Brief Definitive Report

Vaccination with DNA Encoding an Immunodominant Myelin Basic Protein Peptide Targeted to Fc of Immunoglobulin G Suppresses Experimental Autoimmune Encephalomyelitis

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Summary

We explore here if vaccination with DNA encoding an autoantigenic peptide can suppress autoimmune disease. For this purpose we used experimental autoimmune encephalomyelitis (EAE), which is an autoaggressive disease in the central nervous system and an animal model for multiple sclerosis. Lewis rats were vaccinated with DNA encoding an encephalitogenic T cell epitope, guinea pig myelin basic protein peptide 68–85 (MBP68–85), before induction of EAE with MBP68–85 in complete Freund's adjuvant. Compared to vaccination with a control DNA construct, the vaccination suppressed clinical and histopathological signs of EAE, and reduced the interferon γ production after challenge with MBP68–85. Targeting of the gene product to Fc of IgG was essential for this effect. There were no signs of a Th2 cytokine bias. Our data suggest that DNA vaccines encoding autoantigenic peptides may be useful tools in controlling autoimmune disease.

DNA vaccination can generate protective immunity against infectious disease (1) and experimental cancer (2). Potential advantages with DNA vaccination are (*a*) prolonged, endogenous expression of antigen (3), (*b*) long-term immunity with efficient generation of both $CD8^+$ cytotoxic T cells and $CD4^+$ Th cells (1, 4), and (*c*) possibilities to modulate the Th1 or Th2 response by alteration of the vaccination protocol (5). DNA vaccination is also potentially applicable to autoimmune disease, as has been done in one particular model of experimental autoimmune encephalomyelitis (EAE) (4). Various forms of EAE are prototype animal models for Th1 type organ-specific autoimmunity and in many respects resemble human multiple sclerosis (6). The disease can be generated in a number of species by immunization with myelin proteins (7) and has been used extensively for evaluation of preventive as well as therapeutic strategies to alter autoimmune disease (8–11).

In the Pl/J (H-2u) mouse EAE model, in which DNA vaccination has been tested thus far, TCRBV8S2 is dominantly expressed by encephalitogenic T cells, and immunization with plasmid DNA encoding this TCRBV-chain suppresses EAE, probably through induction of Th2 cytokines (4). However, there is no preferential TCR usage in many autoimmune conditions. Furthermore, a Th2 bias is not always beneficial; e.g., in Marmoset monkeys, myelin/oligodendrocyte glycoprotein–induced EAE was aggravated after induction of Th2 immunity (12). Therefore, we wanted to test alternative approaches for DNA vaccination against EAE by targeting the myelin Ag instead of the TCR, and to achieve a potential downregulation of the pathogenic autoimmune responses without a Th2 bias. We used Lewis rat EAE, in which guinea pig myelin basic protein peptide 68–85 (MBP68–85) acts as a strong, disease-inducing T cell epitope (7). We constructed DNA vaccines coding for this amino acid sequence to examine if they were able to alter the course and immune response of a subsequently induced disease. In addition, we explored whether targeting the expressed protein to Fc of IgG by fusion to a protein A analogue (13) could enhance the tolerogenic ability of the construct.

Materials and Methods

Peptides and Mitogen. Greater than 99% pure peptides HYGSLPQKSQRSQDENPV from guinea pig sequence MBP68–85 and VHFFKNIVTPRTP from rat/guinea pig sequence MBP89–101

J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/05/1543/06 $2.00
Volume 187, Number 9, May 4, 1998 1543–1548
http://www.jem.org

were synthesized by the Fmoc/HBTU strategy (A. Engstrom, University of Uppsala, Uppsala, Sweden). Con A was purchased from Sigma Chemical Co. (St. Louis, MO).

