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Immunoprevention of Mammary Carcinoma in HER-2/*neu* Transgenic Mice Is IFN-γ and B Cell Dependent[1]

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A vaccine combining IL-12 and allogeneic mammary carcinoma cells expressing p185^{neu} completely prevents tumor onset in HER-2/*neu* transgenic BALB/c mice (NeuT mice). The immune protection elicited was independent from CTL activity. We now formally prove that tumor prevention is mainly based on the production of anti-p185^{neu} Abs. In the present studies, NeuT mice were crossed with knockout mice lacking IFN-γ production (IFN-$\gamma^{-/-}$) or with B cell-deficient mice (μMT). Vaccination did not protect NeuT-IFN-$\gamma^{-/-}$ mice, thus confirming a central role of IFN-γ. The block of Ab production in NeuT-μMT mice was incomplete. About one third of NeuT-μMT mice failed to produce Abs and displayed a rapid tumor onset. By contrast, those NeuT-μMT mice that responded to the vaccine with a robust production of anti-p185^{neu} Ab displayed a markedly delayed tumor onset. In these NeuT-μMT mice, the vaccine induced a lower level of IgG2a and IgG3 and a higher level of IgG2b than in NeuT mice. Moreover, NeuT-μMT mice failed to produce anti-MHC class I Abs in response to allogeneic H-2^q molecules present in the cell vaccine. These findings show that inhibition of HER-2/*neu* carcinogenesis depends on cytokines and specific Abs, and that a highly effective vaccine can rescue Ab production even in B cell-deficient mice. ***The Journal of Immunology*****, 2004, 173: 2288–2296.**

The ability of spontaneous immune responses to control carcinogenic processes (1) is leading to the concept that a selective stimulation of the immune system could further decrease, possibly abolish, cancer incidence (2). Proof of principle was obtained in various models of induced and spontaneous carcinogenesis using cytokines (3, 4) and vaccines based on DNA, peptides, proteins, or cells expressing tumor Ags (5–11). We have recently shown that a combination of IL-12 and allogeneic tumor cells expressing the membrane protein product of the HER-2/*neu* (p185^{neu}) administered to healthy HER-2/*neu* transgenic mice reduced by 90–100% the risk of mammary carcinoma and more than doubled life expectancy (12). Cytokine release and Ab production appeared to be the immune mechanisms responsible for this efficient and long-term protection, whereas CTLs appeared to play no role (12). This was a provocative finding, because overwhelming experimental evidence shows that CTLs are of pivotal importance in the resistance to transplantable tumor challenges and in the cure of existing cancer lesions (13). In this study, we formally show that prevention of mammary carcinoma with IL-12 and allogeneic cell vaccines expressing p185^{neu} requires intact IFN-γ and B cell responses.

Materials and Methods

Mice

Transgenic BALB/c (H-2^d) female mice, designated here as NeuT mice, overexpressing the transforming activated rat HER-2/*neu* oncogene under control of the mouse mammary tumor virus promoter (12) were bred under specific pathogen-free conditions by Charles River (Calco, Italy). The establishment of *IFN-*γ gene knockout HER-2/*neu* transgenic mice (NeuT-IFN-$\gamma^{-/-}$ mice) has been described previously (12). To obtain B cell-deficient HER-2/*neu* transgenic mice, one female μMT mouse (knockout for the Ig μ-chain gene) on BALB/c genetic background, a kind gift from Dr. T. Blankenstein (Max-Dellbruck Center for Molecular Medicine, Berlin, Germany), was crossed with one NeuT male mouse. Heterozygous knockout/transgenic F_1 male mice were backcrossed with female μMT to obtain mice homozygous for the μ-chain knockout allele and heterozygous for the HER-2/*neu* transgene, designated here as NeuT-μMT. The level of B220$^+$ B cells was routinely monitored by flow cytometry using mAb RA3-6B2 (BD Pharmingen, San Diego, CA). Individually tagged virgin females used in the experiments were treated according to protocols approved by Institutional Review Boards. Mammary pads were inspected weekly, and tumor masses were measured with calipers in two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all 10 mammary glands displayed a tumor or until a tumor exceeded a mean diameter of 1.5 cm, at which time mice were sacrificed for humane reasons.

Cells

TT12 and N202.1E cell clones were derived from mammary carcinomas of FVB-neuN #202 mice (H-2^q), transgenic for the rat HER-2/*neu* protooncogene (14). TT12 cells (referred to as Neu/H-2^q) expressed high levels of p185^{neu}; N202.1E cells (referred to as Neu^{neg}/H-2^q) lacked p185^{neu}. Cells were cultured in Dulbecco's modified minimal essential medium supplemented with 20% FBS (Invitrogen, Milan, Italy) at 37°C in a humidified 5% CO_2 atmosphere. In vaccine experiments, cells were treated with 40 μg/ml mitomycin C (Sigma-Aldrich, Milan, Italy) to block cell proliferation. Surface expression of p185^{neu} and class I H-2^q molecules was assessed by flow cytometry using mAbs 7.16.4 (p185^{neu}; Oncogene Research Products, Cambridge, MA), KH114 (H-2K^q; BD Pharmingen), 34-7-23S (H-2D^q; Cedarlane, Hornby, Ontario, Canada), and 28-14-8S (H-2D/L^q; BD Pharmingen).

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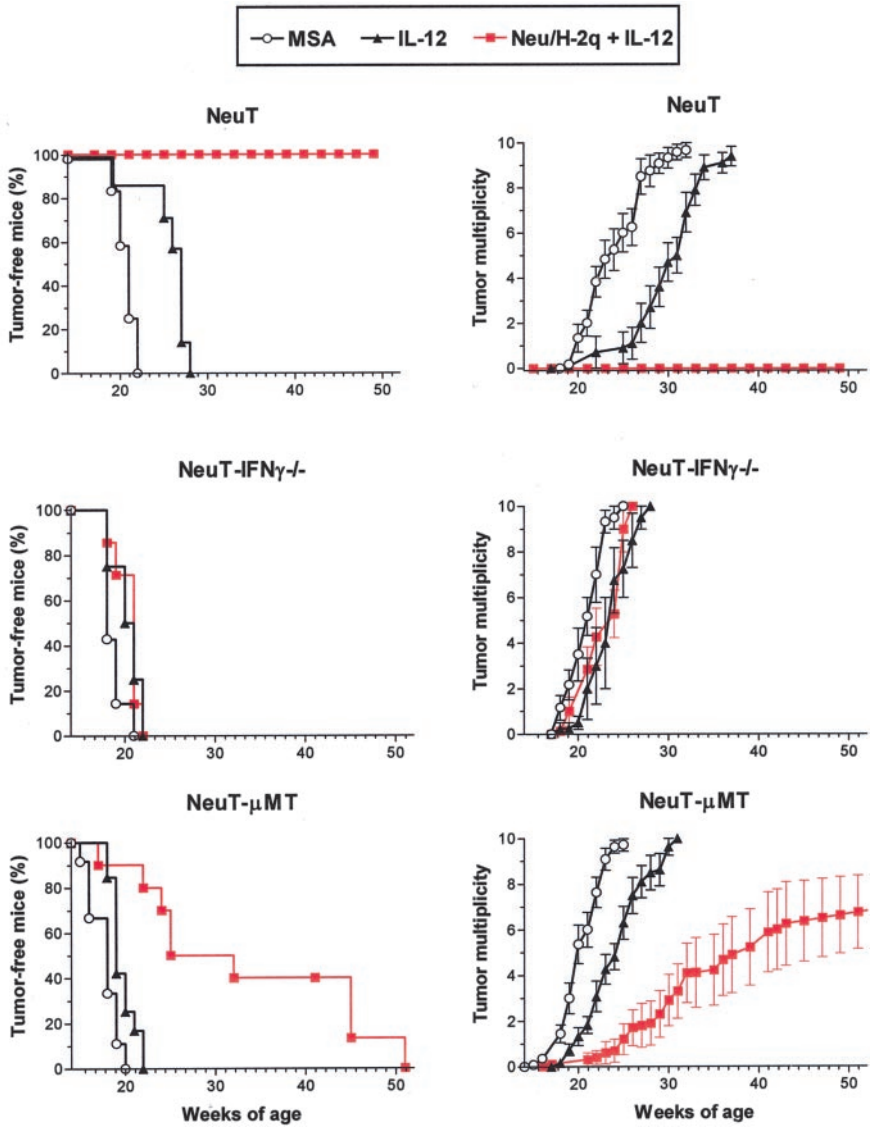

