0.01) by Fc-Aβ (Fig. 1A) and IgL-Aβ vaccinations compared with the controls (Fig. 1B). Aβ depositions in the hippocampus were also decreased. Although the therapeutic protocol seemed to be less effective than the prophylactic one (Fig. 1C), the difference was not statistically significant. Thus, Aβ DNA vaccines had sufficient effect even if the vaccines were administrated after amyloid depositions appeared. We also confirmed that the level of anti-Aβ antibodies in plasma of model mice significantly increased after DNA vaccination. The levels of increase in our system were mild compared with those reported in active immunization.[3] The safety of our vaccines has been established as well as the effects. T cell activation and proliferation, as measured by [³H]-thymidine incorporation of T cells from vaccinated mice, was negative in both wild-type B6 and model mice. Pathological examinations using monoclonal antibodies, CD5 (anti-T cell) and Mac-3 (anti-macrophage), demonstrated no inflammatory lesion in the brain after long-term treatments.

As mentioned above, there are two types of DNA vaccines, viral and non-viral DNA vaccines. We believe that non-viral DNA vaccines are superior to viral DNA vaccines for several reasons. Non-viral DNA vaccines can be prepared in large amounts with standard technology. They are safe because they lack a viral component. One can make the vaccines at a low cost. When considering clinical use, AD patients receive vaccines for the span of their life,
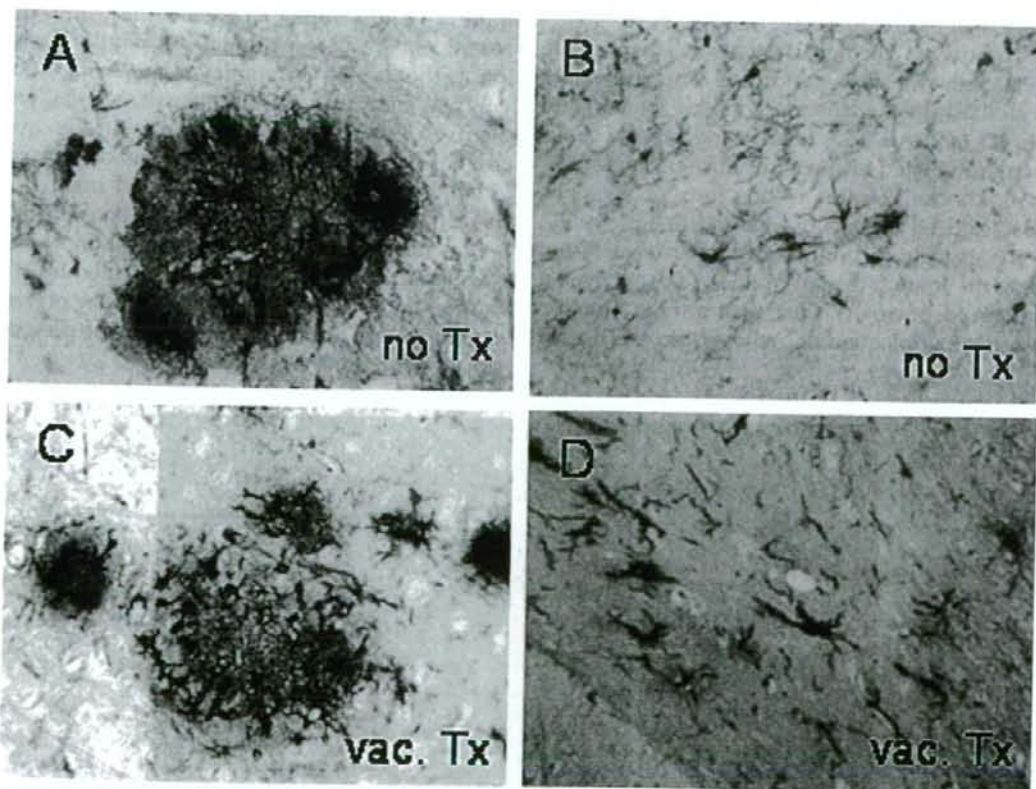

FIG. 2. Double staining with 6F3D (amyloid plaque, blue) and Iba-1 (microglia surface marker, blown) in the brains of treated and untreated mice. Around plaques of untreated mice, there are microglia with rich cytoplasms and processes that show bulbous swellings (A). After the treatment, more microglial cells infiltrated the amyloid plaques (C). In the remote area of non-treated APP23 mice, resting microglia were sparsely distributed (B). In the remote area of treated APP23 mice, microglial cells also increased in number and demonstrate the activated form (D).

and such advantages are quite important factors for the choice of their treatment.

MECHANISMS OF AMYLOID REDUCTION WITH VACCINE THERAPIES

Although Aβ DNA vaccination has significant effects on Aβ reduction in the mouse model, the mechanisms of Aβ clearance remain unclear. There are three hypotheses to explain how anti-Aβ antibodies reduce Aβ deposits in the brain. One possible mechanism is augmentation of Fc-mediated phagocytosis by microglia.[27,28] The second mechanism is direct dissociation by anti-Aβ antibodies.[29,30] The third mechanism is augmentation of Aβ efflux from the brain to the blood circulation.[31,32] We examined whether these mechanisms are operating in our system.

To analyze microglial activation after vaccine treatment, we first performed double-staining with Iba-1 (microglia surface marker, blown) and 6F/3D (anti-Aβ 8-17, blue) using brain sections from vaccinated and control mice. In non-transgenic control B6 mice, resting form of microglia characterized by small soma and fine projection were sparsely distributed in the brain (data not shown). In non-treated model mice, activated amoeboid microglia were seen around amyloid plaques (periplaque area), whose processes deeply

entered into the plaques (Fig. 2A). In the area remote from plaques (remote area), the retesting form of microglia was observed, as was seen in wild-type mice (Fig. 2B). After DNA vaccination, microglia in periplaque area increased in number and clustered around plaques (Fig. 2C). The significant change after the vaccination was the morphological change of microglia in the remote area. Microglia increased in number and switched their form to the amoeboid type, which have long and stringy cytoplasmic processes all over the brain.

To quantitatively analyze the increase of microglia after the vaccination, the number of the microglia was counted both in periplaque and remote areas. At 9 months of age, microglia were increased in number significantly ($p < 0.01$) in both areas after the vaccination. The number of microglia in the periplaque area was larger than that in the remote areas. At 15 months of age, microglia were significantly increased ($p < 0.01$) in number, in similar fashion as that seen in 9 months of age.

While analyzing double-stained sections, we often observed small Aβ deposits inside microglial cells. This phenomenon was confirmed by confocal microscopy. Cy3-labeled microglia (Fig. 3B) enclosed FITC-labeled Aβ deposits (Fig. 3A). A merged image indicates ingestion of Aβ within microglia (Fig. 3C). We confirmed by 3D analysis that this was not a simple overlay of two structures (not shown). The number of phago-
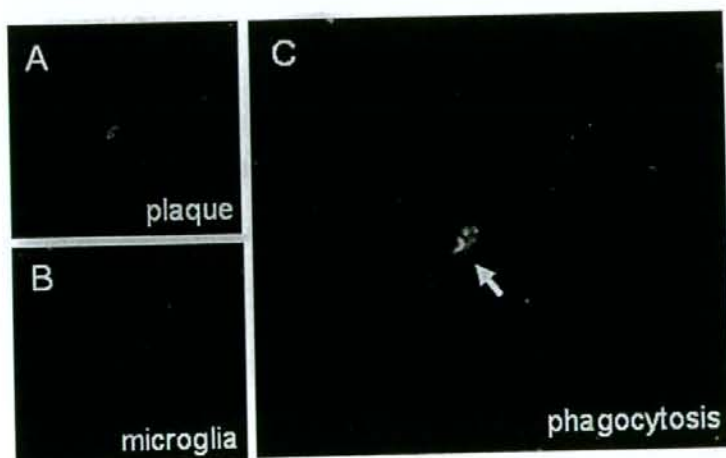

FIG. 3. Phagocytosis of Aβ deposits by activated microglia. Brain sections from treated and untreated APP23 mice were stained with 6F3D (amyloid plaque, green) **(A)** and Iba-1 (microglia, red) **(B)** and observed with a confocal microscope. Solely existed microglia in remote area contain Aβ deposits **(C, arrows)**.

cytosing microglia was significantly increased ($p < 0.01$) after vaccine treatment. The increase of activated microglia in remote areas suggests that clearance of invisible small $A\beta$ aggregates is performed by activated microglia. If this is the case, it is greatly beneficial for AD patients because $A\beta$ oligomers are responsible for AD pathology[33,34] and synaptotoxicity.[35]

We also examined that direct dissociation by anti-$A\beta$ antibodies[36] was operating in our treatment system. It is very difficult to evaluate *in vivo* direct effects of anti-$A\beta$ antibodies on $A\beta$ dissociation. We determined the levels of anti-$A\beta$ antibodies with high affinity by tissue amyloid plaque immunoreactivity (TAPIR) assay using plasma taken from treated and untreated mice. Although the titer from treated mice was slightly higher than that of plasma from untreated mice, the difference was not statistically significant. Thus, direct dissociation of $A\beta$ deposits by the antibodies is not so marked in this system although it may be present.

