Vaccination with Cytoplasmic ErbB-2 DNA Protects Mice from Mammary Tumor Growth Without Anti-ErbB-2 Antibody

Shari A. Pilon,* Marie P. Piechocki,† and Wei-Zen Wei2*†

Wild-type ErbB-2 (E2) positive D2F2/E2 tumors are rejected by active vaccination with ErbB-2 DNA. However, anti-ErbB-2 Ab response can cause cardiac toxicity or interfere with cellular immunity. It will be advantageous to induce only cellular immunity by active vaccination. A panel of E2 DNA vaccines were constructed, and their vaccination efficacy was ranked as E2 > tyrosine kinase-deficient ErbB-2 (E2A) > full-length ErbB-2 targeted to the cytoplasm (cytE2) > tyrosine kinase-deficient cytE2 (cytE2A). E2A is a tyrosine kinase-deficient mutant containing a single residue substitution. CytE2 or cytE2A encodes a full-length protein that is targeted to and rapidly degraded in the cytosol by the proteasomes. Covaccination with cytE2A and GM-CSF or IL-2 DNA resulted in equivalent anti-tumor activity as E2. However, anti-ErbB-2 Ab was induced by E2 or E2A, but not cytE2 or cytE2A. Therefore, cytE2A appears to induce anti-tumor immunity without an Ab response. ErbB-2-specific CTL were detected in mice immunized with cytE2A and GM-CSF and have rejected tumor challenge. Depletion of CD8, but not CD4 T cells reduced anti-tumor immunity, indicating CTL as the effector cells. Covaccination with E2A and cytE2A induced synergistic anti-tumor activity, supporting enhanced peptide presentation from cytE2A, which was further evidenced by superior CTL activation using APCs expressing cytE2 vs E2. Taken together, cytoplasmic ErbB-2 DNA induced anti-tumor CTL, but not humoral response, demonstrating the feasibility of eliciting individual effector mechanism by targeted DNA vaccine. The Journal of Immunology, 2001, 167: 3201–3206.

ErbB-2 or Her-2/neu, a member of the epidermal growth factor receptor family with tyrosine kinase activity, is overexpressed in several human cancers including breast, ovarian, and lung cancers (1, 2). Overexpressed ErbB-2 is associated with aggressive disease and poor prognosis (3). Because ErbB-2-specific Ab and T cells are detected in breast and ovarian cancer patients, ErbB-2 is recognized as a target of immunotherapy (4–7). Hereceptin, a humanized anti-ErbB-2 mAb, has demonstrated clinical benefit in advanced breast cancer patients although cardiac toxicity was exerted particularly when the patients also received anthracyclines or cyclophosphamides (8). Anti-ErbB-2 T cells may not exert such toxicity or other Ab-associated adverse effect. It will be advantageous to control ErbB-2-positive tumors by inducing cellular immunity with active vaccination and administering mAb only as needed. For this purpose, ErbB-2 DNA vaccines were constructed and tested. Full-length ErbB-2 targeted to the cytoplasm (cytE2) and tyrosine kinase-deficient cytE2 (cytE2A) induced anti-tumor CTL without Ab and are excellent candidates for the proposed immunotherapy strategy.

In addition to potential cardiac toxicity, Abs induced by vaccination may have conflicting effects on anti-tumor immunity. Some anti-ErbB-2 mAbs trigger positive signaling events causing enhanced tumor growth (9). Inhibition of T cell activity by tumor-specific Abs has also been described (10, 11). In contrast, neu-specific Abs generated by DNA or cell vaccines contributed to anti-tumor immunity in some Neu-transgenic mice (12–14). Rhesus monkeys immunized with ErbB-2 extracellular domain produce anti-ErbB-2 Abs with inhibitory activity against tumor growth (15). The conflicting reports on Ab activity and the potential cardiac toxicity are causes of concern in generating long-lasting, irreversible Ab response by vaccination.

The efficacy of anti-ErbB-2 T cells also needs clarification. CD8 and CD4 T cells were activated in patients immunized with HLA-A2.1- or HLA-DR-associated ErbB-2 peptides. However, peptide-induced CTL failed to lyse human cancer cells with amplified ErbB-2, leaving in question the efficacy of peptide immunization (16). Vaccination of rats with MHC class II-associated peptides induced anti-neu Ab and T cell immunity, but the anti-tumor efficacy was not clear (17). Improved understanding and manipulation of the various anti-ErbB-2 effector mechanisms will lead to improved clinical trials and is a goal of this study.

