Immunology 1998 **94** 285–289

Immuno-stimulatory effects of bacterial-derived plasmids depend on the nature of the antigen in intramuscular DNA inoculations

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SUMMARY

The CpG motifs of bacterial-derived plasmids augment antigen-specific immune responses and steer those responses towards the T helper 1 (Th1) type. In this study, we have addressed the immuno-stimulatory effect of intramuscular co-administration of CpG motifs containing vector DNA on the modulation of immune responses to the haemagglutinin (HA) and the nucleoprotein (NP) proteins of influenza virus. The co-administration of vector DNA with a HA-encoding plasmid DNA showed a significant enhancement in the total IgG response, the generation of cytotoxic T lymphocyte (CTL), and the T-cell proliferative response. In the case of NP-encoding plasmid DNA inoculations, the co-administration of vector DNA slightly decreased the total IgG response, although the IgG2a/IgG1 ratio and the CTL responses to NP were significantly increased. These observations suggest that the immuno-stimulatory effects of bacterial-derived plasmids depend upon the nature of the co-administered antigen.

DNA immunizations elicit strong immunity to many infectious pathogens including parasites, bacteria, and viruses.[1,2] The most widely used methods for introduction of plasmids that express foreign antigens are intramuscular (i.m.) inoculation and subepithelial inoculation using a gene gun (g.g.).[3,4] Qualitatively different immune responses are induced by these different delivery methods. I.m. inoculation predominantly elicited a T helper 1 (Th1) response with a high level of IgG2a induction, while g.g.-mediated inoculation predominantly elicited a Th2 response with the induction of IgG1.[5] Recently, it has been observed that the inoculation of unmethylated CpG motifs that are derived from bacterial plasmid DNA have the adjuvant effect of stimulating the Th1 response. It was demonstrated that the administration of CpG motif-enriched plasmids or CpG oligonucleotides together with several proteins could promote Th1 immunity, leading to the inducement of IgG2a and interferon-γ (IFN-γ) expression, and to the suppression of immunoglobulin E (IgE) synthesis.[6,7] The immuno-stimulatory functions of these CpG motifs probably resulted from the secretion of various cytokines, such as interleukin-12 (IL-12), IFN-γ, tumour necrosis factor-α (TNF)-α, and IL-6, from macrophages or natural killer (NK) cells in the local environment.[6,8] However, the immuno-stimulatory effects of bacterial-derived plasmid DNA itself have not been clearly determined in DNA inoculation experiments.

In this study, we have investigated the effect of CpG motifs-containing vector DNA on the modulation of immune responses, when it was co-administered by intramuscular DNA inoculations with a plasmid that contained either the membrane-bound haemagglutinin (HA) gene or the intracellular nucleoprotein (NP) gene of influenza A virus. In addition, we compared the resultant immune responses with those from inoculations of antigen-encoding plasmids alone.

To assess the immune responses that are generated in mice, a plasmid that encodes the HA gene of the influenza A/Jap/57 virus was inoculated intramuscularly. In order to determine the dose dependency of plasmid DNA for the generation of humoral immune responses, we inoculated either 5 μg or 100 μg of HA-encoding plasmid (pCIN-HA) and monitored the resulting antigen-specific antibodies by means of enzyme-linked immunosorbent assay (ELISA; Fig. 1a). No meaningful antibody (Ab) response to HA was detected when 5 μg of pCIN-HA was injected; however, a strong Ab response was detected when 100 μg of pCIN-HA was injected, which suggested that a large amount of HA antigen was required to generate an effective humoral response. To investigate the effects of vector DNA co-administration on the modulation of humoral immune responses, we also inoculated 95 μg of vector DNA (pCI-neo; pCIN, Invitrogen, San Diego, CA) together with 5 μg of pCIN-HA. The co-administration of pCIN significantly increased the HA-specific Ab response, making it comparable to the Ab response that was observed with the inoculation of 100 μg of pCIN-HA. All groups of mice that received intramuscular DNA inoculations appeared to predominantly produce IgG2a responses to HA, which is consistent with other reports[5,9] (Fig. 1b). The co-administration of pCIN elicited similar IgG isotype responses

Received 15 April 1998; revised 7 May 1998; accepted 7 May 1998.

