Early Role of CD4\(^+\) Th1 Cells and Antibodies in HER-2 Adenovirus Vaccine Protection against Autochthonous Mammary Carcinomas

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Early Role of CD4⁺ Th1 Cells and Antibodies in HER-2 Adenovirus Vaccine Protection against Autochthonous Mammary Carcinomas

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female mice (20, 21). In this model, CTL activity against HER-2-expressing tumors was not detected in mice protected against tumor by vaccination with an adenoviral vector expressing HER-2, and depletion of CD8+ T cells did not diminish the protection. CD4+ T cells were required only early in the immediate postvaccination period to induce anti-HER-2 Abs, which were both necessary and sufficient for protection. The IgG2a Ig isotype was critical for the induction of HER-2-specific Abs and was required for the development of protective immunity to HER-2-expressing cancers. In contrast to previous approaches that required regular boosts to maintain protective immunity against the relentless action of the oncogene constantly spawning new tumors in this transgenic model (12, 13), the adenovirus vaccine, without the addition of immunostimulatory cytokines, was sufficient to provide long-lasting protection beyond 1 year of age without further boosting after 19 wk of age.

Materials and Methods

Mice

Virgin female BALB-neuT mice (H-2b), BALB/c mice transgenic for the neu oncogene used under the control of mouse mammary tumor virus promoter (22), were bred in our animal facility. Virgin female BALB-neuT mice (C.129 P2(B6)-β2mαmαmαmαm+/−), deficient in the β2m gene and lacking CD8+ T cells, and BALB-INF-γ KO mice (C.129 S7(B6)-Infγαmαm+/−), deficient in the INF-γ gene, were purchased from The Jackson Laboratory. BALB-Igh KO mice (C.129(B6)-Igh-Jααmαm+/−), null for the IgH gene and deficient in B cells, and BALB-FcεR KO mice (C.129(B6)-FcεRαmαmαm+/−), deficient in the FcεR gene and lacking activating IgFcH, FcyRII, were obtained from the Taconic Farms. All mice were maintained in a pathogen-free animal facility. Wild-type and gene KO BALB/c mice were used at 6–10 wk of age, whereas BALB-neuT mice were used at different ages as described in Results. Experiments were conducted in accordance with a protocol approved by the Animal Care and Use Committee of the National Cancer Institute.

Cell lines and media

TUBO is a cloned cell line generated from a spontaneous mammary gland tumor from a BALB-neuT mouse and highly expresses HER-2 protein on the cell membrane (7). TS/A cells were derived from a spontaneous breast cancer of a wild-type BALB/c mouse (23). N202.1A and N202.1E are cell lines derived from a breast cancer from a FVB mouse (H-2q) transgenic for the oncogene expressed under the control of mouse mammary gland tumors (23). N202.1A and N202.1E are cell lines which express NeuR gene and lacking activating IgFc, FcγRII, were obtained from the TUBO and N202.1A cells and is absent on TS/A and N202.1E cells. These cell lines were kindly provided by Dr. P. Nanni (University of Bologna, Italy) and grown in DMEM containing 15% FCS (for TUBO), or RPMI 1640 supplemented with 10% FCS, penicillin, and streptomycin. Human embryonic kidney cells, HEK 293 cells, were obtained from the American Type Culture Collection and cultured in DMEM with 10% FCS. All cell cultures were maintained at 37°C in 5% CO2.

Adenoviral vectors

The E1, E3-deleted adenoviral vectors were generated using the AdMax system (Microbix) and the cDNA encoding the rat neu oncogene lacking the intracellular signaling domain and Ad-EC, expressing the extracellular domain only, and Ad-ECTM, expressing the extracellular and transmembrane domain, were generated in HEK 293 cells by calcium-phosphate precipitation and homologous recombination. Ad-Null, a control vector expressing no transgene, was similarly prepared. All adenoviral vectors were double-plaque purified, expanded in HEK 293 cells, purified on a cesium chloride density gradient, titrated as Pfu per milliliter and stored at −70°C until use.

Vaccination of mice

BALB-neuT mice were immunized with adenoviral vectors three to four times at 3 wk intervals. After optimization of immunization parameters (Fig. 1), 1 × 108 PFU Ad-ECTM was used to immunize BALB-neuT mice i.p. beginning at 6–8 wk of age. Mice similarly vaccinated with Ad-Null were used as a control group. The development of spontaneous mammary gland tumors was followed by palpating each mammary gland twice weekly. Tumor growth was confirmed by magnetic resonance imaging (MRI) in some experiments. In experiments using transplanted tumor cell lines, B6D2F1 mice were immunized i.p. with one dose of 1 × 106 PFU Ad-ECTM or Ad-Null. On the same day except where indicated, the mice were given a s.c. injection of 1 × 106 TUBO cells suspended in 100 μl of PBS and monitored twice weekly for the development of tumors. Tumors were measured using calipers, and tumor size was expressed as the product of the two perpendicular diameters (mm3).

