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Improvement of DNA vaccine immunogenicity by a dual antigen expression system

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Abstract

This study examined whether increased antigen expression resulted in enhanced antigen-specific immune responses in the context of DNA vaccines. To increase antigen expression, two copies of antigen expression cassettes were arranged in a plasmid pDX. BALB/c mice were intramuscularly immunized with various constructs that express influenza antigens and analysed for DNA-raised immunity. The plasmid pDX that expresses two copies of the antigen gene induced stronger antigen-specific immune responses than the plasmid pGA which expresses single antigen gene. To explore the in vivo transgene expression by pDX and pGA, luciferase activity was measured in the muscles transduced with luciferase expression plasmids. The pDX expressing two copies of luciferase induced the highest luciferase activity, which corresponded to the results from vaccination. We concluded that increasing the number of antigen expression cassettes in a vaccine construct improved antigen expression in the transduced tissue, which induced stronger DNA-raised immune responses.
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Vaccines have served to prevent a number of infectious diseases since Jenner's innovation. However, despite the competency of current biotechnology, some pathogens remain in place and exert an overwhelming influence on human health [1]. Although DNA vaccines have been highlighted for their potential to conquer such uncontrolled pathogens, it is also obvious that DNA vaccine immunogenicity must be improved to achieve this goal [2,3]. It has been demonstrated through current vaccinology that the application of immunomodulators and the optimization of the delivery system are effective in manipulating the host immune system for improved vaccine immunogenicity [4,5]. Besides the host immune system, the antigen expression system is also a key in affecting the outcome of DNA immunization. However, the studies on DNA vaccine improvement have particularly concentrated on manipulating the host immune system, and the aspect of antigen expression seems to be thrown aside. To activate the host immune system efficiently, sufficient antigen expression is doubtlessly important as is the choice of optimal immunomodulators. In this regard, we undertake the current study to investigate the relationship between the intensity of immune response and the amount of an antigen produced from a vaccine plasmid. A series of experiments were designed to determine if the increased antigen expression resulted in enhanced antigen-specific immune responses. We herein report that the dual antigen expression system that consists of two antigen expression cassettes induces stronger immune responses than those of an ordinary antigen expression plasmid.

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The findings from this study emphasize the importance of the antigen expression system in the effort to pursue stronger DNA-raised immune responses.

Materials and methods

Expression plasmids for vaccine antigens and reporter genes. Fig. 1 illustrates the plasmid vectors used in the current study. The single promoter plasmid, pGA, is a DNA vaccine vector that consists of cytomegalovirus immediate early promoter, bovine growth hormone polyadenylation site, and *Kan*^{r} [6]. The dual promoter plasmid pDX [7,8], a derivative of pGA that contains two expression cassettes positioned in tandem, is used to increase the antigen expression. Plasmid pDX was constructed by ligating two expression cassettes excised from the pGA vector as described previously [7]. For immunization experiments, vaccine plasmids encoding the nucleocapsid protein (NP) or hemagglutinin (HA) gene of the influenza strain A/PR/8/34 (H1N1) were constructed by insertion of an NP or HA coding region into the pGA or pDX vector. For gene expression analysis, the firefly luciferase (luc) was subcloned from pGL3-control vector (Promega, Madison, WI) as previously described [7]. All plasmid DNA was propagated by growing transformed *Escherichia coli* DH5α in LB broth and purified using the Qiagen Maxi-prep kit (Qiagen, Valencia, CA).

Animals and intramuscular DNA injection. Six- to eight-week-old female BALB/c mice (Japan Clea, Tokyo, Japan) received intramuscular plasmid DNA injection for vaccination and in vivo luciferase expression analysis. The tibialis anterior muscles were injected using 30G needles containing 50 μl of various plasmids in saline. Both the right and left muscles of the mice were injected to reduce the risk of having the needle miss the muscle in the vaccination and to reduce the number of animals used in the expression analysis. The DNA doses in the vaccination and gene expression experiments are shown in Table 1. In vaccination experiments, booster shots were given at 4 and 8 weeks following primary shot. The group alignment and DNA doses were designed to assess the effect of the number of administered plasmids and the number of expression cassettes on the transgene expression and DNA-raised immune responses.

