Suppressive Immunization with DNA Encoding a Self-Peptide Prevents Autoimmune Disease: Modulation of T Cell Costimulation


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Suppressive Immunization with DNA Encoding a Self-Peptide Prevents Autoimmune Disease: Modulation of T Cell Costimulation

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Usually we rely on vaccination to promote an immune response to a pathogenic microbe. In this study, we demonstrate a suppressive form of vaccination, with DNA encoding a minigene for residues 139–151 of myelin proteolipid protein (PLP139–151), a pathogenic self-Ag. This suppressive vaccination attenuates a prototypic autoimmune disease, experimental autoimmune encephalomyelitis, which presents clinically with paralysis. Proliferative responses and production of the Th1 cytokines, IL-2 and IFN-γ, were reduced in T cells responsive to PLP139–151. In the brains of mice that were successfully vaccinated, mRNA for IL-2, IL-15, and IFN-γ were reduced. A mechanism underlying the reduction in severity and incidence of paralytic autoimmune disease and the reduction in Th1 cytokines involves altered costimulation of T cells; loading of APCs with DNA encoding PLP139–151 reduced the capacity of a T cell line reactive to PLP139–151 to proliferate even in the presence of exogenous CD28 costimulation. DNA immunization with the myelin minigene for PLP-altered expression of B7.1 (CD80), and B7.2 (CD86) on APCs in the spleen. Suppressive immunization against self-Ags encoded by DNA may be exploited to treat autoimmune diseases. The Journal of Immunology, 1999, 162: 3336–3341.

Vaccination using DNA is effective in protecting experimental animals against infectious pathogens and cancer (1–5) and recently has been used to prevent autoimmune disease (6). Experimental autoimmune encephalomyelitis (EAE), a prototypic animal model of T cell autoimmunity, reflects many of the clinical and pathologic features of the human disease, multiple sclerosis. We have previously reported that vaccination of H-2b mice with naked DNA, encoding the TCR variable region gene, V8.2, protected mice from EAE. V8.2 encodes the predominant TCR β-chain that is rearranged in myelin basic protein (MBP)-reactive T cells, causing EAE (6). Immunization with the Vβ8.2 gene induced a pattern of Th2 cytokine production by myelin reactive T cells, creating a suppressive environment blocking autoimmunity; T cells reacting to the myelin autoantigen deviated from an aggressive Th1 type to a suppressive Th2 type.

To explore the potential for an Ag-specific therapy of autoimmune disease using DNA immunization, we have designed minigenes encoding the pathogenic fragments of the dominant autoantigen in myelin, the proteolipid protein (PLP). In SJL/J mice, immunization with the synthetic peptides from PLP residues 139–151 or 178–191 in CFA induces EAE (7–10). This mode of immunization leads to the generation of Th1 cells that produce IL-2, IFN-γ, and TNF-α. Furthermore, a peptide analogue of PLP139–151 with substitution of the major TCR contact residues (Leu114/Arg117) acts as a TCR antagonist for PLP139–151-specific T cells and prevents EAE induction and progression (11, 12).

In this study, we describe the use of DNA encoding minigenes of pathogenic peptides to modulate EAE. Our results demonstrate that pathogenic T cell responses to the encephalitogenic peptide PLP139–151 can be attenuated with injections of naked DNA encoding the PLP139–151 epitope. Moreover, we can reduce the incidence and severity of the prototypic Th1-mediated experimental autoimmune disease, EAE.

Materials and Methods

Animals

Six- to eight-week-old female SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antigens

Peptides were synthesized on a peptide synthesizer (model 9050; MilliGen, Burlington, MA) by standard 9-fluorenlymethylcarbonyl chemistry. Peptides were purified by HPLC. Structure was confirmed by amino acid analysis and mass spectroscopy. Peptides used for the experiments were: PLP139–151 (HSLGKWLGHPDKF), PLP139–151 L144/R147 (HSLGKLLGRFDKF), and PLP178–191 (NTWTTGQSIAFPSK). Guinea pig spinal cord homogenate (gSCH) was used after lyophilization.

