Anti–HER-2/neu Immune Responses Are Induced before the Development of Clinical Tumors but Declined following Tumorigenesis in HER-2/neu Transgenic Mice

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ABSTRACT

HER-2/neu oncogene products have been implicated as a potential target of T cell–mediated immune responses to HER-2/neu–induced tumors. Using HER-2/neu transgenic mice (oncomice), we investigated whether, and if so how, anti–HER-2/neu immune responses are induced and modulated in these oncomice from birth to tumor initiation. Female oncomice carrying the activated HER-2/neu oncogene displayed apparent hyperplasia in mammary glands at 10 weeks of age and developed mammary carcinomas around an average age of 26 weeks. Unfractionated spleen cells from 10- to 15-week-old oncomice that were cultured without any exogenous stimuli exhibited cytotoxicity against the F31 tumor cell line established from an HER-2/neu–induced mammary carcinoma mass. The final antitumor effectors were a macrophage lineage of cells. However, this effector population was activated, depending on the stimulation of oncomice CD4+ T cells with oncomouse-derived antigen-presenting cell (APC) alone or with wild-type mouse APC in the presence of F31 membrane fractions, suggesting the presence of HER-2/neu oncomice. These antitumor cytotoxic responses were detected at ~5 weeks of age and peaked at age 10 to 15 weeks. However, the responses then declined at tumor-bearing stages in which the expression of target proteins could progressively increase. This resulted from the dysfunction of CD4+ T cells but not of APC or effector macrophages. These results indicate that an anti–HER-2/neu CD4+ T cell–mediated immune response was generated at the pretumorigenic stage but did not prevent tumorigenesis and declined after the development of clinical tumors.

INTRODUCTION

The HER-2/neu proto-oncogene encodes a receptor-like transmembrane protein with a molecular weight of Mw 185,000 (1). Although the function of HER-2/neu protein is not well defined, overexpression of this protein has been identified in a variety of human cancers, including breast adenocarcinomas (2). HER-2/neu protein is expressed, although in small amounts, in a limited number of fetal and normal adult tissues (3). However, it appears that humans are not rendered tolerant to HER-2/neu protein because in vitro immunization of T cells from normal individuals with peptides derived from HER-2/neu protein has been shown to elicit peptide-specific CTL responses (4).

More direct evidence that tolerance to HER-2/neu protein is not established has been provided as follows: some patients with HER-2/neu–positive breast cancers have been shown to exhibit CD4+ T cell–mediated responses to HER-2/neu protein (5, 6). CD8+ CTLs that recognize HER-2/neu–derived peptides also were found in patients with breast and ovarian cancers overexpressing HER-2/neu protein (7, 8).

Thus, in individuals with an HER-2/neu–overexpressing tumor, tolerance to this oncogenic protein is somewhat circumvented; therefore, HER-2/neu protein is likely to function as the target antigen for antitumor T-cell immunity. Moreover, recent studies (9, 10) showed that HER-2/neu overexpression in animal models is not restricted to HER-2/neu–induced malignant cells such as mammary carcinoma cells. The overexpression of HER-2/neu protein also has been shown in untransformed epithelial cells adjacent mammary carcinoma cells (10) and in mammary epithelial cells exhibiting hyperplasia before tumorigenesis (9). Considering the possibility that overproduction of HER-2/neu protein occurs before malignant transformation of cells expressing the HER-2/neu oncogene, an issue that remains to be resolved is whether an anti–HER-2/neu immune response is generated at the stage of HER-2/neu overexpression before tumorigenesis and, if so, how such a response proceeds along with tumor formation.