Titration of antibody response by ELISA. ELISA was used for titration of the antigen-specific serum IgG responses of the immunized animals. Sera were collected at 4, 8, and 10 weeks following the primary inoculation and stored at −20 °C until they were assayed. The influenza virus A/PR/8/34, purified by sucrose gradient, was used as a capture antigen for the antibodies to influenzas NP and HA. The virus was treated with lysis buffer (0.05 M Tris–Cl, pH 7.8, 0.5% Triton X-100, and 0.6 M KCl) and coated on MaxiSorp plates (Nunc, Roskilde, Denmark). Subsequent to blocking with 3% BSA in PBS, serially diluted antisera were added and incubated at 37 °C for 2 h. Peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology, Barmingham, AL) was used as the detection antibody and plates were developed with ABTS substrate (Southern Biotechnology). Titres are expressed as the reciprocal value of the final detectable dilution, which was defined as 2 SD above the mean optical density at 405 nm of nonimmune sera at the same titration point.

ELISPOT for cytokines produced by ex vivo stimulated splenocytes. The ELISPOT assay was run to measure IFN-γ and IL-4 produced by the restimulated bulk spleen cells harvested from the immunized animals. Two weeks following the second booster shot, splenocytes were harvested and processed as previously described [7,9]. Briefly, multiscreen 96-well plates (Millipore, Bedford, MA) were coated with anti-mouse IFN-γ or IL-4 antibody (R4-6A2 or BVD4-1D11, Pharmingen, San Diego, CA). To stimulate the cells from the NP-immunized

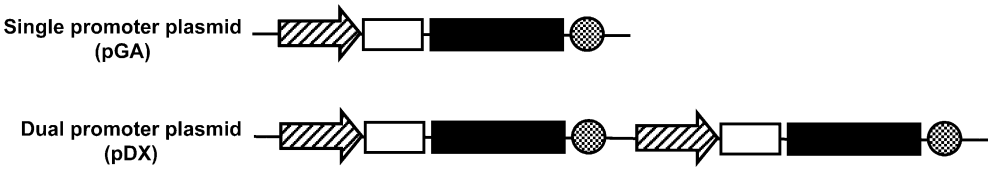

Fig. 1. Schematic diagram of the plasmids. Single promoter plasmid vector, pGA, contains a cytomegalovirus immediate-early promoter (hatched allow) and intron A (open square) to express the transgene (solid square) and a bovine growth hormone polyadenylation signal (dotted circle) for termination of the transcription. Dual promoter plasmid vector, pDX, has two expression cassettes positioned in tandem. Each expression cassette in pDX is identical to that in pGA vector. For immunization, influenza nucleoprotein (NP) or hemagglutinin (HA) gene was inserted as a vaccine antigen. For gene expression analysis, luciferase (luc) was inserted as a reporter gene.

Table 1
Group alignment and the plasmid applied in each experiment

Objective of the experiment	Plasmid administered	Dose[a] (μg)	Number of plasmids[a] ($\times 10^{11}$ copies)	Number of transgenes[a] ($\times 10^{11}$ copies)
DNA vaccination against influenza nucleoprotein	pGA-NP + pGA-luc	24 (12 + 12)	40	20
	2× pGA-NP	24	40	40
	pDX-NP/luc	20	20	20
	pDX-NP/NP	20	20	40
DNA vaccination against influenza hemagglutinin	pGA-HA + pGA-luc	26 (13 + 13)	40	20
	2× pGA-HA	26	40	40
	pDX-HA/luc	20	20	20
	pDX-HA/HA	20	20	40
Transduction of the muscles with reporter luciferase	pGA-luc + pGA-HA	100 (50 + 50)	160	80
	2× pGA-luc	100	160	160
	pDX-luc/HA	80	80	80
	pDX-luc/luc	80	80	160

[a] The values are expressed as an amount for a mouse, but not for the tibialis anterior muscle of each side.

animals, H-2^d-restricted NP class I peptide (TYQRTRALV) and a pool of three class II peptides (FWRGENGRKTRSAYERMCN ILKGK, RLIQNSLTIERMVLSAFDERRNK, and AVKGVGTM VMELIRMIKRGINDRN) were used. For the HA-immunized animals, H-2^d-restricted HA class I peptide (IYSTVASSL) and a pool of five class II peptides (SFERFEIFPKE, HNTNGVTAACSH, CPK YVRSAKLRM, KLKNSYVNKKGK, and NAYVSVVTSNYN RRF) were applied. For ex vivo restimulation, one million cells were incubated in duplicate in the presence of the peptide and anti-CD28 and CD49d Abs (37.51 and R1-2, respectively, both from Pharmingen). A medium containing an irrelevant peptide or PMA plus ionomycin (50 ng/ml PMA and 1 μg/ml ionomycin) was used as negative and positive controls, respectively. The plates were cultured for 40 h at 37 °C, and then washed and biotin-conjugated anti-mouse IFN-γ or IL-4 antibodies (XMG1.2 or BVD6-24G2, Pharmingen) were added and incubated. The plates were subsequently treated with streptavidin–horseradish peroxidase (Vector lab, Burlingame, CA) and then developed with stable diaminobenzidine (Research genetics, Huntsville, AL). The spot forming units (SFU) were counted and normalized for 1×10^6 splenocytes.

