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ErbB-2 is a tumor associated antigen. Vaccination with wild-type ErbB-2 DNA induces effective anti-tumor immunity which includes a strong anti-ErbB-2 antibody response. To activate specific components of the immune response, DNA vaccines were constructed which direct recombinant ErbB-2 to the subcellular compartments of antigen processing and presentation. To induce ErbB-2 specific CTL, a recombinant cytoplasmic ErbB-2 which lacks kinase activity was constructed. CytE2 localizes in the cytoplasm and is rapidly degraded by the proteosome. Vaccination with cytE2 alone does not protect against an ErbB-2 expressing tumor. This may be due to a lack of CD4 T cell induction. To activate or replace ErbB-2 specific CD4 T cells, co-vaccination of cytE2A with cytokine genes was examined. Co-vaccination of cytE2A with either IL-2 or GM-CSF genes induced anti-ErbB-2 CTL and an effective anti-tumor immune response. To induce ErbB-2 specific CD4 T cells, a recombinant ErbB-2 which is targeted to the lysosome for MHC class II processing and presentation was constructed. Co-vaccination with E2-Lamp + GM-CSF induced effective anti-tumor immunity. This study demonstrates the feasibility of eliciting individual effector mechanisms by vaccination with targeted DNA constructs and protection against ErbB-2 expressing tumors without antibody activity.

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INTRODUCTION

The goal of this study is to prevent the growth or recurrence of breast cancer by active vaccination with a tumor antigen, ErbB-2. The specific objectives are to generate and test recombinant DNA vaccines that can induce a strong anti-tumor immune response. Human tumor associated antigens, such as ErbB-2, are generally self antigens and may be associated with transforming activities. In our recombinant vaccines, the transforming activity of ErbB-2 is eliminated by point mutation. Recombinant ErbB-2 molecules are directed to the subcellular compartments of antigen processing and presentation and the generation of an anti-tumor immune response is characterized. Co-vaccination with cytokine genes such as IL-2 or GM-CSF is also tested. The reagents developed in this study will be candidate breast cancer vaccines and the principles established by this study will be applicable to new tumor antigens.

SPECIFIC TASKS

1 Continue to modify and test recombinant cytoplasmic ErbB-2 which are free of tyrosine kinase activity
2 Construct and test recombinant ERBB-2 which is targeted to MHC II antigen processing pathway
3 Enhance vaccine efficacy by local IL-2 secretion and by expression with adenoviral vectors

STUDIES AND RESULTS

Task 1 **Continue to modify and test recombinant cytoplasmic ErbB-2 which are free of tyrosine kinase activity.**

Our lab has reported the generation of several recombinant ErbB-2 (E2) DNA constructs. ErbB-2A (E2A) contains a lysine to alanine point mutation at amino acid 753 to eliminate ATP binding and abolish the tyrosine kinase activity of ErbB-2. To direct recombinant ErbB-2 to the cytoplasm where MHC class I processing occurs, the ER signal sequence was deleted to generate cytoplasmic ERBB-2 (cytE2). Cytoplasmic ErbB-2A (cytE2A) is targeted to the cytoplasm and contains the lysine to alanine point mutation at amino acid 753. To characterize the protein products, the mouse mammary tumor, D2F2, was transfected with the mutant constructs and stable transfectants were selected. Expression of the recombinant proteins was measured by flow cytometry. Transmembrane ErbB-2 and ErbB-2A were readily detected. Cytoplasmic ErbB-2 and cytE2A proteins could be detected only after the transfected cells were incubated overnight with a proteasome inhibitor, indicating degradation shortly after synthesis. ErbB-2 constructs with the lysine to alanine mutation were not phosphorylated as verified by Western Blot. The immunogenicity and vaccination efficacy of cytoplasmic ErbB-2 DNA was further studied and is reported in task 3.