To induce ErbB-2-specific CTL, a panel of human ErbB-2 DNA constructs were generated in our laboratory (18). Tyrosine kinase-deficient ErbB-2 (E2A) encodes full-length ErbB-2 with a single amino acid substitution to replace ATP binding lysine (K) with alanine (A) and to eliminate tyrosine kinase activity. CytE2 has a truncated endoplasmic reticulum (ER) signal sequence and encodes a full-length protein that is released into the cytoplasm rather than transported into the ER as a transmembrane protein. CytE2A is cytE2 with the K-to-A mutation. Plasmid DNA was chosen as the vaccine candidate because it is chemically defined, can be produced in large quantity and purified to homogeneity, and is relatively stable. DNA can be readily modified to encode proteins with the desired biochemical, biological, and thus immunological properties, making it possible to perform mechanistic analysis in a
timely fashion. CytE2 and cytE2A are of particular interest because the proteins are targeted to the cytoplasm and rapidly degraded by the proteasome. Processing of proteins through this pathway should result in a complete repertoire of MHC class I peptides for CD8 T cell recognition. Rapid degradation via the proteasome is associated with enhanced peptide presentation and T cell reactivity (19). However, in our preliminary study, cytE2 or cytE2A vaccination was poorly protective compared with the transmembrane counterparts. In this study, this observation was further analyzed, and profound anti-tumor activity was achieved when GM-CSF or IL-2 DNA was coadministered with cytE2 or cytE2A vaccination. Therefore, CytE2 and cytE2A are candidates for combined DNA vaccination and mAb therapy.

Materials and Methods

Animals and cell lines

BALB/c (6- to 8-wk old) mice were obtained from Charles River Breeding Laboratories (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME). D2F2 is a mouse mammary tumor line derived from a spontaneous mammary tumor that arose in a BALB/c hyperplastic alveolar nodule (HAN) line D2 (20). The human breast cancer cell line SKBR-3, which has amplified ErbB-2, was purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were maintained in vitro in DMEM supplemented with 10% heat-inactivated calf serum (HyClone Laboratories, Logan, UT), 10% NCTC 109 medium (Sigma, St. Louis, MO), 2 mM L-glutamate, 0.1 mM MEM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. D2F2 cells cotransfected with rErbB-2 constructs and prSV2neo: D2F2/E2, D2F2/E2A, D2F2/cytE2, and D2F2/cytE2A, were maintained in medium containing 0.8 mg/ml G418 (Geneticin; Sigma). All tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD) unless otherwise specified.

DNA immunization

The rErbB-2 plasmids pCMV, pCMV/E2 (wild-type ErbB-2, E2), pCMV/E2A (E2A), pCMV/cytE2 (cytE2), and pCMV/cytE2A (cytE2A) have been described previously (18). Plasmids pEFBos/GM-CSF and pEFBos/IL-2, encoding murine GM-CSF and IL-2, were obtained from N. Nishisaki (HyClone Laboratories, Logan, UT), 2 mM L-glutamate, 0.1 mM MEM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. All tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD) unless otherwise specified.

Tumors challenge

At 2 wk after the final DNA vaccination, mice were challenged s.c. in the right flank with 2 × 10^6 D2F2 tumor cells expressing wild-type or mutant ErbB-2. Tumors were measured weekly by a caliper in two dimensions, and mean tumor diameter was calculated. Animals were sacrificed when tumor diameter reached 10 mm.

Measurement of anti-ErbB-2 Abs

Blood was collected from mice 1 wk after the third DNA vaccination or 4 wk after tumor challenge. To measure anti-ErbB-2 Ab, SKBR3 cells were stained using serially diluted mouse serum as the primary Ab. A fluorescein-conjugated goat-antimouse γ-chain of pan IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), IgG1, or IgG2a (Caltag Laboratories, Burlingame, CA) secondary Ab was used to detect bound serum IgG. The mAb TA-1, which recognizes an extracellular domain of ErbB-2, was used as a positive control (Oncogene Research Products, Cambridge, MA). Normal mouse serum or isotype-matched mAb was the negative control. Flow cytometric analysis was performed with a FACSCaliber (BD Biosciences, San Jose, CA). The Ab titer was defined as the highest serum dilution that demonstrated positive staining. Positive results from flow cytometric analysis were verified by immunoprecipitation of ErbB-2 with the anti-erb2 and Western blotting with 3B5 (M. Piechocki, S. Pilon, and W.-Z. Wei, manuscript in preparation).

T cell depletion

To deplete CD4 or CD8 T cells, mice were treated by i.p. injection of 500 μg of GK1.5 or 2.43 (ATCC) hybridoma ascites. Mice were treated for three consecutive days and then every three days thereafter until the completion of the experiment. Six days after the first injection of mAb, animals were challenged s.c. with 2 × 10^6 D2F2/E2 cells. Depletion was verified by FACS analysis of splenocytes 6 days after the first injection (data not shown).

Generation of CTL and CTL assay

Splenocytes from immunized mice were isolated 6 wk after tumor challenge by Ficoll purification and incubated with irradiated stimulator 3T3 cells transfected with K" and ErbB-2 or cytoplasmic ErbB-2. Cultures were maintained in R10: RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamate, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). On day 7, viable cells were analyzed for cytotoxic activity. D2F2 and D2F2/E2 cells were labeled with sodium [111]cIchromate for 2 h at 37°C. In a 96-well round-bottom plate, target cells were incubated with responder cells at different E:T ratios for 4 h at 37°C. Fifty microliters per well of supernatant was transferred to a 96-well plate with 100 μl of Optiphase Supermix scintillation fluid and counted on a TriLux β Scintillation Counter (Wallac, Turku, Finland). The percentage of specific lysis was calculated as 100 × ([experimental release − spontaneous release]/maximum release − spontaneous release). Spontaneous and maximum release were determined in the presence of medium or 1/6 N HCl, respectively.

