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Cyclophosphamide, Doxorubicin, and Paclitaxel Enhance the Antitumor Immune Response of Granulocyte/Macrophage-Colony Stimulating Factor-secreting Whole-Cell Vaccines in HER-2/*neu* Tolerized Mice[1]

Jean-Pascal H. Machiels, R. Todd Reilly, Leisha A. Emens, Anne M. Ercolini, Rachel Y. Lei, Diane Weintraub, Francesca I. Okoye, and Elizabeth M. Jaffee[2]

The Johns Hopkins University School of Medicine, Department of Oncology, Graduate Program in Immunology, Baltimore, Maryland 21231

ABSTRACT

Tumor-specific immune tolerance limits the effectiveness of cancer vaccines. In addition, tumor vaccines alone have a limited potential for the treatment of measurable tumor burdens. This highlights the importance of identifying more potent cancer vaccine strategies for clinical testing. We tested immune-modulating doses of chemotherapy in combination with a granulocyte/macrophage-colony stimulating factor (GM-CSF)-secreting, HER-2/neu (neu)-expressing whole-cell vaccine as a means to treat existing mammary tumors in antigen-specific tolerized *neu* transgenic mice. Earlier studies have shown that *neu* transgenic mice exhibit immune tolerance to the neu-expressing tumors similar to what is observed in patients with cancer. We found that cyclophosphamide, paclitaxel, and doxorubicin, when given in a defined sequence with a GM-CSF-secreting, neu-expressing whole-cell vaccine, enhanced the vaccine's potential to delay tumor growth in *neu* transgenic mice. In addition, we showed that these drugs mediate their effects by enhancing the efficacy of the vaccine rather than via a direct cytolytic effect on cancer cells. Furthermore, paclitaxel and cyclophosphamide appear to amplify the T helper 1 neu-specific T-cell response. These findings suggest that the combined treatment with immune-modulating doses of chemotherapy and the GM-CSF-secreting neu vaccine can overcome immune tolerance and induce an antigen-specific antitumor immune response. These data provide the immunological rationale for testing immune-modulating doses of chemotherapy in combination with tumor vaccines in patients with cancer.

INTRODUCTION

Cytokine-secreting, whole-cell cancer vaccines are currently being investigated for the treatment of solid tumors (1–3). In particular, tumor cells genetically modified to secrete GM-CSF[3] induce a systemic tumor antigen-specific T-cell response potent enough to cure mice with preestablished micrometastases (4). GM-CSF recruits dendritic cells to the vaccine site where they take up and process tumor antigens, subsequently presenting them in a form that can induce effective systemic T-cell responses (5–7). Clinical trials testing both autologous and allogeneic tumor cells engineered to secrete GM-CSF for the treatment of a variety of human cancers have already been completed or are under way (8–11). Although induction of antitumor immunity and clinical responses have been demonstrated in some patients, it is unlikely that this current form of the vaccine is potent enough to be effective in the majority of patients with minimal residual disease or small numbers of metastases (8–11).

Studies aimed at identifying tumor-associated T-cell antigens (12, 13) and understanding antigen-specific T-cell regulation (14–16) have provided new insights into the mechanisms of immune tolerance that may limit the effectiveness of cancer vaccines (17–20). For example, a number of nonmutated, tissue-specific proteins have been identified as T-cell targets recognized on human tumors (12–13, 21, 22). This implies that mechanisms are in place to delete or suppress high avidity T cells specific for these antigens that would otherwise be capable of inducing autoimmunity. This also implies that T cells with lower avidity for these same antigens may have escaped tolerance and are capable of being activated. This would explain reports describing the existence of ineffective antibody and T-cell responses directed at specific antigens expressed by simultaneously progressing cancers in patients (11, 23).

Several groups have observed that some chemotherapeutic agents can modulate the immune response (24–34). A number of reports have demonstrated that some chemotherapeutic agents can enhance the antitumor activity of adoptively transferred T cells (25, 26), tumor vaccines (29, 31), and macrophages (30). For example, it has been known for a long time that pretreatment with agents such as CTX enhances the efficacy of adoptive transfer of antigen-specific lymphocytes and antitumor vaccines (25, 26, 31). The immunopotentiation of T cell-mediated immune response by CTX has been suggested in various animal tumor models as well as in Phase I/II clinical trials (25, 26, 31–33). Mokyr *et al.* (34) demonstrated that the timing between antigen injection and CTX administration is crucial to potentiate the antitumoral immune response. Other studies have revealed the synergistic effect of chemotherapy with passive immunotherapy using the HER-2/neu targeted antibody, trastuzumab (35, 36).

Mice transgenic for the nontransforming rat *neu* proto-oncogene expressed under the control of a mammary-specific promoter (*neu* transgenic mice) develop spontaneous focal mammary adenocarcinomas (37). We described recently the immunological characterization of these mice and found that T-cell tolerance to neu exists in these mice relative to the parental nontransgenic mice (38). Despite the existence of tolerance, it was possible to induce neu-targeted immunity potent enough to overcome this tolerance and significantly delay both transplantable and spontaneously arising tumors. In this report, we have used the *neu* transgenic mouse model to identify chemotherapeutic agents that, when given sequentially with a neu-expressing GM-CSF-secreting whole tumor vaccine, can enhance vaccine efficacy. Our findings show that pretreatment with PTX or CTX increases the vaccine efficacy, in particular the type I cytokine immune response. These results suggest that combined treatment with immune-modulating doses of chemotherapy and the GM-CSF-secreting neu vaccine can overcome immune tolerance and induce an antigen-specific immune response.

MATERIALS AND METHODS

Mice. *neu* transgenic mice developed by Guy *et al.* (Ref. 37; line 202) were bred to homozygosity as verified by Southern blot analysis. FVB/N mice were

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[2] To whom requests for reprints should be addressed, at The Johns Hopkins University School of Medicine, Department of Oncology, Graduate Program in Immunology, 1650 Orleans Street, Blaustein-Bunting Building, Room 4 M07, Baltimore, MD 21231. Phone: (410) 614-4626; Fax: (410) 614-8216; E-mail: ejaffee@jhmi.edu.

[3] The abbreviations used are: GM-CSF, granulocyte/macrophage-colony stimulating factor; CTX, cyclophosphamide; ATCC, American Type Culture Collection; *neu*, HER-2/*neu*; DOX, doxorubicin; CIS, cisplatin; PTX, paclitaxel; IL, interleukin; ELISPOT, enzyme-linked immuno-spot assay; Th, T helper; 3T3, NIH-3T3.