The aforementioned issue may be addressed not in human systems but in animal models using HER-2/neu transgenic mice. Female transgenic mice exhibit a mammary tumor virus-driven overexpression of the transgene in the mammary glands (9–12). Morphologically, atypical hyperplasia was observed in all of the mammary glands at as early as 3 weeks of age in female transgenic mice (13). This hyperplasia progresses for long periods, leading to tumorigenesis at >20 weeks of age (13). Using such an HER-2/neu transgenic line, the present study showed the following: Unfractionated splenocytes from 10- to 15-week-old HER-2/neu transgenic mice generated cytotoxic responses against a tumor cell line established from HER-2/neu–induced mammary carcinoma when they were cultured in vitro without exogenous stimuli. The induction of cytotoxic responses depended on collaboration between HER-2/neu–primed CD4+ T cells and antigen-presenting cell (APC)–processing HER-2/neu protein. However, the direct cytotoxicity was mediated by Mac-1+ cells among splenocytes of transgenic mice and activated with the interaction of CD4+ T cells and APC. The responses were detected in transgenic mice at as early as 5 weeks of age, peaked at age 10 to 15 weeks, but without contributing to the prevention of tumorigenesis, declined at tumor-bearing stages. These results provide important implications for the dynamism of anti–HER-2/neu tumor immune responses in an HER-2/neu–induced tumor model.

MATERIALS AND METHODS

Mice. Male and female transgenic mice (the H-2b FVB/n background) carrying an activated (mutated) rat HER-2/neu oncogene driven by a mouse mammary tumor virus promoter (designated HER-2/neu oncomice or FVB/n–mut neu) were purchased from Charles River Laboratories (Cambridge, MA; ref. 14), bred in our laboratory, and used at >5 weeks of age. Control FVB/n and BALB/c mice were obtained from Clea Animal Supply Center (Kanagawa, Japan) and Shizuoka Experimental Animal Laboratory (Hamamatsu, Japan), respectively. [HER-2/neu oncomouse (FVB/n–mut neu) × BALB/c]F1 and [FVB/n × BALB/c]F1 mice were produced in our laboratory.

Tumor Cell Lines. A mammary carcinoma cell line was established from an HER-2/neu–induced tumor mass and designated F31. CSA1M fibrosarcoma, Meth A fibrosarcoma, and LSTRA leukemia, all of a BALB/c origin, were used.

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All of the tumor cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO2.

Reagents. Anti–HER-2/neu monoclonal antibodies (clone 3B5 and clone 7.16.4) were purchased from Oncogene Research Products (Boston, MA). Anti-CD4 (clone GK1.5; American Type Culture Collection, Manassas, VA) and anti-CD8 (clone 2.43; American Type Culture Collection) monoclonal antibodies were purified from ascitic fluids of the relevant hybridoma cells.

Immunization and Tumor Challenge. F31 or Meth A tumor cells suspended in RPMI 1640 culture medium were treated in vitro with 100 μg/mL mitomycin C (MMC) for 60 minutes. [FVB/n × FVB/n] F1 and FVB/n mice were immunized intraperitoneally with these MMC-treated tumor cells three times as described previously. One day before each immunization, these mice were challenged subcutaneously with 106 viable F31 or Meth A tumor cells. Tumor cells for immunization and challenge were suspended in RPMI 1640 medium.

Depletion In Vitro of CD4+ or CD8+ T Cells. FVB/n mice were immunized with F31 tumor cells three times as described previously. One day before each immunization, the mice were intraperitoneally given 200 μg/mouse of anti-CD4 (GK1.5) or anti-CD8 (2.43) monoclonal antibody in PBS (total, three times/mouse). The depletion of CD4+ and CD8+ T cells in mice 7 days after the last injection of respective monoclonal antibodies was >98% when evaluated by flow cytometric analysis of their splenocytes as described previously (15).