Plasmid Construction. pZZ/MBP68–85: A 94-bp fragment containing a murine heavy chain IgG signal sequence (ss) was ligated upstream and in frame of a 385-bp fragment encoding ZZ (13). Directly downstream of the coding sequence of ZZ and upstream of the stop codon, seven AvaI–AvaI fragments encoding MBP68–85 were ligated in frame. pMBP68–85: A linker, containing AvaI and BbsI sites and stop codon, was ligated downstream and in frame of the ss fragment. Directly upstream of the stop codon, five AvaI–AvaI MBP68–85 fragments were ligated in frame. The ss/ZZ/MBP68–85 fragment and the ss/MBP68–85 fragment were cloned into the eukaryotic expression vector pCI (Promega, Madison, WI). pZZ: A fragment containing ss and ZZ in frame was cloned into pCI. pCIss: The 94-bp ss fragment was cloned into pCI. Expression is driven by immediate/early human CMV enhancer/promoter. *Escherichia coli* host was XL1-Blue (Stratagene Corp., La Jolla, CA).

Plasmid Preparation. Plasmid DNA was prepared by Qiagen plasmid preparation protocol. Endotoxins were removed in an additional step (Endofree buffer set; Qiagen, Santa Clarita, CA).

Plasmid DNA Injections and Cardiotoxin Pretreatment. 5–6-wk-old Lewis (RT1) male rats (Harlan Netherlands, Zeist, The Netherlands) were injected with 100 μl of 10 mM cardiotoxin (Latoxan, Rosans, France) into the *Musculii gastrocnemii.* 7 d later, the rats were injected with 800 μg DNA at 2.0 mg/ml in PBS, divided into four 200-μg injections administered in the *Musculii tibialii* and *M. gastrocnemii* of pZZ/MBP68–85, pZZ, pMBP68–85, or pCIss.

EAE Induction and Clinical Evaluation. 5 wk after DNA vaccination, rats were injected intradermally at the base of the tail with 200 μl inoculum containing 1:1 200-μg MBP68–85 in saline emulsified in CFA, consisting of IFA (Sigma Chemical Co.) and 0.5 mg heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco Laboratories, Detroit, MI). Animals were clinically scored and weighed daily. The symptoms were scored as follows: grade 1, tail weakness or tail paralysis; grade 2, hind leg paraparesis; grade 3, hind leg paralysis; grade 4, complete paralysis (tetraplegy), moribund state, or death.

Determination of MBP68–85–specific IgG and IgG-Isotype Responses. ELISA plates were coated with 10 μg/ml of MBP68–85 in carbonate buffer pH 9.6. Rat sera were diluted 1:10, 1:50, 1:250, 1:1,250, 1:6,250, or 1:31,250 in PBS-M (5% milk powder, 0.2% Tween 20 in PBS). Wells were incubated for 2 h with sera, washed in PBS-T (0.2% Tween 20 in PBS), and incubated for 2 h with 1:1,000 alkaline phosphatase (AKP)–conjugated goat anti–rat IgG (Biosource International, Camarillo, CA), monoclonal AKP-conjugated mouse anti–rat IgG1, IgG2a, or IgG2b (PharMingen, San Diego, CA), respectively, in PBS-M. To determine IgG2c levels, wells were incubated with 1:1,000 biotinylated mouse anti–rat IgG2c (Biosource International) in PBS-M for 1 h followed by a 1-h incubation with 1:1,000 AKP-streptavidin in PBS-T. pNPP (Sigma Chemical Co.) was used as substrate and OD was read at 405 nm.

Cell Preparation and Culture. Inguinal lymph nodes were torn apart in DMEM (GIBCO BRL, Gaithersburg, MD). Mononuclear cells (MNCs) were resuspended in complete medium containing DMEM supplemented with 1% rat serum, 1% penicillin/streptomycin (GIBCO BRL), 1% glutamine (GIBCO BRL), and 50 μM mercaptoethanol (GIBCO BRL), and flushed through a 70-μm plastic strainer (Becton Dickinson, Mountain View, CA), adjusted to 2×10^6 cells/ml, and cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Proliferation Assays. 2×10^5 MNCs were cultivated with or without the relevant antigens in complete medium for 60 h and subsequently pulsed with 0.5 μCi methyl–[^{3}H]thymidine ([^{3}H]TdR, Amersham, Buckinghamshire, UK) for 12 h. DNA was collected on glass fiber filters (Skatron, Sterling, VA) and [^{3}H]TdR incorporation was measured in a beta counter.