MRI of mammary gland tumors

MRI experiments were performed using a 7-tesla horizontal MRI scanner (Bruker Biospin) operating on a Bruker AVANCE platform. Mice were anesthetized with 1.5% isoflurane via a nose cone, and the animal was placed supine and centered in a 72-mm transmit-receive radio frequency coil. Body core temperature was maintained at 37°C using a circulating heated water pad and monitored by means of a rectal temperature probe. Image acquisition was synchronized with the respiration rate to minimize image artifacts due to respiratory motion. A set of scout scans was used to locate the anatomical regions of interest. Subsequently, fast spin echo multiple slice images in the coronal plane were acquired. These slices (10–12 slices) were oriented to visualize dorsal-ventral structures encompassing a region from the upper thorax to the beginning of the tail. Scanning parameters were as follows: matrix size, 256 × 128; number of averages = 8; number of echos = 4; and echo time = 10 ms; field of view = 6.4 × 3.2 cm; slice thickness = 1 mm. The repetition time, dependent on respiration rate, was ~1.5 s making the total acquisition time ~6 min. Furthermore, high-resolution three-dimensional images were acquired with an isotropic resolution of 250 μm (matrix, 256 × 128 × 128), echo time = 6 ms, number of averages = 2, number of echos = 16, total imaging time ~40 min. All MRI were displayed as two-dimensional coronal slices using ParaVision software (Bruker Biospin).

In vivo depletion of T cells and NK cells

Depletion of CD4+ and CD8+ T cells was accomplished by i.p. injection of 500 μg of anti-CD4 or anti-CD8 Ab, respectively. Rat anti-mouse CD4 clone GK1.5 and anti-mouse CD8 clone 2.43 were obtained from the Cancer Research and Development Center (NCI-Frederick). As a control Ab, rat IgG was purchased from ICN Pharmaceuticals. The Abs were injected daily for the first 3 days followed by two more injections over the next week. In kinetic experiments, a single injection of anti-CD4 was sufficient to remove almost all CD4+ T cells. T cell depletion was verified by FACS analysis of PBMCs 1 day after the 3 consecutive day treatments, using FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 Abs (BD Pharmingen). Greater than 95% depletion of the specific T cell subsets was confirmed. To deplete NK cell activity, 50 μg of anti-asialo GM1 (WAKO) diluted to a volume of 400 μl with PBS as recommended by the manufacturer was administered i.p. on days 0, 1, and 2, and then twice weekly until 28 days after tumor challenge. Rabbit serum purchased from Sigma-Aldrich was used in the control mice.

Detection of anti-HER-2 Ab in sera

Serum was diluted 10- to 20-fold in PBS, and the presence of anti-HER-2 Ab was evaluated using FACS analysis using secondary Ab, FITC-conjugated anti-rat Ig or anti-mouse Ig (BD Pharmingen) (clone B.1) and anti-asialo GM1 (WAKO) (clone AK1) expressing N202.1A cells. N202.1A cells plated at 3 × 105 cells/well in U-bottom 96-well microtiter plates were incubated with serially diluted serum followed by the addition of the secondary Ab. After suspension in FACs buffer containing 1 mg/ml propidium iodide to gate out dead cells, anti-HER2/neu Ab was measured using a FACS Calibur (BD Biosciences). T serum was diluted 1:100 in PBS/1% BSA and used as negative controls to detect non-specific binding. The amount of anti-HER-2 Abs present in each serum was expressed as mean fluorescence intensity (MFI) calculated from triplicate wells.
In vitro growth inhibition assay

TUBO cells were cultured at 10^5 cells/well in triplicate in flat-bottom 96-well microtiter plates with pooled serum from BALB/c mice immunized with Ad-Null or Ad-ECTM, serially diluted in DMEM. [3H]Thymidine ([3H]Thy) 10 μCi was added into each well after overnight culture. The plates were harvested 48 h later using a Cell Harvester-96 (Tomtec), and [3H]Thy incorporation was measured using a MicroBeta Trilux liquid scintillation counter (PerkinElmer Wallac). Growth inhibition by anti-HER-2/neu Ab was determined as ([3H]Thy incorporation in Ad-Null serum − [3H]Thy incorporation in Ad-ECTM serum)/([3H]Thy incorporation in Ad-Null serum) × 100.

