

Autologous neu DNA vaccine can be as effective as xenogenic neu DNA vaccine by altering administration route

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Abstract

We examined the therapeutic efficacy of xenogenic human N'-terminal neu DNA vaccine and autologous mouse N'-terminal neu DNA vaccine on MBT-2 tumor cells in C3H mice. Intramuscular injection of xenogenic and autologous neu DNA vaccines produced comparable therapeutic efficacies. Mouse and human N'-neu DNA vaccine induced tumor infiltration of CD8⁺ T cells, while the human vaccine was less effective at stimulating natural killer cells. Depletion of CD8⁺ T cells abolished the therapeutic efficacy of both types of DNA vaccines. On the other hand, xenogenic neu DNA vaccine showed significantly better therapeutic efficacy than autologous DNA vaccine with gene gun immunization. Increased infiltration of CD8⁺ T cells was correlated with enhanced therapeutic efficacy in the human N'-neu group of mice. Therefore, intramuscular injection can enhance the therapeutic efficacy of autologous neu DNA vaccine.

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1. Introduction

The HER-2/ErbB-2/neu proto-oncogene encodes a 185 kDa protein (p185^{neu}) belonging to the epidermal growth factor receptor (EGFR) family. The protein is overexpressed in several types of cancer including breast, gastric, and bladder carcinomas. This involvement in cancer progression and worsening prognosis makes p185^{neu} an attractive target for cancer therapy [1]. Small interfering RNA (siRNA) inhibits the expression of the HER2/neu gene and induces apoptosis of HER-2 positive cancer cells [2].

Passive immunological treatment with Herceptin, a humanized anti-neu antibody, has shown anti-tumor activity in xenograft models and clinical trials [3,4]. The combinational use of chemotherapy and Herceptin can be beneficial [5], although combination therapy involving anthracyclines and Herceptin is associated with cardiac side effects [6,7], which may cause mitochondrial dysfunction in cardiomyocytes [8]. Prolonged administration of HER-2/neu-specific monoclonal antibody has generated neu antigen-negative tumor variants in a transgenic animal model [9].

Active vaccination with either DNA or proteins targeting p185^{neu} provides an alternative therapeutic approach. Vaccination against peptides and domains of p185^{neu} has prevented tumor formation in several animal models [10,11]. Recently, a Phase I clinical trial indicated that a HER-2/neu intracellular domain (ICD) protein vaccine incorporating granulocyte-macrophage colony-stimulating factor as an adjuvant was

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well tolerated and effective in eliciting neu-specific T-cell and antibody immunity [12]. Moreover, HER-2/neu peptide based vaccines induce anti-neu humoral response and cause humoral epitope-spreading in cancer patients [13]. DNA encoding full-length or truncated neu induces protective immunity against neu-expressing mammary tumors [14–19]. The efficacy of HER2/neu DNA as a therapeutic cancer vaccine for established tumors has also been demonstrated *in vivo* [20–23]. Most of these latter studies were performed on tumor cells artificially expressing exogenous p185^{neu} generated either by transgenic injection or by cell transfection [14–23]. Extending these observations, we have demonstrated the therapeutic efficacy of HER2/neu DNA vaccine on mouse tumors that naturally over-express mouse neu [24].

Xenogenic DNA may be required for effective induction of immunity with DNA vaccine against EGFR [25]. However, whether xenogenic DNA is required for DNA vaccines against other antigens including HER2/neu is presently unclear. Several lines of evidence suggest that immunological responses toward ErbB-2/neu may be different from those directed towards ErbB-1/EGFR. Neu-derived peptide epitopes are recognized by cancer-specific cytotoxic T lymphocytes and p185^{neu} antibody in cancer patients [26–29]. Furthermore, autologous human HER2/neu protein and peptide may induce appropriate cellular and humoral immunity in human patients [12].

In this report, we directly compare the therapeutic efficacy of DNA vaccines encoding the extracellular domain of either autologous mouse or xenogenic human p185^{neu} in a mouse tumor naturally overexpressing endogenous p185^{neu}. Our results indicate that autologous mouse neu DNA vaccine is comparably effective as the xenogenic human neu DNA vaccine following intramuscular injection. In contrast, xenogenic neu DNA vaccine is more effective than autologous neu DNA vaccine following gene gun administration.

2. Results

2.1. Construction and characterization of N-terminal extracellular domains of mN'-neu and hN'-neu DNA plasmids

The N-terminal of mouse p185^{neu} was cloned from mRNA of MBT-2 bladder cancer cells with RT-PCR and verified by DNA sequencing. The N-terminal of human p185^{neu} was subcloned from a full-length human p185^{neu} plasmid. Both mouse N'-neu and human N'-neu cDNA was expressed under the control of CMV promoter as described before [24], and named as mN'-neu and hN'-neu, respectively (Fig. 1A). COS-1 cells were transiently transfected with the plasmid and the expression of the extracellular domain of p185^{neu} was demonstrated by immunohistochemical analysis with mon-

