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Advances in Brief

The Collaboration of Both Humoral and Cellular HER-2/neu-targeted Immune Responses Is Required for the Complete Eradication of HER-2/*neu*-expressing Tumors[1]

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Abstract

HER-2/*neu* (*neu*) transgenic mice (*neu-N* mice), which express the nontransforming rat proto-oncogene, demonstrate immunological tolerance to neu that is similar to what is encountered in patients with *neu*-expressing breast cancer. We have shown previously that a significant increase in neu-specific T cells, but no induction of neu-specific antibody, is seen after neu-specific vaccination in *neu-N* mice. In contrast, a significant induction of both neu-specific T-cell and antibody responses is found in nontoleragenic FVB/N mice after vaccination. These mice are fully protected from a s.c. challenge with NT cells, a mammary tumor cell line derived from a spontaneous tumor that arose in a *neu-N* mouse, whereas *neu-N* mice are not. In this study, we demonstrate that $CD4^+$ T cell-depleted FVB/N mice show no induction of neu-specific IgG after vaccination and are unable to reject an NT challenge (0 of 10 mice were tumor free). Conversely, the depletion of natural killer cells has no effect on vaccine-mediated tumor rejection (100% of mice were tumor free). In $CD8^+$ T cell-depleted animals, where vaccine-induced neu-specific IgG titers were normal, NT growth was delayed, but only 10% of mice remained tumor free, demonstrating that neu-specific IgG alone is insufficient for protection from NT challenge. To directly assess the necessity for the combination of neu-specific cellular and humoral immune responses, severe combined immunodeficient mice were given an adoptive transfer of CTLs plus IgG derived from FVB/N mice. Animals that were given CTLs that recognized an irrelevant antigen plus neu-specific IgG developed tumors at a rate similar to $CD8^+$ T cell-depleted FVB/N mice. Animals receiving an adoptive transfer of neu-specific CTLs plus control IgG derived from naive FVB/N mice were only partially protected from NT challenge (50% of animals were tumor free). However, only animals receiving the combination of neu-specific CTLs and neu-specific IgG were fully protected from NT challenge (100% of animals were tumor free). These studies specifically define the immunological requirements for the eradication of neu-expressing tumors in this model system, demonstrating that both cellular and humoral neu-specific responses are necessary for protection from an NT challenge. These data suggest that vaccines optimized to induce maximal T- and B-cell immunity to neu, and possibly to similar putative tumor-rejection antigens, may lead to more potent *in vivo* antitumor immunity.

Introduction

Current vaccine strategies for the treatment of solid tumors tend to focus on the cellular arm of the immune response. However, the success of passive immunotherapy through the administration of monoclonal antibodies that target neu[3] (1), CD19 (2), or the epidermal growth factor receptor (3) has generated renewed interest in the application of humoral immunity in tumor eradication. Trastuzumab, a recombinant humanized monoclonal antibody to neu administered as a single agent or in combination with chemotherapy, produces durable objective responses in women with *neu*-overexpressing breast cancer (4). Similarly, passive immunotherapy with monoclonal antibodies against neu was shown to have a dramatic effect on spontaneous tumor development in transgenic mice expressing rat *neu* (5). We have documented the existence of immunological tolerance to neu in these mice similar to what is observed in patients with *neu*-expressing breast cancers (6). Although *neu-N* mice are capable of generating CTLs against neu after neu-specific vaccination, there is little or no vaccine-mediated induction of neu-specific IgG in mice vaccinated after 8 weeks of age (6). Furthermore, although the growth of *neu*-expressing transplantable tumors in vaccinated *neu-N* mice is significantly delayed relative to control animals, tumor growth is not prevented completely (6). This is in stark contrast to what is seen in the absence of tolerance. In nontransgenic FVB/N mice, a significant induction of both neu-specific CTLs and neu-specific IgG is seen, and mice are completely protected from a transplantable tumor challenge (6). In this study, data are presented demonstrating the induction of neu-specific humoral and cellular immunity in FVB/N mice by active immunization. Furthermore, the combination of neu-specific humoral and cellular immune responses fully protects from a *neu*-expressing tumor challenge, where the absence of either the cellular or humoral arm leads to incomplete protection. These data have important implications for the development of vaccines that induce immunity against antigens that are targets of both B- and T-cell responses.

