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J Immunol 2000; 165:5133-5142; ;
doi: 10.4049/jimmunol.165.9.5133
http://www.jimmunol.org/content/165/9/5133

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

DNA Vaccination Against Rat Her-2/Neu p185 More Effectively Inhibits Carcinogenesis Than Transplantable Carcinomas in Transgenic BALB/c Mice[1,2]

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The ability of vaccination with plasmids coding for the extracellular and the transmembrane domain of the product of transforming rat Her-2/*neu* oncogene (r-p185) to protect against r-p185$^+$ transplantable carcinoma (TUBO) cells and mammary carcinogenesis was evaluated. In normal BALB/c mice, DNA vaccination elicits anti-r-p185 Ab, but only a marginal CTL reactivity, and protects against a TUBO cell challenge. Massive reactive infiltration is associated with TUBO cell rejection. In BALB/c mice transgenic for the rat Her-2/*neu* gene (BALB-neuT), DNA vaccination elicits a lower anti-r-p185 Ab response, no CTL activity and only incompletely protects against TUBO cells, but markedly hampers the progression of carcinogenesis. At 33 wk of age, when control BALB-neuT mice display palpable tumors in all mammary glands, about 60% of immunized mice are tumor free, and tumor multiplicity is markedly reduced. Tumor-free mammary glands still display the atypical hyperplasia of the early stages of carcinogenesis, and a marked down-modulation of r-p185, along with a massive reactive infiltrate. However, BALB-neuT mice protected against mammary carcinogenesis fail to efficiently reject a TUBO cell challenge. This suggests that the mechanisms required for the rejection of transplantable tumors may not coincide with those that inhibit the slow progression of carcinogenesis. ***The Journal of Immunology,*** **2000, 165: 5133–5142.**

The Her-2/*neu* gene encodes a p185 tyrosine kinase growth factor receptor homologous to other members of the epidermal growth factor receptor family (1). Overexpressed or mutated p185 leans toward the formation of homo- or heterodimers with other epidermal growth factor receptor. As these dimers transduce positive growth signals in a ligand-independent way (2), they are involved in the initiation and progression of neoplastic transformation (2, 3).

Overexpression of p185 is frequent in human cancers and correlates with particular aggressiveness (4). In the rat, a single point mutation that replaces the valine residue at position 664 in the transmembrane (TM)[4] domain of p185 with glutamic acid favors p185 homo- and heterodimerization and transforms the Her-2/*neu* protooncogene into a dominant transforming oncogene (5). No such mutation, but only an increased Her-2/*neu* gene copy number and/or excess cell membrane expression of p185, was characterized in human tumors (6). However, recent detection in human breast cancer cells of alternatively spliced forms of Her-2/*neu* that resemble transforming Her-2/*neu* suggests that alternative splicing or mutations may also play a critical role in the development of human cancer (7–9).

The involvement of overexpressed and mutated p185 in the initiation and progression of breast carcinogenesis makes it an interesting target for therapy (10–12) and a docking site for toxins (13) and Ab (14). Normal and rat Her-2/*neu* transgenic mice immunized in various ways to Her-2/*neu* display a protective immune response against transplantable Her-2/*neu* tumors and their artificial metastases (15, 16). Moreover, both specific (17–19) and nonspecific (20, 21) immune reactions elicited in mice transgenic for rat Her-2/*neu* protooncogene and transforming oncogene are variously capable of hindering the development of mammary tumors.

In this paper, we assessed whether DNA vaccination with plasmids coding the TM and extracellular domain (ECD) of the rat p185 (r-p185) elicits a protective immune response. In normal BALB/c mice, r-p185 is a xenogeneic protein, even if mouse p185 and r-p185 differ in <6% of the amino acid residues (22). DNA vaccination elicits complete protection against a lethal challenge of syngeneic carcinoma cells expressing the r-p185 (TUBO cells). In BALB/c mice transgenic for the transforming rat Her-2/*neu* oncogene (BALB-neuT mice), r-p185 is a self-protein. DNA vaccination elicits an incomplete protection against TUBO cells, whereas it protects a significant number of mice against the aggressive progression of the carcinogenesis that takes place in all their mammary glands. An anti-r-p185$^+$ CTL response was never found in these mice, whereas they display a significant titer of anti-r-p185

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Received for publication May 22, 2000. Accepted for publication August 1, 2000.

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[1] This work was supported by the Italian Association for Cancer Research, the Istituto Superiore di Sanità, Special Project Gene Therapy, Consiglio Nazionale delle Ricerche Target Project on Biotechnology, University of Bologna (fund for selected research topics), Ministero dell'Università e della Ricerca Scientifica, and Grant DAMD17-98-1-8030 (to G.F.) from the Department of Army, U.S. Army Medical Research (Fort Detrick, MD).

[2] The information contained in this paper does not necessarily reflect the position or the policy of the U.S. government, and no official endorsement should be inferred.

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[4] Abbreviations used in this paper: TM, transmembrane; β-gal, β-galactosidase; ECD, extracellular domain; neuT, Her-2/*neu*-mutated transforming oncogene; PMN, polymorphonuclear leukocytes; r, rat; rV-neu, recombinant vaccinia virus expressing the r-p185; Spc, spleen cells; TSA-pc, TSA parental cells; V-wt, wild-type Wyeth virus; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage-inflammatory protein-2.

0022-1767/00/$02.00

Ab that may be responsible for the down-modulation of r-p185 observed in preneoplastic mammary cells. However, a TUBO cell challenge is still able to grow progressively in half of the BALB-neuT mice whose mammary carcinogenesis is fully inhibited. This finding suggests that the mechanisms required for the rejection of transplantable tumors may not fully coincide with those that inhibit the slow progression of carcinogenesis and that preneoplastic lesions are particularly sensitive to immune mechanisms elicited by DNA vaccination. It also offers support for the use of DNA vaccination as a new approach in the prevention of tumors expressing oncogenic growth factor receptors on their membrane.

Materials and Methods

Mice

Inbred BALB/c mice overexpressing the transforming rat Her-2/*neu* oncogene ($neuT^+/neuT^-$) driven by the mouse mammary tumor virus promoter (BALB-neuT) and transgene negative ($neuT^-/neuT^-$) (BALB/c) were produced and screened for the presence of the transgene as previously described in detail (20). Groups of individually tagged virgin BALB-neuT and BALB/c females bred under specific pathogen-free conditions by Charles River Breeding Laboratories (Calco, Italy) were treated in accordance with European Union and institutional guidelines. Since all 10 mammary glands of BALB-neuT females undergo carcinogenic transformation with a definite progression (20), these were inspected weekly, and tumor masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all mammary glands displayed a palpable tumor or until a tumor exceeded an average diameter of 10 mm, at which time mice were sacrificed for humane reasons. Except where otherwise specified, surviving BALB-neuT mice were sacrificed at 33 wk (20). Because some immunized mice do not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas per mouse was calculated as the cumulative number of incident tumors/total number of BALB-neuT mice.

