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Plasmid Encoding Interleukin-4 in the Amelioration of Murine Collagen-Induced Arthritis

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Objective. **To evaluate the therapeutic effect of the administration of plasmid encoding interleukin-4 (IL-4) via gene-gun delivery and via intradermal injection on collagen-induced arthritis (CIA).**

Methods. **IL-4 plasmid was administered by gene-gun delivery and intradermal injection to DBA/1 mice immunized with type II collagen (CII). The therapeutic effect on the development of CIA was evaluated clinically with a visual scoring method for arthritis and serologically by enzyme-linked immunosorbent assays and polymerase chain reaction.**

Results. **Treatment with IL-4–expressing plasmid significantly reduced the incidence and severity of CIA, including a reduction in the anti-CII antibody level. In particular, gene-gun delivery had a higher immunosuppressive effect on CIA compared with intradermal injection. As shown by in vitro stimulation assay, the spleen cells from mice immunized with CII and treated with IL-4 plasmid via gene gun exhibited higher Th2 cytokine responses compared with cells treated with control plasmid after in vitro stimulation with CII.**

Conclusion. **The results of this study suggest that treatment with IL-4 plasmid may constitute a new clinical use of cytokine gene therapy for rheumatoid arthritis.**

Rheumatoid arthritis (RA) is characterized by the proliferation of synoviocytes and destruction of cartilage and bone in the joints. It is generally accepted that the proinflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) are critical mediators in the inflammatory process of arthritis (1,2). Anti-TNFα antibodies have been shown to have successful therapeutic effects against inflammation in RA patients (3,4). Others have suggested that the imbalance of Th1/Th2 cytokines is likely to be associated with the perpetuation of chronic inflammation associated with RA (5). IL-4 is crucial in the Th2 response and inhibits the production of proinflammatory cytokines such as IL-1, TNFα, and IL-6 (6,7), and has been shown to suppress Th1 cell activity (8). The level of IL-4 production is often low in the synovial fluid and tissue of RA patients (5).

RA has generally been considered to be a Th1-dominant autoimmune disease in the Th1/Th2 cytokine balance (5). Therefore, the administration of IL-4 to RA patients may act to correct the Th1/Th2 imbalance and may subsequently result in reduced joint inflammation. However, antiarthritis cytokine therapy has several limitations, since systemic administration of these cytokines exerts adverse effects, and daily or weekly administration, due to the short half-life, is necessary to obtain therapeutic effects (8).

Thus, gene therapy is currently being applied as a new approach in the treatment of RA in an experimental model. In this therapy, a viral or nonviral vector is used for gene transfer. Collagen-induced arthritis (CIA) is an experimental animal model that encompasses clinical and pathologic features similar to RA (9). In the present study, we investigated the effect of gene transfer by using a plasmid encoding IL-4 complementary DNA (cDNA) in a murine CIA model.

Several means for introducing DNA into a cell have been developed. These methods include intramuscular or intradermal administration with a needle, and gene-gun delivery (10,11). In the present study, we administered IL-4 plasmid through intradermal injec-

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tion and via gene-gun delivery as routes for DNA transfer in the CIA model. In the administration of IL-4 plasmid, gene-gun delivery has been proven to be a more effective route to induce a therapeutic effect as compared with intradermal injection. This study is the first to demonstrate that therapy using a plasmid vector encoding IL-4 cDNA has a striking effect on the suppression of CIA progression.

MATERIALS AND METHODS

Preparation of plasmid DNA. The control plasmid vector (pCont) used in this study, pCAGGS, which contains a chimeric promoter/enhancer composed of cytomegalovirus early promoter/enhancer and chicken β-actin enhancer driving expression of the heterologous gene, and the plasmid encoding full-length IL-4 cDNA (pIL-4) pCAGGS, were kindly provided by Dr. J. Miyazaki (Osaka University, Osaka, Japan) (11). The pCont and pIL-4 were inserted in *Escherichia coli* XL-1 blue strain, and the high-purity plasmid DNA was extracted by using the Qiagen Mega kit (Qiagen, Tokyo, Japan). The plasmid DNAs were stored at a concentration of 1 μg/μl at −84°C in phosphate buffered saline (PBS) until used.

Mice. DBA/1 mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 8–10 weeks of age. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Hamamatsu University School of Medicine.

Induction and assessment of CIA. Bovine type II collagen (CII; MCK, Tokyo, Japan) was dissolved in 0.05*M* acetic acid and emulsified with an equal volume of Freund's complete adjuvant (Gibco BRL, Grand Island, NY). Mice were immunized intradermally at the base of the tail with 0.1 ml of emulsion containing 100 μg of CII, and 21 days after the primary immunization, the mice received a booster injection with 0.1 ml of emulsion containing 100 μg of CII and Freund's incomplete adjuvant (Gibco BRL) as previously described (9). Thereafter, the course of arthritis was monitored by determining the signs of arthritis, and the date of disease onset was recorded. The clinical scoring of arthritis was assessed from 21 days to 70 days after primary immunization using a visual scoring method, as previously described (12). Briefly, the 4 paws were individually graded from 0 to 3, with a maximum clinical score of 12 per animal, as follows: grade 0 = no swelling; grade 1 = paws with detectable swelling in 1 joint; grade 2 = paws with swelling in more than 1 joint; grade 3 = severe swelling of entire paw and/or ankylosis.

Administration of DNA plasmid. In the CIA model, the administration of plasmid DNA via 2 routes was performed. In the first experiment, 50 μg of pCont or pIL-4 was suspended in 50 μl of PBS and was intradermally injected at the base of the tail on days 0 and 21 after the primary CII immunization.

In the next experiment, gene-gun delivery was used (13). The plasmid DNA–gold particle cartridge for gene-gun delivery was prepared using a Helios Gene Gun Cartridge kit (Bio-Rad, Hercules, CA). Fifty micrograms of pCont or pIL-4 resuspended in 50 μl of PBS was precipitated onto 1.6-μm–diameter gold beads at a density of 2.5 μg of DNA/μg of gold by adding 100 μl of a 100 m*M* spermidine and 100 μl of 1.0*M* $CaCl_2$, as previously described (14). DNA-coated gold particles were washed with ethanol 3 times, and added to 3 ml of ethanol containing 0.02 gm of polyvinylpyrrolidone. The DNA–gold solution was inserted into the tube and dried with N_2. The tube was cut into one segment of tube containing 2 μg of DNA. DNA-coated gold was delivered to the shaved abdomens of mice on days 0 and 21 after the primary CII immunization by using a Helios Gene Gun at a helium discharge pressure of 400 disintegrations per minute.

Measurement of serum anti-CII antibody levels. The levels of serum antibodies to CII were measured by enzyme-linked immunosorbent assay (ELISA) as previously reported (9). The serum samples were collected on day 70 after the primary immunization, for determination of anti-CII IgG antibody levels. Bovine CII was dissolved in 0.1*M* acetic acid at 50 μg/ml and diluted with 0.1*M* sodium bicarbonate at a concentration of 10 μg/ml (pH 9.6). The microtiter plate (96-well; Corning Costar, Cambridge, MA) was coated with 100 μl of CII antigen solution and incubated overnight at 4°C. After washing 3 times with PBS containing 0.05% Tween 20, nonspecific binding was blocked with PBS containing 10% bovine serum albumin (BSA) for 2 hours at room temperature. After 3 washings, 100 μl/well of serum samples serially diluted in PBS/Tween 20/10% BSA, and control serum in the same serial dilution in order to provide the standard titration curves, were also added and incubated for 1 hour at 37°C. After 4 washes, peroxidase-conjugated goat anti-mouse IgG was added and cultures were incubated for 1 hour at 37°C.

In addition, for detection of anti-CII IgG antibody subclasses, peroxidase-conjugated goat anti-mouse IgG1, IgG2a, or IgG2b was added. One hundred microliters of *o*-phenylenediamine dissolved in citrate buffer (pH 5) containing 0.012% H_2O_2 was added to each well, and the reaction was finally stopped using 8*N* H_2SO_4.

The optical density was measured at 500 nm, using a microplate reader. A standard serum sample from a DBA/1 mouse with evident arthritis was also measured in the same serial dilution, and standard curves were generated from this referent. Antibody titers of serum samples were then calculated from this standard curve, and results are expressed as arbitrary units relative to the former standard control serum sample.

In vivo and in vitro cytokine induction assays. Spleen and lymph node cells were obtained on days 28 and 35 after the primary immunization. Single-cell suspensions were prepared and cultured in 48-well plates in Cosmedium (Cosmobio, Tokyo, Japan) at 2×10^6 cells/well in the presence or absence of 50 μg/ml of CII for 48 hours at 37°C in 5% CO_2. The supernatants were then collected and assayed for interferon-γ (IFNγ) and IL-4. IFNγ and IL-4 were measured using a sandwich ELISA kit (Endogen, Boston, MA).

Presence of injected IL-4 plasmid in skin, lymph node, and spleen cells. Skin, lymph node, and spleen cells were obtained after gene-gun delivery, at various time points. These cells were digested at 65°C for 15 minutes and 37°C overnight in 300 μl of 1 mg/ml proteinase K (solution in the buffer containing 10 m*M* Tris HCl [pH 7.4], 10 m*M* EDTA, and 150 m*M* NaCl) and 0.4% sodium dodecyl sulfate. The digestion solutions were heated at 95°C for 20 minutes to inactivate proteinase K, and centrifuged at 13,000 revolutions per minute

Table 1. Effect on the incidence of collagen-induced arthritis (CIA) and arthritis score by gold and control plasmid (pCont) administration via gene-gun (gg) delivery*

	CIA onset rate, no. (%)	Arthritis score	P†
CII	9/14 (64.3)	2.7 ± 1.1	–
CII + gold gg	9/14 (64.3)	3.4 ± 0.9	0.52
CII + pCont gg	10/14 (71.4)	3.3 ± 1.3	0.50

* The CIA onset rate and arthritis scores (mean ± SEM) were observed for 70 days after type II collagen (CII) primary immunization, and their cumulative data on day 70 are indicated.
† Compared with CII immunization alone.

Table 2. Incidence of collagen-induced arthritis (CIA)

	Incidence of arthritis, no. (%)
CII + pCont ID	20/28 (71.4)
CII + pIL-4 ID	6/22 (27.3)*
CII + pCont gg	20/28 (71.4)
CII + pIL-4 gg	2/23 (8.7)†

* The frequency of CIA in mice that received the plasmid encoding full-length interleukin-4 (pIL-4) by intradermal (ID) injection was significantly lower ($P = 0.0039$) compared with those that received the control plasmid vector (pCont) ID injection.
† The frequency of CIA in mice that received pIL-4 gene-gun (gg) delivery was significantly lower ($P < 0.0001$) compared with those that received pCont gg delivery.

for 1 minute. The supernatants were extracted with phenol–chloroform and precipitated with ethanol. The pellets were added with 200 μl of TE buffer containing RNase A (10 mg/ml), and were extracted with phenol–chloroform and precipitated with ethanol.