Adoptive transfer of immune sera

Serum from BALB/c mice immunized with Ad-Null or Ad-ECTM (1 × 10^8 PFU), respectively, was pooled, diluted 3-fold with PBS, sterile filtered and stored at 4°C. HER-2-specific Abs in the serum from Ad-ECTM vaccinated mice were verified by FACS analysis. BALB/c mice were treated with the diluted anti-HER-2 serum (0.3 ml/injection, i.p.) every other day from day 0 to day 16 after TUBO cell injection. Ad-Null serum-treated mice and Ad-ECTM-immunized mice were used as negative and positive controls, respectively.

Statistical analysis

Statistical analysis was performed with JMP statistical software (SAS Institute), using either nonparametric regression analyses for tumor prevention experiments with significance determined by the log-rank test. The comparison of the effect of vaccination on the numbers of tumors among different treatment groups was analyzed by one-way analysis using Tukey-Kramer HSD, and nonparametric Wilcoxon/Kruskal-Wallis tests. A value of p < 0.05 was considered significant.

Results

Prevention of autochthonous carcinomas in BALB-neuT mice

To examine whether tumorigenesis could be prevented with adenoviral vaccines expressing nonfunctional HER-2 protein, BALB-neuT mice were immunized four times at 3–4 wk intervals. Both the Ad-EC- or Ad-ECTM-immunized mice showed a significant delay in the onset of tumors and a decrease in the average number of breast cancers compared with the unvaccinated and Ad-Null-vaccinated groups (p < 0.05, Fig. 1, a and b). At 25 wk of age, 40% of the mice in the Ad-EC-immunized group, and 80% of the Ad-ECTM-immunized mice were tumor free (p < 0.05, Fig. 1c), whereas all the mice in the control groups developed tumors in all 10 mammary glands by this time. Tumor development was confirmed by MRI, although some tumors could not be seen in all 10 mammary glands by this time. Tumor development was significantly delayed by several weeks (Fig. 1c). In contrast, almost complete protection was achieved in mice immunized starting at 7 wk of age (Fig. 2c). Immunization starting at 11 wk of age inhibited tumorigenesis, although some animals developed tumors around 25–30 wk. After 40 wk, tumors developed very quickly in these mice (Fig. 2c). In subsequent experiments, we immunized BALB-neuT mice beginning at 7 wk of age.

CD4+ T cells, but not CD8+ T cells, are important for the induction of antitumor immunity

To investigate the role of T cells in the induction of antitumor immunity in this model, BALB-neuT mice were depleted of T lymphocytes at the time of immunization. Immune responses induced in the absence of CD8+ T cells were as protective against tumor as those induced in their presence (Fig. 3a). However, protection could not be elicited without CD4+ T cells, even though tumor growth was slightly delayed compared with the control groups (Fig. 3a). As expected, when both CD4+ and CD8+ T cells...
were depleted, protective immunity was completely abrogated. Therefore, CD4+ T cells are critical for the induction of protective immune responses to tumor in BALB-neuT mice by Ad-ECTM vaccine. However, CD8+ T cells are not necessary.

Neither CD4+ T cells nor CD8+ T cells are involved in the effector phase of antitumor immune responses

Because CD4+ T cells were simultaneously depleted when mice were immunized with Ad-ECTM (Fig. 3a), it was uncertain whether they are needed to help other immune cells produce the antitumor response or whether they act directly as effector cells against tumor. To examine the timing of CD4 depletion, we needed a model in which one immunization was sufficient. Three to four immunizations were required to prevent spontaneous tumors in BALB-neuT mice (Fig. 2a), making it difficult to perform T cell depletion only at defined times before and after immunization in BALB-neuT mice, because depletion before a boost would also constitute depletion after the previous immunization. Also, recovery from T cell depletion occurs at 3–4 wk after the Ab treatment. In contrast, one immunization with Ad-ECTM was sufficient to prevent growth of the TUBO cells injected into BALB/c mice (Fig. 3, b and c). TUBO is a cloned tumor cell line generated from a spontaneous mammary gland tumor from a BALB-neuT mouse and highly expresses HER-2 protein on the cell membrane (7). Therefore, to allow such timed depletions and also, importantly, to be able to use KO mice not available with the neuT transgene (see below), we conducted parallel studies in the transplantable TUBO tumor model. Wherever possible, findings were confirmed in both models to be sure that they were comparable. To investigate the role of CD4+ T cells as helper cells, BALB/c mice were depleted of CD4+ or CD8+ T cells 1 wk before immunization, followed by a challenge with TUBO cells 1 wk after immunization. Vaccination of mice depleted of CD4+ T cells could not prevent tumor growth, whereas mice depleted of CD8+ T cells were protected (Fig. 3b). As expected, the antitumor immune responses observed in CD8+ T cell-depleted mice were abrogated by depletion of CD4+ T cells (Fig. 3b).