Task 2 **Construct and test recombinant ErbB-2 which is targeted to MHC II antigen processing pathway**

A recombinant ErbB-2 (E2-Lamp) DNA vaccine was generated in our lab to direct ErbB-2 to the lysosome. E2-Lamp encodes a fusion protein with the extracellular and transmembrane domains of ERBB-2 fused to the cytoplasmic tail of the lysosomal-associated membrane protein (lamp-1). This Lamp-1 tail is expected to direct the recombinant protein to the lysosome where it can be degraded for presentation with MHC class II molecules.

To test the efficacy of E2-Lamp, mice were immunized with 100 μg each of E2-Lamp and GM-CSF DNA. Mice received three vaccinations at two week intervals. After vaccination, no anti-ErbB-2 antibody was detected. At two weeks after the final DNA vaccination, mice were challenged with $2x10^5$ EL-4 tumor expressing wild-type ErbB-2. While all of the control mice developed tumors, 80% of the vaccinated mice were protected.

Task 3 **Enhance vaccine efficacy by local IL-2 secretion and by expression with adenoviral vectors**

3.1 Anti-tumor immunity induced by recombinant ERBB-2 constructs

A manuscript has been published in the Journal of Immunology and is attached in Appendix A. Vaccination with E2, E2A, cytE2, or cytE2A resulted in approximately 90, 60, 30 and 10% protection against D2F2/E2 tumor, respectively. Anti-ErbB-2 antibody was induced by immunization with transmembrane, but not cytoplasmic, ErbB-2 DNA. The same pattern, but elevated antibody response was observed in mice bearing mouse mammary tumor D2F2 expressing transmembrane , but not cytoplasmic, ErbB-2. Depletion of CD4 T cells abolished anti-ErbB-2 antibody production. Vaccination with transmembrane ErbB-2 constructs induced anti-ErbB-2 antibody of the IgG2a subset which is indicative of a Th1 response.

Vaccination with cytE2 or cytE2A alone induces very little anti-tumor immunity. The prompt degradation of cytoplasmic ErbB-2 was expected to generate a complete repertoire of antigenic peptides for CD8 T cell recognition. The poor anti-tumor activity of cytE2 DNA vaccination may reflect the lack of antibody or CD4 T cell help. CD4 help may be replaced, at least in part, by cytokine co-vaccination. Co-vaccination with cytE2A and GM-CSF or IL-2 DNA induced 80% protection against D2F2/E2 tumor. No anti-ErbB-2 antibody was detected after vaccination. Co-vaccination with cytE2 and GM-CSF DNA and depletion of CD8 T cells prior to tumor challenge abolished tumor protection. Depletion of CD4 T cells had no effect. These results indicate that cytE2 is an effective vaccine if a cytokine gene is also given. Vaccination with cytE2 or cytE2A activates anti-ErbB-2 CD8 T cells without inducing antibody or activating CD4 T cells.

Growth of D2F2/E2 tumor induces anti-ErB-2 antibody which may contribute to tumor rejection in cytE2 vaccinated mice. To test if tumor rejection is entirely independent of antibody, vaccinated mice were challenged with D2F2 expressing cytoplasmic ErbB-2 (D2F2/cytE2). Vaccination with cytE2 and GM-CSF induced 100% protection against D2F2/cytE2. Interestingly, vaccination with E2 + GM-CSF DNA only protected 63% of mice against D2F2/cytE2. Depletion of CD8 T cells prior to challenge with D2F2/cytE2 abolished tumor rejection, regardless of vaccination. These results indicate that cytE2 + GM-CSF vaccination induced a strong anti-ErbB-2 CD8 T cell response and tumor rejection which is independent of anti-ErbB-2 antibody.

After three vaccinations with cytE2A and GM-CSF DNA, ErbB-2 specific CTL could be detected using a standard chromium release assay against D2F2/E2. CTL were also detected in mice immunized with cytE2A and GM-CSF that rejected D2F2/E2. CTL could not be detected in any tumor bearing mouse regardless of vaccination. Nonspecific killing of D2F2 cells was not detected.