DNA expression vectors and vaccination

The pCMV vector was derived from the pcDNA3 plasmid (Invitrogen, San Diego, CA) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A). The sequence for the ECD and that for the ECD and TM domain of mutated r-p185 were generated from the PCR product using the primers 3′-CGCAAGCTTCATCATGGAGCTGGC-5′ and 3′-CGGAATT CGGGCTGGCTCTCTGCTC-5′ and the primers 3′-CGCAAGCTTCAT GGAGCTGGC-5′ and 3′-ATGAATTCTTTCCGCATCGTGTACTTCT TCCGG-5′, respectively, as previously described (19). PCR products of the expected size were isolated by agarose gel electrophoresis, digested with *Hin*dIII and *Eco*RI, and cloned into the multiple cloning site of the pCMV plasmid to obtain the two plasmids used in this work (ECD and ECD-TM plasmids). The pCMVβ (Clontech Laboratories, Palo Alto, CA) coding for β-galactosidase was used as a control plasmid (β-gal plasmid). *Escherichia coli* strain DH5α was transformed with ECD, ECD-TM, and β-gal plasmids and then grown in Luria-Bertani medium (Sigma, St. Louis, MO) (19). Large-scale preparation of the plasmids was conducted by alkaline lysis using Endofree Qiagen Plasmid-Giga kits (Qiagen, Chatsworth, CA). DNA was then precipitated, suspended in sterile saline at the concentration of 1 mg/ml, and stored in aliquots at −20°C for subsequent use in immunization protocols. Plasmids (100 μg/injection) were injected into the quadriceps muscle through a 28-gauge needle syringe. BALB/c mice were immunized 21 and 7 days before tumor challenge (day 0), starting at the 10th wk of age. BALB-neuT mice were immunized at the 10th and 12th wk of age or at the 6th, 12th, 18th, and 24th wk of age.

Cell lines and recombinant vaccinia virus

TUBO cells are a cloned cell line established in vitro from a lobular carcinoma that arose spontaneously in a BALB-neuT mouse. TSA parental cells (TSA-pc) are an aggressive and poorly immunogenic cell line established from a moderately differentiated mammary adenocarcinoma that spontaneously arose in a BALB/c mouse from Charles River Breeding Laboratories (23). F1-F is a newborn BALB/c mouse-derived skin fibroblast line spontaneously transformed after the 15th in vitro passage (24). Both TUBO and NIH3T3 (American Type Culture Collection, Manassas, VA) cells were cultured in DMEM (BioWhittaker Europe, Verviers, Belgium) supplemented with 20% and 5% FBS (Life Technologies, San Giuliano Milanese, Italy), respectively; TSA-pc and F1-F cells were cultured in RPMI 1640 (BioWhittaker Europe) with 10% FBS.

Flow cytometry

The expression of r-p185 was evaluated by using 7.16.4 mAb (Oncogene Research Products, Cambridge, MA). mAb against $H\text{-}2K^d$ (clone 31-3-4S) $H\text{-}2D^d$ and Ia^d (clone 28-16-8S) were obtained from Cederlane (Hornby, Ontario, Canada). Cells were stained in a standard indirect immunofluorescence procedure with primary Ab followed by a fluorescein-conjugated anti-mouse IgG (Kirkegaard & Perry, Gaithersburg, MD). Cells were resuspended in PBS containing 1 mg/ml propidium iodide to gate out dead cells and evaluated in a FACScan (Becton Dickinson, Mountain View, CA). Flow cytometry showed that TUBO cells are highly positive for r-p185 and class I glycoproteins of the MHC. TSA-pc and F1-F cells express MHC class I, but not class II glycoproteins (23, 24) nor r-p185. To evaluate the presence of Ab capable of binding TUBO cells, sera were collected from six tumor-free BALB-neuT mice before (at 6 wk of age) or after (at wk 33) ECD-TM DNA immunization. A total of 2×10^5 TUBO cells was stained in a standard indirect immunofluorescence procedure with 50 μl of a 1:10 dilution in PBS-azide-BSA of normal or immune sera followed by a fluorescein-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark). The cells were resuspended in PBS-azide-BSA containing 1 mg/ml propidium iodide and evaluated using a FACScan (Becton Dickinson).

Cytotoxicity assays

The CTL activity of lymphocytes from the mice of the various groups was independently tested immediately or after in vitro restimulation in the laboratory of G.F. (Torino, Italy), M.P.C. (Milan, Italy), and P.-L.L. (Bologna, Italy). Lymphocytes (1×10^7) were stimulated for 6 days with 5×10^5 irradiated TUBO cells as described previously (24). To get better stimulation, this basic design was variously changed in the several repeats of the test. Other rat Her-2/*neu* expressing BALB/c target cells were also used as stimulator and target cells. Moreover, the suppressor activity of stimulator rat Her-2/*neu* BALB/c cells was ruled out by adding progressive numbers of third-party TUBO cells in mixed lymphocyte and allogeneic target cell interactions as previously described (25). CTL activity of fresh and restimulated lymphocytes was assayed in 4- and 18-h ^{51}Cr release assays in Milan (26), in 48- and 72-h [^{3}H]TdR release assays in Torino (24, 25), and in 18- and 40-h [^{3}H]proline release assays in Bologna (27) as previously described in detail. In all of these tests, both TUBO cells and other rat Her-2/*neu* expressing BALB/c target cells were highly lysable by allogeneic CTL.

Cell internalization of r-p185

Expression of p185 was evaluated by confocal microscopy. A total of 2×10^5 TUBO cells was suspended in DMEM, incubated with 50 μl of a 1:10 dilution in PBS-azide-BSA of normal or immune sera for 3 h at 4°C or at 37°C, and washed twice with cold PBS-azide-BSA. For detection of cytoplasmic r-p185, TUBO cells were incubated with 1 ml of PBS-4% paraformaldehyde. After 20 min at 4°C, TUBO cells were washed twice with cold PBS-azide-BSA and then incubated with 1 ml of PBS-0.3% Triton X-100. After 30 min at room temperature, TUBO cells were washed twice with cold PBS-azide-BSA. Membrane and cytoplasmic expression of r-p185 TUBO cells was assessed by staining with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Internalization of fluorescent mAb was then measured on a confocal microscope (LFM310; Zeiss, Jena, Germany) (488-nm argon laser and 543-nm helium-neon laser). Green fluorescence was detected after excitation at 488 nm. Images were recorded as TIF files and processed (LSM Image Examiner; Zeiss) to subtract background and enhance lower and middle intensity fluorescence.

Tumor challenge and evaluation of tumor growth

At the times specified, mice were challenged s.c. in the left flank or in the neck region with 0.2 ml of a single-cell suspension of 1×10^5 TUBO or TSA-pc cells or 1×10^4 F1-F cells. These are about the minimal 100% tumor-inducing doses in BALB/c mice (24). The same minimal 100% tumor-inducing dose for TUBO cells was found in both BALB/c and BALB-neuT mice. Subcutaneous neoplastic masses were measured with calipers in two perpendicular diameters. The cages were coded, and the incidence and growth of tumors were evaluated weekly for 60 days in a fashion blind to the group in which they had been treated. Mice tumor free at the end of this period were classed as survivors. Neoplastic masses were measured with calipers in the two perpendicular diameters. Mice with a tumor mass with a mean diameter of >3 mm were classed as tumor bearers. Mice bearing neoplastic masses of >10 mm in mean diameter were killed for humane reasons.

Winn assay

The inhibition of TUBO cell growth in vivo was assayed using the Winn-type neutralization assay as previously described in detail (28). Various numbers of nylon-wool column-purified spleen cells (Spc) were admixed with the minimal lethal dose of TUBO or TSA cells in 0.2 ml of PBS and immediately injected s.c. in the left inguinal region of recipient mice. The ratios of lymphocytes:tumor cells were 1:1, 5:1, and 20:1.