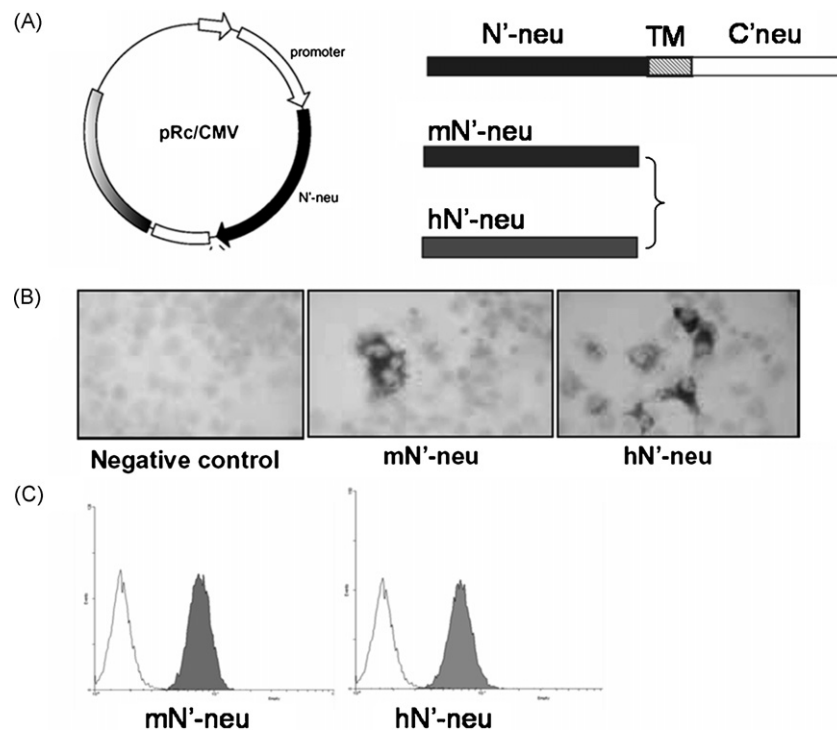


Fig. 1. Characterization of DNA vaccines: (A) schematic diagram of the human N'-neu (hN'-neu) and mouse N'-neu (mN'-neu) expressing vectors; (B) expression of N'-neu *in vitro* evaluated via immunocytochemistry; (C) expression of N'-neu *in vitro* evaluated with flow cytometry. Plasmid-transfected COS-1 cells were stained with mAb against the extracellular domain of mouse and human p185^{neu} followed by FITC-conjugated goat anti-mouse secondary antibody (grey or dark histogram for mouse and human N'-neu, white histogram for vector negative control).

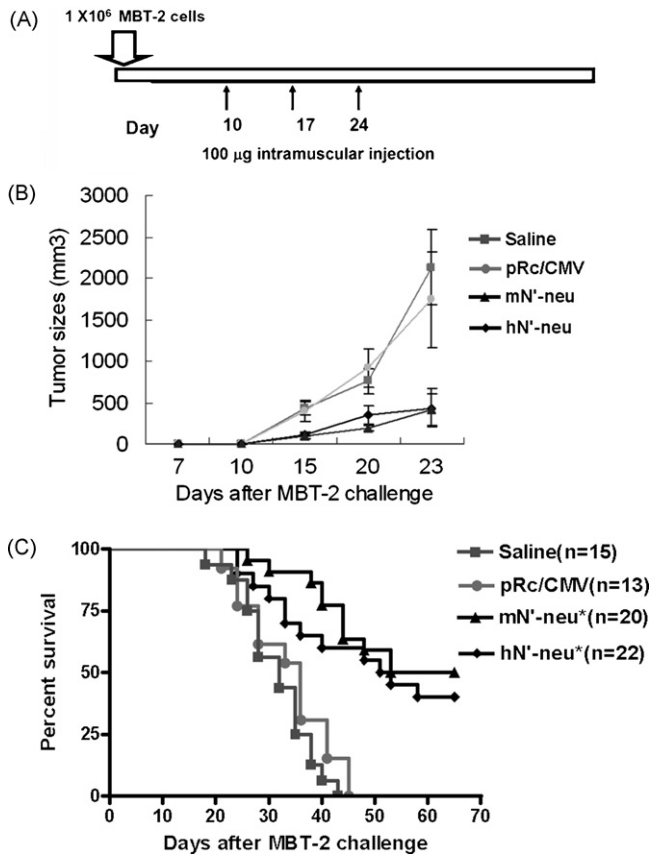


Fig. 2. Therapeutic effects of neu DNA vaccines on established tumor in C3H mice: (A) protocol for DNA vaccination. Ten days after subcutaneous tumor implantation, mice were inoculated with DNA vaccine intramuscularly three times at weekly intervals; (B) tumor volume was measured at the indicated time. Data are means of the animals per group; bars, \pm S.D.; (C) lifespan of C3H mice after subcutaneous challenge with MBT-2 cells. The survival data were subjected to Kaplan-Meier analysis. The digit in the parenthesis is the number of mice in the experiment. The symbol (*) indicates a statistically significant difference when compared with the control saline mice ($P < 0.01$).

oclonal antibody against p185^{neu} (Fig. 1B). The expression of mN'-neu and hN'-neu plasmid was further confirmed by flow cytometry analysis (Fig. 1C).