Aliquots of DNA samples (0.5 μg) were amplified in 20 μl of reaction mixture (50 m*M* KCl/10 m*M* Tris HCl [pH 9.0]/2.0 m*M* $MgCl_2$/0.01% gelatin [weight/volume]/0.1% Triton X-100/200 m*M* of each dNTP/*Taq* polymerase [0.025 units/μl; Takara, Tokyo, Japan]) using 2 sets of primers. The 2 forward primers (the first primer, 5'-GGTTGTTGTGCTGTCTCATC-3', and the second primer, 5'-TAGAGCCTCTGCTAACCATG-3') and the common reverse primer set (5'-CTTCTCCTG-TGACCTCGTTC-3') were used to detect the amplified products of 188 bp and 251 bp using the pIL-4 plasmid template. The 2 forward primer positions were upstream of the inserted IL-4 gene and the reverse primer position was at the coding lesion of the inserted IL-4 gene. The amplification was carried out at 40 cycles (denaturing at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute). The aliquots were separated on 1.5% agarose gel and stained with ethidium bromide.

Statistical analysis. Data were analyzed using the Macintosh StatView software program. Group comparisons were performed using the chi-square test or the Mann-Whitney test for independent samples (nonparametric data).

RESULTS

Influence of gold in gene-gun delivery. In order to determine whether the gold itself used in gene-gun delivery might have a potential influence on CIA onset and severity, gold alone was administered by gene-gun delivery. The incidence of CIA in mice following administration of CII combined with gold gene-gun delivery was comparable with that in mice that received CII alone (64.2% in each group) (Table 1). Moreover, CII administration combined with pCont gene-gun delivery did not significantly affect the incidence of arthritis compared with that with CII alone. Disease severity in mice that received gold gene-gun delivery (mean ± SEM arthritis score 3.4 ± 0.9) and those that received pCont gene-gun delivery (arthritis score 3.3 ± 1.3) showed no statistically significant difference compared with that in control mice immunized with CII alone (arthritis score 2.7 ± 1.1).

Incidence of CIA. DBA/1 mice were immunized with CII and monitored for the development of arthritis for 70 days after primary immunization. As shown in Table 2, the incidence of arthritis was 71.4% in the pCont intradermal injection group and 27.3% in the pIL-4 intradermal injection group, thus showing a sta-

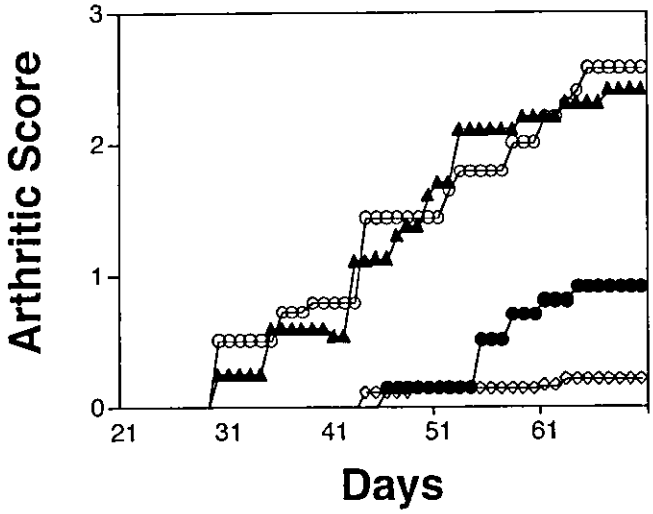

Figure 1. Mean arthritis score as an indicator of the severity of collagen-induced arthritis for 70 days after primary immunization. Mice were immunized with type II collagen (CII) emulsified with Freund's complete adjuvant on day 0 and boosted with CII emulsified with Freund's incomplete adjuvant on day 21. In addition, on days 0 and 21, mice were treated with the control plasmid vector (pCont) by gene-gun delivery (○), the plasmid encoding full-length interleukin-4 (pIL-4) by gene-gun delivery (◇), pCont by intradermal injection (▲), and pIL-4 by intradermal injection (●).

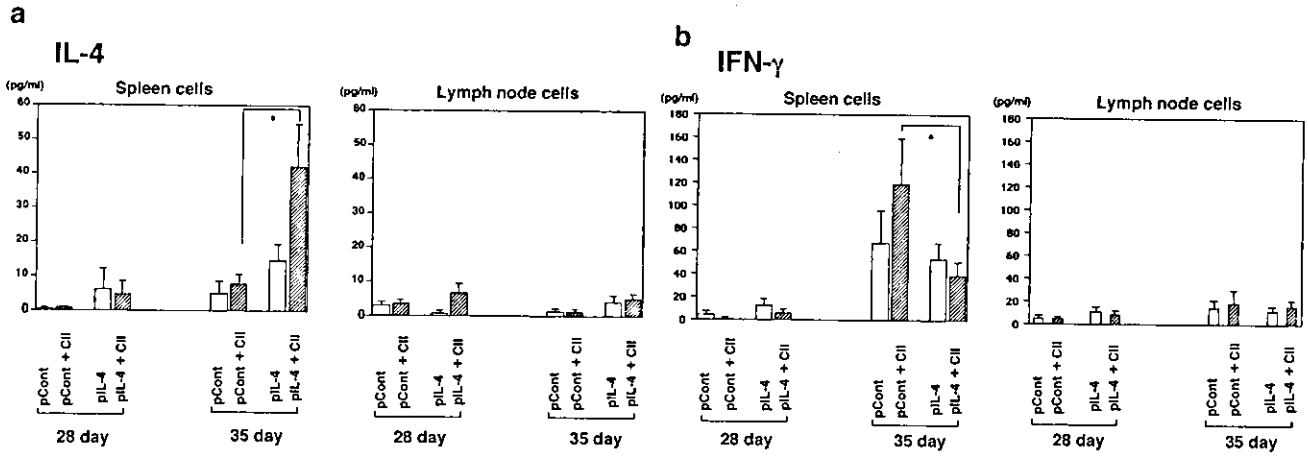

Figure 2. IL-4 (a) and interferon-γ (IFNγ) (b) production by spleen cells and lymph node cells from mice treated with pCont and pIL-4 by gene-gun delivery. Mice were immunized with CII and this was boosted on day 21 after primary immunization. On days 0 and 21, either pCont or pIL-4 was administered by gene-gun delivery. On days 28 and 35 after primary immunization, the spleen and lymph node cells were obtained, and single-cell suspensions (2×10^6 cells/ml) were cultured for 48 hours in the presence or absence of CII (50 μg/ml). Supernatants were tested for IL-4 and IFNγ by enzyme-linked immunosorbent assay. The significance of the differences in cytokine levels in CII-stimulated cells of pCont- and pIL-4–treated mice was analyzed (* = $P < 0.05$). Bars show the mean and SEM. See Figure 1 for other definitions.

tistically significant difference ($P = 0.0039$). After gene-gun delivery, the pIL-4 injection group showed a significantly lower incidence of arthritis onset (8.7%) compared with the pCont injection group (71.4%; $P < 0.0001$).

Severity of arthritis. The severity of arthritis in the mice was assessed on the basis of arthritis scores. Mice treated with pIL-4 intradermally or by gene-gun delivery showed a delayed onset of arthritis and had lower arthritis scores than were observed in mice treated with pCont intradermally or by gene-gun delivery during the period of observation (Figure 1).

Production of cytokines by cultured spleen cells and lymph node cells from mice. In order to investigate the IL-4/IFNγ cytokine balance in CII-stimulated spleen cells and lymph node cells after treatment with pIL-4, the mice administered pCont or pIL-4 by gene-gun delivery were killed on days 28 or 35 after primary immunization. Single-cell suspensions from spleen cells were cultured in the presence or absence of CII, and their supernatants were tested for IFNγ and IL-4.

In spleen cells, higher spontaneous IL-4 production (mean ± SEM 14.7 ± 4.6 pg/ml on day 35; $P = 0.013$) was seen in pIL-4–treated mice compared with pCont-treated mice (Figure 2a), whereas this was not seen in lymph node cells. IL-4 production by CII-stimulated spleen cells was significantly elevated on day 35 in mice treated with pIL-4 via gene-gun delivery (mean ± SEM 42.0 ± 12.7 pg/ml; $P = 0.025$) compared with that in pCont-treated mice, but lymph node cells from mice treated with pIL-4 via gene-gun delivery did not show significantly higher levels of IL-4 production on days 28 and 35 compared with those treated with pCont.

In spleen cells, higher spontaneous IFNγ production was seen on day 35 in pCont-treated mice (67.3 ± 28.3 pg/ml; $P = 0.0006$) and pIL-4–treated mice (52.9 ± 14.0 pg/ml; $P = 0.0006$) compared with IFNγ production on day 28 (Figure 2b). In spleen cells stimulated with CII, IFNγ production on day 35 was higher in the pCont-treated mice (120.5 ± 20.5 pg/ml) than in the pIL-4–treated mice (40.2 ± 11.6 pg/ml) ($P = 0.045$). In lymph node cells, spontaneous IFNγ production was low on both day 28 and day 35, and the levels of IFNγ production after stimulation with CII showed no significant difference compared with medium alone on days 28 and 35. These data show that the gene-gun delivery of pIL-4 induced the development of CII-specific Th2 cytokine in the spleen cells.

Presence of IL-4 plasmid in skin, lymph node, and spleen cells. The pIL-4 plasmid was detected with polymerase chain reaction in cells of the skin, draining lymph node, and spleen. After abdominal gene-gun delivery in the skin, the pIL-4 plasmid was detected with both primer sets on days 2, 4, and 7, but not on day 14 (Figure 3). In addition, the plasmid was detected in the lymph node on days 2–14, and in the spleen on days 4, 7, and 14, but not on day 2.

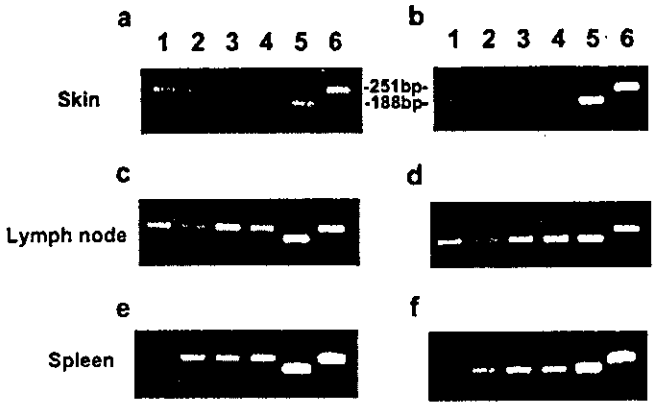

Figure 3. IL-4 plasmid in skin, lymph node, and spleen cells. The cells were obtained after abdominal gene-gun delivery, at various time points. DNA extracted from these cells was amplified using the 2 sets of primers to detect DNA of pIL-4 plasmid. Lane 1, 2 days after abdominal gene-gun delivery of pIL-4; lane 2, 4 days; lane 3, 7 days; lane 4, 14 days; lane 5, the amplified product of 188 bp using the first set of primers, when pIL-4 was used as a template; lane 6, the amplified product of 251 bp using the second set of primers when pIL-4 was used as a template. Lanes 1–4 in **a**, **c**, and **e** represent the amplified products using the first set of primers, and lanes 1–4 in **b**, **d**, and **f** represent the amplified products using the second set of primers. Amplified products were separated on 1.5% agarose gel and stained with ethidium bromide. See Figure 1 for definitions.