We also investigated the role of T cells as effectors in antitumor immune responses by removing T cells after immunization with Ad-ECTM. T cell depletion was done 12 days after immunization and 7 days before the injection of tumor cells. The tumor growth was still prevented both in CD4+ T cell-depleted mice and in CD8+ T cell-depleted mice (Fig. 3c). Moreover, tumor growth was prevented in mice depleted of both CD4+ T cells and CD8+ T cells at later time points (Fig. 3c). These results demonstrate that CD4+ T cells are necessary as helpers for the Ad-ECTM vaccine to induce antitumor immunity, but they do not exert a direct antitumor response, and CD8+ T cells are not needed for any role as either helper or effector cells at any stage.

Protective immunity against tumor growth requires neither NK cells, CD8+ T cells, nor FcRs but does require B cells

To examine the role of NK cells in this model, BALB/c mice were depleted of NK cells by i.p. injections of anti-asialo GM1. To examine the role of Abs, Jh-deficient mice, lacking B cells, were
used. Moreover, using β2m-deficient mice, we confirmed the dispensability of CD8⁺ T cells in antitumor immune responses triggered by the Ad-ECTM vaccine. Tumor growth was not accelerated by the depletion of NK cells compared with the control group. Without NK cell activity, protection against tumor growth was conferred by immunization with Ad-ECTM and was similar to that seen in the presence of NK cells (Fig. 4a). In contrast, B cell-deficient mice were not protected by vaccination with Ad-ECTM (Fig. 4b), but β2m-deficient mice were protected (Fig. 4c). Therefore, we conclude that B cells and probably Abs are necessary for protection, but CD8⁺ T cells and NK cells are not. Ab-dependent cellular cytotoxicity (ADCC) is one of the mechanisms by which Abs act against tumors (24). To examine the role of this mechanism in our model, we used FcγRI/III (high affinity receptor for Fcγ)-KO mice. Tumor growth was inhibited in immunized-FcγRI/III-KO mice (Fig. 4d), suggesting that antitumor immune responses induced by Ad-ECTM are not mediated by FcγR, excluding ADCC as a mechanism of protection by this vaccine.

Anti-HER-2 Abs were detected in Ad-ECTM-vaccinated mice that were protected from tumor growth but not in tumor-bearing mice

Our results in B cell-deficient mice suggested that Abs against HER-2 might play a critical role in preventing tumor growth in BALB-neuT transgenic mice and BALB/c mice challenged with TUBO cells. The mice protected against tumor growth, that is, rat IgG and anti-CD8-treated groups, were found to have anti-HER-2 Abs even 13 wk after immunization with Ad-ECTM (Fig. 4e). However, Ab to HER-2 was not detectable at any significant level in the BALB-neuT mice depleted of CD4⁺ T cells at the time of immunization (Fig. 4e). CD4⁺ T cell-depleted mice were not protected against tumor development (Fig. 3n). Thus, CD4⁺ T cells were needed to induce a high level of anti-HER-2 Abs in BALB-neuT mice immunized with Ad-ECTM.

We also examined anti-HER-2 Ab responses in the TUBO tumor model. Anti-HER-2 Abs were detected in sera from BALB/c mice protected against tumor growth (Ad-ECTM in Fig. 4f) but not significantly in sera from tumor-bearing mice (Ad-Null in Fig. 4f). Anti-HER-2 Abs were not detectable in mice depleted of CD4⁺ T cells, but depleted in CD8⁺ T cell-depleted mice (Fig. 4f). The importance of Ab in tumor rejection was confirmed by detection of anti-HER-2 Abs in serum from mice depleted of NK cells, deficient of CD8⁺ T cells or FcγR, which rejected tumors (Fig. 4g), whereas Ad-ECTM-immunized B cell-deficient mice showed similar tumor growth to control mice, and anti-HER-2 Abs were not detected in these animals (Fig. 4b and g). Thus, immunization with Ad-ECTM protected mice from tumor growth and protection correlated with anti-HER-2 Ab responses in both the spontaneous transgenic mouse tumor model and the TUBO tumor injection model.