Results

Relative efficacy of E2 DNA vaccines

DNA vaccination was tested in six independent experiments (Table I). BALB/c mice were immunized three times at 2-wk intervals with pCMV, pCMV-ErbB-2 (E2), pCMV-ErbB-2A (E2A), pCMV-cytoplasmic ErbB-2 (cytE2), or pCMV-cytoplasmic ErbB-2A (cytE2A). Two weeks after the last vaccination, mice were challenged s.c. with BALB/c mammary tumor D2F2 expressing human ErbB-2 (D2F2/E2). All mice injected with pCMV control vector developed tumors within 2 wk. Six weeks after vaccination, only 8 ± 7% of mice vaccinated with E2 developed tumors, conferring >90% protection. Vaccination with E2A resulted in ~60% protection. CytE2 or cytE2A induced poor anti-tumor immunity, protecting only 30 and

### Table I. Induction of anti-tumor immunity with ErbB-2 DNA vaccines

| Vaccination | D2F2/E2 Tumor Incidence | Total Tumor Incidence (mean ± SD) |
|-------------|-------------------------|---------------------------------|
|             | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 | Exp. 5 | Exp. 6 |                |
| pCMV        | 8/8    | 8/8    | 8/8    | 8/8    | 8/8    | 5/5    | 100 ± 0        |
| E2          | 0/8    | 1/8    | 1/8*   | 4/8    | 3/8*   | 2/5    | 43 ± 7         |
| E2A         | 3/8*   | 4/8    | 4/8    | 4/8    | 6/8    | 4/5    | 89 ± 13        |
| cytE2       | 7/8    | 4/8    | 4/8    | 4/8    | 8/8    | 4/5    | 89 ± 13        |
| cytE2A      | 8/8    | 8/8    | 8/8    | 8/8    | 8/8    | 4/5    | 89 ± 13        |

* Mice were vaccinated with DNA constructs as described in Materials and Methods. The results of six independent experiments are reported.

* Two weeks after the final DNA vaccination, mice were challenged s.c. with 2 × 10^6 D2F2/E2 tumor cells. Tumor was palpated weekly, and D2F2 tumor incidence is reported as total number of animals with palpable tumor at 6 wk/total number of animals challenged with tumor.

* Total tumor incidence is the percentage of all animals from experiments 1–6 with palpable tumor.

* p < 0.05 by the Mantel-Haenszel Log-Rank test as compared to pCMV-vaccinated mice.
10% of immunized mice, respectively. These findings are consistent with our previously reported observation that transmembrane, but not cytoplasmic, ErbB-2 DNA vaccination resulted in significant protection against D2F2/E2 (18). Several mechanisms, including Ab production, may contribute to differential anti-tumor immunity, and this was analyzed.

Induction of anti-ErbB-2 Ab by ErbB-2 derivatives

Sera were collected 2 wk after the third vaccination and serially diluted, and anti-ErbB-2 Ab was measured by its binding to the breast cancer cell line, SKBR3, using flow cytometry. Vaccination with pCMV/E2 induced anti-ErbB-2 IgG in all mice with a titer of 104 ± 55 in eight tested mice (Fig. 1). E2A induced low levels of Ab in some but not all vaccinated mice. CytE2 or cytE2A did not induce Ab in any of the mice. The specificity of anti-ErbB-2 Ab was verified by its binding to D2F2/E2 but not control D2F2 cells. Also, sera from E2-vaccinated mice immunoprecipitated a 185-kDa protein from SKBR3 cells, which was recognized by anti-E2 mAb 3B5 in a Western blot (M. Piechocki, S. Pilon, and W.-Z. Wei, manuscript in preparation). Therefore, cytE2 and cytE2A, which were synthesized in the cytoplasm and degraded promptly by the proteosome, did not induce anti-ErbB-2 Abs. Because the amount of Ab induced by DNA vaccination was low, the differential Ab induction was verified in mice bearing tumors expressing individual mutant ErbB-2 proteins. Mice were injected i.p. with 2 × 105 D2F2 tumor cells transfected with E2, E2A, cytE2, or cytE2A. Expression of rErbB-2 or its derivatives was comparable in all test cells at the time of injection as verified by flow cytometry (data not shown). Sera were collected 4 wk after tumor injection from mice bearing tumors ~5 mm in diameter to ensure equivalent tumor load (Fig. 2). The growth of D2F2/E2 or D2F2/E2A, but not D2F2/cytE2 or D2F2/cytE2A, induced anti-ErbB-2 Abs, consistent with the finding with DNA vaccination. The titers were 4750 ± 1848 and 2425 ± 924 for D2F2/E2- and D2F2/E2A-bearing mice. The large number of growing tumor cells provided abundant Ags to stimulate Ab production. Still, anti-ErbB-2 Ab was not elicited by the growth of D2F2 tumor-expressing cytoplasmic forms of ErbB-2. To test whether Ab to the intracellular domain may have been induced, binding of Ab to fixed and permeabilized SKBR3 cells was tested. No detectable binding to the intracellular domain of ErbB-2 was detected in any of the immunized mice (data not shown).

Induction of anti-ErbB-2 Abs by DNA vaccination.