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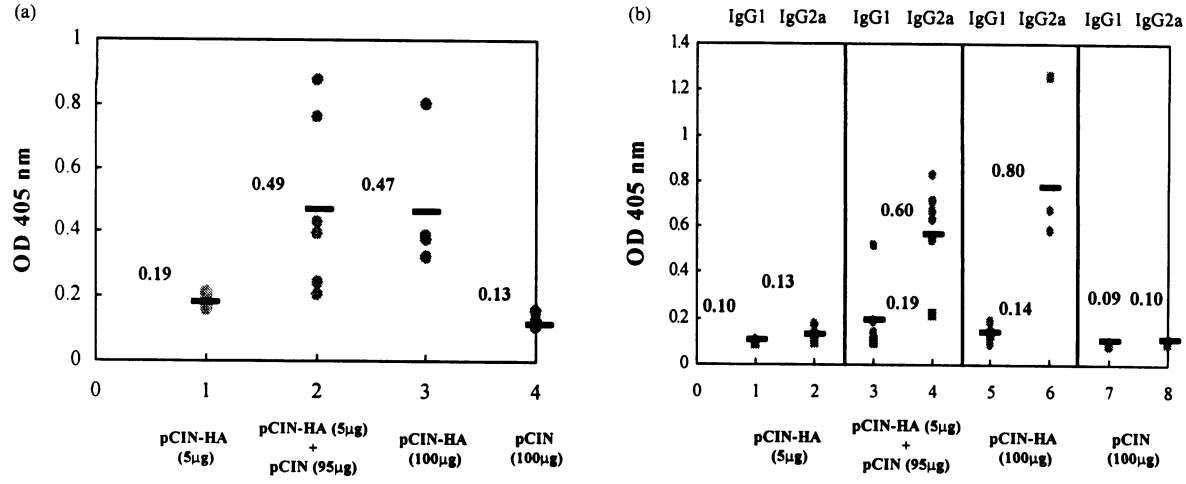

Figure 1. Total IgG responses and IgG isotypes to HA. Six-week-old female BALB/c mice were immunized intramuscularly with the indicated plasmids and sera obtained at week 3 post-immunization (p.i.) were tested by ELISA. Briefly, 96-well plates were coated overnight at 4° with 100 μl (10^6 PFU) of inactivated influenza virus particle (A/Jap/57). The plates were washed twice with phosphate-buffered saline 0·05% Tween-20 (PBST) and incubated with 100 μl of 2% BSA for 1 hr at room temperature. After washing the plates with PBST, 100 μl of 1:100 dilutions of test sera was added to each well and incubated for 2 hr at room temperature. Bound antibodies were detected by the incubation of HRP-conjugated anti-mouse IgG (1:3000) antibodies (Bio-Rad, Hercules, CA) for 1 hr at room temperature. For the determination of antibody isotypes, either HRP-conjugated anti-mouse IgG1 or IgG2a antibodies (Southern Biotechnology, Birmingham, AL) were used as secondary antibodies, respectively. (a) Total IgG responses to HA. (b) Isotyping of anti-HA IgG. The optical density (OD) value is shown for each mouse in the group, and the average values were indicated with lines. The different plasmids that were injected into mice are indicated at the bottom.

to those seen with the inoculation of 100 μg of pCIN-HA, although a slightly lower level of IgG2a response was detected.

