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RESEARCH ARTICLE

Delivery of noncarrier naked DNA vaccine into the skin by supersonic flow induces a polarized T helper type 1 immune response to cancer

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Abstract

Background DNA vaccine is a new and powerful approach to generate immunological responses against infectious disease and cancer. The T helper type (Th)1 immune response is usually required for generating effective anti-tumor responses. A microparticulate bombardment system can induce an immune response using very low amounts of DNA. Using nozzle aerodynamics, a low pressure gene gun has been developed to decrease the noise associated with high pressure gene guns. Particles are propelled by supersonic flow through this novel nozzle. To test whether this gun could inoculate a DNA vaccine that stimulates an anti-tumor Th1 immune response, we examined the effect of direct delivery of naked DNA (i.e. without any carrier) on the anti-tumor immune response of mice.

Methods The luciferase reporter plasmid DNA was delivered using a low-pressure biolistic device and expressed in C3H/HeN, BALB/c, and C57BL/6 mice.

Results Plasmid DNA expression was mainly in the epidermis. Noncarrier naked neu DNA vaccine and gold particle-coated neu DNA vaccine (at 1 μg per mouse) had similar anti-tumor effects in C3H mice. However, cytokine profile examination showed the Th1-bias of the response induced by naked DNA vaccine and the Th2-bias of the response induced by coated DNA vaccine.

Conclusions A shift in the immune response to favour enhanced tumor rejection can be achieved by skin delivery of naked DNA vaccine. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords biolistic; cancer; DNA vaccine; gene gun; neu; Th1 immune response

Introduction

Advances in molecular biology have made it possible to sequence the genomes of primates and infectious agents. This achievement allowed us to identify targets for gene therapy and vaccine development. However, our primary concern is the development of effective and safe methods of delivering gene-based drugs. Although highly efficient viral vectors have been used, their use has many negative consequences (including induction of host immune responses, random integration, and risks of wild-type virus contamination) [1,2]. Nonviral gene transfer provides a safe, superior alternative. Nonviral delivery methods include gene-gun injection, liquid jet injection, intramuscular direct injection, and electroporation, among others [3–5]. Of these methods, the

gene gun, also named the biolistic device, has been demonstrated to deliver genes in an effective and homogeneous fashion. The gene-gun delivery method was originally designed to propel DNA-coated gold particles (using exploding gunpowder as an accelerant) through plant cell walls [6]. High pressure helium replaced gunpowder in most particle-mediated delivery devices [7]. The method is also applied to deliver genes into mammalian cells [8], and may be superior to the lipofectamine method for certain cell lines. In addition, the successful introduction of DNA via skin has been demonstrated *in vivo* [9].

Plasmid DNA vaccine was shown to elicit humoral and cellular immunity [10–12]. The simplicity and stability of DNA vaccines confer advantages over certain current immunological manipulations. DNA vaccines are usually delivered by intramuscular injection or particle-mediated gene-gun transfer. Intramuscular injection induces a predominantly T helper type 1 (Th1) response, whereas gene-gun delivery elicits predominantly T helper type 2 (Th2) responses [13–16]. The difference may be influenced by the amount of DNA, the associated CpG motifs, the nature of the antigen, and the particle used in delivery [17–20]. The advantage of gene-gun delivery over intramuscular injection is that less DNA is used. The Th1 response is usually needed to clear infection and cancer cells [21].

To decrease the noise and cell damage caused by high-pressure (200–400 Psi) gene guns, a low-pressure (30–60 Psi) gene gun was developed (US patent 6436709B1). This low pressure biolistic device can introduce gold particle-coated DNA into animal cells using supersonic flow generated by a rocket nozzle [22,23]. Since supersonic air flow can shoot low density particles through cell walls, we wished to determine whether it could introduce naked DNA into animal skin. Our results demonstrate that this device can deliver naked DNA into mouse skin and thereby provoke Th1-biased immune responses that are effective for cancer therapy.

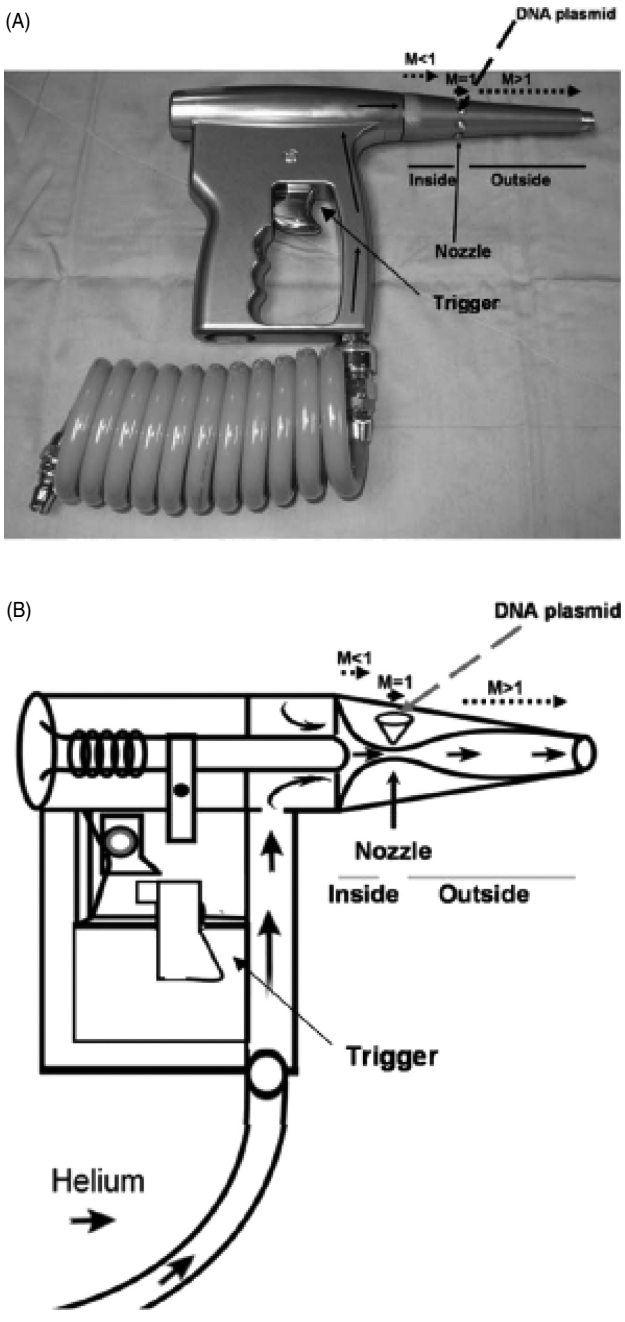

Figure 1. Low-pressure biolistic device. (A) Photograph showing the exterior of the gene delivery device. The plasmid sample is applied through the upper hole. Helium is supplied through an inlet at the bottom. (B) Schematic showing the internal features of the gene delivery device. M, Mach; M <1, subsonic flow; M >1, supersonic flow

Materials and methods

Mice

Female C3H/HeN, BALB/c, and C57BL/6, mice were obtained from the laboratory animal center at National Cheng Kung University. All animal studies were approved by the animal welfare committee at National Cheng Kung University.