Materials and Methods

Mice. *neu-N* mice, 8–10 weeks of age, which express the normal rat proto-oncogene (line N#202; Ref. 7), FVB/N mice (National Cancer Institute, Bethesda, MD), and BALB/c SCID mice (National Cancer Institute) were used. All experiments 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. The *neu*-expressing mouse mammary tumor line, NT, was described previously (6). The 3T3wt cell line (American Type Culture Collection, Rockville, MD), composed of NIH-3T3 cells, and the 3T3-*neu* cell line (CRL-1915; American Type Culture Collection), composed of NIH-3T3 cells expressing rat *neu* (8), were maintained as described (6). NIH-3T3 cells transfected with a plasmid encoding HA were maintained under selection with 1 mg/ml geneticin (Life Technologies, Inc., Grand Island, NY). HA expression was verified by fluorescence-activated cell sorter analysis (data not shown).

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[3] The abbreviations used are: neu, HER-2/neu; IL, interleukin; SCID, severe combined immunodeficient; NK, natural killer; mGM-CSF, murine granulocyte/macrophage-colony stimulating factor; HA, hemagglutinin.

The 3T3wt and 3T3-*neu* lines were genetically modified to express mGM-CSF by retroviral transduction as described (6).

T-Cell Lines. The neu-specific $CD8^+$ T-cell line Fneu-CTL was established from an FVB/N mouse after neu-specific plasmid DNA vaccination, followed by NT challenge. This animal was tumor free for >100 days, after which time the animal was sacrificed and splenectomized. Splenocytes were maintained in culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 0.5% L-glutamate, and 1% penicillin/streptomycin (JRH Biosciences, Lenexa, KS), and 0.1% 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) at 37°C and 5% CO_2. Fneu-CTL cells have been in culture for >24 months and are stimulated every 9 days by coculture with mitomycin-treated 3T3-*neu* cells and irradiated syngeneic splenocytes. The Fneu-CTL line is 100% $CD8^+$, expresses a single T-cell receptor β-chain variable region (Vβ4) as determined by flow cytometry, and shows a high degree of specificity for *neu*-expressing cell lines as determined by chromium release assay (data not shown). Fneu-CTL cells were suspended in sterile HBSS (Life Technologies, Inc.) at a concentration of 4×10^7 cells/ml for adoptive transfer. Control $CD8^+$ T cells specific for HA (FHA-CTL) were similarly obtained from FVB/N mice after vaccination with 3×10^7 plaque-forming units of HA recombinant vaccinia virus. The FHA-CTL line has been maintained *in vitro* for ~1 year and is stimulated as described above for Fneu-CTL except with NIH-3T3 cells transfected with a plasmid encoding HA. These cells are specific for the HA protein and do not lyse *neu*-expressing cell lines (data not shown).

Isolation of Neu-specific IgG. Female FVB/N mice, 8 weeks of age, were primed with a s.c. injection of 5×10^6 neu-expressing NT cells. After 14 days, animals were sacrificed, and blood was obtained by cardiac puncture, pooled, and allowed to coagulate. The serum was then pooled, and the total IgG was obtained by ammonium sulfate precipitation and dialyzed against PBS (Life Technologies, Inc.). neu-specific immunoreactivity of the total IgG was verified by flow cytometry as described previously (6). Control IgG was obtained from naive FVB/N mice. Protein concentrations were determined using the Lowry Assay (Sigma) according to the manufacturer's directions. Total IgG samples were adjusted to a final concentration of 10 mg/ml using HBSS prior to injection.