In Situ Hybridization for Cytokine mRNA Transcribing MNCs. In situ hybridization was performed as previously described (14). As positive controls, Con A–stimulated cells were assessed for cytokine mRNA content.

ELISA to Assess Cytokine Production in Vitro. ELISA kits for detection of secreted IFN-γ, IL-4, and IL-10 were purchased from Biosource International. Supernatants from MNCs, which had been incubated at a concentration of 2×10^6 cells/ml with or without relevant antigens or Con A, were analyzed. The procedure was performed as recommended by the manufacturer.

In Vivo CD8$^+$ T Cell Depletion. Depletion of CD8$^+$ T cells in vivo was performed as previously described (14). 0.5 mg of mAb Ox-8 in 500 μl PBS were injected intraperitoneally on day 0, before induction of disease, and on days 7 and 13 after immunization. Depletion was confirmed by FACS® (Becton Dickinson) analysis on day 18 after immunization.

Histopathological Evaluation. Histological evaluation was performed by standard neuropathological procedures and immunocytochemistry for T cells and macrophages as previously described (15).

Statistics. Abnormally distributed groups were tested with the Mann-Whitney U-test. Normally distributed groups were tested with Student's *t* test.

Results and Discussion

We constructed DNA vaccines encoding MBP68–85 in tandem, with or without fusion to a dimerized synthetic analogue Z of the IgG-binding B domain of staphylococcal protein A (13), pZZ/MBP68–85 (Fig. 1), and pMBP68–85. We hypothesized that binding of the hybrid gene product to IgG could alter the presentation of MBP68–85 and thereby affect its tolerogenicity with consequences for the quality of the ensuing immune response. As negative controls we constructed DNA vaccines with or without ZZ but lacking the MBP68–85 inserts, pZZ and pCIss.

A survey of the effects of DNA vaccination on clinical and histopathological signs of EAE is presented in Table 1. Lewis rats were injected intramuscularly with cardiotoxin and 7 d later with 800 μg of either pZZ/MBP68–85 or pZZ. 5 wk later, the rats were challenged with MBP68–85 in CFA. pZZ-injected control rats displayed a classical monophasic EAE course with disease onset on day 9 after immunization, ascending paraparesis with maximum clinical disease on days 12–14, followed by recovery. In contrast, pZZ/MBP68–85-injected rats showed a drastically altered disease course with reduced mean accumulated EAE score and reduced peak disease severity (Fig. 2 *A* and Table 1).

To study if fusion of MBP68–85 to ZZ was essential for suppression of EAE, rats were treated with pMBP68–85. As control we vaccinated with pCIss. As opposed to the pZZ/MBP68–85 vaccine, treatment with pMBP68–85 failed to significantly suppress EAE (Fig. 2 *B* and Table 1). The exact molecular mechanism for the drastic effect of

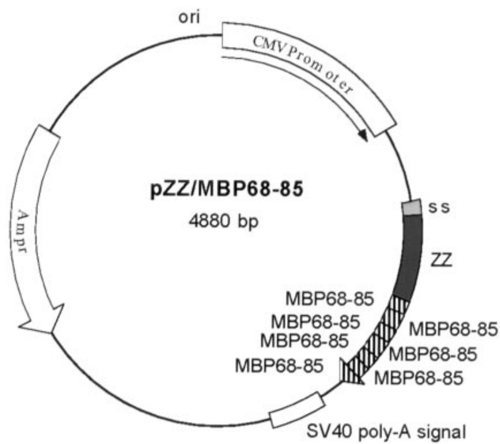

Figure 1. Plasmid map of DNA vaccine pZZ/MBP68–85. Seven repeats of oligonucleotides encoding autoantigen MBP68–85 were cloned downstream of a murine signal sequence fused to a dimerized synthetic analogue of the IgG-binding B domain of staphylococcal protein A gene, ZZ. As a negative control, a corresponding DNA vaccine coding for ZZ was constructed (pZZ). To investigate the role of ZZ in the protection from EAE, a DNA construct lacking ZZ, but encoding autoantigen MBP68–85 in tandem, was constructed (pMBP68–85). pCIss, encoding the signal sequence alone, was used as a negative control for pMBP68–85.