FIGURE 1. Prevention of mammary carcinogenesis in immunocompetent HER-2/*neu* transgenic mice (NeuT) and in transgenic mice deficient in IFN-γ production (NeuT-IFN-$\gamma^{-/-}$) or in B cell immunity (NeuT-μMT). The results of NeuT-IFN-$\gamma^{-/-}$ mice were published previously (12) and are reported here for comparison. Groups of 8–12 mice received MSA, IL-12, or Neu/H-2^q cells plus IL-12 as described in *Materials and Methods*. Tumor multiplicity was calculated as the cumulative number of incident tumors/total number of mice, and is shown as mean ± SEM. By the Mantel-Haenszel test, all tumor-free survival curves of mice vaccinated with Neu/H-2^q cells plus IL-12 (red lines) were significantly different from one another ($p < 0.01$, at least). The survival curves of vaccinated NeuT and NeuT-μMT, but not that of NeuT-IFN-$\gamma^{-/-}$ mice, were significantly different ($p < 0.01$) from those of MSA-treated controls.

Vaccination and IL-12 treatment

Starting at the sixth week of age, mice received successive 3-wk courses of four twice-weekly i.p. vaccinations with 2×10^6 allogeneic Neu/H-2^q mammary carcinoma cells in 0.4 ml of PBS, followed by five daily i.p. administrations of recombinant mouse IL-12 (kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA) in the third week. In the first course, the five injections were of 50 ng of IL-12 in 0.2 ml of PBS supplemented with 0.01% mouse serum albumin (MSA[3]; Sigma-Aldrich); the subsequent injections were of 100 ng of IL-12. After 1 wk of rest, the course was repeated until mice were sacrificed or were 1 year old. Hereafter, mice receiving this combined treatment are referred to as vaccinated mice. Control mice received either mock vaccination or MSA administration. Their tumor progression mirrored that of untreated mice.

Morphologic and immunohistochemical analysis

Tissue samples were processed as described previously for histologic evaluation or for immunohistochemistry (15). The following Abs were used: anti-endothelial cells (anti-CD31, clone mEC-13.324; provided by A. Vecchi, Istituto M. Negri, Milan, Italy); anti-p185^{neu} (C-18; Santa Cruz Biotechnology, Santa Cruz, CA), anti-proliferating cell nuclear Ag (PCNA; Ylem, Milan, Italy), and anti-CD45/B220 (BD Pharmingen).

[3] Abbreviations used in this paper: MSA, mouse serum albumin; PCNA, proliferating cell nuclear Ag; ADCC, Ab-dependent cellular cytotoxicity.

Whole-mount preparation of mammary glands

Whole-mount preparations were performed as reported by Medina (16). Briefly, the skin of euthanized mice was fixed overnight in 10% buffered formalin. The mammary fat pads were scored into quarters and gently scraped from the skin. The quarters were immersed in acetone overnight and then rehydrated and stained in ferric hematoxylin (Sigma-Aldrich), dehydrated in increasing concentrations of alcohol, cleared with histolemon, and stored in methylsalicylate (Sigma-Aldrich). Digital pictures were taken with a Nikon Coolpix 995 (Nital, Turin, Italy) mounted on a stereoscopic microscope (MZ6; Leica Microsystems, Milan, Italy).

IFN-γ production by spleen cells

Spleens were collected from vaccinated and control mice, and single-cell suspensions were prepared, washed in PBS, and resuspended in RPMI 1640 supplemented with 10% FBS. Spleen cells (5×10^5 cells/ml) were then restimulated with mitomycin C-treated target cells (5×10^4 cells/ml) for 6 days at 37°C in RPMI 1640 with 10% FBS containing 10 U/ml recombinant mouse IL-2 (PeproTech, Rocky Hill, NJ). Supernatants were assayed for IFN-γ production by ELISA purchased from Endogen (Woburn, MA).

Ab response

Mice were routinely bled from a lateral tail vein, and sera were stored frozen at −80°C. Analysis of Ab content of sera diluted 1/65 was performed by indirect immunofluorescence followed by flow cytometry. Neu/

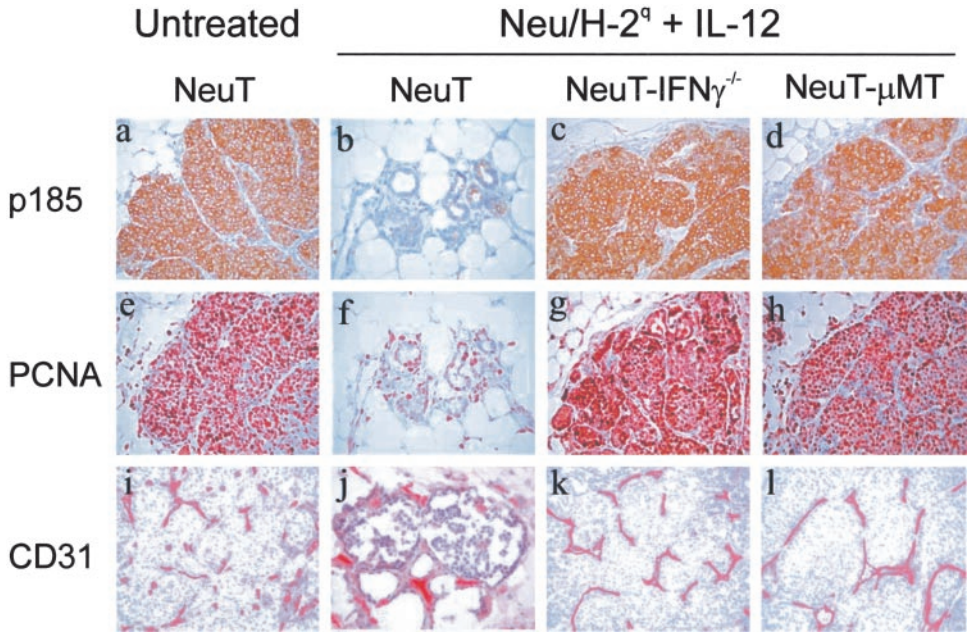

FIGURE 2. Immunohistochemistry of mammary tissue with invasive lobular carcinoma in untreated NeuT, vaccinated NeuT-IFN-$\gamma^{-/-}$, and vaccinated NeuT-μMT mice, and with atypical hyperplasia in vaccinated NeuT mice. Immunostaining with anti-p185^{neu} Ab shows that neoplastic cells of mammary carcinomas developed in untreated NeuT (*A*) and vaccinated NeuT-IFN-$\gamma^{-/-}$ (*C*) and NeuT-μMT (*D*) mice express p185^{neu} in the cytoplasm and on the membrane. The expression of the p185^{neu} is associated with a marked positivity of PCNA (*E*, *G*, *H*). The mammary glands of vaccinated NeuT mice were mainly composed of ductules lined by a single layer of epithelial cells without or with a faint p185^{neu} expression mainly confined in the epithelial cell cytoplasm (*B*); the epithelial proliferation rate was low as assessed by PCNA immunostaining (*F*). A scanty microvessel network supplies the rare foci of mammary hyperplasia found in vaccinated NeuT mice (*J*). On the contrary, untreated NeuT (*I*) and vaccinated NeuT-IFN-$\gamma^{-/-}$ (*K*) mice developed well-vascularized carcinomas. A reduction in microvessel density was observed in vaccinated NeuT-μMT mice (*L*). *A–L*, ×400.