Gene-gun injection

One to 10 μg of naked DNA was dissolved in 20 μl of double-distilled water and added to the loading hole near the nozzle. Pushing the trigger of the low pressure Gene Gun (BioWare Technologies Co. Ltd, Taipei, Taiwan) released the DNA-containing water, which was directly propelled by helium at a pressure of 60 psi into the shaved abdomen of the mouse (Figure 1). To avoid the cross-contamination between each shot, water and ethanol were added sequentially to wash the loading well and the gun was fired to clear the nozzle. Plasmid DNA was precipitated onto gold particles (Bio-Rad, Hercules, CA, USA) at the ratio of 1–2 μg of DNA per mg of gold particles. The total volume (50 μl) was vortexed and sonicated for several seconds before adding equal volumes of 0.05 M spermidine and 2.5 M $CaCl_2$ solution

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with vortexing, and then placed on ice for 10 min. The gold particles were collected by centrifugation at 9000 *g* for 1 min, washed three times with 100% ethanol, resuspended in 20 µl of 100% ethanol, and used as bullets (at a 40 psi pressure of helium) in the same low pressure gene gun used to deliver the naked DNA plasmid.

The efficiency of delivery

The CMV promoter was subcloned from pRc/CMV empty vector (Invitrogen, Carlsbad, CA, USA) by polymerase chain reaction (PCR). Primers were: forward: 5′-GCAATCGGTACCCGATGTACGGGCCAGATATAC-3′; reverse: 5′-GCAATCAAGCTTAATTTCGATAAGCCAGTTAAGC-3′. pCMV-luciferase plasmid was generated by inserting a *Kpn*I/*Hind*III fragment of CMV promoter into the pGL3-basic vector upstream of the luciferase gene. The luciferase activity on mouse skin was detected 48 h after bombardment with pCMV-luciferase. The distribution of luciferase activity in treated mice was visualized using a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany) consisting of a Peltier cooled CCD slow-scan camera mounted on a light-tight specimen chamber. Images were acquired and processed using WinLight software (Berthold Technologies). Just before imaging, the skin was shaved, and 100 µl of D-luciferin (Synchem OHG, Altenburg, Germany) in saline was injected at a dose of 100 mg/kg. Mice then were placed in the chamber, and a gray-scale image was taken with dimmed light. Photon emission was then integrated over a period of 10 min. Luminescence measurements are expressed as the integration of the average brightness/pixel unit expressed as photon counts emitted per second.

Locating sites of gene-gun delivery

Mice were sacrificed 48 h after bombardment with pCMV-EGFP-N1 (Clontech, Palo Alto, CA, USA). Abdominal skin was removed, embedded in paraffin, sectioned (5 µm), and directly observed under an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Detection of CD11c+ GFP-positive cells in inguinal lymph nodes from vaccinated mice

The protocol is modified from that previously reported [24]. In brief, C3H/HeN mice were inoculated with different doses of pCMV-EGFP plasmid (pEGFP-N1; Clontech) via gene-gun bombardment. Inguinal lymph nodes were harvested 48 h later. CD11c+ cells were further enriched from single cell suspensions of isolated inguinal lymph node by CD11c (N418) microbeads (Miltenyi Biotec, Auburn, CA, USA). To increase the purity of the enriched CD11c+ cells, the magnetic separation procedure was repeated using a new column. The purity of the populations was at least 90% as determined by staining with monoclonal anti-CD11c-PE antibody. FACSCalibur flow cytometry (BD Bioscience, Mountain View, CA, USA) was used to determine percentage of GFP positive CD11c + cells in a gated population (electronic gates set for monocytes according to their forward and side scatter characteristics).

Therapeutic efficacy of DNA vaccine with/without coating

Mice were injected subcutaneously in the flank with 1×10^6 MBT-2 cells in 0.5 ml of phosphate-buffered saline (PBS) (day 0). Beginning on day 10, when tumors were palpable, pCMV-human *N*′-neu DNA vaccine [23] or pRc/CMV DNA (Invitrogen) was delivered by gene gun on skin in the shaved abdominal region three times at weekly intervals. Control mice received three injections of water. Tumor size was measured using a caliper twice each week. Tumor volume was calculated using the formula for a rational ellipse: $(m_{12} \times m_2 \times 0.5236)$, where m_1 represents the shorter axis and m_2 the longer axis. Mice were sacrificed when the tumor volume exceeded 2500 mm^3 or the mouse was in poor condition and death was expected shortly. Significance of differences in survival were tested by Kaplan–Meier analysis.

Determination of the expression of extracellular domain of p185neu protein in skin

A 96-well plate was coated with 0.2 µg of rabbit-anti-ErbB-2 antibody (Neomarker, Fremont, CA, USA) in 100 µl of PBS (pH 7.4) and incubated overnight at 4 °C. Nonspecific binding was blocked with PBS containing 1% bovine serum albumin (BSA), followed by three washes with 0.05% Tween 20 in PBS. Skin samples were homogenized 48 h after pCMV-human-*N*′-neu bombardment, while 100 µl of each prepared sample was added to duplicate coated wells and incubated at 37 °C for 2 h. After washing them three times, the following was added to the wells: mouse-anti-ErbB-2 antibody (Ab-20) (Neomarker) (incubated at 37 °C for 90 min), Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Calbiochem, Darmstadt, Germany) (incubated for 45 min at 37 °C), and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (eBioscience, San Diego, CA, USA) for color development. Absorbance was measured at 450 nm with a microplate reader.

