Influence of Antibiotic Treatment on Breast Carcinoma Development in Proto-*neu* Transgenic Mice

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Abstract

The effect of prolonged antibiotic treatments on tumor development was evaluated in proto-*neu* transgenic mice, which spontaneously develop mammary carcinomas. Virgin transgenic mice were treated with metronidazole/ciprofloxacin or gentamicin through the drinking water. The hazard ratio [HR; 95% confidence interval (95% CI)] of breast cancer occurrence in metronidazole/ciprofloxacin-treated mice was more than triple that for controls [3.11 (1.13-8.53); $P = 0.028$], whereas only a slight increase in HR (95% CI) was observed in gentamicin-treated mice [1.39 (0.56-3.47); $P = 0.481$]. Tumor growth rate in gentamicin-treated mice was significantly faster than in untreated control mice ($P = 0.043$). Moreover, mammary glands from mice treated with either antibiotic regimen showed increased lobulization, with more numerous and more developed terminal ductal lobular units than in controls. These results indicate that prolonged exposure to relevant doses of antibiotics affects the mammary glands in this particular model of HER-2/*neu* transgenic mice; further studies to understand the precise mechanism by which antibiotic treatments influence mammary gland differentiation are critical. (Cancer Res 2006; 66(12): 6219-24)

Introduction

Some epidemiologic studies have suggested a positive association between antibiotic use and risk of breast cancer (1, 2). A Finnish population study found that premenopausal women who used antibiotics for urinary tract infections had an elevated risk of breast cancer compared with women who did not use antibiotics; the age-adjusted relative risk [95% confidence interval (95% CI)] was 1.34 (098-1.83) and the risk for women ages <50 years was 1.74 (1.13-2.68; ref. 1). In a North American population study (2), cumulative days of antibiotic use were associated with increased risk of breast cancer, with an estimated odds ratio (95% CI) of 2.07 (1.48-2.88) in women who underwent long-term (>1,001 days) antibiotic treatment. In that study, all classes of antibiotics were associated with increased breast cancer risk and the association persisted after adjustment for factors, such as family history of breast cancer, age at menarche, age at birth of first child, age at menopause, postmenopausal estrogen-replacement therapy, cigarette smoking, alcohol intake, and dietary fat intake (2). Other studies have found either no clear association between breast cancer and antibiotic use (3, 4) or a low association (5) between breast cancer and a specific antibiotic, such as flucloxacillin, which is commonly used to treat breast cancer abscess and mastitis (6). Thus, it remains unclear whether antibiotic use is causally related to breast cancer or whether there are common mediators of antibiotic exposure and breast cancer. Indeed, some women may have underlying immune or inflammatory disorders that predispose them to neoplasia, and antibiotic use would only be a marker for impaired immune function or for underlying infections.

Activation of the HER family of growth factor receptors and subsequent stimulation of their associated intracellular signaling pathways is a significant factor in the genesis of several human cancers (7). Amplification of the HER-2/*neu* gene and consequent protein overexpression occurs in 25% to 30% of primary human breast tumors and is associated with poor prognosis (8, 9). The oncogenic potential of HER-2/*neu* has been confirmed in mammary epithelia of transgenic mice (10, 11). In these mice, the presence of the rat *neu* proto-oncogene driven by the mouse mammary tumor virus promoter/enhancer induces spontaneous multifocal mammary tumors overexpressing the *neu*-encoded p185 protein in all females (12). The stochastic development of the tumors and the long latency period indicate the requirement for additional events in tumor formation. We reasoned that if antibiotic treatment is causally related to breast cancer, antibiotic treatment in an animal model with a genetic predisposition to develop mammary tumors might increase the occurrence of breast carcinoma. We thus evaluated the role of prolonged antibiotic treatments on tumor development in HER-2/*neu* proto-oncogene transgenic female mice.

Materials and Methods

Mice. A colony of transgenic FVB-*neu* mice (12) carrying the rat HER-2/*neu* proto-oncogene under the transcriptional control of the mouse mammary tumor virus 3′ long terminal repeat promoter was established in our animal facilities and maintained under strict inbreeding conditions. Mice were maintained at constant temperature and humidity, with food and water given *ad libitum*. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to the United Kingdom Co-ordinating Committee on Cancer Research guidelines (13).