H-2^q and Neu^{neg}/H-2^q cells were used as targets for Ab binding. Total Ig binding was evaluated using a FITC-conjugated goat anti-mouse IgG (H+L) chains secondary Ab (Euroclone, Milan, Italy). For Ig subclass analysis, the following secondary FITC-conjugated mAbs were purchased from BD Pharmingen: anti-mouse IgG1 clone A85-1, anti-mouse IgG2a clone R19-15, anti-mouse IgG2b clone R12-3, anti-mouse IgG3 clone R40-82, anti-mouse IgM clone R6-60.2, anti-mouse IgA clone C10-3, and anti-mouse IgE clone R35-72.

Complement-mediated and Ab-dependent cellular cytotoxicity (ADCC)

Complement-mediated cytotoxicity test was performed as described previously (12). The ADCC test was performed according to Sung et al. (17). Neu-positive H-2^d mammary tumor cells (12) were plated at 10^4 cells/well in 96-multiwell plates and allowed to attach overnight. Cultures were incubated for 2 h on ice with sera from experimental mice at different dilutions. After washing, spleen cells from normal BALB/c mice were added at 50:1 E:T ratio and plates were incubated at 37°C overnight. Nonadherent cells were carefully washed out, 20 μl of WST-1 solution (Roche Diagnostics, Milan, Italy) was added to each well, and absorbance at 450 nm was read after an additional 4-h incubation.

Immunoprecipitation and Western blot analysis

Neu/H-2^q cells and Neu^{neg}/H-2^q cells were lysed for 30 min on ice with 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 150 mM NaCl plus phosphatase and protease inhibitors, 1 mM PMSF, 1 μg/ml aprotinin, 50 mM NaF, and 10 mM sodium pyrophosphate (all reagents were purchased from Sigma-Aldrich). Cell lysates were normalized at a protein concentration of 4 mg/ml with lysis buffer and diluted 1/3 with TBS (50 mM Tris-HCl (pH 7.5) plus 150 mM NaCl) to decrease detergent concentration. Cell lysates (500 μg of total protein each sample) were immunoprecipitated with 3 μg/ml anti-rat *neu* mAb 7.16.4 (Ab-4; Oncogene Research Products) or with 20 μl of sera from untreated or vaccinated mice for 2 h at 4°C. Immunoprecipitated proteins were eluted following overnight incubation with protein A-agarose (Santa Cruz Biotechnology), washed twice in TBS, and denatured at 100°C for 10 min in 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) containing SDS and 5% 2-ME (Sigma-Aldrich). Proteins were then separated on 7.5% SDS-PAGE gels (Ready Gel; Bio-Rad) and transferred onto polyvinylidene fluoride membranes (Bio-Rad). After blocking with PBS containing 0.1% Tween 20 and 5% nonfat dry milk, membranes were incubated overnight at 4°C with anti-*neu* rabbit polyclonal Ab diluted 1/200 in blocking buffer (C18; Santa Cruz Biotechnology). Finally, the presence of immunoprecipitated HER-2/*neu* proteins was detected by 1-h incubation at room temperature with HRP-linked goat anti-rabbit Ab (Santa Cruz Biotechnology; diluted 1/1000 in blocking buffer), followed by a colorimetric reaction (Opti-4CN Substrate kit; Bio-Rad).

To determine whether vaccination-induced Abs recognize cryptic HER-2/*neu* epitopes, Neu/H-2^q and Neu^{neg}/H-2^q cell lysates were immunoprecipitated with Ab-4 anti-rat *neu* mAb, denatured under reducing conditions, and blotted as described above. Membranes were then blocked with PBS containing 0.1% Tween 20 and 5% BSA and incubated with mice sera diluted 1/250 in PBS containing 0.1% Tween 20 and 2% BSA overnight at 4°C. Bound Abs were detected by HRP-labeled goat anti-mouse IgG Ab (Santa Cruz Biotechnology; diluted 1/1000), followed by the colorimetric reaction.

To study the effect of serum Abs on the phosphorylation status of p185^{neu}, Neu/H-2^q cells were treated for 1 h at 37°C with sera from untreated and vaccinated mice, diluted 1/10 in DMEM without serum. Cell lysates were immunoprecipitated with anti-rat *neu* Ab-4 mAb as described above. Aliquots of immunoprecipitated proteins were analyzed in parallel by Western blotting using C18 rabbit anti-*neu* polyclonal Ab to detect HER-2/*neu* receptor expression level and 1.5 μg/ml biotinylated antiphosphotyrosine Ab (4G10; Upstate, Lake Placid, NY) to detect the phosphorylated HER-2/*neu* receptor.

Results

Immunoprevention is ineffective in mice deficient in IFN-γ or B cells

HER-2/*neu* transgenic mice, referred to as NeuT mice, develop mammary carcinomas in all 10 mammary glands by the age of 32 wk. We found that a combination of MHC allogeneic tumor cells expressing p185^{neu} and systemic rIL-12 was the minimal vaccine that could arrest in all mice mammary carcinogenesis and reduce tumor incidence by 90–100% (12). The major responses elicited

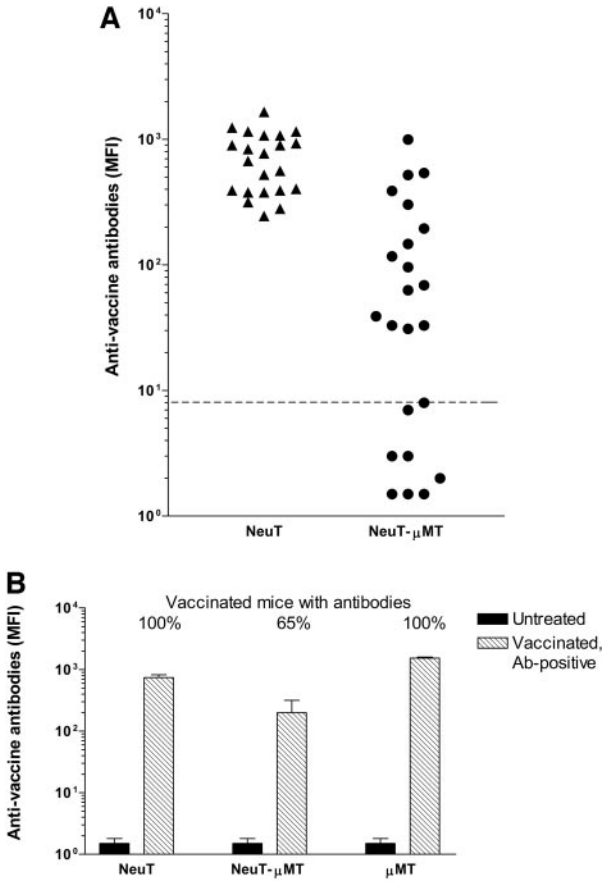

FIGURE 3. Ab response in vaccinated NeuT-μMT mice. *A*, Sera were collected from NeuT or NeuT-μMT mice after three monthly courses of vaccination and tested at 1/65 dilution against Neu/H-2^q cells by indirect immunofluorescence followed by cytofluorometric analysis. Each point represents the mean fluorescence intensity (MFI) of one serum. *B*, Mean Ab levels induced by vaccination of NeuT-μMT mice compared with parental NeuT and μMT mice. Each bar represents the mean $\pm$ SEM of 6–23 individual mice scored as Ab-positive (MFI, >8). The percentage of Ab-positive mice in each vaccinated group is reported above the hatched bars.