Preparation of Various Lymphoid Cell Populations. Spleen cells from onconose and normal mice without active immunization were fractionated in vitro in the following protocols. CD4+ or CD8+ T-cell–depleted populations: Spleen cells were incubated with rat antimouse CD4 or rat antimouse CD8 monoclonal antibody, followed by BioMag goat antirat immunoglobulin-conjugating magnetic particles (Qiagen, Valencia, CA). CD4+ or CD8+ T-cell–depleted populations were obtained by removing cell-bound magnetic particles. Splenic T-cell and APC populations: Spleen cells were depleted of B cells by immunomagnetic negative selection. Briefly, spleen cells were labeled with magnetic microbeads conjugated to antimouse B220 (Miltenyi Biotec, Sunnyvale, CA). Labeled cells were separated from unlabeled cells by magnetic cell sorting using a MiniMACS (Miltenyi Biotec) according to the procedure described previously (16). The magnetic cells (B220+ cells; i.e., B cells) were retained in a MiniMACS column attached to a MiniMACS magnet while the nonmagnetic cells passed through the column. The latter cells were again labeled with magnetic microbeads conjugated to a mixture of antimonutous CD4 plus antimonutous CD8 monoclonal antibodies (Miltenyi Biotec) and then applied to magnetic cell sorting. The cells that were retained in a MiniMACS column and passed through the column were used as T-cell and APC populations, respectively. Cultured T-cell and Mac-1+ cell fractions: Cultured splenocytes were labeled with magnetic microbeads conjugated to a mixture of antimonutous CD4 plus antimonutous CD8 monoclonal antibodies or antimonutous Mac-1 monoclonal antibody (Miltenyi Biotec). The magnetic cells were used as cultured T-cell or Mac-1+ cell populations. The cultured cell fraction depleted of T cells, B cells, and natural killer (NK) cells: Cultured cells were labeled with microbeads conjugated to antimonutous CD4, antimonutous CD8, antimonutous B220, and antimonutous DX5 (Miltenyi Biotec). Labeled cells were similarly separated from unlabeled cells and used as the [T, B, and NK] cell and other cell fractions, respectively.

Splenocyte Cultures. All of the in vitro experiments were performed by using lymphoid cells of untreated mice without vaccination. Unfractionated splenocytes (5 × 107/well), splenocytes depleted of a particular cell population, or a mixture of particular cell-enriched populations were cultured without addition of exogenous tumor antigens in 24-well culture plates (Corning 25820; Corning Glass Works, Corning, NY) in 2 mL of RPMI 1640 medium supplemented with 10% FCS and 2-mercaptoethanol. After incubation in a humidified incubator (5% CO2) for 1 to 5 days, cells and culture supernatants were harvested and submitted to cytotoxicity assays and assays for the determination of cytokine concentrations, respectively.

Cytotoxicity Assays (DNA Fragmentation Assays). Antitumor cytotoxic activity was measured by using a DNA fragmentation assay as described previously (17). Briefly, target tumor cells (2 × 105) were labeled with 200 kBq [3H]thymidine by incubating overnight in a culture dish containing 10 mL of culture medium. Labeled cells (1 × 105) were cultured with different numbers of effector cells for 24 hours (unless otherwise indicated) in 96-well flat-bottomed microwell plates. The plates were harvested on a microtiter plate cell harvester, and the radioactivity trapped on the filters was measured with a liquid scintillation counter. Cytotoxic activity was measured with triplicate wells per group, calculated as follows, and expressed as the mean of % cytotoxicity: % cytotoxicity = [(cpm in effector-free wells – cpm in effector-positive wells)/cpm in effector-free wells] × 100. The SE was excluded from the figures because values were consistently <5%.

Measurement of IFN-γ Concentrations. IFN-γ concentrations in culture supernatants were measured by mouse IFN-γ ELISA as described previously (18).