We next examined the sink effects of anti-$A\beta$ antibodies by measuring the levels of $A\beta$ in plasma taken from treated and untreated mice. At 9 months of age, some treated mice showed relatively high levels of plasma $A\beta$, suggesting that $A\beta$ migration from the brain to the blood is upregulated. However, there was not significant difference at 15 months of age. These findings suggest that the $A\beta$ efflux from the brain to blood (peripheral sink) is not the major route of $A\beta$ reduction after DNA vaccination.

Microglia are thought to play a role in either neuroprotection or neurodamage. We examined the nature of activated microglia in our DNA vaccine therapy. The levels of tumor necrosis factor-alpha (TNF-α), one of cytotoxic cytokines,[37] were determined using brain homogenates of treated and untreated mice. A large quantity of TNF-α was detected in the brain and spinal cord from the positive control, i.e., LPS-treated and MOG-EAE-induced mice. Without DNA vaccine therapy, TNF-α in the brain of wild-type or model mice was nearly zero. After DNA vaccination, the levels of TNF-α in these mice seemed to be slightly increased; however, they were not significantly different from control mice. These findings suggest that activated microglia detected after DNA vaccination may be neuroprotective.

Taken together, in DNA vaccine therapy, Fc-mediated phagocytosis of $A\beta$ deposits by activated microglia is a major route of $A\beta$ reduction. Direct $A\beta$ dissociation and sink effects may be weak in this situation. This information is very important for improvement of DNA vaccines. DNA vaccines are easily reconstructed by adding or changing the sequence in the plasmid vector. We expect that more effective vaccines will be developed and applied for human AD in the near future.

CONCLUSION

Vaccine therapy for AD is a promising strategy if excessive immunoreactions are controlled. We have developed non-viral DNA vaccines for AD that were effective in the animal model without side effects. In our system, Fc-mediated phagocytosis of $A\beta$ depositions by activated microglia is a major route of $A\beta$ reduction. The accumulation of such data helps to develop more effective, safer vaccines that may be clinically applied in the near future.

REFERENCES

1. Citron M. Alzheimer's disease: treatments in discovery and development. Nat Neurosci 2002;5(Suppl): 1055–1057.
2. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci.1991;12:383–388.
3. Schenk D, Barbour R, Dunn W, et al. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 1999;400: 173–177.
4. Morgan D, Diamond DM, Gottschall PE, et al. A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. Nature 2000;408: 982–985.
5. Janus C, Pearson J, McLaurin J, et al. A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature 2000;408:979–982.
6. Orgogozo JM, Gilman S, Dartigues JF, et al. Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. Neurology 2003;61:46–54.
7. Nicoll JA, Wilkinson D, Holmes C, et al. Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. Nat Med 2003;9:448–452.
8. Frenkel D, Maron R, Burt DS, Weiner HL. Nasal vaccination with a proteosome-based adjuvant and glatiramer acetate clears beta-amyloid in a mouse model of Alzheimer disease. J Clin Invest 2005;115:2423–2433.

9. Maier M, Seabrook TJ, Lemere CA. Developing novel immunogens for an effective, safe Alzheimer's disease vaccine. Neurodegen Dis 2005;2:267–272.

10. Maier M, Seabrook TJ, Lazo ND, et al. Short amyloid-beta (Abeta) immunogens reduce cerebral Abeta load and learning deficits in an Alzheimer's disease mouse model in the absence of an Abeta-specific cellular immune response. J Neurosci 2006;26:4717–4728.

11. Masters CL, Beyreuther K. Alzheimer's centennial legacy: prospects for rational therapeutic intervention targeting the Abeta amyloid pathway. Brain 2006;129: 2823–2839.

12. Bard F, Cannon C, Barbour R, et al. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med 2000;6:916–919.

13. Pfeifer M, Boncristiano S, Bondolfi L, et al. Cerebral hemorrhage after passive anti-Abeta immunotherapy. Science 2002;298:1379.

14. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. Nature 1992;356:152–154.

15. Barry MA, Lai WC, Johnston SA. Protection against mycoplasma infection using expression-library immunization. Nature 1995;377:632–635.

16. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993;259:1745–1749.

17. Hoffman SL, Doolan DL, Sedegah M, et al. Nucleic acid malaria vaccines. Current status and potential. Ann NY Acad Sci 1995;772:88–94.

18. Zhang J, Wu X, Qin C, et al. A novel recombinant adeno-associated virus vaccine reduces behavioral impairment and beta-amyloid plaques in a mouse model of Alzheimer's disease. Neurobiol Dis 2003;14: 365–379.

19. Hara H, Monsonego A, Yuasa K, et al. Development of a safe oral Abeta vaccine using recombinant adeno-associated virus vector for Alzheimer's disease. J Alzheimers Dis 2004;6:483–488.

20. Kim HD, Maxwell JA, Kong FK, et al. Induction of anti-inflammatory immune response by an adenovirus vector encoding 11 tandem repeats of Abeta1-6: toward safer and effective vaccines against Alzheimer's disease. Biochem Biophys Res Commun 2005;336:84–92.

21. Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum Gene Ther 2002;13:1935–1943.

22. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Hum Gene Ther 2001;12:861–870.

23. Nishikawa M, Hashida M. Nonviral approaches satisfying various requirements for effective in vivo gene therapy. Biol Pharm Bull 2002;25:275–283.

24. Ghochikyan A, Vasilevko V, Petrushina I, et al. Generation and characterization of the humoral immune response to DNA immunization with a chimeric beta-amyloid-interleukin-4 minigene. Eur J Immunol 2003;33:3232–3241.

25. Schulz JG, Salzer U, Mohajeri MH, et al. Antibodies from a DNA peptide vaccination decrease the brain amyloid burden in a mouse model of Alzheimer's disease. J Mol Med 2004;82:706–714.

26. Okura Y, Miyakoshi A, Kohyama K, et al. Nonviral Abeta DNA vaccine therapy against Alzheimer's disease: long-term effects and safety. Proc Natl Acad Sci USA 2006;103:9619–9624.

27. Bard F, Barbour R, Cannon C, et al. Epitope and isotype specificities of antibodies to beta -amyloid peptide for protection against Alzheimer's disease-like neuropathology. Proc Natl Acad Sci USA 2003;100:2023–2028.

28. Wilcock DM, Munireddy SK, Rosenthal A, et al. Microglial activation facilitates Abeta plaque removal following intracranial anti-Abeta antibody administration. Neurobiol Dis 2004;15:11–20.

29. Bacskai BJ, Kajdasz ST, McLellan ME, et al. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. J Neurosci 2002;22:7873–7878.

30. Solomon B, Koppel R, Frankel D, Hanan-Aharon E. Disaggregation of Alzheimer beta-amyloid by site-directed mAb. Proc Natl Acad Sci USA 1997;94:4109–4112.

31. DeMattos RB, Bales KR, Cummins DJ, et al. Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. Proc Natl Acad Sci USA 2001;98:8850–8855.

32. Dodart JC, Bales KR, Gannon KS, et al. Immunization reverses memory deficits without reducing brain Abeta burden in Alzheimer's disease model. Nat Neurosci 2002;5:452–457.

33. Bucciantini M, Calloni G, Chiti F, et al. Prefibrillar amyloid protein aggregates share common features of cytotoxicity. J Biol Chem 2004;279:31374–31382.

34. Bucciantini M, Giannoni E, Chiti F, et al. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature 2002;416:507–511.

35. Chauhan NB. Intracerebroventricular passive immunization with anti-oligoAbeta antibody in TgCRND8. J Neurosci Res 2007;85:451–463.

36. Kotilinek LA, Bacskai B, Westerman M, et al. Reversible memory loss in a mouse transgenic model of Alzheimer's disease. J Neurosci 2002;22:6331–6335.

37. O'Toole M, Janszen DB, Slonim DK, et al. Risk factors associated with beta-amyloid(1-42) immunotherapy in preimmunization gene expression patterns of blood cells. Arch Neurol 2005;62:1531–1536.

Address reprint requests to:
Yoh Matsumoto
Department of Molecular Neuropathology
Tokyo Metropolitan Institute for Neuroscience
Musashidai 2-6
Fuchu
Tokyo 183-8526
Japan

E-mail: matyoh@tmin.ac.jp

LOOKING AHEAD

Although interrupted, the phase II clinical trial of AN-1792 provides further support for Aβ immunotherapy of Alzheimer's disease. Alternative vaccine therapies, such as nonviral DNA vaccines, are being investigated to reduce excessive immune reactions of the host brain.