by the combined treatment in transgenic mice were an abundant production of IFN-γ by T cells, in particular by CD8$^+$ lymphocytes, and a strong Ab response directed against p185^{neu}. To analyze their importance in the protection from mammary carcinoma afforded by vaccination, NeuT transgenic mice were crossed with knockout mice deficient in IFN-γ release (NeuT-IFN-$\gamma^{-/-}$) or in B cell immunity (NeuT-μMT).

Although the combined treatment protected almost all NeuT mice, none of the NeuT-IFN-$\gamma^{-/-}$ or NeuT-μMT were protected (Fig. 1). Differences between NeuT-μMT and NeuT-IFN-$\gamma^{-/-}$ were apparent in tumor latency and in the number of carcinomas per mouse (tumor multiplicity), which were significantly ($p < 0.01$ at least) reduced by vaccinations in B cell-deficient mice, but not in mice lacking IFN-γ.

p185^{neu} expression and neoangiogenesis in the mammary gland of vaccinated IFN-γ- or B cell-deficient mice

Pathological studies revealed that by the third week of age the mammary glands of NeuT mice developed foci of atypical hyperplasia, which subsequently extended to all mammary glands. Foci of carcinoma in situ, first apparent around the 15th week of age, evolved to invasive carcinomas by the 20th week of age and were present in all the glands 10–12 wk later.

In NeuT mice vaccinated with Neu/H-2^q cells plus IL-12, the mammary glands showed the development of a limited number of atypical hyperplastic foci often surrounded by reactive cells. These hyperplastic foci decreased with time so that, at 45–50 wk of age, the mammary glands were free of hyperplastic foci and tumors. In NeuT-IFN-$\gamma^{-/-}$ and NeuT-μMT mice treated with IL-12 or Neu/H-2^q cells plus IL-12, the invasive lobular carcinomas that eventually developed in mammary glands were histologically very similar to those of untreated NeuT mice.

Importantly, in NeuT mice, the combined treatment resulted in a scarce expression of p185^{neu}, the HER-2/*neu* gene product that, if present, was mainly confined to the cytoplasm of mammary duct epithelial cells, which rarely stained for PCNA, a marker of tumor cell proliferation (Fig. 2, *B* and *F*). By contrast, a marked cytoplasmic and membrane p185^{neu} expression, similar to that found in

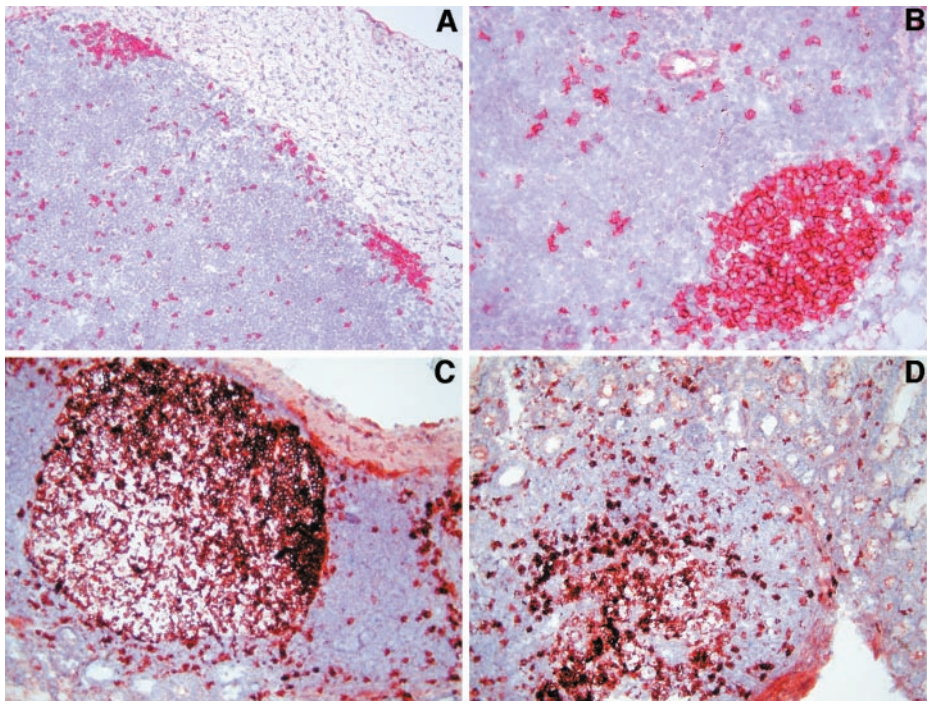

FIGURE 4. Immunostaining with anti-B220 mAb of lymph node (*A*), spleen (*B*), Peyer patch (*C*), and intestinal lymphoid follicle (*D*) from NeuT-μMT mice. Positive B cells are scarce in lymph node and spleen, whereas they are well represented in intestinal lymphoid follicle and numerous in Peyer patch. *A*, $\times$200; *B–D*, $\times$400.

Table I. *B cell content of peripheral blood and lymphoid organs of NeuT and NeuT-μMT mice*[a]

Lymphocyte Origin	NeuT	NeuT-μMT	
		Ab-negative	Ab-positive
Peripheral blood	50.0 ± 5.1	0.5 ± 0.2	1.4 ± 0.5
Spleen	45.5 ± 2.5	2.2 ± 0.3	5.0 ± 0.4[b]
Mesenteric lymph node	29.5 ± 3.3	0.5 ± 0.1	0.6 ± 0.2
Inguinal lymph node	26.2 ± 3.7	0.5 ± 0.2	0.6 ± 0.3
Peyer patches	60.7 ± 1.2	14.5 ± 1.5	25.2 ± 1.2[b]

[a] The percentage of B220-positive cells, evaluated by FACS analysis of gated lymphocytes, is expressed as the mean ± SEM of three to five mice per group.
[b] Significantly different from Ab-negative NeuT-μMT ($p < 0.05$, Student's *t* test).

hyperplastic and neoplastic lesions of untreated mice (Fig. 2*A*), was displayed by neoplastic cells of carcinomas developed in both NeuT-μMT and NeuT-IFN-$\gamma^{-/-}$ mice (*C* and *D*). Expression of p185^{neu} was associated with a marked expression of PCNA (Fig. 2, *E*, *G*, and *H*).

The microvessel count by anti-CD31 immunostaining (Fig. 2, *I–L*) revealed a significant ($p \leq 0.005$) decrease in the number of microvessels supplying hyperplastic or neoplastic lesions in vaccinated NeuT and NeuT-μMT mice when compared with that of untreated NeuT mice (vessel count in vaccinated NeuT mice, 6.1 ± 1.7; in vaccinated NeuT-μMT, 9.8 ± 1.2; in vaccinated NeuT-IFN-$\gamma^{-/-}$, 11.7 ± 1.9; in untreated NeuT mice, 14.4 ± 2.2). A similar reduction in microvessel density was observed in NeuT and NeuT-μMT mice treated with IL-12 only (9.0 ± 1.8 and 9.2 ± 1.5, respectively).