**FIGURE 4.** Protective immunity against tumor growth requires B cells, but neither NK cells nor CD8⁺ T cells, and depends on anti-HER-2 Abs but is not dependent on high affinity FcγR. a, BALB/c mice (n = 5) were injected with TUBO cells and depleted of NK cell activity (△, ●) with anti-asialo GM1 or treated with rabbit serum (△, △) for a control group. They were vaccinated with Ad-ECTM (●, △) or with Ad-Null (△, ○). Mice (n = 5) deficient of B cells (b), deficient of β2m (c), or deficient of FcγRIII (d) were immunized with Ad-ECTM (□, ○, □, □), or with Ad-Null (□, □, □, □) and diluted 20-fold. Anti-HER-2 Ab detection was performed by FACS analysis using N202.1A cells. TS/A cells were used as negative control together with N202.1E (1), to detect any nonspecific binding activity in anti-HER-2, the positive control sera from mice treated with Ad-ECTM and rat IgG. Serum in mice treated with Ad-Null was included as a negative control. The average amount of anti-HER-2 Abs (± SD) in each group is expressed as MFI. f, Sera (II) were collected 3 wk after immunization from each group of BALB/c mice (n = 5) in Fig. 3b or, for g, from each group of mice (n = 5) in a–d and diluted 20-fold. Anti-HER-2 Ab detection was performed and depicted as in e. In e and f, all animals treated with Abs were also immunized with Ad-ECTM. In g, open and closed bars represent animals immunized with Ad-Null vs Ad-ECTM, respectively. The results (a–g) are representative of three independent experiments showing similar results.
FIGURE 5. CD4+ T cell presence for <2 days at the time of immunization was sufficient to induce protective immunity against tumor growth. a, BALB/c mice (n = 5) were treated with anti-CD4 Ab at various time points: 7 days before (○), day 0 (□), 2 days (▼), 3 days (■), 5 days (▲), or 8 days (×) after immunization with Ad-ECTM. Non-immunized (■) and immunized/anti-CD4-un-treated mice (●) were included as control groups. Mice were injected with TUBO cells (10⁶ cells, s.c.) on day 0. The mean sizes of tumors (mm² ± SD) in each group are expressed. This is a representative of two independent experiments showing similar results. b, BALB/c mice (n = 5) were treated with anti-CD4 Ab from 6 h (○), 12 h (■), 18 h (▲), 24 h (▼), or 36 h (○) after or simultaneously (□) with TUBO cell injection and immunization with Ad-ECTM, except for Ad-Null immunized group (●). The mean sizes of tumors (mm² ± SD) in each group are expressed. This is a representative of three independent experiments showing similar results. c, CD4 depletion as a function of time after treatment with GK1.5 anti-CD4 mAb. To ascertain the resolution of these depletion experiments, the kinetics of depletion were studied by monitoring the level of CD4+ T cells in peripheral blood over time after treatment. Peripheral blood cells were stained with RM4-4 Ab, chosen to bind a different epitope to GK1.5 so as to be able to detect cells even when GK1.5 was bound. Depletion (relative to 30% CD4+ cells before depletion) was complete within <1 h, and remained complete for at least 12 days after treatment. d, Anti-HER-2 Abs (●) were measured in serum samples after 10X dilution, collected at day 20 after immunization with Ad-ECTM, from mice (n = 5) treated with anti-CD4 Ab at the time shown as in a. The mean sizes of tumors (mm² ± SD) in each group are shown at day 26 after tumor challenge. The average amount of anti-HER-2 Abs (with ± SD) is expressed as MFI.

Presence of CD4+ T cells <2 days after immunization was sufficient for antitumor immune responses to be induced

