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Prevention of Spontaneous Breast Carcinoma by Prophylactic Vaccination with Dendritic/Tumor Fusion Cells[1]

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Genetically modified mice with spontaneous development of mammary carcinoma provide a powerful tool to study the efficacy of tumor vaccines, since they mimic breast cancer development in humans. We used a transgenic murine model expressing polyomavirus middle T oncogene and mucin 1 tumor-associated Ag to determine the preventive effect of a dendritic/tumor fusion cell vaccine. The MMT (a transgenic murine model) mice developed mammary carcinoma between the ages of 65–108 days with 100% penetrance. No spontaneous CTL were detected. However, prophylactic vaccination of MMT mice with dendritic/tumor fusion cells induced polyclonal CTL activity against spontaneous mammary carcinoma cells and rendered 57–61% of the mice free of the disease at the end of experiment (180 days). Furthermore, the level of CTL activity was maintained with multiple vaccinations. The antitumor immunity induced by vaccination with dendritic/tumor fusion cells reacted differently to injected tumor cells and autochthonous tumor. Whereas the injected tumor cells were rejected, the autochthonous tumor evaded the attack and was allowed to grow. Collectively these results indicate that prophylactic vaccination with dendritic/tumor fusion cells confers sufficient antitumor immunity to counter the tumorigenesis of potent oncogenic products. The findings in the present study are highly relevant to cancers in humans. ***The Journal of Immunology*****, 2003, 170: 1980–1986.**

Genetic predisposition plays a major role in breast cancer development. The identification of oncogenes and tumor suppressor genes associated with cancer development provides an opportunity for immunologic manipulation to target these gene products, so that the onset of cancer development will be inhibited. Ideally, these studies should be conducted in animal models that mimic human cancer development. Although the transplantable tumor models have been the primary screening tools for cancer vaccine development, they do not fit this criterion, since the tumor in these models grows very quickly without the multiple stages of cancer development found in human cancers.

The advent of genetically engineered mice with a targeted gene mutation that mimics the gene alteration in human cancers provides a powerful tool to study the efficacy of vaccines. One of the transgenic murine models (MMT) developed by us (P. Mukherjee and S. Gendler, unpublished observation) expresses the polyomavirus middle T (PyMT)[3] oncogene under the transcriptional control of the mouse mammary tumor virus promoter long terminal repeats (1) and the human mucin 1 (MUC1) in a tissue-specific fashion (2). Although PyMT Ag is not associated with carcinogenesis in humans, it binds signal transduction proteins such as the c-Src family (3–5), phosphatidylinositol 3′-kinase (6), Ras (7, 8), and c-Myc (9–12). These proteins are altered in human cancers. The association with and activation of the tyrosine kinase activity of these signal transduction proteins by PyMT Ag promote cell growth and/or survival and result in widespread transformation of the mammary epithelia and rapid production of multifocal mammary adenocarcinomas in 100% of female mice (1, 13). The majority of the mice develop metastases in the lungs (1).

MUC1 is a high m.w. glycoprotein that is overexpressed in human breast cancers (14, 15). Aberrant glycosylation of the MUC1 core in breast carcinoma cells results in the generation of distinct epitopes not found in normal tissues (16, 17). Studies have demonstrated that these cryptic epitopes are recognized by CTL in patients with breast carcinoma (18, 19) and in animal models (20–23). Taken together, these findings suggest that the MUC1 Ag may represent an appropriate target for immunotherapy of breast carcinomas.

In the present study we vaccinate MMT mice at varying time points of tumor development with dendritic cells (DC) fused with spontaneous mammary carcinoma cells (FC/MMT). We show that vaccination of MMT mice with fusion cells in the early stage of tumorigenesis induces immunity that is sufficient to block or delay tumor development. This inhibition of tumor development is associated with the induction of polyclonal CTL and Ag-specific Ab. These results indicate that prophylactic vaccination with fusion cells elicits sufficient immune response to counter the tumorigenesis of potent oncogenic products.

Materials and Methods

Mice

Female C57BL/6 mice, 6–8 wk old, were purchased from Taconic Farms (Germantown, NY). The transgenic mice include 1) MT mice expressing polyomavirus middle T (PyMT) oncogene driven by the mouse mammary tumor virus long terminal repeat and developing spontaneous mammary carcinoma (1), 2) MUC1 transgenic mice (MUC1.Tg) expressing MUC1 Ag in a tissue-specific fashion similar to that in humans (2), and 3) MMT

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[3] Abbreviations used in this paper: PyMT, polyomavirus middle T; DC, dendritic cell; FC/MT, DC fused with MT tumor cells; FC/MMT, DC fused with spontaneous mammary carcinoma cells; MUC1, mucin 1.

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mice expressing PyMT and human MUC1 double transgenes and developing spontaneous mammary carcinoma. MT mice were generated by breeding the female wild-type C57BL/6 strain with male MT mice. MMT mice were generated by crossing the female C57BL/6 strain of MUC1.Tg mice with male MT mice. All mice are congenic on the C57/BL6 background more than 10 generations. The mice were selected for expression of the PyMT oncogene and/or MUC1 by PCR (2, 24). Only female mice either positive for MT (MT mice) or MT/MUC1 double transgenes (MMT mice) were used for the experiments. The mice were maintained in microisolator cages under specific pathogen-free conditions.