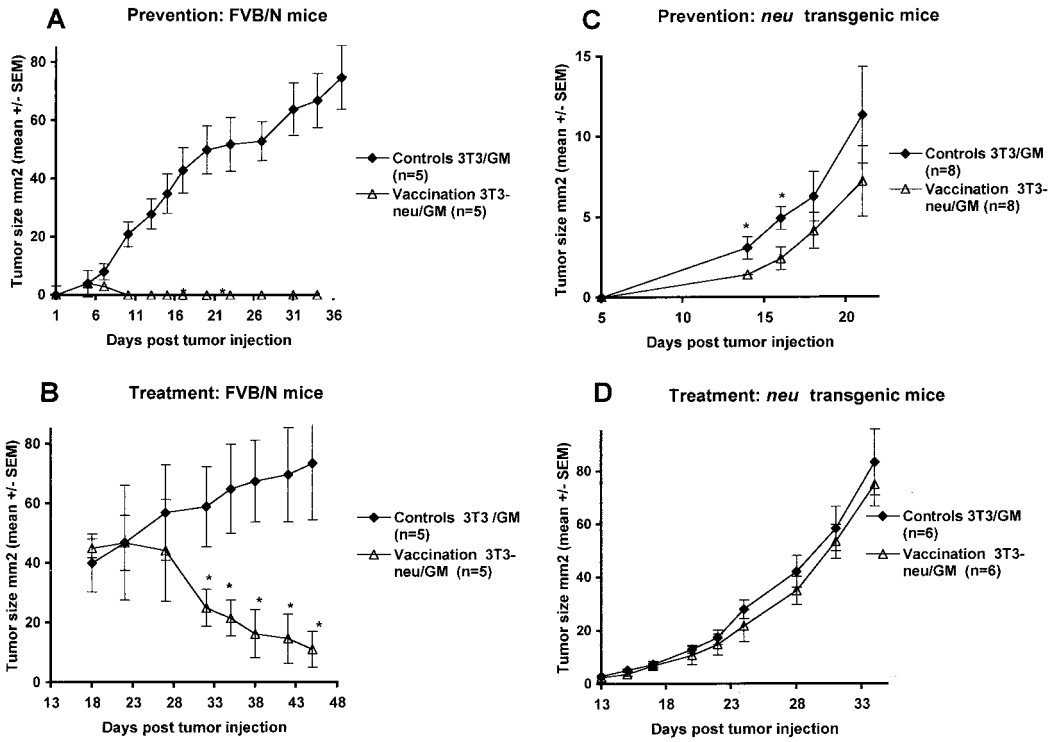

Fig. 1. neu-specific vaccination can prevent and treat neu-expressing tumors in the parental FVB/N but not in *neu* transgenic mice. Parental FVB/N (*A*) and *neu* transgenic (*C*) mice were vaccinated with three simultaneous s.c. injections of 10^6 3T3-*neu*/GM vaccine cells (right and left hind limbs and left upper limb) on day 0 and challenged with 5×10^6 (FVB/N mice) or 5×10^4 (*neu* transgenic mice) NT cells into the right upper mammary fat pad on day 14. In a treatment experiment, a second group of parental FVB/N (*B*) and *neu* transgenic (*D*) mice were first implanted with 5×10^6 (FVB/N) or 5×10^4 (*neu* transgenic mice) NT cells into the right upper mammary fat pad. FVB/N mice were vaccinated 2 weeks later, and *neu* transgenic mice were vaccinated 1 day later with three simultaneous s.c. injections of 10^6 3T3-*neu*/GM into the right and left hind limbs and left upper limb. All mice were monitored twice a week for a change in tumor growth. Plotted is the mean of the products of the two perpendicular diameters (mm^2) for five to eight mice/group as a function of days after tumor implantation; *bars*, SE. Control mice in each study received similar injections with the 3T3/GM mock vaccine. Similar results were obtained in four independent experiments. ♦, controls 3T3/GM (mock vaccine); △, vaccination 3T3-*neu*/GM. *, $P < 0.05$ as determined by unpaired Student's *t* test.

obtained commercially from The Jackson Laboratory. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Cell Lines and Media. NT cells were derived from spontaneous mammary tumors of female *neu* transgenic mice as described previously (Ref. 38; NT cells are from a neu-expressing tumor cell line derived from spontaneous tumor of *neu* transgenic mice; NT-B7 are NT cells that human the B7 costimulatory molecule). The NT cell line overexpresses the rat *neu* cDNA, and these levels remain stable (38). The NT cell line was grown in our defined Breast Media, which consists of RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 20% FBS (Hyclone, Logan, UT), 1% L-glutamine (JRH Biosciences, Lenexa, KS), 1% non-essential amino acids, 1% sodium pyruvate, 0.5% penicillin/streptomycin, 0.02% Gentamicin (Sigma Chemical Co., St. Louis, MO), and 0.2% insulin (Lilly, Indianapolis, IN), and maintained at 37°C in 5% CO_2 atmosphere. The NT cell line was expanded to large numbers to produce master cell banks of each line to avoid extensive *in vitro* passage, thereby maintaining reproducibility of each *in vivo* study. Production was performed at the NIH cGMP facility (Frederick, MD). neu and MHC class I levels were tested by fluorescence-activated cell sorter and confirmed to be stable before and after freezing. NT-B7 cells were produced via retroviral transduction of NT cells with a human B7-1 encoding retrovirus as described previously (4). NT-B7 was maintained in our defined Breast Media supplemented with neomycin (Life Technologies, Inc.). NIH-3T3 cells (3T3; ATCC, Rockville, MD) were grown in DMEM (Life Technologies, Inc.) with 10% bovine calf serum (Hyclone) 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 0.5% penicillin/streptomycin at 37°C and 10% CO_2 atmosphere. The NIH-3T3 derivative, ATCC CRL-1915 (3T3-*neu*; ATCC), which overexpresses the rat HER-2/*neu* proto-oncogene, was grown in 3T3 media + 0.3 μM methotrexate at 37°C in 10% CO_2 atmosphere. NIH-3T3 cells and 3T3-*neu* were genetically modified to express the murine cytokine GM-CSF using the retroviral vector MFG as described previously (4), resulting in 3T3/GM and 3T3-*neu*/GM cell lines, respectively (3T3/GM are 3T3 cells that produce murine GM-CSF; 3T3-*neu* are NIH-3T3 cells that express rat HER-2/*neu* cDNA; 3T3-*neu*/GM are 3T3-*neu* cells that produce murine GM-CSF). Murine GM-CSF production was tested with a commercially available ELISA kit (Endogen, Woburn, MA) and was determined to be between 200 and 250 ng/10^6 cells/24 h for 3T3/GM and 3T3-*neu*/GM. GM-CSF bioactivity was confirmed using the GM-CSF-dependent cell line, NFS-60, as described previously (4). Production of GM-CSF by untransduced NT cell line is not detected as determined by ELISA.

Chemotherapeutic Agents. PTX (Bristol-Myers Squibb, Princeton, NJ), DOX (Gensia, Irvine, CA), and CIS (Bristol-Myers Squibb) were diluted in HBSS before injection. CTX (Bristol-Myers Squibb) was diluted in sterile water before injection. PTX, CTX, and CIS were injected i.p.; DOX was injected i.v.