For quantitation of neu-specific serum IgG, serum samples were obtained by tail bleed 1 day prior to tumor challenge (*neu-N* and FVB/N mice) or 1 week after the transfer of neu-specific serum (SCID mice) and neu-specific IgG titers determined as described (6).

Whole-Cell Vaccinations. Vaccinations with 3T3/GM cells, composed of 3T3wt cells producing mGM-CSF, or 3T3-*neu*/GM cells, composed of 3T3-neu cells producing mGM-CSF, were performed as described (6).

Chromium-Release Assays. Animals were vaccinated with 3T3-*neu*/GM or 3T3/GM and CTL prepared as described (6). Lytic function was determined against 3T3wt, 3T3-*neu*, and NT cells in a 4-h ^{51}Cr-release assay. The percentage of neu-specific lysis was determined by the following formula: % neu-specific lysis = (% lysis against 3T3-*neu* targets) − (% lysis against 3T3wt targets).

Depletion Studies. The depletion of $CD4^+$ and $CD8^+$ T-cell subsets and NK cells was accomplished by i.p. injection of 500 μg of GK1.5 (anti-CD4) or 2.43 (anti-CD8) antibody or pk136 (anti-NK) antibody, respectively, as described (6).

Tumor Challenge Experiments. Tumor challenge consisted of 5×10^4 (for *neu-N* mice), 1×10^6 (for SCID mice), or 5×10^6 (for FVB/N mice) NT cells s.c. in the right hind limb 14 days after receiving a whole-cell vaccine (*neu-N* and FVB/N mice) or 1 day prior to adoptive transfer (SCID mice). Animals were monitored twice weekly for the development of palpable (>5 mm in diameter) tumors. Animals were sacrificed before tumors reached a diameter >12 mm.

Adoptive Transfer Experiments. One day after s.c. tumor challenge, female SCID mice, 8 weeks of age, received 2×10^7 T cells via tail vein injection and/or passive infusion of 1 mg of IgG given i.p. T cells were maintained *in vivo* by daily i.p. injections of 20,000 international units of recombinant human IL-2 (Chiron, Emeryville, CA). IgG injections were given weekly throughout the experiment. Animals were monitored as described above.

Statistical Analyses. Statistical analyses were performed using the Statview software program (SAS Institute Inc., Cary, NC). Kaplan-Meier nonparametric regression analyses for tumor challenge experiments were performed, and significance was determined using the Mantel-Cox log-rank test.

Results and Discussion

We previously described the existence of tolerance to neu in *neu-N* mice (6). One measure of neu-specific tolerance in *neu-N* mice is the absence of an inducible neu-specific humoral response above low baseline levels of neu-specific IgG (6). In addition, splenocytes from *neu-N* mice taken 14 days after neu-specific vaccination, although capable of neu-specific lysis of 3T3-*neu* cell targets (6), cannot lyse NT cells in a 4-h ^{51}Cr-release assay (data not shown). The net result of this is incomplete protection from tumor challenge and partial prevention of spontaneous tumor formation in vaccinated animals. In contrast, FVB/N mice, in which tolerance to the rat neu protein does not exist, generate robust neu-specific antibody and CTL responses to vaccination and are fully protected from NT challenge (6). In this model of *neu*-expressing tumor eradication, successful antitumor immunity appears to be associated with the induction of both neu-specific humoral and cellular immunity in nontolerized mice. Therefore, we used the nontoleragenic FVB/N mice to define the immunological requirements for the successful rejection of *neu*-expressing tumors.