Morphological analysis

Groups of three BALB-neuT mice were sacrificed at the indicated times each week until the 33rd wk. For histological evaluation, tissue samples were fixed in 10% neutral-buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with anti-dendritic cells (NLDC 145; Cederlane), anti-CD4 and anti-CD8a (Sera-Lab, Crawley Down, Sussex, U.K.), anti-Mac-1 (anti-CD11b/CD18), anti-Mac-3 and anti-Ia (Boehringer Mannheim, Milan, Italy), anti-polymorphonuclear leukocytes (PMN) (RB6-8C5, provided by R. L. Coffman, DNAX, Palo Alto, CA), anti-asialo GM1 (Wako Chemicals, Dusseldorf, Germany), anti-endothelial cells (mEC-13.324), anti-ELAM-1 (E-selectin; both provided by A. Vecchi, Istituto M. Negri, Milan, Italy); anti-ICAM-1 (CD54), anti-VCAM-1 (PharMingen, San Diego, CA), anti-IL-4, anti-IL-6, anti-IL-10, anti-IL-12, and anti-monocyte chemoattractant protein-1 (MCP-1) (PharMingen); anti-macrophage-inflammatory protein-2 (MIP-2; Walter Occhiena Srl, Torino, Italy); anti-RANTES (PeproTech, Rocky Hill, NJ); anti-IL-1β (Genzyme, Cambridge, MA), anti-TNF-α (Immuno Kontact, Frankfurt, Germany), anti-IFN-γ (provided by S. Landolfo, University of Turin, Torino, Italy), and anti-inducible NO synthase (Transduction Laboratories, Lexington, KY) Ab. To evaluate the expression of r-p185 Ag, paraffin-embedded sections were tested with anti-neu Ab (C-18)-G (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, they were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit or horse anti-goat Ig (Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ab was removed by washing and the slides were incubated with ABC complex/AP (Dako). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blind fashion. From mice with multiple tumors, one sample per tumor growth area and 10 randomly chosen fields in each sample were evaluated for each point determination. Positive cells were counted under a microscope ($\times$400 field, $\times$40 objective, and $\times$10 ocular lens; 0.180 mm^2/field). The expression of adhesion molecules, cytokines, and mediators was defined as absent ($-$), scarcely ($+/-$), moderately ($+$), and frequently ($++$) present on cryostat sections tested with the corresponding Ab.

ELISA titration of anti-r-p185 Ab

Serum samples were titrated for the presence of anti-r-p185 Ab by ELISA and immunoprecipitation followed by Western blot analysis as described previously (29). NIH3T3 fibroblasts were infected with the V-Wyeth virus (wild-type control virus, V-wt) or with the recombinant vaccinia virus expressing the r-p185 (rV-neu), both kindly provided by Therion Biologics (Cambridge, MA). The r-p185 recombinant protein was detected by Western blot analysis using the Ab-1 polyclonal Ab at 1 mg/ml as described below. For ELISA, 5×10^4 NIH3T3 cells/well were allowed to adhere overnight in 96-well culture plates. After washing with PBS, sucrose gradient-purified rV-neu and V-wt viruses were added for 12–18 h at 10 PFUs/well. Plates were then dried overnight and nonspecific binding was blocked by a 1-h incubation with 5% BSA in PBS. Ab-4 mAb or mouse myeloma protein (MOPC21; Cappel, West Chester, PA) at 1 μg/ml or mouse serum pool (1:5/1:50/1:250/1:1250 dilutions) were added for 3 h at 30°C. After washing, HRP-conjugated goat anti-mouse IgG plus IgM (Life Technologies, Rockville, MD) was added, and the bound Ab was detected with *o*-phenylenediamine dihydrochloride (Sigma). The reaction was stopped with 25 μl of 4 N H_2SO_4, and the absorbance at 492 nm was evaluated with an automatic ELISA reader. The specific absorbance of each sample was calculated by subtracting its absorbance from that of V-wt NIH3T3 cells. The titer of the serum was defined as the highest dilution reaching a specific binding with an OD of 0.3. The contribution of each isotype to the anti-r-p185 titer was evaluated using a 1:500 and a 1:50 dilution of BALB/c and BALB-neuT sera, respectively, and a sera Mouse Typer Isotyping kit (Bio-Rad, Richmond, CA) as previously described (30). The percentage was calculated as the ratio of the specific OD 492-nm values of each isotype and that of all isotypes $\times$100.

Western blots

For immunoprecipitation, NIH3T3 cells were infected with 10 PFU/cell of either V-wt or rV-neu and cultured at 37°C for 18 h. Cell lysates were prepared in lysis buffer (10 mM sodium phosphate (pH 7.4), 100 mM NaCl, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) containing 100 μg/ml aprotinin and 1 mM PMSF. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad) according to Bradford (31).

One microgram of Ab-4 mAb or purified MOPC-21 mouse myeloma protein or 3 ml of mouse serum and 20 μl of protein G-Sepharose were reacted with 300 μg lysate of V-wt- or rV-neu-infected NIH3T3 cells for 3 h at 4°C. The beads were washed with Staph A buffer, and the pellets were denatured by boiling for 5 min in 30 μl of sample buffer (100 mM Tris (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 50 mM 2-ME). Electrophoresis of immunoprecipitates or protein lysates (100 μg/lane) was conducted in denaturing 8% Tris-glycine polyacrylamide gels (SDS-PAGE). Gels were then processed for immunoblotting using Ab-1 polyclonal Ab at 1 μg/ml and bound Ab were visualized as previously described (30).

Statistical analysis

Differences in tumor incidence were evaluated using the Mantel-Haenszel log rank test, differences in tumor/mouse numbers using Wilcoxon's rank sum test, and differences in the number of tumor-infiltrating cells by Student's *t* test.

Results

r-p185 expressed by TUBO cells is poorly immunogenic in normal BALB/c mice

TUBO cells are a cloned line established in vitro from a BALB-neuT mouse mammary carcinoma. They display membrane class I H-2^d MHC glycoproteins and r-p185 neu proteins (Fig. 1, *upper panels*). In BALB/c mice, r-p185 is a xenogeneic Ag that differs from mouse r-p185 in $<$6% of the amino residues (22). Despite these differences, a challenge of 1×10^5 TUBO cells grew progressively in all BALB/c mice (Table I) and gave rise to lobular carcinomas histologically similar to those that appear in BALB-neuT-transgenic mice (Figs. 1*e* and 2*c*). The reactive cell infiltrate associated with TUBO cell growth was marginal and not stronger than that of the fully syngeneic TSA-pc mammary carcinoma (Table II). No anti-TUBO cell CTL, nor IFN-γ, nor GM-CSF release were found when Spc from BALB/c mice bearing 3- or 10-mm mean TUBO tumors were tested immediately or after 6 days in in vitro restimulation with TUBO cells as previously described in detail (24, 25) (data not shown). Moreover, no anti-r-p185 Ab were detected in the sera of mice receiving saline or immunized with β-gal plasmid (Fig. 3). Despite their high membrane expression of the xenogeneic r-p185, growing TUBO cells appear to trigger a marginal or no immune reaction in BALB/c mice.

DNA vaccination induces protective immunity against TUBO cells in BALB/c mice

A significant and specific inhibition of TUBO cells was found in BALB/c mice immunized with either ECD or ECD-TM plasmids 21 and 7 days before TUBO cell challenge (Table I). TUBO cells initially formed small cell aggregates infiltrated by reactive leukocytes in close contact with severely injured tumor cells (Fig. 1*h*). Rejection was associated with a marked influx of PMN, $CD4^+$ and $CD8^+$ T lymphocytes, and an increase in the number of dendritic cells, macrophages, and NK cells (Table II). Induction and increased expression of endothelial cell adhesion molecules was also evident in tumor vessels. IFN-γ and MCP-1 were expressed, while overexpression of IL-1β, TNF-α, and IL-10 was also evident. No TUBO cell inhibition was found in BALB/c mice injected with saline only or immunized with β-gal plasmids. Since the results from these two treatment groups were consistently similar, hereafter both groups are cumulatively denominated as controls.