2.2. Efficacy of mN'-neu and hN'-neu DNA vaccines in mice with established tumors

The protocol for inoculation of DNA vaccine is shown in Fig. 2A. We found that vaccination of mN'-neu or hN'-neu slowed the rate of growth of MBT-2 tumors compared with saline-treated mice. However, there was no difference between mN'-neu and hN'-neu vaccinated groups (Fig. 2B). In addition, the survival rate of vaccinated mice results also indicated that mN'-neu DNA vaccine showed similar efficacy as hN'-neu DNA vaccine (Fig. 2C). Xenogenic neu DNA vaccine did not show better efficacy than autologous mouse N'-neu DNA vaccine, as reported for EGFR DNA vaccine [25].

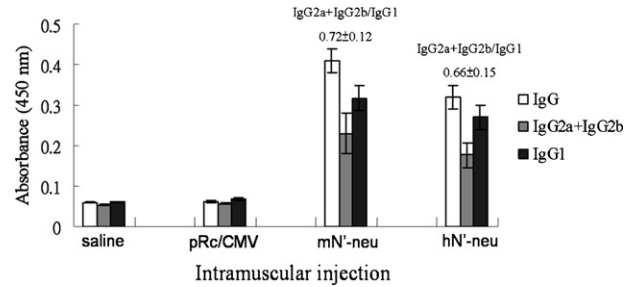


Fig. 3. Neu-specific IgG and IgG subclass titers from mice immunized using the intramuscular vaccination. The titers of anti-mouse p185^{neu} IgG and IgG subclass in sera of mice were determined with ELISA on dishes coated with the extracellular domain of mouse p185^{neu}. The overall IgG2a + IgG2b/IgG1 ratios (mean \pm standard deviations) for extracellular domain of mouse p185^{neu} antigen are shown above the bars.

2.3. Cellular and humoral immunity

To examine the immunological responses induced by N'-neu DNA vaccine, we measured the titer of total IgG anti-p185^{neu} antibody in mouse serum. mN'-neu DNA vaccine induced higher anti-p185^{neu} antibody response compared to the hN'-neu DNA vaccine, although the difference is statistical insignificant ($P = 0.12$) (Fig. 3). We further examined the IgG subtype of anti-neu antibody induced by DNA vaccine, the ratio of IgG2a + IgG2b/IgG1 was not significantly different between the mN'-neu and hN'-neu groups (Fig. 3). For the cellular immunity, we examined the infiltration of lymphocytes at tumor sites (Table 1). No macrophages were detected by immunostaining (data not shown). Massive infiltration of natural killer cells was observed in the mice vaccinated with mN'-neu DNA vaccine. Infiltration of CD4⁺ T cells and CD8⁺ T cells were observed in both mN'-neu and hN'-neu groups of mice. We observed considerably more increased infiltration of CD8⁺ T cells in the hN'-neu group of mice, although the increases did not reach statistical significance compare to the mN'-neu groups ($P = 0.09$) (Table 1). We further evaluated the cellular immunity with cytotoxic response to MBT-2 cells using splenocytes isolated vaccinated mice (Fig. 4). The splenocytes isolated from both mN'-neu and hN'-neu groups of mice lysed MBT-2 cells with equal potency.

Table 1
Infiltrated lymphocytes at tumor sites within cryosectioned samples

Vaccine group	CD4 ⁺ T cells	CD8 ⁺ T cells	NK cells
Saline (IM)	2 \pm 1	1 \pm 0	1 \pm 1
Vector (IM)	2 \pm 2	2 \pm 1	1 \pm 1
Mouse N'-neu (IM)	40 \pm 8	31 \pm 4	27 \pm 6*
Human N'-neu (IM)	32 \pm 7	41 \pm 7	7 \pm 2

Note: Cell count was performed at 400 \times magnification. Three samples and five randomly chosen fields/sample were evaluated. Results are expressed as mean \pm standard deviation of immunohistochemical positive cells in the cryosection. IM: intramuscular injection. The symbol (*) indicates a statistically significant difference when compared with the human N'-neu group ($P < 0.01$).

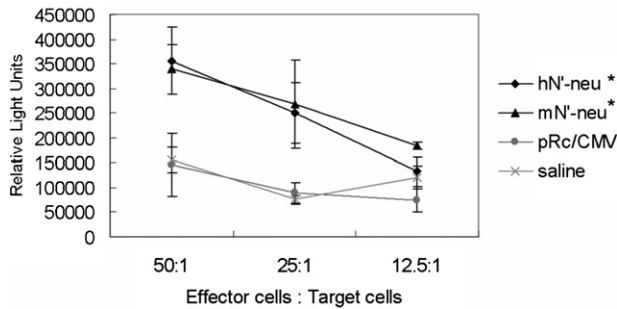


Fig. 4. The role of cellular immunity in immunological defense. Lysis of MBT-2 cells with splenocytes derived from mice inoculated with various vaccines or saline. Splenocytes were incubated with serial dilution of MBT-2 cells expressing luciferase. The release of luciferase upon lysis of MBT-2 cells was measured by means of a luminometer. The symbol (*) indicates a statistically significant difference when compared with the control saline mice ($P < 0.01$).

2.4. Influence of CD8⁺ T cells on induction of anti-tumor immunity

CD8⁺ T cells play a major role in defending MBT-2 cells in a C3H mice animal model [28]. To confirm this role, C3H mice were depleted of CD8⁺ T cells by a regimen of weekly procedures (Fig. 5A). Depletion completely abolished the therapeutic efficacy of mN'-neu (Fig. 5B) and hN'-neu DNA vaccines (Fig. 5C).