PLP peptide expression vector

Three minigenes, each one encoding a PLP epitope, were constructed by annealing two oligonucleotides with a 16-mer overlapping complementary sequence (underlined) and extending with DNA polymerase and dNTPs: PLP178–191, 5'-GTGAGACCATGCATTGTTTGGGAAAATGGCTAGGACATCCCGAC9AAGTTTTCTAGATAGCTA-3'; PLP139–151, 5'-CTCGGAGACCAAGACATACCTGGACACCTGCCAGTCTATTGCCCTTCCTAGACAGTGATCTAGCTAGCTA-3'; PLP139–151 L144/R147, 5'-CTCGGAGACCAAGACATACCTGGACACCTGCCAGTCTATTGCCCTTCCTAGACAGTGATCTAGCTAGCTA-3'; PLP139–151, 5'-CTCGGAGACCAAGACATACCTGGACACCTGCCAGTCTATTGCCCTTCCTAGACAGTGATCTAGCTAGCTA-3'; PLP139–151 L144/R147, and 5'-CTCGGAGACCAAGACATACCTGGACACCTGCCAGTCTATTGCCCTTCCTAGACAGTGATCTAGCTAGCTA-3'; PLP139–151 L144/R147, and 5'-CTCGGAGACCAAGACATACCTGGACACCTGCCAGTCTATTGCCCTTCCTAGACAGTGATCTAGCTAGCTA-3'
FIGURE 1. Anti-PLP139–151, and anti-gpSCH IgG Ab titers in SJL/J mice after DNA immunization with the PLP minigene. Sera of SJL/J mice, injected with the plasmid DNA coding for the PLP139–151 peptide, were taken 7 days after the second intramuscular injection and tested by ELISA for the presence of anti-gpSCH (A) or anti-PLP139–151 (B) IgG Ab titers. After incubation with the diluted sera, goat anti-mouse IgG conjugated to alkaline phosphatase was added. Results are expressed as the OD of individual samples in a group of 10 animals. OD values for preimmune sera were: dilution 1:10, 0.12; dilution 1:20, 0.08; and dilution 1:40, 0.03.

GACCATGCAATTGGGAAACTCTAGACGACGCCCCAAGTTT
TTCTAGAGCTA-3′. These oligonucleotide duplexes were designed to incorporate XhoI and XbaI restriction sites.

The products were cloned into the multiple cloning region of pTARGET Vector (Promega, Madison, WI), a mammalian expression vector driven by the CMV promoter. Positive clones were identified by color screening and correct orientation of the inserts was confirmed by DNA automatic sequencing. Purification of the plasmid DNA was done by Wizard plus Maxiprep (Promega) according to the manufacturer’s instructions.

DNA immunization protocol
Experimental animals were injected in the left quadriceps with 0.1 ml of 0.25% bupivacaine-HCl (Sigma, St. Louis, MO) in PBS. Two and 10 days later, mice were injected with 0.05 ml of plasmid DNA (1 mg/ml in PBS), in the same muscle.

ELISA for anti-PLP139–151 or anti-gpSCH Ab titers
Polyethylene 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with 0.1 ml of either peptide or gpSCH, and diluted in PBS at a concentration of 0.01 mg/ml in PBS. After blocking with PBS + 0.5% FCS (Life Technologies, Grand Island, NY) and 0.05% Tween 20 (Bio-Rad, Hercules, CA), mouse sera were incubated for 2 h at room temperature and Ab binding was tested by the addition of alkaline phosphatase-conjugated goat anti-mouse-IgG (Southern Biotechnology Associates, Birmingham, AL). After the addition of the enzyme substrate, plates were read at 405 nm in an ELISA reader.

EAE induction
PLP130–151 peptide was dissolved in PBS to a concentration of 2 mg/ml and emulsified with an equal volume of IFA supplemented with 4 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI). Mice were injected s.c. with 0.1 ml of the peptide emulsion and on the same day and 48 h later, were injected i.v. with 0.1 ml of 4 μg/ml Bor-
detella Pertussis toxin in PBS. Experimental animals were scored as follows: 0, no clinical disease; 1, tail weakness or paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness or paralysis; and 5, moribund or dead animal.

Lymph node cell proliferation assays
Draining lymph nodes were removed from mice after the acute phase of disease, and lymph node cells (LNC) were tested in vitro for specific proliferative responses to the PLP139–151 peptide. Cultures were prepared in flat-bottom 96-well microtiter plates in a volume of 0.2 ml/well at a cell concentration of 2.5 × 10^5/ml. The tissue culture media for the assay consisted of RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5 × 10^-5 M), and 1% autologous fresh normal mouse serum. After 72 h of incubation at 37°C, cells were pulsed for 18 h with 1 μCi/well of [3H]thymidine. Plates were harvested and [3H]thymidine incorporation was measured in a scintillation counter.