Anti-Aβ Vaccination as a Promising Therapy for Alzheimer's Disease

*by Yoshio Okura
and Yoh Matsumoto*

Summary

Alzheimer's disease is the most common cause of dementia characterized by progressive neurodegeneration. Recently, a vaccine therapy for Alzheimer's disease was developed as a curative treatment. Although clinical trials of active vaccination for Alzheimer's disease were halted due to the development of meningoencephalitis in some patients, the clinical and pathological findings of treated patients suggest that the vaccine therapy is effective. Hence, newly designed vaccines are being invented to control excessive T-cell immune reactions after the human clinical trial. In this article, we will review conventional vaccine therapies and newly developed vaccine therapies, mainly DNA vaccines, for possible clinical application in the near future. © 2007 Prous Science. All rights reserved.

Alzheimer's disease, first reported by Alzheimer 100 years ago, is most common cause of age-related cognitive decline, affecting more than 12 million people worldwide.[1] The disease is characterized in its earlier stage by progressive memory impairment and cognitive decline, altered behavior and language deficit. Later, patients develop global amnesia and slowing of motor function, and finally die typically within 9 years after diagnosis. Current drug therapies such as donepezil hydrochloride slow cognitive decline; however, the effect is limited. Recently, it is generally believed that accumulation of amyloid beta (Aβ) is the first event in the pathogenesis of Alzheimer's disease. In other words, Aβ deposition is an upstream event of tau phosphorylation, tangle formation and

neuronal death (amyloid cascade hypothesis).[2] Based on this hypothesis, vaccine therapy has been developed for curative treatment of Alzheimer disease by targeting the underlying cause.

Antiamyloid immunotherapy

Schenk et al. for the first time demonstrated the effect of Aβ vaccines. Monthly inoculation with synthetic Aβ in complete Freund's adjuvant (CFA) could lead to high anti-Aβ antibody titers (Fig. 1A), and dramatic reductions of amyloid deposition in

PDAPP transgenic mice.[3] The vaccine was able to slow or reverse amyloid deposit formation, even if administrated after amyloid deposition occurred. Neuritic plaques and astrocytic reactions were also decreased by the vaccination. They speculated that acceleration of Fc receptor-mediated microglial phagocytosis plays a major role in plaque reduction. Subsequent studies demonstrated that clearance of Aβ depositions following immunization
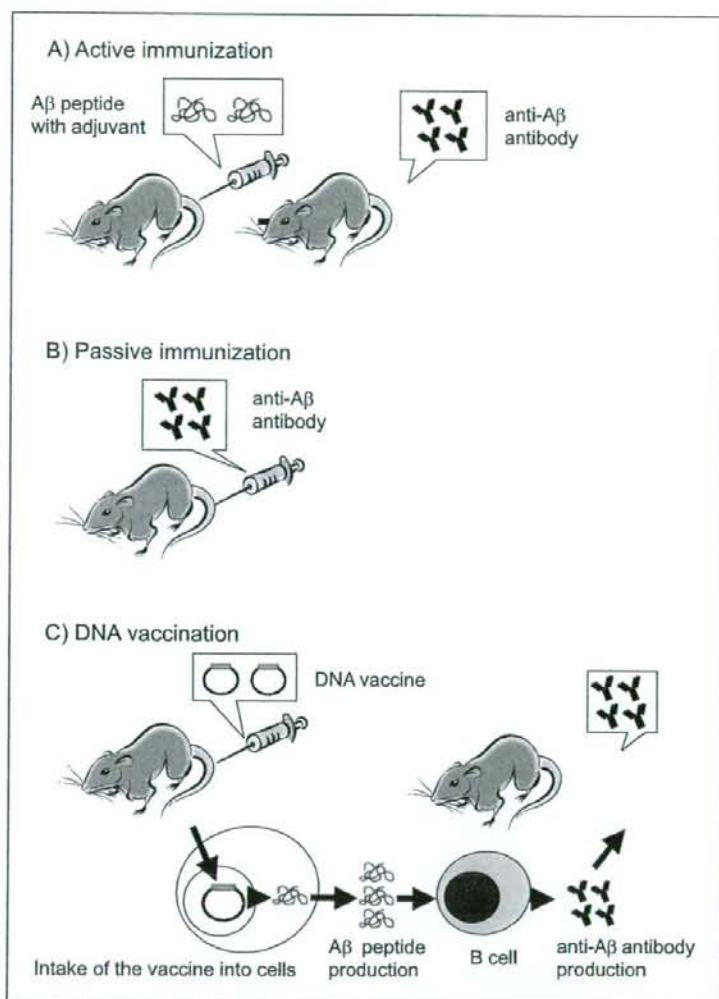
Correspondence: Y. Matsumoto, matyoh@tmin.ac.jp

Fig. 1. Types of vaccine therapies for Alzheimer's disease. A) Active immunization: $A\beta_{1-42}$ peptides are administered with an adjuvant to induce anti-Aβ antibodies. B) Passive immunization: anti-Aβ antibodies are administrated directly. C) DNA vaccination: plasmid vectors encoding $A\beta_{1-42}$ gene are injected to generate Aβ peptides and subsequent anti-Aβ antibodies. Aβ peptide production is continued for a certain period.

cells with anti-Aβ antibodies on brain slices from Tg mice induced the clearance of Aβ.[9]

The second mechanism is a direct effect of antibodies on Aβ leading to dissolution of amyloid fibrils or neutralization of Aβ oligomers (Fig. 2B). Direct injection of F(ab')$_2$ antibodies into the brain equally mediated a decrease in Aβ.[10] Antibodies raised against the N-terminal region (1-28) of the Aβ peptide binded to *in vitro*-formed Aβ assemblies, leading to disaggregation and increased solubility of Aβ fibrils.[11]

The third mechanism, the peripheral sink hypothesis, postulates that administration of anti-Aβ antibodies to the circulation results in a net efflux of Aβ from the brain to the plasma (Fig. 2C).[7] Rapid improvement in cognition was observed in animals after intravenous injection of antibodies and increased plasma concentrations of Aβ.[12] Injection of an agent that has high affinity for Aβ (gelsolin or GM1) reduced the level of Aβ in the brain.[13]

A clinical trial of amyloid vaccination

Based on the promising results obtained using transgenic mice, clinical trials with $A\beta_{42}$ (AN-1792) in conjunction with the T helper (Th) 1 adjuvant QS-21 were initiated. The phase I studies using single or multiple doses of the vaccine demonstrated good immunological responses and tolerability to the vaccine. However, a phase IIa study performed in 375 patients at several sites was halted because meningoencephalitis developed in 18 patients.[14] It was suggested that vaccination with the Aβ peptide vaccine in a Th1 type adjuvant induced T-cell responses against Aβ. However, the autopsy case showed apparent clearance of Aβ plaques from large areas of the neocortex as well as a decrease in plaque-associated astrocytes and neuritic dystrophy.[15] Thus, the clinical trial clearly demonstrated benefits of vaccine therapy. Taken together, the results indicate that the vaccine therapy is potentially effective for human
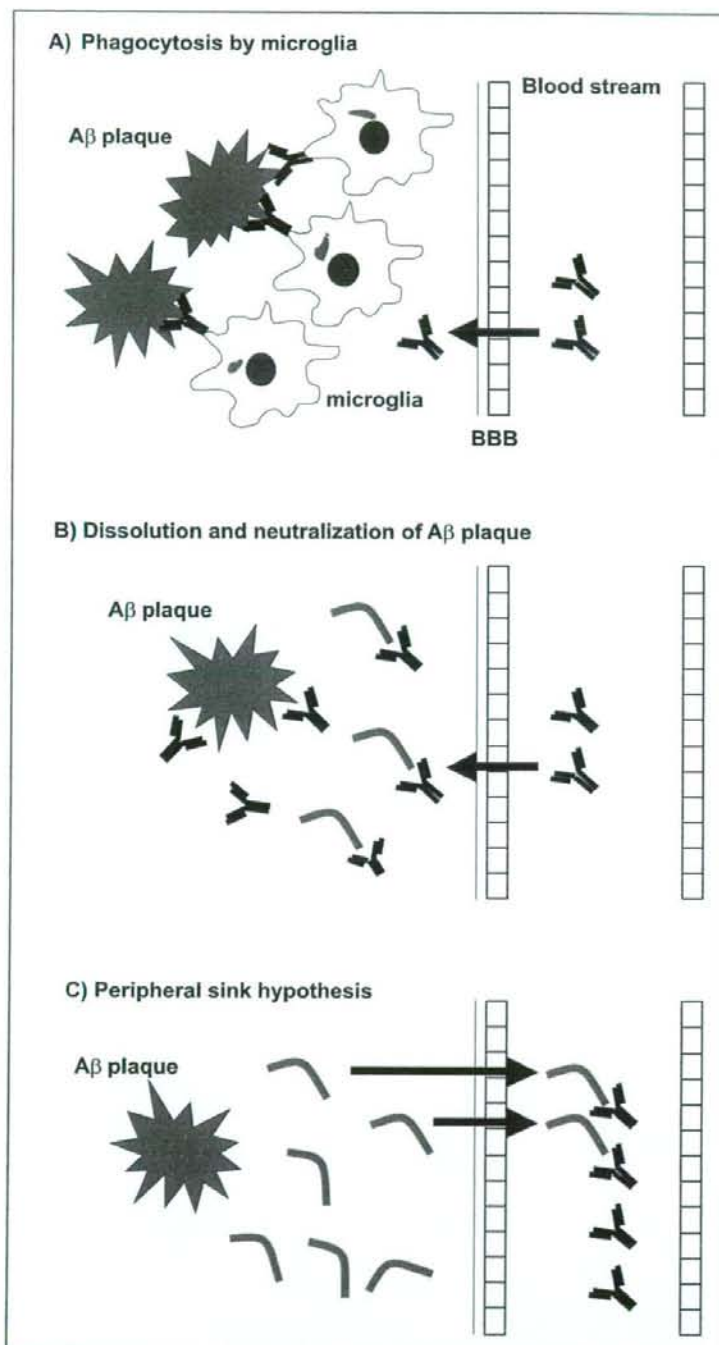
protected APP-Tg mice from developing memory deficits.[4,5] Clearance of Aβ depositions and improvement of memory were also observed after passive administration of antibodies against Aβ (Fig. 1B).[6,7]

