		Mice DNA electroporated with	
	Empty PcDNA3 plasmids	p185EC-TM plasmids	Empty pcDNA3 plasmids	p185EC-TM plasmids
Cytotoxicity (LU_{20})[b]	4.9 ± 2.5	30.4 ± 6.1	14.8 ± 1.6	44.4 ± 9.0
IFN-γ producing $CD8^-$ cells[c]	N.D.	N.D.	5–8	25–36
IFN-γ producing $CD8^+$ cells[c]	N.D.	N.D.	7–19	33–42
ADCC[d]	4.1	8.5	3.9	32.0

[a] BALB-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.
[b] Mean ± SE of LU_{20} 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 either vaccinated or electroporated = $P < 0.05$; Student's *t* test). 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%.
[c] Percentage of cells secreting IFN-γ (range) in the spleen cells from three mice from each treatment group after anti-CD3 and -CD28 monoclonal antibody stimulation.
[d] Lysis percentage of TUBO target cells in an antibody-dependent cellular cytotoxicity (*ADCC*) test. Pools of sera were from five mice. The data are from one representative experiment out of three performed independently with sera from distinct mice. N.D., not done.

($P < 0.0001$), and IgG3 ($P < 0.003$) from that observed in the vaccinated mice. Because these IgG isotypes mediate antibody-dependent cellular cytotoxicity, their ability to guide Spc lytic activity was assayed *in vitro*. The sera from electroporated mice guided a marked antibody-dependent cellular cytotoxicity against $rp185^{neu+}$ target cells (Table 1).

Considering that sequential courses of DNA electroporation are required to restrain the progression of the preneoplastic lesions, the kinetics of antibody titer were evaluated. Eight weeks after each electroporation course, the titer of anti-$rp185^{neu}$ antibodies decreased, whereas it was boosted ($P < 0.0001$) by the following course (Fig. 4*C*). An increase in $CD4^+$ 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. 3*H*), 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).

DNA Electroporation Does Not Protect BALB-neuT/γKO and BALB-neuT/μKO Mice. The production of antibodies to $rp185^{neu}$ 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-$rp185^{neu}$ antibodies were detectable in the sera of any BALB-neuT/μKO mice 2 weeks after the electroporation course (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*

Spleens from BALB-neuT mice	CD4[a] (%)	CD8[a] (%)	IFN-γ production[b]
14-week-old untreated	22 ± 2	9 ± 2	5 ± 1
14-week-old, one DNA electroporation course	24 ± 2	9 ± 4	41 ± 2
44-week-old, four DNA electroporation courses	33 ± 3	12 ± 3	176 ± 3

[a] Percentage of positive spleen cells as evaluated by cytofluorimetry.
[b] Spleen cells from three mice from each treatment group were recovered from a 6-day with $rp185^{neu+}$ cells and incubated for 24 h with TUBO cells. The IFN-γ released in the supernatants was evaluated in an ELISA test (U/ml).

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.

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 $rp185^{neu}$ that occurs in suckling BALB-neuT mice (10) hinders the induction of a protective immunity against a challenge of $rp185^{neu+}$ transplantable tumors (7). Nevertheless, repeated DNA electroporations of p185EC-TM plasmids begun when multiple *in situ* carcinomas were present slowly cleared the preneoplastic lesions and maintained all 1-year-old BALB-neuT mice free from detectable disease.

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

	Mean lesion size (μm^2) ± SE	Number of lesions > 150 μm^2 ± SE	Tumor index
Donors electroporated with empty pcDNA3 plasmids[a]			
Sera	392.4 ± 43.3	3.55 ± 0.9	1372
CD3 T lymphocytes[b]	348.7 ± 63.6	3.3 ± 0.5	1151
Sera + CD3 T lymphocytes	361.8 ± 60.1	2.78 ± 1.0	1013
Donors electroporated with the p185EC-TM plasmids			
Sera	84.0 ± 15.4	0.5 ± 0.2	42
CD3 T lymphocytes	121.2 ± 27.8	0.9 ± 0.2	108
Sera plus CD3 T lymphocytes	30.0[c] ± 13.5	0.1 ± 0.01	3

[a,c] Values significantly different ($P \leq 0.01$; 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 or CD3 T lymphocytes separately. At least 30 mammary glands of 17-week-old recipient BALB-neuT mice were evaluated for each treatment group. The mean lesion sizes were measured by computer-assisted image analysis in ≥100 randomly selected points for each mammary gland perpendicular to duct direction. A 0 value was assigned to points without lesions. The tumor index is the product of the mean lesion size and number.
[b] The percentage of $CD3^+$ T cells was > 85% as evaluated by fluorescence-activated cell sorter analysis.