PCR

The mice were examined for MUC1 and MT gene expression with PCR analysis. Ten-microgram aliquots of tail and mammary tumor tissue were digested with proteinase K, and DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). PCR was conducted in a total volume of 50 μl in PerkinElmer Gene Amp tubes (Norwalk, CT) with the following reagents: 5 μl of 10× PCR buffer including 15 mM $MgCl_2$, 0.02% formamide, 0.2 mM dNTP, 100 nM 5′-CTTGCCAGCCATAGCACCAAG-3′ (bp 745–765) forward primer, and 100 nM 5′-CTCCACGTCGTGGACATTGATG-3′ (bp 1086–1065) reverse primer for the MUC1 gene; 100 nM 5′-AGTCACTGC TACTGCACCCAG-3′ (bp 282–302) and 100 nM 5′-CTCTCCTCAGTTC CTCGCTCC-3′ (bp 817–837) primer for the MT gene; 1.25 U of *Taq* polymerase; 2 μl of tail DNA (~500 ng), and reagent quality H_2O. The amplification program consisted of one cycle of 10 min at 94°C and 40 cycles of 30 s each at 94, 61, and 72°C. The PCR product of each reaction was analyzed by size fraction through a 1% agarose gel. Amplification of MUC1-positive DNA resulted in a 500-bp fragment (2), and amplification of MT-positive DNA resulted in a 491-bp fragment (24).

Cell culture and fusion

DC were obtained from bone marrow culture of C57BL/6 mice as described previously (25). Mammary carcinoma cells were isolated from MMT or MT mice. Briefly, spontaneous mammary tumors removed from female MMT or MT mice were teased into single cells. The tumor cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 U/ml penicillin, and 100 μg/ml streptomycin. After overnight culture, the nonadherent and dead cells were removed, and fresh medium was added. On days 2–3 of culture, the viability and phenotype of mammary carcinoma cells were checked. DC and mammary carcinoma cells were collected from the above primary cultures and placed in tubes at a 10:1 ratio. Fusion was conducted with 50% polyethylene glycol in Dulbecco's PBS without Ca^{2+} or Mg^{2+} at pH 7.4 (26, 27). DC were fused with MT tumor cells (FC/MT, MUC1-negative) or MMT tumor cells (FC/MMT, MUC1-positive). The percentage of fused cells was checked by cell surface Ag expression.

FACS analysis

The phenotypes of DC, MMT carcinoma cells, and FC/MMT cells were analyzed by flow cytometry. The cells were washed with PBS and incubated with FITC-conjugated-mAb and HMPV (anti-MUC1; BD PharMingen, San Diego, CA) for 30 min on ice. After washing twice with PBS, PE-conjugated mAb M5/114 (anti-MHC class II; BD PharMingen) or CD86 (anti-B7; BD PharMingen) was added for another 30 min on ice. Cells were washed, fixed, and analyzed by FACScan (BD Biosciences, Bedford, MA). In some experiments the fused cells were selected by FITC-HMPV (anti-MUC1) and PE-M5/114 (anti-MHC II) double-colored fluorescence cell sorting using MoFlo (Cytomation, Fort Collins, CO) with Summit version 3.0 analysis software.

Vaccination

Groups of MT or MMT mice were vaccinated s.c. with 5×10^5 FC/MT or FC/MMT cells (irradiated with 30 Gy) at the age of 15 days or younger or at 16–30 days. Vaccination was repeated monthly four additional times. The control groups consisted of mice immunized with irradiated MMT or MT tumor cells, DC mixed with tumor cells, or DC alone or mice injected with PBS. The mice were followed for up to 180 days. Mammary tissue was palpated twice a week before tumor development and every other day after the appearance of tumor. Progressively growing mass was regarded as tumor and was measured by calipers in two perpendicular diameters. The mice were sacrificed if the tumor was >2 cm. The mice were cared for according to institutional animal care and use committee guidelines.

Histologic and immunohistochemical staining

Groups of MT or MMT mice were sacrificed at varying ages. The mammary glands were harvested and fixed in 2% paraformaldehyde. Sections (5 μm) were prepared, stained with H&E, and examined under microscopy. To determine MUC1 expression in MMT mammary tissue, the section was also stained with anti-MUC1 mAb (BD PharMingen) for 30 min at room temperature and then subjected to indirect immunoperoxidase staining using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Humoral immune response

Sera were obtained from MMT mice immunized with 5×10^5 FC/MMT cells. Serum from MMT mice injected with PBS was used as control. Microtiter plates were precoated overnight at 4°C with 100 μl/well of MUC1 Ag (50 U/ml in PBS, pH 7.4). MUC1 Ag was purified from the ZR75 human breast cancer cell line (28). Each well was washed three times with PBS/Tween (0.05% Tween 20, v/v) and blocked with 120 μl/well 5% horse serum in PBS for 1 h at room temperature. After washing, 4-fold dilutions of mouse serum were added to each well for 2 h. The plates were washed and incubated with sheep anti-mouse IgG conjugated to HRP (Amersham Pharmacia Biotech, Piscataway, NJ). Ab complexes were detected by development with *o*-phenylenediamine (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA microplate Autoreader EL310 at OD 490 nm.