Antibiotic treatment. Two different antibiotic regimens were given: (*a*) gentamicin, an aminoglycoside antibiotic, which is not absorbed in the intestinal tract, and (*b*) metronidazole, given alone or together with ciprofloxacin. The effect of antibiotic treatment on mammary gland morphogenesis was evaluated in female transgenic FVB-*neu* mice that received gentamicin (70 mg/L) or metronidazole (500 mg/L) plus sucrose (20 g/L), through the drinking water, starting at age 60 days. Controls received drinking water plus sucrose. The antibiotic solution was changed every 3 days. The effect of antibiotic treatment on tumor appearance was assessed in 25 (gentamicin experiment) and 24 (metronidazole/

Note: A. Rossini and C. Rumio contributed equally to this work.

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Table 1.

(A) Breast tumor frequency in experimental and control mice

Group	Total no. mice	No. mice with single tumor	No. mice with two tumors	No. mice with three tumors	Total no. tumors
Experiment 1					
Gentamicin	13	4	2	1	11
Controls	12	4	2	0	8
Experiment 2					
Metronidazole/ciprofloxacin	10	7	2	0	11
Controls	12	6	0	0	6

(B) Statistical comparison of breast tumor occurrence in treated and control mice

	HR (95% CI)	*P* (likelihood ratio test)
Gentamicin vs control	1.39 (0.56-3.47)	0.481
Metronidazole/ciprofloxacin vs control	3.11 (1.13-8.53)	0.028

ciprofloxacin experiment) female transgenic mice, ages 30 to 50 days, randomly divided into two groups and either treated with antibiotics or untreated. Gentamicin (70 mg/L) or metronidazole (500 mg/L)/ciprofloxacin (125 mg/L) was given with sucrose (20 g/L) through the drinking water *ad libitum*. Mammary glands were inspected twice weekly. Once tumors appeared, the date of disease onset was recorded and tumor growth was assessed by measuring the two perpendicular diameters (in mm) of the tumor mass. Tumor volume was calculated as $\pi / 6 \times \text{length} \times \text{width}^2$.

Histology. Whole mounts of inferior mammary glands obtained from antibiotic-treated and control mice were prepared as described in http://ccm.ucdavis.edu/tgmouse/HistoLab/wholmt1.htm and stained with ferric hematoxylin. Images of whole-mount preparations were obtained with a digital camera mounted under a light microscope.

For histologic evaluation, inferior mammary glands were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E.

To evaluate the expression of rat $p185^{neu}$, paraffin-embedded sections were tested with anti-*neu* antibody (C-18, Santa Cruz Biotechnology, Santa Cruz, CA). After washing, sections were overlaid with biotinylated goat anti-rabbit immunoglobulin (DAKO, Carpinteria, CA) for 30 minutes, washed to remove unbound antibody, and incubated with peroxidase-conjugated streptavidin (DAKO).

Statistical methods. Survival analysis methods were used in which the time variable was mouse age at time of tumor onset; in tumor-free mice, time was censored at age at death or at latest age of assessment (260 days in the gentamicin-treated mice and 240 days in the metronidazole/ciprofloxacin-treated mice). Because some mice developed multiple tumors, the Prentice et al. marginal approach (14), which is an extension of the Cox model and suitable for analysis of repeated events, was used. The effect of antibiotic administration compared with controls was then estimated in terms of hazard ratio (HR), with a corresponding 95% CI and two-sided Wald's *P*. *P*s below the 5% threshold were considered significant.

This approach enabled estimation of both event-specific and overall HRs, allowing for heterogeneous and homogeneous treatment effects, respectively, between repeated events. Because model fitting to the data, as assessed by the Akaike Information Criterion, always favored the models assuming homogeneous treatment effects, only the overall HR estimate is given for each experiment.

All analyses were carried out using SAS software (SAS Institute, Inc., Cary, NC, 2000).

