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RESEARCH ARTICLE

Exacerbation of Viral and Autoimmune Animal Models for Multiple Sclerosis by Bacterial DNA

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Theiler's murine encephalomyelitis virus (TMEV) infection and relapsing-remitting experimental allergic encephalomyelitis (R-EAE) have been used to investigate the viral and autoimmune etiology of multiple sclerosis (MS), a possible Th1-type mediated disease. DNA immunization is a novel vaccination strategy in which few harmful effects have been reported. Bacterial DNA and oligodeoxynucleotides, which contain CpG motifs, have been reported to enhance immunostimulation. Our objectives were two-fold: first, to ascertain whether plasmid DNA, pCMV, which is widely used as a vector in DNA immunization studies, could exert immunostimulation *in vitro*; and second, to test if pCMV injection could modulate animal models for MS *in vivo*. We demonstrated that this bacterially derived DNA could induce interleukin (IL)-12, interferon (IFN)γ (Th1-promoting cytokines), and IL-6 production as well as activate NK cells. Following pCMV injections, SJL/J mice were infected with TMEV or challenged with encephalitogenic myelin proteolipid protein (PLP) peptides. pCMV injection exacerbated TMEV-induced demyelinating disease in a dose-dependent manner. Exacerbation of the disease did not correlate with the number of TMEV-antigen positive cells but did with an increase in anti-TMEV antibody. pCMV injection also enhanced R-EAE with increased IFNγ and IL-6 responses. These results caution the use of DNA vaccination in MS patients and other possible Th1-mediated diseases.

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Introduction

Multiple sclerosis (MS) is the major cause of neurological disability among young adults. The etiology is largely unknown. Clinical and experimental evidence suggests, however, that MS is a virus-induced disease and/or an autoimmune disease (6, 7, 25, 50). Theiler's murine encephalomyelitis virus (TMEV) infection and relapsing-remitting experimental allergic (autoimmune) encephalomyelitis (R-EAE) have been used to study the possible viral and autoimmune mechanisms for MS, respectively (20, 50). TMEV belongs to the family *Picornaviridae* and central nervous system (CNS) infection causes an inflammatory demyelinating disease in its natural host, the mouse. R-EAE can also be induced in mice with a subcutaneous injection of CNS antigen emulsified with complete Freund's adjuvant (CFA) or adoptive transfer of CNS antigen-specific CD4+ Th1 cells (22). The 2 models are similar to MS clinically and histologically where cellular and humoral immune responses are believed to play important roles in the pathogenesis (20, 50).

DNA immunization (or vaccination) is the *in vivo* administration of naked DNA molecules encoding antigen(s) of interest. DNA immunization is a potential means of mimicking the *de novo* production of correctly folded antigens and major histocompatibility complex (MHC) class I-restricted antigen presentation normally observed with live vaccines, without the risks associated with the use of infectious agents (9). Thus, DNA immunization can induce both cellular and humoral immune responses in many experimental systems. Moreover, DNA immunization is highly effective at inducing long-lived memory responses. Both DNA and immune responses induced by DNA immunization have been detected more than one year after immunization (reviewed in (49)).

In contrast to RNA molecules, such as interferon (IFN)-inducing Poly I-C, the DNA vector backbone itself was believed to be immunologically inert. Recently, however, there is growing evidence that non-vertebrate DNA, or sequences derived from it, could provoke powerful immune responses (16, 28, 49). These effects result from an "immunostimulatory sequence (ISS)" consisting of an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines ("CpG motif") within the DNA. Non-vertebrate DNA, which contains CpG motifs more frequently than vertebrate DNA, and CpG oligodeoxynucleotides have been shown to induce the production of Th1-promoting cytokines, such as interleukin (IL)-12 and IFNγ; polyclonal B cell activation mediated by IL-6; and natural killer (NK) cell activation. Therefore, in DNA immunization, not only the protein-encoding region of plasmid DNA (transcription unit) but also the plasmid backbone itself can modulate immune response, since many of the plasmid vectors contain CpG motifs (40, 49).

Experimentally, exposure of immune cells to antigen in the presence of bacterial or CpG DNA biases the immune response to Th1 and protects against Th2-type responses, such as asthma (14) and *Leishmania major* infection (63). However, initiation of a Th1-type response has the potential danger of inducing Th1-mediated pathology, such as Th1-associated autoimmune diseases. Segal *et al*. (42) demonstrated *in vitro* that bacterial DNA or CpG oligodeoxynucleotides converted quiescent myelin basic protein-specific T cells into effector cells capable of transferring EAE, a CD4+ Th1 cell-mediated disease. Furthermore, Schwartz *et al*. (41) showed that intratracheal instillation of bacterial DNA caused inflammation in the lower respiratory tract of mice. Bacterial DNA injection could also trigger toxic shock mediated by tumor necrosis factor (TNF)-α, which is released by macrophages in mice treated with D-galactosamine (43). Currently, experiments using DNA immunization have been conducted not only in Th2-mediated diseases, but also in Th1-mediated diseases, including animal models for MS (4, 21, 52, 55). In addition, clinical trials of DNA immunization in human patients have also been initiated (47).

Our objectives were two-fold: first, to ascertain whether bacterial plasmid DNA, pCMV, which is widely used as a vector in DNA immunization studies (52), could exert immunostimulatory effects *in vitro*; and second, to test whether pCMV injection alone could modulate viral and autoimmune models for MS *in vivo*. We found that pCMV could induce IL-12, IFNγ, and IL-6 production as well as activate NK cells *in vitro*. Next, we investigated the dose-dependent effect of pCMV inoculation on the development of TMEV-induced demyelinating disease. We found that pCMV injection could potentiate TMEV-induced demyelinating disease in a dose-dependent manner. Finally, we observed that 3 pCMV injections could exacerbate R-EAE induced with 2 encephalitogenic epitopes, myelin proteolipid protein $(PLP)_{139\text{-}151}$ or $PLP_{178\text{-}191}$. We propose there may be potential dangers associated with DNA inoculation in MS patients and others with possible Th1-mediated diseases.