Mechanisms of amyloid reduction with vaccine therapies

Although Aβ peptide vaccination has effects on Aβ reduction in the mouse model, the mechanisms of Aβ clearance remain unclear. There are three hypotheses to explain how anti-Aβ antibodies reduce Aβ depositions in the brain (Fig. 2). One possible mechanism is that anti-Aβ antibodies enhance Fc receptor-mediated phagocytosis of Aβ by microglial cells (Fig. 2A). Following peripheral administration of anti-Aβ antibodies, activated microglia were found surrounding the plaques.[8] The culture of microglial

A) Phagocytosis by microglia

Aβ plaque

Blood stream

microglia

BBB

B) Dissolution and neutralization of Aβ plaque

Aβ plaque

C) Peripheral sink hypothesis

Aβ plaque

Fig. 2. Mechanisms of amyloid reduction with a vaccine treatment. A) Phagocytosis by microglia: anti-Aβ antibodies traverse blood–brain barrier (BBB) and attach to Aβ deposits, which leads to Fc receptor-mediated phagocytosis by microglia. B) Dissolution and neutralization of Aβ plaque: antibodies bind N-terminal end of Aβ depositions and dissolve amyloid fibrils or neutralize Aβ oligomers. C) The peripheral sink hypothesis: anti-Aβ antibodies in the circulation induce a net efflux of Aβ from the brain to the plasma.

Alzheimer's disease if excessive immune reactions are minimized to avoid unwanted neuroinflammation.

After further improvement of peptide vaccines, there are two phase I clinical trials of active immunization with minimum side effects using ACC-001 (Elan and Wyeth), which contains Aβ 1-7 derivatives, and CAD-106 (Novartis), which consists of an Aβ fragment coupled to a carrier.[16] However, there is still the possibility of meningoencephalitis in active immunization because adjuvants are necessary for peptide vaccination.

Passive immunization

Passive transfer of anti-Aβ antibodies is an alternative strategy (Fig. 1B), which is as effective as active immunization in the mouse model of Alzheimer's disease. Peripheral administration of antibodies against Aβ peptide was sufficient to reduce amyloid burden. Despite relatively modest serum levels, the passively administered antibodies were able to enter the central nervous system, decorate plaques and induce clearance of pre-existing amyloid.[6] Direct injection of antibodies into the brain induced rapid parenchymal Aβ clearance.[10] Passive immunization is more acceptable than active vaccination because it does not need adjuvant injection and does not elicit the hazardous cellular responses observed in the clinical trial of active immunization. Moreover, the dose can be controlled easily. However, this approach will require caution in the conduct of human trials. Long-term adoptive transfer of the antibodies in old APP transgenic mice reduced amyloid loads, but doubled the number of microhemorrhages in 27-month-old APP mice treated for 5 months with an N-terminal specific anti-Aβ monoclonal antibody.[17] Moreover, after passive immunization, *in vivo* production of neutrizing antibodies such as anti-idiotype antibodies must be considered. A serious disadvantage of passive immunization is the cost of monoclonal antibodies. An enormous number of patients cannot afford the expensive medical costs.

Although there are some problems to be solved, passive administration of an Aβ-specific humanized monoclonal antibody (bapineuzumab; Elan and Wyeth) is currently in a phase II clinical trial in patients with Alzheimer's disease.[16]

Development of new vaccine therapies for the next generation (DNA vaccines)

Among alternative vaccine therapies, DNA vaccination may open up a new avenue for the treatment of Alzheimer's disease because it is simple, easily modified and can be used without adjuvant (Fig. 1C).[18,19] The immune responses of the host induced by DNA vaccination are generally Th2 type.[18,20,21]

Initially, Aβ DNA vaccines were developed using adeno-associated virus (AAV) vectors[22,23] or adenovirus vector.[24] A single administration of the AAV vaccine induced a prolonged and strong production of Aβ-specific serum IgG in Tg mice and resulted in improved ability of memory and cognition, decreased Aβ depositions in the brain, and a resultant decrease in plaque-associated astrocytosis.[22] Much higher titers of antibodies against Aβ were obtained when an adenovirus vector encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) was co-administered with the vector encoding 11 tandem repeats of $A\beta_{1-6}$.[24] However, the ability to scale up the AAV vector production severely restricts the commercialization and use of AAV vectors.[25] Moreover, a viral replication could not be completely excluded when the adenovirus vector is used for vaccines. Thus, the clinical application of DNA vaccines with virus vectors seems to be difficult at present.

We and others have focused on plasmid vectors. DNA vaccines with plasmid vectors have many advantages over those with virus vectors because the vaccines can be mass produced at a low cost[18,19] and have no possibility of viral infection or transformation.[26,27] Ghochikyan et al. developed an $A\beta_{1-42}$ DNA vaccine

with Th2 cytokine sequence (IL-4) and confirmed the generation of anti-Aβ antibodies after vaccination in wild-type B6 mice.[28] Schulz et al. developed and Aβ DNA vaccine with a secretary signal, tissue-type plasminogen activator (tPA). With simultaneous use of low dose Aβ peptide, DNA vaccine therapy reduces amyloid plaque in a mouse model.[29] However, significant reduc-

tions of Aβ deposition were not obtained with these DNA vaccines alone.

We also focused on the benefit of the plasmid vector and prepared three types of Aβ DNA vaccines using a mammalian expression vector.[30] The sequence of $A\beta_{1-42}$ and additional sequences were inserted in the plasmid, as shown in Figure 3A. The first
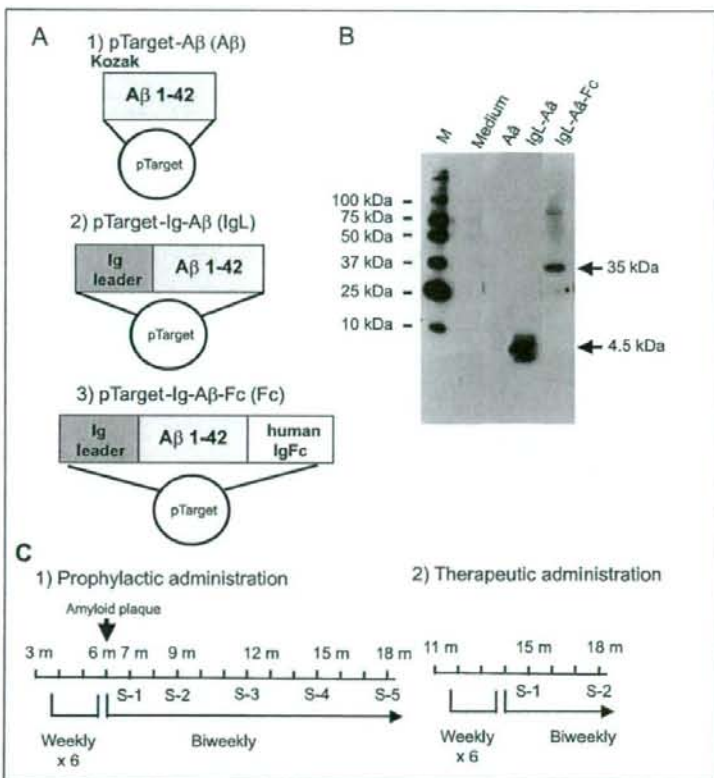