To directly compare tumor growth rates of antibiotic-treated and control mice, growth data were logarithmically transformed and a best-fit linear regression was determined for each tumor. Slopes of these lines were compared using an unpaired *t* test.

Differences in the number of buds and ducts in female transgenic mice treated with gentamicin plus sucrose, metronidazole plus sucrose, or sucrose alone were compared using Dunnett's test.

Results

Tumor latency. To evaluate the effect of antibiotic treatments on tumor appearance, 13 female transgenic mice received gentamicin and sucrose (experiment 1) and 12 received metronidazole and sucrose (experiment 2) through the drinking water starting at age 30 to 50 days. Metronidazole was used in association with ciprofloxacin, an antibiotic active on Gram-negative bacteria, as frequently done in the clinical setting (15). For each experiment, 12 control mice randomized from the same litters received drinking water plus sucrose. Two of the 12 metronidazole/ciprofloxacin-treated mice died probably due to treatment toxicity, at age 136 and 144 days, respectively, after ~3 months of antibiotic administration; thus, metronidazole/ciprofloxacin treatment was stopped at that time in the remaining 10 mice. Gentamicin was given until the end of the experiment (day 260).

Table 1A lists the frequency of breast tumors according to the number of mice in each experiment and total number of tumors detected in each group. Overall, 19 tumors were detected in the first experiment and 17 tumors in the second experiment. Figure 1*A* and *B* shows tumor latency in the two experiments. Statistical analysis indicated that the HR (95% CI) of breast cancer occurrence in metronidazole/ciprofloxacin-treated mice was more than triple that in controls [3.11 (1.3-8.53); $P = 0.028$], whereas a slight increase in breast cancer HR (95% CI) was observed in gentamicin-treated mice [1.39 (0.56-3.47); $P = 0.481$; Table 1B].

Tumor growth rate. To directly compare the tumor growth rate in the experimental groups with controls, tumor growth data were logarithmically transformed and a "best-fit" linear regression was determined for each tumor. No statistical difference between metronidazole/ciprofloxacin-treated and untreated mice ($P = 0.7527$, unpaired *t* test; Fig. 2*A*) was detected, whereas tumors in gentamicin-treated mice grew significantly faster than those in untreated control mice ($P = 0.043$, unpaired *t* test; Fig. 2*B*).

Mammary gland morphology in antibiotic-treated mice. Antibiotic treatment might influence morphogenesis of mammary

gland, which is one of the few organs in which major morphogenic changes take place after birth. Preliminary light microscopic analysis of whole mounts of mammary glands from gentamicin-treated, metronidazole/ciprofloxacin-treated, and control mice, sacrificed at different times (for advanced tumor mass or at the end of the experiments), revealed an increase in ductal branching and terminal buds in antibiotic-treated mice compared with control mice. To better determine the effect of antibiotic treatments on mammary gland development, three groups of female transgenic mice were treated through the drinking water with gentamicin plus sucrose, metronidazole plus sucrose, or sucrose alone, respectively, starting at age 60 days. To avoid toxicity, ciprofloxacin was not used in conjunction with metronidazole treatment. At age 7 to 9 months, four mice per group were sacrificed for mammary gland whole-mount preparation, whereas mammary tissue from two mice per group was used for histology and immunohistochemistry. The whole-mount preparations from gentamicin- or metronidazole-treated mice revealed an increase in ductal branching and in the number of buds versus control mice. Fig. 3 shows representative whole-mount preparations from each experimental group. Indeed, the number (mean $\pm$ SD) of buds per microscopic field was 100.8 $\pm$ 29.0 in gentamicin-treated mice, 218.4 $\pm$ 25.4 in metronidazole-treated mice, and 56.3 $\pm$ 11.5 in control mice (P = 0.04 in gentamicin-treated versus control mice and P < 0.0001 in metronidazole-treated versus control mice; Fig. 3).