Determination of serum anti-neu antibody titer

Recombinant human-ErbB2 protein (0.2 µg/100 µl; R&D Systems, Minneapolis, MN, USA) was added to 96-well flat-bottom plate, which was incubated overnight

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at 4 °C, blocked with PBS buffer containing 1% BSA (room temperature 1–2 h), and washed with PBS containing 0.05% Tween 20 for three to five times. Mouse anti-ErbB-2antibody (Ab-20) was used to generate the standard curve, and the background value calculated from absorbance read in control wells containing an irrelevant antibody (i.e. anti-SV40 large T antigen; Oncogene Science, Cambridge, MA, USA). The titer of human anti-p185neu antibody in test sera was determined by serial dilution and addition to the plates. HRP-conjugated anti-mouse IgG (Calbiochem) was used to detect total mouse IgG and TMB substrate was for colour development. Absorbance was read at 450 nm with a microplate reader.

Spleen cell-mediated cytotoxicity assay for targeting MBT-2 cells

The protocol is modified from that previously reported [23,25]. 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 grown in RPMI 1640 with 25 mM Hepes and L-glutamate (GibcoBRL, Rockville, MD, USA), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 50 mM 2-mercaptoethanol, 100 U/ml penicillin, and 10% fetal bovine serum (FBS). In addition, 10 μg/ml of recombinant extracellular domain of neu protein, amino acids 1–652 (R&D Systems) was added. After 5 days of incubation, non-adherent cells were harvested as effector cells and plated with MBT-2 luciferase cells as target cells [23]. Target cells of 5×10^3 cells/well were incubated for 18 h in triplicate at 37 °C with effector cells serial diluted 50 : 1, 25 : 1, or 12.5 : 1. 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, USA). One hundred μl of the culture medium was mixed with 100 μl of the substrate luciferin (dual luciferase reporter system; Promega). The mixture was then placed into an EG & G MiniLumat LB9506 luminometer (Berthold Technologies). Light emission was recorded for 10 s, with triplicate measurements being performed for each sample. The percentage-specific lysis was calculated using the following formula:

$$\%\ \text{lysis} = (\text{test RLU [relative light units]} - \text{spontaneous RLU}/\text{max RLU} - \text{spontaneous RLU}) \times 100.$$

Cytokine emzyme-linked immunosorbent assay (ELISA)

Lymphocytes (5×10^6) obtained from peripheral lymph nodes 1 week after the last vaccination were cultured for 48 h in a 24-well plate containing 10 μg/ml of recombinant human ErbB2 protein (R&D Systems) in 1 ml of RPMI 1640 with 10% FBS. The supernatants were harvested and assayed for the presence of cytokines using the mouse ELISA Ready-SET-Go kits (eBioscience) according to the manufacturer's instructions.

Reverse transcriptase (RT)-PCR

Total RNA was extracted from lymphocytes by TRIZOL (Invitrogen). cDNA was synthesized from 2 μg of RNA using MMLV-Reverse Transcriptase according to the manufacturer's directions. Primers were: IL-12p40 forward: 5′-TGC TGG TGT CTC CAC TCA TGG C-3′; IL-12p40 reverse: 5′-TTT CAG TGG ACC AAA TTC CAT T-3′; IFN-γ forward: 5′-AAC GCT ACA CAC TGC ATC TTG G-3′; IFN-γ reverse: 5′-CAA GAC TTC AAA GAG TCT GAG G-3′; IL-4 forward: 5′-GAA TGT ACC AGG AGC CAT ATC-3′; IL-4 reverse: 5′-CTC AGT ACT ACG AGT AAT CCA-3′; IL-10 forward: 5′-CGG GAA GAC AAT AAC TG-3′; IL-10 reverse: 5′-CAT TTC CGA TAA GGC TTG-3′; hypoxanthine phosphoribosyltransferase (HPRT) forward: 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′; HPRT reverse: 5′-GAT TCA ACT TGC GCT CAT CTT AGG C-3′. cDNA was amplified by Protaq (Protech, Taipei, Taiwan). PCR amplifications were carried out in 50 μl volumes containing 20–60 pmol of each primer, Protaq buffer (Protech), 200 μM each of dNTP, and 5 U of ProTaq polymerase. PCR was performed on a PCR thermal cycler (MJ Research, Watertown, MA, USA). The PCR reaction commenced at 94 °C for 2 min, followed by 30–40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining under ultraviolet light.

Determination of DNA degradation by gene-gun firing

One μg of naked pCMV-Her-2/neu DNA or gold particles coated with 1 μg of pCMV-Her-2/neu DNA was shot into a circular region, equal in area to the area at the exit of the gene-gun nozzle, on 6-μm-pore-size filter paper (Advantec, Tokyo, Japan) at a pressure of 60 or 40 psi, respectively. The DNA on the bombarded filter paper was recovered by placing the paper in a PD column (Geneaid, Taiwan) and immersing it in 100 μl of ddH_2O for 5 min. The DNA was eluted by centrifuging (15300g for 5 min) the column. After agarose gel electrophoresis of 0.3 μg of the eluted DNA, DNA integrity was evaluated from video images by densitometry using Visionworks LS software (UVP, Upland, CA, USA).

Statistical analysis

SE values were calculated from each triplicate set and *t*-tests were performed using GraphPad Prism 4 software (GraphPad Software; San Diego, CA, USA). A

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p-value <0.05 was considered statistically significant. Kaplan–Meier analysis was carried out and the graphs plotted using GraphPad Prism 4, and curves were compared using the log-rank test.

Results

Biolistic gene delivery device

A photograph of the low-pressure gene delivery device is shown in Figure 1A. Low pressure helium is supplied through the bottom inlet. The DNA sample is applied to the top hole and propelled into the target tissue via a nozzle. The internal features are illustrated in Figure 1B. The inner nozzle is convergent, whereas the outer nozzle is divergent. The trigger momentarily releases helium gas. A supersonic flow is generated when the pressure difference between the inner and outer nozzle is greater than 1.9 atm and can accelerate particles to extremely high speed. Previous results from our group and others indicated that supersonic flow can safely and effectively deliver gold particle-coated DNA into animals and generate immune responses delaying tumor growth [22,23].