To explore how long CD4+ T cells are required to achieve a successful vaccination against HER-2, we depleted CD4+ cells at various time points relative to the initiation of vaccination. Anti-CD4 treatment 7 days before or on the day of immunization and tumor challenge abrogated antitumor immunity against TUBO cell growth (Fig. 5a), as expected from the results shown in Fig. 3. However, antitumor immune responses were not significantly affected when CD4+ T cells were depleted beginning day 2 after immunization. Similar protection was observed from mice treated with anti-CD4 on day 3 to day 8 after immunization (Fig. 5a). To narrow down the time more precisely, we treated BALB/c mice with anti-CD4 beginning 6 h after immunization. Tumor growth was delayed in mice treated at 6 h after immunization and prevented in some mice treated at 12, 18, and 24 h, but the effect was not consistent (Fig. 5b). Protection against TUBO cell growth was observed in mice treated with anti-CD4 36 h after immunization or later (Fig. 5b). Thus, CD4+ T cell help surprisingly was required only for the first 36 h after immunization. To verify the kinetics of in vivo depletion of CD4+ T cells, splenocytes were prepared at each time point after anti-CD4 Ab treatment and analyzed by FACS. The Ab used for detection was not blocked by the Ab used for depletion (data not shown), indicating that masking of CD4 was not the mechanism of rapid disappearance of CD4+ T cells. Indeed, the CD4+ T cell depletion was fast and substantial after one injection of anti-CD4 Ab. Even after 1 h, splenic CD4+ T cells were decreased from the normal level of 30% to 2% and hardly detectable at days 4–5 (Fig. 5c). The CD4+ T cells took >4 wk to recover to the normal pretreatment level in the spleen. Thus, the brief 36-h requirement for CD4+ T cells to provide help did not artificially appear shorter than actually needed because of a delay in depletion of the CD4+ cells.

In parallel with protection, the level of anti-HER-2 Ab was increased proportional to the duration of CD4+ T cell presence after immunization (Fig. 5d), with no Ab observed in mice depleted of CD4+ T cells at day −7, some Ab in mice depleted 2 days after immunization, and the highest levels in mice depleted of CD4+ T cell presence after 3–12 days following immunization. The means of tumors (mm² ± SD) in each group are shown at day 26 after tumor challenge. The average amount of anti-HER-2 Abs (with ± SD) is expressed as MFI.

FIGURE 6. IgG2a anti-HER-2 Abs was important for effective rejection of tumor growth and IFN-γ was necessary but IL-4 was not. a, BALB/c mice (○, n = 5), IFN-γ KO mice (□, ■, n = 5), or IL-4 KO mice (▲, ▲, n = 5) were injected with TUBO cells (10⁶ cells, s.c.). They were vaccinated with Ad-ECTM (●, ●; 10⁷ PFU, i.p.) or with Ad-Null (○, ○) on the same day as tumor injection. The mean sizes of tumors (mm² ± SD) in each group are shown. After 3 wk, sera were collected from each group in a and diluted 20-fold with PBS. Subtypes (b) or total Ig levels (c) of anti-HER-2 Abs were measured using secondary Abs specific for murine IgG1, IgG2a, IgG2b, or IgM and total Ig by FACS analysis using N202.1A cells. The average amount of anti-HER-2 Abs (± SD) in each group is expressed as MFI. This result (a–c) is a representative of three independent experiments showing similar results.
T lymphocytes, not CD8+ T cells on day 8 after immunization. Tumor regression was delayed or less efficient in mice depleted of CD4+ T cells at early time points compared with that of mice depleted of CD4+ T cells at day 6 or day 8 after immunization (Fig. 5a). Thus, the kinetics of CD4+ T cell depletion indicated that Ad-ECTM vaccination required CD4+ T cell help only during the first 2 days after immunization but not afterwards, and the antitumor activity was correlated with the level of anti-HER-2 Ab produced.

Importance of IFN-γ and Ig IgG2a isotype for protection

To determine whether the CD4+ T cell help played a qualitative role as well as a quantitative one, we asked whether altering Th1 vs Th2 cytokine profiles or the Ig isotypes they control played a role in protection. Whereas IL-4 KO mice were protected by Ad-ECTM immunization just as well as wild-type BALB/c mice, indicating a lack of need for Th2 help, IFN-γ KO mice showed diminished protection compared with the wild-type mice (Fig. 6a). When sera from these animals were examined for Ig isotype, although no significant difference was seen in levels of HER-2-specific IgG1, IgG2b, or IgM (Fig. 6b) or in total anti-HER-2-specific Ig levels (Fig. 6c) between either KO strain and the wild type, a near absence of IgG2a isotype anti-HER-2 Ab was found in the IFN-γ KO mice (Fig. 6b). Thus, the only isotype loss that correlated with the loss of protection in the IFN-γ KO mice was that of IgG2a. (Note that one can compare mice for the same isotype, but not compare quantities of different isotypes by this assay because the detecting reagents were different.) Although it is clear that the remaining isotypes can give partial protection in the absence of IgG2a, the fact that similar total levels of HER-2-specific Ig were not sufficient for complete protection in the absence of IgG2a isotype implies that this isotype is probably the most effective, even when it is a minor component of the total. Although it was not feasible to purify enough Ab of individual isotypes to test this interpretation directly, the correlation provides strong indirect evidence for a major role of IgG2a. Because we had found that CD4+ T cells were necessary only to provide help for Ab production and not for an effector cell function, we conclude that the importance of the Th1 cytokine IFN-γ lies in its necessity to produce substantial levels of the critical IgG2a Ig isotype.