Histologic examination revealed adipose tissue surrounding small ducts lined with unilayered, isoprismatic epithelial cells in control mammary tissue; the ducts were surrounded by myoepithelial cells and connective tissue. Mammary glands from gentamicin-treated group displayed the increased ductal branching observed in whole mounts, with the ducts lined by a single layer of epithelial cells. In metronidazole-treated mice, ductal branching was also increased, but the ducts and terminal buds were lined by multilayered epithelium and the lumen of the buds was no longer visible because of the thick epithelial tissue (Fig. 4). The number (mean $\pm$ SD) of ducts per microscopic field in the gentamicin- or metronidazole-treated mice exceeded that of controls (11.9 $\pm$ 0.7 for gentamicin-treated mice, 22.5 $\pm$ 1.6 for metronidazole-treated mice, and 5.8 $\pm$ 0.9 for untreated mice; P = 0.02 in gentamicin-treated versus control mice and P = 0.001 in metronidazole-treated versus control mice; Fig. 4). Immunohistochemical analysis revealed few mammary epithelial cells expressing the rat *neu* proto-oncogene, with no evident differences between untreated and antibiotic-treated groups (Fig. 5).

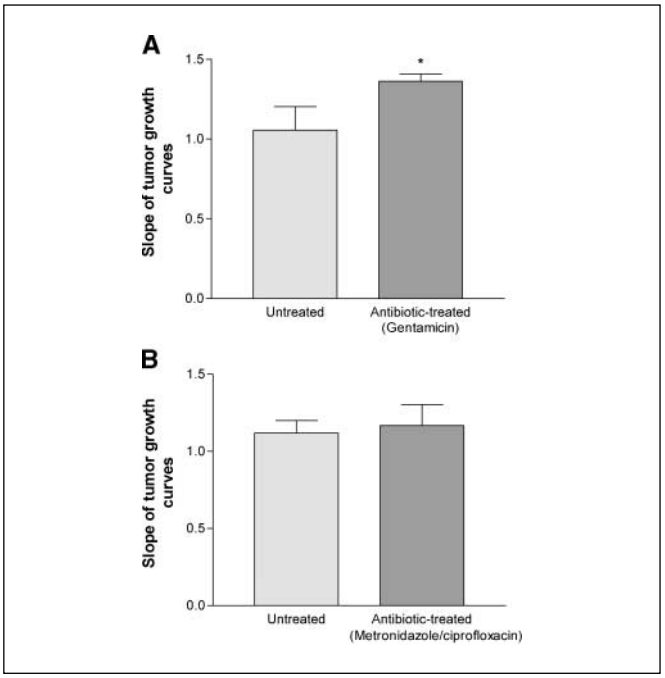

Figure 2. Effect of gentamicin (*A*) or metronidazole/ciprofloxacin (*B*) treatment on spontaneous mammary tumor growth rate. Tumor growth data were transformed on a logarithmic scale and the "best-fit" linear regression was plotted. Slopes of the tumor growth curves were compared using an unpaired *t* test. Rates of tumor growth differed significantly between gentamicin-treated and untreated mice (*, P = 0.043) but not between metronidazole/ciprofloxacin-treated and untreated mice (P = 0.7527).