3.2 **Generation of adenoviral vector with cytE2 and verification of protein expression.**

The plan to construct adenovirus expressing potentially oncogenic ErbB-2 was abandoned due to the high risk. Rather, ErbB-2 DNA will be modified to contain a fragment of an exogenous antigenic sequence. It is expected that this construct will enhance T cell responses and overcome potential tolerance.

KEY RESEARCH ACCOMPLISHMENTS

- Anti-ErbB-2 antibody of the IgG2a subset was inducted by vaccination with transmembrane, but not cytoplasmic, ErbB-2.
- Anti-tumor immunity against D2F2/E2 was induced by vaccination with transmembrane ErbB-2 or by co-vaccination with cytE2 and IL-2 or GM-CSF genes. The latter does not induce anti-ErbB-2 antibody production.
- Vaccination with cytE2A and GM-CSF DNA was superior to E2 vaccination in protection against tumors expressing cytoplasmic ErbB-2.
- Rejection of D2F2/E2 tumor after vaccination with cytE2A and GM-CSF was dependent on CD8, but not CD4, T cells.
- Anti-ErbB-2 CTL was detected after vaccination with cytE2 + GM-CSF and in mice which had rejected tumor.
- Anti-tumor immunity was induced after vaccination with E2-Lamp + GM-CSF genes in the absence of antibody production.

REPORTABLE OUTCOMES

Publications

1. Pilon, S.A., Piechocki, M.P., and Wei, W.Z. 2001. Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody. *Journal of Immunology* 167: 3201.

2. Wei, W. Z., Shi, W. P., Galy, A., Lichlyter, D., Hernandez, S., Groner, B., Heilbrun, L.,and Jones, R. F. 1999. Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA. *Int.J.Cancer* 81, *1-7.*

Abstracts

1. Pilon, S., Kelly, C., Marriott, E., and Wei, W.Z. 1999. Protection against mammary tumor growth by vaccination with recombinant ERBB-2 DNA encoding transmembrane or cytoplasmic protein. *The Faseb Journal* 13, A645.

2. Pilon, S.A., Piechocki, M.P., and Wei, W.Z. 2000. IL-2 or GM-CSF substitute for CD4 T cell help in cytERBB-2 DNA vaccination to induce anti-tumor immunity. *AACR Proceedings* 41, 5060.

3. Pilon, S.A., and Wei, W.Z. 2001. CD4 T cells are required for epitope spreading against tumor antigens. *The Faseb Journal*

Presentations

1. Anti-tumor immunity induced by recombinant ERBB-2 DNA Vaccines. DOD 2000 Era of Hope Meeting, "New Investigators: The Future of Breast Cancer Research", Atlanta, GA

2. Anti-tumor immunity induced by cytoplasmic ErbB-2 DNA vaccination. 2000 Graduate Student Research Day, Wayne State University, Detroit, MI (3rd place in Oral Competition)

List of personnel receiving pay from research effort:

Shari A Pilon, PhD candidate

CONCLUSIONS

ErbB-2 is a tumor associated antigen. Vaccination with wild-type ErbB-2 DNA induces effective anti-tumor immunity which includes a strong anti-ErbB-2 antibody response. To activate specific components of the immune response, DNA vaccines were constructed which direct recombinant ErbB-2 to the subcellular compartments of antigen processing and presentation. To induce ErbB-2 specific CTL, a recombinant cytoplasmic ErbB-2 which lacks kinase activity was constructed. CytE2 localizes in the cytoplasm and is rapidly degraded by the proteosome. Vaccination with cytE2 alone does not protect against an ErbB-2 expressing tumor. This may be due to a lack of CD4 T cell induction. To activate or replace ErbB-2 specific CD4 T cells, co-vaccination of cytE2A with cytokine genes was examined. Co-vaccination of cytE2A with either IL-2 or GM-CSF genes induced anti-ErbB-2 CTL and an effective anti-tumor immune response. To induce ErbB-2 specific CD4 T cells, a recombinant ErbB-2 which is targeted to the lysosome for MHC class II processing and presentation was constructed. Co-vaccination with E2-Lamp + GM-CSF induced effective anti-tumor immunity. This study demonstrates the feasibility of eliciting individual effector mechanisms by vaccination with targeted DNA constructs and protection against ErbB-2 expressing tumors without antibody activity.