Chromium-51 cytotoxicity assay

Splenocytes were isolated from MT or MMT mice immunized with 5×10^5 FC/MT or FC/MMT cells by Ficoll separation. Splenocytes from nonvaccinated MT or MMT mice were used as controls. The target cells (MT and MMT tumor cells or MC38 and MC38/MUC1 carcinoma cell lines) were prelabeled with chromium 51 for 1 h at 37°C and added to the wells of 96-well, V-bottom plates with T cells (effector) for 5 h at 37°C. The supernatants were assayed for chromium-51 release in a gamma counter, and CTL activity was determined at the indicated E:T cell ratios. The percentage of specific chromium-51 release was determined by the following equation: percent specific release = [(experimental − spontaneous)/(maximum − spontaneous)] × 100.

Tumor cell challenge

Groups of MMT mice at the age of 38, 65, or 92 days were vaccinated three times with 5×10^5 FC/MMT at 7-day intervals. Five days after the third vaccination, the mice were challenged s.c. in the flank near the base of tail with mammary carcinoma cells isolated from MMT. As controls, littermates were injected with PBS and then challenged with mammary carcinoma cells. The mice were followed for up to 30 days after inoculation of tumor cells. Tumor growth was checked and measured daily using calipers.

Statistical analysis

Statistical significance was analyzed using χ^2 and Student's *t* tests.

Results

Characterization of MMT mice

The MMT mice were generated by crossing female C57BL/6 strain of MUC1.Tg with male MT mice that expressed PyMT oncogene. PCR was used to detect the bitransgenes. Amplification of MUC1- and MT-positive DNA resulted in 500- and 491-bp fragments, respectively. Whereas the MT gene occurred in tail and mammary tissues from MMT and MT mice, the MUC1 gene was present only in samples from MMT mice (Fig. 1*A*). To determine tumorigenesis, groups of MMT and MT mice were sacrificed at multiple time points, and mammary tissue was collected. Histologic examination revealed that MMT and MT mice developed mammary carcinoma in roughly three stages that arose sequentially over the lifetime of the mouse. Normal mammary glands expressed MUC1, yet were morphologically asymptomatic until 3 wk of age. Focal hyperplasia, beginning to appear in the fourth week, evolved into mammary intraepithelial neoplasms, carcinoma in situ, and finally diffuse invasive tumors (Fig. 1*B*). The mammary tumors in MMT and MT mice consisted of glandular adenocarcinoma with some variation. Most tumors were sclerotic, with dense connective tissue stroma separating the tumor cells. Cribriform and solid tumors were also observed. Immunohistologic examination demonstrated MUC1 expression in mammary tissue and/or tumors from MMT, but not MT, mice. Whereas MUC1 was detected on the apical surface of epithelial cells lining

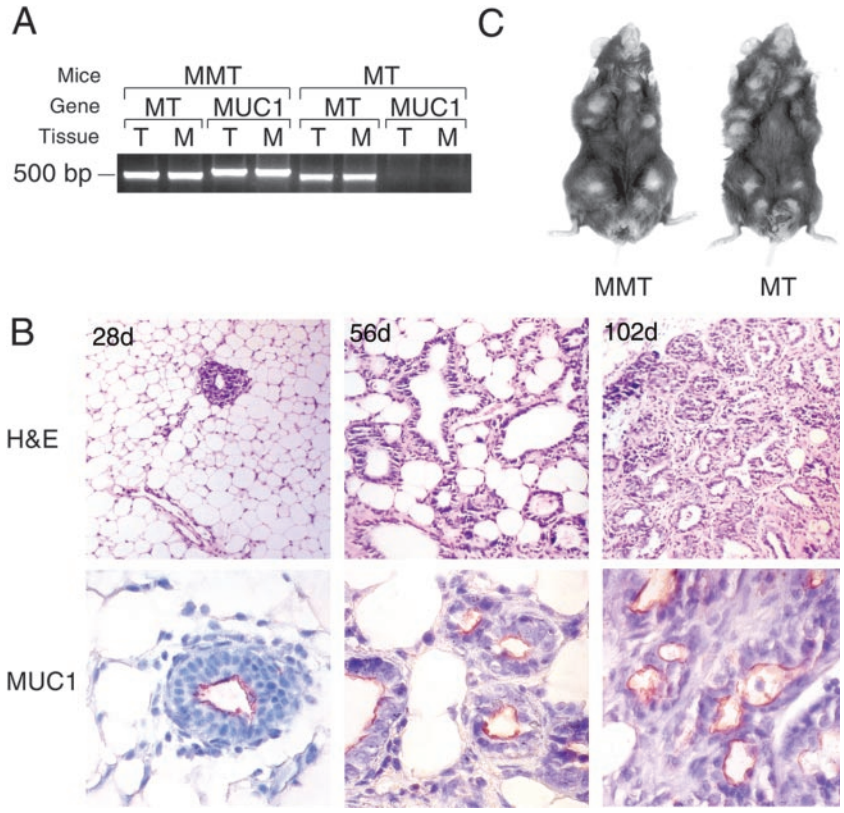

FIGURE 1. Development of spontaneous carcinoma in MMT and MT mice. *A*, PCR analysis for MT genes and MUC1 in tail tissue (T) and mammary tumor (M) of MMT and MT mice. Amplification of MUC1-positive DNA results in a 500-bp fragment, and amplification of MT-positive DNA results in a 491-bp fragment. *B*, Histologic examination with H&E staining of spontaneous mammary tumor in MMT mice at different ages (magnification, ×10). Immunohistochemical staining with anti-MUC1 mAb to detect MUC1 expression in mammary tissue or tumor (magnification, ×40). *C*, Development of multiple mammary carcinomas in MMT and MT mice by the age of 102 days.

the lumen in mammary tissue, strong MUC1 staining was found throughout mammary tumor cells (Fig. 1*B*).