Materials and Methods

Plasmid construction. We used plasmid pCMV, which was derived by excision of the β-galactosidase gene from pCMVβ (Clontech, Palo Alto, CA) (61). Plasmid pCMV contains the immediate early gene promoter/enhancer from human cytomegalovirus, an intron (splice donor/splice acceptor) and polyadenylation signal from simian virus 40, an ampicillin resistance gene, and 20 CpG motifs (GenBank Database, accession #U02451). To remove endotoxin, plasmid DNA was purified with EndoFree Plasmid Maxi Kit (endotoxin level < 0.6 ng/mg of plasmid DNA, Quiagen, Chatsworth, CA).

Cytokine Assays. Whole spleen cells from a naïve female SJL/J mouse were suspended in Tris-buffered 0.83% ammonium chloride to lyse erythrocytes and then washed 3 times. We cultured spleen cells at 2×10^6 cells/ml in 6-well plates in the presence or absence of pCMV (5 μg/ml). We also isolated mononuclear cells (MNCs) from $PLP_{139\text{-}151}$ peptide-induced EAE mice following with either three saline or pCMV injections. Five mice from each group were sacrificed 24 days after EAE induction when mice showed the first relapse. The inguinal lymph nodes and spleens were removed and pooled, and MNC suspensions were prepared. MNCs were cultured with 50 μg/ml $PLP_{139\text{-}151}$ peptide. Culture supernatants were harvested 24, 48 or 72 hours after stimulation. We measured IL-4, IL-6, IL-12 (p70) and IFNγ using the enzyme-linked immunosorbent assay (ELISA) system, OptEIA™ Set (Pharmingen, San Diego, CA), according to the manufacturer's instruction. The lower limit of sensitivity of each cytokine assay was 3.9 pg/ml for IL-4, 7.8 pg/ml for IL-6, and 15.6 pg/ml for IL-12 (p70) and IFNγ.

Assay for NK cell activity. We incubated spleen cells from a normal SJL/J mouse at a concentration of 2×10^6

cells/ml with or without 5 μg/ml pCMV DNA for 6 days. The cells were collected, washed 3 times and used as effector cells. Target cell lysis was assessed by the ^{51}Cr-release assay (58, 59). NK cell-sensitive YAC-1, and NK cell-resistant P815 and EL4 (American Type Culture Collection, Rockville, MD) target cells were labeled with $Na_2{}^{51}CrO_4$ (New Life Science Products, Inc., Boston, MA). YAC-1 and P815 were kindly provided by Dr. Raymond M. Welsh (Department of Pathology, University of Massachusetts Medical Center, Worcester, MA). The target cells were placed in wells of 96-well round-bottomed microtiter plates, and various quantities of effector cells were added. After a 5-hour incubation, the radioactivity released from the cells was measured by a model 20/20 gamma counter (Iso-Data, Inc., Palatine, IL). The percentage of target cell lysis was calculated using the following equation: % lysis = (experimental release cpm - spontaneous release cpm)/(maximal release cpm - spontaneous release cpm) × 100.

Animal experiments. Female SJL/J mice were purchased from either the Jackson Laboratory (Bar Harbor, ME) or National Cancer Institute (Bethesda, MD). We injected 100 μg of pCMV intramuscularly into the hind limbs 1 to 3 times at 1-week intervals with each leg receiving 50 μg of pCMV in 50 μl of phosphate-buffered saline (PBS) or normal saline. Control mice were injected with PBS or normal saline. Two weeks following the pCMV inoculation(s), TMEV disease or R-EAE was induced in now 7-week-old mice. We injected 2×10^5 plaque-forming units (PFU) of the DA strain of TMEV intracerebrally. R-EAE was induced with subcutaneous injection with 100 nmol of modified $PLP_{139\text{-}151}$ (2) or $PLP_{178\text{-}191}$ peptide (8) in CFA in the tail base. TMEV-infected mice were viewed for clinical signs of disease: a loss in righting reflex, a waddling gait and spastic paralysis (20). R-EAE mice were followed for clinical signs of disease according to the following scale (52): 0, no clinical disease; 0.5, loss of tonicity of the distal half of the tail; 1, complete loss of tail tonicity; 2, mild hind leg paresis; 3, moderate hind leg paralysis; 4, complete paraplegia; and 5, quadriplegia, moribund state or death.

Histology. Two to three months after TMEV infection or R-EAE induction, mice were killed under halothane anesthesia and perfused with 4% paraformaldehyde. Spinal cords were divided into 10 to 12 coronal slabs and were embedded in paraffin by standard methods (51). Spinal cord sections were stained

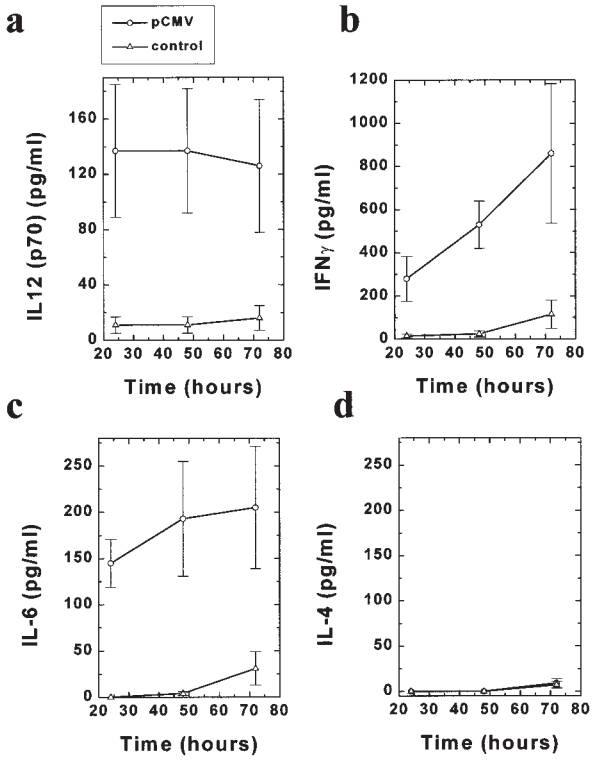

Figure 1. Cytokine production of spleen cells after stimulation with plasmid DNA, pCMV. Whole spleen cells (2×10^6 cells/ml) from a SJL/J mice were stimulated with (O) or without (Δ) pCMV for 24, 48, or 72 hours. Cytokine activity in the culture supernatants was assayed by quantitative ELISA. pCMV-stimulated spleen cells produced IL-12 (p70) (**a**), IFNγ (**b**) and IL-6 (**c**), but no IL-4 (**d**). All assays were performed in duplicate. Values are the means ± SEM of 2 independent experiments.

with luxol fast blue. Histologic scoring was performed as described by Rodriguez *et al*. (33, 36) with slight modifications (52). Each spinal cord section was divided into quadrants: the ventral column, the dorsal column and each lateral column. Any quadrant containing meningitis, demyelination or perivascular cuffing was given a score of 1 in that pathologic class. The total number of positive quadrants for each pathologic class was determined, then divided by the total number of quadrants present on the slide and multiplied by 100 to give the percent involvement for each pathologic class. An overall pathologic score was also determined by giving a positive score if any lesions were present in the quadrant. TMEV-antigen positive cells were detected by the avidin-biotin peroxidase complex (ABC) technique (53, 54) using hyperimmune rabbit serum to DA virus (64). Enumeration of TMEV-antigen positive cells was carried out with a light microscope at a magnification of 200 (11).