Production of anti-CII IgG antibodies in mice. To examine the effect of pIL-4 administered by gene-gun delivery or intradermal injection in the production of anti-CII–specific IgG antibodies, serum samples were collected on day 70 after primary immunization, and CII-specific IgG levels were measured by ELISA. The levels of anti-CII IgG antibodies following both gene-gun delivery and intradermal injection in mice treated with pIL-4 were significantly lower compared with those in mice treated with pCont ($P = 0.037$ and $P = 0.049$, respectively) (Figure 4).

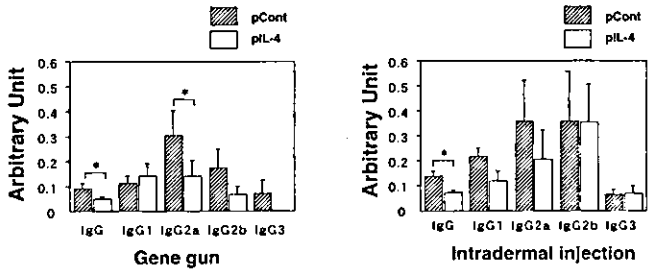

Figure 4. Production of anti-CII IgG antibodies. DBA/1 mice were immunized with CII and treated with pCont or pIL-4 on days 0 and 21, and killed on day 70. The serum samples from day 70 were analyzed for CII-specific IgG antibodies as described in Materials and Methods. Bars show the mean and SEM arbitrary units. * = $P < 0.05$. See Figure 1 for definitions.

In addition, CII-specific IgG1, IgG2a, IgG2b, and IgG3 levels were measured. CII-specific IgG2a levels in mice receiving pIL-4 by gene gun were significantly lower ($P = 0.016$) compared with those in mice treated with pCont by gene gun. CII-specific IgG2a, IgG2b, and IgG3 levels after intradermal administration and CII-specific IgG2b and IgG3 levels after gene-gun delivery were also lower in the pIL-4–treated mice compared with those in the mice treated with pCont, although no significant difference was seen. It is noteworthy that among the IgG subclasses analyzed after gene-gun delivery, IgG1 levels were increased in the pIL-4 treatment group as compared with the pCont group, although there was no statistically significant difference. This tendency was consistent with the fact that IL-4 is associated with the IgG1 class switch during antibody production (15). In contrast, intradermal inoculation did not significantly enhance the production of CII-specific IgG1.

DISCUSSION

In this study, the plasmid encoding IL-4 cDNA had a marked regulatory effect on CIA by reducing the incidence and severity of arthritis. Previous studies demonstrated that IL-4 acts as a suppressive agent on CIA development (8,16–18), has an ability to induce a Th2 cytokine shift in the Th1/Th2 balance, and regulates Th1 cytokine production (8). The protective effect of IL-4 on CIA has been considered to be associated with blocking of the production of the proinflammatory cytokines IL-1 and TNFα, which might augment CIA development, and with the production of IL-1 receptor antagonist, as previously described (6,7,18). Lubberts et al observed that local IL-4 treatment prevented joint damage and bone erosion, despite severe inflammation (19). The protective effect was associated with decreased formation of osteoclast-like cells and down-regulation of IL-17, IL-6, IL-12, and osteoprotegrin ligand in the synovium. However, therapy using antiinflammatory cytokines is limited, since it requires toxic levels to achieve effective biologic responses, and these cytokines generally have a short half-life (20). Gene therapy may be able to overcome this problem by producing its effects over a long period.

Several routes and methods of DNA administration, such as gene-gun delivery of DNA precipitated on gold beads or intradermal or intramuscular injection of DNA (21–23), have been applied in experimental animal

models. The present study demonstrated that both intradermal injection and gene-gun delivery of plasmid encoding IL-4 cDNA prevented the onset of CIA and reduced the clinical symptoms. It is especially striking that the gene-gun delivery of plasmid expressing IL-4 had a markedly suppressive effect on CIA as compared with intradermal injection. Previous studies have demonstrated production of protein in the treated muscle or tissue of animals treated with plasmids through the intramuscular or intradermal route (23,24).

Interestingly, it has been shown that plasmid delivered by gene gun into the abdominal skin is taken up by subcutaneous dendritic cells, such as Langerhans cells, which subsequently migrate to lymphoid tissue and produce the encoding protein (25,26). In our study, after the intradermal injection of plasmid encoding IL-4 cDNA at the tail base or gene-gun delivery in the abdominal skin of mice, IL-4 production was detected in serum, although its levels were very low, being close to the minimum detectable level (data not shown). In in vitro CII stimulation assays of CII-treated mice, spleen cells after gene-gun delivery of IL-4 plasmid showed markedly elevated IL-4 production compared with those after gene-gun delivery of the control plasmid. IL-4 production by CII-stimulated spleen cells was more elevated in pIL-4–treated mice than in pCont-treated mice, but lymph node cells from pIL-4–treated mice did not show significantly higher levels of IL-4 production compared with those from pCont-treated mice.

In order to examine whether the pIL-4 plasmid might not migrate to inguinal lymph nodes from the abdominal skin, we analyzed the presence of the plasmid in skin, inguinal lymph node, and spleen cells by polymerase chain reaction at various time points after gene-gun delivery. The plasmid transfected to the skin was demonstrated to migrate to the draining lymph node on day 2 and to the spleen on day 4, and to remain on day 14. Sallusto et al showed that memory T cells were divided into 2 distinct subsets. CCR7+ memory cells, which are called central memory, express lymph node homing receptors but lack immediate effector function, such as the production of IL-4, IL-5, and IFNγ (27). In contrast, CCR7− memory cells, which are called effector memory, express receptors for migration to inflamed tissues and display immediate effector function. If a lymph node is categorized to central memory, and a spleen to effector memory, the lower response of lymph node cells to CII stimulation compared with that of spleen cells may be understandable. The higher IL-4 production by spleen cells stimulated with CII on day 35 compared with day 28 after first immunization with CII is associated with the proliferation of CII-reactive T cells, as we have described in a previous report (9).

It is of interest that the gene-gun delivery of pIL-4 has remarkable therapeutic efficacy in CIA development compared with intradermal injection, while the amount of plasmid DNA administered by gene gun is 50-fold less than that of intradermal injection. From this result, gene-gun delivery may be considered an effective route of plasmid administration as a therapeutic tool for the management of arthritis.

IL-4 has been reported to have the potential to induce IgG1 antibody production, and to reduce IgG2a antibody production (15). In this study, the results following gene-gun delivery of pIL-4 were compatible with these data. Continuous administration of exogenous IL-4 in a CIA model delays the onset of arthritis and suppresses clinical symptoms (8). In our study, the intradermal administration of IL-4 plasmid reduced the CII-specific IgG2a level but did not up-regulate the CII-specific IgG1 subclass level, although there was no statistically significant difference in the IgG2a:IgG1 ratio. The reason for this is not clear, but the route of plasmid administration, including gene-gun versus intradermal injection, may be associated with the antibody-production mechanism on the immune system. Feltquate et al demonstrated that immunization by the gene-gun delivery of plasmid induces a Th2 immune response as compared with the intramuscular or intravenous route (14). Horsfall et al suggested that the antiinflammatory properties of IL-4 are mediated in part by the down-regulation of the Th1 response rather than the up-regulation of the Th2 response (8).

In order to suppress CIA by IL-4 protein, repeated administration of IL-4 is necessary, because cytokines disappear with a short half-life in vivo (8). Cytokine gene therapy circumvents this problem by maintaining constant blood levels of the encoding cytokines.

Currently, some types of viral vector, including retrovirus and adenovirus, have been used in gene therapy applications. For the treatment of murine CIA, successful results using adenovirus vector encoding IL-4 have been reported (28,29). There is, however, a report with contradictory findings, in that the adenovirus vector was demonstrated to generate a replication-competent adenovirus subpopulation due to recombination with the host cell genome (30). The retrovirus vector has been reported to cause T cell neoplasm after infection in rodents (31) and requires dividing cells to deliver the vector efficiently (20). Thus, viral vectors may have

several disadvantages for clinical use, including pathogenicity.

Nonviral vectors such as plasmids are nonpathogenic, but the efficiency of the gene transfer to the target cells is lower than that of viral vectors. However, in our study, gene-gun plasmid delivery resulted in efficient protein expression of the encoding gene. In particular, one of the advantages of the gene gun is that it can be used to transfer in nondividing cells, and DNA–gold beads for gene transfer can be easily prepared. The efficiency of gene transfer to the skin by gene-gun delivery is high, and the adverse effects of the plasmid vectors themselves are fewer as compared with those of viral vectors.

Several types of gene transfers have been shown to have beneficial effects in arthritis models (28,32–34). The present study is the first to demonstrate that delivery of the plasmid vector encoding IL-4 by systemic administration has an in vivo therapeutic effect in the CIA model. Taken as a whole, these results suggest that the IL-4 plasmid may act as a potent immunosuppressive antiarthritis agent in the treatment of RA.

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Cytotoxic T-Lymphocyte-, and Helper T-Lymphocyte-Oriented DNA Vaccination

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ABSTRACT

DNA vaccines have advantages over other types of vaccines in that they can induce strong cellular immune responses, namely cytotoxic T lymphocytes (CTL) and helper T lymphocytes (Th). DNA vaccines are therefore considered a promising alternative to attenuated live vaccines in the field of infectious diseases. So far, various DNA vaccines have been generated and tried to induce a particular cellular immune response by virtue of recombinant DNA technology. DNA vaccines have been designed for efficient transcription and translation of target genes by a variety of strategies. Also, various DNA vaccine strategies for induction of specific CTL and Th have been reported by taking into consideration antigen presentation pathways and the strategies have been shown to be effective to elicit particular T-cell responses. In this paper, we have reviewed these strategies, including our study on epitope-specific T-cell induction by DNA vaccination against *Listeria monocytogenes* infection. From this review, it has been surmised that, to induce strong immune responses by DNA vaccines, the immunization route and the immunization regimen, such as heterologous "prime-boost" regimen, should also be considered.

INTRODUCTION

DNA VACCINATION IS A VACCINATION method by the direct inoculation of a eukaryotic expression plasmid encoding antigen molecules of interest into host animals. The vaccines have been intensively studied in the past decade. It was surprising to know the paper by Wolff and colleagues (1990) describing that direct intramuscular injection of plasmid DNA allows the expression of plasmid-encoded proteins in the tissues of injected mice, because even expression of plasmid DNA in cultured cells needs some expertise in transfection methodology, such as the calcium phosphate precipitation method or the liposome method, etc. Subsequently, Tang and colleagues (1992) showed that injection of plasmid DNA directly into the skin with gene gun bombardment effectively induces specific antibody production. These early important studies have been reviewed in Chattergoon *et al.* (1997) and Liu and Ulmer (2000). Among the various vaccination strategies tried so far, DNA vaccines have been shown to have advantages over other types of vaccines by inducing cellular immune responses, namely, cytotoxic T lymphocytes (CTL) and type 1 helper T lymphocytes (Th1), without utilizing live organisms. In addition, DNA vaccines offer several other advantages such as their relatively easy design and construction with enormous flexibility using recombinant DNA technology, their chemical stability, and relatively low cost.