Acronym and Symbol Definition

CTL	Cytotoxic T cell
CytE2	ERBB-2 lacking ER signal sequence
cytE2A	ERBB-2 lacking ER signal sequence and lacking tyrosine kinase activity
E2A	ERBB-2 lacking tyrosine kinase activity
ER	Endoplasmic reticulum
ErbB-2	Transmembrane protein
E2	Gene encoding transmembrane protein ErbB-2
GM-CSF	Granulocyte-macrophage colony stimulating factor
IL-2	Interleukin 2
MHC	Major histocompatibility complex

Appendix A

Vaccination with Cytoplasmic ErbB-2 DNA Protects Mice from Mammary Tumor Growth Without Anti-ErbB-2 Antibody[1]

Shari A. Pilon,* Marie P. Piechocki,† and Wei-Zen Wei[2]*†

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). Herceptin, 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)[3] 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

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[2] Address correspondence and reprint requests to Dr. Wei-Zen Wei, Karmanos Cancer Institute, 110 E. Warren, Detroit, MI 48201. E-mail address: weiw@karmanos.org

[3] Abbreviations used in this paper: cytE2, full-length ErbB-2 targeted to the cytoplasm; ER, endoplasmic reticulum; E2, wild-type ErbB-2; E2A, tyrosine kinase-deficient ErbB-2; cytE2A, tyrosine kinase-deficient cytE2.

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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 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 cosmic 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 lines cotransfected with rERBB-2 constructs and pRSV2/neo: 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 (Osaka University, Osaka, Japan). BALB/c mice at 6–8 wk of age received i.m. injections of plasmid DNA 1–2 μg/μl suspended in saline with 50 μl in each thigh. Vaccination was repeated three times at 2-wk intervals.

Tumor challenge

At 2 wk after the final DNA vaccination, mice were challenged s.c. in the right flank with 2×10^5 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 anti-mouse 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 antiserum 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 2×10^5 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 separation and incubated with irradiated stimulator 3T3 cells transfected with K^d 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 [^{51}Cr]chromate 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[a]	D2F2/E2 Tumor Incidence[b]						Total Tumor Incidence[c] (mean ± SD)
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	
pCMV	8/8	8/8	8/8	8/8	8/8	5/5	100 ± 0
E2	0/8*	1/8*	1/8*				8 ± 7
E2A	3/8*	4/8		4/8	3/8*	2/5	43 ± 7
cytE2	7/8		4/8				69 ± 27
cytE2A	8/8			8/8	6/8	4/5	89 ± 13

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

[b] Two weeks after the final DNA vaccination, mice were challenged s.c. with 2×10^5 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.

[c] 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 SKBR 3 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 s.c. with 2×10^5 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 SKBR-3 cells was tested. No detectable binding to the intracellular domain of ErbB-2 was detected in any of the immunized mice (data not shown).

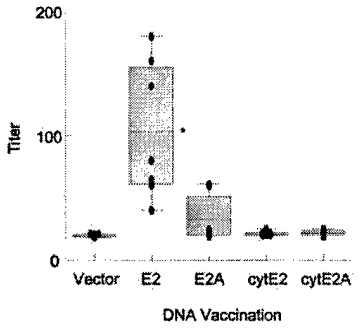

FIGURE 1. Induction of anti-ErbB-2 Abs by DNA vaccination. BALB/c mice ($n = 8$) were immunized three times at 2-wk intervals with 100 μg of wild-type or mutant ErbB-2 DNA as indicated. Sera were collected after the third DNA vaccination and serially diluted. Anti-ErbB-2 IgG Ab was measured by its binding 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.