Anti-r-p185 Ab were detected in the sera of both ECD- and ECD-TM-immunized mice. They are mainly IgG2a, IgM, and IgG1 (Fig. 3). All sera pools scored positive immunoprecipitated

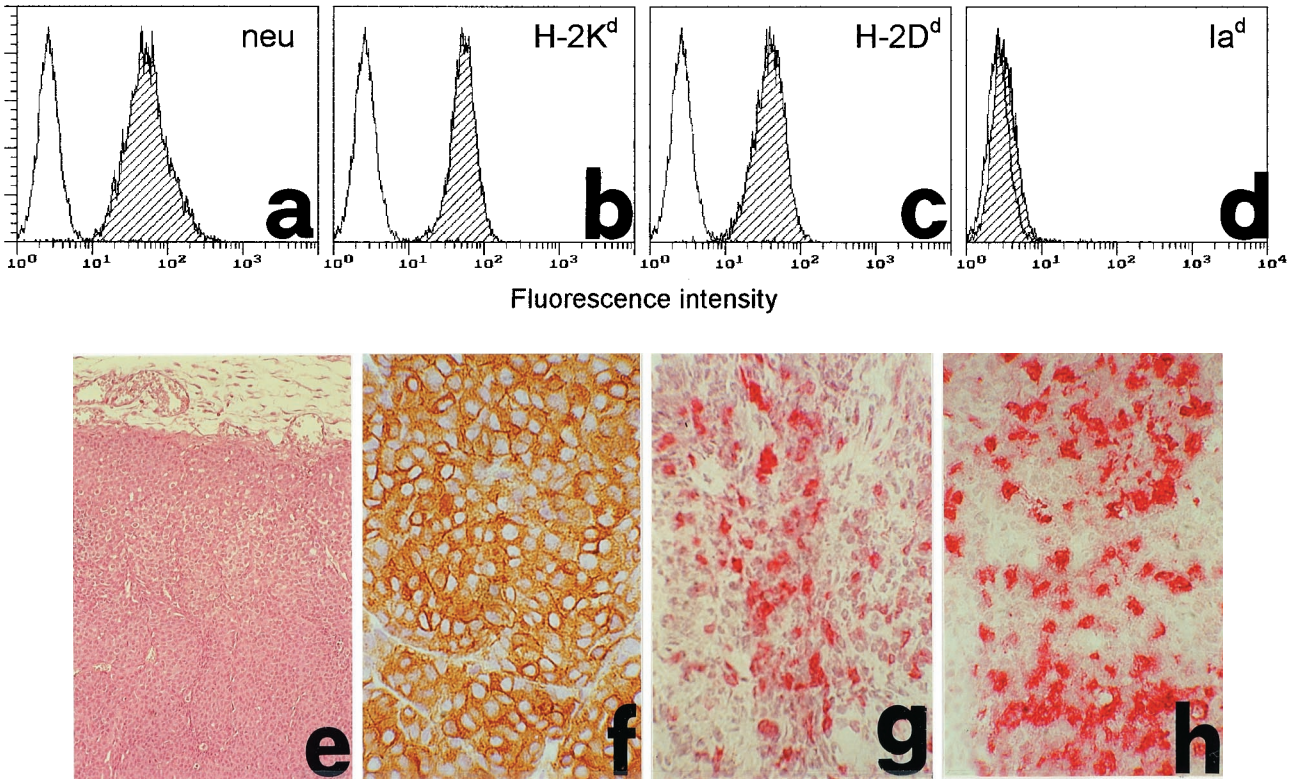

FIGURE 1. Features of TUBO cells. *Upper panels*, Flow cytometric analysis of the expression r-p185 protein (*a*), MHC class I (H-2K^d, *b*; H-2D^d, *c*), and MHC class II (Ia^d; *d*) by TUBO cells. Open profiles, Cells stained with secondary Ab; shaded profiles, cells stained with anti-r-p185 or anti-MHC Ab. In each graph, the ordinate represents the number of cells and the abscissa reports the fluorescence intensity in logarithmic arbitrary units. *Lower panels*, TUBO cells injected s.c. in BALB/c mice gave rise to lobular carcinomas histologically similar to the mammary carcinomas arising in BALB-NeuT mice (*e*) that homogeneously express r-p185 on their cell membrane (*f*). TUBO cell rejection area in BALB/c mice immunized with ECD-TM plasmid was heavily infiltrated by CD4$^+$ T lymphocytes (*g*) and PMN (*h*). Original magnification: *e*, ×200; *f–h*, ×630).

the r-p185 from the NIH3T3 cells infected with rV-neu and not those with V-wt virus. Those scored negative did not (data not shown). The anti-r-p185 Ab titer did not substantially increase after a subsequent challenge and rejection of TUBO cells. In contrast with both the Ab response and the marked cell reaction at tumor site, a marginal CTL activity and no IFN-γ release against TUBO cells were found in Spc from ECD- or ECD-TM-immunized BALB/c mice collected 7 days after the last immunization and tested immediately or after 6 days in in vitro restimulation with TUBO cells (data not shown). Moreover, both fresh and in vitro restimulated Spc did not block TUBO cell growth in a Winn assay (28), and only a slight delay of tumor growth was found at a 20:1 lymphocyte:tumor cell ratio.

DNA vaccination induces a partial protection against TUBO cells in BALB-neuT mice

In female BALB-neuT mice, r-p185 is a self-protein markedly expressed in terminal ductal-lobular structures of the mammary glands as early as the third week of age (Fig. 2*a*). To evaluate whether DNA vaccination induces a protective response to TUBO cells, BALB-neuT mice were immunized with the ECD-TM plasmid two or four times. Vaccination on week 10 and 12, when hyperplasia of the terminal ductal-lobular structures is already evident, protects a few mice against a TUBO challenge 7 days after the second immunization (Table III). However, the latency of the tumors that eventually grew was extended in immunized as compared with control mice. Partial protection, too, was found in mice immunized four times and challenged with TUBO cells at 33 wk of age (Table III). Immunohistochemistry showed that the tumor area of the TUBO challenge in ECD-TM-immunized mice presents a significant increase in the number of CD8$^+$ cells and PMN as compared with control mice (Table II). No difference in the expression of endothelial adhesion molecules, cytokines, and mediators was found.

No anti-r-p185 Ab were found in the sera from mice challenged with TUBO cells only or immunized with saline or β-gal plasmids. The titer of those found in sera from ECD-TM-immunized mice was higher in animals that received four vaccinations (Table IV).

DNA vaccination effectively halts carcinogenesis in BALB-neuT mice

Since DNA vaccination elicited a partial resistance against TUBO cells, its ability to hamper the aggressive carcinogenesis that takes

Table I. *Growth and rejection of TUBO cells in control, ECD-immunized, and ECD-TM-immunized BALB/c mice*

BALB/c Mice Immunized with[a]	Tumor Take/Mice Challenged with		
	TUBO cells	TSA-pc	F1-F cells
Saline only	12/12 (0%)[b]	6/6 (0%)	6/6 (0%)
β-gal plasmid	6/6 (0%)	6/6 (0%)	6/6 (0%)
ECD plasmid	0/6[c] (100%)	ND	6/6 (0%)
ECD-TM plasmid	0/12[c] (100%)	6/6 (0%)	6/6 (0%)

[a] Performed at days −21 and −7 before challenge (day 0).
[b] Percentage of survival in parentheses.
[c] $p \leq 0.001$ as compared to BALB/c mice receiving saline only.