Anti-HER-2 serum inhibited tumor growth both in vivo and in vitro

Because ADCC did not appear to be responsible, we explored the mechanism of Ab-mediated protection by examining whether tumor growth could be inhibited with anti-HER-2 Ab. In vitro TUBO cell growth was inhibited by anti-HER-2 Ab addition (Fig. 7a). [3H]Thy incorporation was decreased by ~90% when TUBO cells were cultured with anti-HER-2 serum at 1/10 dilution compared with control mouse serum, and the inhibition was dose dependent. To see the effect of anti-HER-2 Ab on in vivo tumor growth, mice were treated with anti-HER-2 serum every other day from day 0 to day 16 after tumor injection. In vivo tumor growth in BALB/c mice was completely prevented by treatment with anti-HER-2/neu serum (Fig. 7b), and it was as effective in protection of mice from tumor growth as Ad-ECTM immunization (Fig. 7b). These results demonstrate that anti-HER-2 Ab is both necessary and sufficient to protect against HER-2-expressing mammary tumor growth in Ad-ECTM-immunized mice and may act directly on the tumor cells.

Discussion

In this study, we show that spontaneous autochthonous breast cancers in HER-2 transgenic mice could be completely prevented with an adenoviral vaccine expressing non-signaling HER-2 and that Ab and helper CD4+ T lymphocytes, not CD8+ T cells, played a critical role in inducing antitumor immunity. We report that BALB-neuT mice (Figs. 2 and 3, n = 25) were completely protected against spontaneous mammary tumor growth until at least 55 wk of age, when treated with Ad-ECTM (1 × 106 PFU) four times every 3–4 wk beginning at 7 wk of age and ending on week 19. As the transforming HER-2 oncogene is embedded in the genome of BALB-neuT mice, a unique dynamic relationship between the continuous generation of tumors by neoplastic stem cells and inhibitory immune response is occurring. The adenoviral vaccine is able to elicit sustained protection even 30 wk after the last boost. Such a prolonged and effective immune memory has never been induced in this devastating model of autochthonous cancer (25). Effective tumor protection lasting a whole year has not been achieved except when immunization with the allogeneic tumor vaccine plus IL-12 (14) or with the DNA plasmid vaccine (12) were continuously repeated. We consider two possible explanations for the long-lasting protection induced by the adenoviral vaccine. One is that the immune memory is more durable. However, a second possibility is that the Abs elicited by the vaccine given at 7 wk of age are sufficient to permanently eliminate the neoplastic mammary stem cells, so that the neoplastic process due to the transgenic oncogene is actually halted, rather than just kept in check. Further studies on the neoplastic stem cells, beyond the scope of the present study, will be required to test this latter possibility.

Considering that BALB-neuT mice develop palpable tumors beginning at 14–15 wk of age and boosting immunizations are required for long-term antitumor immunity, immunization beginning at 11 or 15 wk of age might be too late to induce effective immune responses against the tumor (or the neoplastic stem cells), as studied in FVB mice that display a much slower tumor progression (26). BALB-neuT mice highly express HER-2 protein on the surface of the terminal ductular-lobular units of all the mammary glands by the 3rd week of age. Moreover, atypical mammary hyperplasia is already observed between 3 and 6 wk of age (11). Thus, immunization was optimal when started at 6–9 wk of age.
Immune responses are generally enhanced by repeated immunizations. However, there is a concern that responses to vaccination with adenoviral vectors might be attenuated by pre-existing anti-adenoviral immunity as a result of prior infection with adenovirus (27–29). In our experiments using an adenoviral vector as an antitumor vaccine, four immunizations with Ad-ECTM completely protected BALB-neuT mice from tumorigenesis, whereas three doses did not. Therefore, secondary immune responses against HER-2 were not hindered by anti-adenoviral immunity presumably induced by repeated immunizations. Indeed, prior exposure to adenovirus did not always significantly decrease the protective antitumor responses to adenoviral vaccine (30–32). Thus, pre-existing anti-adenoviral immunity may not be an absolute barrier to the subsequent use of adenoviral vaccines to induce antitumor immune responses.