Cytokine determination
Draining LNC (10^5 cells/ml) from experimental animals were taken after the acute phase of the disease and stimulated in vitro with varying concentrations of Ag. After 24 and 48 h of stimulation, supernatants were collected and tested by sandwich ELISA.

Ribonuclease protection assay
For mRNA detection, tissue RNA samples from experimental animals were tested using the MultiProbe RNase Protection Assay System, RibOQuant (PharMingen, San Diego, CA), according to the manufacturer’s instructions.

Fluorocytometric analysis
Spleen cells (5 × 10^6/ml) from naive SJL/J mice were incubated in the presence of plasmid DNA coding for the PLP139–151 sequence (0.01 mg/ml) at 37°C. After 24 h, cells were collected and analyzed on FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The following Ab conjugates were used: FITC anti-mouse CD80, clone 16–10A1; FITC anti-mouse CD86, clone GL1; FITC anti-mouse I-A^d, clone 10–3.6; R-PE (phycoerythrin)-conjugated anti-mouse B220, clone RA3–6B2; R-PE-conjugated anti-mouse CD11b, clone M1/70; and PE-conjugated anti-mouse CD4, clone GK 1.5. All Abs were purchased from PharMingen.

Results and Discussion
The minigene coding for the PLP130–151 peptide was cloned into an expression vector and injected intramuscularly into SJL/J mice twice at 1-wk interval. Ten days after the last injection, experimental animals were bled and their sera were tested for the presence of specific Abs. As shown in Fig. 1, anti-PLP139–151 IgG titers can be detected in the mice previously injected with the PLP139–151 minigene. Thus, specific serological immune responses are induced with this particular construct.

To determine whether injection of DNA containing PLP sequences can be effective in protecting mice from EAE induction, the PLP139–151 minigene construct was injected intramuscularly twice at 1-wk interval. Ten days after the last injection, mice were

<table>
<thead>
<tr>
<th>DNA Injected</th>
<th>Percent Incidence</th>
<th>Mean Disease Score (day 11)</th>
<th>Mean Day of Disease Onset</th>
<th>Mean Peak Disease Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP130–151</td>
<td>68 (13/19)*</td>
<td>0.9 ± 0.3</td>
<td>11.5 ± 0.5</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.0005)</td>
<td>(p &lt; 0.008)</td>
<td>(p &lt; 0.005)</td>
<td>(p &lt; 0.007)</td>
</tr>
<tr>
<td>PLP139–151</td>
<td>70 (14/20)</td>
<td>0.6 ± 0.2</td>
<td>11.5 ± 0.4</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0035)</td>
<td>(p &lt; 0.007)</td>
<td>(p &lt; 0.01)</td>
</tr>
<tr>
<td>PLP130–151(L/R)</td>
<td>85 (17/20)</td>
<td>1.2 ± 0.3</td>
<td>11.6 ± 0.5</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.009)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.01)</td>
</tr>
<tr>
<td>pTARGET</td>
<td>90 (18/20)</td>
<td>2.7 ± 0.3</td>
<td>10.1 ± 0.27</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>Nonplasmid</td>
<td>100 (10/10)</td>
<td>2.1 ± 0.7</td>
<td>9.9 ± 0.4</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

* Means given as mean ± SEM.

a Numbers in parentheses denote sick animals over tested animals.

b All p values given as comparison to pTARGET by Student’s t test.
challenged with the PLP 139–151 peptide emulsified in CFA. As shown in Table I, amelioration of acute clinical disease is observed in the animals vaccinated with the PLP 139–151 plasmid vector, as compared with the control plasmid group. The onset of disease was delayed compared with the control plasmid group (11.5 ± 0.5 days, \( p < 0.008 \)), mean peak disease severity was reduced (\( p < 0.005 \)), and mean disease score was reduced (\( p < 0.0005 \)). In addition, other groups were injected with either 1) a plasmid containing a minigene encoding the altered peptide ligand PLP p139–151 (W144L, H147R) or 2) a plasmid containing a minigene encoding the PLP epitope p178–191. Onset of disease was delayed (11.6 ± 0.5 days, \( p < 0.009 \)), and mean peak disease score was reduced (\( p < 0.02 \)) with the minigene encoding the altered peptide ligand (W144, H147). Also, onset of disease was delayed (11.5 ± 0.4 days, \( p < 0.003 \)), mean peak disease severity was reduced (\( p < 0.007 \)), and mean disease score was reduced (\( p < 0.0001 \)) with the minigene encoding the PLP peptide p178–191.