2.5. Xenogenic hN'-neu DNA vaccine had better therapeutic efficacy with gene gun delivery

As the vaccination method of DNA vaccine affects the immunological response [22,30,31], we tested the efficacy of mN'-neu and hN'-neu DNA vaccines applied using a gene gun (Fig. 6A). Human N'-neu DNA vaccine significantly delayed tumor growth (Fig. 6B) and prolonged mouse survival as compared with mN'-neu when administered by gene gun (Fig. 6C).

2.6. Humoral and cellular immunity

Both mN'-neu and hN'-neu groups of mice induced approximately similar amount of specific anti-mouse p185^{neu} antibody (Fig. 7); however, gene gun inoculation induced much more antibody titer than the intramuscular injection (Figs. 3 and 7). Furthermore, we examined the IgG subtype of anti-neu antibody induced by DNA vaccine, the result showed that IgG subtype pattern in gene gun injection of hN'-neu and mN'-neu DNA vaccine were not different (Fig. 7). The gene gun vaccination induces a significant lower IgG2a + IgG2b/IgG1 ratio compare to the intramuscular injection of neu DNA vaccine (Figs. 3 and 7). As for the cellular immunity, the sparse infiltration of NK cells was observed at tumor sites both in the mice vaccinated with mN'-neu or hN'-neu DNA vaccine (Table 2). On the other hand, significant infiltration of CD8⁺ T cells was

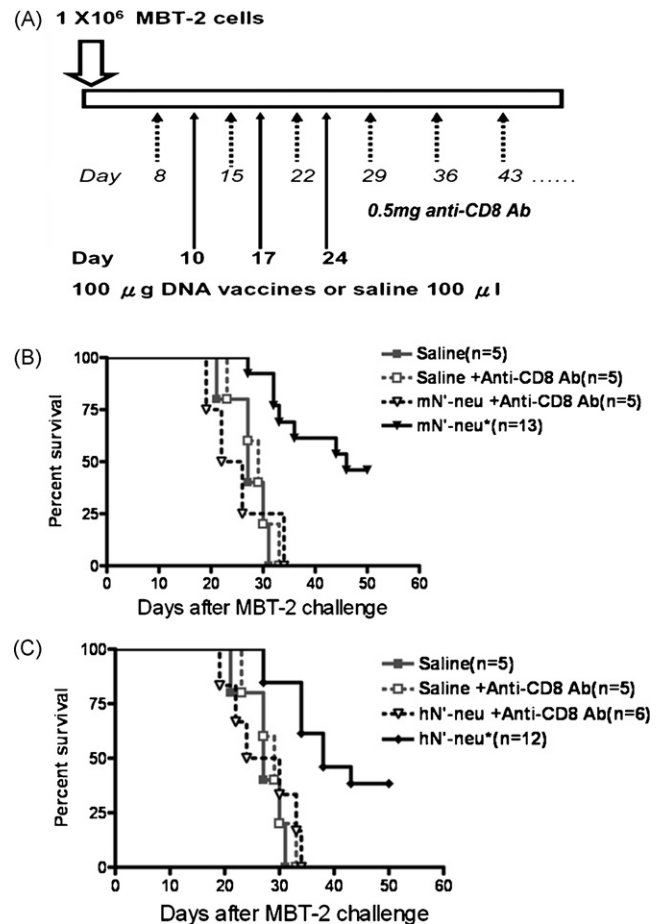


Fig. 5. CD8⁺ T cells are essential for the therapeutic effect. (A) Protocol for depletion of CD8⁺ T cells *in vivo*. Tumor-bearing mice were injected intraperitoneally with 500 µg of anti-CD8 antibody at weekly intervals starting from 2 days before the first inoculation of DNA vaccine. Lifespan of C3H mice after subcutaneous challenge with MBT-2 cells is depicted in (B). mN'-neu; and (C) hN'-neu depletion of CD8⁺ T cells. The survival data were subjected to Kaplan-Meier analysis. The symbol (*) indicates a statistically significant difference ($P < 0.01$), when compared with the vaccinated mN'-neu or hN'-neu mice without depletion of CD8⁺ T cell.

observed in the mice vaccinated with hN'-neu DNA vaccine (Table 2) ($P < 0.01$). Hence, the increased cytotoxic T cells were correlated with the therapeutic efficacy by vaccinating autologous or xenogenic DNA vaccine with gene gun.

Table 2
Infiltrated lymphocytes at tumor sites within cryosectioned samples

Vaccine group	CD4 ⁺ T cells	CD8 ⁺ T cells	NK cells
Saline (GG)	1 ± 1	0	0
Vector (GG)	1 ± 1	1 ± 1	0
Mouse N'-neu (GG)	13 ± 4	59 ± 7	2 ± 1
Human N'-neu (GG)	21 ± 6	77 ± 4*	2 ± 1

Note: Cell count was performed at 400× magnification. Three samples and five randomly chosen fields/sample were evaluated. Results are expressed as mean ± standard deviation of immunohistochemical positive cells in the cryosection. GG: gene gun. The symbol (*) indicates a statistically significant difference when compared with the mouse N'-neu group ($P < 0.01$).