Novel biolistic gene gun can deliver noncarrier naked DNA into mouse epidermis

Initially, we investigated whether naked DNA could be delivered into the skin of C3H/HeN mice, BALB/c mice, and C57BL/6 mice. Luciferase was used as a reporter gene to monitor the expression of the DNA vaccine plasmid; imaging detected luciferase activity *in vivo* after delivery of 1 μg of naked DNA plasmid in the three strains of mice. However, the activity was significantly higher after vaccination with 1 μg of coated DNA than with 1, 5, or 10 μg of naked DNA in C3H/HeN, BALB/C, and C57BL/6 mice (Figure 2A). Quantification of luciferase expression is shown in Figure 2B. To further examine the localization of the plasmid, the coated pCMV-EGFP DNA or naked pCMV-EGFP DNA was used to inoculate C3H/HeN mice. Their skins were sectioned 48 h after inoculation and examined by immunofluorescence microscopy. Coated or naked pCMV-EGFP DNA was mainly detected in the epidermis (Figure 2C). Several studies have demonstrated that, following intradermal immunization, dendritic cells migrate to draining lymph nodes where they play a major role in priming and stimulating antigen-specific T cells [26–28]. Therefore, it is important to determine whether dendritic cells express the reporter genes in draining lymph nodes when naked DNA is administered. The numbers of CD11c+ and GFP-positive cells in the inguinal lymph nodes are similar whether vaccination is with gold particles coated with 1 μg of pCMV-EGFP DNA or 1 μg of naked pCMV-EGFP DNA. Interestingly, we observed a greater percentage of GFP-positive CD11c+ cells in lymph nodes from mice injected with 5 and 10 mg of naked DNA than in lymph nodes from mice vaccinated with either 1 mg gold particles-coated pCMV-EGFP DNA or 1 mg of naked pCMV-EGFP DNA (Figure 2D).

Cancer therapy with naked DNA delivered by a low-pressure biolistic device

To examine whether noncarrier DNA vaccine had a cancer therapeutic effect, MBT-2 bladder tumor cells in C3H/HeN mice were used as a tumor model [29]. Overexpression of endogenous p185neu was observed in MBT-2 cells. We had previously demonstrated that low-pressure gene-gun propelled, skin delivery of gold particles coated with DNA encoding the extracellular portion of p185neu (pCMV-human-*N*′-neu) inhibited MBT-2 tumor growth in C3H/HeN syngeneic mice [23]. One million MBT-2 tumor cells were implanted subcutaneously at day 1. Gold particle-coated or noncarrier naked pCMV-human-*N*′-neu DNA (1 μg) was inoculated three times at weekly intervals after the tumors became palpable (day 10). The tumor volume was measured (Figure 3A) and the survival data were subjected to Kaplan–Meier analysis (Figure 3B). One μg of pCMV-human-*N*′-neu DNA could delay tumor progression and extend lifespan. In addition, 1 μg of naked DNA vaccine had the same therapeutic efficacy as 1 μg of the coated DNA vaccine. ELISA was used to detect the extracellular portion of p185neu in skin. The amount of the *N*-terminal portion of human p185neu produced by 1 μg of naked DNA vaccine was much lower than that produced by 1 μg coated DNA vaccine (Figure 4A). The amount of anti-p185neu neu antibody in serum was the measure of humoral immunity. The antibody response was weaker to naked DNA vaccine than to coated DNA vaccine, which is consistent with the expression of pCMV-human-*N*′-neu (Figure 4B). Cell-mediated toxicity to MBT-2 targets was the measure of anti-tumor cellular immunity in the spleen cell population. Both vaccines elicited similar cell-mediated cytotoxic immune responses (Figure 4C). Therefore, we hypothesized that the naked DNA vaccine may activate a Th1-biased immune reaction and lead to cellular immune responses. RT-PCR assay of the Th1/Th2 cytokine profile of mice inoculated with naked versus coated DNA vaccine revealed significant induction of IFN-γ by the naked DNA vaccine but not by the coated DNA vaccine. By contrast, interleukin (IL)-4 and IL-10 was significantly induced by the coated DNA vaccine, suggesting a Th2 immune response (Figure 4D). ELISA of IL-4 and IFN-γ protein expression reflected the RT-PCR results; however, there was less of a difference in IFN-γ (Figure 4E). These results suggest that compared to the coated vaccine, the naked vaccine induces more of a Th1-type immune response.

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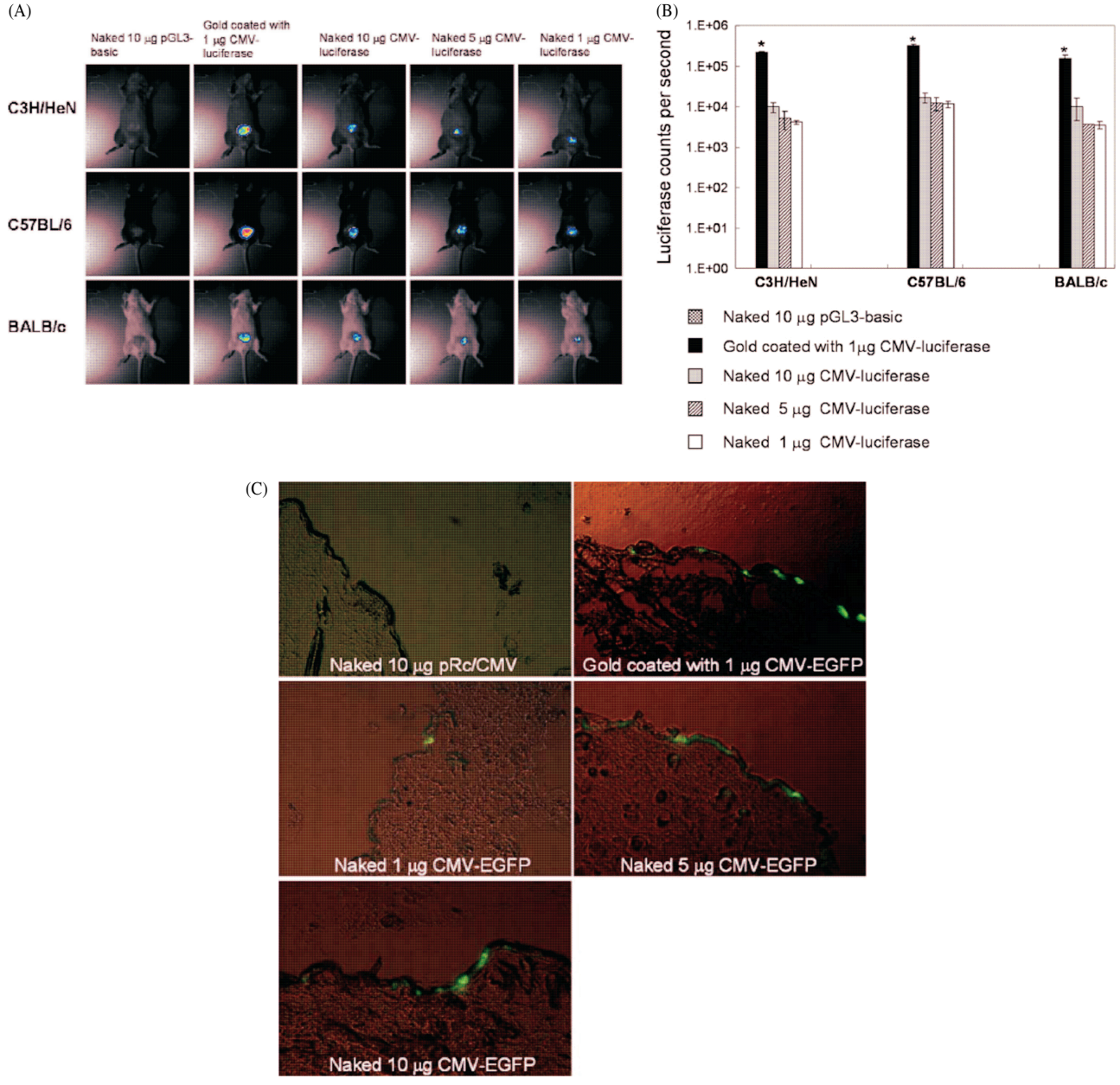