Vaccination elicits Ab production in B cell-deficient NeuT-μMT mice

It has been reported that B cells from μMT mice, in particular on a BALB/c genetic background, can bypass the IgM defect and undergo class switch leading to Ig secretion (18–21). We found that only one third of NeuT-μMT mice receiving the vaccine was truly unresponsive, whereas the remaining two thirds of mice produced abundant high-titer Abs against the vaccine (Fig. 3*A*). We then compared the serum Ab levels of Ab-proficient NeuT-μMT mice with those of the two parental mouse lines, NeuT and μMT (Fig. 3*B*). The frequency of Ab production in response to the vaccine was 100% both in NeuT and μMT mice, and the levels of serum Abs were also similar. It could be concluded that an effective vaccine elicited very high levels of Abs even in B cell-deficient μMT mice. Only when the two genetic lesions were combined in the same mouse (i.e., in NeuT-μMT mice), we observed a lack of anti-vaccine Abs production in about one third of mice and reduced Ab levels in the remaining two thirds. This may be attributed to the fact that HER-2/*neu* expression makes NeuT-μMT mice tolerant to HER-2/*neu* gene product p185^{neu} (9).

An extensive examination of lymphoid organs of NeuT-μMT mice by immunohistochemistry (Fig. 4) and flow cytometry (Table I) revealed the presence of small proportions of B220$^+$ B cells in the spleen and in lymph nodes, and of substantial proportions of B cells in Peyer patches (19) and in intestinal lymphoid follicles. Secondary lymphoid organs of vaccinated NeuT and neuT-μMT mice were comparatively larger than those of untreated mice, and cellularity was increased (data not shown). The proportion of B220$^+$ B cells in spleen and Peyer patches of Ab-positive NeuT-μMT mice was significantly higher than in Ab-negative mice, but remained well below the proportion found in NeuT mice (Table I). Most B220$^+$ B cells of NeuT-μMT mice expressed surface immunoglobulins, and the level of surface Ig was similar to that of B cells from NeuT mice (data not shown).

Ab dependence of tumor prevention in NeuT-μMT mice

The bimodal distribution of Ab responses among vaccinated NeuT-μMT mice prompted us to re-evaluate the survival curves shown in Fig. 1. A clear difference in survival became evident when vaccinated NeuT-μMT mice were stratified according to their Ab response (Fig. 5). Mice that did not produce Igs in response to vaccination were prone to early onset of mammary carcinoma with a slight delay in comparison to untreated mice. On the contrary, tumor onset was significantly delayed in vaccinated NeuT-μMT mice able to mount a sizeable Ab response (Fig. 5). The analysis of whole-mount preparation of mammary glands before the onset of macroscopic carcinomas (16-wk-old mice) showed the presence of diffuse atypical hyperplasia and occasional in situ carcinomas in both NeuT and Ab-deficient NeuT-μMT mice, whereas in the mammary glands of Ab-proficient NeuT-μMT mice, hyperplastic nodules were rare and in situ carcinomas were absent (Fig. 6).

IFN-γ production by NeuT-μMT lymphocytes

The survival curves and tumor multiplicities of Ab-deficient NeuT-μMT clearly showed a residual protection from mammary carcinoma that was completely absent in NeuT-IFN-$\gamma^{-/-}$ mice (compare Figs. 1 and 5). IFN-γ, besides its well-known activities on B and T cells, can directly inhibit the growth of preneoplastic and neoplastic mammary cells (12); moreover, it can hamper tumor growth through the induction of the antiangiogenic chemokines monokine induced by IFN-γ and IFN-γ-inducible protein 10.

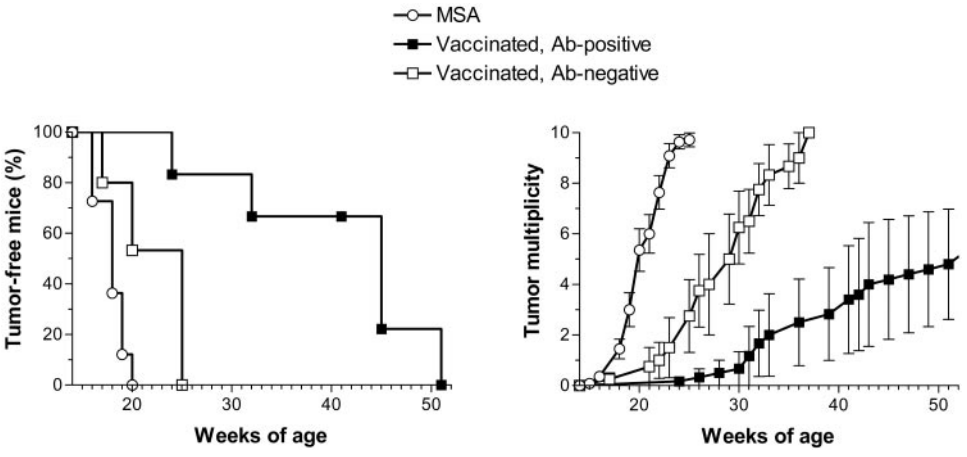

FIGURE 5. Immunoprevention of mammary carcinoma in NeuT-μMT mice is Ab dependent. Vaccinated NeuT-μMT mice shown in Fig. 1 were stratified according to Ab production in response to vaccination (see Fig. 3). Tumor-free survival curve of vaccinated, Ab-positive mice was significantly different from that of vaccinated, Ab-negative mice ($p < 0.01$ by the Mantel-Haenszel test).