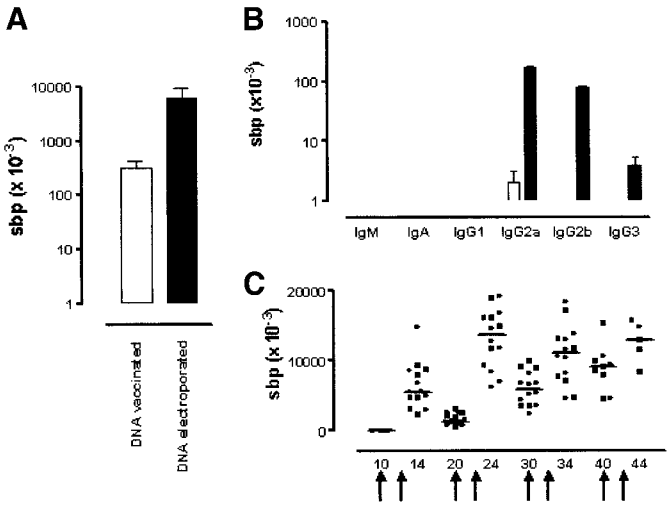

Fig. 4. Anti-rp185^{neu} antibodies in the sera of DNA vaccinated and electroporated BALB-neuT mice. Two weeks after the first course, the antibody titer is much higher in the individually tested sera of 14 electroporated mice than sera of mice vaccinated i.m. (*A*). The isotype titration was performed in four different pools of sera from i.m. vaccinated (*white columns*) and electroporated (*black columns*) mice (*B*). Vertical bars, SD. The antibody titer was also sequentially evaluated before and 2 weeks after each electroporation course (*C*). It increased after each course. Eight weeks later, it was lower but rose again 2 weeks after each electroporation. ○, the 29 values of single sera; *horizontal bars*, median value. In *A–C*, values are expressed as specific binding potential (*sbp*).

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 antigen available for the induction of immune responses. Transfection of other cells, such as antigen-presenting cells, may also be facilitated. Direct application of an electric field to the tissue could result in an inflammatory response that may further enhance the immunogenicity 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-rp185^{neu} antibody plays a significant protective role, presumably because rp185^{neu} is both the target tumor antigen and a receptor regulating cell growth (22). By down-regulating rp185^{neu} 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 rp185^{neu}, 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-rp185^{neu} antibody, which may also activate complement-mediated lysis as suggested by their isotype. The ability of anti-rp185^{neu} 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-rp185^{neu} 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-CD3 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 p185^{neu+} cells, as assessed in ELISPOT (data not shown). Intratumor IFN-γ also directly inhibits the proliferation of p185^{neu+} tumor cells and their production of proangiogenic factors (27).

As numerous peptides potentially fitting in the H-2^d glycoproteins are already present in the protein encoded by p185EC-TM plasmid (28), plasmids coding for the full-length rp185^{neu} 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 rp185^{neu+} tumors transplanted in wild-type BALB/c mice, for which rp185^{neu} is a xenogeneic nontolerated model tumor antigen (14). Although vaccination cured all wild-type BALB/c mice, no cure was observed in the absence of antibody, in FcγRI/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 necroscopied 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-rp185^{neu} antibodies cross-reacted with mouse p185^{neu}. 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 p185^{neu} 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 rp185^{neu} 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 rp185^{neu} 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 p185^{neu} or rp185^{neu} 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-

genesis (32). These receptors are suitable targets for both T-cell and antibody immune reactivity. Moreover, their interaction with antibody may regulate tumor cell behavior in ways not shared by conventional tumor antigens with no biological role in malignancy (22). As amplification of $p185^{neu}$ expression in premalignant lesions starts at the hyperplasia stage (33), the most important advantage to be gained comes from attacking the target receptor before initiation of the series of genetic and epigenetic events that lead to genetic instability and result in invasive and metastatic malignancy.

The high efficacy of DNA electroporation (19–21) makes it an attractive regimen for extrapolation to a clinical setting. Although the efficacy of i.m. vaccination can be enhanced by the concurrence of costimulatory molecules and cytokines (9, 34), DNA electroporation 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