Figure 2. Naked DNA can be delivered into mouse skin. (A) Expression of luciferase in skin. Mice were inoculated with 1 μg of gold particle-coated pCMV-luciferase DNA or 1, 5, 10 μg of naked pCMV-luciferase DNA. *In vivo* images showing luciferase activity at 48 h after inoculation (taken with a Night Owl imaging unit). (B) Histogram showing the quantification of luciferase activity. *Statistically significant difference compared to the 10-μg naked CMV-luciferase group ($p < 0.05$) (C) Expression of GFP in the epidermis. C3H/HeN mice were inoculated with 1 μg of gold particle-coated pCMV-EGFP DNA or 1, 5, or 10 mg of naked pCMV-EGFP DNA. The mouse skin was removed 48 h later, fixed, paraffin-embedded, and sectioned for green fluorescence observation. (D) Migration of GFP-positive CD11c+ dendritic cells into lymph node. Mice were inoculated with 1 μg of gold particle-coated pCMV-EGFP DNA or 1–10 μg of naked pCMV-EGFP DNA, and the inguinal lymph nodes were removed 48 h later. The ratio of GFP positive to total CD11c+ dendritic cells was measured using flow cytometry. The CD11c+ dendritic cell population was first enriched using CD11c (N418) microbeads and then by flow cytometry gated for monocyte-like cells. *Statistically significant difference compared to the control DNA plasmid ($p < 0.05$)

Influence of gene-gun firing on DNA plasmid integrity

Finally, we monitored the integrity of DNA plasmids before and after supersonic flow (i.e. coated or naked pCMV-human-HER-2/neu DNA propelled by supersonic flow onto filter paper at 40 or 60 psi, respectively). The DNA was recovered from the filter paper and evaluated by gel electrophoresis. Compared with the control (DNA simply spotted on filter paper), DNA propelled by supersonic flow was somewhat degraded at air pressures of 40 psi (coated DNA) or 60 psi (naked DNA). Semi-quantitative analysis of video images revealed 65–70% shearing of the naked DNA and 20–25% shearing of the coated DNA (Figure 5). However, approximately one-third of the DNA remained apparently intact. The residual intact

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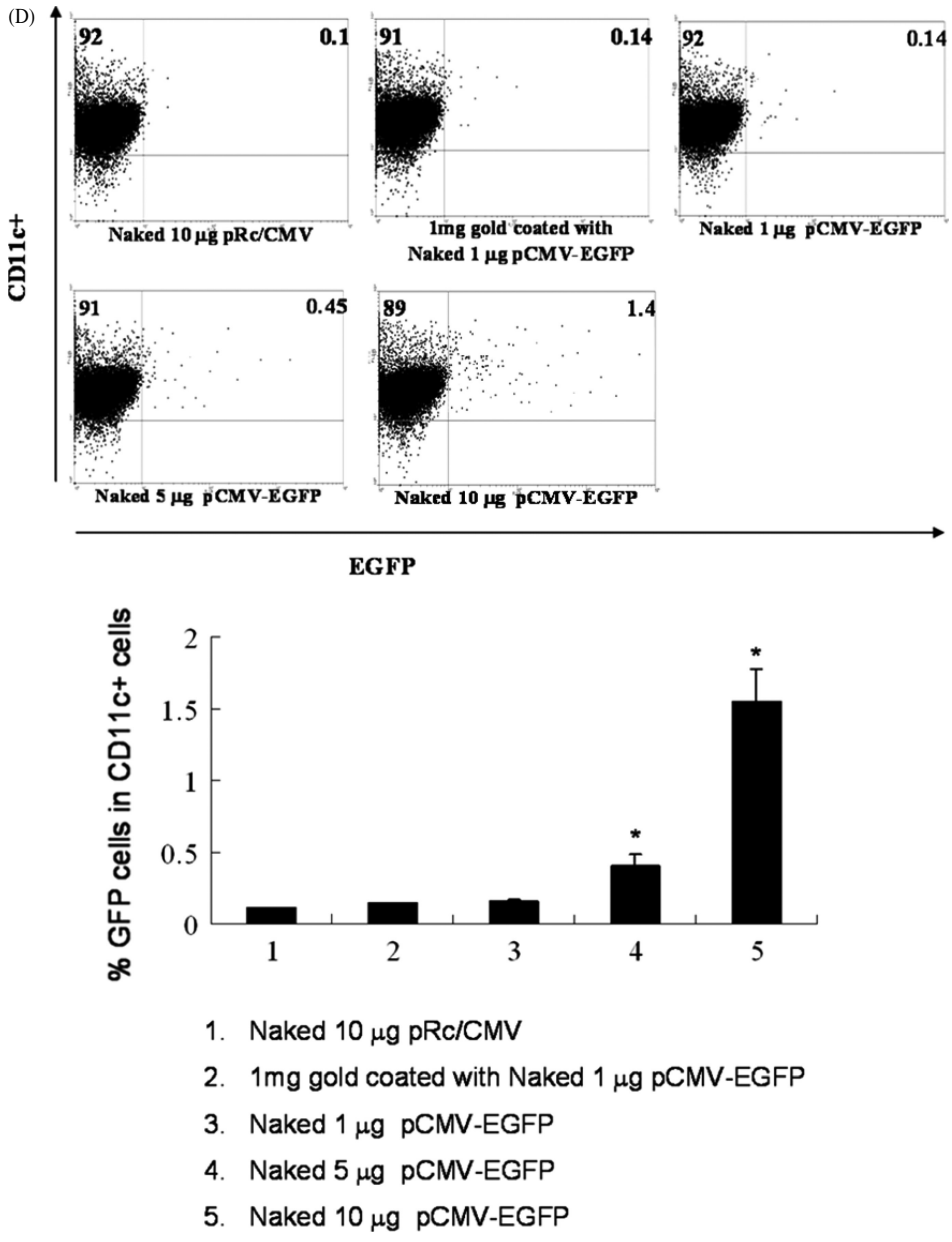