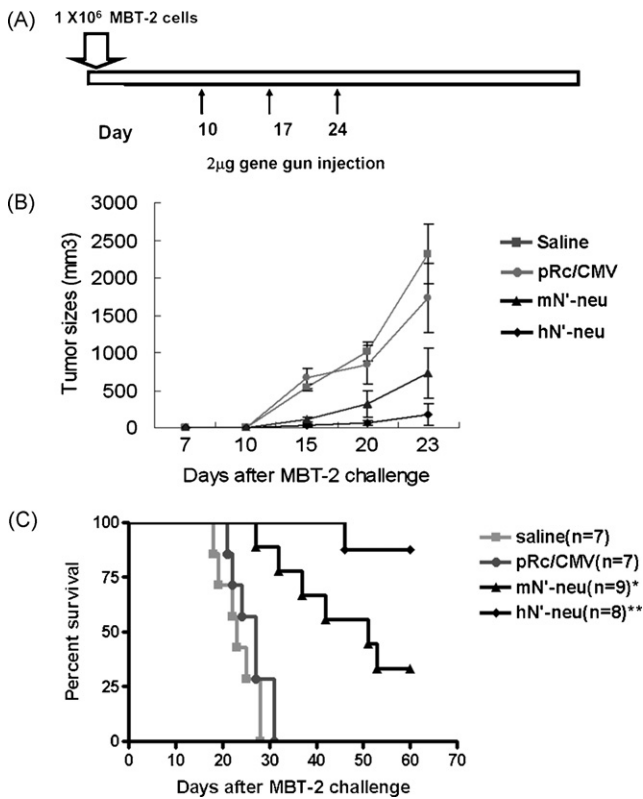


Fig. 6. Human *N'*-neu has significantly better therapeutic efficacy than mouse *N'*-neu with gene gun delivery: (A) the protocol used is depicted. Two μg DNA was used in the gene gun approach; (B) tumor volume was measured at the indicated time. Data are means of the animals per group; bars, \pm S.D. (C) Lifespan of C3H mice after subcutaneous challenge with MBT-2 cells. The survival data were subjected to Kaplan-Meier analysis. The digit in the parenthesis is the number of mice in the experiment. The symbol (*) indicates a statistically significant difference when compared with the control saline mice ($P < 0.01$). The symbol (**) indicates a statistically significant difference when compared with the control saline mice ($P < 0.01$) and statistical significant difference when compared with mN'-neu group of mice ($P < 0.05$).

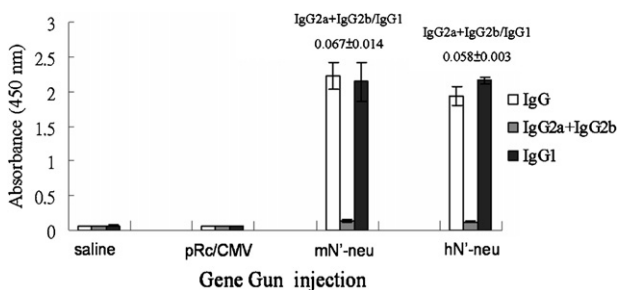


Fig. 7. Neu-specific IgG and IgG subclass titers from mice immunized using the gene gun. The titers of anti-mouse p185^{neu}-specific IgG and IgG subclass in sera of mice were determined with ELISA on dishes coated with the extracellular domain of mouse p185^{neu}. The overall IgG2a + IgG2b/IgG1 ratios (mean \pm standard derivations) for extracellular domain of mouse p185^{neu} antigen are shown above the bars.

3. Discussion

In this report we have examined the therapeutic efficacy of xenogenic and autologous neu DNA vaccines on a mouse tumor model naturally overexpressing p185^{neu}. Autologous neu DNA vaccine had the same therapeutic efficacy as xenogenic neu DNA vaccine upon intramuscular immunization. T-cell depletion experiment indicates that CD8⁺ T cell plays a major role in the immunological defense afforded by either xenogenic or autologous neu DNA vaccine. NK cells and anti-neu antibody may provide additional immune defense based on an increase in the tumor infiltration of NK cells and higher specific anti-mouse neu antibody in the serum. Furthermore, we demonstrate that xenogenic human neu DNA vaccine provides stronger therapeutic efficacy when administered by gene gun. The increased therapeutic efficacy correlates with the significantly enhanced tumor infiltration of CD8⁺ T cells. This confirms our previous work [24] and reinforces the view that CD8⁺ T cells may well be essential for immunological therapeutics for neu DNA vaccine.

Pupa et al. have previously demonstrated that the xenogenic DNA vaccine could inhibit mammary carcinoma development in HER2/neu transgenic mice [19]. In their model, xenogenic human neu DNA vaccine can induce anti-mouse p185^{neu} antibody response which may be responsible for the inhibition of tumor progression. Similar observation was made in our study that xenogenic human neu DNA can induce anti-mouse p185^{neu} antibody. However, cellular immunity including T cells and NK cells may be more important in mediating tumor rejection in our transplantable animal tumor model. The tumor progression in transgenic mice is much more slower than transplantable tumor, and the titer of antibody generated may be sufficient in inhibiting the transition from dysplasia to carcinoma with the targeting of both normal and cancer cells [19]. On the other hand, the apoptosis of tumor cells mediated by cellular immunity is essential in targeting tumor destruction and delay the tumor progression or even eradication in transplantable animal model. The timing of induction of immunity is important for both types of animal model. Inoculation of neu DNA vaccine will have no effect if later than three months in transgenic animal [19], on the other hand, inoculation of DNA vaccine will have little therapeutic effect when the tumor is too large, for example, three weeks after transplantation (unpublished observation).