Vaccination and Tumor Challenge. On the day of vaccination, vaccine cells grown *in vitro* were trypsinized, washed three times in HBSS (pH 7.4; Life Technologies, Inc.), and counted. The cells were resuspended in HBSS at 10^7 cells/ml and irradiated with 50 Gy from a ^{137}Cs source (Nordion, Toronto, Ontario, Canada), discharging 1400 rad/min. Eight-week-old *neu* transgenic or FVB/N mice were given three simultaneous 100-μl s.c. injections (right and left hind limbs and left arm) using a 1-ml tuberculin syringe with a 27-gauge needle (39). The mice in the vaccine group received three simultaneous injections of 10^6 3T3-*neu*/GM cells. To insure that the effect observed was *neu* specific, all control mice as well as the mice in the chemotherapy group alone received a mock vaccination consisting of three s.c. injections of 10^6 3T3/GM cells. 3T3/GM mock vaccination did not delay the occurrence of tumor growth compared with mice injected only with HBSS (data not shown). On the day of the tumor challenge, NT cells thawed from frozen bank stores and grown *in vitro* for 1 or 2 weeks were trypsinized, washed three times in HBSS, and injected into the right upper mammary fat pad. Mice were challenged with 5×10^4 (*neu* transgenic) or 5×10^6 (FVB/N) NT tumor cells. In treatment experiments, mice were challenged with NT cells on day 0 and vaccinated on day 3 unless otherwise specified. In prevention experiments, mice were challenged with NT cells 2 weeks after vaccination. Tumor occurrence (shown as the tumor-free probability) or changes in tumor growth were monitored twice a week. Changes in tumor growth (mm^2) were determined by multiplying the two perpendicular diameters.

Table 1 *Dose- and schedule-dependent associations between chemotherapy and the GM-CSF secreting whole-cell vaccine in FVB/N mice*[a]

	T cell count (nadir) number/ μl ± SD (normal range, 4000–9000)[b]	Chemotherapy 1 day before vaccine	Chemotherapy 7 days after vaccine
CTX			
50 mg/kg	6128 ± 847	+[c]	−
100 mg/kg	5120 ± 1033	+	−
150 mg/kg	1559 ± 356	+	NT
200 mg/kg	1100 ± 478	+/−	NT
250 mg/kg	989 ± 122	+/−	NT
PTX			
20 mg/kg	4365 ± 501	+	−
30 mg/kg	4200 ± 675	+	NT
35 mg/kg	3600 ± 543	+/−	NT
40 mg/kg	3451 ± 345	+/−	NT
DOX			
4 mg/kg	6265 ± 1298	+/−	+/−
8 mg/kg	5586 ± 945	+/−	+/−
15 mg/kg	4180 ± 501	−	−
CIS			
2 mg/kg	6320 ± 903	+/−	+/−
3 mg/kg	6200 ± 674	+/−	+/−
5 mg/kg	3679 ± 455	−	−
10 mg/kg	3400 ± 697	−	−

[a] FVB/N mice were implanted with 5×10^6 NT cells in the right upper mammary fat pad on day 0. Groups of 5–10 mice received either: (*a*) HBSS as a control; (*b*) 3T3-*neu*/GM vaccine cells; (*c*) chemotherapy; or (*d*) chemotherapy and 3T3-*neu*/GM vaccine cells. 3T3-*neu*/GM vaccine cells were injected s.c. at three sites (10^6 cells/site), the left and right hind limb and left upper limb, on day 3. All mice were monitored twice a week for a change in tumor growth as determined by multiplying the two perpendicular diameters. *P* was determined by unpaired Student's *t* test.

[b] Blood was drawn 4 days after chemotherapy administration (nadir), and T cells were automatically counted (ANTECH Diagnostics, New York). Data shown are the mean of T cell counts for three mice at day 4 after chemotherapy injection (nadir).

[c] +, additional effect (vaccine/chemotherapy group statistically superior ($P < 0.05$) to each treatment modality alone); +/−, no effect (vaccine/chemotherapy group statistically superior ($P < 0.05$) to chemotherapy group but not to vaccine group); −, inhibition effect (no statistical difference between vaccine/chemotherapy group and chemotherapy group); NT, not tested.

T-Cell Assays. neu-specific, IFN-γ- or IL-4-producing T cells were quantified by ELISPOT analysis. *neu* transgenic mice were given a s.c. challenge with NT cells, followed 3 days later by vaccination with 3T3-*neu*/GM or 3T3/GM with or without chemotherapy. On day 12 after vaccine, T cells were isolated from splenocytes by Ficoll (Amersham, Uppsala, Sweden) separation and passed over a nylon wool column to remove B cells and macrophages. $CD4^+$ cells were positively selected with Dynabeads and Detachbead mouse CD4, according to the manufacturer's instructions (Dynal, Lake Success, NY). After one round of $CD4^+$-positive selection, >98% of cells were shown to be $CD4^+$ by fluorescence-activated cell sorter. neu-specific IFN-γ or IL-4 production was determined by a standard ELISPOT protocol and as described previously (38, 40). IFN-γ-treated NT-B7 cells (10^4 per well) were used as stimulators, and serial dilutions of unfractionated lymphocytes or $CD4^+$ T cells were added to the wells for an 18-h incubation at 37°C. Each condition was tested in triplicate. Reagents and materials used in the assay were the following: 96-well filtration plate (MA1PS4510, Millipore, Molsheim, France), rat antimouse IFN-γ at 10 μg/ml (PharMingen, San Diego, CA), rat antimouse biotin IFN-γ (Biosource International, Camarillo, CA), rat antimouse IL-4 at 10 μg/ml (PharMingen), rat antimouse biotin IL-4 (Biosource International), avidin-alkaline phosphatase at 2 μg/ml (Sigma Chemical Co., St. Louis, MO), and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical Co.).

Statistical Analysis. Unpaired Student's *t* tests were performed to analyze tumor size and ELISPOT data. Kaplan-Meier analyses were used to analyze tumor-free survival, and the log-rank test was used for comparisons. $P < 0.05$ was considered as statistically significant.