Figure 2. (*Continued*)

nucleic acid might be quantitatively and qualitatively sufficient to generate a Th1-biased immune response in the skin.

Discussion

In the present study, we demonstrate that naked DNA can be delivered via supersonic flow into mouse skin using a low-pressure biolistic device. The naked DNA can induce Th1-biased immunity and be used as a cancer therapeutic agent. The expression of DNA-encoded protein was DNA dose-dependent, as demonstrated by luciferase imaging *in vivo*, and was mainly in the epidermis. Furthermore, the noncarrier naked pCMV-human-*N′*-neu DNA vaccine and gold particle-coated pCMV-human-*N′*-neu DNA vaccine had comparable therapeutic value as demonstrated by the significant delay in tumor progression and extension of mouse lifespan. Less antigen was expressed in response to the naked DNA vaccine than to the coated DNA vaccine, and the antibody response induced by the naked DNA vaccine was weak. By contrast, both vaccines elicited comparable cell-mediated cytotoxicity responses of spleen cells to MBT-2 cells. The reason both vaccines have comparable anti-tumor effects might be that eradication of MBT-2 tumor overexpressing p185neu is mainly through CD8+ T cells [23,29], with the antibody response apparently playing only a minor role.

The Th1/Th2 profile was influenced by multiple factors including the method, route, amount of gene-gun delivered DNA, and the nature of the antigen. The Th1 type response is mainly attributed to immunostimulatory DNA sequences containing the CpG motif. Although DNA

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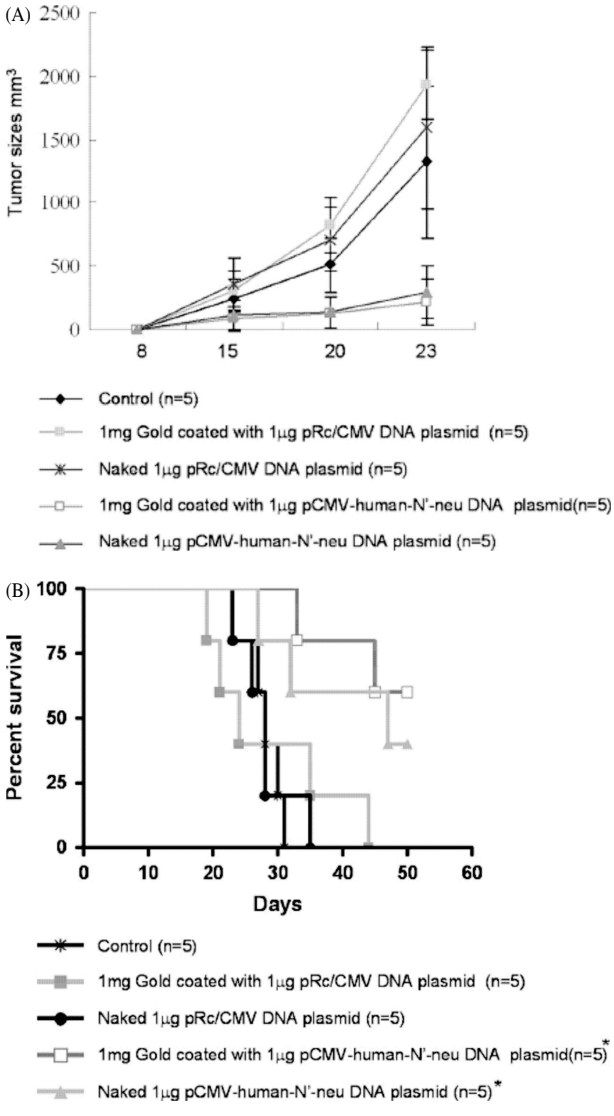

Figure 3. Cancer therapy with gene-gun delivery of naked or coated pCMV-human-*N′*-neu DNA vaccine. (A) Time course of tumor volume change. The average tumor volume was measured until sacrifice due to excess tumor burden. (B) Lifespan of C3H/HeN mice after subcutaneous challenge with MBT-2 cells. The number of mice in the experiment are in parenthesis. *Statistically significant difference compared to the control saline mice ($p < 0.05$). The survival data were subjected to Kaplan–Meier analysis

dose as low as 1 μg can stimulate Th1 responses by muscle injection [28], this low dose (1 μg) may not be enough to induce a Th1 response by skin vaccination using a gene gun. On the other hand, gold particle DNA vaccines were demonstrated to have mainly Th2-promoting effects [30,31]. In this report, we demonstrated that 1 μg of gene-gun inoculated naked DNA might be sufficient to produce a Th1-biased response, suggesting that the amount of DNA plays only a minor role in Th1/Th2 biasing and that the carrier of the DNA may have a greater role.

Evidence indicates that Th1 immunity is critical for the induction of specific cell-mediated cytotoxic cells such as tumor-specific cytotoxic T lymphocytes in tumor-bearing mice [32,33]. In the present study, naked DNA vaccine induced a stronger Th1-biased response than coated DNA vaccine, but the spleen cell-mediated cytotoxic responses to naked and coated DNA were similar (Figure 4C). One explanation for this result is that coated DNA may more efficiently transfect skin keratinocytes, which affect the magnitude of immune response by presenting the protein products of transfected genes through a process [34] that culminates in the induction of antigen-specific CD8+ T cells

In the spleen cell-mediated cytotoxic response to MBT-2 tumor cells, lymphocytes are activated by recombinant HER-2/neu protein, suggesting that the cytotoxic response is probably directed towards those MBT-2 cells overexpressing HER-2/neu. However, we cannot exclude nonspecific immune cells such as natural killer cells or macrophages in the spleen from playing a role in the anti-MBT-2 cell response. Hence, whether nonspecific immune cells contribute to tumor cell killing and whether the DNA formulation can affect this role should be examined.