It is interesting to note that many more NK cells were detected at tumor sites when mice were intramuscularly inoculated with autologous mouse neu DNA vaccine. The increase of NK cells may partly explain the equal efficacy observed for autologous and xenogenic neu DNA vaccine with intramuscular injection, since NK cells play an important role in immunological defense. Three factors may determine the infiltration of NK cells at tumor sites. The first is the differential activation toward NK cells by various dendritic cells (DC). For example, monocyte-derived

DCs are more potent stimulators of NK cells than Langerhan cells in the skin [32]. Secondly, CpG motifs in the large amounts of DNA with intramuscular injection may provide stronger signals for activating NK cells [33,34]. Thirdly, specific anti-mouse neu autoantibody at tumor sites may further enhance the adhesion of NK cells [35,36]. We have further studied the subtypes of IgG with xenogenic and autologous DNA vaccine, but the IgG1/IgG2a + IgG2b ratio did not alter. It is possible that the epitopes recognized by anti-p185^{neu} antibody is more important in determining the protection from tumor formation in our animal model.

Biolistic delivery of DNA into skin using gene gun usually directly delivers the DNA into Langerhan cells in the skin, and the activated DCs migrate into lymph node, activating an immune response [37,38]. Direct presentation of DNA vaccines by Langerhan cells may play a major role in eliciting the immune response. On the other hand, DNA vaccine delivered with intramuscular injection may go through direct presentation or cross-presentation. The DNA vaccine may be presented by myocytes, and cross-presented by DCs residing or infiltrating into the muscle site. Cross presentation can cause cross-tolerance or cross-priming [39,40]. Environmental signals, such as inflammatory signals or CpG motifs in the large amounts of DNA with intramuscular injection, may switch the outcome of cross-presentation from cross-tolerance to cross-priming [39]. It is possible that local inflammation induced by intramuscular injection may cause a similar conversion to cross-priming, and thus enhance the therapeutic efficacy of the autologous neu DNA vaccine.

Langerhan cells are more potent stimulators of cytotoxic T lymphocytes than monocyte derived DCs [41]. However, Langerhan cells are less phagocytotic [42], and may be less dependent on cross-presentation. Therefore, xenogenic DNA vaccine induces a very strong immunological response with direct presentation in Langerhan cells. Self-peptide missense mutations enhance the binding of MHC class I molecules and enhance TCR signaling. The stimulation can activate the naïve T cells not only react to the mutated self-peptides and parental non-mutated peptides in tumor cells [43,44]. Xenogenic DNA may employ a similar mechanism to achieve activation of T cells, since xenogenic DNA has a natural missense mutation compared to that of autologous neu DNA.

Recently, Smorlesi et al. [30] have evaluated the therapeutic efficacy of neu DNA vaccine with different immunization routes, and found that the intramuscular injection with electroporation provided the best therapeutic efficacy in transgenic animal model system. On the other hand, gene gun delivery method may provide better therapeutic efficacy when xenogenic DNA vaccine is used in our animal model. The discrepancy may be due to the differences of tumor progression in these two different animal systems: the progression of tumor growth is much more slowly in transgenic mice. The Th1 type antibody response (IgG2a + IgG2b) is important for inhibiting tumor progression in transgenic tumor model [19], and the gene gun method appears to induce a Th2 type (IgG1)

antibody response ([30] and this study). Therefore, intramuscular injection is a better route for slow-progression native tumor. In contrast, to inhibit the fast-growing transplantable tumor may require the high titer IgG1 antibody induced by gene gun method.

In contrast to the present findings, a prior study on EGFR supports the view that xenogenic human DNA is essential for inducing immunological protection or therapeutics with intramuscular administration [25]. This discrepancy may be due to an intrinsic property of self-antigens. EGFR immunological response has only been identified recently in cancer patients [45,46], which suggests that EGFR may be a poorer immunogen than HER2/neu. The conversion from cross-tolerance to cross-priming may be more difficult for EGFR. On the other hand, the MBT-2/C3H mouse tumor model may be more prone to immunological stimulation than the B16 melanoma cells and C57BL/6 animal model. Therefore, autologous neu DNA vaccine can successfully induce immunological therapeutic effects.

Altogether, the present study provides a basis for the clinical application of neu DNA vaccine. The route of immunization affects the selection of xenogenic DNA vaccine or autologous DNA vaccine. It will be of paramount importance to study the immunological interactions between resident DCs in various organs and the inflammatory cytokines induced by vaccines or tumors. This interaction may play an important role in determining the outcome of active immunization of DNA vaccines against neu or other tumor-associated antigens. The efficacy of neu DNA vaccine is only sufficient to delay the tumor progression, but is not sufficient to completely eradicate the existed tumor in our animal system. Further modification of vector and delivery method or combination with other therapeutic module is required to achieve complete response. Synthetic vectors can be modified much more flexibly than viral vectors [47]. Conjugation with cytokines [24], usage of tissue specific promoter [48], or combination of apoptosis-related molecules [49] may provide additional versatile immunological choice for gene therapy in the future.