The tumor incidence data indicate that the mammary tumors could be palpated when the mice were ~65 days old. Approximately half the mice developed mammary tumors at 80–90 days. Almost all the tumors were multiple, with synchronous kinetics in MT and MMT mice (Fig. 1*C*). The tumors progressed very rapidly, and the mice became moribund and were sacrificed 3–4 wk after the appearance of mammary carcinoma.

Collectively, these findings indicate that the expression of PyMT oncogene results in transformation of mammary epithelia and rapid production of mammary carcinomas in MT and MMT mice. More important, multiple stages of tumor development, similar to those in human cancers, are observed. Thus, MMT mice provide a better model for studies of tumorigenesis and vaccine development. The consistent expression of MUC1 in mammary carcinomas represents a potential target for immunotherapy and a marker for measuring the immune response.

Generation of DC/spontaneous mammary carcinoma fusion cells

To develop a DC/tumor fusion cell vaccine, spontaneous mammary carcinoma cells were isolated from MMT and MT mice. The tumor cells were cultured in vitro for 2 days and fused with syngeneic DC generated from wild-type mice. To assess the formation of fusion cells, two-colored flow cytometry was used. Whereas MUC1 was detected in the mammary carcinomas of MMT tumor, and MHC class II and costimulatory molecules were detected in DC, fusion of DC with mammary carcinoma cells from MMT mice (FC/MMT) resulted in dual expression of MUC1 and MHC class II or MUC1 and costimulatory molecules (Fig. 2). In contrast, there was no MUC1 expression on DC or fusion of DC with mammary carcinoma cells from MT mice (FC/MT; data not shown). These results indicate that fusion of DC with spontaneous mammary carcinomas results in the expression of tumor Ags in the context of costimulatory signals and MHC molecules.

Prevention of mammary carcinomas in MMT and MT mice by fusion cell vaccination

Previous data indicate that immunization of mice with FC/MUC1 induces antitumor immune responses that provide protection against the challenge of MUC1-positive tumor cells (26, 29). However, we do not know whether prophylactic vaccination with fusion cells can block the development of mammary carcinomas in a genetically altered model prone to breast cancer. To address this issue, two groups of MMT mice were immunized with FC/MMT. The vaccination was commenced in the first group of mice at the age of 15 days or younger and in the second group of mice at the age of 16–30 days. The immunization was repeated four times at

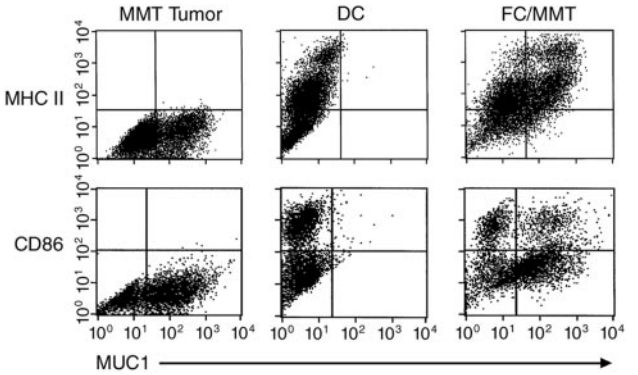

FIGURE 2. Phenotype of FC/MMT fusion cells. Expression of MUC1 and MHC class II or CD86 on FC/MMT determined by two-color flow cytometric analysis.

monthly intervals. All MMT mice treated with irradiated MMT tumor cells, DC mixed with tumor cells, DC alone, or PBS developed mammary carcinomas between the age of 65–108 days and were usually sacrificed after becoming moribund around 90–120 days (Fig. 3*A*). In contrast, immunization with FC/MMT fusion cells rendered 57–61% of the mice free of disease at the end of the experiment (180 days). It appears that the group with earlier vaccination faired better, with 61% protection (<15 days old; $n = 18$) compared with 57% protection in groups vaccinated at the age of 16–30 days ($n = 42$). The percentage of tumor-free mice from both groups is statistically significant compared with those of the control groups ($p = 0.001$). However, there is no statistically significant difference between the two experimental groups ($p = 0.5$; Fig. 3*A*). A similar trend, yet less positive results, were obtained in MT mice. Vaccination with FC/MT provided 41% protection for the mice with earlier vaccination and 33% protection for mice with later vaccination (Fig. 3*B*). Histologic examination of mammary tissue from vaccinated MMT mice at the end of the experiment revealed no tumor formation (Fig. 3*C*). These results indicate that prophylactic vaccination with DC/tumor fusion cells induces potent antitumor immunity to prevent or delay the development of mammary tumors in genetically altered mice prone to breast cancer.