In the present study, skin delivery of 1 μg of gold particle-coated or naked pCMV-EGFP DNA generated similar numbers of GFP-positive CD11c+ dendritic cells in lymph nodes. Interestingly, the percentage of these cells in lymph nodes is much higher when the naked pCMV-EGFP-DNA dose is 10 μg. The result could be attributed to two possible factors. Results indicating DNA vaccine coadministered with plasmids carrying anti-apoptotic factors can increase the percentage of dendritic cells migrated from epidermis to lymph node [24] suggest that the survival (after bombardment with naked DNA versus coated DNA) and migration of dendritic cells into lymph nodes may be increased because the dendritic cells are less damaged by gene-gun transfer of naked DNA than by gene-gun transfer of coated DNA. The second possibility is that naked DNA may be more easily taken up by dendritic cells in the epidermis than coated DNA. Uptake of coated DNA might be less efficient because, when introduced into the epidermis, the DNA is in the proximity of, but not injected directly into, the dendritic cells. Uptake is therefore dependent on endocytosis and related processes, which may be more efficient for naked DNA than coated DNA. Since the gold coating limits the amount of DNA uptake and not all 10 μg of gold-coated DNA is taken up [35], we cannot exclude the possibility that delivery of 10 μg of coated DNA would not elevate the percentage of GFP-positive cells to a similar extent. The mechanism of this interesting phenomenon requires further investigation.

The mechanism by which DNA penetrates the skin barrier is unknown. First, the force of supersonic flow may cause transient skin permeability to large molecules such as plasmid DNA. Second, the DNA (rather than penetrate) may first adhere to the skin surface and subsequently be absorbed through the skin barrier. The latter possibility is less likely because treatment of skin

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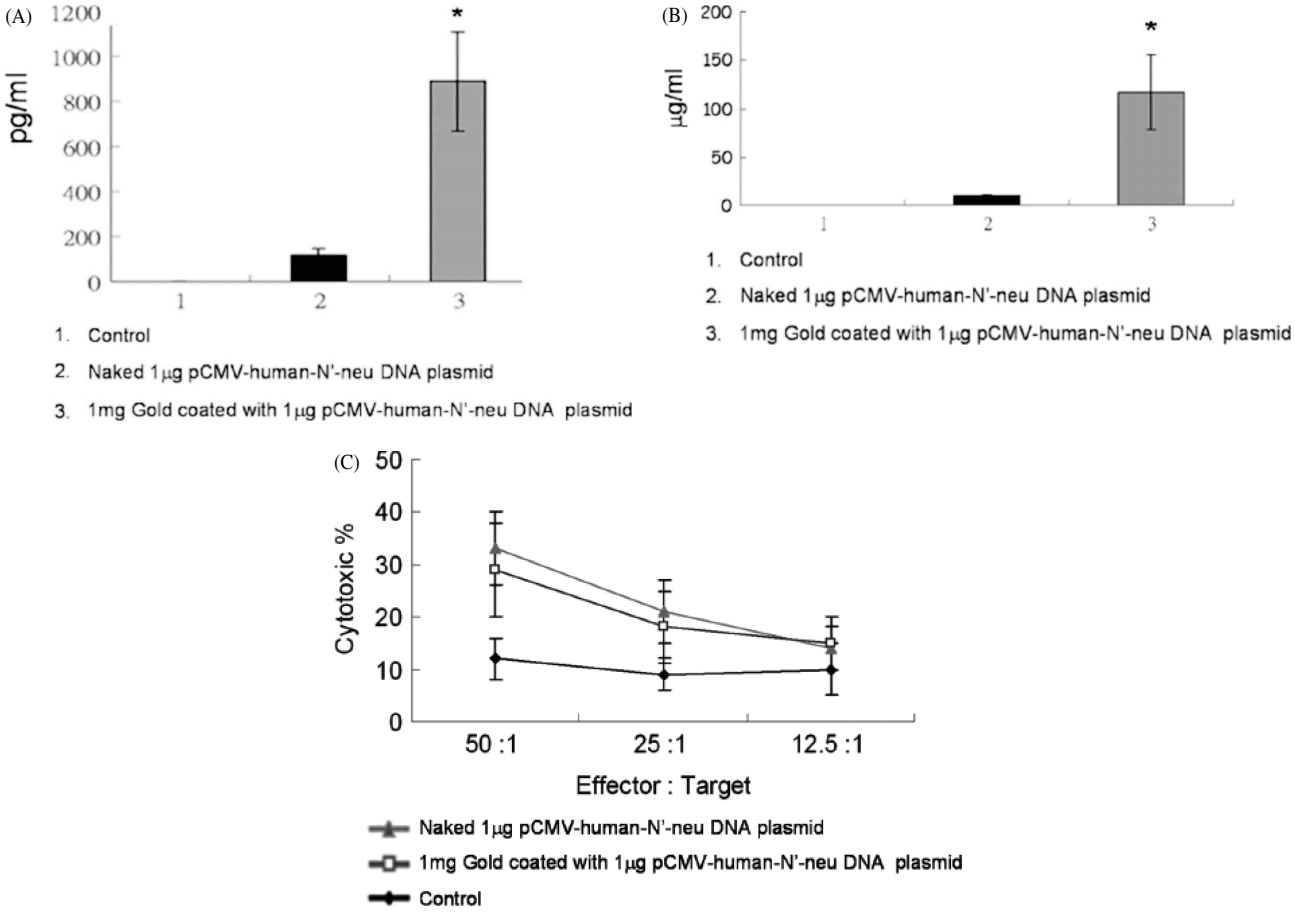

Figure 4. The immune responses to naked or coated pCMV-human-*N′*-neu DNA vaccine. (A) The expression of the extracellular domain of p185neu in skin measured with ELISA. (B) Anti-p185neu antibody titers in sera measured with ELISA. The data are average titers in the sera of three mice. (C) Spleen cell-mediated cytotoxicity assay for targeting MBT-2 cells in inoculated mice. Target cells were MBT-2-luciferase cells cultured *in vitro*. Effector cells were lymphocytes from mice treated with naked or coated *N′*-neu DNA vaccine. Cytotoxicity was determined by the luciferase release. Each point represents the average of triplicate wells. (D) Pooled splenocytes from each group were stimulated with p185neu antigen. RNA was extracted and RT-PCR was performed to assess cytokine mRNA levels. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal control. (E) Supernatants were collected 2 days after stimulation and the concentration of IFN-γ and IL-4 were measured with ELISA. *Statistically significant difference compared to the 1-μg naked pCMV-human-*N′*-neu DNA vaccine group ($p < 0.05$)

with DNase immediately after bombardment had no effect on luciferase expression (data not shown). A recent report on gene-gun transfer of naked DNA [36] suggested that the shockwave generated by a gene gun can cause transient permeability to outside substances, which is similar to our first proposed mechanism.