Mice injected with DNA and further challenged with the encephalitogenic peptide PLP 139–151 were sacrificed after resolution of the acute phase of the clinical disease. Draining LNC were restimulated in vitro with the PLP139–151 peptide and tested for their proliferative responses and cytokine production. Fig. 2 shows that LNC from mice injected with DNA coding for the PLP 139–151 peptide had lower proliferative responses when compared with the LNC from control animals (\( p < 0.01 \)). Fig. 3A shows that when stimulated with the PLP 139–151, LNC from mice immunized with the plasmid DNA coding for the PLP 139–151 region secrete lower levels of IL-2 and IFN-\( \gamma \) in comparison with control groups. To assess levels of cytokine mRNA transcripts in inflamed brain, we used a ribonuclease protection assay on mRNA isolated from brain tissue. Fig. 3B reveals a reduction in mRNA levels of IFN-\( \gamma \), and IL-15 in mice immunized with the minigene encoding the PLP 139–151 region. Therefore, a correlation between low incidence of clinical disease, reduced cellular responses, and low levels of IL-2, IL-15, and IFN-\( \gamma \) is evident in the PLP 139–151 DNA-vaccinated mice. The relative expression levels of cytokine mRNA bands shown in Fig. 3B were measured by densitometry. To correct for loading differences, the values were normalized according to the level of expression of the housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH), within each sample. Densitometric analysis confirmed reduction of expression level of the tested cytokines in brains of mice vaccinated with the plasmid DNA coding for the PLP 139–151 determinant compared with \( \text{pTargetT} \) and PLP 139–151 (L/R) plasmid DNA-vaccinated mice (data not shown).

To elucidate a mechanism for decreased T cell responses, we tested in vitro the effect of APCs cultured in the presence of DNA on the proliferative responses of PLP 139–151-specific T cells.
Splenocytes were incubated either with plasmid DNA coding for the PLP<sub>139–151</sub> segment or with the PLP<sub>139–151</sub> peptide and used as a source of APC to stimulate L139 cells, a PLP<sub>139–151</sub>-specific T cell line. Proliferative responses of the L139 T cell line to the above APCs were compared in the presence or absence of anti-CD28 Ab-coated beads. As shown in Table II, L139 cells responded to syngeneic APCs preincubated with the synthetic peptide PLP<sub>139–151</sub> (8512 mean cpm). This response is increased with the addition of anti-CD28 Abs (127281 mean cpm). However, when the APCs were incubated with the plasmid DNA containing PLP<sub>139–151</sub> coding sequence, L139 cells were unable to respond to APCs (3358 mean cpm), even in the presence of anti-CD28 Abs (4532 mean cpm). This down-regulation was not an effect of the plasmid itself, because APCs incubated with plasmid containing an irrelevant sequence did not affect the proliferative response of the PLP<sub>139–151</sub> peptide (3358 mean cpm), or control plasmid (3358 mean cpm). This down-regulation was not an effect of the APCs (3358 mean cpm), even in the presence of anti-CD28 Abs (127281 mean cpm). However, as a source of APC to stimulate L139 cells, a PLP<sub>139–151</sub>-specific T cell line, L139, were added to each well. After 48 h of further incubation, plate was labeled with H<sup>3</sup>thymidine and proliferation was assessed by harvesting 18 later and counting [H]thymidine incorporation. To demonstrate that the exogenously applied naked DNA is taken up by the splenocytes and is expressed, we used the RT-PCR technique. Total RNA was purified from the splenocytes using the Rneasy total RNA kit (Quiagen, Valencia, CA). RT-PCR was performed using the Access RT-PCR System (Promega) and oligonucleotide primers specific for the PLP<sub>139–151</sub> minigene. Vector-specific primers were used in a separate RT-PCR to exclude the possibility of DNA contamination. A single band corresponding to the PLP<sub>139–151</sub> minigene was amplified from total RNA purified from splenocytes loaded with the PLP<sub>139–151</sub> plasmid DNA (data not shown).