To determine the relative importance of the various T-cell subsets in the antitumor response, FVB/N mice were depleted of $CD4^+$ T cells, $CD8^+$ T cells, or NK cells by antibody injection (GK1.5, 2.43, and pk136, respectively). The animals were then vaccinated, challenged 2 weeks later with NT cells, and monitored for tumor development. In addition, serum samples were obtained prior to tumor challenge, and neu-specific IgG titers were determined. These data, summarized in Table 1, demonstrate an absolute requirement for both $CD4^+$ T cells as well as $CD8^+$ T cells in mediating tumor-free survival. The results confirm recently reported data demonstrating a similar $CD4^+$ and $CD8^+$ T-cell dependence for protection from an NT challenge in *neu-N* mice (6). The tumor-free survival of CD4-depleted mice is essentially indistinguishable from that of immunocompetent mice receiving a mock vaccination ($P = 0.53$ *versus* no deplete, 3T3/GM). In the absence of $CD4^+$ T-cell help, these animals would be incapable of generating either a T-cell or B-cell effector response; the lack of neu-specific B-cell effectors is confirmed by the absence of a neu-specific IgG response in these animals. Mice depleted of $CD8^+$ T cells, where $CD4^+$ T-cell help is still in place,

Table 1 *Effects of lymphocyte subset depletion on tumor-free survival in FVB/N mice*

FVB/N mice were depleted of $CD4^+$ T cells, or NK cells as described in "Materials and Methods." Depletion was verified by flow cytometric analysis of splenocytes (data not shown), and depletion was maintained by twice-weekly injections of the depleting antibody. Animals were then vaccinated with 3T3/GM (control vaccine) or 3T3-*neu*/GM (neu-specific vaccine), followed 14 days later by s.c. NT challenge. The mice were monitored for the development of palpable tumors (>5 mm diameter). The average tumor-free survival in days ± SD as well as the number of animals tumor free at the completion of the experiment (day 60 after challenge) are reported for each group. In addition, serum samples were taken from each animal 1 day prior to tumor challenge and analyzed for neu-specific IgG as described in "Materials and Methods." The titer reported is the greatest dilution of serum for which a shift in mean fluorescence intensity of binding to 3T3-*neu* cells is seen relative to an irrelevant control antibody. The values shown are the average titer ± SD from several ($n \geq 4$) from each group. These data are combined from two separate experiments.

Group	Neu-specific IgG (titer ± SD)	Mean tumor-free survival (days ± SD)	No. of tumor-free/ Total animals
No deplete, 3T3/GM	None detected	16 ± 6	0/10
CD4 deplete, 3T3-*neu*/GM	None detected	16 ± 5	0/20
CD8 deplete, 3T3-*neu*/GM	160 ± 21	33 ± 10[a]	2/20
NK deplete, 3T3-*neu*/GM	157 ± 24	60[a]	20/20
No deplete, 3T3-*neu*/GM	163 ± 27	60[a]	20/20

[a] The estimate of the mean tumor-free survival is biased because animals that were tumor free at the conclusion of the experiment were assigned a tumor-free survival of 60 days.

develop neu-specific IgG at titers that are identical to that obtained in undepleted animals receiving a neu-specific vaccination (no deplete, 3T3-*neu*/GM). Additionally, these mice demonstrate a significant delay in the appearance of palpable tumors relative to the mock vaccine controls ($P < 0.001$ *versus* no deplete, 3T3/GM). However, only 10% of the $CD8^+$-depleted animals remained tumor free beyond day 60 after challenge, whereas all undepleted animals receiving a neu-specific vaccination were tumor free.