Sera were collected 2 wk after the third vaccination and serially diluted, and anti-ErbB-2 Ab was measured by its binding to the breast cancer cell line, SKBR3, using flow cytometry. Vaccination with pCMV/E2 induced anti-ErbB-2 IgG in all mice with a titer of 104 ± 55 in eight tested mice (Fig. 1). E2A induced low levels of Ab in some but not all vaccinated mice. CytE2 or cytE2A did not induce Ab in any of the mice. The specificity of anti-ErbB-2 Ab was verified by its binding to D2F2/E2 but not control D2F2 cells. Also, sera from E2-vaccinated mice immunoprecipitated a 185-kDa protein from SKBR3 cells, which was recognized by anti-E2 mAb 3B5 in a Western blot (M. Piechocki, S. Pilon, and W.-Z. Wei, manuscript in preparation). Therefore, cytE2 and cytE2A, which were synthesized in the cytoplasm and degraded promptly by the proteosome, did not induce anti-ErbB-2 Abs. Because the amount of Ab induced by DNA vaccination was low, the differential Ab induction was verified in mice bearing tumors expressing individual mutant ErbB-2 proteins. Mice were injected i.p. with 2 × 105 D2F2 tumor cells transfected with E2, E2A, cytE2, or cytE2A. Expression of rErbB-2 or its derivatives was comparable in all test cells at the time of injection as verified by flow cytometry (data not shown). Sera were collected 4 wk after tumor injection from mice bearing tumors ~5 mm in diameter to ensure equivalent tumor load (Fig. 2). The growth of D2F2/E2 or D2F2/E2A, but not D2F2/cytE2 or D2F2/cytE2A, induced anti-ErbB-2 Abs, consistent with the finding with DNA vaccination. The titers were 4750 ± 1848 and 2425 ± 924 for D2F2/E2- and D2F2/E2A-bearing mice. The large number of growing tumor cells provided abundant Ags to stimulate Ab production. Still, anti-ErbB-2 Ab was not elicited by the growth of D2F2 tumor-expressing cytoplasmic forms of ErbB-2. To test whether Ab to the intracellular domain may have been induced, binding of Ab to fixed and permeabilized SKBR3 cells was tested. No detectable binding to the intracellular domain of ErbB-2 was detected in any of the immunized mice (data not shown).

Induction of anti-ErbB-2 Abs by DNA vaccination.

Sera were collected 2 wk after the third vaccination and serially diluted, and anti-ErbB-2 Ab was measured by its binding to the breast cancer cell line, SKBR3, using flow cytometry. Vaccination with pCMV/E2 induced anti-ErbB-2 IgG in all mice with a titer of 104 ± 55 in eight tested mice (Fig. 1). E2A induced low levels of Ab in some but not all vaccinated mice. CytE2 or cytE2A did not induce Ab in any of the mice. The specificity of anti-ErbB-2 Ab was verified by its binding to D2F2/E2 but not control D2F2 cells. Also, sera from E2-vaccinated mice immunoprecipitated a 185-kDa protein from SKBR3 cells, which was recognized by anti-E2 mAb 3B5 in a Western blot (M. Piechocki, S. Pilon, and W.-Z. Wei, manuscript in preparation). Therefore, cytE2 and cytE2A, which were synthesized in the cytoplasm and degraded promptly by the proteosome, did not induce anti-ErbB-2 Abs. Because the amount of Ab induced by DNA vaccination was low, the differential Ab induction was verified in mice bearing tumors expressing individual mutant ErbB-2 proteins. Mice were injected i.p. with 2 × 105 D2F2 tumor cells transfected with E2, E2A, cytE2, or cytE2A. Expression of rErbB-2 or its derivatives was comparable in all test cells at the time of injection as verified by flow cytometry (data not shown). Sera were collected 4 wk after tumor injection from mice bearing tumors ~5 mm in diameter to ensure equivalent tumor load (Fig. 2). The growth of D2F2/E2 or D2F2/E2A, but not D2F2/cytE2 or D2F2/cytE2A, induced anti-ErbB-2 Abs, consistent with the finding with DNA vaccination. The titers were 4750 ± 1848 and 2425 ± 924 for D2F2/E2- and D2F2/E2A-bearing mice. The large number of growing tumor cells provided abundant Ags to stimulate Ab production. Still, anti-ErbB-2 Ab was not elicited by the growth of D2F2 tumor-expressing cytoplasmic forms of ErbB-2. To test whether Ab to the intracellular domain may have been induced, binding of Ab to fixed and permeabilized SKBR3 cells was tested. No detectable binding to the intracellular domain of ErbB-2 was detected in any of the immunized mice (data not shown).

**FIGURE 1.** Induction of anti-ErbB-2 Abs by DNA vaccination. BALB/c mice (n = 8) were immunized three times at 2-wk intervals with 100 μg of wild-type or mutant ErbB-2 DNA as indicated. Sera were collected after the third DNA vaccination and serially diluted. Anti-ErbB-2 IgG Ab was measured by its binding to SKBR3 cells and was measured by flow cytometry. Whole anti-ErbB-2 IgG was detected. The results are expressed as the titer of individual samples, and the mean value of each group was indicated by the cross bar in the shared block. *, p < 0.01 by Student’s t test as compared with pCMV-vaccinated mice.