We also examined the modulation of antigen-specific cytotoxic T lymphocyte (CTL) responses by pCIN co-administration in intramuscular DNA inoculations. HA-specific CTL responses were increased when 100 μg of pCIN-HA was inoculated, as compared with when 5 μg of pCIN-HA was inoculated (Fig. 2a). At 10:1 effector:target (E:T) ratios, the specific lysis of P815 target cells reached approximately 37% by the effector cells from mice that were inoculated with 100 μg of pCIN-HA. However, the effector cells from mice that were inoculated with 5 μg of pCIN-HA required five-fold higher E:T ratios to reach a comparable level of specific lysis. The co-administration of 95 μg of pCIN with 5 μg of pCIN-HA induced a significantly higher CTL response to HA than did the inoculation of 5 μg of pCIN-HA alone. CTL activity was increased from 16% to 45% at 10:1 E:T ratios by the co-administration of pCIN. As a control, spleen cell from each of the immunized mice showed no significant CTL activities in response to P815 target cells that were infected with recombinant vaccinia virus that encoded β-galactosidase (rVV-LacZ) (Fig. 2b). In addition, the spleen cells from mice that were inoculated with 100 μg of pCIN alone showed about 10% lysis activity to P815 targets at 50:1 E:T ratios.

To further elucidate the modulation of cellular immune response that resulted from pCIN co-administration, we performed a T-cell proliferation assay with splenocytes from pCIN-HA-immunized mice (Fig. 2c). Splenocytes from mice that were inoculated with 100 μg of pCIN alone had a non-specific stimulation index (SI) of approximately 2 when inactivated influenza virus particles (A/Jap/57; 3×10^6 PFU/ml) were added to the culture. Antigen-specific responses were detected in the splenocytes from mice that were inoculated with either 100 μg or 5 μg of pCIN-HA, with SI values of approximately 5·2 and 3·5, respectively. Furthermore, the co-administration of 95 μg of pCIN induced the highest T-cell proliferative response, which reached an SI value of about 7·5, suggesting that the co-administration of pCIN augmented T-helper-cell response. Taken together, these results demonstrate that the co-administration of vector DNA with pCIN-HA in intramuscular DNA inoculations can enhance humoral as well as cellular immune responses to HA.

To test if the immuno-stimulatory effects of vector DNA that were seen in the co-administration experiments with the HA gene are applied to a different antigen, we also performed DNA inoculations with a plasmid that encoded the NP gene of the influenza A/PR/8 virus. The inoculation of 5 μg of pCIN-NP was sufficient to elicit a strong Ab response to NP (Fig. 3a). However, the inoculation of 100 μg of pCIN-NP did increase the NP-specific Ab response. In contrast to what was observed with the inoculation of pCIN-HA, the co-administration of pCIN with pCIN-NP elicited a slightly lower level of total IgG response to NP than did the inoculation of 5 μg of pCIN-NP alone. Interestingly, the lower IgG2a/IgG1 ratio [mean optical density (OD) value of IgG2a/mean OD value of IgG1] was detected in mice that were inoculated with 5 μg of pCIN-NP than was detected in mice that were inoculated with 100 μg of pCIN-NP. However, this IgG2a/IgG1 ratio was significantly increased by the co-administration of 95 μg of pCIN (Fig. 3b).

The NP-specific CTL activity induced by the inoculation of 100 μg of pCIN-NP (about 27%) was shown to be lower