Fig. 1. Progression of mammary carcinogenesis through hyperplasia in untreated female FVB/n mice and establishment of a mammary carcinoma line F31. A, kinetics of tumor occurrence in 83 female FVB/n mice. R, histology of mammary tissue from 10- and 20-week-old FVB/n mice and a 10-week-old normal FVB/n mouse; H&E staining, ×400. C, phase-contrast microscopic image of F31 tumor cells. D, lysates from Meth A, CSA1M, and F31 tumor cells were subjected to immunoprecipitation, followed by immunoblot analysis with anti–HER-2/neu monoclonal antibody.
Preparation of Plasma Membrane Fraction of Tumor Cells. Cells were suspended in hypotonic buffer [25 mMol/L HEPES-buffered saline (pH 7.4), 10 mMol/L KCl, and 1 mMol/L EDTA] for 10 minutes at 4°C and homogenized using a Dounce homogenizer (20 strokes). Intact cells, nuclei, and other debris were pelleted by centrifugation at 270 × g for 5 minutes. The particulate (membrane) fraction was separated from the soluble fraction by centrifugation at 100,000 × g for 30 minutes.

Solubilization of Tumor Cell Membrane Fractions, Immunoprecipitation, and Immunoblot Analysis. F31 membrane fractions were solubilized with ice-cold lysis buffer (1% digitonin, 20 mMol/L Tris-HCl, 150 mMol/L NaCl, 2 mMol/L EDTA, 5 mMol/L lodoacetamide, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, and 1 mMol/L phenylmethylsulfonyl fluoride). After preclearing with normal rat IgG prebound to protein G-Sepharose (Amersham Biosciences, Piscataway, NJ), the lysate was immunoprecipitated with mouse anti–HER-2/neu monoclonal antibody (clone 7.16.4) prebound to protein G-Sepharose. To elute proteins, they were boiled in SDS-containing sample buffer and resolved on 6% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). For immunoblot analysis, membranes were blocked in PBS containing 5% nonfat dry milk, and 0.1% Tween-20 and sequentially incubated with mouse anti–HER-2/neu monoclonal antibody (clone 3B5) and horseradish peroxidase-conjugated antimmunoglobulin IgG F(ab')2 (Amersham Biosciences). Detection was performed using enhanced chemoluminescence (Amersham Biosciences).

RESULTS

HER-2/neu–Transgenic Mice (FVB/n-neu) and F31 HER-2/neu–Induced Mammary Carcinoma Cells. Fig. 1A shows the kinetics of mammary tumor occurrence in 83 female FVB/n-neu oncomice. Clinical tumors were detected after 20 weeks of age and developed in all of these oncomice by 32 weeks of age. Microscopic examination of mammary tissue revealed hyperplasia of mammary glands in 10-week-old FVB/n-neu but not in control (WT) FVB/n mice (Fig. 1B, top). Even though 20-week-old FVB/n-neu mice were free of clinical tumors, a tumor nodule was microscopically observed in mammary glands (Fig. 1B, bottom).

A cloned line of HER-2/neu–induced mammary carcinoma cells was established from a tumor mass in an FVB/n-neu oncomouse and designated F31. A representative image of F31 cells is shown in Fig. 1C. These tumor cells and HER-2/neu–unrelated control tumor cells (Meth A and CSA1M fibrosarcomas) were lysed, and lysates were examined for the expression of HER-2/neu protein by immunoprecipitation followed by immunoblot analysis with anti–HER-2 monoclonal antibody. Fig. 1D shows that F31 cells, but not other control tumor cells, express HER-2/neu protein with an approximate molecular weight of M, 185,000.