**FIGURE 2.** Induction of anti-ErbB-2 Abs after tumor growth. BALB/c mice (n = 4) were injected with 2 × 105 D2F2 tumor cells overexpressing wild-type or mutant ErbB-2 proteins. One group of mice were depleted of CD4 T cells 6 days before the injection of 2 × 105 D2F2/E2 cells. Depleted state was maintained by i.p. injections of anti-CD4 Ab (GK1.5) every three days. Sera were collected 4 wk after tumor injection. Whole anti-ErbB-2 IgG was measured by flow cytometry. *, p < 0.01 by Student’s t test as compared with mice receiving D2F2 parental tumor.

To test whether CD4 T cells were required for Ab production, mice were injected i.p. with anti-CD4 mAb GK1.5 at 6 days before D2F2/E2 tumor injection; this was continued every 3 days for 4 wk until sera were collected. Anti-ErbB-2 Abs were detected in untreated mice but not in mice depleted of CD4 T cells (Fig. 2). Therefore, induction of anti-ErbB-2 Ab is a CD4 T cell-dependent process.

The subclass of IgG production is determined by CD4 T cells. Th1 cells, characterized by the production of IFN-γ, induce B cell isotype switch and IgG2a production, and Th2 cells induce IgG1 secretion (21). In E2 DNA-vaccinated mice, IgG2a was the predominant Ab, indicating Th1 activation (Fig. 3A). D2F2/E2 tumor growth in naive mice induced primarily IgG1, indicating Th2 activation (Fig. 3B).

**Inhibition of tumor growth by vaccination with cytoplasmic ErbB-2 and cytokine DNA**

The prompt degradation of cytoplasmic ErbB-2 or ErbB-2A was expected to generate a complete repertoire of antigenic peptides for CD8 T cell recognition (19). The poor anti-tumor activity of cytE2 and cytE2A DNA vaccination may reflect the lack of Ab or CD4 T cell help. CD4 help may be replaced, at least in part, by cytokine covaccination. To test whether exogenous cytokine can
provide the necessary help during cytE2A DNA vaccination, mice were vaccinated with a combination of cytE2A and cytokine DNA. Mice were immunized three times at 2-wk intervals with pCMV/cytE2A and pEFBos/IL-2 or pEFBos/GM-CSF (Fig. 4). Of the 10 immunized mice, 8 were protected from D2F2/E2 tumor growth whether they received the covaccination with IL-2 or GM-CSF DNA. Immunization with pCMV/cytE2A only protected one mouse, consistent with our earlier finding. None of the mice receiving pCMV, pEFBos/IL-2, or pEFBos/GM-CSF were protected. Anti-ErbB-2 Ab was not detected in any of the mice after DNA vaccination (data not shown), and protection against tumor growth may be largely the result of CD8 T cell activation.

Next, the induction of cytotoxic T cells by cytE2A and GM-CSF DNA vaccination was examined. Mice were sacrificed 6 wk after tumor challenge, and splenocytes were prepared and stimulated in vitro. In our experience BALB/c mammary tumor cells were very poor APCs and generally caused death of cocultured lymphocytes (data not shown). This may be due, at least in part, to the expression of Fas ligand on their surface (our unpublished results). To provide appropriate in vitro stimulation to CTL, APC were engineered. BALB/c 3T3 cells were transfected with E2 and Kd. Cell clones with stable expression of both ErbB-2 and Kd were selected.

CTL activity was measured by the 51Cr release assay after splenocytes were cultured with irradiated 3T3 stimulator cells for 5–7 days. Lysis of D2F2/E2 was observed at an E:T ratio of 10:1 or higher using CTL from mice that were immunized with pCMV/cytE2A and pEFBos/GM-CSF, and that had rejected D2F2/E2 tumor challenge (Fig. 5A). Control D2F2 cells were not lysed (Fig. 5B). The mice that were similarly immunized but failed to reject tumor did not demonstrate CTL activity. Mice immunized with control pCMV, pCMV/cytE2A, or pEFBos/GM-CSF developed tumor from the challenge, and CTL was not detected. These results indicated the expansion of CTL in cytE2A- and GM-CSF-vaccin-ated mice following tumor rejection.

To determine whether CD4 or CD8 T cells were required for tumor rejection, mice were vaccinated three times with cytE2A and GM-CSF DNA. One week after the final DNA vaccination or 1 wk before tumor challenge, mice were injected i.p. with mAb 2.43 to deplete CD8 T cells or GK1.5 to deplete CD4 T cells. T cell depletion was maintained for the remainder of the experiment by Ab injection every three days. Control pCMV-injected mice all developed tumor (Fig. 6). In 80% of mice vaccinated with cytE2A and GM-CSF, D2F2/E2 tumors were rejected. Depletion of CD4 T cells had no effect on tumor rejection, and 80% of the mice remained tumor free. Depletion of CD8 T cells resulted in <40% protection, indicating that CD8, but not CD4 T cells were required for tumor rejection.