Polyclonal CTL induced by vaccination with FC/MMT fusion cells

To define in part the basis of antitumor immunity induced by vaccination with FC/MMT, we measured CTL activity against syngeneic mammary carcinoma cells at varying time points in groups of different ages. Fig. 4*A* shows increased CTL activity against MMT mammary carcinoma cells after vaccination. The CTL activity was elevated after the second vaccination and peaked after the third and fourth vaccinations. It appears that the age of the mice had little impact on the CTL activity. In contrast, there was little, if any, CTL activity in nonvaccinated mice, regardless of whether they were tumor-bearing. A similar trend of CTL activity was found with splenocytes from immunized MT mice (Fig. 4*B*). Moreover, the CTL from immunized MMT mice lysed not only the MMT tumor cells from which the fusion cells were constructed, but also the syngeneic MC38/MUC1 carcinoma and MT tumor cells (Fig. 4, *C* and *D*), indicating that polyclonal CTL were induced. These results indicate that there are no spontaneous CTL in naive MMT and MT mice and that vaccination with FC/MMT or FC/MT induces polyclonal CTL against the relevant tumor cells with shared tumor Ags.

Induction of anti-MUC1 humoral response in MMT mice vaccinated with FC/MMT fusion cells

To determine the level of MUC1-specific Ab, MMT mice were vaccinated s.c. with 5×10^5 FC/MMT. The vaccination was repeated three additional times at monthly intervals. Nonvaccinated MMT mice were used as controls. The sera from MMT mice vaccinated with FC/MMT at different ages were collected at multiple time points and analyzed for the presence of anti-MUC1 Ab by ELISA. Vaccination with FC/MMT induced an anti-MUC1 humoral response in MMT mice. The level of anti-MUC1 Ab increased after the third and fourth vaccinations (Fig. 5*A*). However,

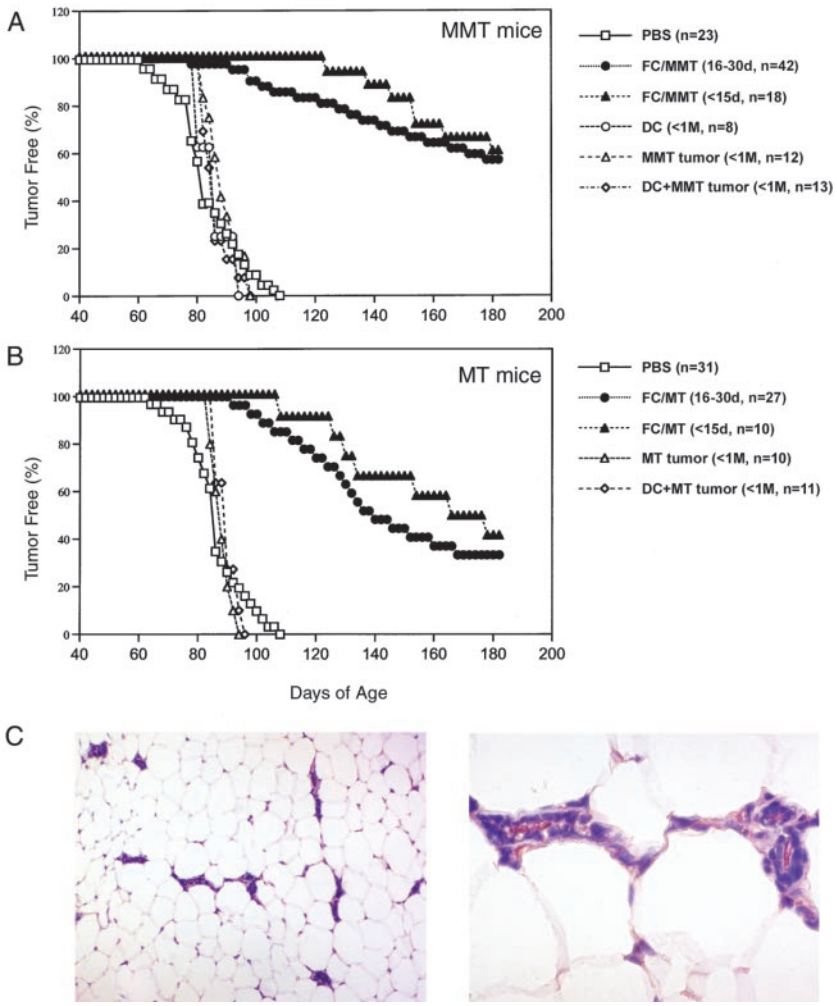

FIGURE 3. Prevention of spontaneous mammary tumor in vaccinated MMT and MT mice. *A*, Female MMT mice were vaccinated s.c. with 5×10^5 FC/MMT fusion cells at the base of tail at the age of 15 days or younger (▲) or at 16–30 days (●). Vaccination was repeated four times at monthly intervals. Mice <1 mo old were injected with irradiated MMT tumor cells (△), DC mixed with MMT tumor cells (◇), DC alone (○), or PBS (□) as controls. *B*, Female MT mice were vaccinated s.c. with 5×10^5 FC/MT fusion cells at the age of 15 days or younger (▲) or at 16–30 days (●). Vaccination was repeated four times at monthly intervals. Mice <1 mo old were injected with irradiated MT tumor cells (△), DC mixed with MT tumor cells (◇), or PBS (□) as controls. The mice were followed for up to 180 days, at which time the number of mice free of tumor was determined. *C*, Photomicrograph of immunohistochemical stained sections of mammary tissue removed from a vaccinated MMT mouse at the age of 180 days (*left panel*, ×10; *right panel*, ×40).