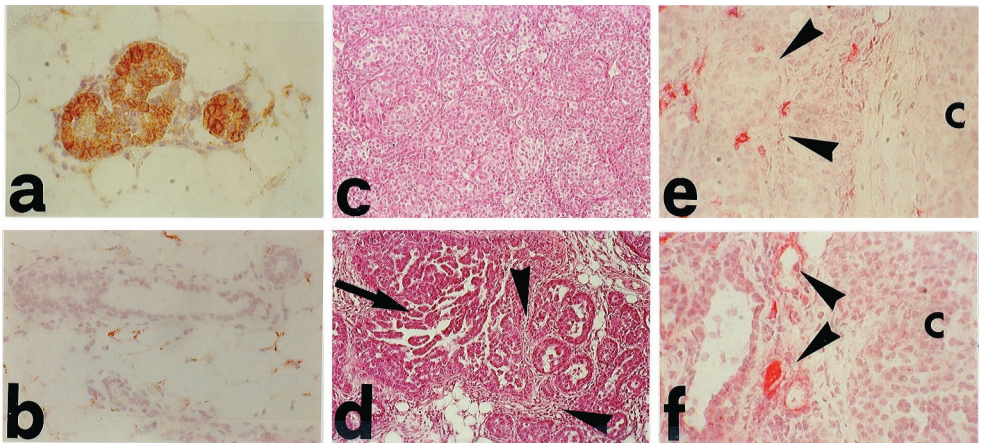

FIGURE 2. Morphological events associated with the inhibition of carcinogenesis in BALB-neuT mice. r-p185 is markedly expressed (in brown) in the hyperplastic terminal ductal-lobular structures of the mammary glands of females 3-wk-old BALB-NeuT mice (*a*). At 33 wk of age, all mammary glands of control BALB-NeuT mice show invasive lobular carcinomas (*c*), whereas several glands from ECD-TM-immunized mice display areas composed of numerous ductules lined by a monolayer of epithelial cells without membrane or cytoplasmic r-p185 expression (*b*). Serial sections indicate that the majority of these ductules do not end in differentiated terminal ductal-lobular structures. Some glands show areas of atypical hyperplasia with the interposed stroma (arrowheads) markedly infiltrated by reactive cells and foci of desegregated lobular carcinoma (arrow) with loosely cohesive tumor cells (*d*). Hyperplastic areas are infiltrated by $CD8^+$ lymphocytes (arrowheads, *e*) while microvessels expressed VCAM-1 adhesion molecule (arrowheads, *f*). When areas of hyperplasia (*left*) and lobular carcinoma (*c*, *right*) are present in the same gland (*e* and *f*), infiltrating $CD8^+$ lymphocytes (*e*, arrowheads) and VCAM-1 expression (*f*, arrowheads) are evident in hyperplastic foci and almost absent in carcinomas. Original magnification: *a*, *b*, *e*, and *f*, ×400; *c* and *d*, ×200.

place in all of the mammary glands of BALB-neuT mice was assessed. Mice were immunized at the 6th, 12th, 18th, and 24th wk of age with the ECD-TM plasmid, and then inspected weekly to follow tumor onset and growth. At 33 wk, when all 10 mammary glands of control mice presented an evident palpable mass, 57% of the immunized mice were still completely free (Fig. 4, *upper panel*). A significant reduction in tumor multiplicity was also evident (Fig. 4, *bottom panel*).

Pathological observations showed that at 33 wk of age control mice uniformly display invasive lobular carcinomas in all 10 glands (Fig. 2*c*). By contrast, three distinct patterns were displayed by the glands from ECD-TM-immunized mice. Those without a palpable mass showed numerous ductules lined by a monolayer of epithelial cells. In about 60% of cases, serial sections displayed truncated ductules that did not end in differentiated terminal ductal-lobular structures (Fig. 2*b*), and the remaining structures

Table II. *Reactive cell content, expression of endothelial adhesion molecules and production of cytokines and mediators at the tumor area 7 days after TSA-pc or TUBO challenge of BALB/c and BALB-neuT mice immunized with ECD-TM plasmid*

	BALB/c Mice Challenged with		ECD-TM-Immunized BALB/c Mice Challenged with TUBO Cells	BALB-neuT Mice Challenged with TUBO Cells	ECD-TM-Immunized BALB-neuT Mice Challenged with TUBO Cells
	TSA-pc	TUBO cells			
Reactive cells[a]					
Dendritic cells	14 ± 4	ND	6 ± 1[b]	−	−
Macrophages	20 ± 4	20 ± 5	55 ± 15[b]	19 ± 5	14 ± 4
PMN	14 ± 4	7 ± 2	76 ± 19[b]	6 ± 2	14 ± 3[b]
$CD8^+$ lymphocytes	5 ± 2	9 ± 3	30 ± 6[b]	4 ± 2	8 ± 2[c]
$CD4^+$ lymphocytes	ND	ND	40 ± 9[b]	1 ± 1	3 ± 2
NK cells	ND	10 ± 3	30 ± 5[b]	7 ± 2	8 ± 3
Endothelial adhesion molecules					
ICAM-1	+[d]	+	++	+	+
ELAM-1	−	−	+	+/−	+
VCAM-1	−	−	+/−	−	−
Cytokines and mediators					
IL-1β	−	+	++	+/−	+/−
TNF-α	+	+/−	+	+	+
IFN-γ	−	−	+	+/−	+/−
IL-4	−	−	−	ND	ND
IL-10	+	+	++	ND	ND
IL-12	−	−	−	ND	ND
MCP-1	−	−	+	−	−
MIP-2	+	+	+	+	+
RANTES	ND	ND	ND	+/−	+/−

[a] Cell counts performed at ×400 in a 0.180-mm^2 field. At least 3 samples (1 sample/tumor growth area) and 10 randomly chosen fields/sample were evaluated. Results are expressed as mean ± SD of positive cells/field evaluated on cryostat sections by immunohistochemistry.

[b] Values significantly different ($p < 0.001$) from corresponding values in untreated mice.

[c] Values significantly different ($p < 0.005$) from corresponding values in untreated mice.

[d] The expression of adhesion molecules, cytokines, and mediators was defined as absent (−), scarcely (+/−), moderately (+), and frequently (++) present on cryostat sections decorated with the Ab.

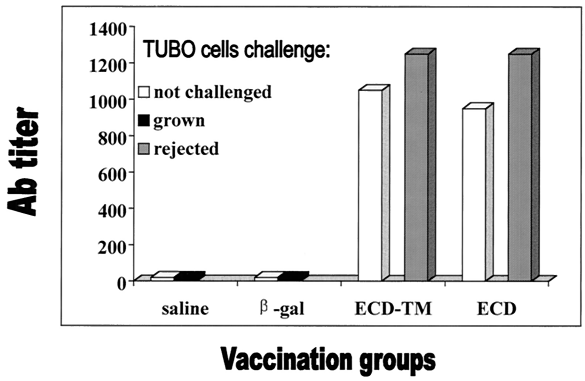

FIGURE 3. Titer of Ab to r-p185 in ECD- and ECD-TM-immunized BALB/c mice. Mice were immunized at days −21 and −7 before challenge (day 0), and sera collected at day 0 (before TUBO challenge) or 2 wk later. Sera from six mice were pooled and the titer was defined by ELISA as the reciprocal of the dilution reaching an OD of 0.3. The percentage of various isotype relative to the total anti-r-p185 Ig in the sera of ECD-TM-immunized mice that have rejected TUBO cells was: IgG2a, 59%; IgM, 26%; and IgG1, 12%.

showed a reduced tendency to give rise to foci of lobular atypical hyperplasia. Massive reactive cell infiltration of their stroma was associated with the induction and increased expression of IL-1β, TNF-α, and IFN-γ and of endothelial cell adhesion molecules (Table V).