1. Klein G, Sjogren HO, Klein E, Hellstrom KE. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. Cancer Res 1960;20:1561–72.
2. Boon T. Antigenic tumor cell variants obtained with mutagens. Adv Cancer Res 1983;39:121–51.
3. Berzofsky JA, Ahlers JD, Belyakov IM. Strategies for designing and optimizing new generation vaccines. Nat Rev Immunol 2001;1:209–19.
4. Qin Z, Richter G, Schuler T, Ibe S, Cao X, Blankenstein T. B cells inhibit induction of T cell-dependent tumor immunity. Nat Med 1998;4:627–30.
5. Finn OJ, Forni G. Prophylactic cancer vaccines. Curr Opin Immunol 2002;14:172–7.
6. Forni G, Lollini PL, Musiani P, Colombo MP. Immunoprevention of cancer: is the time ripe? Cancer Res 2000;60:2571–5.
7. Rovero S, Amici A, Di Carlo E, et al. DNA vaccination against rat Her-2/neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. J Immunol 2000;165:5133–42.
8. Boggio K, Nicoletti G, Di Carlo E, et al. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. J Exp Med 1998;188:589–96.
9. Cappello P, Triebel F, Iezzi M, et al. LAG-3 enables DNA vaccination to persistently prevent mammary carcinogenesis in HER-2/neu transgenic BALB/c mice Cancer Res 2003;63:2518–25.
10. Di Carlo E, Diodoro MG, Boggio K, et al. Analysis of mammary carcinoma onset and progression in HER-2/neu oncogene transgenic mice reveals a lobular origin. Lab Investig 1999;79:1261–9.
11. Muller WJ, Sinn E, Wallace R, Pattengale PK, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 1988;54:105–15.
12. Nagata Y, Furugen R, Hiasa A, et al. Peptides derived from a wild-type murine protooncogene c-erbB-2/HER2/neu can induce CTL and tumor suppression in syngeneic hosts. J Immunol 1997;159:1336–43.
13. Diodoro MG, Di Carlo E, Zappacosta R, et al. Salivary carcinoma in HER-2/neu transgenic male mice: an angiogenic switch is not required for tumor onset and progression. Int J Cancer 2000;88:329–35.
14. Curcio C, Di Carlo E, Clynes R, et al. Nonredundant roles of antibody, cytokines and perforin for the immune eradication of established Her-2/neu carcinomas. J Clin Investig 2003;111:1161–70.
15. Ercolini AM, Machiels JP, Chen YC, et al. Identification and characterization of the immunodominant rat HER-2/neu MHC Class I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice. J Immunol 2003;170:4273–80.
16. Nanni P, Nicoletti G, De Giovanni C, et al. Combined allogeneic tumor cell vaccination and systemic IL-12 prevents mammary carcinogenesis in HER-2/neu transgenic mice. J Exp Med 2001;194:1195–205.
17. Trinchieri G. Biology of natural killer cells. Adv Immunol 1989;47:187–376.
18. Cavallo F, Giovarelli M, Gulino A, et al. Role of neutrophils and $CD4^+$ T lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene transfection. J Immunol 1992;149:3627–35.
19. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. Nat Biotechnol 1998;16:867–70.
20. Mir LM, Breau MF, Gehl J, et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci USA 1999;96:4262–7.
21. Widera G, Austin M, Rabussay D, et al. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. J Immunol 2000;164:4635–40.
22. Lollini P-L, Forni G. Cancer immunoprevention: tracking down persistent tumor antigens. Trends Immunol 2003;24:62–6.
23. Katsumata M, Okudaira T, Samanta A, et al. Prevention of breast tumour development in vivo by downregulation of the p185neu receptor. Nat Med 1995;1:644–8.
24. Klapper LN, Vaisman N, Hurwitz E, Pinkas-Kramarski R, Yarden Y, Sela M. A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. Oncogene 1997;14:2099–109.
25. Musiani P, Modesti A, Giovarelli M, et al. Cytokines, tumor-cell death and immunogenicity: a question of choice. Immunol Today 1997;18:32–6.
26. Forni G, Fujiwara H, Martino F, et al. Helper strategy in tumor immunology: expansion of helper lymphocytes and utilization of helper lymphokines for experimental and clinical immunotherapy. Cancer Metastasis Rev 1988;7:289–309.
27. Cavallo F, Quaglino E, Cifaldi L, et al. IL12-activated lymphocytes influence tumor genetic programs. Cancer Res 2001;61:3518–23.
28. Chen Y, Hu D, Eling DJ, Robbins J, Kipps, TJ. DNA vaccines encoding full-length or truncated Neu induce protective immunity against Neu-expressing mammary tumors. Cancer Res 1998;58:1965–71.
29. Reilly RT, Machiels JP, Emens LA, et al. The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors Cancer Res 2001;61:880–3.
30. Bowne WB, Srinivasan R, Wolchok JD, et al. Coupling and uncoupling of tumor immunity and autoimmunity. J Exp Med 1999;190:1717–22.
31. Cordaro TA, De Visser KE, Tirion FH, Schumacher TN, Kruisbeek AM. Can the low-avidity self-specific T cell repertoire be exploited for tumor rejection? J Immunol 2002;168:651–60.
32. Blume-Jensen P, Hunter T. Oncogenic kinase signaling. Nature 2001;411:355–65.
33. Xu R, Perle MA, Inghirami G, Chan W, Delgado Y, Feiner H. Amplification of Her-2/neu gene in Her-2/neu-overexpressing and -nonexpressing breast carcinomas and their synchronous benign, premalignant, and metastatic lesions detected by FISH in archivial material. Mod Pathol 2002;15:116–24.
34. Rovero S, Boggio K, Di Carlo E, et al. Insertion of the DNA for the 163–171 peptide of IL1beta enables a DNA vaccine encoding p185(neu) to inhibit mammary carcinogenesis in Her-2/neu transgenic BALB/c mice. Gene Ther 2001;8:447–52.
35. Tollefsen S, Vordermeier M, Olsen I, et al. DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. Scand J Immunol 2003;57:229–38.

[7] http://www.genetronics.com/press/pr20000503.html.