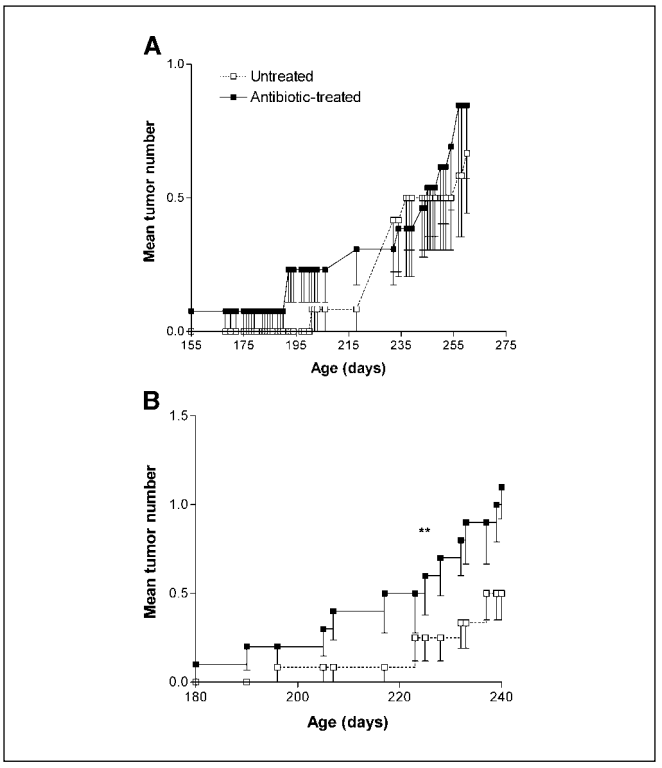

Figure 1. Effect of gentamicin (*A*) or metronidazole (*B*) treatment on number of spontaneous mammary tumors in FVB-*neu* transgenic female mice. *Points,* mean number of palpable mammary carcinomas per mouse/total number of mice; *bars,* SE. **, P = 0.0014.

Discussion

Our data indicate a significantly higher risk of tumor development in metronidazole/ciprofloxacin-treated mice (HR, 3.11; 95% CI, 1.13-8.53) but not in gentamicin-treated mice (HR, 1.39; 95% CI, 0.56-3.47) compared with controls. However, tumors in the gentamicin-treated group grew significantly faster than those in untreated controls. Note that the metronidazole/ciprofloxacin regimen was suspended due to treatment toxicity before the appearance of the first tumor, whereas gentamicin treatment was continued to the end of the experiment. Our data also indicate increased lobulization of mammary glands, with more numerous ducts and more developed terminal buds, in both groups of antibiotic-treated mice. Mammary glands from metronidazole-treated mice presented even ducts and terminal buds lined by

multilayered epithelium, with most bud lumens obscured by the thick epithelial tissue, whereas mammary glands from gentamicin-treated mice showed only increased ductal branching and bud number.

The observed influence of antibiotic treatment on HER-2/*neu*-positive breast cancer risk in mice might rest in a reduced capacity of intestinal microflora to metabolize compounds implicated in or with known inhibitory roles at several points in the carcinogenesis pathway (16–19). In HER-2/*neu* proto-oncogene transgenic mice, estrogens have been reported to be involved in the development and growth of cells that are targets for HER-2/*neu* signaling pathways involved in tumor formation (20). Similarly, increased ductal density is associated with increased breast cancer risk in women (21, 22). Because the presence of intestinal microflora is essential for the production of equol and other phytoestrogen metabolites (23, 24) that are thought to play an important role in mammary development potentially through antiestrogenic mechanisms (25, 26), an antibiotic-induced reduction in intestinal flora might underlie the increased lobulization of the mammary glands observed in mice treated with our antibiotic regimens, both of which have a strong killing action on intestinal flora. Velicer et al. (2) found that all types of antibiotics, independent of their action on a specific class of gut microflora, were associated with an increased risk of breast cancer. Thus, the relationship between antibiotic treatment and increased risk of breast carcinoma might reflect a quantitative rather than a qualitative alteration of intestinal flora. The antibiotic-induced decrease in intestinal bacterial load might even increase tumorigenesis by compromising activation of the innate immune system cells, which has been suggested to play a role in the development of the mammary ductal tree (27) and in preventing cancer through the activity of natural killer cells (28). An inverse relationship between bacterial infections and cancer is supported by considerable historical evidence documenting recovery of cancer patients after an acute bacterial