Cytoplasmic ErbB-2 may be more effective than the transmembrane ErbB-2 at producing MHC class I peptides. It may be advantageous to include the cytoplasmic form of ErbB-2 in all vaccine regimens to enhance CD8 T cell activation. This hypothesis was tested by immunizing mice with 50 μg each of E2A and cytE2A (Fig. 7). Control groups received 100 μg of E2A or cytE2A. All mice that received the combination vaccine rejected tumor growth, whereas 50 and 20% of mice rejected tumor after they were immunized with E2A and cytE2A, respectively. The synergistic anti-tumor effect of covaccination with E2A and cytE2A is consistent with the notion that cytoplasmic ErbB-2 enhanced CTL activation.

To compare directly the presentation of MHC class I-associated peptides from ErbB-2 vs cytoplasmic ErbB-2, splenocytes were prepared from mice that rejected D2F2/E2 tumor after vaccination with cytE2A and GM-CSF DNA. Immune splenocytes were cultured for 7 days with irradiated 3T3 cells that were transfected with Kd and E2 or cytE2. CTL activity against D2F2/E2 cells was measured by chromium release assay (Fig. 8). CTL stimulated with either 3T3/Kd/E2 or 3T3/Kd/cytE2 lysed D2F2/E2, but not D2F2 cells. However, the lytic activity was much higher in CTL stimulated with 3T3/Kd/cytE2. At an E:T ratio of 40:1, ∼41 ± 6 and

FIGURE 4. Covaccination with cytE2A and cytokine DNA. Mice (n = 10 per group) were vaccinated three times at 2-wk intervals with 100 μg of the indicated plasmid DNA. Mice were challenged with 2 × 105 D2F2/E2 cells, and the percentage of tumor-free animals was recorded weekly for six consecutive weeks.

FIGURE 5. ErbB-2-specific CTL are present in tumor-free mice vaccinated with cytE2A and GM-CSF. BALB/c mice were vaccinated three times at 2-wk intervals with pCMV, GM-CSF, cytE2A, or cytE2A + GM-CSF. Two weeks after final DNA vaccination, mice were challenged with 2 × 105 D2F2/E2 tumor. At 6 wk after tumor challenge, splenocytes were isolated and incubated for 7 days with irradiated 3T3/Kd/E2 stimulator cells and used in a 4h chromium-release assay. D2F2/E2 (A) and D2F2 (B) were used as target cells. *, p < 0.01 as compared with lysis by pCMV-vaccinated mice. This experiment was repeated two times with similar results.

FIGURE 6. Requirement of CD8 T cells in tumor rejection. Mice were vaccinated three times at 2-wk intervals with 100 μg of pCMV or cytE2A + GM-CSF. CytE2A and GM-CSF vaccinated mice were further divided into three groups and were not treated, depleted of CD4 T cells, or depleted of CD8 T cells as described in Materials and Methods. There were eight mice in each group. Tumor incidence was recorded weekly for eight consecutive weeks.
15 ± 1% of D2F2/E2 cells were lysed by CTL incubated with 3T3/K\textsubscript{E2} and 3T3/K\textsubscript{E2A}, respectively. The levels of ErbB-2 or cytoplasmic ErbB-2 proteins in 3T3/K\textsubscript{E2} and 3T3/K\textsubscript{E2A} were comparable, as determined by flow cytometry (data not shown). These results strongly demonstrated an increased presentation of MHC class I peptides from cytoplasmic ErbB-2 when compared with ErbB-2.

Taken together, these results demonstrated that Ab-independent anti-tumor immunity was achieved by covaccination with DNA encoding cytoplasmic ErbB-2, which was efficiently processed and presented via the MHC class I pathway.

**Discussion**

Vaccination with E2, E2A, cytE2, or cytE2A resulted in ~90, 60, 30, and 10% protection against D2F2/E2 tumor, respectively (Table I). All recombinant proteins contained the entire ErbB-2 structural sequence, but the subcellular localization, membrane stability, and tyrosine kinase activity significantly affected their immunogenicity. Although anti-ErbB-2 Ab induced by E2 may contribute to the rejection of D2F2/E2 tumors, covaccination with cytE2 and cytokine DNA, which did not induce Ab, was also highly effective against D2F2/E2 tumors. CTL detected in immunized mice after they rejected D2F2/E2 tumors contributed to tumor rejection (Fig. 5) because depletion of CD8, but not CD4, T cells significantly reduced tumor protection (Fig. 6). Anti-tumor activity that could not be eliminated by CD4 or CD8 depletion may be a result of nonspecific effectors recruited to the tumor site. Enhanced presentation of CTL-reactive peptides from cytE2 was supported by synergistic anti-tumor activity after covaccination with E2A and cytE2A. Direct and unequivocal evidence of enhanced peptide presentation was provided by the significantly greater CTL-stimulating activity of APCs expressing cytE2 when compared with those expressing E2.