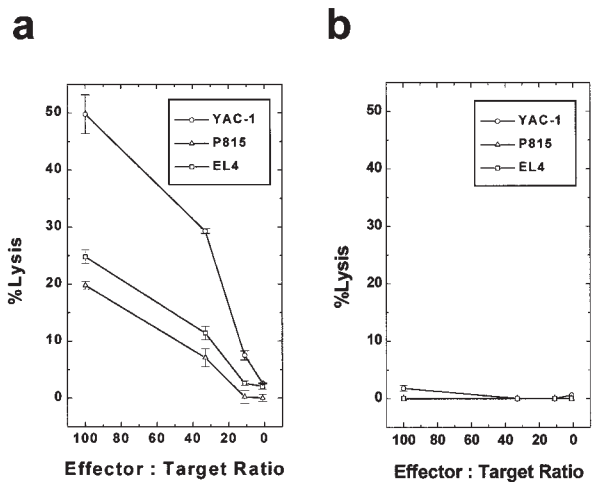

Figure 2. NK activity of spleen cells from SJL/J mice. Naive spleen cells were incubated *in vitro* for 6 days in the presence (**a**) or absence (**b**) of plasmid pCMV, followed by 5-hour ^{51}Cr-release assay on target cells, YAC-1 (O), P815 (Δ), and EL4 (□). (**a**) pCMV incubation induced moderate NK-sensitive YAC-1 killing and NK-resistant P815 and EL4 killing. (**b**) In contrast, SJL/J spleen cells did not show NK activity without stimulation. Results are representative of 2 independent experiments.

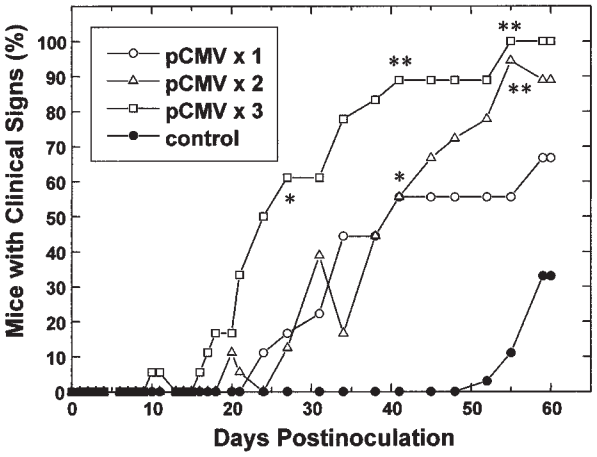

Figure 3. Clinical course of TMEV disease following plasmid pCMV injection. Mice were injected intracerebrally with TMEV following plasmid pCMV, once (O), twice (Δ), or 3-time (□) injection. We observed clinical signs in the mice during the course of disease, including a loss in righting reflex, waddling gait and spastic paralysis. Plasmid injection groups showed clinical symptoms earlier than control mice without plasmid injection (●). The influence appeared in a dose-dependent manner on the number of plasmid injections. The values represent the percentage of mice with clinical signs in all mice examined. Each experiment group consisted of 5 to 9 mice. ** $p < 0.05$*, *** $p < 0.01$*, ANOVA, compared with control.

Serum anti-TMEV and anti-PLP peptide antibody assays. TMEV-infected mice and R-EAE mice were bled 2 and 3 months after disease induction, respectively. A quantitative ELISA was used to measure the level of serum anti-TMEV or anti-PLP peptide immunoglobulin (Ig) as described previously (18, 52, 54). Ninety-six well plates were coated with DA virus antigen or PLP peptide overnight. After blocking, serial dilutions of murine sera was added to the plates and incubated for 90 minutes (min). After washing, a peroxidase-conjugated goat anti-mouse IgG1, IgG2a or IgG2b (Caltag Laboratories, South San Francisco, CA) antibody was added for 90 min. The plates were then colorized with *o*-phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, MO) and were read at 492 nm on a Titertek Multiskan Plus MK II spectrophotometer (Flow Laboratories, McLean, VA).

Results

In vitro induction of cytokine production. Non-vertebrate DNA or oligodeoxynucleotides, encoding CpG motifs, are known immunostimulants both *in vivo* and *in vitro*. Immunostimulatory DNA has been reported to induce normal spleen cells to produce IL-12, IFNγ and IL-6, but no IL-4 (15). We first tested whether our plasmid construct pCMV, a widely used DNA vector, could elicit cytokine production *in vitro* from lymphoid cells from SJL/J mice. Naïve spleen cells from SJL/J mice were cultured in the presence of pCMV and monitored for cytokine production. As seen in Figure 1, pCMV-stimulated spleen cells produced IL-12 (p70), IFNγ and IL-6 as early as 24 hours post-treatment, while limited production of the cytokines was measured in supernates from lymphoid cells without pCMV treatment. No IL-4 was detected either in the presence or absence of pCMV.

In vitro augmentation of NK cell activity. Bacterial DNA has been reported to augment NK cell activity (57). We demonstrated that pCMV could enhance NK cell activity of naïve SJL/J spleen cells *in vitro*. NK cell assays were conducted using lymphoid cells incubated with or without pCMV. pCMV-stimulated spleen cells showed moderate killing of the NK cell-sensitive YAC-1 cells and reduced killing of NK cell-resistant P815 and EL4 cell lines (Figure 2a). In contrast, spleen cells did not show any NK cell activity without pCMV stimulation (Figure 2b). This is consistent with the low NK cell activity normally found in SJL/J mice (12).