Induction of F31-Specific Immunity in HER-2/neu Transgenic Mice (FVB/n-neu) by Immunization with F31 Tumor Cells. We first investigated whether immunization of FVB/n-neu mice with HER-2/neu–induced tumor cells (F31) results in F31-specific protective immunity. [FVB/n-neu × BALB/c]F1 mice were immunized intraperitoneally with MMC-treated F31 (FVB/n background) or Meth A (BALB/c background) tumor cells three times. One week after the final immunization, the mice were challenged subcutaneously with either type of viable tumor cells (Fig. 2A and B). The results show that immunization with F31 or Meth A tumor cells induced protective immunity against the corresponding tumor. Anti-F31 immunity was similarly induced in the parental oncomouse strain FVB/n-neu (data not shown) and the wild-type (WT) strain FVB/n (Fig. 2C). In the latter, the depletion of CD4+ T cells by injecting anti-CD4 monoclonal antibody during the immunization completely inhibited the induction of anti-F31 immunity, whereas the induction was not affected by CD8+ T-cell depletion. Thus, these results suggest that FVB/n-neu mice are not rendered tolerant to HER-2/neu and
production and cytotoxic responses by oncomouse splenocytes depend on T cell–APC interactions. Purified T cells from unvaccinated 10-week-old oncomice were cultured with various populations of APC prepared from unvaccinated 10- to 15-week-old oncomice or control mice. Fig. 4A showed that IFN-γ production is induced when FVB/n c-neu oncomouse T cells are cocultured for 3 days with APC from FVB/n c-neu oncomice but not from FVB/n non-oncomice. Along with IFN-γ production, anti-F31 cytotoxicity was generated by the coculture of [FVB/n c-neu × BALB/c] F1 oncomouse T cells with APC from FVB/n c-neu or [FVB/n c-neu × BALB/c] F1 oncomice but not with APC from BALB/c or [FVB/n × BALB/c] F1 non-oncomice (Fig. 4B). The coculture of non-oncomouse F1 T cells and oncomouse APC failed to induce IFN-γ production and cytotoxic responses (data not shown). Moreover, the cells obtained by 5-day cultures of CD4⁺ T cell-depleted splenocytes from unvaccinated FVB/n c-neu mice no longer exhibited cytotoxicity against F31 targets (Fig. 3C, right). The IFN-γ production also was abrogated when CD4⁺ T cells were depleted from FVB/n c-neu splenocytes before culture (Fig. 3C, left). In contrast, the induction of cytokine production and cytotoxic effector cells was not changed and rather enhanced, respectively, by CD8⁺ T-cell depletion (Fig. 3C). These results indicate the CD4⁺ T cell-mediated nature of the responses.

**Stimulation of Oncomouse CD4⁺ T Cells with WT APC Pulsed with F31 Membrane Fractions.** We examined whether unvaccinated oncomouse CD4⁺ T cells are stimulated by non-oncomouse (WT) APC in the presence of F31-derived molecules. [FVB/n × BALB/c] F1 (WT)-derived APC and [FVB/n c-neu × BALB/c] F1 oncomouse T cells were cultured in the presence of F31 or Meth A membrane fractions. The results of Fig. 5 show that cytotoxic responses (A) and IFN-γ production (B) were generated only when oncomouse F1 T cells were cultured with WT APC and F31 membranes and that the levels of response are comparable with or slightly higher than those generated by coculture with oncomouse APC in the absence of F31 membranes. Together with the results of Figs. 3 through 5, these findings suggest that the generation of cytotoxic and IFN-γ-producing responses from unvaccinated oncomouse splenocytes depends on collaboration between HER-2/neu–primed CD4⁺ T cells and APC-presenting HER-2/neu protein.

**Nature of Anti-F31 Cytotoxic Effector Cells Generated from FVB/n c-neu Splenocyte Cultures.** The results of Fig. 3C suggest that anti-F31 effector cells generated from FVB/n c-neu splenocyte cultures are not ordinary CD8⁺ CTL. To exclude the T-cell nature of cytotoxic effector cells in this model, effector cells obtained 5 days after the culture of unvaccinated FVB/n c-neu splenocytes were separated into various cell fractions as described in Materials and Methods to assess

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**Fig. 3.** CD4⁺ T cell–mediated or –induced IFN-γ production and anti-F31 cytotoxic responses in cultures of unfractionated splenocytes from unvaccinated 10-week-old FVB/n c-neu mice. Unfractionated (A and B) or CD4⁺ or CD8⁺ T cell–depleted splenocytes (C) from unvaccinated 10-week-old FVB/n c-neu mice were cultured for 1 to 5 days without exogenous stimuli. Culture supernatants and cultured splenocytes were examined for IFN-γ production and cytotoxic responses.