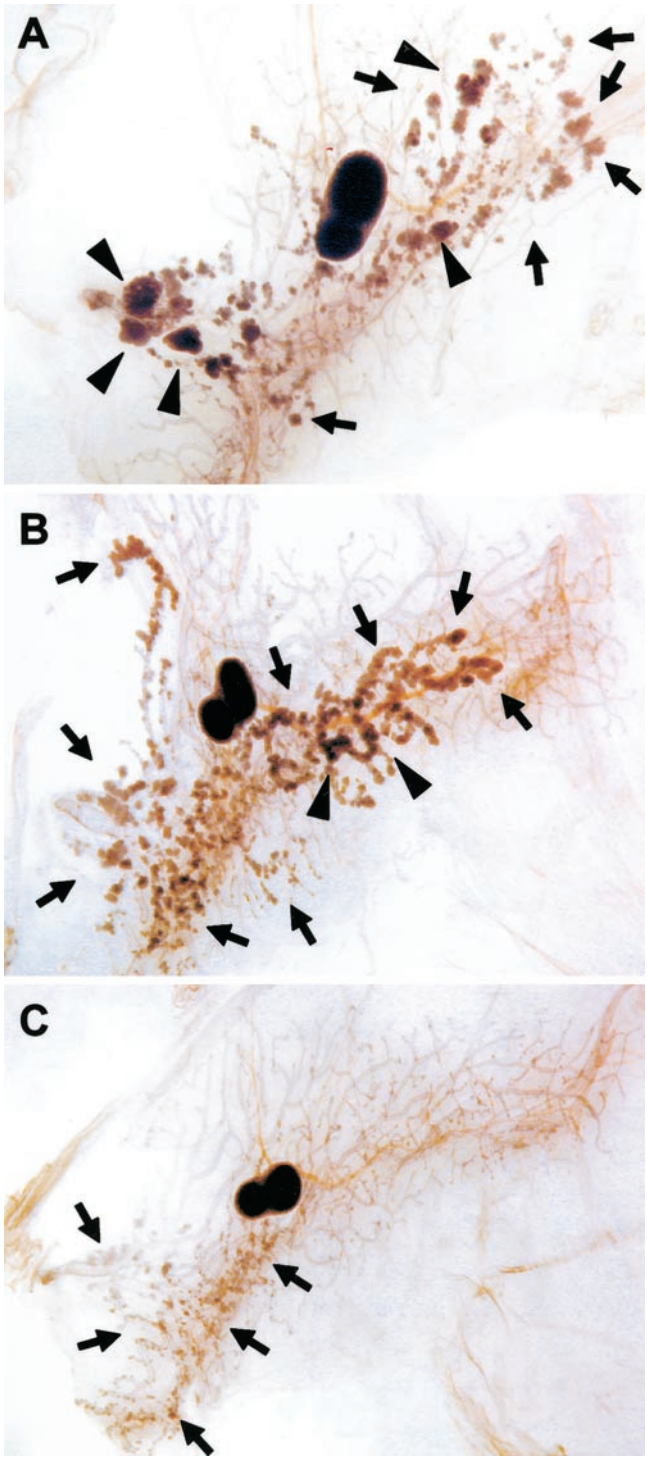

FIGURE 6. Whole mounts of inguinal mammary glands from one untreated (*A*) and two vaccinated NeuT-μMT mice, one Ab deficient (*B*), the other Ab proficient (*C*) at the age of 16 wk. The black oval in the center of each picture is the inguinal lymph node. In *A* and *B* numerous and large atypical hyperplastic foci (arrows) and a few established mammary carcinomas (arrowheads) were present, whereas a dramatic reduction in diffusion and dimension of hyperplastic foci and the absence of carcinoma were evident in *C*.

We found that IFN-γ release by spleen cells of vaccinated NeuT-μMT mice was clearly detectable in vitro, although at a level lower than that of vaccinated NeuT mice (Fig. 7). After in vitro restimulation with the vaccine, splenocytes from both NeuT and NeuT-

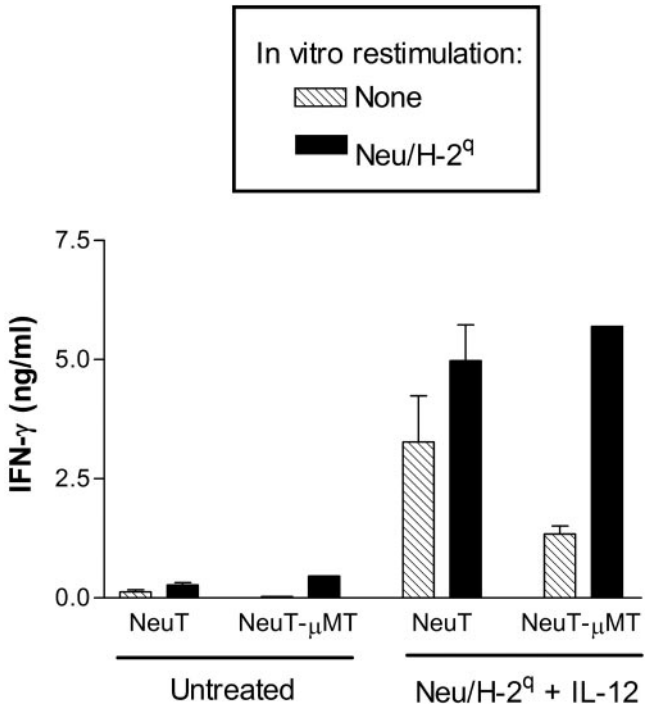

FIGURE 7. Release of IFN-γ by NeuT-μMT spleen cells. Spleens were obtained from untreated mice or from mice vaccinated with Neu/H-2^q cells plus IL-12. IFN-γ release by spleen cells was assessed by ELISA after 6 days in vitro in the presence or absence of stimulator vaccine cells.

μMT mice released comparable amount of IFN-γ. The residual protection from mammary carcinoma produced by vaccination in Ab-negative NeuT-μMT mice provides an estimate of the relative importance of non-B cell-mediated effects of IL-12 and IFN-γ in this system.

Specificity, isotype, and activity of Abs elicited by vaccination in NeuT-μMT mice

Mammary carcinogenesis was significantly delayed by vaccination in Ab-proficient NeuT-μMT mice; however, all mice eventually succumbed to progressive tumors, unlike NeuT mice that remained tumor free for $>$1 year. This suggests that the Ab response of B cell-deficient NeuT-μMT mice was lacking in comparison with that of NeuT mice. We have already shown that anti-vaccine Ab level of NeuT-μMT mice was quantitatively inferior to that of NeuT mice (Fig. 3). Western blot analysis of Ab specificity showed that sera from Ab-proficient NeuT-μMT mice specifically recognized and immunoprecipitated p185^{neu} molecules from HER-2/*neu*-positive transgenic cell lysates; however, a strong quantitative difference was evident in comparison to sera from NeuT mice (Fig. 8*A*). Sera from Ab-proficient NeuT-μMT mice showed a marginal activity against denatured p185^{neu}, and sera from Ab-deficient mice were again negative (Fig. 8*B*), thus indicating the absence of Abs against cryptic determinants. We never observed a significant difference between sera of untreated and vaccinated mice when tested on cell lysates of p185^{neu}-negative cells.

We then studied the spectrum of Ig isotypes produced by NeuT, NeuT-IFN-$\gamma^{-/-}$, and NeuT-μMT mice in response to vaccination (Fig. 9). We reported previously (12) that the absence of tumor prevention in NeuT-IFN-$\gamma^{-/-}$ mice was associated with the lack of IgG2a, IgG2b, and IgG3 subclasses, whereas IgG1 production was similar to that of NeuT mice. The isotype spectrum of NeuT-μMT mice was clearly distinct from that of NeuT-IFN-$\gamma^{-/-}$; however, it showed some subtle differences in comparison with NeuT

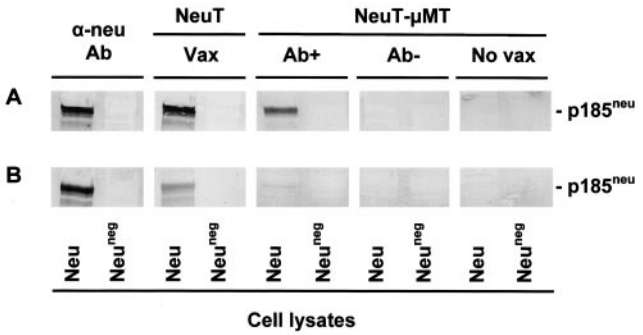

FIGURE 8. Ab response of vaccinated NeuT-μMT mice analyzed by immunoprecipitation and Western blot. *A*, Immunoreactivity of sera against native p185^{neu} protein. Lysates of Neu/H-2^q and Neu^{neg}/H-2^q cells (here referred to as Neu and Neu^{neg}, respectively) were immunoprecipitated with pooled sera obtained from four vaccinated Ab-proficient (Ab+) or -deficient (Ab−) NeuT-μMT mice or from untreated NeuT-μMT mice (No vax). Immunoprecipitation with Ab4 anti-rat *neu* mAb (α-*neu* Ab) or with sera from vaccinated NeuT mice (NeuT/Vax) are shown for comparison. The C18 anti-p185^{neu} rabbit Ab was used for Western blot. *B*, Immunoreactivity of mice sera against cryptic epitopes of p185^{neu} protein. p185^{neu} was immunoprecipitated with Ab4 mAb, denatured, and blotted using sera as above or C18 Ab (α-*neu* Ab). A HRP-linked goat anti-mouse IgG Ab was used for detection.

mice. In particular, both IgG2a and IgG2b were produced by NeuT-μMT mice, but the IgG2a:IgG2b ratio was reversed in comparison with NeuT mice. IgG3 production was low in vaccinated NeuT mice, but was completely absent in NeuT-μMT mice.