Glands with no palpable tumors or with small tumor masses showed foci of in situ lobular carcinomas. In this case, however, the majority of tumor cells were desegregated and loosely cohesive (Fig. 2*d*). Reactive leukocytes and expression of proinflammatory cytokines were both more marked than in carcinomas of control mice and much less numerous than in the hyperplastic foci (Table V). These differences were also evident when both carcinoma and hyperplasia were present in contiguous areas of the same mammary gland (Fig. 2, *e* and *f*). This suggests that hyperplasia is open to the immune reactivity, but becomes much less susceptible once it has progressed to carcinoma. In both cases, reactive leukocytes were in the stroma surrounding hyperplastic and neoplastic lobules and did not penetrate the basal membrane (Fig. 2*d*). Finally, mammary glands with >4-mm mean diameter tumor masses displayed

Table III. *Growth and rejection of TUBO cells in control and ECD-TM-immunized BALB-neuT mice*

BALB-neuT Mice Immunized and Challenged		TUBO Cell Growth		
Vaccination plasmids	Week of vaccination	Week of TUBO cell challenge	Takes/ challenged mice	Latency time (days)
Saline only	10, 12	13	6/6 (0)[a]	22 ± 3
β-gal plasmid	10, 12	13	6/6 (0)	17 ± 1
ECD-TM plasmid	10, 12	13	4/6 (33%)	58 ± 17[b]
ECD-TM plasmid	6, 12, 18, 24	33	3/6 (50%)	20 ± 2

[a] Percentage of survival in parentheses.
[b] $p \leq 0.001$ as compared to BALB-neuT mice receiving saline only.

invasive carcinomas indistinguishable from control mice (data not shown).

Immune activities associated with the inhibition of carcinogenesis

Here, too, the marked infiltrate observed in pathological specimens did not correlate with the induction of a CTL response in Spc from immunized mice tested either fresh or after in vitro restimulation nor with their ability to inhibit the growth of TUBO cells in a Winn test (data not shown). By contrast, a significant anti-r-p185 Ab response, mainly represented by IgG2a, IgG1, IgM, and IgA (Table IV), was detected in sera from immunized mice. When incubated with TUBO cells, these sera induced stripping of r-p185 from the membrane and its cytoplasmic internalization (Fig. 5*g*). A similar impressive down-modulation of r-p185 membrane expression and its cytoplasmic expression only were observed in most hyperplastic lesions from ECD-TM-immunized mice (Fig. 5*h*). Their reduced r-p185 cell surface expression was accompanied by a diminished nuclear positivity of proliferating cell nuclear antigen (PCNA; data not shown). When the lesions progressed to carcinoma in situ, areas with high membrane expression of r-p185 and marked nuclear PCNA positivity alternated with others with only intracytoplasmic r-p185 and no PCNA expression (data not shown). No more r-p185 down-modulation but a similarly marked r-p185 membrane staining was evident in most neoplastic cells of invasive mammary carcinomas in immunized mice or in control BALB-neuT mice (data not shown).

Table IV. *Ab to r-p185 in ECD-TM-immunized BALB-neuT mice challenged with or without TUBO cells*

BALB-neuT Mice Immunized with	No. of Mice	Week of Vaccination	TUBO Cell Challenge	Growth of a TUBO Tumor (>3 mm diameter)	Week of Bleeding	Dominant Stage of the Mammary Glands	Titer of Ab to r-p185[a]
Saline	6	10, 12	NC[b]	−	13	Carcinoma in situ	<5
Saline	6	10, 12	Yes[c]	+	16	Carcinoma in situ	<5
β-gal plasmid	6	10, 12	NC	−	16	Carcinoma in situ	<5
β-gal plasmid	6	10, 12	Yes[c]	+	16	Carcinoma in situ	<5
β-gal plasmid	6	6, 12, 18, 24	NC	−	33	Invasive carcinoma	11
ECD-TM plasmid	4	10, 12	Yes[c]	+	24	Normal/Atypical hyperplasia	85
ECD-TM plasmid	2	10, 12	Yes[c]	−	24	Normal/Atypical hyperplasia	73
ECD-TM plasmid	4	6, 12, 18, 24	NC	−	33	Normal/Atypical hyperplasia	240[d]
ECD-TM plasmid	3	6, 12, 18, 24	NC	−	33	Atypical hyperplasia/Invasive carcinoma	200
ECD-TM plasmid	3	6, 12, 18, 24	Yes[e]	−	38	Atypical hyperplasia	200
ECD-TM plasmid	3	6, 12, 18, 24	Yes[e]	+	38	Atypical hyperplasia	230

[a] Ab titer was defined in ELISA as in Fig. 3.
[b] NC, Not challenged.
[c] Challenged with 1×10^5 TUBO cells at 13 wk of age.
[d] Ig isotypes of anti-r-p185 Ab: IgG2a, 32%; IgG1, 24%; IgM, 22%; and IgA, 13%.
[e] Challenged with 1×10^5 TUBO cells at 33 wk of age.

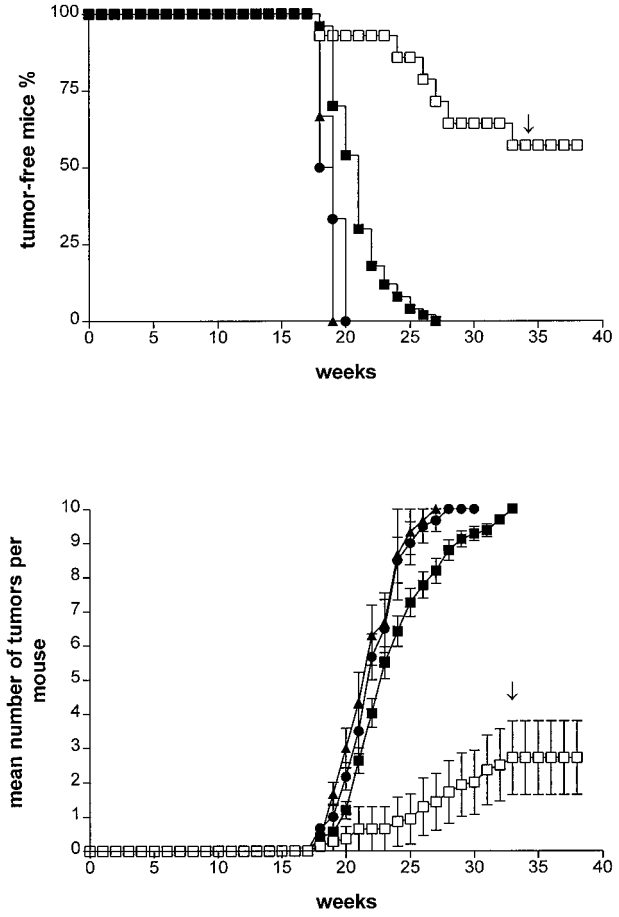

FIGURE 4. Effect of vaccination with ECD-TM plasmid on carcinogenesis in BALB-neuT mice. Time of appearance of the first tumor (*upper panel*) and mean number of palpable mammary carcinomas per mouse (*lower panel*) in the group of 50 untreated mice (■), of 6 mice treated with saline only (▲), and β-gal (●), and in a group of 14 mice immunized with ECD-TM (□). Arrow shows the week in which ECD-TM-immunized mice without any palpable tumors were challenged in the neck with TUBO cells.

BALB-neuT mice in which carcinogenesis is inhibited are still susceptible to TUBO cells

Six DNA-vaccinated BALB-neuT mice that did not display any palpable tumor at week 33 were challenged s.c. in the neck with TUBO cells (Fig. 4, arrow). Three rejected the challenge. In the other three TUBO cells grew after latency similar to the control BALB-neuT mice (Table III). No spontaneous mammary carcinoma progressed and became palpable in any of these six mice during the 35 days that followed the TUBO cell challenge, showing that inhibition of carcinogenesis can exceed 38 wk, when the mice were sacrificed.