Anti-ErbB-2 Ab induced by E2 or E2A DNA vaccination was primarily IgG2a, indicating the activation of Th1 cells. IgG1 was induced in tumor-bearing mice, indicating a Th2 response (Fig. 3). It is not clear whether different Ab isotypes render different anti-tumor activity, although Th1 responses have been associated with anti-tumor effect (22). Anti-ErbB-2 Abs may exert anti-tumor activity via classical pathways such as complement fixation and Ab-dependent cell-mediated cytotoxicity or by inducing apoptosis via truncated signaling (23). But Abs have also been shown to interfere with anti-tumor immune T cell activity, implicating Ab production as a negative factor in anti-tumor activity (10, 11). With a comprehensive immune response to ErbB-2 that activates all effector arms, it is not possible to dissociate the roles of each component. Here we have demonstrated the feasibility of inducing effective anti-tumor cellular immunity without anti-ErbB-2 Ab. If Abs to a particular epitope prove to be safe and beneficial, it will be advantageous to elicit such Ab with defined ErbB-2 peptide fragments rather than whole protein (24).

Consistent with our earlier findings, ErbB-2 vaccines with lysine to alanine substitution at amino acid 753 in the intracellular domain (E2A and cytE2A) were less effective than their native counterparts. The single point mutation eliminated tyrosine kinase activity and correlated with decreased membrane stability of ErbB-2A. The expression level of E2A in transfected cells was about half that of E2 when measured by flow cytometry and Western blotting (data not shown). It is possible that interaction between E2A and chaperon proteins, such as grp94 in the ER (25) and heat shock protein 90 in the cytoplasm (26), was altered by the mutation, resulting in reduced stability. The mutation may also alter the interaction between E2A and the ubiquitin ligase, c-Cbl, to accelerate E2A degradation (27). Any of these mechanisms may reduce stability and alter processing of E2A for T cell activation.

It is not clear how membrane-associated ErbB-2 is processed through the MHC class II processing pathway for CD4 T cell activation. Transmembrane ErbB-2 shed from tumor cells may be phagocytosed and reprocessed by APCs. When ErbB-2 is activated by heterodimerization with other members of the ErbB-2 family, the complexes are endocytosed and may be directed to the lysosome for degradation (28). In the lysosome, ErbB-2 may be degraded into peptides that can be presented with MHC class II molecules to ErbB-2-specific CD4 T cells. Because of its cytosolic localization and rapid degradation, cytoplasmic ErbB-2 will not be targeted to the lysosome and will not be a candidate for the MHC class II processing pathway and, therefore, be unable to activate CD4 T cells.

Presentation of MHC class I peptides without costimulation signals may result in suppressed or anergized anti-tumor CTL. Garza et al. (29) have shown in a lymphocytic choriomeningitis virus glycoprotein transgenic system, immunization with lymphocytic choriomeningitis virus glycoprotein peptide could induce activation and expansion of Ag-specific CTL. In the absence of activated APCs, these activated T cells were rapidly deleted and tolerance was induced. In the current study, vaccination with cytoplasmic ErbB-2 may be comparable to vaccination with the entire repertoire of MHC class I-associated peptides. Without costimulation signals, a short-lived CTL response may be induced. Only by covaccination with a cytokine gene was an effective anti-tumor response observed. Expression of IL-2 at the site of vaccination may provide signals for CTL survival and expansion. Coexpression of GM-CSF may recruit and activate APC to process and present ErbB-2 epitopes for full CTL activation.
Results from this study demonstrated the feasibility of turning on anti-tumor CTL without the involvement of Abs. With this test system, the positive and negative effect of anti-ErbB-2 Abs in tumor rejection can be defined without ambiguity. ErbB-2-based vaccination and immunotherapy can be designed rationally with these tools and knowledge. The same principles can be applied to improve the efficacy of most vaccines.

References

1. Inglehart, J., M. Kraus, B. Langton, G. Huper, B. Kerns, and J. Marks. 1990. Increased erbB-2 gene copies and expression in multiple stages of breast cancer. Cancer Res. 50:6701.

2. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, and M. F. Press. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707.

3. Slamon, D. J., G. M. Clark, S. G. Wong, J. W. Levin, A. Ullrich, and W. L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177.

4. Disis, M. L., S. M. Papa, J. R. Gralow, R. Dittadi, S. Menard, and M. A. Cheever. 1997. High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. J. Clin. Oncol. 15:3563.

5. Disis, M. L., E. Calanoff, M. Calaforni, A. E. Murphy, W. Chen, B. Groner, M. Jeschke, N. Lydon, E. McGlynn, R. B. Livingston, et al. 1994. Existence T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res. 54:16.

6. Peoples, G. E., P. S. Goedegebuure, R. Smith, D. C. Linehan, I. Yoshino, and T. J. Eberlein. 1995. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc. Natl. Acad. Sci. USA 92:432.

7. Fisk, B., B. Anderson, K. R. Gravitt, C. A. Ogg, S. Misra, K. L. Rock. 1995. Rate of tumor rejection in mice treated with MCA: a comparison of HER-2/neu transgenic mice. Cancer Res. 55:2931.

8. Ewer, M. S., H. R. Gibbs, J. Swafford, and R. S. Benjamin. 1999. Cardotoxicity in patients receiving Trastuzumab (Herceptin): primary toxicity, synergistic or sequential stress, or surveillance artifact? Semin. Oncol. 26(Suppl. 12):96.

9. Hurwitz, E., I. Stancovski, M. Sela, and Y. Yarden. 1995. Suppression and protection of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. Proc. Natl. Acad. Sci. USA 92:5533.