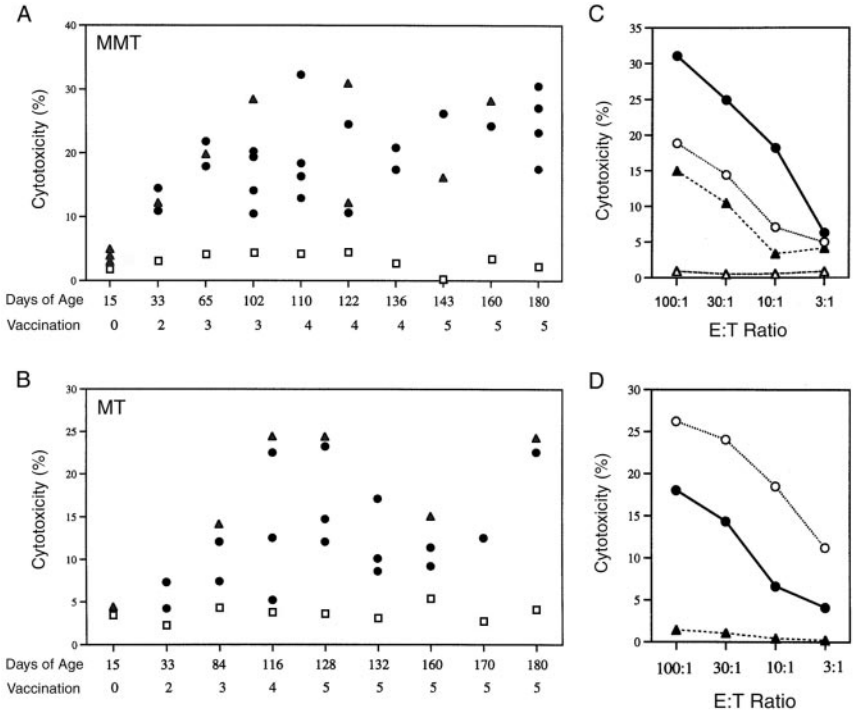

FIGURE 4. Antitumor response induced by FC/MMT and FC/MT fusion cell vaccination. *A* and *B*, Dot plot illustration of CTL activity induced by vaccination with fusion cells in MMT and MT mice. *A*, Splenocytes isolated from MMT mice at various ages that had been vaccinated at different times with 5×10^5 FC/MMT were incubated with MMT spontaneous mammary tumor cells at a 100:1 ratio. Each dot represents CTL activity in an MMT mouse at the indicated age and number of vaccinations. CTL activity was determined in splenocytes from MMT mice vaccinated with FC/MMT at the age of <15 days (▲) or at 16–30 days (●) or injected with PBS (□). *B*, Splenocytes isolated from MT mice at various ages vaccinated at different times with 5×10^5 FC/MT were incubated with MT spontaneous mammary tumor cells at a 100:1 ratio (●). CTL activity was determined in splenocytes from MT mice vaccinated with FC/MT at the age of <15 days (▲) or at 16–30 days (●) or injected with PBS (□). *C* and *D*, Specificity of CTL from immunized mice. Splenocytes isolated from MMT mice vaccinated with FC/MMT (*C*) and MT mice vaccinated with FC/MT (*D*) were incubated with MC38/MUC1 (▲), MC38 (△), and spontaneous mammary tumor cells from MMT mice (●) and MT mice (○) at the indicated E:T cell ratio. CTL activity was determined by the standard ^{51}Cr release assay.

a low level of anti-MUC1 Ab was detected in nonvaccinated MMT mice (Fig. 5*B*). In contrast, there was no anti-MUC1 Ab in MT mice immunized with FC/MT (Fig. 5, *A* and *B*). These results indicate that immunization with FC/MUC1 is associated with the production of anti-MUC1 Ab in MMT mice.