ZZ/MBP68–85 fusion on tolerogenicity is currently under study. Potentially, ZZ might affect distribution and half life of MBP68–85. ZZ binds to B cells in vivo (16). Uptake of the secreted fusion protein and subsequent presentation on MHC class I and II molecules on B cells might alter the ensuing immune response after subsequent immunogen challenge (17). Indeed, targeting of MBP peptide to B cell surface–exposed IgD suppresses EAE (10), and conjugating Ag to Fc of IgG induces tolerance (18).

Clinical signs of EAE and degree of inflammation within the central nervous system (CNS) may dissociate (19). Therefore it was also important to study the degree of concomitant CNS inflammation. On day 12 after immunization, 8 pZZ/MBP68–85-treated and 8 pZZ-treated rats were killed for histopathological evaluation. A semiquantitative evaluation of coded brains and spinal cords revealed that the mean inflammatory index in the spinal cord of the rats treated with pZZ/MBP68–85 was strongly reduced; 1.1 compared to 4.0 in the pZZ-treated control group (Table 1). The pathology was characterized by perivenous inflammation. In both groups, inflammatory infiltrates consisted of T cells and, to a lesser extent, macrophages. The inflammatory infiltrates were present in highest density in the spinal cord and brain stem, whereas forebrain areas and peripheral nerves were less affected.

To study the immune mechanisms involved in suppression of EAE after DNA vaccination, we next studied the profile of T cell reactivity to MBP68–85. MBP-specific IFN-γ–secreting $CD4^+$ T cells are crucial for disease induction of MBP-induced EAE (20). Antigen-induced IFN-γ and proliferative responses in vitro of lymph node cells (LNCs) from MBP68–85-immunized and DNA-vaccinated rats were measured on day 12 after immunization. Interestingly, the proliferative response to MBP68–85 of T cells derived from the regional lymph nodes in the pZZ/MBP68–85-treated and the pZZ-treated groups was not significantly different (Fig. 3 *A*). On the contrary, IFN-γ

Table 1. *Effect of DNA Vaccination on Clinical and Histopathological Signs of EAE*

			Clinical data				Histopathology	
Experiment	Treatment	*n*	Mean accumulated EAE score	*P*	Mean maximum EAE score	*P*	Mean inflammatory index	*P*
1	pZZ/MBP68–85	8	3.4	0.0001	1.1	0.02		
	pZZ	8	15.2		2.6			
2	pZZ/MBP68–85	8	4.5	0.001	0.8	0.028		
	pZZ	8	14.6		2.6			
	pZZ/MBP68–85	8	ND		ND		1.1	0.04
	pZZ	8	ND		ND		4.0	
	pMBP68–85	8	9.1	NS	1.4	NS		
	pCIss	8	12.0		2.4			
	pZZ/MBP68–85*	8	5.4	0.006	0.6	0.028		
	pZZ*	8	13.6		2.1			

The experiments were done on two occasions. Rats received the DNA vaccines 5 wk before induction of EAE with MBP68–85 in CFA. In experiment 2, 16 rats were killed on day 12 after immunization for evaluation of histopathology and immune responses. Mean accumulated EAE score is the cumulative disease score on day 21 after immunization (score obtained daily [0–4]). Mean maximum EAE score designates the average peak score of clinical disease at any time during the disease course, here on days 12–14 after immunization. Mean inflammatory index measures the degree of inflammation in spinal cord of rats killed on day 12 after immunization. *P* values were calculated with Mann-Whitney's test. *NS*, not significant at 0.05 level.
*Rats were injected with mAb OX-8 on days 0, 7, and 13 after immunization to deplete $CD8^+$ cells in vivo.