RESULTS

A neu-targeted Vaccine Is Highly Effective at Preventing and Treating *neu*-expressing Tumors in Parental FVB/N but not in *neu*-Transgenic Mice. We have shown previously that *neu* transgenic mice demonstrate immune tolerance to neu; the dose of tumor cells required for tumor growth in 100% of animals was at least 100-fold lower for the *neu* transgenic mice when compared with parental FVB/N mice (38). Nontransgenic and *neu* transgenic mice were compared for their ability to respond to a neu and GM-CSF expressing whole-cell vaccine (3T3-*neu*/GM) given before or after a tumor challenge with NT tumor cells. Nontransgenic FVB/N mice vaccinated once with 3T3-*neu*/GM demonstrated an impressive anti-tumor response capable of preventing and treating large tumor burdens (Fig. 1, *A* and *B*). *neu* transgenic mice given the same vaccine demonstrated a small but significant and reproducible delay in transplantable tumor growth when the tumor challenge was given 2 weeks after the vaccine (Fig. 1*C*). However, in treatment experiments, a statistical difference in the rate of tumor growth between the control and the vaccine groups was not detected in *neu* transgenic mice, even if the vaccine was administered as early as 1 day after the tumor challenge (Fig. 1*D*). These results provide further evidence that the *neu* transgenic mice demonstrate an immune tolerance to neu.

Identification of a Chemotherapy Dose Range and Sequence of Administration That May Enhance the Antitumor Effects of the 3T3-neu/GM Vaccine. The data presented in Fig. 1 show that our vaccine approach is potent enough to eradicate large established tumor burdens in a host that does not exhibit antigen-specific immune tolerance to tumor. In contrast, the same vaccine is not potent enough to prevent tumor development in the *neu* transgenic mice (38, 41). Therefore, immune-modulating agents that can overcome the mechanisms of tolerance may enhance the effectiveness of neu-specific immunization.

One immune-modulating approach is to test selected chemotherapeutic agents at non-immune-suppressing doses for their ability to enhance the potency of the vaccine. We therefore tested four chemotherapeutic agents (CTX, DOX, PTX, and CIS) in combination with the 3T3-*neu*/GM vaccine. These four drugs were chosen for evaluation for three reasons: (*a*) these agents are commonly used for the treatment of human cancers; (*b*) each drug represents a different class of chemotherapeutic agents and would be expected to interact with the vaccine by distinctly different mechanisms; and (*c*) there are data in the literature suggesting that each of these agents have immune-modulating effects (24–34, 42, 43).

Initially, feasibility studies were performed in FVB/N mice to identify a dose range and optimal sequence of administration for each chemotherapeutic agent when combined with the vaccine. Mice were given 5×10^6 NT cells inoculated into the right upper mammary fat pad on day 0 and vaccinated 3 days later with three simultaneous s.c. injections of 1×10^6 irradiated 3T3-*neu*/GM cells given into the left and right hind limbs and the left upper limb. Each of the four chemotherapeutic agents were given either 1 day prior to vaccination (at the time of immune priming) or 7 days after vaccination (at the time of initial T-cell activation and expansion). Table 1 summarizes the dose range for each agent used as well as the type of effect observed.

When either PTX (dose range between 20 and 30 mg/kg) or CTX (dose range between 50 and 150 mg/kg) were given prior to the vaccine, the combination of chemotherapy plus vaccine was better to control tumor growth than treatment with either modality alone (Table 1). However, when these two chemotherapeutic agents were given at the same doses 7 days after vaccination, the combination chemotherapy/vaccine was not superior to chemotherapy alone. In contrast, DOX (dose <10 mg/kg) and CIS (dose <5 mg/kg) neither inhibited nor significantly enhanced the potency of the vaccine when given either 1 day before or 1 week after vaccination.

CTX, PTX, and DOX Enhance the Antitumor Effects of the Vaccine and Significantly Delay Transplantable Tumor Progression in *neu*-Transgenic Mice. Next, we tested the dose and schedule of each chemotherapeutic agent found to be effective in the nontolerized mice for the ability to enhance the potency of the vaccine in the *neu*