Fig. 3. Construction of DNA vaccines (A), *in vitro* characterization (B) and the treatment protocol (C). A) Three DNA vaccines were produced using a mammalian expression vector. DNA encoding the $A\beta_{1-42}$ sequence was inserted in XhoI/KpnI site of the plasmid (K-Aβ vaccine) (A-1). In the second vaccine, the signal sequence of mouse Igκ is added to the 5' end to improve the secretive efficiency (IgL-Aβ vaccine) (A-2). The third vaccine possesses the Fc portion of human immunoglobulins to improve the stability of the secreted protein (Aβ-Fc vaccine) (A-3). B) Western blot analysis revealed that translated Aβ proteins were detected in supernatants of cultured cells transfected with IgL-Aβ and Aβ-Fc vaccines. C) The protocol of vaccine treatment. To examine the prophylactic effect of DNA vaccines, the vaccines were administrated to APP23 mice from 3-4 months of age before the appearance of amyloid depositions. The mixture of one of the vaccines (100 mcg) and bupivacaine (0.25 mg) was injected intramuscularly on a weekly basis for the first 6 weeks. Then, the vaccine without bupivacaine was injected every 2 weeks thereafter. Mice were sampled at 7, 9, 12, 15 and 18 months of age (C-1). For therapeutic treatment, the vaccines were administered to APP23 mice from 12 months of age, after the appearance of amyloid plaques. Samplings were performed at 15 and 18 months of age (C-2). (Reproduced from Okura, Y., Miyakoshi, A., Kohyama, K. et al. *Nonviral Abeta DNA vaccine therapy against Alzheimer's disease: Long-term effects and safety.* Proc Natl Acad Sci U S A 2006, 103: 9619–24. © 2006 National Academy of Sciences, U.S.A.)

one contains only the $A\beta_{1-42}$ sequence with the Kozak sequence at the 5' end (referred to as K-Aβ vaccine) (Fig. 3A-1). To the second, the Igκ signal sequence of murine immunoglobulin was added to improve the secretion ability (IgL-Aβ vaccine) (Fig. 3A-2), and the third possesses the Fc portion of human immunoglobulin at the 3' end to maintain stability (Fc-Aβ vaccine) (Fig. 3A-3). Before *in vivo* administration, these DNA vaccines were transfected to HEK295T cells and the secretion of $A\beta_{1-42}$ peptide into the culture supernatant was assayed with Western blotting (Fig. 3B). The production of intracellular $A\beta_{1-42}$ peptide was confirmed in all three vaccines by ELISA (data not shown). It was clearly demonstrated that the supernatants of cultured cells that were transfected with IgL-Aβ and Aβ-Fc vaccines contained translated proteins (4.5 and 35 kDa, respectively), whereas K-Aβ-transfected cells did not secrete the peptide into the extracellular space. These findings indicate that the addition of the leader sequence is important for transportation of the protein to the extracellular space and that this event is critical for the effects of DNA vaccines.

We employed two types of regimens, prophylactic and therapeutic, to examine the effect of Aβ DNA vaccination. For the prophylactic protocol, vaccine administration was started from 3 to 4 months of age, before the appearance of amyloid deposition. APP23 mice received 6 weekly and subsequent biweekly injections of the vaccines and were examined at 7, 9, 12, 15 and 18 months of age (Fig. 3C-1). At 7 months of age, granular amyloid depositions were recognized in the frontal cortex in the control groups (empty vector-administrated and untreated mice) (Fig. 4B). At this stage, Aβ plaques were not detected in the hippocampus. In sharp contrast, cortical Aβ depositions in mice treated with Aβ-Fc (Fig. 4A), IgL-Aβ and Aβ vaccines were significantly reduced ($p < 0.01$). The Aβ burden was reduced to approximately 15–30% that of the untreated groups (Fig. 4E). At 12 months of age, amyloid depositions in

untreated mice were increased and some of them became large (> 50 μg) in the frontal cortex of the untreated mice (Fig. 4D). Aβ depositions in the hippocampus were also equally decreased ($p < 0.01$). It was shown that the suppressive effect of Aβ-Fc vaccine was almost equal to that of IgL-Aβ vaccine. However, K-Aβ vaccine

was less effective than the former two (Fig. 4E and 4F) and was not used in subsequent experiments. At 15 and 18 months of age, the plaques in untreated groups were rapidly increased. Untreated APP23 mice showed an age-dependent increase of amyloid plaques in the cerebral cortex (Fig. 5, open square) and hippocampus. The
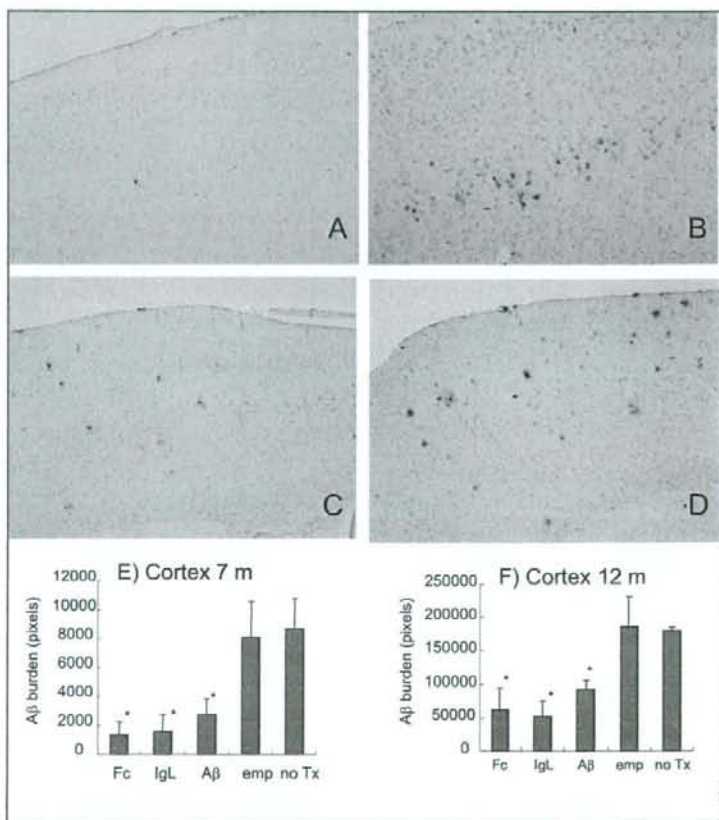

Fig. 4. Reduction of Aβ burden in APP23 mice at 7 months after DNA vaccination starting from 4 months (A, B and E). Immunohistochemical examinations revealed that granular amyloid depositions were detected in the frontal cortex of untreated mice at 7 months of age (B). In mice vaccinated with Aβ-Fc vaccine, amyloid plaques in the frontal cortex were reduced (A). Quantitative analysis demonstrated that the cortical Aβ burden at 7 months was significantly decreased ($p < 0.01$) after the prophylactic treatment with Aβ-Fc (15.5% of untreated controls), IgL-Aβ (18.2%) and Aβ vaccine (31.4%) than those found in untreated and empty vector-vaccinated mice (E). Reduction of Aβ burden in APP23 mice at 12 months after DNA vaccination starting from 4 months (C, D and F). Many Aβ deposits were observed in the frontal cortex of control mice (D), but were significantly reduced after treatment with Aβ-Fc (C) vaccines. Quantitative image analysis of Aβ burden in the cortex at 12 months of age revealed that Aβ deposits were significantly reduced (*$p < 0.01$) in mice with prophylactic treatment with Aβ-Fc (33.7% of untreated mice), IgL- Aβ- (28.6%) and K-Aβ (51.3 %) vaccines (F). K-Aβ vaccine was less effective than the former two. Magnification A-B ×62, C-D ×24. (Reproduced from Okura, Y., Miyakoshi, A., Kohyama, K. et al. *Nonviral Aβ DNA vaccine therapy against Alzheimer's disease: Long-term effects and safety.* Proc Natl Acad Sci U S A 2006, 103: 9619–24. © 2006 National Academy of Sciences, U.S.A.)
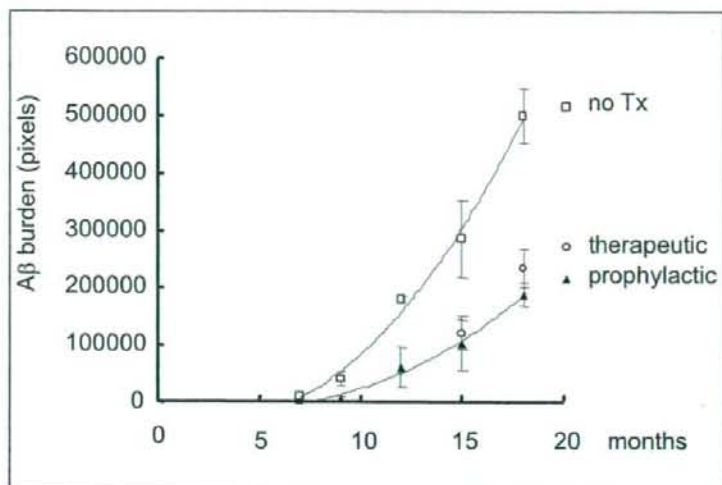
Fig. 5. The overall quantitative analysis. The amyloid deposition was first detected in untreated mice at 7 months of age and rapidly increased after 15 months of age (open squares). Prophylactic administration of Fc-Aβ vaccine prevented the Aβ deposition to 10–30% of that in untreated animals before 12 months of age and to 40–50% after 15 months (closed triangles). The effects of therapeutic administration (open circles) were almost the same as those of prophylactic administration (closed triangles).

the vaccines were administrated after amyloid depositions appeared.