In vivo potentiation of TMEV-induced demyelinating disease. Since we demonstrated immunostimulatory effects of plasmid pCMV *in vitro*, we next evaluated the effects of pCMV injection on a virus-induced animal model for MS, TMEV-induced demyelinating disease. In many DNA immunization studies, both cellular and humoral immune responses have been shown to increase following boosting with plasmid DNA (61). To test whether pCMV injection(s) modulated TMEV disease in a dose-dependent fashion, we inoculated SJL/J mice with plasmid pCMV once, twice or 3 times followed by infection with TMEV. During the acute phase of TMEV infection, 1 to 2 weeks postinfection, there were no obvious clinical differences among the groups. Later, during the chronic demyelinating phase, mice without plasmid injection generally showed clinical signs, such as ataxia and hind limb paralysis, 2 months after TMEV infection (Figure 3). In contrast, pCMV-injected mice had an earlier clinical onset of disease and more severe signs of demyelinating disease than control mice. Mice injected with pCMV 3 times developed the most severe clinical disease with the earliest onset. Thus, the modulation of clinical disease occurred in a dose-dependent fashion contingent on the number of plasmid injections. pCMV injection(s) alone without TMEV infection caused no disease (data not shown).

CNS pathology of TMEV infection. During the chronic phase of TMEV infection, mice develop inflammatory demyelinating disease with virus persistence in the spinal cord white matter (20, 50). Lesions are seen most frequently in the ventral column, ventral root entry zone and lateral columns with minimal dorsal column involvement (32). We examined the spinal cords of pCMV-injected mice 2 months after TMEV infection. The extent of disease/pathology was quantified. In all pathologic categories studied, CNS pathology was enhanced by pCMV in a dose-dependent manner (Figure 4). In the mice injected with pCMV once or twice, dorsal column involvement was minimal (Figure 5a). However, in the mice injected with pCMV 3 times, we could see extensive dorsal column involvement, most frequently in the cuneate fasciculus, while the gracile fasciculus was relatively spared (Figure 5b). No CNS changes were found in mice injected with plasmid alone (data not shown).

To see whether enhancement of the disease correlated with an increase in virus persistence, we compared the number of TMEV-antigen positive cells among the pCMV-injected groups. Interestingly, there was no relationship between the number of TMEV-antigen positive

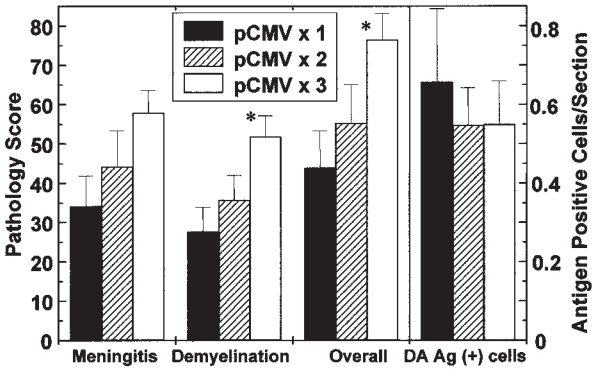

Figure 4. Pathologic scores and the number of TMEV-antigen (Ag) positive cells in the spinal cord of TMEV infected mice following plasmid pCMV injection once (closed column), twice (hatched column) or 3 times (open column). Two weeks after final plasmid injection, mice were infected with DA strain of TMEV intracerebrally. CNS pathology was scored 2 months after TMEV inoculation as described in the Materials and Methods section. DA virus Ag positive cells were detected by immunohistochemistry; the average number of Ag positive cells in 1 coronal section was calculated. All pathology scores increased plasmid dose-dependently. There was no difference, however, in the number of DA virus Ag positive cells among the groups. Values are expressed as mean ± SEM. Each experimental group consisted of 9 mice. *$*p < 0.05$*, ANOVA, compared with that of the single plasmid injection group.

cells and spinal cord pathology (Figure 4). Mice injected with pCMV once had the most number of TMEV-antigen positive cells among the groups (Figure 5c, e). Despite the severest lesions in the mice injected with pCMV 3 times, no increase in the number of virus antigen positive cells in and around the lesions was observed (Figure 5d, f). These results suggest that an increase in virus antigen containing cells was not a contributing factor for the enhanced TMEV disease observed.

Anti-TMEV IgG isotype responses. In addition to virus persistence, the immune response against TMEV is a contributing factor to the disease. Bacterial DNA can influence humoral immune responses (17). CpG motifs have been reported to cause polyclonal activation of B cells by induction of IL-6 (15) and immunoglobulin class switching most likely by the induction of Th1-promoting cytokines (3, 37). Based upon these findings, we tested whether pCMV injection could modulate the anti-TMEV IgG isotype responses. As seen in Figure 6, we found that all anti-TMEV IgG isotypes were increased in a dose-dependent fashion contingent on the number of plasmid injections. This suggests that the