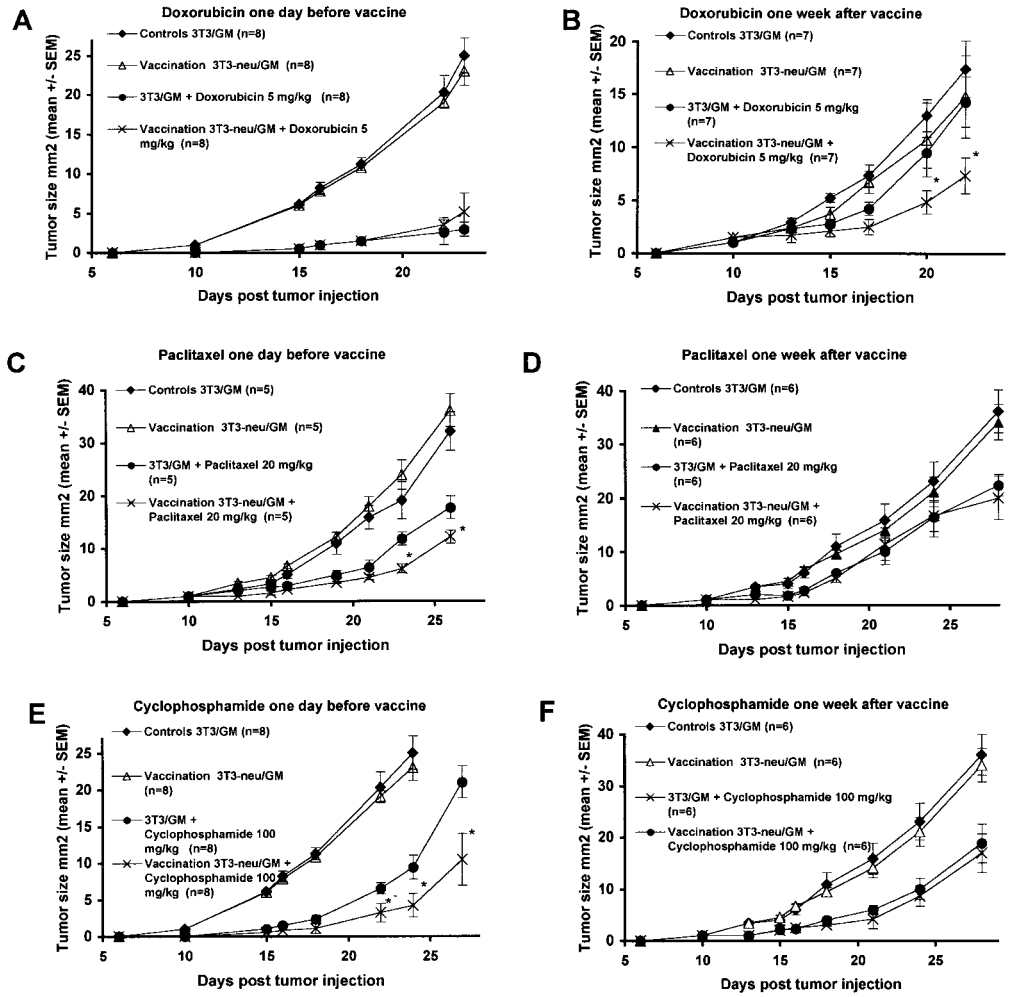

Fig. 2. DOX, PTX, and CTX can enhance the antitumor effect of the neu-targeted vaccine in *neu*-transgenic mice when given in proper sequence. Between five and eight mice/group received either: (*a*) mock vaccination (3T3/GM) as control; (*b*) 3T3-*neu*/GM vaccine alone; (*c*) chemotherapy and a mock vaccine (3T3/GM); or (*d*) chemotherapy and 3T3-*neu*/GM vaccine. In all experiments, 5×10^4 NT cells were implanted in the right mammary fat pad on day 0, and mice were vaccinated on day 3. Vaccination consisted of either 3T3-*neu*/GM or 3T3/GM given s.c. at three sites (10^6 cells/site), the left and right hind limbs and left upper limb. All mice were monitored twice a week for a change in tumor growth. Plotted is the mean of the products of the two perpendicular diameters (mm^2) for five to eight mice/group as a function of days after tumor implantation; *bars*, SE. Similar results were obtained in two independent experiments. *A*, 5 mg/kg DOX was given i.v. on day 2, 1 day before vaccination. *B*, 5 mg/kg DOX was given i.v. on day 10, 1 week after vaccination. *C*, 20 mg/kg PTX was given i.p., on day 2, 1 day before vaccination. *D*, 20 mg/kg PTX was given i.p. on day 10, 1 week after vaccination. *E*, 100 mg/kg CTX was given i.p. on day 2, 1 day before vaccination. *F*, 100 mg/kg CTX was given i.p. on day 10, 1 week after vaccination. ♦, controls-3T3/GM (mock vaccine); △, vaccination 3T3-*neu*/GM; ●, 3T3/GM (mock vaccine) + chemotherapy; *X*, vaccination 3T3-*neu*/GM vaccine + chemotherapy. ∗, $P < 0.05$ as determined by unpaired Student's *t* test between the chemotherapy group alone and the chemotherapy and vaccine group.

transgenic mice. Mice were inoculated with 5×10^4 NT cells in the right upper mammary fat pad on day 0 and vaccinated 3 days later. As shown in Fig. 2, when either CTX or PTX was given 1 day before vaccination, they enhanced the potential of the vaccine to delay tumor growth. In contrast, DOX had no effect when administered prior to vaccination but could enhance the antitumor effect when administered 1 week after the vaccination. CIS was the only drug of the four that did not appear to enhance the antitumor immune response of the vaccine at all at the dose range and schedules studied (data not shown).

Because CTX and PTX enhance the effect of the vaccine at a different time point than DOX, it is likely that the mechanisms by which they interact with the vaccine also differ. If this is indeed the case, then it is possible that administering either CTX or PTX in sequence with both the vaccine and DOX would further enhance the antitumor immune response in the *neu* transgenic mice. We therefore tested the combination of CTX (100 mg/kg) and DOX (5 mg/kg) given, respectively, 1 day before and 7 days after vaccination. This polychemotherapy regimen induced a mild leukopenia ranging between 4000 and 5000 WBCs (normal between 8,000 and 12,000). As shown in Fig. 3, the association of CTX/DOX and vaccine was significantly more effective at controlling the tumor occurrence than either treatment modality alone. This polychemotherapy/vaccine regimen cured 20% of the *neu* transgenic mice in two similar experiments and was better to control tumor growth than CTX/vaccine or DOX/vaccine (data not shown).

Chemotherapy Appears to Enhance the Potency of the neu-targeted Vaccine through a Mechanism Distinct from Direct Tumor Lysis. Prevention experiments were performed to determine whether the mechanism by which the chemotherapeutic agents enhance the efficacy of the vaccine is through direct tumor killing or through amplification of the antitumor immune response. Mice received three simultaneous s.c. injections of 10^6 3T3-*neu*/GM vaccine in the right and left hind limb and right upper limb on day 0. PTX or CTX were given i.p. 1 day prior and DOX was given i.v. 7 days after vaccine. All mice were challenged with 5×10^4 NT cells 14 days after vaccination. This experimental design makes it unlikely that the chemotherapy can directly reduce the tumor burden because the chemotherapy dose was administered 7 days prior to tumor challenge for DOX and 15 days prior to tumor challenge for PTX and CTX. As

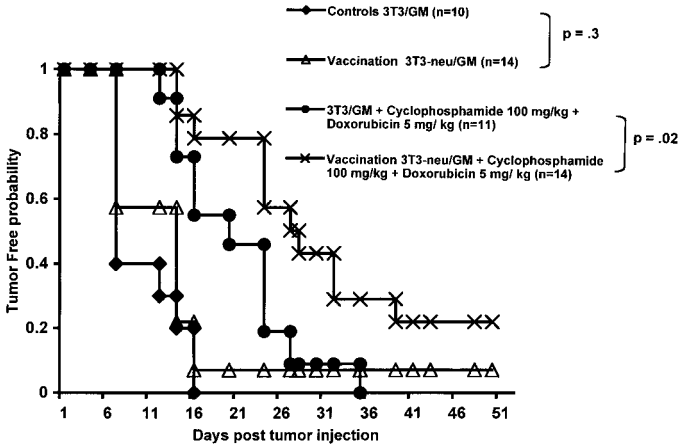

Fig. 3. Polychemotherapy can enhance the antitumor effect of the neu-targeted vaccine in *neu* transgenic mice. Between 10 and 14 mice/group (pooled from two independent experiments) received either: (*a*) controls 3T3/GM (mock vaccine); (*b*) 3T3-*neu*/GM vaccine alone; (*c*) polychemotherapy and 3T3/GM (mock vaccine); or (*d*) polychemotherapy and 3T3-*neu*/GM vaccine. NT cells (5×10^4) were implanted in the right mammary fat pad on day 0. Vaccination (3T3-*neu*/GM or 3T3/GM) was given at three sites (10^6 cells/site), the left and right hind limbs and left upper limb, on day 3. The chemotherapy consisted of i.p. 100 mg CTX on day 2 (1 day prior to the vaccine) and i.v. 5 mg/kg DOX on day 10 (7 days after the vaccine). All mice were monitored twice a week for tumor occurrence. ♦, controls 3T3/GM (mock vaccine); △, vaccination 3T3-*neu*/GM; ●, 3T3/GM (mock vaccine) + 100 mg/kg CTX + 5 mg/kg DOX; *X*, vaccination 3T3-*neu*/GM vaccine + 100 mg/kg CTX+ 5 mg/kg DOX.

shown in Fig. 4, CTX and vaccine combination induced a more potent antitumor response than either vaccine or chemotherapy alone. Similar results were obtained with PTX and DOX (data not shown). These data therefore support the hypothesis that these chemotherapeutic agents increase the potency of the vaccine via mechanisms that are distinct from direct tumor lysis.