Recently, it was reported that the intracellular Aβ deposition in cortical pyramidal neurons is the first neurodegenerative event in Alzheimer's disease.[31] Therefore, we counted the number of neurons containing intracellular Aβ depositions in the cortex of Aβ-Fc vaccine-administered and control mice. Aβ-deposited neurons were significantly decreased with both the prophylactic (50.2% of untreated control, $p < 0.01$) and therapeutic (59.5%, $p < 0.05$) treatments at 15 months of age (Fig. 7).

The titers of plasma anti-Aβ antibodies after the treatment were determined by ELISA. The levels of anti-Aβ antibodies were significantly increased compared with the untreated
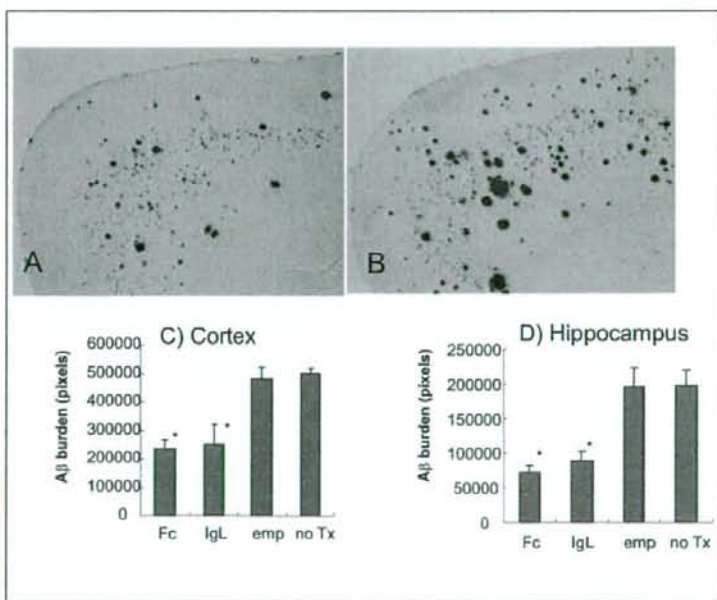
prophylactic protocol, using Aβ-Fc vaccine, revealed that the final reduction rate of Aβ burden in the cerebral cortex at 18 months of age was approximately 38.5% that of untreated groups (Fig. 5, closed triangles). These results demonstrated that two of three vaccines produced in this study were effective in the prophylactic treatment.

When considering the clinical applications, it is critical to know the effects of the vaccines in therapeutic application. For this purpose, the vaccination was started at 12 months of age, 6 months after the start of Aβ deposition and the brains were examined at 15 and 18 months (Fig. 3C-1). In therapeutic treatments, amyloid plaques in the cortex were significantly decreased ($p < 0.01$) by Aβ-Fc and IgL-Aβ vaccination (Fig. 6A) compared with the controls (Fig. 6B). Aβ depositions in the hippocampus were also decreased ($p < 0.01$) (Fig. 6D). Although the therapeutic protocol (Fig. 5, open circle) seemed to be less effective than the prophylactic one (Fig. 5, closed triangle), the difference was not significant. Thus, Aβ DNA vaccines had sufficient effects even if

Fig. 6. Aβ burden reduction at 18 months of age after therapeutic treatment starting from 12 months. While large Aβ deposits (> 100 μm) were observed in the frontal cortex of control mice at 18 months of age (B), significant reduction was observed after 6-month therapeutic administration of the IgL-Aβ vaccine (A). Quantitative image analysis of Aβ burden in the cortex at 18 months of age revealed that Aβ deposits were significantly reduced (*p < 0.01) in mice with therapeutic treatment of Aβ-Fc (47.0% of untreated mice) and IgL-Aβ (49.9%) vaccines. Aβ deposits in the hippocampus were also significantly reduced (*p < 0.01) after Aβ-Fc (38.0% of the control) and IgL-Aβ (46.0%) vaccine treatment (D). Magnification A and B, ×24.
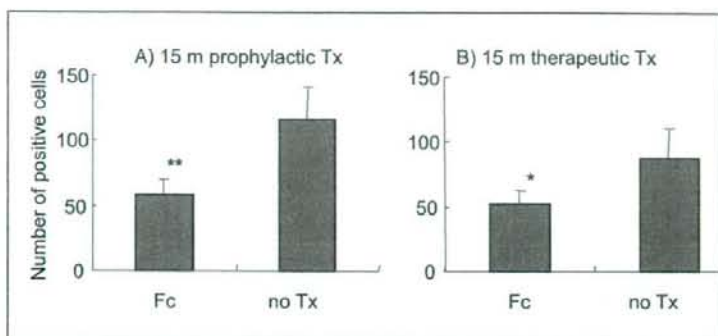
Fig. 7. Quantitative analysis of intracellular Aβ depositions after the prophylactic (A) and therapeutic (B) treatment with Aβ-Fc vaccine at 15 months of age. Microphotographs of the cerebral cortex (8 fields/mouse) were taken and neurons containing Aβ depositions were counted in a blinded manner. The numbers of positive neurons in vaccinated mice were significantly reduced compared with those in untreated mice ($*p < 0.05$; $**p < 0.01$).

and empty vector-vaccinated mice. Double staining with 6F/3D (anti-Aβ) and Iba-1 (antimicroglia) demonstrated the increase of phagocytizing microglia in the cerebral cortex of vaccine-treated Tg mice (unpublished data). It suggests that Aβ phagocytosis by activated microglia is a major pathway of Aβ clearance during nonviral DNA vaccine therapy.

The safety of our vaccines has been established as well as the effects. T-cell activation and proliferation, [³H]-thymidine incorporation of T cells from vaccinated mice was negative in both wild-type B6 and APP23 Tg mice strain. Pathological examinations using monoclonal antibodies, CD5 (anti-T cell) and Mac-3 (anti-macrophage) demonstrated no inflammatory lesion in the brain after long-term treatments (data not shown). Thus, our nonviral Aβ DNA vaccines are highly effective and safe and promising as vaccine therapy against human Alzheimer's disease.

Conclusions

Although interrupted, the phase II clinical trial of AN-1792 provides further support for Aβ immunotherapy of Alzheimer's disease. Alternative vaccine therapies have been investigated and developed to reduce excessive immune reactions of the host brain. As discussed in this article, nonviral DNA vaccines are being introduced as a

promising therapy against human Alzheimer's disease.

References

1. Citron, M. *Alzheimer's disease: Treatments in discovery and development.* Nat Neurosci 2002, 5(Suppl): 1055–7.
2. Hardy, J. and Allsop, D. *Amyloid deposition as the central event in the aetiology of Alzheimer's disease.* Trends Pharmacol Sci 1991, 12: 383–8.
3. Schenk, D., Barbour, R., Dunn, W. et al. *Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse.* Nature 1999, 400: 173–7.
4. Morgan, D., Diamond, D.M., Gottschall, P.E. et al. *A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease.* Nature 2000, 408: 982–5.
5. Janus, C., Pearson, J., McLaurin, J. et al. *A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease.* Nature 2000, 408: 979–82.
6. Bard, F., Cannon, C., Barbour, R. et al. *Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease.* Nat Med 2000, 6: 916–9.
7. DeMattos, R.B., Bales, K.R., Cummins, D.J. et al. *Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease.* Proc Natl Acad Sci U S A 2001, 98: 8850–5.
8. Wilcock, D.M., Munireddy, S.K., Rosenthal, A. et al. *Microglial activation facilitates Abeta plaque removal following

intracranial anti-Abeta antibody administration.* Neurobiol Dis 2004, 15: 11–20.
9. Bard, F., Barbour, R., Cannon, C. et al. *Epitope and isotype specificities of antibodies to beta-amyloid peptide for protection against Alzheimer's disease-like neuropathology.* Proc Natl Acad Sci U S A 2003, 100: 2023–8.
10. Bacskai, B.J., Kajdasz, S.T., McLellan, M.E. et al. *Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy.* J Neurosci 2002, 22: 7873–8.
11. Solomon, B., Koppel, R., Frankel, D. and Hanan-Aharon, E. *Disaggregation of Alzheimer beta-amyloid by site-directed mAb.* Proc Natl Acad Sci U S A 1997, 94: 4109–12.
12. Dodart, J.C., Bales, K.R., Gannon, K.S. et al. *Immunization reverses memory deficits without reducing brain Abeta burden in Alzheimer's disease model.* Nat Neurosci 2002, 5: 452–7.
13. Matsuoka, Y., Saito, M., LaFrancois, J. et al. *Novel therapeutic approach for the treatment of Alzheimer's disease by peripheral administration of agents with an affinity to beta-amyloid.* J Neurosci 2003, 23: 29–33.
14. Orgogozo, J.M., Gilman, S., Dartigues, J.F. et al. *Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization.* Neurology 2003, 61: 46–54.
15. Nicoll, J.A., Wilkinson, D., Holmes, C. et al. *Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: A case report.* Nat Med 2003, 9: 448–52.
16. Masters, C.L. and Beyreuther, K. *Alzheimer's centennial legacy: Prospects for rational therapeutic intervention targeting the Abeta amyloid pathway.* Brain 2006, 129: 2823–39.
17. Pfeifer, M., Boncristiano, S., Bondolfi, L. et al. *Cerebral hemorrhage after passive anti-Abeta immunotherapy.* Science 2002, 298: 1379.
18. Tang, D.C., DeVit, M. and Johnston, S.A. *Genetic immunization is a simple method for eliciting an immune response.* Nature 1992, 356: 152–4.
19. Barry, M.A., Lai, W.C. and Johnston, S.A. *Protection against mycoplasma infection using expression-library immunization.* Nature 1995, 377: 632–5.
20. Ulmer, J.B., Donnelly, J.J., Parker, S.E. et al. *Heterologous protection against influenza by injection of DNA encoding a viral protein.* Science 1993, 259: 1745–9.
21. Hoffman, S.L., Doolan, D.L., Sedegah, M. et al. *Nucleic acid malaria vaccines. Current status and potential.* Ann NY Acad Sci 1995, 772: 88–94.
22. Zhang, J., Wu, X., Qin, C. et al. *A novel recombinant adeno-associated virus vaccine reduces behavioral impairment and beta-amyloid plaques in a mouse model of

Alzheimer's disease. Neurobiol Dis 2003, 14: 365–79.