**Fig. 4.** IFN-γ production and generation of cytotoxicity occur via interactions between oncomouse T cells and APC from oncomice but not from WT mice. A. Purified T cells from 10-week-old unvaccinated FVB/n c-neu or WT FVB/n mice were cultured for 3 days with APC from 10- to 15-week-old unvaccinated FVB/n (WT) or FVB/n c-neu oncomice. Culture supernatants were examined for IFN-γ production. B. Purified [FVB/n c-neu × BALB/c] F1 T cells were cultured with APC from the mice indicated. Effector cells were examined for cytotoxic responses against F31 tumor cells.

**Fig. 5.** Stimulation of [FVB/n c-neu × BALB/c] F1 T cells by WT APC together with F31, but not with Meth A membrane fractions. Purified T cells from 10-week-old [FVB/n c-neu × BALB/c] F1 oncomice were cultured for 5 days with APC from 10-week-old [FVB/n × BALB/c] (WT) F1 mice in the presence of F31 or Meth A membrane fractions or with FVB/n c-neu oncomouse APC alone. Effector cells and culture supernatants were assayed for anti-F31 cytotoxicity (A) or IFN-γ production (B).
the cytotoxicity of each fraction against F31 target cells. As shown in Fig. 6A, the cell fraction containing T, B, and NK cells failed to exhibit cytotoxicity, whereas the [T, B, and NK]−-depleted cell fraction displayed much greater cytotoxicity than unfractionated effector cells. A Mac-1+ cell–enriched fraction also exhibited enhanced cytotoxicity, but the T cell–enriched fraction did not kill target cells (Fig. 6B), suggesting that Mac-1+ cells such as macrophages are the major effector cells. This was supported by additional experiments: The expression of cytotoxic activity by unfractionated effector cells required relatively longer periods of coculture with target cells (Fig. 6C), which contrasts with the notion that CD8+ CTLs can display high levels of cytotoxicity during short-term (mostly 4 to 6 hours) cocultures. Moreover, unfractionated effector cells exhibited cytotoxic activity toward various (but not all of the) types of tumor target cells, including cells expressing totally different MHC molecules (Fig. 6D). Collectively, these observations strongly suggest that the final effectors are not T cells but rather cells of the macrophage lineage.

**Inducibility of Antitumor Cytotoxicity and IFN-γ Production by Splenocytes from Various Ages of FVB/n-neu Mice.** In the preceding experiments, antitumor cytotoxic responses and IFN-γ production were induced in splenocytes from 10- to 15-week-old FVB/n-neu oncomice. Finally, we examined at which postnatal stages oncomice exhibit these immune responses and how the responses change toward the tumor-bearing stages. As summarized in Fig. 7, both responses induced by the culture of unvaccinated FVB/n-neu splenocytes were detected at age 5 weeks, peaked at ∼10 to 15 weeks of age, and declined at the tumor-bearing stage.

To investigate whether the decline of antitumor immune responses at tumor-bearing stages is ascribed to a dysfunction of the responding CD4+ T cells or stimulating APC, cocultures of T cells and APC from FVB/n-neu oncomice at various ages were conducted in combination. The results show that the antitumor cytotoxicity generated by the culture of T cells from 10-week-old FVB/n-neu oncomice increases depending on the age of the FVB/n-neu oncomice from which the APCs are derived (Fig. 7C, right). In contrast, the T cells from 25-week-old FVB/n-neu mice exhibited a reduced responsiveness irrespective of the age of the mice from which the APC were derived (data not shown). The age-dependent reduction in IFN-γ production also was ascribed to the dysfunction of T cells in aged FVB/n-neu mice expressing HER-2-neu protein in mammary glands before mammary carcinomas develop, but the responses decline with tumor development as a result of dysfunction at the T cell but not APC level.