PTX and CTX Given in Sequence with the *neu*-targeted Vaccine Results in an Increase in *neu*-specific T Cells in *neu*-Transgenic Mice. IFN-γ ELISPOT analysis was used to quantitate neu-specific T-cell induction in *neu* transgenic mice after 3T3-*neu*/GM vaccine with and without chemotherapy. Mice were challenged with NT cells, followed 3 days later with either a 3T3-*neu*/GM vaccine or a mock vaccination (3T3/GM). CTX, PTX, and DOX were given either 1 day before the vaccine or 1 week after the vaccine. The mice were sacrificed 12 days after vaccine administration, and unfractioned T cells were isolated from the spleen as described in "Materials and Methods." As shown in Fig. 5, CTX and PTX administered 1 day before the vaccine increased the number of neu-specific T cells when compared with mice that received 3T3-*neu*/GM vaccine alone. PTX and CTX injected after the vaccine significantly decreased the number of neu-specific T cells when compared with the mice that received vaccine alone. In contrast, DOX given 1 day before the vaccine or 1 week after the vaccine did not decrease or increase the number of neu-specific T cells. This supports the hypothesis that the DOX given after the vaccine increased its efficacy through a different mechanism than PTX or CTX.

CTX and PTX Appear to Specifically Enhance the Th1 Response of the 3T3-*neu*/GM Vaccine in *neu*-Transgenic Mice. We have described previously the importance of $CD4^+$ T cells in orchestrating the host response to tumor after vaccination with whole-cell vaccines engineered to secrete GM-CSF (4, 38, 44). To study the Th1 and Th2 balance in *neu* transgenic mice given chemotherapy in sequence with the vaccine, IFN-γ and IL-4 ELISPOT analyses were performed on $CD4^+$ T cells isolated and purified from spleen 12 days after vaccination. As shown in Fig. 6, the Th1 but not the Th2 response was increased when PTX or CTX were given before a 3T3-*neu*/GM vaccine compared with the group that received the vaccine only. In contrast, DOX given after the vaccine did not increase or decrease the Th1 or Th2 response (data not shown). These data therefore suggest that CTX and PTX, when given prior to the vaccine, enhance the Th1 T-cell response.

DISCUSSION

The data presented in this study support the following two conclusions: (*a*) CTX, PTX, and DOX, when given in a defined sequence with a murine GM-CSF secreting neu-expressing whole-cell vaccine, enhance the potential of the vaccine to delay tumor growth in tolerized *neu* transgenic mice. The optimal immune-modulating dose for each chemotherapeutic agent appears to be just above doses that begin to induce cytopenias; and (*b*) the enhanced antitumor response appears to be mediated, at least in part, by an increase in number and function of antigen-specific T cells (CTX and PTX), in particular the Th1 response. These findings suggest that combined treatment with immune-modulating doses of chemotherapy and the GM-CSF-secreting neu vaccine can overcome immune tolerance and induce a more potent antigen-specific antitumor immune response than vaccine alone.

neu transgenic mice offer the opportunity to test vaccine strategies in the context of tumor-specific immune tolerance (38). Our previous studies have demonstrated that *neu* transgenic mice exhibit a neu-specific immune tolerance similar to what is observed in patients with breast cancers that overexpress HER-2/*neu* (38). Although neu-targeted vaccination was able to eradicate large burdens of preestablished tumors in the nontolerized parental mice in this study, these same vaccines could only significantly delay the development of transplantable neu-expressing tumors in a prevention model in the *neu* transgenic mice (38). Furthermore, we did not observe a significant difference in tumor growth between the control and vaccine groups in the treatment experiments. This reinforces data reported previously demonstrating that tumor vaccines alone have a limited potential for the treatment of measurable tumor burdens and highlights the importance of identifying more potent vaccine strategies for clinical testing.

We evaluated the possible integration of chemotherapy and vaccine to treat transplantable mammary tumors in *neu* transgenic mice. We found that, when given in the proper sequence and at immune-modulating doses, systemic administration of CTX, PTX, and DOX can enhance rather than inhibit the antitumor immunity generated by the vaccine. The fact that this finding is also observed in prevention experiments in which

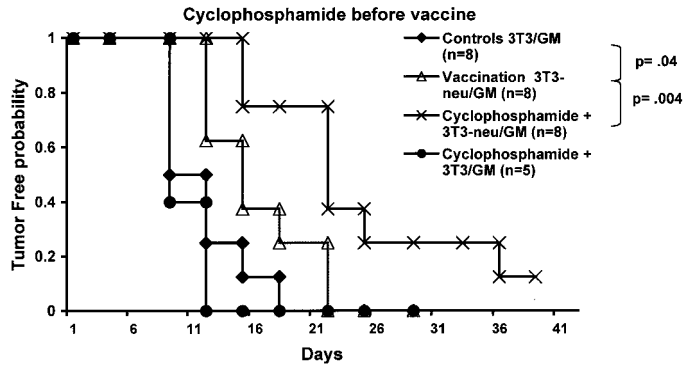

Fig. 4. Chemotherapy enhances the potency of neu-specific vaccine through a mechanism distinct from direct tumor lysis. Between 5 and 8 *neu* transgenic mice were vaccinated s.c. with 3T3-*neu*/GM cells given at three sites (10^6 cells/site), the left and right hind limbs and left upper limb, with and without chemotherapy. Two weeks after vaccination, mice were challenged into the mammary fat pad with 5×10^4 NT cells. Mice in the control group received a mock vaccination (3×10^6 3T3/GM). Mice were observed three times a week for tumor occurrence. Results are shown as tumor-free probability (*Y axis*) on days after tumor challenge (*X axis*). Similar results were obtained in two independent experiments. ♦, controls-3T3/GM (mock vaccine); △, 3T3-*neu*/GM vaccine only; ●, 100 mg/kg CTX 1 day before 3T3/GM (mock vaccine); *X*, 100 mg/kg CTX 1 day before a 3T3-*neu*/GM vaccine.