Costimulatory signal was delivered by adding anti-CD28-coated beads (5000 per well) together with the T cells. Anti-CD28 Ab (clone 37.51) was obtained from PharMingen. Sulfate polystyrene latex microspheres of 5 × 0.1 μm in diameter were obtained from Interfacial Dynamics (Portland, OR). Beads (6 × 10<sup>5</sup>) were suspended in 6 ml of PBS and incubated with 24 μg of anti-CD28 Ab for 1.5 hours at 37°C. Beads were washed extensively with PBS and resuspended in RPMI 1640 and 10% FCS and allowed to block for ≥30 min at room temperature. Results are expressed as mean cpm of triplicate wells.

The ability of a myelin minigene construct to down-regulate the costimulatory effect of anti-CD28 Abs on a PLP-specific T cell line emphasizes its capacity to modulate APC-T cell interactions. Fluorocytometric analyses were conducted to determine whether DNA immunization influences the surface expression of CD28 ligands on APCs. After 24 h of incubation with the plasmid DNA, splenocytes were stained with either anti-B7.1 (CD80) or anti-B7.2 (CD86) Abs. As shown in Fig. 4, up-regulation of B7.1 and B7.2 is observed in Mac-1-positive cells, but not in B220<sup>+</sup> cells in which down-regulation of B7.2 was observed. I-A<sup>a</sup> expression in spleen cells also increased in both Mac-1 and B220-positive cells upon incubation with DNA. Similar up-regulation of costimulatory molecules has been observed in vivo in PBLs and spleen cells of animals inoculated with DNA expression cassettes coding for the HIV core protein 55 (14). In contrast with this observation, we found that in autoimmune responses to PLP<sub>139–151</sub> the changes of expression of costimulatory molecules after DNA immunization exert a protective effect by modulation of the proliferative potential and cytokine production of autoreactive T cells. Recently it has been reported that in EAE, there is enhancement of B7.1 expression relative to B7.2 in the splenic environment, a finding that can help explain how the immune system tilts toward immunotolerance, rather than immunological ignorance of self (15). Interestingly, B7.2 increases in the central nervous system during active EAE and during relapses (15). Down-regulation of B7.2 correlates with remission. Changes in B7.1 and B7.2 expression upon uptake of DNA by APCs could be a key factor in regulating T cell responses toward self-Ags in autoimmune diseases.

DNA vaccines have been effective in generating protective immune responses in several models of cancer and of viral, bacterial, and parasitic infections (16). Although generation of Th1-like responses may be a property of DNA vaccines targeting nonself Ags (17), Th1 responses elicited to self with DNA vaccination have not
been achieved. Biological effects of DNA motifs, like unmethylated CpG dinucleotides in particular base contexts (CpG-S motifs), may modulate innate immune responses when injected to animals. Although we cannot discard a possible effect of such sequences in the PLP139–151 and PLP 139/151 (L/R) constructs, the CG motifs in these inserts do not fulfill the complete criteria for a CpG-S motif (18). Nevertheless, further testing should be performed to explore the potential immunomodulatory effects of such motifs in our system. Suppression of EAE has been reported in Lewis rats by previous immunization with DNA encoding an immunodominant MBP peptide in tandem with IgG Fc receptor. Vaccination suppressed clinical and histopathological signs of EAE and reduced the IFN-γ production after challenge with MBP 68–85 peptide (19). Vaccination was unsuccessful without inclusion of the tandem IgG Fc construct. In the experiments presented in this study, there was apparently no need for any tandem construct in conjunction with the myelin minigene. In both the present paper and in the experiments using DNA with the Fc IgG construct, defective Th1 immunity to self was observed. In contrast, our laboratory has reported induction of protective Th2-type responses by DNA immunization in EAE (6). Therefore, the immune response to a DNA vaccine-encoding self might be very different from what is observed with DNA vaccination to foreign Ags. It might be predicted that immune responses induced by self Ags encoded in DNA vaccines will parallel what has been observed for immunization with the same self Ag in peptide or protein form. Our results suggest that a self Ag encoded in a DNA vector can anergize self-reactive T cells, and prevent an autoimmune attack. Costimulation of T cells by DNA encoding self Ags can anergize self-reactive T cells, and prevent an autoimmune attack. Our observations in the EAE suggest a model in which DNA immunization can be used for treatment of autoimmune disease.

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References