Regarding the mechanism of protective immunity, most studies of antitumor immunity have focused on the role of CD8\(^+\) T lymphocytes, although there are reports describing a critical role of CD4\(^+\) T lymphocytes (33, 34). CD4\(^+\) T cells can function as helper or effector cells in antitumor immune responses. Our data demonstrate that CD4\(^+\) T lymphocytes are crucial for the induction of antitumor immunity by the adenoviral HER-2 vaccine. In contrast, prevention of tumorigenesis was not significantly impaired by depletion of CD8\(^+\) T lymphocytes or in β\(_m\)-deficient mice. CTL are difficult to detect in BALB/c mice (7, 11) or C57BL/6 mice (8) immunized with DNA encoding HER-2 protein, and we also could not detect CTL activity against HER-2-expressing target cells even in Ad-ECTM immunized HER-2-transgenic and wild-type mice (data not shown). Moreover, the TUBO model confirmed that CD8\(^+\) T cells were not needed for antitumor immune responses in either the priming or effector phase, as demonstrated by protection of BALB/c mice from tumor growth with CD8\(^+\) T cells depleted either before or after immunization, as well as by protection in β\(_m\)-KO mice. In this respect, our present data that the mammary tumor-preventing antitumor immune response was not dependent on CD8\(^+\) T cells contrasts with other studies (9, 16) in which CD8\(^+\) T cells were required to protect mice from growth of tumors expressing HER-2, implying that different mechanisms can be operative with different vaccines.

In our current studies, in contrast to the other vaccine approaches, tumor protection appeared to be solely Ab mediated. Tumor growth was not inhibited in B cell-deficient mice immunized with the vaccine. Furthermore, adoptive transfer of anti-HER-2 serum collected from immunized mice showed that anti-HER-2 Abs directly inhibited TUBO cell growth in vitro and also prevent tumor growth in vivo. These results suggested that antitumor protection induced by Ad-ECTM was mediated directly by Ab against HER-2, which was both necessary and sufficient and required CD4\(^+\) T lymphocytes only as helper cells in the priming phase of B cells. Moreover, the kinetics of allowable CD4\(^+\) T cell depletion after immunization for inhibition of tumor growth correlated closely with the kinetics of CD4\(^+\) T cell depletion for Ab responses. Thus, this adenoviral vaccine appears unique in producing long-lasting protection against the growth of mammary tumors that is purely mediated by Abs without any need for either CD8\(^+\) T cells or effector CD4\(^+\) T cells. Besides the requirement for CD4\(^+\) help for the quantity of Ab produced, CD4\(^+\) T cells played a role in the quality of the Ab, because the Th1 cytokine IFN-γ was required for optimal protection, and the presence of IFN-γ correlated with the ability to make a critical Ab isotype, IgG2a. Although IgG2a can bind to as well as complement Fcγ receptors, the preference specifically for IgG2a and not IgG2b did not correlate clearly with either FcR binding or complement fixation ability (15, 35). Also, studies in FcγR-KO mice excluded ADCC as a mechanism for protection, in contrast to the requirement for FcγRs for the protective activity of many human mAbs against human tumor xenografts in nude mice (24). As the Ab inhibits growth of a pure population of tumor cells in vitro, the Ab may act directly on the tumor cells to deprive them of the growth signals mediated through HER-2. Thus, further studies will be required to determine the basis for the preference for the IgG2a isotype. A mAb with similar direct inhibitory activity on HER-2 activation, in contrast to trastuzumab (Herceptin), has been described to inhibit signaling and tumor growth (36). A vaccine inducing such Abs could be even more effective.

In summary, we have achieved durable protection using a HER-2-expressing adenoviral vaccine against both spontaneous mammary carcinomas in HER-2 transgenic mice and a HER-2-expressing mammary carcinoma cell line in wild-type BALB/c mice. Surprisingly, the protection appeared to be primarily Ab mediated. Abs were both necessary and sufficient for this protection, whereas CD8\(^+\) T cells were not required at all and CD4\(^+\) T cells were necessary only as helper cells during the initial 36–48 h priming phase after immunization but not as direct antitumor effector cells at the time of challenge. These results are in accord with the efficiency of an anti-HER-2 mAb treatment in HER-2-positive human breast cancer, and with the finding that vaccine based on a single HER-2 B cell epitope peptide can partially protect HER-2 transgenic mice against development of mammary tumors (37). A vaccine inducing endogenous Ab production may perhaps be more effective than repeated administration of an exogenous mAb. These promising findings bode well for the use of such an anti-HER-2 adenoviral vaccine in the prevention of breast cancer in high-risk individuals or in the prevention of metastasis after local treatment of breast cancer patients.

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Disclosures

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References