23. Hara, H., Monsonego, A., Yuasa, K. et al. *Development of a safe oral Abeta vaccine using recombinant adeno-associated virus vector for Alzheimer's disease.* J Alzheimers Dis 2004, 6: 483–8.

24. Kim, H.D., Maxwell, J.A., Kong, F.K. et al. *Induction of anti-inflammatory immune response by an adenovirus vector encoding 11 tandem repeats of Abeta1-6: Toward safer and effective vaccines against Alzheimer's disease.* Biochem Biophys Res Commun 2005, 336: 84–92.

25. Urabe, M., Ding, C. and Kotin, R.M. *Insect cells as a factory to produce adeno-associated virus type 2 vectors.* Hum Gene Ther 2002, 13: 1935–43.

26. Nishikawa, M. and Huang, L. *Nonviral vectors in the new millennium: Delivery barriers in gene transfer.* Hum Gene Ther 2001, 12: 861–70.

27. Nishikawa, M. and Hashida, M. *Nonviral approaches satisfying various requirements for effective in vivo gene therapy.* Biol Pharm Bull 2002, 25: 275–83.

28. Ghochikyan, A., Vasilevko, V., Petrushina, I. et al. *Generation and characterization of the humoral immune response to DNA immunization with a chimeric beta-amyloid-interleukin-4 minigene.* Eur J Immunol 2003, 33: 3232–41.

29. Schiltz, J.G., Salzer, U., Mohajeri, M.H. et al. *Antibodies from a DNA peptide vaccination decrease the brain amyloid burden in a mouse model of Alzheimer's disease.* J Mol Med 2004, 82: 706–14.

30. Okura, Y., Miyakoshi, A., Kohyama, K. et al. *Nonviral Abeta DNA vaccine therapy against Alzheimer's disease: Long-term effects and safety.* Proc Natl Acad Sci U S A 2006, 103: 9619–24.

31. Fernandez-Vizarra, P., Fernandez, A.P., Castro-Blanco, S. et al. *Intra- and extracellular Abeta and PHF in clinically evaluated cases of Alzheimer's disease.* Histol Histopathol 2004, 19: 823–44.

Yoshio Okura and Yoh Matsumoto* work in the Department of Molecular Neuropathology at the Tokyo Metropolitan Institute for Neuroscience in Japan. *Correspondence: Yoh Matsumoto, Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Musashidai 2-6, Fuchu, Tokyo 183-8526, Japan. Tel.: +81-423-25-3881, ext. 4719; Fax: +81-423-21-8678; E-mail:matyoh@tmin.ac.jp.

Differential effects of decoy chemokine (7ND) gene therapy on acute, biphasic and chronic autoimmune encephalomyelitis: Implication for pathomechanisms of lesion formation

Il-Kwon Park, Keiko Hiraki, Kuniko Kohyama, Yoh Matsumoto *

Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan

Abstract

Multiple sclerosis (MS) exhibits several clinical subtypes such as the relapsing–remitting (RR) and secondary progressive (SP) forms. In accordance with this, formation of demyelinating plaques in the central nervous system (CNS) occurs by different mechanisms. In the present study, we induced acute, biphasic and chronic (RR or SP) EAE in rats and examined the effects of decoy chemokine (7ND) gene therapy, which inhibits the migration of macrophages, to address the above issue. Interestingly, it was demonstrated that the clinical signs of acute EAE and the first attack of biphasic EAE were minimally affected, whereas chronic EAE and the relapse of biphasic EAE were completely suppressed with 7ND treatment. In the CNS, the number of infiltrating macrophages was reduced in all the stages of the three types of EAE. These findings suggest that in acute EAE and in the first attack of biphasic EAE, where anti-macrophage migration therapy was almost ineffective, pathogenic T cells are mainly involved in lesion formation. In contrast, the relapse of biphasic EAE and chronic EAE macrophages play a major role in the disease process. Thus, the mechanisms of lesion formation are not uniform and immunotherapy should be performed on the basis of information about the pathomechanisms of autoimmune diseases.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Acute; Biphasic; Chronic EAE; Macrophage; 7ND; Gene therapy

1. Introduction

The pathogenesis of multiple sclerosis (MS) and its related disorders such as neuromyelitis optica (NMO) is still poorly understood. One of the reasons for this is that there are many variants in terms of the clinical course (Lublin and Reingold, 1996) and pathology (Lucchinetti et al., 2000). Recent progress has shown that the clinical course of MS consists of the early inflammatory phase and late neurodegenerative phase (Sospendra and Martin, 2005). Moreover, even in the early inflammatory phase, the predominant population of infiltrating cells varies considerably (Lucchinetti et al., 2000; van der Goes et al., 2005). Therefore, it is possible that similar neurological deficits are produced by the different mechanisms. The present study aimed to clarify the precise pathomechanisms of lesion formation by treating different types of experimental autoimmune encephalomyelitis (EAE). For this purpose, we performed the treatment experiments with decoy chemokine gene (7ND) to inhibit macrophage infiltration into the central nervous system (CNS). We reasoned that if certain lesions are formed mainly by macrophages, then clinical and pathological conditions would be greatly improved or completely suppressed by anti-macrophage migration therapy. In contrast, the lesions formed mainly by T cells would be minimally affected or unaffected.

7ND was first developed by Zhang et al. as a potent inhibitor of MCP-1/CCL2, as evidenced by the finding that 7ND specifically inhibits MCP-1/CCL2-mediated monocyte chemotaxis (Zhang et al., 1994). On the basis of these data, 7ND has been used for the treatment of various disease models such as vascular diseases

---

(Egashira, 2003; Egashira et al., 2000), arthritis (Gong et al., 1997) and pulmonary hypertension (Ikeda et al., 2002). In all these diseases, macrophages play an essential role in lesion formation and 7ND treatment was found to be effective.

In a previous study, we demonstrated that the neutralization of MCP-1/CCL2 with decoy chemokine receptor gene encoding the binding site of CCR2, which is a receptor for MCP-1/CCL2, significantly suppresses the relapse, but not the first attack, of biphasic EAE (Matsumoto et al., 2005). This suggested that EAE lesions in the spinal cord at the first attack are formed by pathomechanisms different from that during the relapse. In the present study, we examined the effects of 7ND, a potent inhibitor of MCP-1/CCL2, on acute, biphasic and chronic EAE to elucidate the differences in the pathomechanisms among three types of EAE. Consequently, we found that acute EAE and the first attack of biphasic EAE were minimally affected by 7ND treatment, whereas the relapse of biphasic EAE and chronic EAE were completely suppressed in the majority of immunized rats by the same treatment. These findings suggest that T cells play a major role in formation of the former lesions and that macrophages are essential effectors for the latter lesion formation. Thus, immunotherapy should be performed on the basis of information about the pathomechanisms of lesion formation of autoimmune diseases.

2. Materials and methods

2.1. Animals

LEW.1AV1 rats were kindly provided by Dr. R. Gold, Department of Neurology, Wuerzburg University, Germany and maintained in our animal facility. Lewis (LEW) and DA rats were purchased from Japan SLC Inc. (Shizuoka). All rats used were 8–12 weeks of age.

2.2. Reagents

Recombinant rat MOG was prepared as described previously (Sakuma et al., 2004). Briefly, the gene coding the extracellular domain (amino acid 1–125) of MOG was amplified using primers specific for the corresponding MOG sequence. The PCR products were then digested with Sph I and Hind III and subcloned into pQE30 (QIAGEN, Tokyo, Japan) for large-scale preparation. Recombinant MOG in transformed *E. coli* was isolated under denaturing conditions and purified using Ni-NTA Agarose (QIAGEN). Then, purified MOG was diluted and refolded in PBS containing 1 M L-arginine, 2 mM glutathione (reduced form) and 0.2 mM glutathione (oxidized form). The obtained protein contained endotoxins less than 10 EU/1 mg protein as determined with a Toxinometer ET-2000 (Wako, Tokyo, Japan).