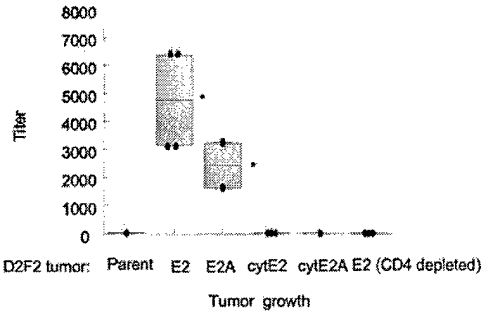

FIGURE 2. Induction of anti-ErbB-2 Abs after tumor growth. BALB/c mice ($n = 4$) were injected with 2×10^5 D2F2 tumor cells overexpressing wild-type or mutant ErbB-2 proteins. One group of mice were depleted of CD4 T cells 6 days before the injection of 2×10^5 D2F2/E2 cells. Depleted state was maintained 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. 3*A*). D2F2/E2 tumor growth in naive mice induced primarily IgG1, indicating Th2 activation (Fig. 3*B*).

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

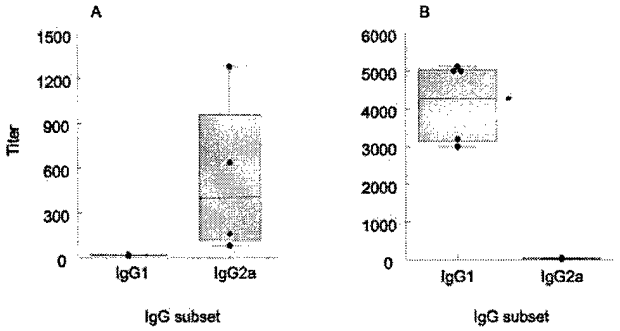

FIGURE 3. Anti-ErbB-2 IgG subsets induced by E2 vaccination or tumor growth. *A*, Sera ($n = 4$) were collected 1 wk after the third pCMV/E2 DNA vaccination as described. IgG1 and IgG2a subtypes were measured. *B*, Sera ($n = 6$) were collected 4 wk after the injection of 2×10^5 D2F2/E2 cells. IgG1 and IgG2a subsets were measured. The results are expressed as the titer of individual samples. *, $p < 0.001$ by Student's t test as compared with naive mice.

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 K^d. Cell clones with stable expression of both ErbB-2 and K^d were selected.

CTL activity was measured by the ^{51}Cr 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. 5*A*). Control D2F2 cells were not lysed (Fig. 5*B*). 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-vaccinated 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 K^d and E2 or cytE2. CTL activity against D2F2/E2 cells was measured by chromium release assay (Fig. 8). CTL stimulated with either 3T3/K^d/E2 or 3T3/K^d/cytE2 lysed D2F2/E2, but not D2F2 cells. However, the lytic activity was much higher in CTL stimulated with 3T3/K^d/cytE2. At an E:T ratio of 40:1, ~41 ± 6 and

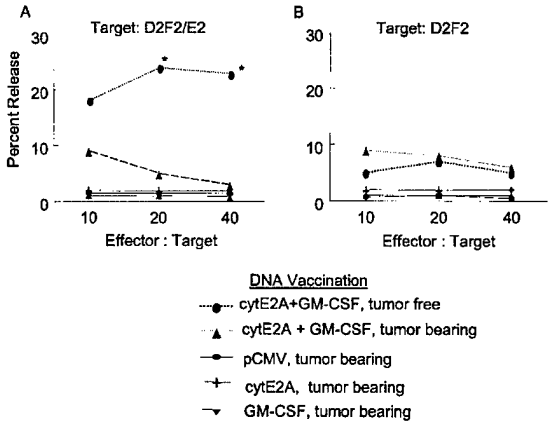

FIGURE 5. ErbB-2-specific CTL are present in tumor-free mice vaccinated with cytE2A and GM-CSF. BALB/c mice were vaccinated three times at 2-wk intervals with pCMV, GM-CSF, cytE2A, or cytE2A + GM-CSF. Two weeks after final DNA vaccination, mice were challenged with 2×10^5 D2F2/E2 tumor. At 6 wk after tumor challenge, splenocytes were isolated and incubated for 7 days with irradiated 3T3/K^d/E2 stimulator cells and used in a 4-h 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.