**DISCUSSION**

Recent studies have shown that HER-2/neu oncoprotein is overexpressed in a variety of human malignancies including breast and ovarian cancers (2, 21) and that CD4+ T cell–mediated immunity is detectable in some patients with HER-2/neu–overexpressing tumors (5, 6). However, such a CD4+ T-cell response also was observed in healthy individuals (6). Together with the fact that HER-2/neu protein is essentially a self-protein, these observations raise the question of whether anti HER-2/neu reactivity represents antitumor protective immunity or whether HER-2/neu protein can actually induce effective T-cell responses against HER-2/neu–overexpressing tumors. This issue could be addressed in an HER-2/neu transgenic mouse model because females of this line exhibit overexpression of the transgene in the mammary gland for long periods and ultimately develop mammary carcinomas (9–12).

The present results obtained using unvaccinated HER-2/neu transgenic mouse line show that at the pretumorigenic stage, APC process and present HER-2/neu protein to stimulate anti–HER-2/neu CD4+ T cells, and consequently anti–HER-2/neu CD4+ T cells, are primed in vivo. Therefore, when unfractionated splenocytes including CD4+ T cells and APCs are cultured even without exogenous HER-2/neu protein, the activation of anti–HER-2/neu CD4+ T cells occurs and then leads to cytokine production and cytotoxic responses. Regarding the latter, Mac-1+ cells activated via interactions with these anti–HER-2/neu CD4+ T cells were found to exhibit cytotoxicity against HER-2/neu–induced mammary carcinoma cells (F31). Such a CD4+ T-cell immunity was detectable at 5 weeks of age and peaked at ∼10 to 15 weeks of age. CD4+ T-cell responses failed to prevent tumorigenesis and actually declined after tumorigenesis. CD8+ T-cell responses were not detected in HER-2/neu transgenic mice throughout their life. These observations indicate that HER-2/neu expression can induce detectable CD4+ T-cell responses. However, these responses are not only insufficient to prevent tumorigenesis but also are reduced after the formation of clinical tumors.

The present observations should be discussed from the following several aspects. First, the results showed that CD4+ T cells of these transgenic mice are primed to HER-2/neu protein at pretumorigenic stages during which epithelial hyperplasia (9, 13) and HER-2/neu overexpression (9, 10) are seen in mammary glands. The priming of
CD4⁺ T cells to HER-2/neu was shown by their capacity to respond to freshly isolated APC from HER-2/neu transgenic mice, but not from control WT mice in the absence of exogenous HER-2/neu tumor membrane fractions, or to respond to WT APC in the presence of such membrane fractions. The induction of positive immune responses in HER-2/neu transgenic mice is consistent with previous studies (13, 22). It also is similar to the observations that splenic APCs harvested from tumor-bearing mice have already processed tumor antigens (23) and coexist in splenocytes with CD4⁺ T cells that are primed in vivo to tumor antigens through interactions with such APCs (19, 20). Despite the sensitization in vivo of anti-HER-2/neu CD4⁺ T cells, transgenic mice still developed tumors. They also failed to reject an inoculum of 10⁶ HER-2/neu⁺ tumor cells (F31).

In relation to the aforementioned findings, anti-HER-2/neu CD4⁺ T-cell responses have been detected in some but not all of the mammary carcinoma patients (5). However, the levels of these responses are rather low, comparable with those observed in normal individuals (6). However, the biological significance of anti-HER-2/neu responses cannot be totally excluded: The finding that mammary carcinomas are induced in HER-2/neu transgenic mice, but not HER-2/neu- or neu⁻/⁻ mice. cultured with APC from 5-, 10-, or 25-week-old oncomice (C and D). Cultured splenocytes and culture supernatants were assayed for anti-F31 cytotoxicity (A and C) or IFN-γ production (B and D); TB, tumor-bearing.