We have reported to construct DNA vaccines able to elicit only a particular T-cell subset against T-cell epitopes derived from *Listeria monocyotogenes* and examined their efficacy. We review here strategies to induce specific T-cell subsets by naked DNA immunization with an emphasis on our results on DNA vaccines against *L. monocytogenes* as a model of DNA vaccination against intracellular pathogens.

OUTLINE OF DNA-MEDIATED IMMUNIZATION

Typical eukaryotic expression plasmids can be utilized for DNA vaccines. DNA vaccines are composed of (1) an antigen-encoding gene whose expression is driven by (2) a strong eukaryotic promoter such as cytomegalovirus immediate-early (CMV I.E.) promoter/enhancer, (3) a polyadenylation termination sequence such as the sequence derived from simian virus

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40 (SV40) or bovine growth hormone (BGH) gene, and (4) a prokaryotic selective marker such as ampicillin resistance gene to facilitate selection of *Escherichia coli* clones carrying the plasmid. In addition, plasmids for DNA vaccines should contain special nucleotide sequences for enhancing the immunogenicity, namely an unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide with appropriate flanking regions. In mice, the optimal flanking region is composed of two 5′ purines and two 3′ pyrimidines (Van Uden and Raz, 2000). An ampicillin resistance gene contains the CpG, but a kanamycin resistance gene does not have it (Sato *et al.*, 1996). The CpG motif stimulates the innate immune system through Toll-like receptor 9 to produce a series of immunomodulatory cytokines such as interleukin (IL)-12 and interferon (IFN)-γ, which promote the development of Th1 cells (Krieg *et al.*, 1995; Klinman *et al.*, 1996; Roman *et al.*, 1997).

Major immunization methods for DNA vaccines tried so far are, (1) intramuscular injection into the hind leg quadriceps or tibialis anterior, (2) gene gun bombardment of DNA-coated gold particles into the epidermis, (3) intradermal DNA immunization (Raz *et al.*, 1994), and (4) topical application of DNA vaccines (Fan *et al.*, 1999) that have also been reported to be able to induce immunization effects. Furthermore, several "carrier"-mediated DNA vaccine administration methods have been reported such as (5) liposomes, (6) microparticle encapsulation, and (7) attenuated bacteria. These methods are briefly reviewed in Gurunathan *et al.* (2000).

In intramuscular immunization, primary cells that plasmid DNA is transferred into are myocytes. As myocytes are not professional antigen-presenting cells (APC), the mechanisms of DNA vaccines have been controversial, but bone marrow-derived APC has been suggested to be involved in antigen presentation in DNA vaccines (Corr *et al.*, 1996; Iwasaki *et al.*, 1997).

It is of particular interest that gene gun DNA immunization requires 100 to 1000 times less DNA than muscle DNA inoculation to generate equivalent antibody responses (Pertmer *et al.*, 1995, 1996). In addition, gene gun DNA immunization appeared to bring about highly reproducible and reliable results in antibody production and induction of specific CTL and IFN-γ production from immune splenocytes (Yoshida *et al.*, 2000). It has been suggested that muscle DNA immunization tends to raise predominant Th1 responses, while gene gun DNA immunization is apt to produce Th2 responses (Feltquate *et al.*, 1997). The difference is considered to be mainly due to the difference of plasmid amounts used for the vaccinations. This difference may affect (1) the amount of antigen produced from the plasmids and (2) the amount of CpG motif present in plasmid DNA vaccines.

DNA VACCINE DESIGN FOR INCREASING ANTIGEN LEVEL IN APC

Investigators have tried to optimize DNA vaccination by increasing the expression level of antigenic molecules in APC. A variety of factors affects the expression level. We would like to review strategies for enhancing antigen expression level in APC.

Choice of eukaryotic promoter

Cheng and colleagues (1993) assessed the activities of five viral and five cellular promoters in different rat tissues by using gene gun bombardment. Their results demonstrated that CMV I.E. enhancer/promoter activity was consistently the highest in each tissue. Hence, the promoter has been used intensively for DNA vaccines. For the specific expression in myocytes, desmin promoter, which works specifically in myocytes, was also used for DNA vaccination via intramuscular injection (Kwissa *et al.*, 2000). Promoters that are preferentially active in professional APC, like scavenger receptor gene promoter, MHC class II promoter or dectin-2 promoter (Zhi *et al.*, 1997; Corr *et al.*, 1999; Takashima and Morita, 1999, respectively) have been examined, but several reports showed that they did not induce satisfactory immune responses (Zhi *et al.*, 1997; Corr *et al.*, 1999).

Inclusion of the Kozak consensus translational initiation sequence

For the efficient translation of target genes, a Kozak consensus translational initiation sequence around ATG translation start codon (CCA/GCCATG) have been shown to be important (Kozak, 1987). An and colleagues (2000) suggested that the Kozak consensus sequence should be added in minigene DNA vaccine constructs. Strugnell and colleagues (1997) reported that the DNA vaccine construct for expression of *Chlamydia trachomatis* major outer membrane protein (MOMP) with the most optimal Kozak sequence ("A" at −3 position relative to the initiation codon) showed the high expression of MOMP protein when transfected into Cos-7 cell line. Nevertheless, the plasmid injected into mice did not result in satisfactory antibody production. In addition, any "ATG" sequences in the 5′-untranslated region of antigen genes should be removed because the sequence may work as the translation start codon. The distance between the promoter and the open reading frame would also affect the expression level of antigens.

Codon optimization

Interspecies difference of codon usage is one of the major obstacles for effective induction of specific immune responses against pathogens by DNA vaccination. When genes derived from pathogens such as bacteria, protozoa, and some viruses, codon usage is one of problems for the expression in eukaryotic cells. We constructed a plasmid DNA vaccine harboring a wild-type DNA sequence of a dominant CTL epitope of *L. monocytogenes* derived from hemolysin, listeriolysin O (LLO), LLO 91–99 (p91wt), and then tried immunization of mice with the DNA vaccine by intramuscular injection. However, we could not clearly induce LLO 91–99-specific CTL in BALB/c mice (Uchijima *et al.*, 1998). One of the reasons for the failure of the induction may be the difference of the codon usage between mammalian cells and *L. monocytogenes*. *L. monocytogenes* genome is highly A+T-rich. In contrast, the mammalian genome is G+C-rich. This difference may affect the efficiency of *L. monocytogenes* gene expression in mammalian cells. So, we constructed a DNA vaccine using the LLO 91–99 gene, whose codons were optimized to those of the mammalian cells

(p91mam). The DNA vaccine gave an excellent CTL induction by intramuscular immunization (Uchijima *et al.*, 1998). We further evaluated the "codon optimization effect" on CTL induction by the DNA vaccine (Nagata *et al.*, 1999). In that study, we analyzed in mammalian cultured cells the translation efficiency of several genes composed of different levels of optimization to mammalian cells, but encoding an identical CTL epitope derived from *L. monocytogenes*, LLO 91–99 or a murine malaria parasite, *Plasmodium yoelii*, PyCSP 281–290, and showed that the codon optimization level of the genes is not precisely proportional to, but does correlate well, with the translation efficiency in mammalian cells. The results also correlated well with the induction level of specific CTL response in vivo (Nagata *et al.*, 1999; Fig. 1). In that study, we used the relative synonymous codon usage (RSCU) and the codon adaptation index (CAI) values as the indices of the codon optimization level. These values have been explained by Sharp and Li (1987). The RSCU values of codons used in *L. monocytogenes* and the malaria parasite (*Plasmodium* spp.) showed the opposite relationship to the RSCU values of codons used in mice and humans, indicating that native codons frequently used in these organisms are rarely used in mice and humans. However, such a relationship is not necessarily applicable in all pathogens. For example, RSCU values of codons used at high frequency in *Mycobacterium tuberculosis* genes are quite similar to those in *Mus musculus* genes (Nakamura *et al.*, 2000). In such a case, codon optimization for DNA vaccine constructs may not be necessary.

A similar effect of codon optimization was noted in a murine DNA immunization model against human immunodeficiency virus type 1 (HIV-1) infection, by using the genes encoding HIV-1 gp120 (André *et al.*, 1998), gp160 (Vinner *et al.*, 1999) or gag (Deml *et al.*, 2001), and also in a murine malaria DNA vaccine encoding *Plasmodium falciparum* merozoite protein (Narum *et al.*, 2001).

Facilitation of intercellular spreading of antigens

To facilitate intercellular spreading of antigens in vaccinated animals, Hung and colleagues (2001) reported a unique interesting approach using VP22 protein. VP22 is a unique herpes simplex virus-1 protein that has been demonstrated the remarkable property of intercellular transport and shown to be capable of facilitating the spread of fused proteins to surrounding cells (Eliott and O'Hare, 1997). Hung and colleagues constructed a DNA vaccine of VP22-human papillomavirus type 16 E7 chimeric protein and showed that the DNA vaccine successfully spread the fused protein *in vivo* and enhanced MHC class I presentation of the antigen.

Targeting DNA to APC and enhancing antigen presentation ability of APC

Induction of specific T cells requires antigen presentation by professional APC. Therefore, introduction of target genes specifically into professional APC by DNA immunization may be useful for inducing T cells efficiently. To realize this intention, the gene gun immunization method would be the best, as this method allows the genes to enter dermal dendritic cells and Langerhans cells directly.

Some investigators have reported elegant DNA vaccine strategies for the localization of antigens to professional APC efficiently. Boyle and colleagues (1998) reported that DNA vaccines for human immunoglobulin attached to CTLA-4 molecule, which allows targeting the antigen to APC, enhanced antibody production against the immunoglobulin, although the same approach did not enhance CTL responses (Deliyannis *et al.*, 2000). In another approach, You *et al.* (2001) evaluated a DNA vaccine for a model hepatitis B virus *e* antigen fused to an IgG Fc fragment. They showed that the secreted fusion proteins are efficiently captured and processed by dendritic cells via receptor-

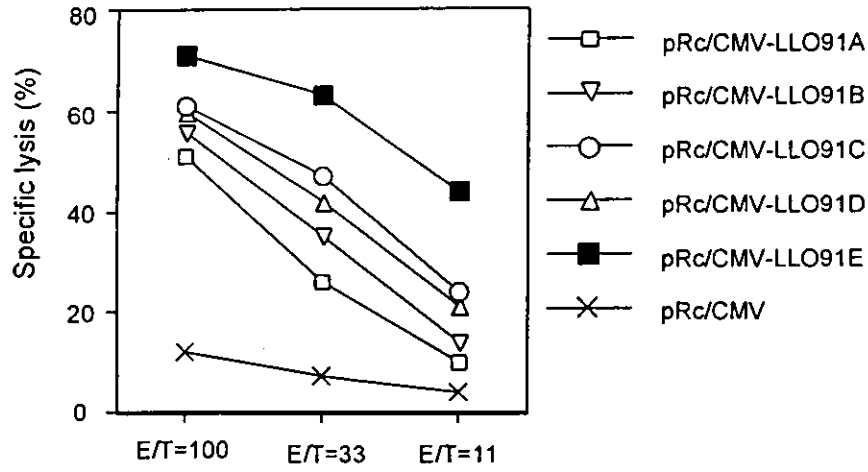

FIG. 1. Codon optimization effect on specific CTL induction by DNA vaccination. BALB/c mice were immunized by gene gun with several plasmids for expression of LLO 91–99, whose codons were optimized to the mouse to the different extent, pRc/CMV-LLO91 **A, B, C, D,** or **E.** Immune spleen cells were stimulated *in vitro* with LLO 91–99-pulsed syngeneic splenocytes for 5 days and subjected to CTL assay. The percentage of the specific lysis was determined using BALB/3T3 cells pulsed with LLO 91–99 peptide as target cells (reproduced from Nagata *et al.*, 1999).

mediated endocytosis and then presented to the major histocompatibility complex (MHC) class I and class II molecules.