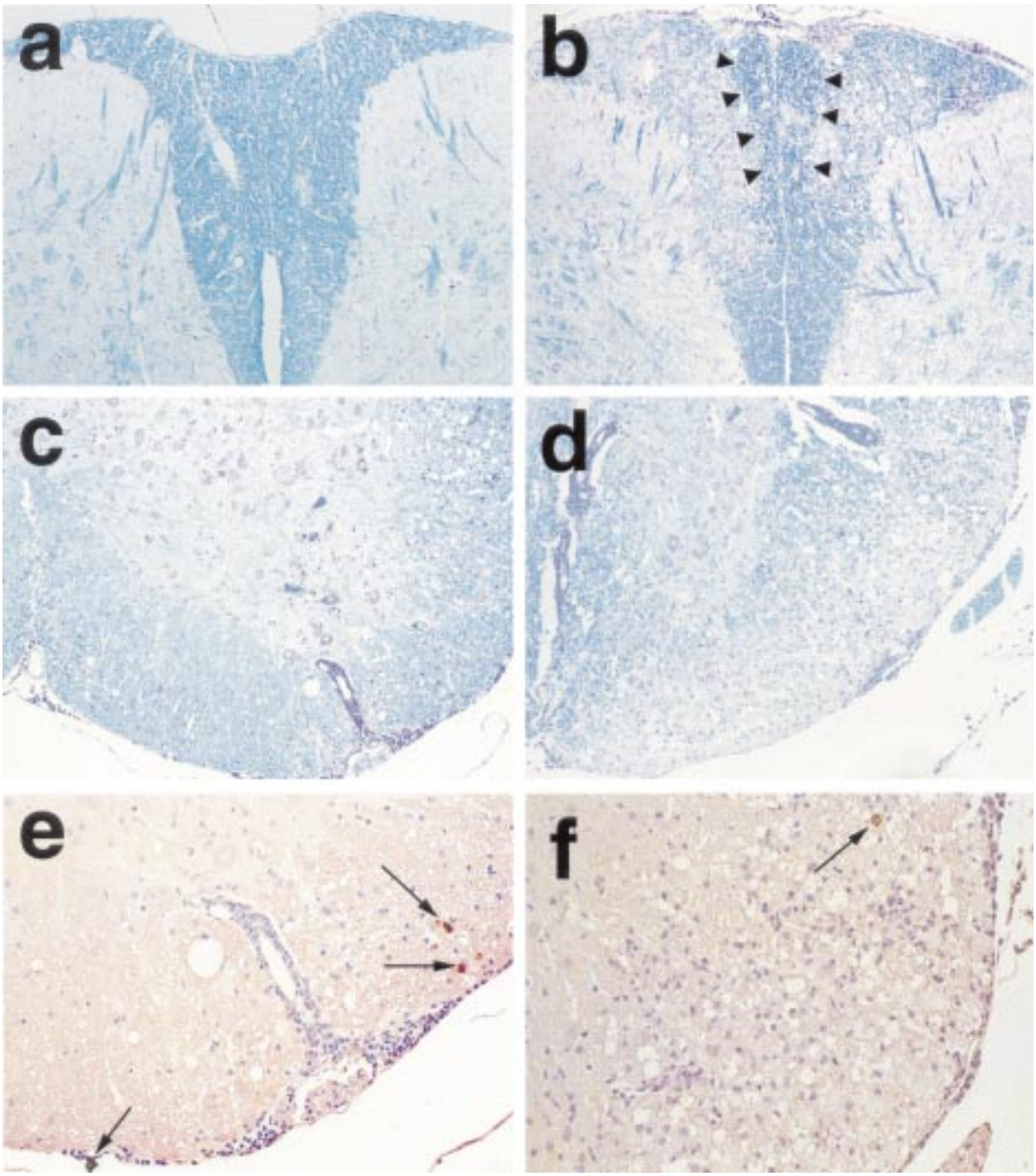

Figure 5. Histopathology of the spinal cord of TMEV infected mice following plasmid pCMV injection. Mice were killed 2 months after TMEV injection. Little dorsal column involvement was seen in the mice injected once with pCMV (**a**). Those injected 3 times with pCMV, however, showed intense cuneate fasciculus involvement (**b**), while the gracile fasciculus was relatively spared (arrowhead). In the ventral root entry zone, mice injected once with pCMV showed less inflammatory demyelinating lesion (**c**) than those injected 3 times with pCMV (**d**). In consecutive sections, however, we detected more TMEV-antigen positive cells (arrow) in mice injected once with pCMV (**e**) than those injected 3 times with pCMV (**f**). (**a-d**) Luxol fast blue stain, × 70. (**e, f**) Immunohistochemistry with anti-TMEV antibody, × 35.

Priming Antigen[a]	Treatment[b]	Acute EAE			Chronic EAE		
		Onset Day	Clinical Score[c]	EAE/Total Mice[d]	Onset Day	Clinical Score	EAE/Total Mice
$PLP_{139-151}$	PBS	13.8	2.1 ± 0.6	8/8	24.5	0.9 ± 0.6	5/8
	pCMV	13.4	2.4 ± 1.0	10/10	25.3	1.7 ± 1.5	8/10
$PLP_{178-191}$	PBS	14.0	0.4 ± 0.4	1/5	-	-	0/5
	pCMV	14.5	1.0 ± 0.3	4/5	28.3	1.4 ± 0.6*	3/5

[a] For relapsing-remitting experimental allergic encephalomyelitis (R-EAE) induction, SJL/J mice were injected subcutaneously with myelin proteolipid protein $(PLP)_{139-151}$/ CFA or $PLP_{178-191}$/CFA 2 weeks after the final plasmid inoculation.
[b] Mice were injected intramuscularly either with PBS or plasmid pCMV 3 times in 1-week intervals.
[c] Mean maximum clinical score ± SEM from all mice examined.
[d] Number of mice with clinical symptoms of EAE/total number of mice examined.
* $p < 0.05$ (*t* test).

Table 1. Effect of Plasmid DNA Inoculation on the Course of R-EAE.

augmentation of anti-TMEV immune responses by pCMV could contribute to increased TMEV disease.

Exacerbation of PLP-induced R-EAE by plasmid injection. Since 3 injections of pCMV was most effective in increasing TMEV disease, a virus-induced demyelination model of MS, we decided to test whether 3 DNA injections could also exacerbate an autoimmune model of MS, R-EAE. Following 3 pCMV injections, we induced R-EAE in SJL/J mice using 2 widely used encephalitognic peptide, $PLP_{139-151}$ or $PLP_{178-191}$ peptides (2, 8). In $PLP_{139-151}$-induced R-EAE, during the chronic phase, we detected a slight increase of clinical disease by plasmid injection (Table 1). On the other hand in $PLP_{178-191}$-induced R-EAE, pCMV increased both the acute and chronic EAE clinical signs compared with control animals.

Histology of PLP-induced R-EAE. Three months after R-EAE induction, we compared the histology between pCMV-injected and control mice. In both $PLP_{139-151}$- and $PLP_{178-191}$-induced R-EAE, demyelinating lesions were mainly found in the ventral root entry zone and the gracile fasciculus of the dorsal column (52) (Figure 7). The cuneate fasciculus and the corticospinal tract were usually spared. This contrasted with the lesion distribution of TMEV disease, in which the cuneatus fasciculus was frequently involved (Figure 5b). As seen in Figure 8, we could find an increase of pathologic scores in the $PLP_{178-191}$-induced R-EAE group following pCMV injection versus the PBS-injected control R-EAE group (Figure 8b). In $PLP_{139-151}$-induced R-EAE, plasmid injection also increased demyelinating lesions compared with the control group ($p < 0.01$, Figures 7, 8a). Except in their severity, the distribution of lesions in the spinal cord were the same between the pCMV-injected mice and control mice.