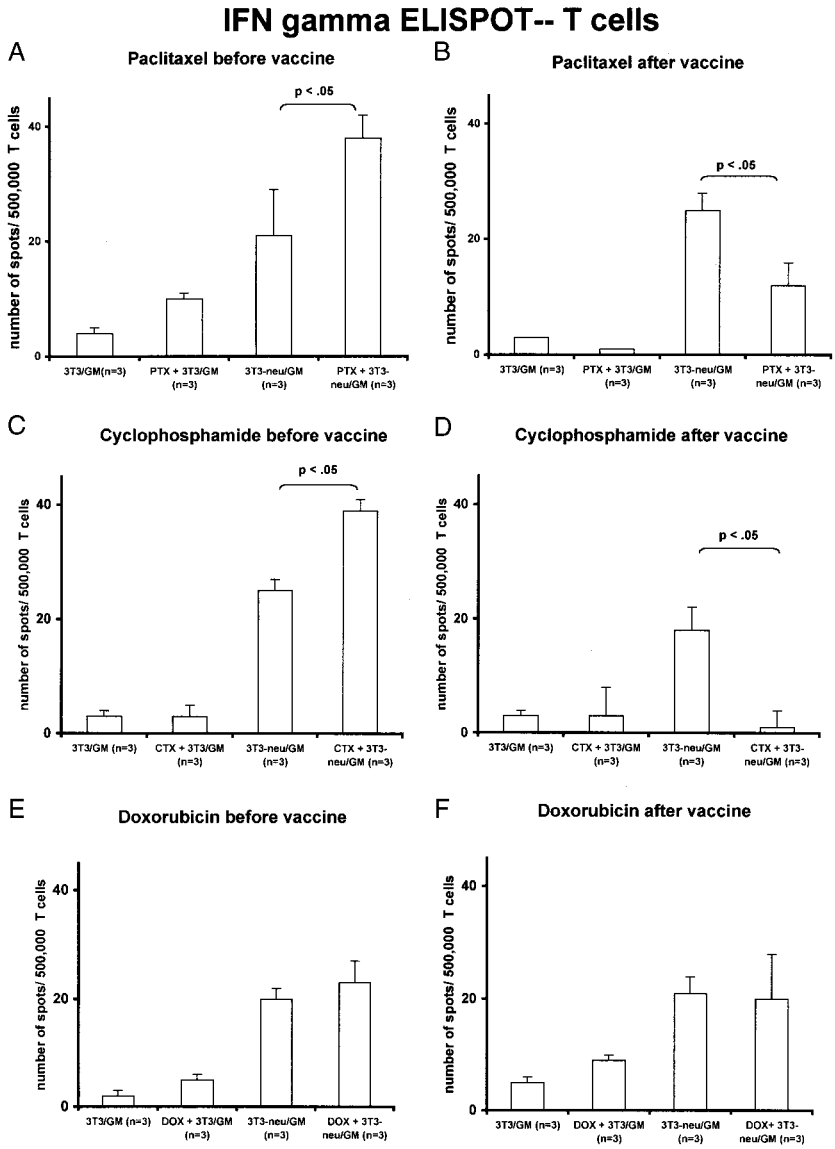

Fig. 5. CTX and PTX but not DOX given in sequence with the neu-targeted vaccine results in an increase in neu-specific T cells in *neu* transgenic mice. IFN-γ ELISPOT analysis was used to determine the number of neu-specific T cells induced in *neu* transgenic mice with a 3T3-*neu*/GM vaccine with or without chemotherapy. NT tumor cells (5×10^4) were implanted in the right mammary upper fat pad on day 0, and mice were vaccinated on day 3. Vaccination (3T3-*neu*/GM or 3T3/GM) was given s.c. at three sites (10^6 cells/site), the left and right hind limbs and left upper limb. Mice were sacrificed 12 days after the administration of the vaccine, and the T cells were isolated from spleen as described in "Materials and Methods." ELISPOT analysis was performed as described in "Materials and Methods." Three mice per group received either: (*a*) controls 3T3/GM (mock vaccine); (*b*) 3T3-*neu*/GM vaccine alone; (*c*) chemotherapy and 3T3/GM (mock vaccine); and (*d*) chemotherapy and 3T3-*neu*/GM vaccine. Plotted are the mean (three wells/condition) of the number of spots counted in the wells containing the T cells and the stimulator cells minus the number of spots counted in the well containing the T-cell alone; *bars*, SD. NT-B7 stimulator cells do not give any background (data not shown). *P* was determined by unpaired Student's *t* test between the vaccine group and the chemotherapy + vaccine group.

the tumor challenge is given 7 days after the last dose of chemotherapy suggests that the antitumor effect cannot be explained only by a direct chemotherapy-induced cytolytic effect on the tumor cells. Rather, CTX, PTX, and DOX appear to also have a direct immune augmenting effect. This immune-enhancing effect appears to be attributable in part to an augmentation of the number and activity of antigen-specific T cells. Furthermore, the data suggest that PTX and CTX may amplify the Th1 T-cell response. In contrast to CTX and PTX, DOX does not appear to significantly enhance the number of neu-specific T cells in our model. It is still possible that it acts by enhancing T-cell function. However, alternative mechanisms, including recruitment and activation of professional antigen-presenting cells, and enhancement of innate immune responses also require consideration.

Previous studies have already demonstrated that pretreatment with CTX prior to T-cell adoptive transfer enhances T-cell efficacy (25–26). There are also reports suggesting that CTX can enhance the antitumor immune response of whole-cell vaccination in the clinic (31) and induce a Th1 immune response in tumor models (45). Other studies have suggested that pretreatment with CTX can overcome tolerance (46, 47). Yoshiba *et al.* (46) successfully provoked significant delayed-type hypersensitivity footpad reactions against syngeneic and autologous testicular cells in mice pretreated with CTX. In addition, Polak *et al.* (47) demonstrated that acquired tolerance to 2,4-dinitrochlorobenzene can be reversed by a single treatment with CTX just prior to administration of the allergen. Our results are consistent with these earlier findings and confirm that CTX can break tolerance and augment the antigen-specific antitumor immune response induced by a GM-CSF-secreting whole-cell vaccine in a murine model that exhibits tumor-specific tolerance. However, an earlier study performed by our group failed to demonstrate a synergistic effect between pretreatment with CTX and immunization with a GM-CSF-secreting whole-cell vaccine in the murine CT26 colorectal carcinoma model (29). The discrepancy between the results of the earlier study and this current study may be explained in part by the difference in the tumor models, because tolerance has not been demonstrated in the CT26 tumor system. In fact, the interactions of each chemotherapeutic agent with vaccine were more evident in the *neu* transgenic mice than in the parental FVB/N mice. The differences may also be explained by the timing and dose of CTX tested in the two studies (29).

The exact mechanisms by which CTX enhances antitumor immunity are still undergoing debate. Many studies have reported that CTX may delete or inhibit tumor-induced suppressor or immunoregulatory T cells (48, 49). Others have suggested that CTX may release soluble factors, which may sustain the proliferation, survival, and activity of the transferred immune T cells (26). Recently, Schiavoni and colleagues (50, 51)