Efficient antigen presentation requires accessory molecules expressed on APC. Latouche and Sadelain (2000) showed that B7.1, ICAM-1, and LFA-3 molecules are most important for antigen presenting capacity. Coadministration of these accessory molecules will improve the immunization effects of DNA immunization. In an analogous manner, the activation of T cells was facilitated by coadministration of plasmids encoding CD40 ligand and/or cytokines (Gurunathan *et al.*, 1998).

ANTIGEN PROCESSING AND PRESENTATION REQUIRED FOR INDUCTION OF T-CELL SUBSETS (CTL AND Th)

The types of effective immune responses against infectious diseases depend on the location of pathogens responsible. For example, extracellularly located pathogens are, in general, vulnerable to antibody-mediated effector mechanisms. On the other hand, protection against intracellularly located pathogens depends on induction of specific cell-mediated immunity (Kaufmann, 1993; Hess *et al.*, 2000). Two major arms of cellular immunity come into play in the protection. CTL is a main effector against pathogens located in the cytoplasm of host cells, such as viruses, *Rickettsia* spp. or *L. monocytogenes*, while Th1 plays a pivotal role in the protection against infections with intracellular pathogens located in vacuolar compartments, such as *Mycobacteria* spp. or *Salmonella* spp. Therefore, induction of effective resistance to infection depends on vaccines being capable of eliciting certain effectors. Both cellular immune responses have been shown to be effectively induced with DNA vaccines (Chattergoon *et al.*, 1997; Donnelly *et al.*, 1997; Alarcon *et al.*, 1999; Gurunathan *et al.*, 2000; Shedlock and Weiner, 2000).

Both CTL and Th have T-cell receptor molecules on their surface in common, but CTL and Th in general have CD8 molecules and CD4 molecules on their surface, respectively. $CD8^+$ CTL are presented antigens (antigenic peptides) in association with MHC class I molecules on the surface of APC, and $CD4^+$ Th are presented antigens in association with MHC class II molecules. Therefore, efficient induction of CTL and Th requires efficient presentation of antigenic molecules through MHC class I and class II antigen processing and presentation pathway, respectively.

MHC class I molecules have been shown to be expressed in almost all somatic cells except for neurons and germ cells. To prime CTL, antigenic peptides must be presented on MHC

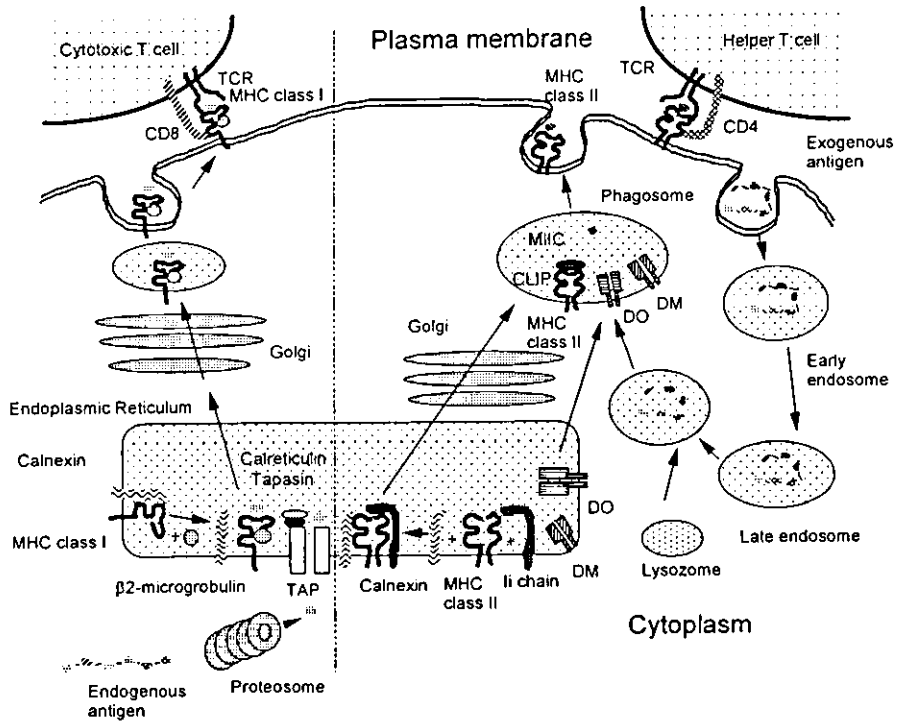

FIG. 2. Antigen processing and presentation pathways through MHC class I and class II molecules. Endogenous antigens are thought to be degraded by large, ATP-dependent proteasome complex. The resulting peptides are then translocated into the lumen of the ER by TAP transporters. In the ER, antigenic peptides bind to the groove of MHC class I molecules. The peptide–MHC class I complexes are transported through the Golgi to the cell surface. Exogenous antigens are phagocytosed by phagocytes such as macrophages. Then, the antigens are degraded into peptides in endosome/lysosome compartments. MHC class II molecules associate with Ii molecules in the ER and the complex moves to endosome/lysosome compartments. Antigenic peptides bind to MHC class II molecules in the compartment named MIIC with the help of DM and DO molecules. The peptide–MHC class II complexes are then displayed on the cell surface.

class I molecules on the surface of professional APC, which possess special accessory molecules. In general, proteins located in the cytoplasm of the APC (endogenous antigens) are processed with the proteasome complex and selected peptides go into the endoplasmic reticulum (ER) through transporters associated with antigen processing (TAP) molecules. Antigenic peptides are supposed to bind to the groove of MHC class I molecules in the ER. Then, they go to the cell surface and are presented to $CD8^+$ T cells [Fig. 2; reviewed in Pamer and Cresswell (1998) and Rock and Goldberg (1999)]. On the other hand, MHC class II molecules are expressed only in professional APC having specific antigen presenting capacity, such as macrophages, dendritic cells, and B cells. Basically, MHC class II molecules are able to present antigenic peptides derived from the outside of the APC (exogenous antigens). Exogenous proteins are phagocytosed into APC and localized in the vesicles called phagosomes separated from the cytoplasm by lipid bilayer membranes. Phagosomes then fuse with lysosomes, which are also vesicular compartments full of peptidases. After the fusion, exogenous proteins are degraded into smaller pieces of peptides. MHC class II molecules associate with invariant chain (Ii) molecules in the ER and the complex goes to the endosomes by virtue of endosomal targeting signals located in the cytoplasmic regions of Ii molecules and also of MHC class II molecules. In the endosomes, Ii molecules are gradually degraded and only a small portion designated class II associated Ii peptides (CLIP), which is located in the groove of MHC class II molecules, remained. Then, antigenic peptides exchange with CLIP with the help of MHC class II-like molecules, H2-DM and H2-DO (mice), or HLA-DM and HLA-DO (humans) under the influence of local acidic pH and finally go into the groove of MHC class II molecules. They are then finally presented to $CD4^+$ T cells on the surface of APC [Fig. 2; reviewed in Germain (1999); Bryant *et al.* (2002)]. Targeting of antigens into endosomal compartments is a key factor to facilitating induction of antigen-specific $CD4^+$ T cells.

T-CELL RESPONSES TO *LISTERIA MONOCYTOGENES* INFECTION—DNA VACCINE STUDY AGAINST INTRACELLULAR PATHOGENS

We have been working on DNA vaccines against *L. monocytogenes* as a model of intracellular bacteria. *L. monocytogenes* is a Gram-positive facultative intracellular bacterium. Murine *L. monocytogenes* infection system has been studied as a good model system for intracellular bacteria infection (Cossart and Mengaud, 1989; Kaufmann, 1993). The bacterium has been known to induce MHC class I-restricted $CD8^+$ T-cell responses in addition to MHC class II-restricted $CD4^+$ T-cell responses since the bacterium is capable of escaping from phagocytic vesicles into the cytoplasm of the host cells, thereby introducing the bacterial proteins into the MHC class I antigen processing pathway. Both $CD8^+$ CTL and $CD4^+$ Th1 have been shown to be amplified at listerial infection and to play a critical role in protective immunity in experiments of depletion and adoptive transfer of specific T-cell subsets (Kaufmann *et al.*, 1985; Czuprynski and Brown, 1990; Sasaki *et al.*, 1990) or by analyses of mutant mice with a genetic defect in β2-microglobulin or H2-Aβ gene (Roberts *et al.*, 1993; Ladel *et al.*, 1994).