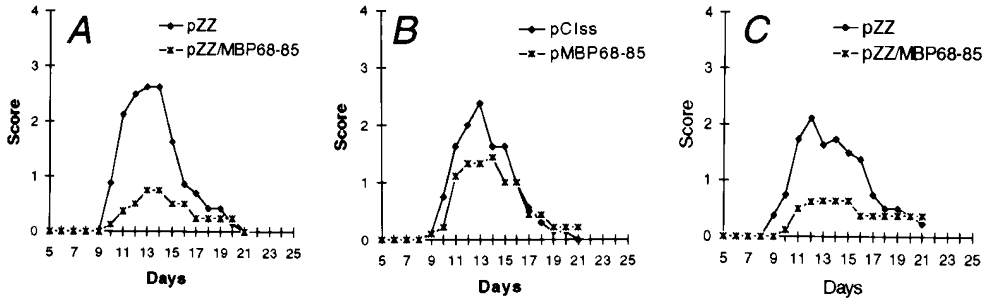

Figure 2. (*A*) Effect of DNA vaccination on mean clinical EAE score after treatment with pZZ/MBP68–85 or pZZ and subsequent induction of EAE with MBP68–85. (*B*) Effect of DNA vaccine lacking ZZ gene. Mean clinical EAE score of rats after treatment with DNA vaccine encoding MBP68–85 but not ZZ, pMBP68–85, or negative control pClss after subsequent induction of EAE (n = 8/group) with MBP68–85. (*C*) Effect of $CD8^+$ cell depletion on mean clinical EAE score of rats after treatment with pZZ/MBP68–85 or pZZ after subsequent induction of EAE with MBP68–85. $CD8^+$ cell depletion with mAb OX-8 was performed on days 0, 7, and 14 after immunization.

production in response to MBP68–85 was dramatically reduced in the protected pZZ/MBP68–85-treated rats, both at the level of the numbers of cells transcribing IFN-γ mRNA (P <0.0001; Fig. 3 *B*) and of the amount of IFN-γ in cell culture supernatants from MBP68–85-exposed T cells (P <0.001; Fig. 3 *C*), compared to the pZZ-treated rats. Furthermore, we measured IL-4 and IL-10 production in response to MBP68–85 to assess if the observed effects of pZZ/MBP68–85 could be due to induction of a Th2-biased autoimmune response. However, we could not detect any differences in the number of cells transcribing IL-4 or IL-10 mRNA (data not shown) or in the levels of IL-10 (Fig. 3 *D*) or IL-4 (undetectable) in cell culture supernatants between rats treated with pZZ/MBP68–85 or with pZZ, indicating no measurable alteration of the Th1/Th2 balance of encephalitogenic T cells in our system.