Abs against p185^{neu} may affect tumor growth in several ways, including direct inhibition of p185^{neu}, complement-mediated cytotoxicity, and ADCC (22–24). In vitro exposure of Neu/H-2^q cells to sera from vaccinated mice did not affect p185^{neu} phosphorylation status (data not shown), but determined a decrease up to 50% of total p185^{neu} level. This is in agreement with the increased ubiquitin-mediated degradation induced by *neu*-specific Abs (22). The study of complement-mediated cytotoxicity mediated by sera of vaccinated mice, showed a decreased activity in sera from immunodeficient mice, with the lowest levels in NeuT-IFN-$\gamma^{-/-}$ (Fig. 10*A*). Only sera from immunocompetent NeuT mice determined significant levels of ADCC (Fig. 10*B*).

Finally, a conspicuous hole was found in the repertoire of Abs induced by vaccination in NeuT-μMT mice. The vaccine contains

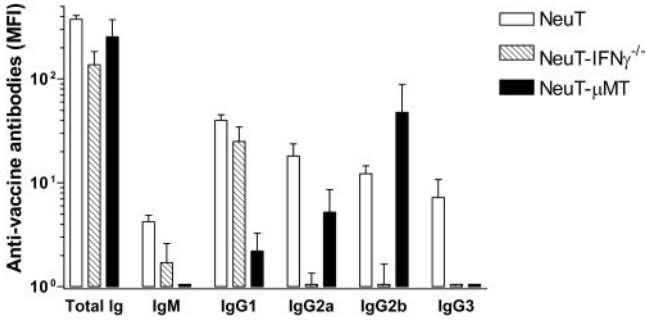

FIGURE 9. Subclasses of Abs induced by vaccination in NeuT, NeuT-IFN-$\gamma^{-/-}$, and NeuT-μMT mice. The results of NeuT-IFN-$\gamma^{-/-}$ mice were previously published (12) and are reported here for comparison. Cytofluorometric analysis of serum binding to Neu/H-2^q vaccine cells with secondary anti-mouse isotype Abs is reported. IgA and IgE Abs were absent from all sera (data not shown). Each bar represents the mean ± SEM of sera (1/65) from three to nine mice at the 17th week of age bled after the third course of vaccination.

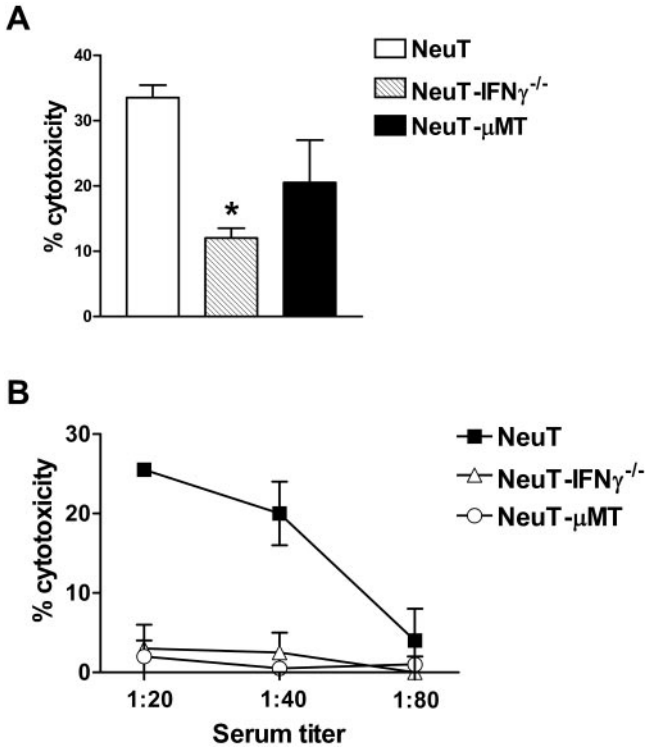

FIGURE 10. Complement-mediated cytotoxicity (*A*) and ADCC (*B*) with sera of mice vaccinated with Neu/H-2^q cells plus IL-12. Each point or bar represents the mean ± SEM of sera from three to four mice bled after the third course of vaccination (17 wk of age). ∗, Significantly different from NeuT mice ($p < 0.01$, Student's *t* test).

MHC-allogeneic mammary carcinoma cells; therefore, it is expected to induce, in addition to p185-specific Abs, other Abs recognizing H-2^q class I glycoproteins and other Ags (e.g., mammary tissue-specific Ags). NeuT-μMT mice, at variance with NeuT mice, failed to produce Abs recognizing a p185-negative, H-2^q class I-positive mammary carcinoma clone (Fig. 11*A*) deriving from a HER-2/*neu* transgenic mammary carcinoma (14). In complement-mediated cytotoxicity tests, sera of Ab-proficient NeuT-μMT mice did not lyse allogeneic lymphocytes of the H-2^q haplotype (Fig. 11*B*). This indicates that the allogeneic Ab response of NeuT-μMT mice is defective, and suggests that the response against subdominant Ags present in the vaccine could be also impaired.

Discussion

HER-2/*neu* transgenic mice lacking either IFN-γ or B cells were not protected from mammary carcinoma by a highly effective vaccination combining IL-12 and MHC allogeneic cells expressing the p185^{neu} Ag.

IFN-γ release was the most conspicuous T cell response present in vaccinated HER-2/*neu* transgenic mice, in the absence of detectable CTL (12). The complete disappearance of protection from mammary carcinoma in transgenic mice lacking IFN-γ confirmed the pivotal role of this cytokine. Morphological data indicated that IFN-γ production by reactive cells was fundamental to elicit an efficient antitumor response (12). Its absence, in fact, resulted in a scarce intratumoral recruitment of reactive cells, which were also unable to produce proinflammatory cytokines and chemokines (data not shown). Inhibition of tumor neoangiogenesis, mainly induced by IL-12-elicited IFN-γ, contributed to the effectiveness of the combined treatment, because the vascular network supplying

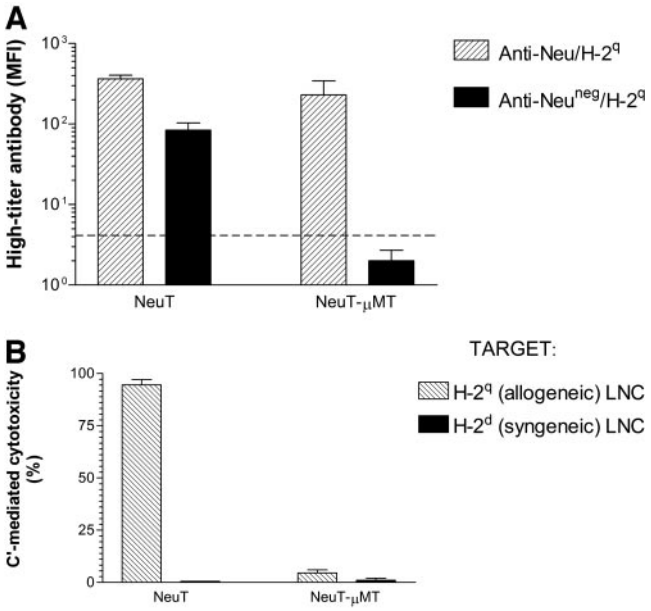

FIGURE 11. Non-p185^{neu} Abs elicited in NeuT-μMT mice by vaccination with Neu/H-2^q cells plus IL-12. *A*, Cytofluorometric analysis of serum binding to vaccine cells or to a HER-2/*neu* transgenic, p185-negative mammary carcinoma clone (Neu^{neg}/H-2^q). Each bar represents the mean ± SEM of sera (1/65) from three to four mice at the 17th week of age bled after the third course of vaccination. *B*, Complement-mediated cytotoxicity against syngeneic lymph node cells (LNC) from NeuT (H-2^d) mice or against MHC allogeneic H-2^q LNC.

mammary hyperplasia in NeuT or carcinomas in NeuT-μMT mice was reduced in comparison with untreated NeuT and treated NeuT-IFN-$\gamma^{-/-}$ mice.