So far, several T-cell epitopes (MHC-binding antigenic peptides) in listerial antigens have been reported (Fig. 3). Pamer and colleagues (Pamer, 1997; Busch *et al.*, 1999) have reported four different *L. monocytogenes* epitopes presented by MHC class I ($H2\text{-}K^d$) molecules to CTL; those are listeriolysin O (LLO) 91–99, p60 (murein hydrolase) 217–225, p60 449–457, and mpl (metalloprotease) 84–92. Two of these four epitopes, LLO 91–99 and p60 217–225, have been demonstrated to induce dominant immune responses. First identified MHC class II binding peptide in *L. monocytogenes* is LLO 215–226, an $H2\text{-}E^k$ binding peptide (Ziegler *et al.*, 1994; Safley *et al.*, 1995). Then, p60 301–312 was identified as an $H2\text{-}A^d$ binding peptide (Geginat *et al.*, 1998). Recently, Geginat *et al.* (2001) tried

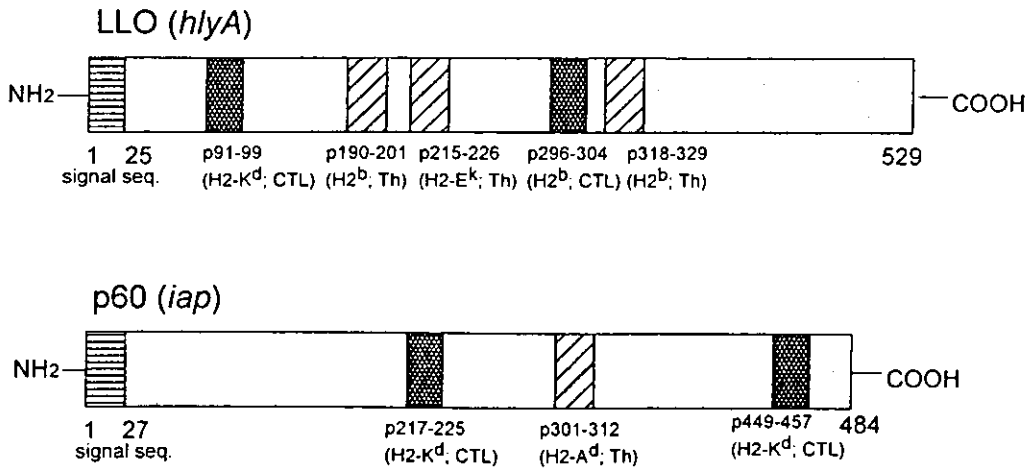

FIG. 3. Major protective antigens of *Listeria monocytogenes*, listeriolysin O (LLO), and p60. LLO and p60 molecules of *L. monocytogenes* have been shown to be major protective antigens. So far, several $CD8^+$ CTL and $CD4^+$ Th epitopes in these antigens have been reported.

to identify $CD4^+$ and $CD8^+$ T-cell epitopes in LLO and p60 molecules based on the screening of peptide spot libraries with splenocytes derived from *Listeria*-infected BALB/c and C57BL/6 mice. They confirmed all known $CD4^+$ and $CD8^+$ T cell epitopes in LLO and p60 molecules and additionally identified six new $H2^d$-, and six new $H2^b$-restricted T cell epitopes, containing $H2^b$-restricted LLO 190–201, LLO 318–329 ($CD4^+$ T-cell epitopes) and LLO 296–304 ($CD8^+$ T-cell epitopes) (Fig. 3). The adoptive transfer of LLO 91–99-specific $CD8^+$ CTL (Harty and Bevan, 1992) or p60 301–312-specific $CD4^+$ Th (Geginat *et al.*, 1998) conferred protection against lethal listerial infection, suggesting that induction of T cells specific to these T-cell epitopes is a prerequisite for protection against listerial challenge.

CTL-ORIENTED DNA VACCINES

A variety of studies have been conducted on the efficient induction of CTL of a particular specificity and is summarized in Figure 4.

CTL-Epitope minigene DNA vaccines and the effect of the addition of the leader sequence on CTL induction

As mentioned previously, we have demonstrated that the minigene DNA vaccine, encoding only a dominant CTL epitope of *L. monocytogenes*, LLO 91–99, was effective for inducing CTL *in vivo* by gene gun-mediated DNA immunization (Uchijima *et al.*, 1998; Nagata *et al.*, 1999). These results suggest that the DNA vaccine plasmids are directly incorporated in APC and that the APC present target peptides to T cells by DNA immunization, although Cho *et al.* (2001) suggested that cross-priming is a predominant mechanism for inducing $CD8^+$ T-cell responses in gene gun DNA immunization. Some CTL epitopes were reported to be modified to have more immunogenic capacity by substituting several amino acid residues (epitope enhancement; Berzofsky, 1993). Berzofsky *et al.* (2001) suggested that epitope sequences can be modified in three ways: (a) for increasing the affinity of binding to an MHC molecule; (b) for increasing the affinity of the peptide–MHC complex for the T-cell antigen receptor; or (c) for achieving a more broadly crossreactive T-cell responses.

Ciernik *et al.* (1996) added a DNA sequence encoding adenovirus E3 leader sequence to mutant p53 minigene (more precisely, not the minimal CTL epitope, but CTL epitope-covering portion) and constructed the minigene plasmid and showed that the plasmid DNA vaccination is capable of eliciting CTL against mutant p53 efficiently. Addition of the leader sequence will localize the target polypeptide in the ER, bypassing the need for the TAP transporter. Subsequently, the MHC class I binding peptide (the CTL epitope) in the mutant p53 molecule would efficiently bind to MHC class I molecules there. They reported that the addition of the leader sequence dramatically induced the specific CTL activity. As mentioned earlier, they used the mutant p53 peptide, which contains 18 amino acids, instead of the minimal CTL epitope in it. The peptide has to be trimmed before binding to MHC class I molecules in the ER. In such a case, accumulation of the peptide in the ER by virtue of the leader sequence will facilitate the binding of the peptide and MHC class I molecules in the ER. However, the LLO 91–99 peptide used in our study (Uchijima *et al.*, 1998; Nagata *et al.*, 1999) is a 9-mer peptide. In this case, further trimming is not required for binding to MHC class I molecules. The difference would explain the

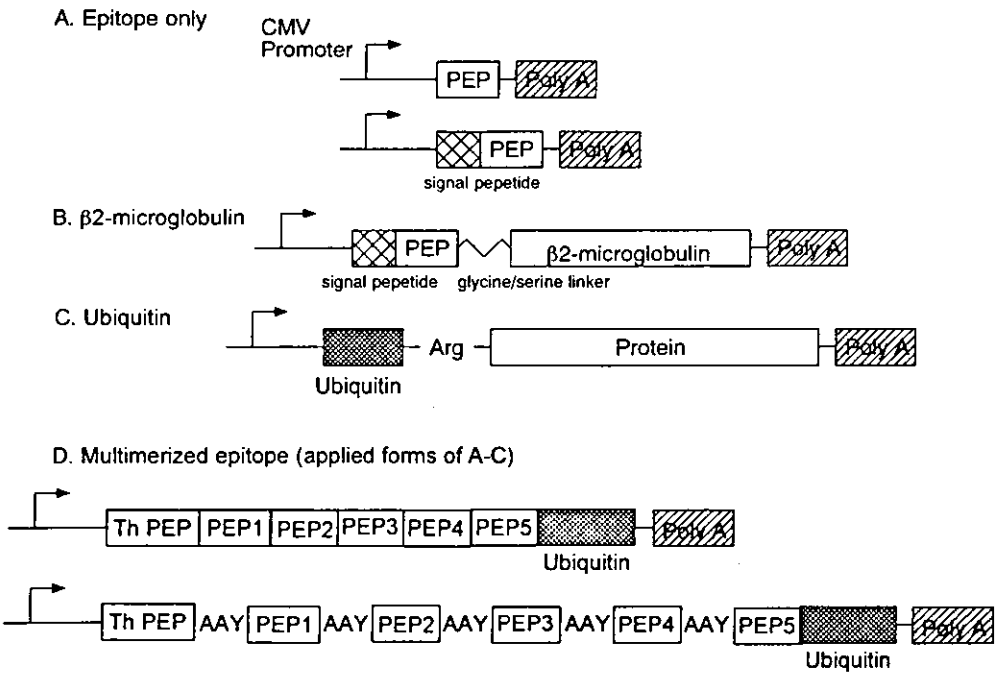

FIG. 4. Schematic presentation of several CTL-oriented DNA vaccines. Gene structures of CTL-oriented DNA vaccines are shown. See following references, Uchijima *et al.* (1998), Ciernik *et al.* (1996) for (**A**), Uger and Barber (1998) for (**B**), Wu and Kipps (1997) for (C), and Velders *et al.* (2001) for (**D**).

reason why CTL minigene DNA vaccine induces specific CTL efficiently without the addition of the leader sequence in our studies, although Iwasaki *et al.* (1999) reported that the addition of the leader sequence still enhances the magnitude of the CTL responses by a minimal CTL epitope DNA vaccine with both intramuscular and gene gun-mediated administration.

Fusion with β2-microglobulin

Uger and Barber (1998) prepared an expression plasmid of influenza virus nucleoprotein (NP) 366–374 (H2-D^b-restricted CTL epitope) fused with β2-microglobulin molecule by a glycine/serine short linker. They introduced the plasmid into a murine cultured cell line, and showed the efficient loading of the CTL epitope on MHC class I molecules. Their study is not an *in vivo* study, but the plasmid may be usable in DNA vaccine for efficient CTL induction.

Ubiquitination of target genes

In general, intracellular protein antigens are processed into the peptides for antigen presentation via MHC class I molecules, as mentioned previously. The peptides have been reported to be generated mainly via the ubiquitin (Ub)-proteasome pathway although the Ub-independent pathways have also been suggested (Murakami *et al.*, 1992; Jariel-Encontre *et al.*, 1995). Ub is a 76-amino acid peptide involved in controlling the normal protein intracellular turnover in the cytoplasm of eukaryotic cells. Ubiquitination of cellular proteins occurs in lysine residues of the proteins in an ATP-dependent manner, and the ubiquitinated proteins have been reported to be sensitive to degradation by proteasomes. The identity of the N-terminal residue of proteins is a determinant of whether the proteins are sensitive or resistant to degradation by proteasomes (N-end rule; Varshavsky, 1992). For example, if the N-terminal residue is methionine, the protein is stable, and if the residue is arginine, the protein is destabilizing.

So far, DNA vaccines that enhance the ubiquitin-proteasome degradation of target antigens have been reported. Wu and Kipps (1997) prepared an expression plasmid for chimeric β-galactosidase (β-gal)-*LacI* partial segment containing two lysine residues (served as a substrate of ubiquitination) and also an arginine residue was added in the N-terminal region (pUb-Arg-*lacZ*). They used this fusion plasmid as a DNA vaccine. This linear fusion of one Ub to chimeric β-gal-*LacI* molecule is supposed to be cleaved after the last residue of the Ub portion *in vivo* by Ub-specific processing proteases (Backmair *et al.*, 1986). After the cleavage, the Arg residue becomes the utmost N-terminal residue of the processed molecule, which is subsequently subjected to ubiquitination and degradation. As expected, immunization with the DNA vaccine showed efficient induction of β-gal-specific CTL. pUb-Arg-*lacZ*-transfected P815 cells (a murine mastocytoma cell line) did not have β-gal enzymatic activity, probably due to degradation by proteasomes. Whitton's group (Rodriguez *et al.*, 1997; Whitton *et al.*, 1999) also showed similar results by using expression plasmids for ubiqitinated NP derived from lymphocytic choriomeningitis virus. These results indicate that the strategy of antigen ubiquitination is useful to generate enhanced CTL activity and to reduce the production of antigen-specific antibodies. However, Fu *et al.* (1998) reported that the modifications of NP DNA vaccine with ubiquitin conjugation did not affect their ability to induce specific CTL responses, suggesting ubiquitin conjugation alone does not guarantee the improved targeting of endogenously synthesized antigens to MHC class I antigen processing pathway.