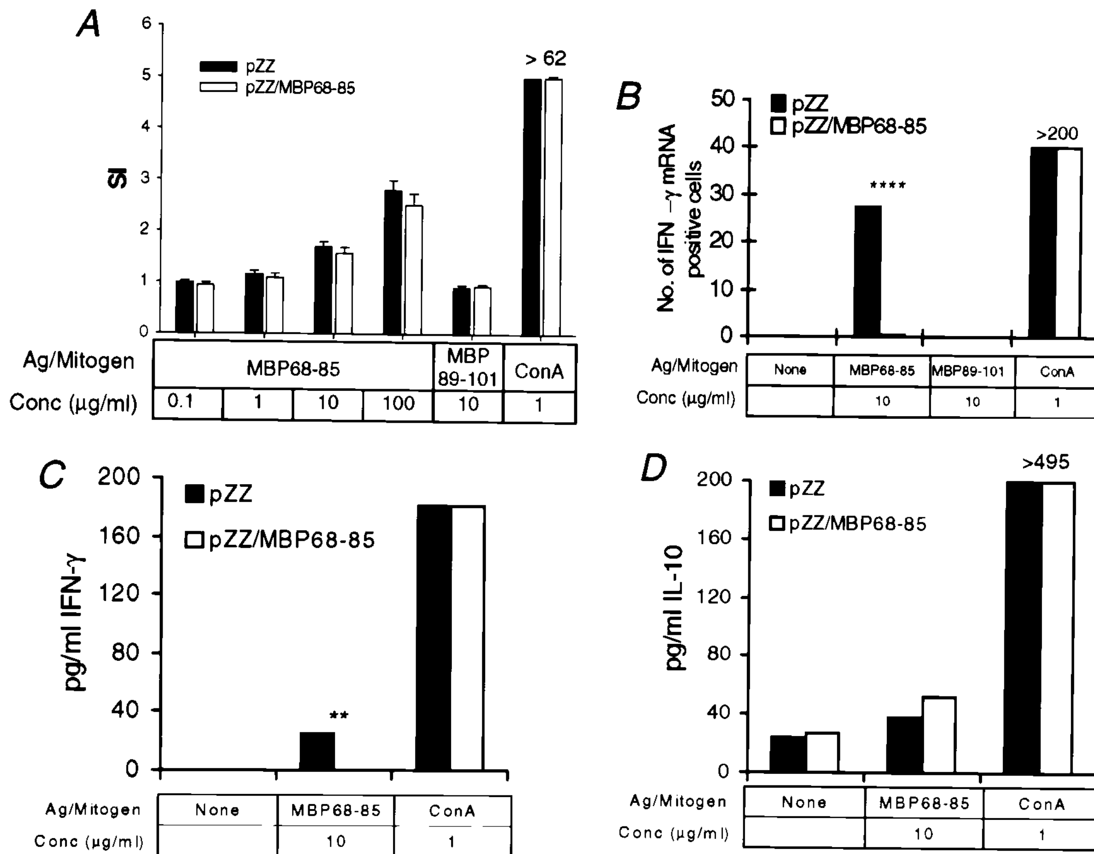

Figure 3. (*A*) T cell proliferation ± SEM after 72 h of in vitro exposure to antigen MBP68–85 (0.1–100 μg/ml) or Con A at 1 μg/ml. Peripheral LNCs were collected at 12 d after immunization from rats treated with pZZ/MBP68–85 or pZZ before immunization with MBP68–85. *SI*, Stimulation index. *Conc* designates concentration. (*B*) IFN-γ mRNA transcription 12 d after immunization of rats treated with pZZ/MBP68–85 or pZZ before immunization with MBP68–85. ****P <0.0001. Measurement of the number of IFN-γ transcribing cells per 10^5 LNCs by in situ hybridization after a 72-h exposure to MBP68–85, MBP89–101, or Con A. (*C*) IFN-γ and (*D*) IL-10 expression at 12 d after immunization of rats treated with DNA vaccine pZZ/MBP68–85 or pZZ before immunization with MBP68–85. Supernatants from LNCs exposed in vitro to MBP68–85 or Con A for 72 h were tested in cytokine ELISA for the presence of IFN-γ and IL-10, respectively. **P <0.001.

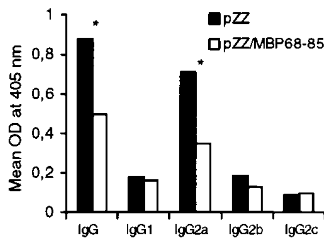

Figure 4. Mean MBP68–85-specific IgG and IgG isotype responses at 1:50 serum dilution. Rats received the DNA vaccines 5 wk before induction of EAE with MBP68–85. Rat sera were collected from rats killed on day 12 after immunization. *$P < 0.03$.

To study the effects of DNA vaccination on B cell immunity, we next measured MBP68–85-specific serum antibodies. At day 12 after immunization, total serum levels of IgG and IgG2a MBP68–85-specific antibodies were reduced and MBP68–85-specific IgG1, IgG2b, and IgG2c responses were similar in the pZZ/MBP68–86-treated group compared with controls (Fig. 4). The MBP68–85-specific IgG isotype data further strengthened the lack of a Th2 shift, since the IgG1 levels in pZZ/MBP68–85-treated compared with pZZ-treated rats were not enhanced. Our data suggest that the functional differentiation of MBP68–85-specific encephalitogenic $CD4^+$ T cells is altered by the preceding DNA vaccination. pZZ/MBP68–85 did not reduce expansion of MBP68–85-specific immunocompetent cells, but changed their ability to secrete the proinflammatory cytokine IFN-γ after Ag challenge. Since the encephalitogenicity of MBP68–85-specific T cells in an immunocompetent host strongly depends on their ability to secrete proinflammatory cytokines (20), the alteration in responsiveness to secrete IFN-γ after Ag-exposure might be one important reason for the protection of rats vaccinated with pZZ/MBP68–85. Our findings are partly consistent with a recent study by Marusic and Tonegawa where intraperitoneal injection of MBP peptide 1–17 in MBP 1–17 TCR transgenic mice induced tolerance by dampening Th1 type as well as Th2 type responses (21). Potentially, the lack of induction of a Th2 type response with our tolerogenic protocol is advantageous, since Th2 type responses can result in increased levels of secreted autoantibodies, which can be detrimental under certain conditions (12).