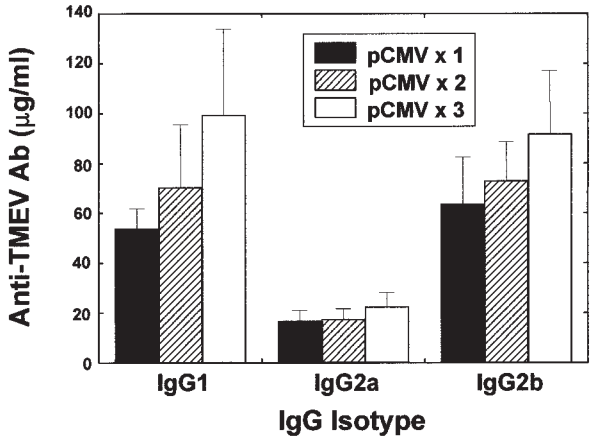

Figure 6. Serum anti-TMEV IgG isotype responses of mice following plasmid pCMV injection. Mice were injected with pCMV, once (closed column), twice (hatched column), or 3 times (open column) before TMEV intracerebral inoculation. Mice were bled 2 months after TMEV injection. ELISA was used to measure the level of serum anti-TMEV antibody (Ab). All anti-TMEV IgG isotype responses increased plasmid dose-dependently. Data represent the mean ± SEM of 9 samples.

Anti-PLP IgG isotype responses. We also determined how pCMV injection modulates anti-PLP IgG isotype responses in R-EAE mice (Figure 9). In $PLP_{139-151}$-induced R-EAE, control R-EAE mice developed a predominant anti-PLP IgG1 isotype response ($p < 0.05$, Wilcoxon signed rank test), while pCMV injection elicited similar anti-PLP IgG1 and IgG2b isotype responses (Figure 9a). In contrast, in $PLP_{178-191}$-induced R-EAE, control R-EAE mice produced more anti-PLP IgG2b antibody than that of IgG1 ($p < 0.05$), while pCMV injection elicited similar anti-PLP IgG1 and IgG2b isotype responses (Figure 9b). A slight increase of the anti-PLP IgG2a isotype response was seen in pCMV-injected groups immunized with either $PLP_{139-151}$ or $PLP_{178-191}$ peptide.

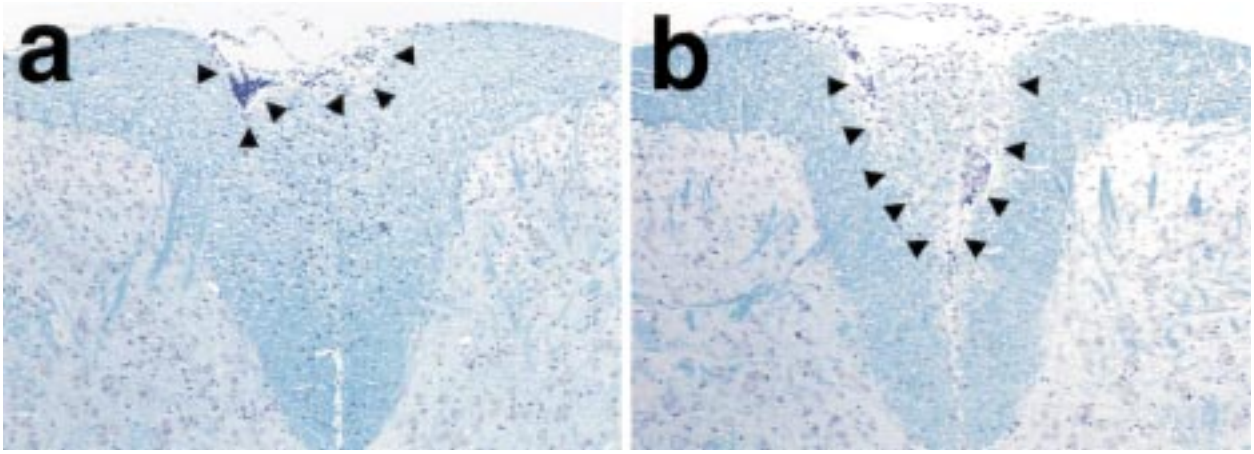

Figure 7. Representative histopathology of R-EAE induced with $PLP_{139-151}$ peptide following PBS (**a**) or pCMV infection (**b**). pCMV-injected mice showed more severe demyelination than control mice. In the dorsal column, the demyelination was usually seen in the gracile fasciculus (arrowhead), but not in the cuneate fasciculus or in the corticospinal tract. Luxol fast blue stain, × 70.

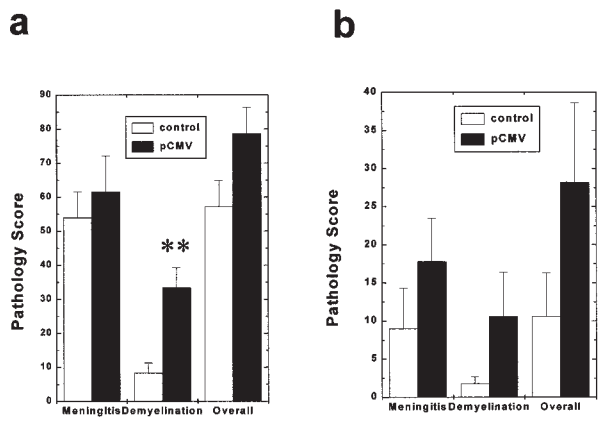

Figure 8. Effect of pCMV injection on spinal cord pathology in R-EAE. Mice were immunized subcutaneously with $PLP_{139-151}$/CFA (**a**) or $PLP_{178-191}$/CFA (**b**) 2 weeks after PBS (open column) or 3-times pCMV injection (closed column). CNS pathology was scored 3 months after R-EAE induction as described in the Materials and Methods section. In both $PLP_{139-151}$- and $PLP_{178-191}$-induced R-EAE, pCMV injection increased the demyelinating lesions. Values are expressed as mean ± SEM of 5 to 10 mice. ** $p < 0.01$, *t* test.