Discussion

Present data show that vaccination with plasmids coding either ECD-TM or ECD of r-p185 protects BALB/c mice against a challenge with TUBO mammary carcinoma cells that express the xenogeneic r-p185 on their membrane. In transgenic BALB-neuT, r-p185 is a self-protein that is already overexpressed by the mammary gland at the third week of life. Nonetheless, vaccination with the ECD-TM plasmid protects a smaller, but significant portion of BALB-neuT mice against TUBO cells, and significantly inhibits the progression of their aggressive mammary carcinogenesis. At 33 wk, large lobular carcinomas are evident in all mammary glands of the controls (20, 32). At this time point, more than half of the immunized BALB-neuT mice are tumor free, and the mean number of glands with a palpable carcinoma is much lower than in the controls. Interestingly, not all mice whose carcinogenesis is fully inhibited reject a subsequent TUBO cell challenge.

These data show that DNA vaccination manages the aggressive progression of Her-2/*neu* mammary carcinogenesis more effectively than the nonspecific reactivity elicited by systemic IL-12 (20). The best IL-12 regimen markedly delays, but rarely inhibits this carcinogenesis (21). The mechanisms involved are also different. IL-12 induces the release of a series of downstream mediators that trigger nonspecific cellular immunity and impair the vascular proliferation associated with carcinogenesis (20, 33). Its antiangiogenic and angiotoxic activity is mostly effective on the fragile capillary sprouts associated with the shift from atypical hyperplasia to carcinoma (20). By contrast, ECD-TM vaccination results in inhibited progression unaccompanied by areas of ischemic-hemorrhagic necrosis or signs of vascular damage.

At 33 wk of age, three prototypic scenarios are displayed by the mammary glands of ECD-TM-immunized mice. Atypical hyperplasia is displayed by tumor-free mammary glands. In control BALB-neuT mice, this lesion connotes a much earlier phase of carcinogenesis. At variance with controls, the hyperplasia in immunized mice is accompanied by pronounced infiltration of dendritic cells, macrophages, PMN, and T lymphocytes and the local presence of proinflammatory cytokines. The high expression of adhesion molecules by vessel endothelial cells accounts for this marked reactive cell infiltration. Furthermore, the terminal ductal-lobular structures and hyperplastic foci were less prosperous, formed of epithelial cells that express cytoplasmic but not membrane r-p185, and displayed a reduced cell proliferation. In vitro, r-p185 stripping from TUBO cell membrane and its cytoplasmic internalization is induced by the Ab present in the sera of these mice.

Other mammary glands display evident in situ lobular carcinomas. By contrast with the much earlier corresponding lesions in the controls, carcinoma cells are desegregated and loosely cohesive and produce empty lacunar spaces. Areas with high membrane expression of r-p185 and marked nuclear PCNA positivity alternate with others with only intracytoplasmic r-p185 and no PCNA expression. Down-modulation of membrane r-p185 correlates with a restrained neoplastic proliferation. Finally, there were no pathological differences between the invasive carcinomas observed in the immunized mice and the controls. Although 38 wk is a significant period in the life of a mouse, the evidence on the presence of microscopic preneoplastic and neoplastic lesions in the mammary glands of vaccinated mice has spurred ongoing experiments to evaluate how long further boosting vaccinations inhibit the progression of carcinogenesis during the aging of mice.

Apparently both leukocyte infiltration and the release of proinflammatory cytokines decline as the hyperplasia progresses to carcinoma. The immune mechanisms elicited by ECD-TM vaccination reach hyperplastic lesions and halt their progression, but are incapable of dealing with an established carcinoma whose extracellular matrix (34, 35), neovessels (36), positive pressure (37), and release of many suppressive factors (38) secure its resistance to immune attack. These factors may also account for the poor ability of ECD-TM vaccination to inhibit TUBO cell challenges. TUBO cells, like transplantable tumors in general, very quickly give rise to solid tumor masses that are highly vascularized and refractory to most immune mechanisms (39).

The discrepancy between the impressive ability of immunized BALB-neuT mice to halt the progression to carcinoma and their relatively minor ability to inhibit the takes of transplantable TUBO

Table V. *Reactive cell content, expression of endothelial adhesion molecules, and production of cytokines and mediators in the mammary glands of control or ECD-TM-immunized BALB-neuT mice*

	Carcinomas in β-gal Immunized Mice (week 13)	Carcinomas in ECD-TM-Immunized Mice (week 33)	Atypical hyperplasia in ECD-TM-Immunized Mice (week 33)
Reactive cells			
Dendritic cells	1 ± 0^a	7 ± 2^b	10 ± 3^b
Macrophages	11 ± 3	22 ± 4^b	23 ± 4^b
PMN	3 ± 2	12 ± 3^b	$23 \pm 4^{b,c}$
$CD8^+$ lymphocytes	3 ± 1	8 ± 2^b	$30 \pm 6^{b,c}$
$CD4^+$ lymphocytes	2 ± 1	1 ± 0	$12 \pm 4^{b,c}$
NK cells	2 ± 1	5 ± 2^b	8 ± 2^b
Endothelial adhesion molecules			
ICAM-1	$+^d$	++	++
ELAM-1	−	+	++
VCAM-1	−	−	+
Cytokines and mediators			
IL-1β	−	+/−	+
TNF-α	+/−	+	++
IFN-γ	−	+/−	+
MCP-1	−	−	+/−
MIP-2	+/−	+	++

[a–d] As for Table II.

cells may be due to the type of reaction mechanisms activated by vaccination. ECD- and ECD-TM-vaccinated mice, in fact, never displayed a marked CTL response, despite all the in vitro restimulation attempts performed independently in three of the laboratories involved in this study. This provocative finding may suggest that in our system DNA vaccination was unable to break T killer cell tolerance against r-p185, a xenogeneic protein that shares >94% homology with mouse p185. This is somewhat surprising, since various peptides from r-p185 bind the grooves of H-2^d class I and class II glycoproteins and display amino acid sequences different from those of mouse p185 (22). Despite the repeated efforts in CTL assessment, it is virtually impossible to rule out any potential flaws in our in vitro analyses. Moreover, the absence of detectable in vitro T cell activity does not necessarily correlate with an effective T cell-dependent antitumor immunity in vivo (28). In contrast, pathological findings indicated that reactive cells were in the stroma surrounding hyperplastic and neoplastic terminal ductal-lobular structures of the mammary, but they never penetrated the basal membrane and were found intermingled with neoplastic epithelial cells.