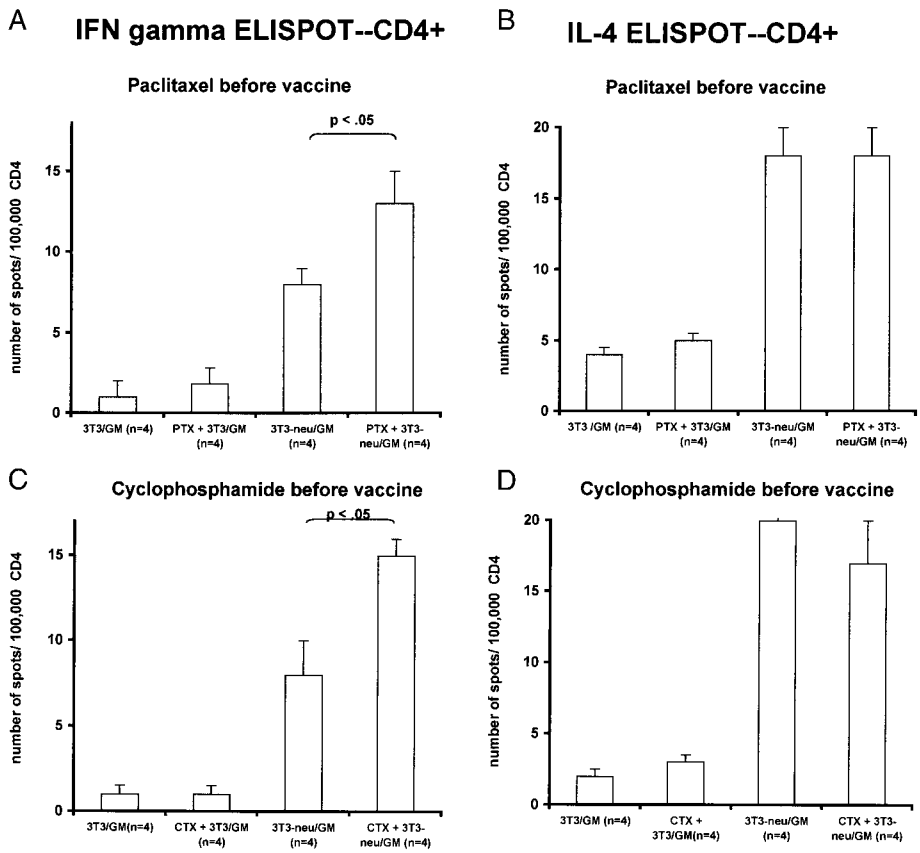

Fig. 6. PTX and CTX appear to enhance the Th1 response of a 3T3-*neu*/GM vaccine in *neu* transgenic mice. To study the Th1 and Th2 balance in *neu* transgenic mice given chemotherapy in sequence with the vaccine, IFN-γ (*A* and *C*) and IL-4 (*B* and *D*), ELISPOT analyses were performed on $CD4^+$ T cells. NT tumor cells (5×10^4) were implanted in the right upper mammary fat pad on day 0, and mice were vaccinated on day 3. Vaccination (3T3-*neu*/GM or 3T3/GM) was given s.c. at three sites (10^6 cells/site), the left and right hind limbs and left upper limb. Mice were sacrificed 12 days after the administration of the vaccine, and the $CD4^+$ T cells were isolated from spleen as described in "Materials and Methods." ELISPOT analysis was performed as described in "Materials and Methods." Four mice per group received either: (*a*) controls-3T3/GM (mock vaccine); (*b*) 3T3-*neu*/GM vaccine alone; (*c*) chemotherapy and 3T3/GM (mock vaccine); or (*d*) chemotherapy and 3T3-*neu*/GM vaccine. Plotted are the mean (three wells/condition) of the number of spots counted in the wells containing the T cells and the stimulators cells minus the number of spots counted in the well containing the T cells alone; *bars*, SD. NT-B7 stimulator cells do not give any background (data not shown). *P* was determined by unpaired Student's *t* test between the vaccine group and the chemotherapy + vaccine group.

demonstrated that CTX induces type 1 IFN secretion *in vivo* and enhances the number of T cells exhibiting the $CD44^{hi}$ memory phenotype.

To our knowledge, this is the first study to evaluate the potential synergy between PTX and an antigen-specific whole-cell vaccine for the ability to induce T-cell responses. As with CTX, we observed that PTX was synergistic with the vaccine only when given prior to vaccination. Multiple immunostimulatory functions have been previously attributed to PTX *in vitro* and *in vivo* (30, 52, 53). PTX can enhance the tumoricidal activity of murine macrophages by inducing nitric oxide production and secretion of TNF-α, IL-1β, and superoxide anions (54). PTX has also been reported to enhance macrophage IL-12 production, a Th1-type cytokine (54). This finding provides one explanation for the observed PTX/vaccine induced increase in a number of neu-specific Th1 cells in our studies. The fact that PTX inhibited the *in vivo* activity of the vaccine when given after vaccination is not surprising because PTX has been shown to impair the proliferation of T cells by stabilization of the microtubules (55). Importantly, the observed abrogation of *in vivo* activity also correlated with a lack of Th1 induction when PTX was given after vaccination.

Among the chemotherapeutic drugs tested, DOX was the only one that enhanced the *in vivo* antitumor response when given after the vaccine. This observed *in vivo* response could not be correlated with an increase in the number of neu-specific T cells. Although an earlier report from our group suggested that DOX could enhance tumor-specific T-cell activity, this finding was only based on an observed increase in CTL activity *in vitro* (29). Others have reported that splenic and tumor-infiltrating mature T cells were completely insensitive to DOX cytotoxicity and showed increased CTL activity when examined *ex vivo* (56). However, CTL activity is not quantitative and has not been rigorously evaluated for its ability to correlate with *in vivo* antitumor activity. Other reports have suggested that DOX can modulate monocyte/macrophage activity in an antigen-independent manner (24). Mihich and colleagues (57, 58) have demonstrated a 2-fold increase in the number of splenic macrophages as early as 5 days after DOX administration. DOX has also been shown to increase macrophage tumoricidal activity (59). Early studies from our group have also defined a non-antigen-dependent role for macrophages induced by the GM-CSF whole-cell vaccine (4, 44). Macrophages have been shown to infiltrate the site of tumor challenge as early as 1 day after immunization (4). These macrophages release nitric oxide and probably collaborate with other immune cells infiltrating the site to cause its destruction (44). Further investigation of the effects of DOX on macrophages when given with the GM-CSF-secreting vaccine is under way.

We successfully combined immune-modulating doses of chemotherapy and an antigen-specific vaccine to treat neu-expressing tumors in *neu* transgenic mice. The doses of chemotherapy that appear to enhance the vaccine are clearly inferior as tumor lytic agents to the conventional cytoreductive doses currently used in the clinic. Further studies are needed to evaluate the impact of conventional chemotherapy doses on the potency of antigen-specific vaccines.

In conclusion, our data support a role for immune-modulating doses of chemotherapy in overcoming immune tolerance when combined with antigen-specific vaccination. These data provide the rationale for testing immune-modulating doses of chemotherapy in sequence with antigen-specific cancer vaccines in patients with cancer.

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Cyclophosphamide, Doxorubicin, and Paclitaxel Enhance the Antitumor Immune Response of Granulocyte/Macrophage-Colony Stimulating Factor-secreting Whole-Cell Vaccines in HER-2/*neu* Tolerized Mice

Jean-Pascal H. Machiels, R. Todd Reilly, Leisha A. Emens, et al.

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