PLP-specific cytokine responses in R-EAE mice. Cytokine milieu is known to affect immunoglobulin class switching (1). Thus, the altered IgG isotype responses in R-EAE mice injected with pCMV supports the hypothesis that pCMV could modulate cytokine production *in vivo*, leading to exacerbation of R-EAE. Following pCMV or saline injection, we induced R-EAE with $PLP_{139-151}$ peptide and sacrificed the mice on day 24, when mice first showed relapse. We isolated MNCs from spleens and lymph nodes and compared PLP-specific cytokine production between the groups. As seen in Figure 10, we found a significant increase in IFNγ and IL-6 production in MNC cultures from the pCMV injected group compared with control. Little if any IL-12 and IL-4 was detected in either group. The lack of induction of IL-12, macrophage-derived cytokine, is most likely due to the nature of the lymphoproliferative assay which induces mainly MHC class II-restricted antigen-specific T cell responses.

Discussion

In this paper, we first showed that bacterial plasmid DNA, pCMV, which is widely used as a vector in DNA immunization, could elicit proinflammatory cytokine production *in vitro*. Although we have not definitively identified the DNA motifs that are responsible for the immunostimulation, our data support the hypothesis that CpG motifs are likely one of the contributing factors. Our data are compatible with those of other investigators, who used CpG DNA as a stimulator of spleen cells (15, 37, 42). We also demonstrated NK cell activation in SJL/J mice by pCMV, another functional hallmark of the immunostimulatory effect induced by CpG DNA (57). Thus, the immunostimulatory effects seen in our studies likely result from the 20 CpG motifs encoded in pCMV, although it remains possible that other structural characteristics of pCMV unrelated to the CpG motif could also induce the effects.

In TMEV-induced demyelinating disease, both virus persistence in the spinal cord and an immune-mediated mechanism have been suggested to play important roles (reviewed in (20, 50)). There are some discrepancies

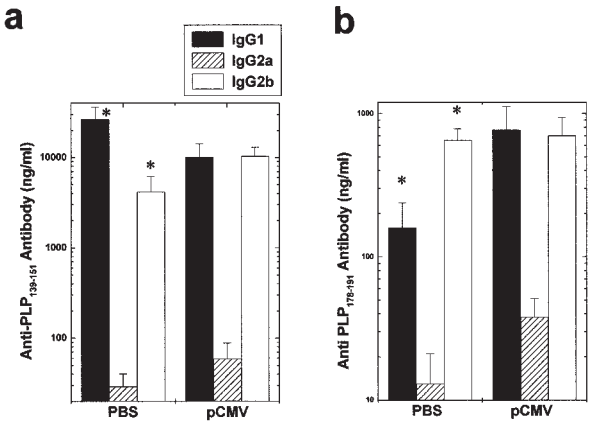

Figure 9. Serum anti-PLP IgG isotype titers in R-EAE mice following plasmid pCMV injection. Mice were injected intramuscularly with either PBS or pCMV 3 times in 1-week intervals. Two weeks after final injection, mice were immunized subcutaneously with either $PLP_{139-151}$ (**a**) or $PLP_{178-191}$ peptide (**b**) emulsified with CFA. Three months following R-EAE induction, blood from 5 mice was collected in each group. (**a**) In $PLP_{139-151}$-induced R-EAE, control mice elicited higher anti-$PLP_{139-151}$ IgG1 isotype response than that of IgG2b (*$p < 0.05$, Wilcoxon signed rank test), while pCMV-injected mice showed similar IgG1 and IgG2b isotype responses. (**b**) In $PLP_{178-191}$-induced R-EAE, we detected a higher anti-$PLP_{178-191}$ IgG2b isotype response than that of IgG1 in control R-EAE mice injected with PBS (*$p < 0.05$), while pCMV inoculation elicited similar IgG1 and IgG2b isotype responses. Slight increase of anti-PLP IgG2a isotype responses were seen in pCMV-injected groups immunized with either $PLP_{139-151}$ or $PLP_{178-191}$ peptide. Values are expressed as mean ± SEM of anti-$PLP_{139-151}$ antibody titers in $PLP_{139-151}$-induced R-EAE mice and anti-$PLP_{178-191}$ antibody titers in $PLP_{178-191}$-induced R-EAE mice.

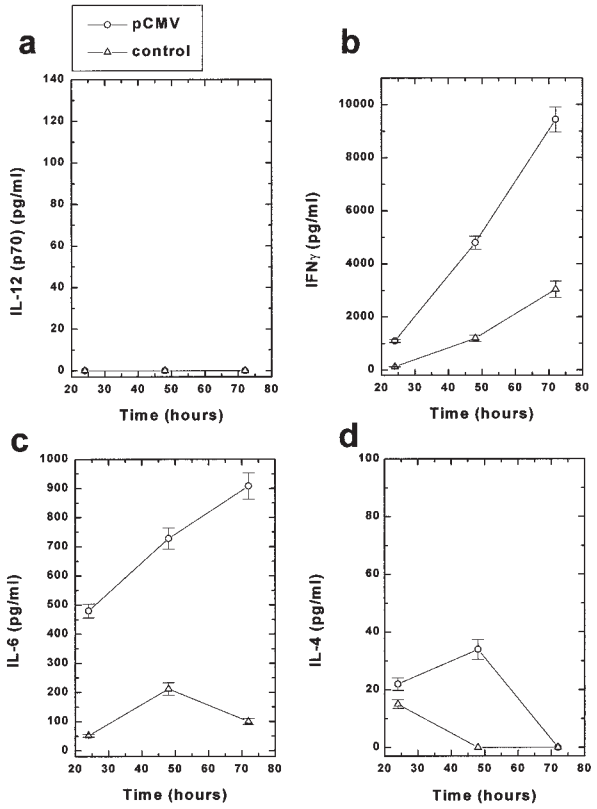

Figure 10. Modulation of cytokine production by plasmid DNA, pCMV. Inguinal lymph nodes and spleens from 5 R-EAE mice induced with $PLP_{139-151}$ peptide following three-time pCMV (O) or saline injection (Δ) were pooled. MNCs were isolated and stimulated with $PLP_{139-151}$ peptide for 24, 48 or 72 hours. IL-12 (p70) (a), IFNγ (b), IL-6 (c) and IL-4 (d) production in the culture supernatant was assayed by quantitative ELISA. Enhancement of IFNγ and IL-6 production was noted in pCMV injection group.

between the experimental systems. The amount of TMEV RNA and/or number of antigen positive cells have been shown to correlate with disease severity, while others have reported no correlation between TMEV persistence and demyelination (reviewed in (56)). In our system we demonstrated that pCMV injection exacerbated TMEV disease in a dose-dependent fashion both clinically and histologically. The enhancement of the disease did not correlate with the number of TMEV-antigen positive cells but did with an increase in anti-TMEV antibody. These results suggest that modulation of anti-TMEV immune responses by pCMV, but not an increase of virus persistence, is a contributing factor for the exacerbation. As noted above, we confirmed that pCMV could induce naïve spleen cells to produce IL-12, IFNγ and IL-6, all of which are known as proinflammatory cytokines (46). Thus, plasmid DNA pretreatment could also alter the cytokine microenvironment *in vivo*, leading to the exacerbation of demyelinating disease induced by a subsequent TMEV infection.