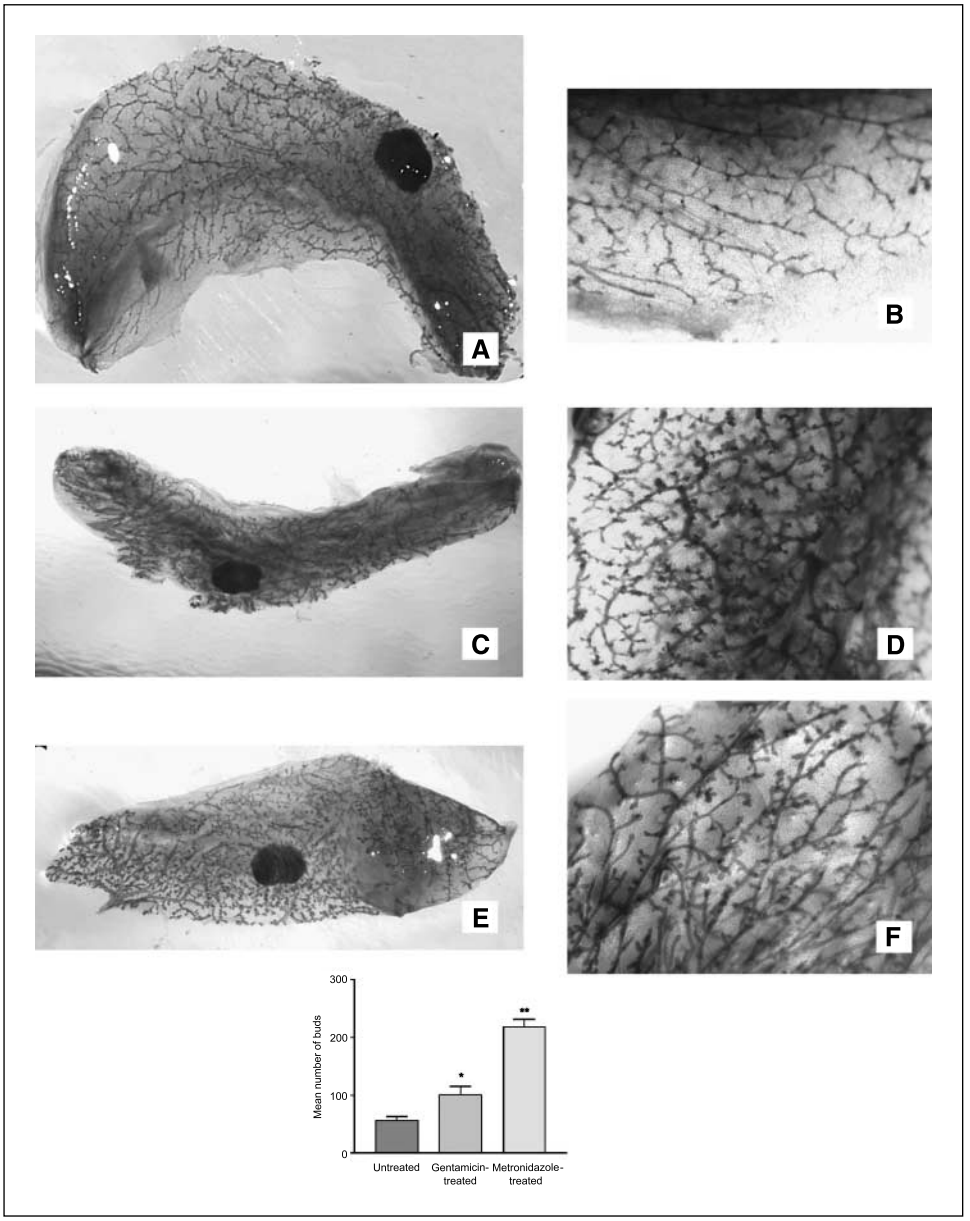

Figure 3. Light microscopy analysis of mammary gland ductal network in (*A* and *B*) untreated, (*C* and *D*) metronidazole-treated, and (*E* and *F*) gentamicin-treated mice. Terminal ductal lobular units were more developed and more numerous in mammary glands from mice treated with either antibiotic regimen. Original magnification, ×400. *Columns,* mean number of buds evaluated in four digital images per mouse (at least two mammary glands per mouse; four mice per group); *bars,* SD. *, $P = 0.04$, gentamicin versus untreated; **, $P < 0.0001$, metronidazole versus untreated.