Two questions that are mutually related then may be raised. One is why anti-HER-2/neu CD4⁺ T-cell responses fail to prevent tumorigenesis and the other is why once activated, T-cell responses decline at tumor-bearing stages. The former may be explained, in part, by considering the failure of HER-2/neu expression to induce the activation of anti-HER-2/neu CD8⁺ effector T cells. CD8⁺ CTLs generally are the major antitumor effector population. Although anti-HER-2/neu CD8⁺ T cells were present in a tumor-infiltrating lymphocyte population, the detection of these CTLs required repeated in vitro stimulation of the population with autologous tumor cells (8). Therefore, the lack of CD8⁺ CTL responses in HER-2/neu-expressing individuals may be caused by the low frequencies of anti-HER-
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2neu CD8+ CTL precursors. Although anti-HER-2/neu CD4+ T cells function to activate Mac-1+ cytotoxic cells instead of CD8+ CTL, anti-HER-2/neu immunity without involvement of CD8+ CTL could be insufficient to prevent tumorigenesis. How and why the frequency of anti-HER-2/neu CD8+ T-cell precursors is reduced compared with that of CD8+ T-cell precursors could be a biologically crucial aspect of an HER-2/neu tumor model.

Beside T-cell function, it is shown that anti-HER-2/neu antibodies (especially IFN-γ-dependent IgG subclass) produced by vaccination and tumor challenge play a crucial role in protective immunity in transgenic mice bearing HER-2/neu oncogene (26–30). In contrast, nontransgenic normal mice vaccinated previously are shown to exhibit protective immunity against HER-2/neu tumor challenge in the absence of specific antibody (31, 32). Thus, in the transgenic mouse model, eradication of HER-2/neu tumor cells may require anti-HER-2/neu antibody in addition to T cells and macrophages. Importantly, it has been shown that FVB/n transgenic mice with an activated HER-2/neu oncogene, the same strain as our FVB/n c-neu, give rise to a significant specific antibody after vaccination and tumor challenge, whereas they fail to produce anti-HER-2/neu antibody before vaccination (33). Therefore, an inadequate amount of anti-HER-2/neu antibody in unvaccinated FVB/n-neu mice may explain why the transgenic mice spontaneously develop tumors.

If the host’s immune system fails to prevent tumorigenesis, an established clinical tumor then could have deleterious effects on various lymphoid populations including T cells and dendritic cells. It has been documented that tumor secretes a number of immunosuppressive factors such as transforming growth factor β (34, 35) and vascular endothelial growth factor (36). These factors exert their immunoregulatory effects on antitumor T-cell responses by acting directly on T cells (37) or indirectly on dendritic cells (36, 38). For example, vascular endothelial growth factor has been reported to affect the maturation of dendritic cells (36, 38). Because dendritic cells are the APC most responsible for the activation of CD8+ CTL via a cross-presentation pathway (39–42), their dysfunction could cause the activation of T cells, particularly of CD8+ CTL precursors, to be down-regulated.

The present results illustrate that HER-2/neu oncoprotein functions as a target tumor antigen for CD4+ T cells capable of inducing antitumor cytotoxic responses in collaboration with Mac-1+ cells. These CD4+ T-cell responses are induced at pretransmigrogenic stages in individuals with mammary glands expressing HER-2/neu protein but decline once a clinical tumor is established. Moreover, HER-2/neu protein fails to efficiently induce CD8+ CTL activation under conditions in which CD4+ T cells are activated. These T-cell dysfunctions could account, in part, for why tumor cells expressing immunogenic oncoprotein continue to grow without being rejected. Thus, an effort to activate an anti-HER-2/neu immune response using cancer vaccine strategies should consider the possibility that hosts fail to induce tumor rejection responses despite the presence of a manifest target tumor antigen. Rather, elucidating the mechanism underlying this failure and attempting to correct it may be essential and a prerequisite to tumor immunotherapy in HER-2/neu–induced tumor models.

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