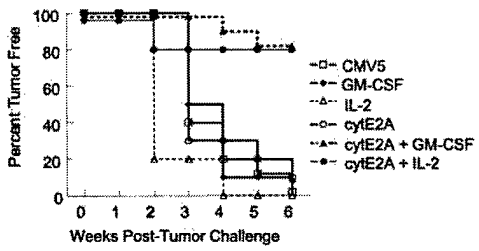

FIGURE 4. Covaccination with cytE2A and cytokine DNA. Mice (n = 10 per group) were vaccinated three times at 2-wk intervals with 100 μg of the indicated plasmid DNA. Mice were challenged with 2×10^5 D2F2/E2 cells, and the percentage of tumor-free animals was recorded weekly for six consecutive weeks.

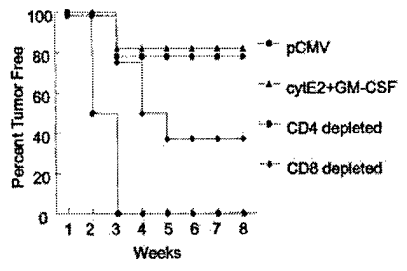

FIGURE 6. Requirement of CD8 T cells in tumor rejection. Mice were vaccinated three times at 2-wk intervals with 100 μg of pCMV or cytE2A + GM-CSF. CytE2A and GM-CSF vaccinated mice were further divided into three groups and were not treated, depleted of CD4 T cells, or depleted of CD8 T cells as described in *Materials and Methods*. There were eight mice in each group. Tumor incidence was recorded weekly for eight consecutive weeks.

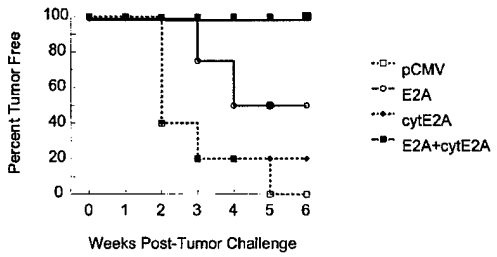

FIGURE 7. Covaccination with E2A and cytE2A results in enhanced tumor protection. BALB/c mice were vaccinated three times at 2-wk intervals with 100 μg of pCMV, E2A, cytE2A, or 50 μg each of cytE2A and E2A. Two weeks after the third DNA vaccination, mice were challenged with 2×10^5 D2F2/E2 cells. Tumor incidence was measured weekly.

15 ± 1% of D2F2/E2 cells were lysed by CTL incubated with 3T3/K^d/cytE2 and 3T3/K^d/E2, respectively. The levels of ErbB-2 or cytoplasmic ErbB-2 proteins in 3T3/K^d/cytE2 and 3T3/K^d/E2 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.

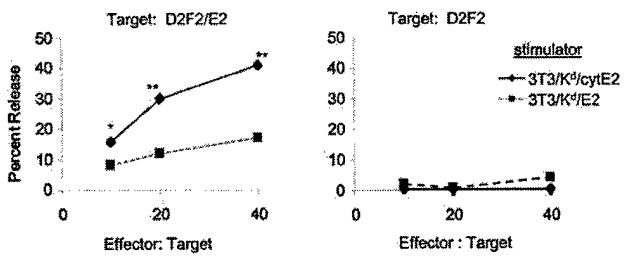

FIGURE 8. Stimulation of ErbB-2-specific CTL by APCs expressing ErbB-2 or cytoplasmic ErbB-2. Splenocytes were isolated from mice which were immunized with cytE2A and GM-CSF DNA and rejected D2F2/E2 tumors. Cells were incubated for 7 days with irradiated 3T3/K^d/cytE2 or 3T3/K^d/E2 cells, and CTL activity was tested in a 4-h chromium release assay. D2F2 and D2F2/E2 were used as target cells. **, $p < 0.001$; *, $p < 0.05$ when compared with lysis by CTL activated with 3T3/K^d/cytE2 cells.

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.

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