The fact that FVB/N mice depleted of $CD8^+$ T cells were not fully protected from NT challenge, despite the presence of normal neu-specific IgG titers, suggested that a neu-specific antibody response, although growth inhibitory, was not sufficient for tumor eradication. To more directly assess the necessity for neu-specific antibody in tumor rejection, we sought to reconstitute the neu-specific immune response of FVB/N mice in a SCID model system. SCID mice were given a s.c. tumor challenge on day 0. Animals were then divided into groups receiving either: (*a*) Fneu-CTL cells plus daily IL-2; (*b*) total IgG derived from FVB/N mice primed against rat *neu*; (*c*) Fneu-CTL cells plus neu-specific IgG plus IL-2; or (*d*) total IgG derived from naive FVB/N mice plus FHA-CTL plus IL-2. An *in vitro* analysis of the lytic ability of the Fneu-CTL line in a 4-h ^{51}Cr-release assay demonstrated excellent specificity for neu (Fig. 1*A*). A similar study of the lytic ability of the FHA-CTL line showed excellent specificity for HA-expressing cells with no recognition of neu-expressing targets (Fig. 1*B*). The dose of Fneu-CTL used in these experiments was based on studies in which FVB/N mice received a s.c. NT challenge followed by the adoptive transfer of either 1×10^7, 2×10^7, or 3×10^7 Fneu-CTL. In these experiments, there was no statistical difference in the tumor-free survival of the animals (data not shown). Six days after the first injection of neu-specific IgG, SCID mice had serum antibody titers of ~150 (data not shown), similar to what we have reported in

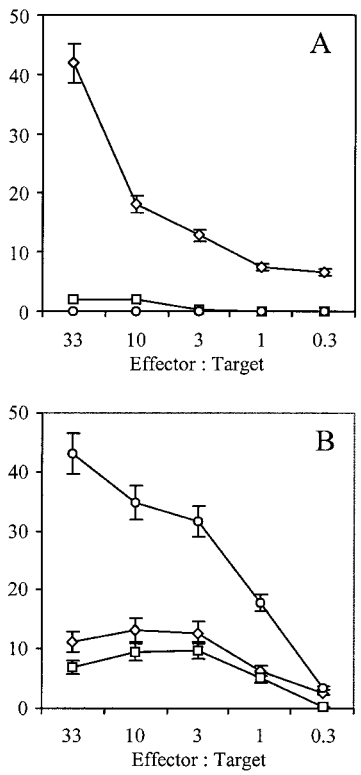

Fig. 1. The Fneu-CTL and FHA-CTL T-cell lines are specific for neu and HA, respectively. Fneu-CTL (*A*) and FHA-CTL (*B*) $CD8^+$ T cells were used in a 4-h ^{51}Cr-release assay at the E:T ratios indicated. The percentage of lysis is indicated for incubation with 3T3-wt (□), 3T3-neu (◇), and 3T3-HA (○; NIH-3T3 cells expressing HA). Data were obtained in triplicate. The mean value is shown.

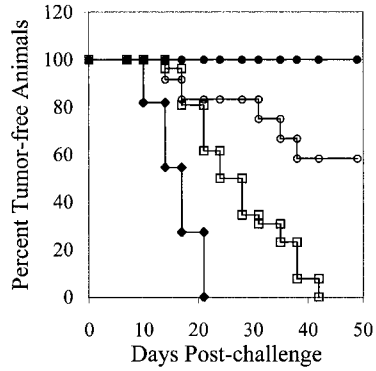

Fig. 2. SCID mice require both neu-specific CTLs and neu-specific IgG for tumor eradication. SCID mice were given an s.c. NT challenge and then 2×10^7 neu-specific $CD8^+$ T cells (○), 1 mg/week IgG from neu-primed mice (□), or both (●). Control animals received HA-specific T cells, 1 mg/week IgG from naive mice, and IL-2 (◆). This experiment was repeated twice ($n \geq 5$ animals/group/experiment) with similar results. The cumulative data are shown.