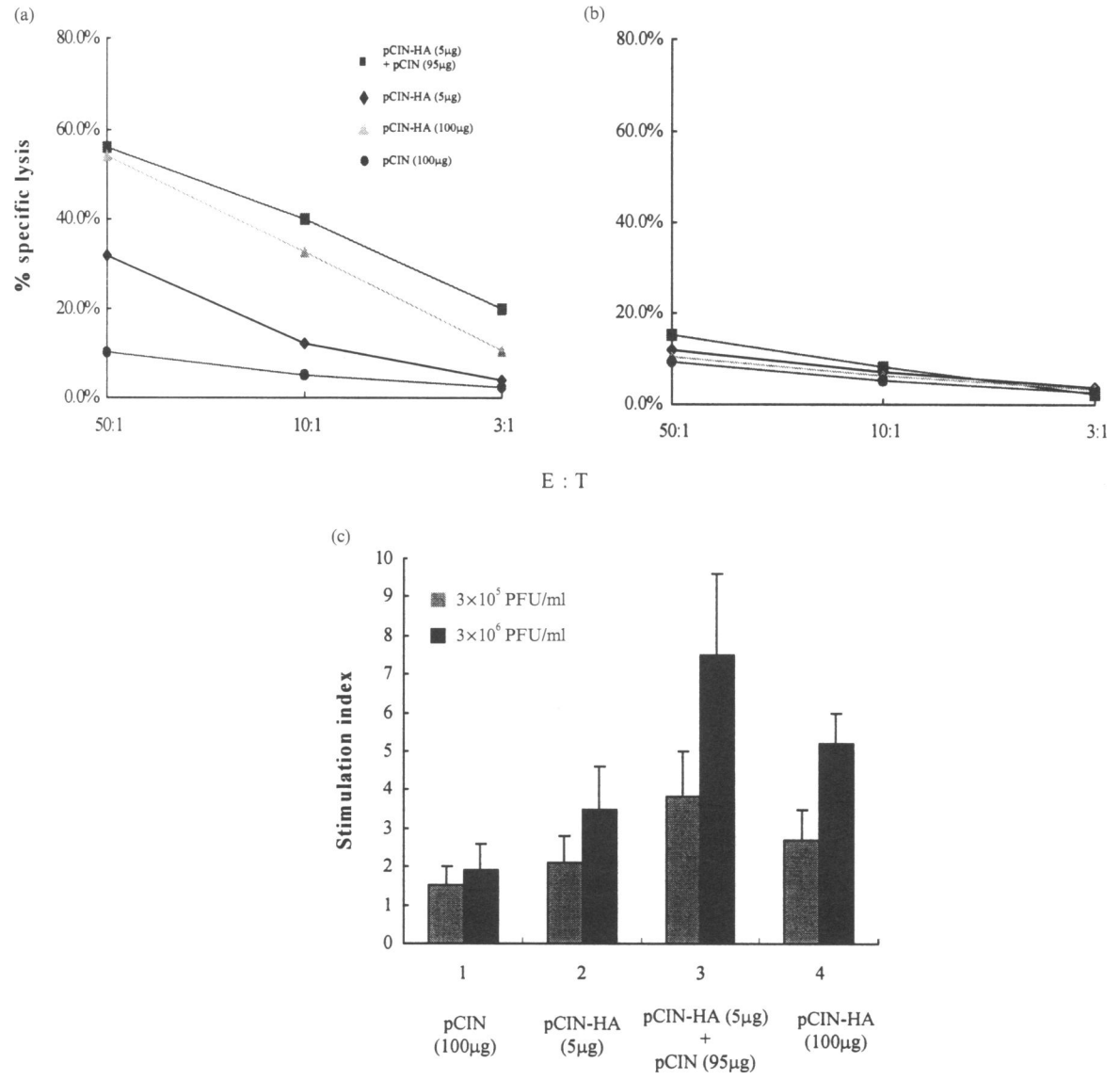

Figure 2. CTL responses to HA that were generated by intramuscular DNA inoculation. Pooled spleen cells that were obtained week 3 p.i. were maintained in RPMI-1640 medium that was supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 50 μM of β-mercaptoethanol, and 10 U/ml of recombinant murine IL-2 (Pharmingen, San Diego, CA). Responder cells were cultured for 7 days at $1{\cdot}5 \times 10^7$ cells/ml in the presence of 5×10^6 naive splenocytes that had been infected with 10 multiplicity of infection (M.O.I.) of recombinant vaccinia virus that encoded the HA gene of the A/Jap/57 virus (rVV-HA) and were treated with mitomycin C for 30 min at 37°. P815 ($H\text{-}2^d$) target cells were also infected with either 5 M.O.I. of rVV-HA (a) or rVV-LacZ (b) and labelled with ^{51}Cr Na_2CrO_4 (Amersham). Target cells at 10^4 per well were incubated for 5 hr in triplicate at 37° with various dilutions of effector cells. The maximum and spontaneous release of ^{51}Cr were determined from wells that were treated with either 2% Triton-X 100 or medium alone, respectively. (c) T-cell proliferative responses to HA. Spleen cells were treated with ACK lysis buffer in order to clear RBC.