Injection of a single CTL epitope minigene DNA will generate a single CTL epitope peptide, which is supposed to enter the ER through TAP transporters. Our results (Uchijima *et al.*, 1998; Nagata *et al.*, 1999) indicated such a single minigene DNA immunization without any modification induced specific CTL efficiently. Interestingly, Rodriguez *et al.* (1998) showed even such a minigene DNA immunization improved by ubiquitination in terms of induction of high frequency of memory CTL, suggesting that ubiquitination allows more effective delivery of the minigene to the proteasome. They used a somewhat longer minigene encoding 32 amino acid residues covering two MHC class I binding peptide-encoding regions. In such a case, further trimming of peptides is necessary for the binding on MHC class I molecules as in the aforementioned minigene DNA vaccine construct described in Ciernick *et al.* (1996). This may be a reason why ubiquitination improved CTL induction after their minigene DNA vaccination. Our preliminary results show that such minigenes whose translation products do not have the exact size that fits in the groove of MHC class I molecules (8-, to 9-mer), but have some extra length, very inefficiently present the antigen to T cells (Aoshi, unpublished observation). We speculate that polypeptides bigger than minimal antigenic peptides, but smaller than the size of common proteins (e.g., around 20-mer peptides) are very resistant to proteasomal degradation. Proteasome complex may be facilitated for degradation of unfolded or misfolded large proteins, but may not be used for degradation of such artificially small peptides. In such a case, the ubiquitination of such small peptides dramatically increase the proteasomal degradation of the peptides.

Another intriguing strategy for enhancing proteasomal localization of antigens was reported by Hung and colleagues (2003). The centrosome has been shown to be a subcellular compartment rich in proteasomes (Antón *et al.*, 1999). They reported a DNA vaccine for a chimeric molecule composed of γ-tubulin (an established centrosomal marker) and human papillomavirus type 16 E7 (a model tumor antigen). They showed that the DNA vaccination targeted the antigen to centrosomal compartments and showed that the DNA vaccination induced enhanced MHC class I presentation and a marked increase in the number of E7-specific $CD8^+$ T-cell precursors.

Fusion with a carrier protein

Describing one particular approach for the efficient induction of $CD8^+$ T-cell subset, Wolkers *et al.* (2002) recommended a carboxyl-terminal fusion of CTL epitope to a carrier protein of foreign origin. They constructed their DNA vaccines encoding a carboxy-terminal fusion of either H2-D^b-restricted CTL epitope, NP 366–374 derived from influenza virus, or H2-D^b-restricted E7 49–57 derived from human papilloma virus to green fluorescent protein (GFP) and showed that the DNA vaccines induced much larger clonal size of antigen-specific $CD8^+$ CTL by intramuscular immunization of these DNA vaccines compared with the clonal size induced by these epitope minigene DNA vaccination. The purpose of the GFP fusion strategy was

the provision of $CD4^+$ T-cell help through recognition of GFP-encoded $CD4^+$ T cell epitopes. Maecker *et al.* (1998) also showed that CTL induction by both intramuscular and intradermal DNA administration is dependent upon the generation of $CD4^+$ T-cell help via a class II MHC-dependent pathway. Our results showed that CTL minigene DNA vaccination with gene gun induced specific CTL without any $CD4^+$ T-cell help (Yoshida *et al.*, 2001). We speculate that the route of naked DNA immunization is critical for requirement of CD4 T-cell help. So far, intramuscular or intradermal DNA administration was carried out in many reports which show that T-cell help is important for CTL induction. On the other hand, gene gun-based DNA immunization was carried out in many reports which show that CTL induction occurred independent of CD4 T-cell help.

Multimerized CTL epitope DNA vaccines

As a corollary of a single CTL epitope minigene DNA vaccination, several reports tried multimerized CTL epitope DNA vaccines (polyepitope DNA vaccines). The polyepitope vaccine was first evaluated by Whitton *et al.* (1993). They generated a recombinant vaccinia virus system for expression of CTL–epitope minigenes tandemly fused in a "string-of-beads" manner and showed that this "string-of-beads" vaccine can induce CTL specific to each different epitope and protect vaccinated animals against infections. Subsequently, Thomson *et al.* (1998b) constructed a DNA vaccine plasmid containing 10 contiguous minimal CTL epitopes, which were restricted by five MHC alleles and derived from five viruses (influenza virus, adenovirus, murine cytomegalovirus, Sendai virus, and lymphocytic choriomeningitis virus), a murine malaria parasite (*Plasmodium berghei*), and a tumor model antigen (ovalbumin). They injected mice with the plasmid by intramuscular injection or gene gun-mediated intradermal injection. They showed that the DNA vaccination successfully induces each epitope-specific CTL activity. Results of our single CTL–epitope DNA vaccine indicated that a single dominant CTL epitope is sufficient for the induction of protective immunity (Yamada *et al.*, 2001) suggesting that the selection of the most dominant CTL epitope for each pathogen is critical for the efficacy of DNA vaccines.

Although some reports suggest that flanking sequences of a CTL epitope are important for the precise processing of the CTL epitope *in vivo* and that some CTL epitopes will interfere with other epitope function (Del Val *et al.*, 1991), a majority of reports showed that immunization with multimerized CTL–epitope DNA without any spacer successfully induces CTL specific to each CTL epitope. But, some reports (e.g., Velders *et al.*, 2001) suggest the importance of defined flaking sequences around epitopes and addition of ubiquitin. Ishioka *et al.* (1999) evaluated minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes employing HLA class I transgenic mice. Such studies are useful as pilot experiments to evaluate DNA vaccines before progressing to studies on human subjects.

HELPER T-CELL ORIENTED DNA VACCINES

A variety of studies have been performed on the efficient induction of Th of a particular specificity and is summarized in Figure 5.

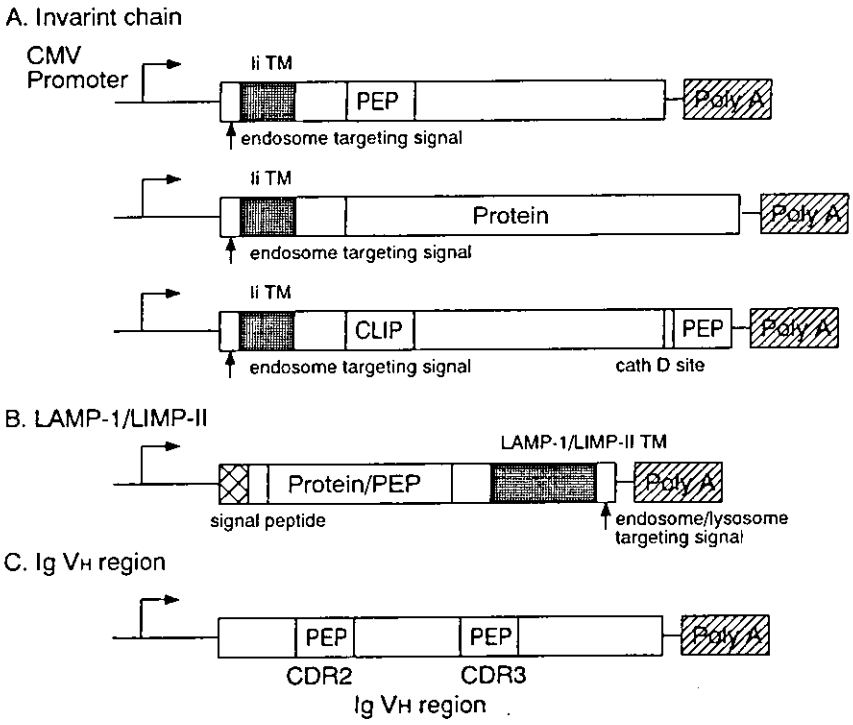

FIG. 5. Schematic presentation of several Th-oriented DNA vaccines. Gene structures of reported Th-oriented DNA vaccines are shown. See following references, for example, Nagata *et al.* (2001, 2002), Toda *et al.* (2002), van Bergen *et al.* (1997) for (A), Thomson *et al.* (1998a), Rodriguez *et al.* (2001) for (B), and Casares *et al.* (1997a, 1997b) for (C).

Th epitope/CLIP-exchanged DNA vaccines

Plasmid DNA is supposed to enter directly into APC, especially in gene gun-based vaccination. So, basically, the antigen presentation system through MHC class I molecules may be a primary antigen presentation route for DNA vaccination. However, efficient MHC class II antigen presentation is indispensable for efficient induction of $CD4^+$ helper T cells. Several groups have reported that MHC class II-positive cultured cells transfected with Ii cDNA, in which the CLIP region of the Ii molecule was replaced with a Th epitope of interest, efficiently stimulate specific T-cell lines (van Bergen *et al.*, 1997; Malcherek *et al.*, 1998; Fujii *et al.*, 1998, and reviewed in van Bergen *et al.*, 1999). We applied this strategy for Th-oriented DNA vaccines. We constructed the recombinant Ii DNA vaccine for OVA323-336 (Nagata *et al.*, 2001) or LLO 215–226 (Nagata *et al.*, 2002) Th epitope and evaluated the immunogenicity of the DNA vaccines. We observed the epitope-specific T-cell proliferation and IFN-γ production by spleen cells derived from the recombinant Ii DNA vaccines (Fig. 6). Some mice immunized with OVA 323–336-Ii DNA vaccine induced OVA 323–336-specific antibodies in the sera as well as the epitope-specific $CD4^+$ T cells (Nagata *et al.*, 2001). Recently, DNA vaccines of this type have been reported from other laboratories (van Tienhoven *et al.*, 2001; Toda *et al.*, 2002).

Utilization of endosomal/lysosomal targeting signals

Wu *et al.* (1995) reported a vaccine strategy for endogenous MHC class II presentation of antigens by taking advantage of the lysosome-associated membranous protein-1 (LAMP-1) molecule with the vaccinia virus system. The molecule is a type-1 transmembrane protein located predominantly in lysosomes and late endosomes involving the MHC class II antigen processing pathway. The cytoplasmic domain of LAMP-1 contains the amino acid sequence, Tyr-Gln-Thr-Ile, which is important for endosomal/lysosomal protein targeting. *In vitro* activation of Epstein-Barr virus-, and influenza virus-specific $CD4^+$ memory CTL was successfully demonstrated by infection of cultured cells with the recombinant vaccinia viruses for the polyepitope–LAMP-1 fusion proteins (Thomson *et al.*, 1998a). DNA immunization using this strategy has been examined for induction of a specific $CD4^+$ T-cell subset. Ji *et al.* (1999) showed that targeting human papillomavirus type 16 E7 to the endosomal/lysosomal compartments by gene-gun immunization enhances the antitumor immunity enough to protect mice against the challenge from virus protein-expressing tumors and to eradicate the preexisting tumor cells. We also tried a DNA vaccine for a chimeric molecule, where an antigenic peptide (LLO 215–226) was fused with the endosomal/lysosomal targeting signal in the LAMP-1 molecule. We observed that the LLO 215–226–LAMP-1 DNA vaccine gave induction of the epitope-specific Th induction and par-