FVB/N mice after 3T3-*neu*/GM vaccination (Table 1). The tumor-free survival of these mice is shown in Fig. 2. Consistent with our observations in the FVB/N depletion studies, SCID mice given neu-specific IgG alone showed a significant delay in the appearance of palpable tumors ($P < 0.001$ relative to IL-2 alone); however, no animals remained tumor free. This study also confirmed the importance of a potent neu-specific CTL response because 50% of animals given neu-specific $CD8^+$ T cells were tumor free beyond the 50-day end point of the experiment. Additional control groups in which animals received either FHA-CTL cells or total IgG derived from naive FVB/N mice were indistinguishable from animals receiving IL-2 alone (data not shown). Furthermore, animals receiving neu-specific T cells plus IgG from naive mice were indistinguishable from animals receiving only neu-specific T cells (data not shown). Most striking, however, was the finding that the combination of neu-specific IgG and neu-specific CTLs gave significantly better protection from tumor challenge than either treatment alone; all animals in the CTL/IgG combination group were tumor free beyond the end point of the experiment. These data demonstrate that, although neu-specific CTLs play a dominant role in tumor rejection, the greatest level of protection is seen when both neu-specific $CD8^+$ T cells and antibodies are present.

There is a large body of evidence in the literature to support the idea that antibodies directed against neu can inhibit the growth of *neu*-expressing tumors (9–11) through several mechanisms, including antibody-dependent cell cytotoxicity and the inhibition of signal transduction through neu. Thus, it is likely that the neu-specific antibody response in CD8-depleted mice is mediating the growth-inhibitory effects in these studies. The fact that NK-depleted mice are fully protected from tumor challenge suggests that NK-mediated mechanisms of immunity do not play a significant role in the delayed tumor growth seen in the CD8-depleted group. This does not, however, exclude a role for macrophage- or monocyte-mediated antibody-dependent cell cytotoxicity in addition to complement fixation and/or the direct growth inhibition of NT cells by antibody-mediated blockade of signaling through neu. Clynes *et al.* (10) reported recently that the *in vivo* growth-inhibitory activity of trastuzumab, a Food and Drug Administration-approved monoclonal antibody against the human *neu* gene product, is only partially mediated by the direct interaction of the antibody with surface neu on a *neu*-overexpressing target tumor cell line. In addition to the antibody-mediated tumor growth inhibition, the data demonstrated a major role for Fc receptor-dependent mechanisms in tumor eradication (10). Although the data did not exclude a specific role for NK cells in tumor rejection, a dependence on cells expressing

both FcγRIIb and FcγRIII (*i.e.*, monocytes and macrophages) was demonstrated. This is consistent with our observation that NK cells are not required for protection from s.c. tumor challenge in vaccinated FVB/N mice (Table 1).

The Fneu-CTL line used in the adoptive transfer experiments described above was derived from an FVB/N mouse and demonstrates significant lysis of *neu*-expressing targets. However, the neu-specific T-cell repertoire of *neu-N* mice is functionally distinct from that of FVB/N mice in its lytic ability.[4] Similarly, patients with neu-positive tumors would be expected to express a neu-specific T-cell repertoire that reflects tolerance to this "self" protein. It is possible that under these conditions of more limited neu-specific CTL lytic ability, the presence of significant neu-specific IgG titers is even more important. Whether the effect of the antibody and CTL interaction is synergistic or simply additive remains to be determined.

neu-specific CTL and antibody responses have been found in patients with neu-expressing tumors (12–15). In addition, several groups have demonstrated vaccine-induced neu-specific CTL and antibody responses in animal model systems with vaccine-mediated protection from neu-expressing tumor challenge as well as spontaneous tumor formation (16–20). However, there has been no evidence to date correlating protective antitumor immunity with both antigen-specific CTL and antibody responses. Here we show that the induction of neu-specific CTL and IgG responses in nontolerized mice after rat neu-specific vaccination is potent enough to fully protect these animals from challenge with a rat *neu*-expressing tumor line. Significantly, we have also demonstrated that optimal antitumor immunity is achieved only when both neu-specific CTLs and neu-specific IgG are present. Together, these studies suggest that vaccines optimized to induce maximal T-cell and B-cell immunity to neu, and possibly to similar putative tumor rejection antigens, may lead to more potent *in vivo* antitumor immunity.

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