Assay for in vivo luciferase expression. The muscles injected with various luciferase expression plasmids were removed at three days following injection and soaked in muscle lysis buffer (10 mM Tris–HCl, pH 7.8, 2 mM $MgCl_2$, and 0.5% Tween 20). The muscles then underwent three freeze–thaw cycles and were subsequently homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The suspension was spun down at 1500*g* for 15 min and the saved supernatant was subjected to the luciferase assay. Luciferase activity was determined with an assay kit (Promega) using a Lumicounter 700 chemiluminometer (Microtech, Chiba, Japan) and expressed as relative light units (RLU)/s per mg protein, with protein contents being determined by the BioRad DC protein assay kit (BioRad, Hercules, CA).

Statistics. Statistical analyses were performed by Kruskal–Wallis test and significance was defined at $p < 0.05$.

Results

Humoral immune response

BALB/c mice were intramuscularly immunized three times (at weeks 0, 4, and 8) with one of the various plasmids encoding influenza NP or HA. Anti-NP or anti-HA antibodies in sera collected from the mice at weeks 4, 8, and 10 were titrated by ELISA. All immunized mice produced antibodies specific to influenza NP or HA. These were first detected at low levels 4 weeks after primary injection and continued to increase as shown in Fig. 2. Up to the eighth week, no significant differences in antibody titre among groups were observed. However, at 10 weeks, the sera from the animals immunized with the dual promoter plasmid expressing two antigen genes showed a higher antibody titre than the others. Interestingly, the second highest antibody titre was seen in the sera from the animals immunized with the dual promoter plasmid having an antigen and a mock insert expression cassette, although the number of antigen expression cassettes in those groups was smaller than that of 2× single promoter vaccine groups. The sera immunized with a single promoter vaccine construct showed a similar level of antibody titre, regardless of the number of antigen expression cassettes in an inoculation. The above-mentioned inclination was preserved in both NP and HA immunized animals. The immunoglobulin isotype profile was also analysed. The IgG2a response was consistently stronger than the IgG1 response in all groups and the IgG2a antibody titres showed similar trends to those in Fig. 2 (data not shown). To compare the persistency of the antibody production in the mice immunized with various vaccine constructs, some mice from the HA immunized group were maintained until 24 weeks and sera were collected at 12, 16, 20, and 24 weeks. In all groups, the antibody titres showed a gradual decline and reached background levels at 24 weeks (data not shown).

Cell-mediated immune response

Mice were immunized with various vaccine plasmids as described above and the spleens were harvested at 10 weeks after the priming. The ELISPOT assay was performed to evaluate the antigen-specific

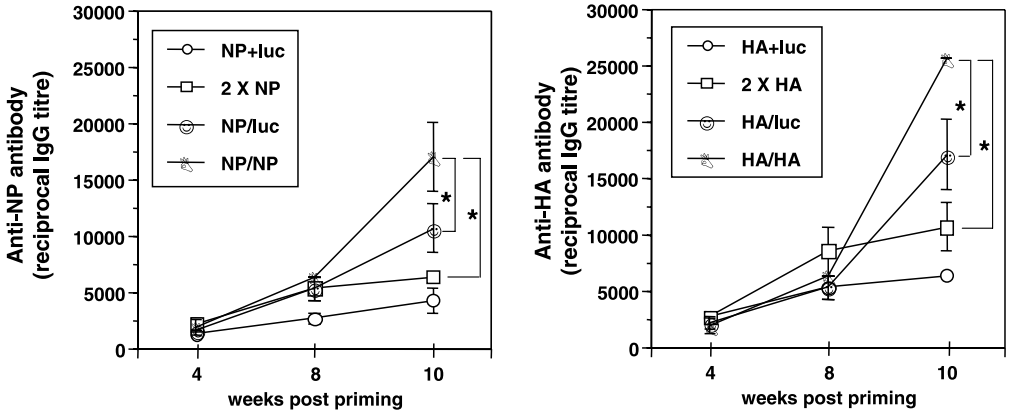

Fig. 2. Time kinetics of the serum antibody titre to influenza nucleoprotein (left) and hemagglutinin (right) of the mice immunized with the indicated single or dual promoter vaccine plasmid. The results are expressed as means ± standard errors ($n = 5$) in terms of reciprocal IgG titre and the * indicates statistical significance. Similar results were obtained in a repeat experiment (not shown).