4. Materials and methods

4.1. Animals, cell lines, and antibodies

Inbred female C3H/HeN mice (6–8 weeks of age) weighing 18–20 g were used. Animal experiments were approved by the National Cheng Kung University animal welfare committee. MBT-2 is a mouse transitional cell carcinoma cell line established by inducing mouse with the carcinogen *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide [24]. Monoclonal antibody (mAb) against the extracellular domain of mouse p185^{neu} (Ab-2; clone 9G6, Oncogene Science, Cambridge, MA) was used to detect surface expression of p185^{neu} in MBT-2 cells, via flow cytometry [24], and to detect the expression of N'-neu DNA vaccine in COS-1 cells.

4.2. Preparation of human and mouse *N'*-neu DNA expression vectors

MBT-2 cells were harvested and total RNA was isolated using a total RNA extraction system (Viogene-Biotek Corp., Hsichih, Taiwan) according to the manufacturer's instructions. The RNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR) for amplification of the extracellular domain of the mouse neu gene (mN'-neu) using the primers GCAATCGCAAGCTTATGGAGCTG-GCGGCCTGGTG and GCAATCGCGCGGCCGCTACT-GCTCTGCTGGGCAGCCTC. The extracellular domain of the human neu gene (hN'-neu) was generated from the PCR product of pSV2-neu (human) using the primers GCACCCGCAAGCTTATGGAGCTGGCGGCCTTGTG and TAAATATAGCGGCCGCTACTCGGCGGGGCAGC-CCT. The amplified products were cloned into the multiple cloning site of pRc/CMV (Invitrogen, San Diego, CA) to construct the expression vectors, pRc/CMV-mN'-neu and pRc/CMV-hN'-neu. All the constructs were confirmed by DNA sequencing.

4.3. Flow cytometric analysis of the expression of *N'*-neu DNA vaccine *in vitro*

The expression of DNA vaccine was confirmed in transfected COS-1 cells by flow cytometric analysis [24]. Transfected cells were monodispersed, washed with phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde at room temperature for 10 min, and permeabilized with a buffer containing saponin (1%), Na₃N (1%), and fetal bovine serum (FBS; 1%) for 10 min on ice. The fixed and permeabilized cells were stained with mAb against the N-terminal domain of mouse p185^{neu} (Ab-2; Oncogene Science, Cambridge, MA) and FITC-conjugated goat against mouse secondary Ab (Chemicon International, Temecula, CA). Normal mouse IgG mAb was used as the negative control.

4.4. Preparation and evaluation of DNA vaccines

Plasmid DNA was purified with Endofree Qiagen Plasmid Mega Kits (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. DNA was then precipitated using isopropanol and resuspended in sterile saline at the concentration of 1 mg/ml. The endotoxin content was less than 20 U/mg of DNA, as determined by the *Limulus* amoebocyte lysate assay (Sigma Chemical Co., St. Louis, MO).

4.4.1. Intramuscular injection

Mice were injected subcutaneously in the flank with 1×10^6 MBT-2 cells in 0.5 ml PBS (day 0). Beginning on day 10 when tumors were palpable, 100 μ g of DNA vaccine in 0.1 ml sterile saline was administered intramuscularly in the upper thigh at weekly interval for three times. Control mice received three injections of only 0.1 ml of saline. Tumor

size was measured using a caliper two times a week. Tumor volume was calculated by the formula of a rational ellipsoid: ($m_1^2 \times m_2 \times 0.5236$), where m_1 represents the shorter axis and m_2 the longer axis. Mice were sacrificed when the tumor volume exceeded 2500 mm³ or the mouse was in poor condition and death was expected shortly. Significant differences were revealed by Kaplan-Meier analysis of survival rates.

4.4.2. Gene gun injection

Plasmid DNA was precipitated onto gold particles (Bio-Rad 1652263, Bio-Rad, Hercules, CA) for gene gun vaccination at the ratio of 1–2 μ g DNA per milligram gold particles. The gold particles and DNA solution were vortexed and sonicated for several seconds before adding 0.05 M spermidine and 2.5 M CaCl₂ solution with vortexing. This solution was placed on ice for 10 min. Gold particles were collected by centrifugation and washed three times with 100% ethanol. The particles were resuspended in 100% ethanol as bullets with appropriate volume. Plasmid human-neu and mouse-neu DNA-coated gold particles were delivered to the shaved abdominal region of mice at a helium pressure of 50 psi using a low pressure-accelerated gene gun (BioWare Technologies Co. Ltd., Taipei, Taiwan).

4.5. Construction and transfection of *Sec*-mouse *N'*-neu/myc/His (*Sec*-mN-tag)

The extracellular domain of the mouse neu gene containing signal peptide was generated from the PCR product of pRc/CMV-mN'-neu using the primers GCAATCGCAAGCT-TATGGAGCTGGCGGCCTGGTG and GCAATCGCGCG-GCCGCTGCTGCTGGGCAGCCTC. The amplified products were cloned into the multiple cloning site of pcDNATM3.1/myc-His B (Invitrogen, San Diego, CA) to construct the expression vectors, Sec-mN-tag. The constructs were confirmed by DNA sequencing. Lipofectamine 2000 reagent was purchased from Invitrogen Technologies. Cos-7 cells were transfected with Sec-mN-tag plasmid. Transfected cells were passed into medium containing 800 μ g/ml of G418 (Promega) at 24 h after transfection. Positive colonies were further cloned by limiting of dilution.