DNA vaccines are efficient inducers of $CD8^+$ T cell–mediated immunity (1), and $CD8^+$ cells can be protective in EAE (14). For this reason we were interested to study the role of $CD8^+$ cells in conferring protection after DNA vaccination with pZZ/MBP68–85. We depleted the $CD8^+$ cells of pZZ or pZZ/MBP68–85-treated rats after immunogen challenge with MBP68–85 in CFA on days 0, 7, and 13 after immunization with the mAb Ox-8 in vivo (14). On day 18 after immunization, $CD8^+$ cells were reduced to 14% of the numbers of nontreated controls, as assessed by FACS® analysis. The Ox-8 treatment did not affect the protective effect of pZZ/MBP68–85 on MBP68–85-induced EAE. We did not rule out that $CD8^+$ cells could not have any impact on the initial priming of the protective immunity after injection of the DNA vaccines, but we demonstrated that the suppression after active immunization with MBP68–85 was not mediated by $CD8^+$ cells.

Bacterial immunostimulatory DNA sequences (ISS) can function as Th1-promoting adjuvants (22) and are necessary for effective DNA vaccination against β-galactosidase (23). The vector backbone of our DNA vaccines contain three ISS with the AACGTT sequence, known to induce IFN-γ production by NK cells and IL-12 production by B cells, and to induce IL-12 and TNF-α production by macrophages in vitro (24). The dramatic reduction of IFN-γ production after vaccination with pZZ/MBP68–85 makes it unlikely that these ISS would induce IL-12 production. However, preliminary data (Lobell, A., unpublished observations) reveal that the protective effect of pZZ/MBP68–85 is abolished after replacing pZZ/MBP68–85s vector backbone with a vector lacking the AACGTT motifs. Studies regarding the potential of ISS in protection against autoimmune disease are warranted, and are ongoing in our laboratory.

The vaccination effect discussed in this paper was recorded for one particular immunodominant T cell epitope in the Lewis rat. We will determine if this vaccination protocol also affects the encephalitogenic responses to other epitopes of MBP or even other myelin antigens, as has been described in oral tolerance (8) or in altered peptide ligand therapy (9). In these studies, there is circumstantial evidence that production of type 2 cytokines (8, 9) and TGF-β (8) is instrumental for a disease downregulatory response resulting in dampening of disease-promoting cells of whatever specificity. The lack of induction of a Th2-biased immune response in our system would predict epitope specificity and no bystander suppression. If true, successful application of the principle of DNA vaccination to the treatment of human autoimmune disease would then require knowledge of a particular, disease-relevant T cell epitope. It is also possible that the autoimmune disease may be driven by a set of different epitopes even from different myelin proteins (25). In view of the MHC influence on many human organ-specific inflammatory diseases, it is possible that a hierarchy of a restricted set of different epitopes may be relevant to disease. If so, and if they are defined, the present DNA vaccination protocol does allow construction of vaccines covering broad ranges of different epitopes.

This work was supported by The European Union (Biomed 2), the Swedish Medical Research Council, The Swedish Association of Neurologically Disabled, the Swedish Cancer Society, Pharmacia & Upjohn, and SBL vaccin AB. R. Weissert is a postdoctoral fellow of the Deutsche Forschungsgemeinschaft (We 1947/1-1).

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Received for publication 16 December 1997 and in revised form 20 February 1998.

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