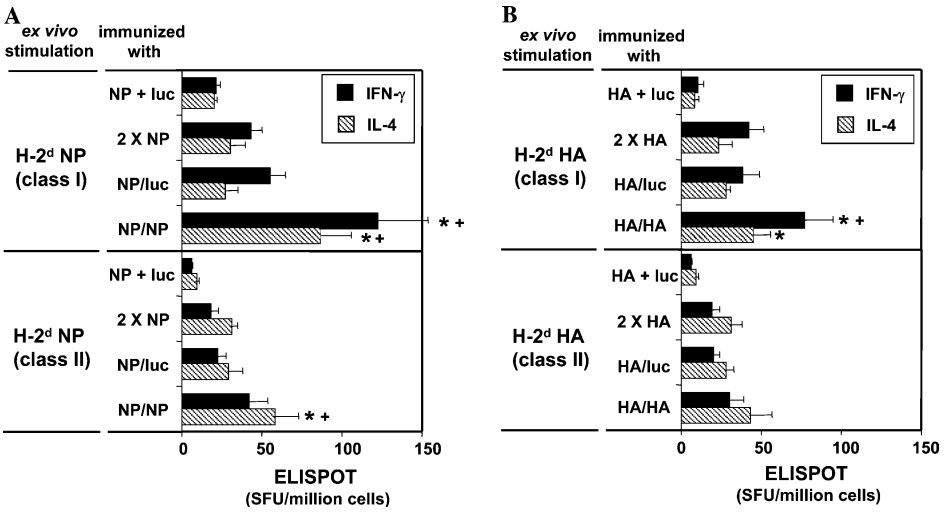

Fig. 3. Influenza nucleoprotein (A) and hemagglutinin (B)-specific IFN-γ (solid bar) and IL-4 (hatched bar) ELISPOT responses of mice immunized with the indicated single or dual promoter vaccine plasmid. Splenocytes were harvested 2 weeks following the second booster shot and received ex vivo stimulation with H-2^d-restricted class I or II peptides. The results are expressed as means ± standard errors ($n = 5$) in terms of spot forming units (SFU) per million splenocytes. Backgrounds for cells stimulated with an irrelevant peptide (<20 SFU/10^6 cells) have been subtracted from the ELISPOT data. The * indicates significant difference between the two groups immunized with pDX vector, whereas the + indicates significant difference between a dual antigen group (NP/NP or HA/HA) and 2× single antigen group (2× NP or 2× HA).

IFN-γ and IL-4 producing CD8 (measured following stimulation with class I peptide) and CD4 (measured following stimulation with class II peptide) T lymphocytes. As shown in Fig. 3A, the mice immunized with the dual promoter vaccine expressing two NP genes (NP/NP) yielded a prominent number of IFN-γ and IL-4-producing CD8 cells, which were two to three times more frequent than that of the dual promoter plasmid expressing NP and luc (NP/luc) or the 2× single promoter NP vaccine (2× NP) groups. A lesser extent of IFN-γ and IL-4-producing CD4 cell responses was observed, however, the total number of cytokine producing cells in each group exhibited similar trends to those seen in CD8 cells. The results from ELISPOT for HA-specific CD8 and CD4 cells producing IFN-γ and IL-4 (Fig. 3B) paralleled those obtained for NP-specific cytokine production, although NP-immunized animals yielded a stronger response than HA-immunized animals. Ex vivo stimulation with class I peptide elicited a dominant IFN-γ response whereas class II peptide induced IL-4 dominance, irrespective of the antigen administered. This trend is identical to that seen in the previous studies involving the ELISPOT assay to detect NP- or HA-specific cytokine responses.