Rejection of challenge MMT mammary carcinoma cells in vaccinated MMT mice

Our previous data demonstrate that prophylactic vaccination with fusion cells rendered more than half the mice free of tumors for up to 180 days. Yet the remaining mice still developed tumors, although their appearance was delayed (Fig. 3). One possibility is that the host CTL are exhausted or have developed tolerance/ignorance. To explore this possibility we determined the existence of antitumor immunity by challenging MMT mice vaccinated at various ages with MMT mammary carcinoma cells. Three colonies of mice were vaccinated with FC/MMT. The vaccination was commenced in mice at the ages of 38, 65, and 96 days ($n = 4$/group). The vaccination was repeated three times at weekly intervals. Five days after the last vaccination, the mice were challenged s.c. with 5×10^5 MMT tumor cells in the flank near the base of tail. There was no tumor growth in the challenge sites of all vaccinated MMT mice regardless of age (Fig. 6, *A–C*). In contrast, all nonvaccinated littermates ($n = 4$/group) developed MMT tumor at the challenge sites (Fig. 6, *A–C*). The group of MMT mice vaccinated at the age of 65 days rejected the challenge tumor, but developed spontaneous mammary carcinoma, although its appearance was delayed (Fig. 6*B*). These results indicate a differential immune response to injected and autochthonous tumors. Moreover, even though mammary tumors had already been palpated in the group of mice at the age of 96 days when vaccination was initiated, the tumor-bearing mice were still able to mount an effective antitumor immune response against the injected MMT tumor (Fig. 6*C*). Antitumor immunity is induced in MMT mice regardless of age and presence of tumor at the time of vaccination. The CTL (Fig. 4) are functional in response to the tumor cell challenge; however, they fail to inhibit or eliminate autochthonous tumors. Taken together, these results indicate that fundamental differences exist in the immune response against challenge or autochthonous tumors.

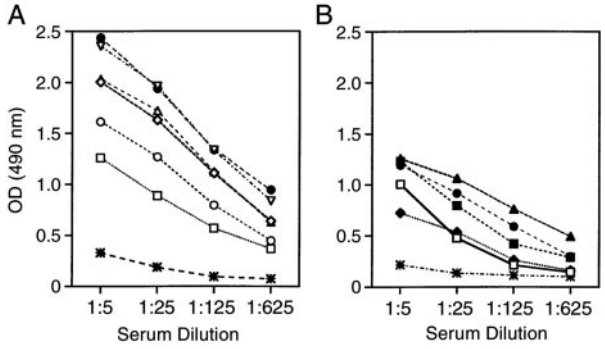

FIGURE 5. Humoral response induced by vaccination with FC/MMT in MMT mice. Sera were collected from vaccinated MMT mice at various ages ($n = 3$ in each group). Anti-MUC1 Ab was detected by ELISA assay. *A*, Anti-MUC1 Ab detected in FC/MMT-vaccinated MMT mice at the age of 36 (□), 65 (○), 102 (◇), 111 (△), 160 (▽), and 182 (●) days. *B*, MUC1-specific Ab from nonvaccinated MMT mice at the age of 46 (□), 110 (♦), 120 (■), 132 (▲), and 142 (●) days. Sera from MT mice (*) were assayed as control.

Discussion

DC/tumor fusion cells have induced potent antitumor immunity in a variety of models (26, 29–32). However, this is the first time that such a study has been conducted in a genetically modified model of spontaneous breast cancer. The use of genetically modified mice (MMT) offers several advantages over the transplantable tumor models: 1) the mice carry genetic alterations that interfere with signal transduction in a manner similar to that in human breast cancers; 2) the mammary carcinoma develops in multiple stages, as does human cancer; 3) the tumor develops in a competent immune system; 4) the mammary carcinoma progresses much more slowly than transplanted tumor, thus giving the host sufficient time

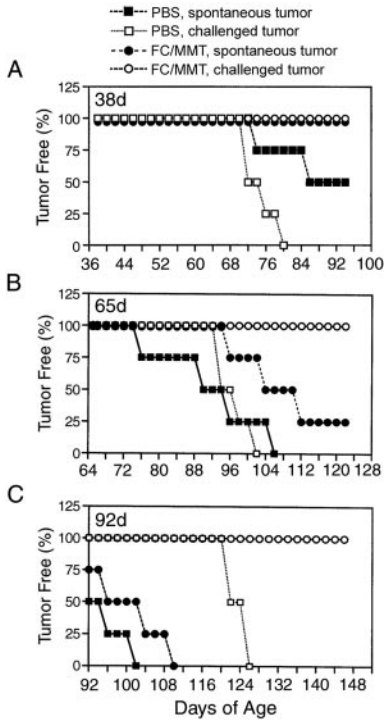

FIGURE 6. Rejection of injected mammary carcinoma cells in vaccinated MMT mice. MMT mice at different ages were vaccinated three times s.c. with 5×10^5 FC/MMT. Five days after the third vaccination, the mice were challenged s.c. with 5×10^5 MMT tumor cells in the flank near the base of the tail. The mice were followed for 30 days. Growth of spontaneous mammary tumor from mice vaccinated with FC/MMT (●) or from their littermates injected with PBS (■) and growth of injected MMT tumor from vaccinated mice (○) or from their littermates vaccinated with PBS (□) were determined. *A*, Percentage of tumor-free mice when vaccination was commenced at the age of 38 days. *B*, Percentage of tumor-free mice when vaccination commenced at the age of 65 days. *C*, Percentage of tumor-free mice when vaccination commenced at the age of 92 days.

to mount an effective immune response; 5) 100% of the mice develop mammary tumors within a reasonable time, making this a reliable tumor model; and 6) the expression of MUC1 provides a useful target for immunotherapy as well as a marker for measuring the immune response.