However, from a heuristic point of view, the pleiotropic action of IFN-γ, which is simultaneously a regulatory molecule in most antitumor immune responses and a direct effector acting on tumor cells themselves, did not allow a clear dissection of further immune mechanisms.

We found that different immune treatments were able to delay mammary carcinogenesis (25), but the jump from delay to effective prevention of carcinoma was invariably accompanied by the appearance of anti-p185^{neu} Abs (6, 12), thus indicating a fundamental role for the B cell response in combination with T cell-derived cytokines (8, 22, 26). Abs directed against membrane oncoproteins like p185^{neu} may play multiple antitumor roles that include block of mitogenic signal transduction through inhibition of receptor dimerization and induction of internalization and recycling along with immune-mediated functions like complement-mediated cytotoxicity and ADCC (8, 12, 22–24, 27, 28).

To formally demonstrate the role of Abs in cancer prevention, we vaccinated B cell-deficient, HER-2/*neu* transgenic NeuT-μMT mice. To our surprise, two thirds of NeuT-μMT mice responded to vaccination with a copious production of specific Abs of various Ig subclasses. This result is in general agreement with recent findings that μMT mice of BALB/c background are capable of mounting "natural" and Ag-induced Ab responses (20, 21). In μMT mice, Abs are produced by plasma cells deriving from B cells that completed development either via surface expression of IgD or possibly through premature switching to IgG1 or other isotypes. It has been reported that immunization with either T cell-dependent or -independent Ags induces Ab responses only in about one third of μMT mice (20), whereas our vaccine was able to induce specific Abs in 100% of μMT mice. This finding points to the great potency of this combination of IL-12, allogeneic MHC, and p185^{neu}.

At variance with μMT mice, NeuT-μMT mice were not uniformly able to mount Ab responses to our vaccine. HER-2/*neu* transgenic mice are tolerant to the p185^{neu} protein (9); thus, the difference in the proportion of vaccine responses between μMT and NeuT-μMT mice may result from the immunological tolerance of the latter. This dichotomous behavior of NeuT-μMT mice in response to our vaccine was exploited to investigate long-term prevention of mammary carcinoma in the absence of Ab response. Without protective Abs, the preventive effect of the vaccine vanished, whereas a high Ab response went along with a delayed onset of mammary carcinomas.

The mean latency of tumors in vaccinated, Ab-deficient NeuT-μMT mice was about 1 month longer than in untreated mice. A similar delay (3 wk) was induced by the administration of IL-12 alone, without allogeneic cells. These data can be compared with the complete inefficiency of vaccination in NeuT-IFN-$\gamma^{-/-}$ mice, allowing us to dissect immune-mediated from direct antitumor effects of IFN-γ. It could be concluded that the major contribution of IFN-γ in the prevention of mammary carcinoma was its activity of immune mediator, in particular for what concerns the induction of a switch to Th1-type Igs. On the contrary, direct antitumor activities, such as the induction of antiangiogenic chemokines (monokine induced by IFN-γ and IFN-γ-inducible protein 10), or the inhibition of preneoplastic cell proliferation, in the absence of Abs, produced only a short delay in tumor appearance.

Vaccination induced a significant delay of mammary carcinogenesis in Ab-proficient NeuT-μMT mice, but all mice eventually developed tumors. By contrast, NeuT mice were completely protected by vaccination. This suggests that the B cell deficit extant in Ab-proficient NeuT-μMT mice precluded a complete prevention of mammary carcinoma, a fact that could shed further light on the nature of protective Abs. As mentioned above, quantitative differences in Ab titers may play a role in lowering the protection. Even Ab-proficient NeuT-μMT mice had significantly lower levels of anti-p185^{neu} Abs than NeuT mice. Major qualitative differences among NeuT, NeuT-IFN-$\gamma^{-/-}$, and NeuT-μMT were evident in the IgG subclasses elicited by vaccination. An abundant IgG1 response in NeuT-IFN-$\gamma^{-/-}$ mice, which were not protected by vaccination, indicates that the γ_1 chain was not involved in prevention. NeuT-IFN-$\gamma^{-/-}$ mice lacked anti-vaccine IgG2a and IgG2b, two Ab subclasses that were produced both by NeuT and by Ab-proficient NeuT-μMT mice. In NeuT mice, the IgG2a:IgG2b ratio was skewed in favor of IgG2a, whereas in NeuT-μMT, the reverse was true. It has been shown that, in the absence of μ-chains, the γ_{2b} chain can support B cell development (29, 30); thus, the imbalance found in NeuT-μMT mice could be the consequence of the alternative pathways leading to B cell survival and differentiation in this knockout mouse (20). From the point of view of cancer prevention, the imbalance found in NeuT-μMT suggests that a response skewed to IgG2a might provide a better protection from mammary carcinoma. IgG3 were induced by vaccination in NeuT mice, but not in NeuT-IFN-$\gamma^{-/-}$ or in NeuT-μMT mice. Because μMT mice are specifically deficient in IgG3 production (20, 21), the NeuT-μMT model could not be used to assess the relative importance of this isotype in cancer prevention.

An intriguing alteration in the specificity of nonprotective, "bystander" Abs was evident. We found that vaccination induced protective Abs directed against p185^{neu} both in NeuT and in NeuT-μMT mice; thus, we believe that quantitative and isotypic differences discussed above explain the lower degree of cancer prevention obtained in Ab-proficient NeuT-μMT mice. However, the combined vaccine is expected to induce a wide range of Abs recognizing determinants other than p185^{neu}. In particular, the expression of allogeneic MHC molecules by cells in the vaccine

should elicit anti H-2^q class I Abs. Such Abs are a byproduct devoid of preventive efficacy, because mice receiving IL-12 plus allogeneic cells lacking $p185^{neu}$ are not protected from mammary carcinoma (12). The very low level of anti-class I Abs found in vaccinated, Ab-proficient NeuT-μMT mice indicates a profound alteration in the ability to respond to multiple Ags. A possible explanation is that the selection caused by the $p185^{neu}$ Ag irreversibly shapes the development of B cells in deficient NeuT-μMT mice (31, 32).

In conclusion, we showed that B cell responses regulated by IFN-γ were a key mechanism mediating prevention of mammary carcinoma in mice vaccinated with allogeneic tumor cells and IL-12. This conclusion is important both to direct further investigation of the B cell-mediated mechanisms at work in the HER-2/*neu* system (e.g., Ag presentation, cytokine release, complement-mediated cytotoxicity, ADCC, direct down-modulation of $p185^{neu}$) and to design similar attempts at preventing cancer in humans.

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