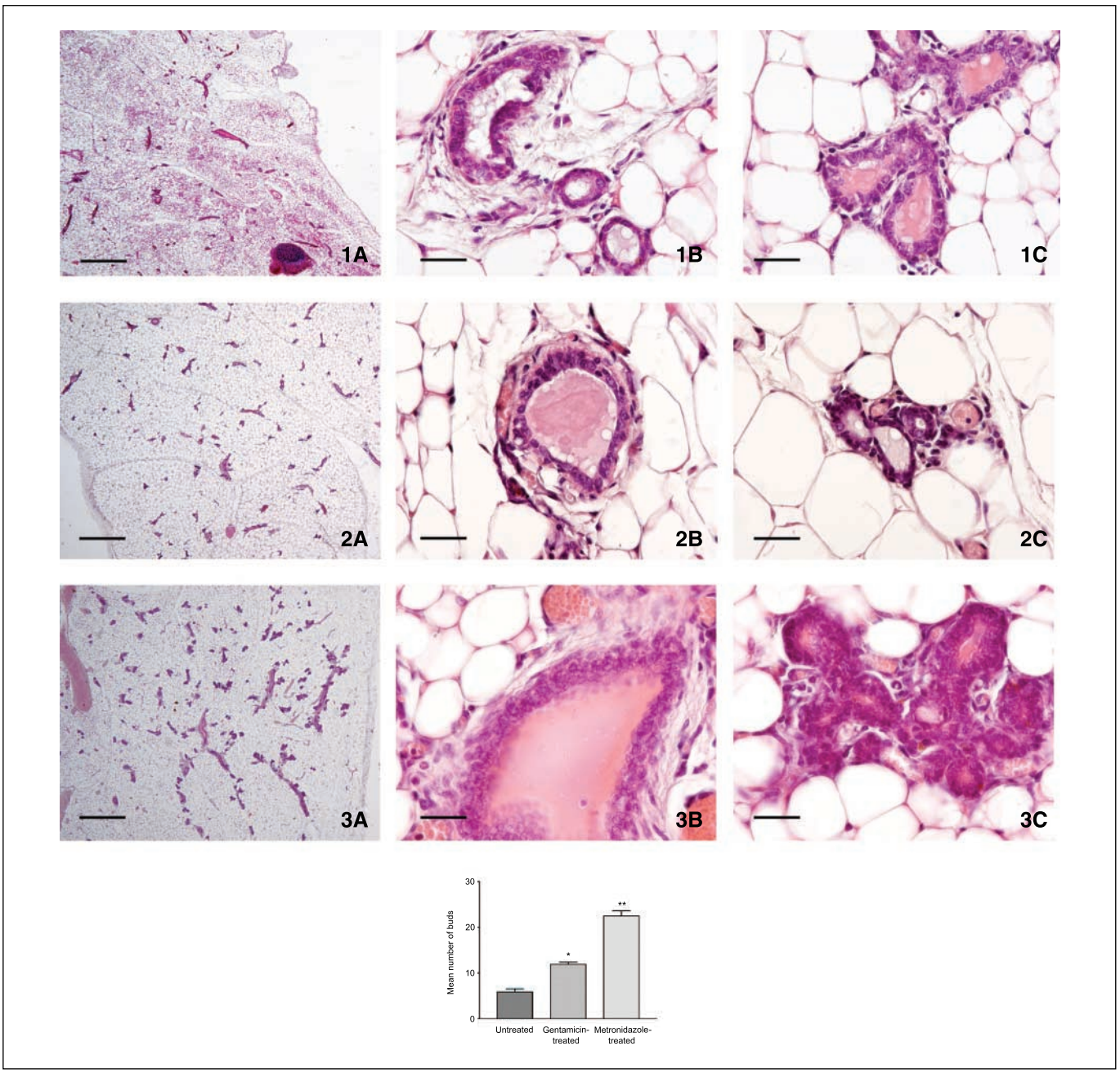

Figure 4. Histologic examination of mammary glands from control mice with low magnification showing normal ductal branching (*1A*), transverse section of duct showing single-layer epithelium (*1B*), and terminal buds surrounded by single-layer epithelium (*1C*); gentamicin-treated mice (2), with low magnification showing increased ductal branching (*2A*), transverse section of ductal branching showing single-layer epithelium (*2B*), and terminal buds surrounded by single-layer epithelium (*2C*); and metronidazole-treated mice with low magnification showing increased ductal branching (*3A*), transverse section of duct with lumen obscured by surrounding multilayer epithelium (*3B*), and terminal buds surrounded by multilayer epithelium, with reduced lumen (*3C*). Bar, 120 μm (low magnification) and 30 μm (high magnification). *Columns,* mean number of ducts evaluated in 30 digital images per mouse (at least two mammary glands per mouse; two mice per group); *bars,* SD. *, $P = 0.02$, gentamicin versus untreated; **, $P = 0.001$, metronidazole versus untreated.

infection as well as the lower incidence of cancer in patients with tuberculosis. Since the pioneering work of Coley (29) using streptococcal extracts, Bacillus Calmette-Guerin has been used in experimental model systems and in humans as an approach to activating nonspecific anticancer immunity. We recently reported that continuous activation of the innate immune system through treatment with CpG oligonucleotides prevented or delayed the onset of mammary carcinoma in