Our results demonstrate that naked DNA can be introduced through the skin in sufficient quantity to generate a cellular response capable of delaying tumor progression. Naked DNA vaccines may have several advantages over coated DNA vaccines, including ease of development, minimal preparation costs, and reduced skin damage. The level of antigen expression in the skin and the antibody response indicate that naked DNA is less efficiently delivered than coated DNA. Therefore, naked DNA may be useful in treating cancer or diseases that depend on the cell-mediated immune response, but not the antibody-mediated response. In addition, naked DNA may be useful in treating diseases, such as Dengue virus infection, where preventing inadvertent antibody responses is desirable. The development of severe dengue hemorrhagic fever/dengue shock syndrome after vaccination suggests that anti-virion antibodies induced by current Dengue virus vaccines may enhance infection [37].

Our experiments further extend the use of biolistic technology to deliver naked DNA into skin. The key feature of this low-pressure gene gun is the converging-diverging nozzle, which is used in rocket engines. The force generated by the nozzle is determined by three parameters: (i) the gas speed; (ii) length of the terminal spray tube; and (iii) type of gas used. Alteration of these parameters may further expand the range of gene-gun usage. For example, naked DNA may be delivered into surgically exposed organs in the future. Furthermore, since supersonic flow apparently caused approximately three-fold more damage to naked DNA than coated DNA, finding ways to protect DNA molecules may further enhance its laboratory or clinical use.

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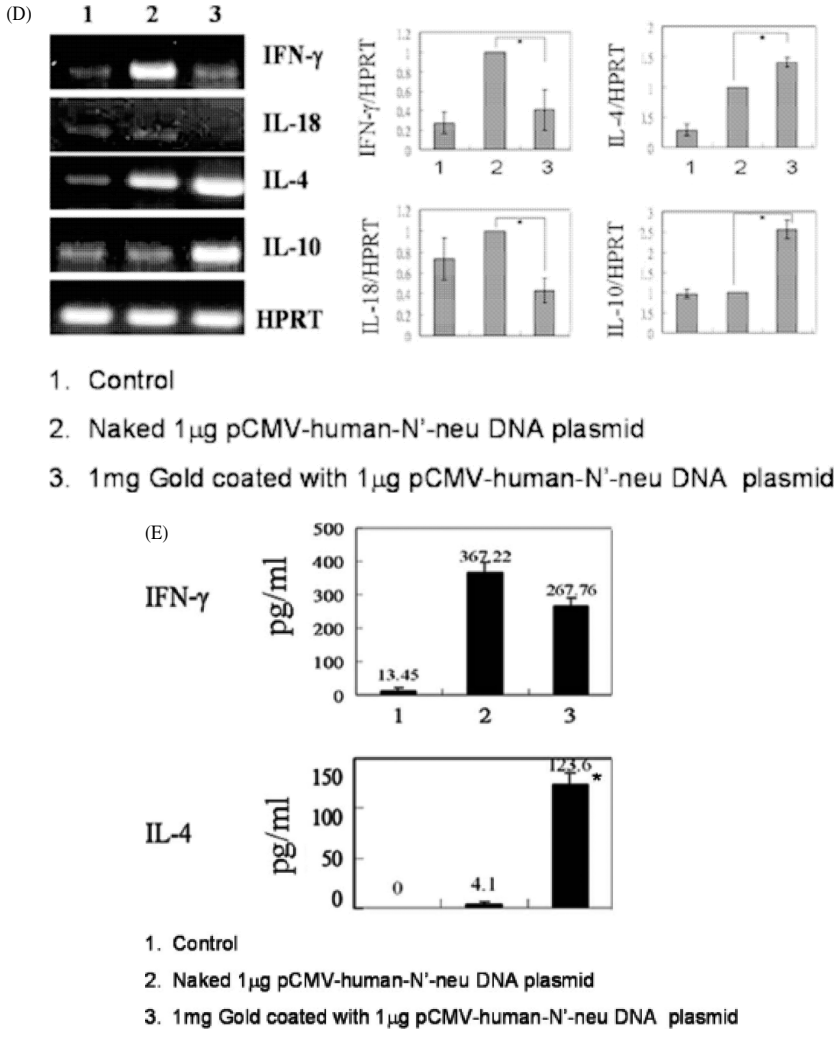

Figure 4. (*Continued*)

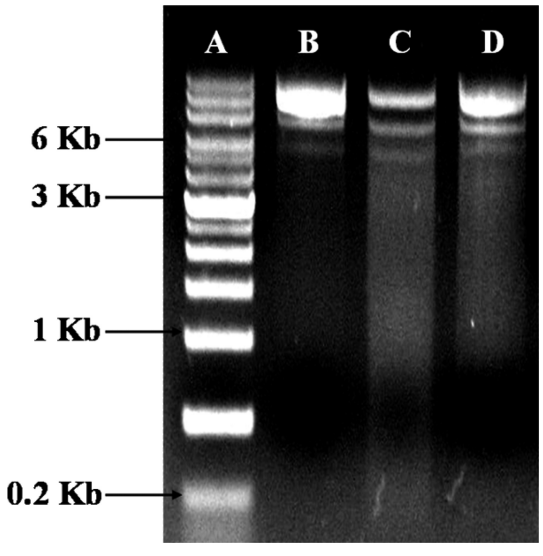

Figure 5. Influence of supersonic flow on plasmid DNA integrity. The integrity of plasmid DNA after gene-gun delivery was analysed by electrophoresis through a 1% agarose gel. Lane A: molecular weight marker; lane B: HER-2/neu DNA plasmid before delivery by gene gun; lane C: Naked HER-2/neu DNA plasmid after delivery by gene gun (60 psi); lane D: Gold particle coated with HER-2/neu DNA plasmid after delivery by gene gun (40 psi). 0.3 μg of DNA was loaded per lane

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