[11] Splenocytes were added to each well of a round-bottomed 96-well plate, and inactivated virus particles were added to the wells in triplicate at concentrations of 3×10^5 or 3×10^6 PFU/ml. After 3·5 days in culture, the cells were pulsed with 1 μl of [^{3}H]thymidine for 18 hr. The plates were harvested with MultiScreen assay system (Milipore, Bedford, MA) and the amount of incorporated radioactivity was measured. The stimulation index (SI) was calculated as the mean counts per minute (C.P.M.) of stimulated wells divided by the mean C.P.M. of wells that received only media.

than what was induced by the inoculation of 5 μg of pCIN-NP (about 51%) at E:T ratios of 25:1 (Fig. 3c), suggesting that the expression of large amounts of NP antigen may inhibit the generation of a maximal CTL response. Our results are consistent with a previous report by Ulmer *et al.*[10] that showed that the intramuscular injection of either 1 μg or 10 μg of NP-expressing plasmid induced higher CTL activities than did the injection of 100 μg of this plasmid DNA. As expected, the co-administration of 95 μg of pCIN greatly increased the NP-specific CTL activity up to approximately 61% at 5:1 E:T ratios. As a control, spleen cells from each of the immunized mice showed less than 15% of specific lysis to P815 targets that had been pulsed with control peptide (Fig. 3d). Taken together, these observations suggest that the