10. Qin, Z., G. Richter, T. Schuler, S. Ibe, X. Cao, and T. Blankenstein. 1998. B cells inhibit induction of T cell-dependent tumor immunity. Nat. Med. 4:627.

11. Feldman, J. 1972. Immunological enhancement: a study of blocking antibodies. Adv. Immunol. 15:167.

12. Chen, Y., D. Hu, D. J. Eling, J. Robbins, and T. J. Kipps. 1998. DNA vaccines encoding full-length or truncated neu induce protective immunity against neu-expressing mammary tumors. Cancer Res. 58:1965.

13. Rovero, S., A. Amici, E. DeCarlo, R. Bei, P. Nanni, E. Quaglini, P. Porcedda, K. Boggio, A. Smorlesi, P. L. Lollini, et al. 2000. Inhibition of carcinogenesis by expressing mammary tumors. Adv. Immunol. 15:167.

14. Reilly, R. T., M. B. Gottlieb, A. M. Ercolini, J. H. Machiels, C. E. Kane, F. I. Okoye, W. J. Muller, K. H. Dixon, and E. M. Jaifee. 2000. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. Cancer Res. 60:3569.

15. Fendly, B. M., C. Kotts, W. L. T. Wong, I. Figari, W. Harel, L. Staib, M. E. Carver, D. Vetterlein, M. S. Mitchell, and H. M. Shephard. 1993. Successful immunization of rhesus monkeys with the extracellular domain of p185HER2: a potential approach to human breast cancer. Vaccine Res. 2:129.

16. Zaks, T. Z., and S. Rosenberg. 1998. Immunization with a peptide epitope (p369–377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. Cancer Res. 58:4902.

17. Disis, M. L., J. R. Gralow, H. Bernhard, S. L. Hand, W. D. Rubin, and M. A. Cheever. 1996. Peptide-based, but not whole protein, vaccines elicit immunity to HER-2/neu, oncogenic self protein. J. Immunol. 156:3151.

18. Wei, W. Z., W. P. Shi, A. Galy, D. Lichlyter, S. Hernandez, B. Groner, L. Heilbrun, and R. F. Jones. 1999. Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA. Int. J. Cancer 81:1.

19. Grant, E. P., M. T. Michalek, A. L. Goldberg, and K. L. Rock. 1995. Rate of antigen degradation by the ubiquitin-proteasome pathway influences MHC class I presentation. J. Immunol. 155:3750.

20. Mahoney, K. H., B. E. Miller, and G. H. Heppner. 1985. FACS quantitation of leucine aminopeptidase and acid phosphatase on tumor associated macrophages from metastatic and nonmetastatic mouse mammary tumors. J. Leukocyte Biol. 38:573.

21. Toellner, K., S. A. Luther, D. M. Sze, D. M. Sze, R. K. Choy, D. R. Taylor, S. Habu, H. Tashiro, M. Sato, and A. Ohta. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. J. Exp. Med. 187:1193.

22. Nishimura, T., K. Iwakabe, M. Sekimoto, Y. Ohmi, T. Yahata, M. Nakui, T. Sato, H. Tashiro, M. Sato, and A. Ohta. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. J. Exp. Med. 190:617.

23. Sliwkowski, M. X., J. A. Lodgren, G. D. Lewis, T. E. Hotaling, B. M. Fendly, and J. A. Fox. 1999. Nonclinical studies addressing the mechanism of action of Trastuzumab (Herceptin). Semin. Oncol. 26:60.

24. Dakappagari, N. K., D. B. Douglas, P. L. Tirozzi, V. C. Stevens, and P. T. P. Kaumaya. 2000. Prevention of mammary tumors with a chimeric HER-2 B-cell epitope peptide vaccine. Cancer Res. 60:3782.

25. Chavany, C., E. Mimnaugh, P. Miller, R. Bitton, P. Nguyen, J. Trepel, L. Whitesel, R. Schmurt, J. D. Moyer, and L. Necker. 1996. p185neu encoded human ErbB-2 DNA vaccination. J. Immunol. 155:3750.

26. Schaff, T. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. J. Exp. Med. 190:617.

27. Soga, S., L. M. Necker, T. W. Schulte, Y. Shiotsu, K. Akasaka, H. Narumi, T. Agatsuma, Y. Ikana, C. Murakata, T. Tamaoki, and S. Akinaga. 1999. KF25706, a novel oxime derivative of radicicol, exhibits in vivo antitumor activity via selective depletion of Hsp90 binding signaling molecules. Cancer Res. 59:2931.

28. Klappper, L. N., H. Waterman, M. Sela, and Y. Yarden. 2000. Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquination of HER-2. Cancer Res. 60:3384.

29. Gilboa, L., R. Ben-Levy, Y. Yarden, and Y. I. Henis. 1995. Roles for a cytoplasmic tyrosine and tyrosine kinase activity in the interaction of neu receptors with coated pits. J. Biol. Chem. 270:7091.

30. Garza, K. M., S. M. Chan, R. Suri, L. T. Nguyen, B. Odermatt, S. P. Schoenberger, and P. S. Ohashi. 2000. Role of antigen-presenting cells in mediating tolerance and autoimmunity. J. Exp. Med. 191:2021.