4.6. Preparation of *secE2* for ELISA

The methods of preparing recombinant Sec-mN-tag protein as previously described [50]. Briefly, the medium was replaced with serum free hybridoma-Max (Gibco BRL) when COS-7-Sec-mN-tag cells were grown to 80% confluence. Cells were cultured for additional 4–5 days. Cell culture supernatants were harvested and filter through molecular-porous membrane (spectrum Laboratories Inc.) 2 days in 4 °C. A 10 \times concentrate of culture supernatants was prepared using freeze dryer (Labconco) according to the manufacturer's guidelines. One unit of recombinant Sec-mN-tag was defined as 1 ml of the 10-fold concentrated cell culture supernatant.

4.7. Determination of serum anti-neu antibody titer

Antibody titers were determined using an enzyme-linked immunosorbent assay (ELISA). A total of 50 U recombinant Sec-mN-tag protein was added to each well. The plate was incubated overnight at 4 °C. Nonspecific binding was blocked with 1% BSA in PBS, followed by three washes with PBS containing 0.05% Tween 20. Test sera were serially diluted (IgG and IgG1, 1:10,000; IgG2a and IgG2b, 1:25) and added to the plates to determine the titer of mouse anti-p185^{neu} antibody. For detection of mouse IgG, HRP-conjugated anti-mouse IgG (Calbiochem, Darmstadt, Germany) was used; detection of mouse IgG1, HRP-conjugated anti-mouse IgG1 (Pharmingen); detection of mouse IgG2a/IgG2b, HRP-conjugated anti-mouse IgG2a+IgG2b (Pharmingen) was used. Color development was facilitated using 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. Absorbance was read at 450 nm with a microplate reader (Dynatech MR5000 plate reader).

4.8. Histological analysis of lymphocyte infiltration

Tumor tissues removed from vaccinated mice one week after the third vaccination were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Inc., USA) and then frozen in liquid nitrogen. Cryosections (5 µm) were fixed with 3.7% formaldehyde and acetone, washed with PBS three times, and incubated overnight at 4 °C with anti-CD4 (GK 1.5; BD Biosciences Pharmingen, San Jose, CA), anti-CD8 (53-6.7; Pharmingen), anti-macrophage (rm C5-3 for CD14; Pharmingen), or anti-pan-NK (DX5; Pharmingen) antibodies. After an additional reaction with peroxidase-conjugated secondary antibody, an aminoethylcarbazole substrate kit (Zymed Laboratories, San Francisco, CA) was used for color development. Immunohistochemical staining was analyzed independently in a blinded fashion by two observers. For quantification of immune infiltrating cells, we used a light microscope with a 10× eyepiece and a 40× objective lens. The total number of cells in five high-power fields was counted. Three samples from three mice were analyzed.

4.9. Generation and selection of stable transfected MBT-2-luciferase cell lines

The luciferase gene was cloned into the plasmid pCMV vector as previously described [24]. MBT-2 cells were transfected with the plasmid, and selected with G418 at the concentration of 800 µg/ml.

4.10. In vitro CTL induction and activity

Female C3H/HeN mice (6–8 weeks old) were injected with DNA vaccine three times as described above. A week after the third DNA vaccination, spleen cells were harvested and were grown in RPMI 1640 with 25 mM HEPES and L-glutamate (GibcoBRL, Rockville, MD), supplemented with

penicillin (100 U/ml), streptomycin (100 µg/ml), 50 mM 2-mercaptoethanol (ME), 100 U/ml penicillin, and 10% FBS. In addition, 500 U Sec-mN-tag protein were added. After 5 days of incubation, non-adherent cells were harvested as effector cells and plated with MBT-2 luciferase cells as target cells. Target cells of 5×10^3 /well were incubated for 18 h in triplicate at 37 °C with serial dilutions (50:1, 25:1, 12.5:1) of effector cells. After 18 h, cells were recovered by centrifugation and 100 µl of supernatant was obtained. The specific lysis was assessed in the supernatant using a conventional luciferase detection system (Promega, Madison, WI). One hundred microliter of the culture medium was mixed with 100 µl of the substrate (luciferin). The mixture was then placed into an EG & G (Berthold) MiniLumat LB9506 luminometer. Light emission was recorded for 10 s. Triplicate measurements were performed for each sample.

4.11. Depletion of CD8⁺ T cells

To deplete CD8⁺ T cells, murine anti-mouse CD8 (2.43; 500 µg), or control antibody (purified rat IgG; 500 µg) was injected intraperitoneally to mice. The first injection took place 2 days prior to DNA vaccination and at intervals of 7 days thereafter. The efficacy of depletion was evaluated by flow cytometry [24]. Single-cell suspension splenocytes (10^5) were obtained from anti-CD8 mAb-treated mice for immunostaining or PE-conjugated anti-CD8 mAb (53-6.7; Pharmingen), respectively.

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