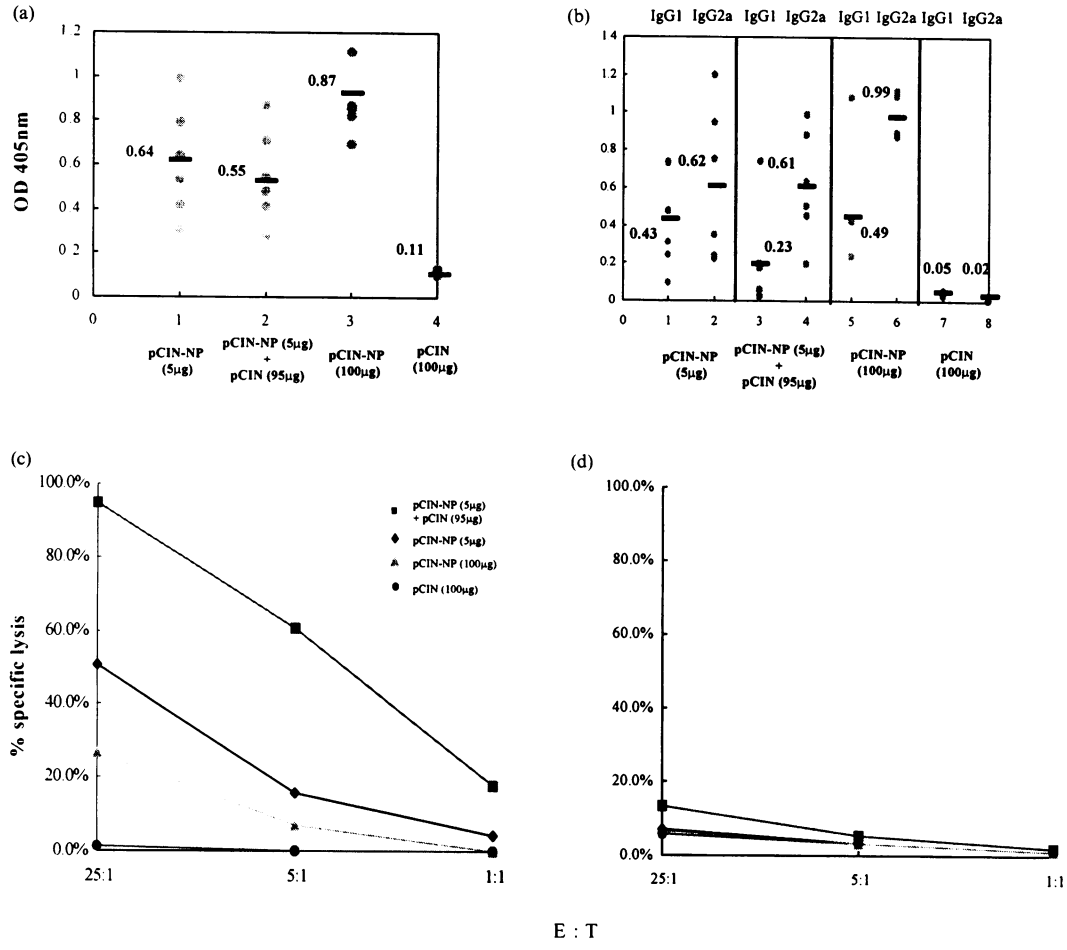

Figure 3. (a) Total IgG responses to NP and (b) isotyping of anti-NP IgG. Each well of a 96-well plate was coated with 100 ng of NP that was purified from *Escherichia coli*. ELISAs were carried out as previously described. (c) CTL responses to NP. Responder spleen cells were cultured for 6 days at 2×10^7 cells/ml in the presence of 10 μM of $NP_{147-155}$ peptide (TYQRTRALV) and 10 U/ml of recombinant murine IL-2. Cytotoxicity was assayed against P815 cells that were pulsed with 10 μM of $NP_{147-155}$ peptide and labelled with ^{51}Cr Na_2CrO_4. 10^4 target cells per well were incubated for 5 hr in triplicate at 37° with various dilutions of effector cells. (d) Cytotoxicity was assayed against P815 cells that were pulsed with 10 μM of control peptide (gp120 V3 peptide of human immunodeficiency virus type 1; RIQRGPGRAFVTIGK).

NP-specific immune responses were further shifted by the co-administration of vector DNA to Th1-type cellular immune response.

The mechanism by which intramuscular DNA inoculation induces immune responses is not yet clear. Recent observations have suggested that immune responses could be elicited by the movement of free DNA and the release of antigens from injected muscles into the distal lymph node or the spleen.[12,13] The co-inoculation of vector DNA with antigen-encoding plasmids may be able to make the effective cytokine milieu in the local environment that allows for immune responses to be generated by specific antigens. This is probably because of the stimulation of macrophages, B cells, and NK cells.[6,8,14] However, our observation that the co-administration of vector DNA could differentially modulate Ab responses to NP and HA suggests that the immuno-stimulatory effects of vector DNA are likely to depend on the nature of the co-administered antigen. In addition, the immune responses that are elicited by the inoculation of 100 μg of antigen-encoding plasmid were somewhat different from the co-administration of vector DNA, even if the total amount of plasmid was consistent and all the constructs had the same backbone. These findings suggest that the immuno-stimulatory function of bacterial plasmid DNA can be affected by the amount of expressed antigen in the local environment. Recently, the importance of CTL responses for the control of acute influenza virus infection has been demonstrated.[15,16] We have shown that the inoculation of large amounts of plasmid DNA hampered the generation of CTL to NP, but not to HA, which suggests that the dose of plasmid DNA that is used in inoculations has to be carefully determined. In addition, our data suggest that the co-administration of vector DNA will be an important strategy for optimizing the efficacy of influenza DNA vaccines.

ACKNOWLEDGMENTS

We express great thanks to Sang Chun Lee for conscientious animal care, and to Jin Won Yun and Chu Hee Lee for technical supports. We also extend thanks to Drs T. J. Braciale and Y. S. Hahn, the

University of Virginia, VA, USA for the kind gift of influenza A/Jap/57 virus and rVV-HA.

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