The present study demonstrates that vaccination with DC/tumor fusion cells confers sufficient antitumor immunity to block or delay mammary tumor development in a genetically altered model prone to breast cancer. The advantage of using DC/tumor fusion cells are 3-fold. First, the fusion cells are capable of expressing the whole repertoire of tumor Ags from an individual tumor. Thus, tumor-specific polyclonal CTLs are induced. Second, the fusion cells express the tumor Ags in the context of costimulatory signals and MHC class I and II molecules. Therefore, both arms of cell-mediated immunity are activated, and the immune response is greatly enhanced (27). Third, fusion cells are capable of processing and presenting tumor Ag, including those that are unidentified, thus circumventing the necessity of defining the tumor Ags. The findings that prophylactic use of DC/tumor fusion cells blocks or delays the development of spontaneous mammary tumor in the present study further support the idea that DC/tumor fusion cells may represent a promising alternative in the prevention and treatment of breast cancer.

One commonly shared tumor Ag is MUC1, which is expressed in 72% of cancers (33). MUC1 has been recognized as a multifunctional protein that plays a role in the protection and lubrication of mucous membrane, signal transduction, and modulation of the immune system (34). MUC1 is not required for mammary carcinogenesis in MMT mice. However, our study shows that vaccination with FC/MMT provides better protection than that with FC/MT (Fig. 3, *A* and *B*). These results indicate that MUC1 is an immunogenic Ag capable of eliciting immune response to reject MUC1-positive tumors when properly presented.

MUC1 is a tumor-associated Ag expressed in a variety of normal tissues. Theoretically, the anti-MUC1 immune response can be detrimental to the healthy organs expressing MUC1. However, we failed to observe any autoimmune disease in animal studies. Vaccinated MMT mice have been followed for >1 yr. They have survived in a healthy state without manifestation of any autoimmune disease (our unpublished observations). The difference in the expression of MUC1 between cancer and normal tissue may be attributed to the differential response. The spontaneous mammary cancer overexpressed MUC1 diffusely (Fig. 1). In contrast, MUC1 expression in mammary tissue was limited to the apical surface of the epithelium facing the lumen, which is not accessible to the immune system. The MUC1 expressed by cancer cells is also underglycosylated, thus exposing the protein core. The unmasking of the core protein may reveal the peptide epitopes that are recognized by CTL (22). Collectively, these results suggest that MUC1 is a preferred target for a cancer vaccine.

Vaccination leads to life-long protection against infectious disease. However, tumor Ag elicits an immune response of only a short duration. To determine whether CTL can be induced and then maintained, we vaccinated the MMT and MT mice multiple times at monthly intervals. A comparable level of CTL was demonstrated in multiply vaccinated mice, indicating the CTL are maintained. Furthermore, these CTL are functional and reject injected tumor cells in vivo. We have also shown the induction of competent antitumor immunity regardless of tumor burden by the host, indicating that tumor burden in mice may not be translated into systemic immune suppression. However, we failed to detect MUC1-specific CTL in tumor-bearing naive MMT mice. The lack of spontaneous MUC1-specific CTL may be attributed to the fact that tumor cells are not professional APC. The differences in spontaneous tumor models may also dictate whether spontaneous CTL are induced. No naturally occurring MUC1-specific CTL have been found in tumor-bearing MMT mice (P. Mukherjee and S. J. Gendler, unpublished observations).

The goal of vaccine development has been to prevent the disease; thus, its use is prophylactic. Most studies for tumor vaccine are focused on the treatment of tumors. Few studies have been conducted for prophylactic use, partly due to the lack of suitable tumor models. In the present study the prophylactic use of FC/MMT fusion cell vaccine rendered 57–61% mice free of mammary tumors at the end of the experiment. The result raises hope that a tumor vaccine can be developed to prevent the disease in populations with a high risk of breast cancer. Our results also indicate that 40% of the mice still develop mammary carcinoma, although its inception is delayed. Several mechanisms may contribute to this situation. Tumor cells are known to evade the immune system by down-regulation of tumor Ag/MHC molecules (35, 36), by Ag presentation in the absence of costimulatory molecules (37, 38), or by the development of tolerance and/or anergy of T cells (39). Our data, however, suggest that differential immune response by the host to injected and autochthonous tumor may be responsible for the development of spontaneous mammary carcinomas in our model. The vaccinated mice reject the transplanted mammary tumor cells, thus indicating the existence of functionally competent CTL; yet they still develop autochthonous

mammary tumors. It appears that the autochthonous tumor is ignored by the CTL. Of particular interest, such ignoring develops late in tumor development, since the CTL or antitumor immunity induced by the vaccination with fusion cells in all the mice is effective in delaying the development of mammary tumor. These results contradict the findings by Rovero et al. (40). In their report immunization with DNA vaccine against rat Her-2/neu p185 rendered six BALB/c mice expressing the Her-2/neu oncogene free of spontaneous mammary tumors, whereas three of the mice developed challenge tumors. We attribute the discrepancy to differences in the animal models used, the potency of oncogenes, the vaccines, and the vaccination regimens. It remains to be investigated whether the autochthonous tumor develops a shield to escape attack by CTL and/or whether the CTL have lost the ability to kill tumor cells. Alternatively, stimulation by the oncogene product may be too powerful to be inhibited in the long term, since PyMT is a potent oncogene (1, 41). Nevertheless, the finding that vaccination with fusion cells doubles the latency period of mammary carcinoma in a model expressing such a potent oncogene is encouraging. Our next goal is to improve the long term efficacy of the vaccine.

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