However, the contribution of each cytokine for exacerbation of TMEV disease in this experiment is unclear, since the role of cytokines in unmodulated TMEV infection is still controversial. Both IL-12 and IFNγ are Th1-promoting cytokines, (1, 24) and Th1-type immune responses are believed to play a pathogenic role in TMEV disease (10, 39). On the other hand, anti-IFNγ neutralizing antibody treatment has been reported to exacerbate TMEV disease (29, 34). Rodriguez *et al*. (35) showed that IL-6 administration suppressed chronic TMEV disease with increases in anti-TMEV antibody titers. Moreover, CpG DNA is known to induce production of other cytokines, such as, IL-1β, TNF-α, IFNα/β, IL-18 and macrophage-inflammatory protein (MCP)-2 (37, 41, 45, 49). Thus, to better understand the mecha-

nism of enhancement in TMEV disease by pCMV injection, one needs to elucidate the entire cytokine and chemokine networks during the course of regular TMEV infection, and then compare it with that of pCMV-injected mice before and after TMEV infection. To this end we are currently investigating the cytokine and chemokine profiles during the acute and chronic phases of TMEV infection (48).

Also we demonstrated that plasmid DNA injection could exacerbate the autoimmune demyelinating disease, R-EAE, clinically and histologically. In the plasmid-injected R-EAE mice, we found increased PLP-specific IFNγ and IL-6 production in MNC culture compared with cultures from the control group (Figure 10). EAE is known to be mediated by CNS antigen-specific CD4+ Th1 cells, and proinflammatory cytokines are known to be involved in the pathomechanisms of the disease (42, 46). Thus, in this experiment, pCMV injection most likely could elicit proinflammatory cytokine production, leading to exacerbation of R-EAE. In addition, in both R-EAE induced with $PLP_{139\text{-}151}$ and $PLP_{178\text{-}191}$ peptides, we noted altered IgG1 and IgG2b antibody responses against PLP peptides between control and pCMV-injected groups. These results also support the idea that pCMV injection can alter the cytokine milieu *in vivo*, since Th1/Th2 balance is known to affect immunoglobulin class switching (1).

Alternatively, plasmid DNA injection might affect a common disease pathway shared in TMEV infection and R-EAE. In both MS models, apoptosis of encephalitogenic lymphocytes has been suggested to contribute to disease remission, while apoptosis of myelin-forming oligodendrocytes can contribute to demyelination (27, 38, 50, 53, 54). Bacterial DNA has been shown to stimulate macrophages to produce TNF-α, (45) which is known to induce oligodendrocyte apoptosis (5). However, CpG DNA can protect lymphocytes against apoptosis *in vitro* (60). Taken together, plasmid DNA injection could cause oligodendrocyte apoptosis and/or protect encephalitogenic lymphocytes against apoptosis, leading to potentiation of demyelinating disease.

This study provides explanations for a number of epidemiological findings related to MS. Considering possible multifactorial causes of MS (50), it is important to note that bacterial plasmid DNA injection could exacerbate demyelinating diseases regardless of their cause, either virus or autoimmunity as demonstrated in this paper. Viral and bacterial infections have been related to the initiation or an exacerbation of MS (25, 30, 44). To date no single virus or bacteria infection can explain the etiology of MS. Common factor(s) or pathways among the virus and bacterial infections might contribute to the pathogenesis. Our results suggest that immunostimulation by viral and bacterial CpG DNA could be one of the contributing factors for exacerbation of MS (13, 59). Similarly, it is attractive to hypothesize that exposure to bacterial DNA before puberty could contribute to the susceptibility to MS, where exposure to environmental factors before the age of 15 are believed to play a pathogenic role (19).

Aside from exogenous non-vertebrate CpG DNA, endogenous or genomic CpG has also been suggested to be involved in the pathogenesis of autoimmune diseases (16). In vertebrate genomic DNA, the cytosine nucleotides in most CpG dinucleotides are methylated at the 5 position. DNA methylation can affect gene expression and changes in cellular DNA methylation have been implicated in differentiation. Young *et al*. (62) demonstrated that the CpG dinucleotide within the IFNγ promoter is not methylated in IFNγ-producing Th1 clones, but is methylated in Th2 clones. Interestingly, genomic T cell DNA in patients with systemic lupus erythemotosus and rheumatoid arthritis has been shown to be hypomethylated, possibly as a result of decreased activity of T cell methyltransferase (31). These studies provide possible linking of unmethylated CpG DNA to pathogenesis of autoimmune diseases. It will be of further interest to determine whether immune modulating genes, such as cytokine promoter regions, show differences in their methylation patterns in immune cells of the patients with possible autoimmune diseases including MS; although Richardson *et al*. (31) demonstrated that there was no significant difference in DNA methylation between 7 MS patients and controls.

Here, we show that plasmid DNA injection could exacerbate virus induced and autoimmune animal models for MS. We also found that plasmid DNA could induce the production of proinflammatory cytokines, including IFNγ, whose administration is known to exacerbate MS (26). Moreover, proinflammatory and Th1 cytokines are believed to be involved in the pathogenesis of MS (23). While no serious side effects have been reported in clinical trials of DNA immunization, some constructs used in DNA immunization may contain sequences from bacterial plasmids containing CpG motifs. One may argue that vector DNA encoding anti-inflammatory or Th2-promoting molecules may override the immunostimulatory effect of CpG plasmid DNA, even though the vector DNA contains multiple CpG motifs. However, potential immunomodulatory effects should be considered since the control group would be administered vector DNA, lacking the coding

region of the test molecule, as a placebo in clinical trials. This study provides a caution to the clinical application of not only DNA immunization but also other therapies involving the administration of non-vertebrate DNA, including DNA virus vector and antisense gene therapy, particularly to the people susceptible to MS and other possible Th1-mediated diseases.

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