The only in vitro finding that correlates with the protection in vivo and the evidence of immune reactions at challenge sites or in

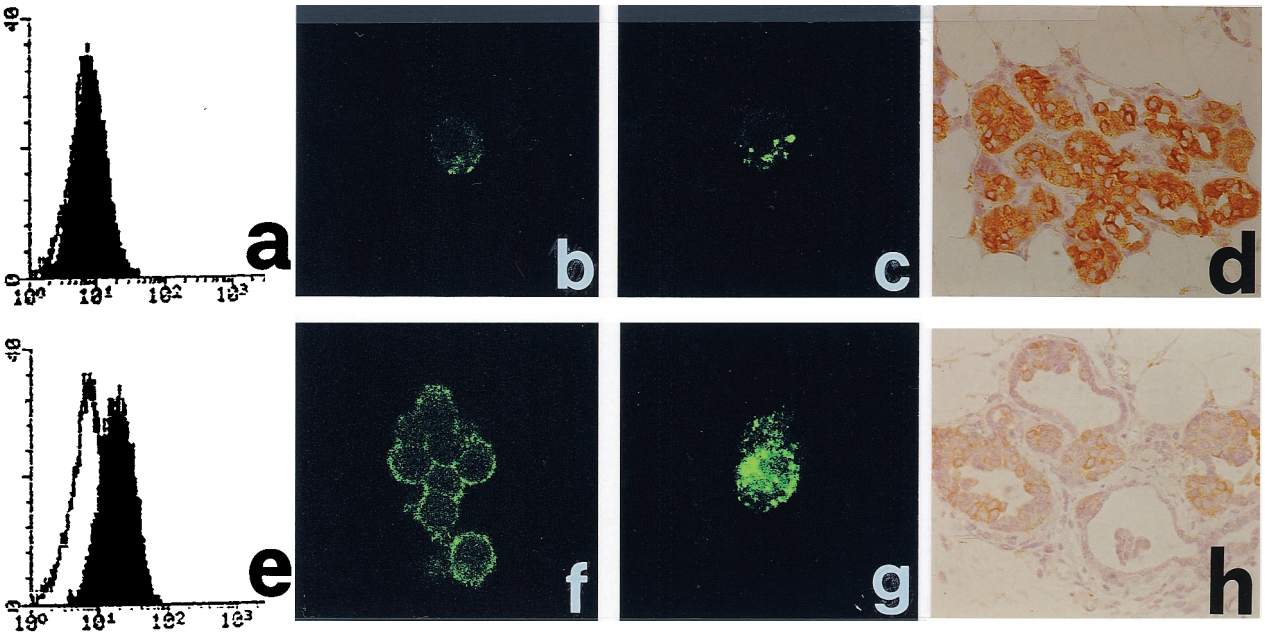

FIGURE 5. Down-modulation of cell membrane expression of r-p185. *a* and *e*, Flow cytometric analysis of the ability of sera to bind TUBO cells. Open profiles, Cells stained with secondary Ab; shaded profiles, cells stained with sera pool from BALB-neuT mice before (*a*) or after DNA vaccination (*e*). The ordinate represents the number of cells and the abscissa reports the fluorescence intensity in arbitrary logarithmic units. *b*, *c*, *f*, and *g*, Confocal analysis of r-p185 expression after a 3-h incubation of TUBO cells at 4°C (*b* and *f*) or at 37°C (*c* and *g*) with sera from BALB-neuT mice before (*b* and *c*) and after DNA vaccination (*f* and *g*). Immunohistochemistry reveals that the majority of epithelial cells in hyperplastic lesions from ECD-TM-immunized mice at 33 wk of age express the r-p185 only in the cytoplasm (*h*), whereas a clear membrane and cytoplasm r-p185 positivity are evident in similar lesions displayed much earlier (about at 6–10 wk of age) by control BALB-neuT mice (*d*). Original magnification: *d* and *h*, × 400.

the mammary glands is the production of anti-r-p185 Ab. Although proliferating TUBO cells expressing high amounts of r-p185 on their membrane are unable to elicit a detectable Ab response in both BALB/c and BALB-neuT mice, a significant titer of anti-r-p185 Ab was found in the sera from our immunized mice. It increased when four vaccinations were given instead of two, although BALB-neuT mice consistently produced lower Ab than BALB/c mice. Ab titer is not markedly affected by the challenge and rejection of TUBO cells nor by the development of mammary carcinomas.

Anti-r-p185 Ab induces a functional block of r-p185 receptor function (14), down-regulates its expression on the cell membrane (14, 40), impedes its ability to form the homo- or heterodimers that spontaneously transduce proliferative signals to the cells (40, 41), and blocks its ability to bind ligands (42), as has been observed with anti-Her-2/*neu* mAb. These mAb also significantly suppress the growth of transplantable $p185^+$ tumors (43, 44) and the onset of mammary carcinomas in Her-2/*neu*-transgenic mice (40), and delay tumor growth in patients with Her-2/*neu*-positive tumors (45). The morphological features of inhibited proliferation associated with marked membrane down-modulation of r-p185 and diminished nuclear positivity of PCNA characterizing the progression of both preneoplastic lesions and incipient carcinomas point to direct inhibitory activity on the part of anti-r-p185 Ab. A reduced r-p185 expression could be sufficient for the reversal of their transformed phenotype into a more normal one (14, 40). The high and homogeneous r-p185 membrane expression in advanced lobular carcinomas grown in ECD-TM-immunized and control BALB-neuT mice suggests that its down-regulation becomes less inducible as carcinogenesis progresses.

The success of DNA vaccination in the inhibition of $p185^+$ carcinomas appears to mostly depend on an Ab response to a growth factor receptor whose down-regulation slows the preneoplastic cell proliferation and tumor development. This inhibition mechanism is different from immunological destruction of the malignant cells. However, in immunized mice, leukocytes present at the tumor growth site may also play an important regulatory role (28, 33, 39). Moreover, TUBO cells are rejected by ECD-TM-immunized BALB/c mice that display a high titer of IgG2a, IgM, and IgG1 anti-r-p185 and a massive cellular infiltrate. These Ab isotypes activate PMN and other cells to mediate Ab-dependent cell-mediated cytotoxicity (44–48) and complement-dependent cytotoxicity (IgG2a and IgM), and inhibit the growth of the $p185^+$ tumor in vivo (44). In BALB-neuT mice, ECD-TM plasmid immunization elicits a much lower titer of IgG2a, IgG1, IgM, and IgA anti-r-p185. In these mice, only a partial resistance to a TUBO challenge but major impairment of the progression of carcinogenesis was found. In both BALB/c- and BALB-neuT-immunized mice, CTL do not appear to play a major role, whereas IgG, IgM, and IgA may synergistically promote Ab-dependent cell-mediated cytotoxicity (44, 47–49).

In BALB-neuT mice, our results also fail to show a direct correlation between the titer of anti-p185 Ab and protection from TUBO challenge and inhibition of Her-2/neu carcinogenesis. Several issues may make this correlation less linear such as, for instance, the epitopes recognized by anti-r-p185 Ab, the isotypes of the Ig-elicited and the Ab-dependent mechanisms that are mostly responsible for protection from tumor formation. To definitively address the role of anti-r-p185 Ab in the inhibition of Her-2/*neu* carcinogenesis, we are currently breeding BALB-neuT mice devoid of B cells functions (BALB-neuT/μMT) (50).

The present findings extend and corroborate in a much more aggressive model of carcinogenesis our earlier demonstration that DNA vaccination halts the slower and more limited Her-2/*neu* carcinogenesis taking place in FVB mice (19). Despite the similarity of mammary carcinogenesis in BALB-neuT mice and women (32), the mechanisms of tolerance to r-p185 could be different from those to self p185 in women. However, the r-p185 amino acid sequence is very similar to that of mouse p185, and ECD-TM DNA vaccination and in vitro restimulation never elicited a significant CTL response. Even if the data from BALB-neuT-transgenic mice cannot be directly translated to humans, they show that tolerance to an Ag already markedly expressed during the third wk of age can be partially broken. ECD-TM plasmid immunization appears to be more effective than other forms of anti-r-p185 vaccination (17, 18). Considering that Her-2/*neu* is overexpressed by a substantial proportion of human mammary carcinomas and that many women with a high risk of cancer are being recruited in ongoing epidemiological, genetic, molecular, and radiological screening programs, DNA vaccination could be envisaged as a new prospect in the prevention of carcinogenesis due to the overexpression of oncogenic growth factor receptors (51).

Acknowledgments

We thank Prof. John Iliffe for editing this manuscript.

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