In vivo luciferase expression

Luciferase reporter expression was also measured to assess the extent of gene expression by both single and dual promoter expression systems. Fig. 4 shows the luciferase activity of the muscle injected with single and dual promoter luciferase expression vectors. Although

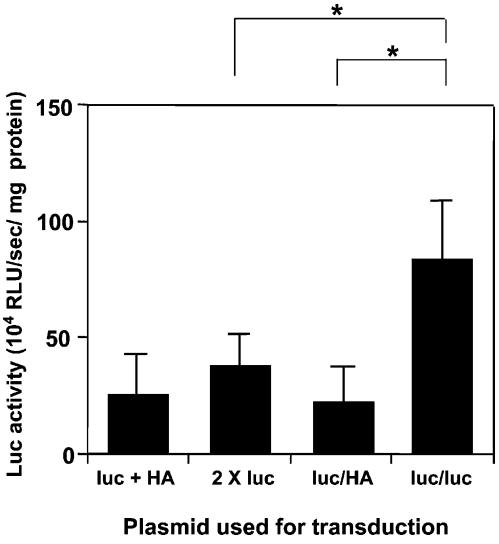

Fig. 4. Effect of single and dual promoter plasmids on in vivo luciferase expression. The luciferase activity of the tibialis anterior muscles transduced with 50 μg (single promoter group) or 40 μg (dual promoter group) of the indicated luciferase expression plasmid by saline injection. Two days following DNA injection, the muscles were resected for luciferase assay. Each bar represents the mean ± standard errors ($n = 10$) and the * indicates statistical significance.

the luciferase activity varied widely, the muscles of all groups produced a detectable level of luciferase. The dual promoter vector for two luciferase genes (luc/luc) gave the highest luciferase activity among the four groups, whereas the other three groups showed lower expression levels. The level of intramuscular luciferase expression directly correlates with the results from vaccination.

Discussion

The central finding from the current study is that a vaccine plasmid having two antigen expression cassettes induced stronger immune responses than an ordinary vaccine plasmid, even when an equivalent number of antigen expression cassettes were administered to the animal. This suggests that increased antigen expression resulted in enhanced antigen-specific immune responses. This finding also supports the previous observations that magnitude of immune responses reflected the strength of antigen expression [10–12]. Enhancement of antibody response by the use of dual antigen expression plasmid was observed both in NP- and HA-immunized animals. By contrast, enhancement of T cell response by dual antigen expression plasmid was evident only in the NP-immunized animals. This discrepancy was possibly caused by the divergent immunogenicity of NP and HA [13,14], as our previous study indicated that NP preferentially elicits cell-mediated immunity whereas HA readily induces antibody response [7,8]. A few studies have been attempted to improve DNA vaccines by improving antigen expression through the alteration of promoter [15–17] and the induction of muscle regeneration [15,18,19]. However, to our knowledge, there has been no study pursuing increased antigen expression by increasing the copies of antigen expression cassettes in a plasmid vector.

We hypothesized that a dual promoter plasmid that expresses two copies of an antigen can double the amount of antigen produced in a transduced cell, which results in enhanced DNA-raised immune responses. To explore the aspect of gene expression underlying the induction of immune responses by a dual promoter DNA vaccines, in vivo transgene expression was analysed using reporter luciferase. The plasmid pDX, which carries two copies of luciferase genes, yielded the strongest luciferase expression. Increasing the copies of transgene in a plasmid is a simple strategy, however, it is proved to be effective in enhancing the transgene expression in vivo. The results from luciferase assay corresponded to the outcome of vaccination, which implied that increasing the copies of antigen gene brought about improved antigen expression and antigen-specific immune responses.

Interestingly, a greater antibody titre was observed in antigen/luc groups than in 2× antigen groups in both NP- and HA-immunized animals. As for the T cell response, a comparable level of cytokine production was observed in both antigen/luc and 2× antigen groups, regardless of the antigen administered. Similarly, no significant differences were observed between luc/HA and 2× luc group in luciferase assay. It should be noted that the number of vaccine plasmids and the number of antigen expression cassettes per animal in antigen/luc group were half those of the 2× antigen group as displayed in Table 1. The total amount of DNA administered to an animal, in terms of micrograms per mouse, was also smaller in antigen/luc groups than in 2× antigen groups. Accordingly, the size of vaccine plasmid could affect the antibody response from DNA vaccination, the larger plasmids are presumably advantageous in eliciting greater antibody titre. For clarification, further studies exclusively designed to address this issue are necessary, since various antigens should be tested for generalization and no rationale is found behind the advantage of larger plasmids.

The current study highlights the number of antigen expression cassettes in a vaccine plasmid in eliciting DNA-raised immune responses. We conclude that the dual antigen expression system, as used in this study, constitutes a simple and straightforward strategy to improve antigen expression for enhanced DNA vaccine immunogenicity.

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