HER-2/*neu* transgenic mice (30). If the same immune control of mammary tumorigenesis occurs in humans, antibiotics given in combination with bacterial products, such as CpG or endotoxins, might be a strategy to maintain innate immunity on alert even during antibiotic treatments.

Although both antibiotic regimens influenced mammary gland structure, only metronidazole/ciprofloxacin treatment led to significantly accelerated of tumor development. We cannot exclude that this finding might be related to some additional activity of

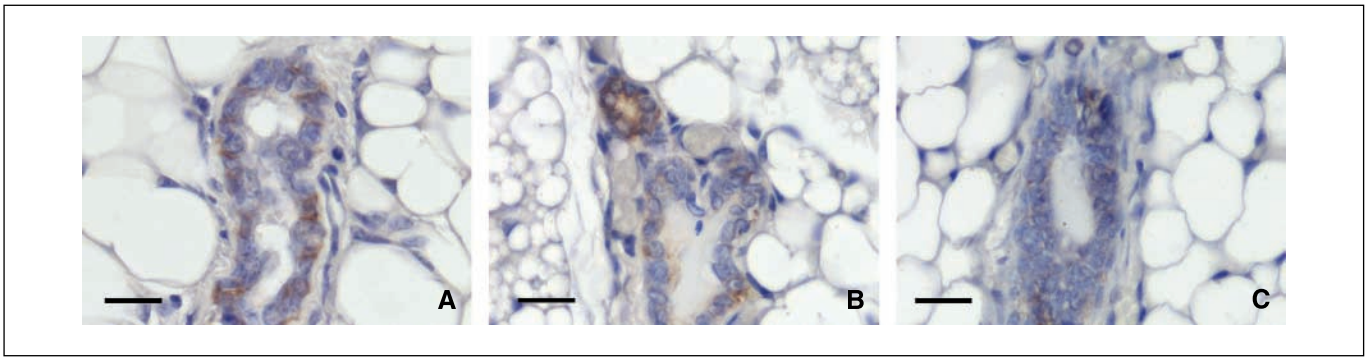

Figure 5. Immunohistochemical reactivity of anti-rat *neu* antibody on mammary glands from (*A*) untreated mouse, (*B*) gentamicin-treated mouse, and (*C*) metronidazole-treated mouse.

metronidazole. In fact, lifetime treatment with metronidazole has been reported to increase the incidence of benign tumors, such as lung adenomas in mice and mammary fibroadenomas in female rats, suggesting a potential genotoxic action (31–34). However, the significant acceleration of tumor development in metronidazole-treated but not in gentamicin-treated mice is more likely associated with the greater hyperplasia of ducts and terminal buds induced by metronidazole.

In conclusion, our data show that prolonged exposure to relevant doses of antibiotics affects the mammary glands in this particular model of HER-2/*neu* transgenic mice; further studies to understand the precise mechanism by which antibiotic treatments influence mammary gland differentiation are critical.

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