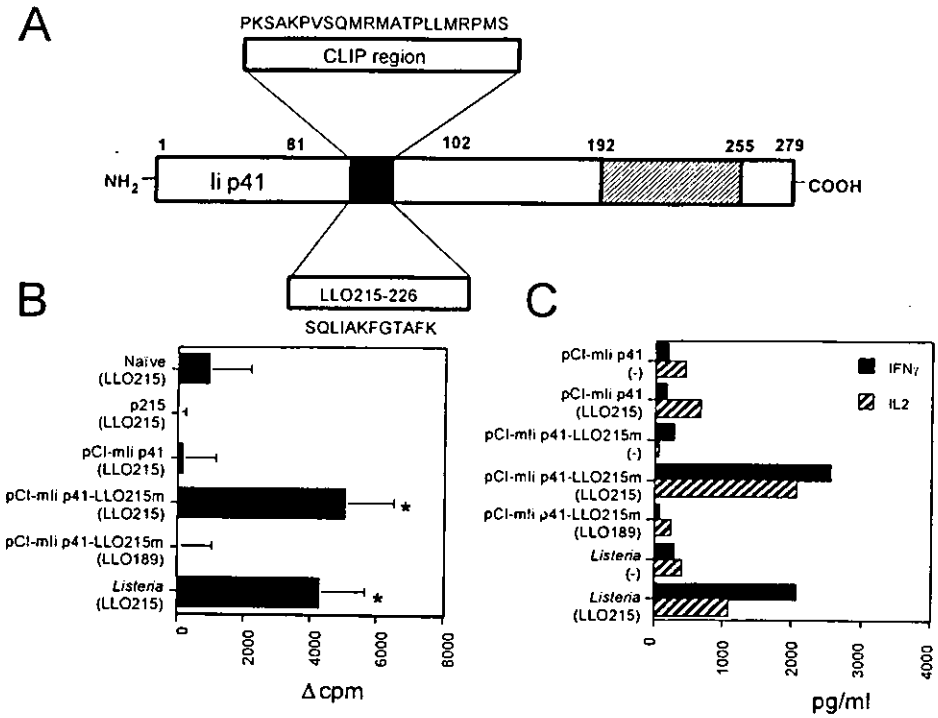

FIG. 6. Induction of LLO 215–226-specific Th by vaccination of plasmid for CLIP-replaced Ii molecule. (**A**) Schema of CLIP/LLO 215–226-replaced Ii molecule (mIi p41–LLO215m) whose expression plasmid was used for DNA vaccine. (**B**) LLO 215–226-specific splenocyte proliferation. C3H/He mice were immunized with the plasmid by gene gun bombardment four times. Immune spleen cells were harvested and cultured *in vitro* with indicated peptides (shown in parenthesis) for 2 days and then [^{3}H] thymidine was added for the last 12 h. Asterisks indicate statistical significance ($P < 0.001$) compared with the value of naive mice. (**C**) LLO 215–226-specific IFNγ and IL-2 production by immune spleen cells. The immune spleen cells were cultured with indicated peptides (shown in parenthesis) for 4 days (for IFN-γ) or 1 day (for IL-2). IFN-γ and IL-2 concentrations in the supernatants were qualified by sandwich enzyme-linked immunosorbent assay (modified figures of Nagata *et al.*, 2002).

tial protection against *L. monocytogenes* challenge, although the magnitude of the immunogenicity was somewhat lower than that by LLO 215–226/CLIP-replaced Ii DNA vaccine (Uchiyama *et al.*, 2002; Fig. 7). Vidalin *et al.* (1999) tried a DNA vaccine utilizing hepatitis C core protein–LAMP-1 fusion protein. Unfortunately, induction of the hepatitic C virus-specific T cells failed. They used a plasmid encoding the full-length hepatitis C virus core protein for the immunization, in which case the amounts of the MHC class II binding peptides produced would be low, as the protein must be further subjected to antigen processing to produce the peptides.

Recently, Rodriguez *et al.* (2001) reported successful $CD4^+$ T-cell induction by immunization of Th–epitope minigene plasmid DNA by utilizing the lysosomal targeting signal located in the carboxyl terminal tail of the lysosomal integral membrane protein-II (LIMP-II). They showed that the immunological consequences varied depending on the Th–epitope examined. As a result, a LAMP-1 or LIMP-II DNA vaccine may be useful for the targeting of proteins as well as peptides to the endosomal/lysosomal compartments, whereas the CLIP-replaced type of Ii DNA vaccine can afford to deliver only a small peptide, Th epitope, in theory. However, the immunological consequences of LAMP-1 or LIMP-II DNA vaccine may be weaker than the CLIP-replaced type of Ii DNA vaccine, and will also depend on target genes, as suggested in Rodriguez *et al.* (2001).

Utilization of a carrier protein structure-recombinant immunoglobulin molecule

Casares *et al.* (1997a, 1997b) reported on a DNA vaccine utilizing a recombinant immunoglobulin molecule, where the complementarity determining region (CDR) 3 and CDR2 regions of the heavy chain variable region were exchanged with an immunodominant Th epitope (HA 110–120) and a major B-cell epitope (HA 150–159) of influenza virus, respectively. They showed that immunization of mice with the DNA vaccine successfully induced influenza virus-specific Th and antibodies. This approach used a structurally rigid immunoglobulin molecule as the backbone molecule for the DNA vaccine.

Effect of the leader sequence on CD4 Th induction

Akbari *et al.* (1999) showed that vaccination with a DNA construct encoding the nonsecreted form (without the leader sequence) of the fifth component of the complement (C5) protein induces strong, long-lived $CD4^+$ T-cell responses. They showed that a small number of dendritic cells take up the plasmid DNA and migrate to the draining lymph nodes, and suggested that these dendritic cells are necessary for induction of long-lived $CD4^+$ T-cell responses. $CD4^+$ T-cell responses are generally thought to be induced by antigen presentation of "exogenous antigens" by dendritic cells. But this report showed that vaccination with a DNA construct encoding a protein that is not secreted is capable of inducing strong, long-lived $CD4^+$ T-cell responses.

Use of altered peptide ligand to increase peptide affinity for MHC molecules

Alexander *et al.* (1994) reported an artificial Th epitope that is capable of being presented on multiple HLA-DR molecules, by inserting anchor residues for diverse HLA-DR molecules in a polyalanine backbone and charged or bulky residues in the positions interacting with the TCR. This strategy may be applied for construction of helper T-cell-oriented DNA vaccines.

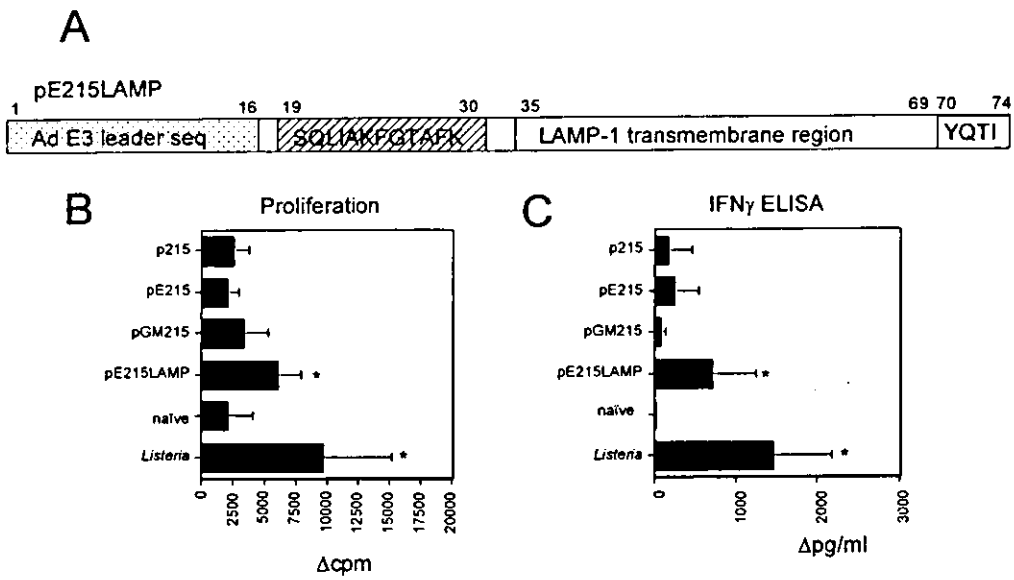

FIG. 7. Induction of LLO 215–226-specific Th by vaccination of plasmid for LLO 215–LAMP fusion molecule. (**A**) Schema of LLO 215–LAMP fusion molecule whose expression plasmid (pE215LAMP) was used for DNA vaccine. (**B**) LLO 215–226-specific splenocyte proliferation. C3H/He mice were immunized with the plasmid by gene gun bombardment four times. Immune spleen cells were harvested and cultured in vitro with LLO 215–226 peptide for 2 days and then [^{3}H] thymidine was added for the last 12 h. Asterisks indicate statistical significance ($P < 0.001$) compared with the value of naive mice. C. LLO 215–226-specific IFNγ production by immune spleen cells. The immune spleen cells were cultured with LLO 215–226 peptide for 5 days. The IFNγ concentration in the supernatants was qualified by sandwich enzyme-linked immunosorbent assay. Asterisks indicate statistical significance ($P < 0.005$) compared with the value of naive mice (modified figures of Uchiyama *et al.*, 2002).

IMPROVEMENT OF IMMUNIZATION REGIMEN: PRIME-BOOST METHOD FOR DNA IMMUNIZATION

In addition to the design of DNA vaccine plasmids, immunization protocols also need to be taken into consideration for enhancing immunological effects. Recent evaluation of DNA immunization indicates that DNA immunization alone has a limitation in terms of overall effects, and that the combination of different immunization methods gives better end results. As a pilot study, Li *et al.* (1993) reported that the combination of priming with recombinant influenza virus expressing the CTL epitope derived from *P. yoelii* circumsporozoite protein (PyCSP) and boosting with PyCSP-expressing vaccinia virus yielded more protection than immunizing with either PyCSP-expressing influenza virus or vaccinia virus alone, or the opposite order of prime-boost regimen (with vaccinia-PyCSP first and the recombinant influenza second). Similarly, a DNA heterologous prime-boost regimen, that is, primed with naked DNA vaccination and boosted with recombinant viral vectors such as vaccinia virus and adenovirus, has been shown to evoke superior levels of immunity (McShane, 2002; Ramshaw and Ramsay, 2000). Such a regimen has been evaluated in several infection models including malaria, HIV-1, tuberculosis, and herpes simplex virus (HSV) (Kent *et al.*, 1998; Schneider *et al.*, 1998; Sedegah *et al.*, 1998; Hanke *et al.*, 1999; Allen *et al.*, 2000; Sullivan *et al.*, 2000; McShane *et al.*, 2001). So far, the mechanisms of prime-boost vaccination are not clear. Even so, the relatively low-level, but persistent, expression of immunogenic proteins *in vivo* by naked DNA vaccines is suggested by Ramshaw and Ramsay (2000) to be important to prime immunological responses and to induce enhanced cellular immunity. Interestingly, Eo *et al.* (2001) reported that mucosal immunological responses were optimal when animals were primed with recombinant vaccinia virus vector and boosted with a naked DNA vaccine, which is an opposite approach compared with the regimen for systemic immunological responses.

SUMMARY

CTL and Th are key effectors in cell-mediated immunity. Here, we reviewed a variety of trials to induce specific T-cell subsets efficiently by DNA immunization. One of the excellent features of DNA vaccines is its extraordinary flexibility in designing the constructs by virtue of recombinant DNA technology. Various modifications of DNA vaccines have been examined and reported. It is important to develop excellent vaccines capable of inducing only particular favorable immune responses while preventing adverse responses by taking advantage of the flexibility in DNA vaccine design.

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