The myelin fraction was extracted from bovine spinal cords as described previously with a few modifications (Agrawal et al., 1972; Casado et al., 1988). Briefly, spinal cord tissue was homogenized and washed in 0.32 M sucrose and the suspension was overlaid on 0.84 M sucrose. After centrifugation, the interface was collected, washed with Milli Q water and homogenized.

Using fractions other than the interface, this process was repeated and the interface was collected. These preparations were lyophilized and kept at −80 °C until use. Western blot analysis revealed that the purified myelin preparation contained myelin basic protein (MBP), proteolipid protein (PLP) and MOG (data not shown). Guinea pig, bovine and rat MBP were prepared as described previously (Deibler et al., 1972).

2.3. Preparation of 7ND plasmid and gene therapy

Total RNA was isolated from the spleen of a normal Lewis rat using RNAzol ™ B (TEL-TEST INC.). The RNA was subjected to reverse transcription using ReverTra Ace-α- ™ (TOYOBO, Osaka, Japan) and amplification with specific primers (sense, GACTCGAGACCATGCAGGTCACCTGCTGCTAT; antisense, GAGCGGCCGCTCAAGTCTTCGGAGTTTGGG) was performed as shown in previous studies (Egashira et al., 2000; Zhang et al., 1994). The PCR fragment was then cloned into the pCR^{R}4Blunt-TOPO cloning vector (Invitrogen, Tokyo, Japan) and sequenced to confirm that the plasmid contains the insert with the right sequence. The resulting pCR^{R}4Blunt-TOPO 7ND was subjected to enzymatic digestion with Xho I-Not I (210 bp) and ligated into the Xho I-Not I site of pTARGET (Promega, Tokyo, Japan), which was used for the experiments.

7ND at a dose of 100 μg was administered intramuscularly three times a week between days 0 and 21 post-immunization. Control rats were given an empty vector at the same dose with the same protocol.

2.4. EAE induction and clinical evaluation

Three types of EAE, acute, biphasic and chronic EAE were induced by the following methods. Acute EAE was induced in LEW rats by immunization with MBP and biphasic EAE was induced in DA rats by immunization with myelin (Matsumoto et al., 2005). In both cases, the onset of the disease and maximal severities were relatively uniform. Chronic EAE was induced in LEW.1AV1 rats by immunization with recombinant MOG. The clinical course of the disease was variable and the majority of rats showed the relapsing–remitting (RR) or the secondary progressive (SP) form as described previously (Sakuma et al., 2004). Clinical signs were evaluated as the total score of the degree of paresis of each limb and tail (partial paresis, 0.5; complete paresis, 1.0). Therefore, the clinical score of complete paralysis of four limbs plus tail or the moribund conditions was 5.

2.5. Histological and immunohistochemical examinations

The optic nerve, cerebrum, brain stem, cerebellum and the cervical, thoracic and lumbar spinal cord were routinely examined. The tissues were fixed in 4% paraformaldehyde and processed for paraffin embedding. Six μm sections were cut and stained with hematoxylin and eosin (H&E) and with Kruever and Barrera's (K–B) method. Inflammatory lesions were graded using sections stained with H&E and W3/13 for T cells into four categories (Grade 1, leptomeningeal and adjacent subpial cell

infiltration; Grade 2, mild perivascular cuffing; Grade 3, extensive perivascular cuffing; Grade 4, extensive perivascular cuffing and severe parenchymal cell infiltration). Demyelinating lesions were graded using sections stained with the K–B method into five categories (Grade 1, trace of perivascular or subpial demyelination; Grade 2, focal demyelination; Grade 3, demyelination involving a quarter of tissues examined; Grade 4 massive confluent demyelination involving half of the tissue; Grade 5, extensive demyelination involving the entire tissues. Macrophage infiltration was evaluated by counting ED1-positive cells under high magnification and expressed as %

ED1-positive cells (ED1-positive cells/ED1-positive plus ED1-negative cells × 100).

Single immunoperoxidase staining was performed as described previously (Matsumoto and Fujiwara, 1987; Ohmori et al., 1992). Briefly, paraffin-embedded sections were deparaffinized and rehydrated. After blocking the endogenous peroxidase activity with methanol containing 0.3% hydrogen peroxide, the sections were incubated with mAb W3/13 (Dainippon Pharm, Osaka, Japan) for T cell-staining or ED1 (purified from the hybridoma supernatant) for macrophage-staining. After washing, the sections were incubated with
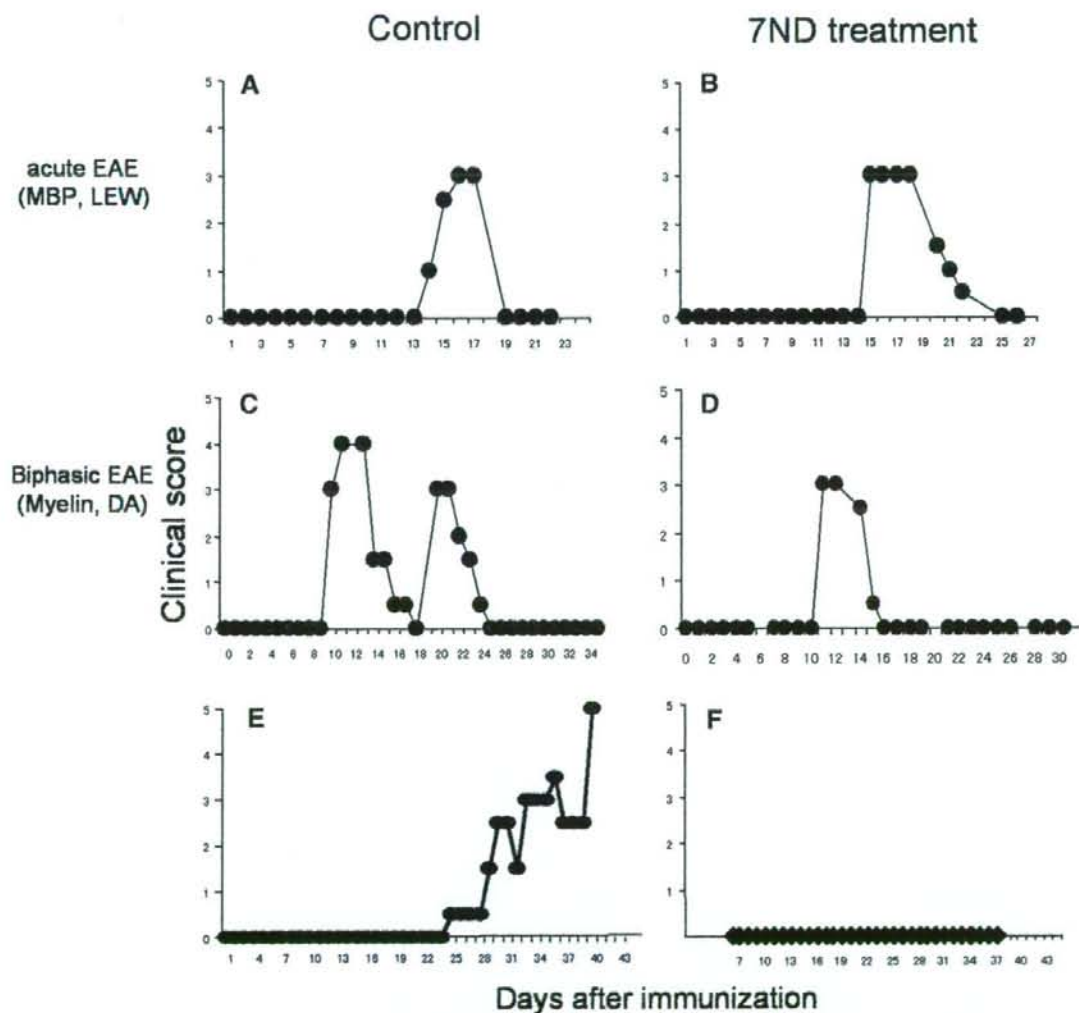

Fig. 1. Modulation of the clinical course of acute, biphasic and chronic EAE by 7ND treatment. In acute EAE, there is no significant change in the clinical course between control (A) and 7ND-treated (B) rats. Treatment with 7ND of rats immunized with myelin results in complete suppression of the relapse (D) found in control rats (C), while the first attack is suppressed slightly. In chronic EAE, treated rats (F) did not develop EAE at all, while the control rats had severe and continuous clinical signs (E). The representative clinical courses of individual rats with acute, biphasic and chronic EAE are illustrated. Acute and biphasic EAE showed relatively uniform clinical courses and did not show any further relapse by day 60 (unpublished observations). In contrast, LEW.1AV1 rats immunized with MOG developed the relapsing–remitting or secondary progressive form of chronic EAE. In (E), secondary progressive EAE is shown.

biotinylated anti-mouse IgG (Vector, Burlingame, CA) followed by horseradish peroxidase (HRP)-labeled VECTSTAIN Elite ABC Kit (Vector). HRP-binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide. To confirm the specificity of the staining, the primary antibodies were omitted or replaced with normal mouse IgG. The controls did not show any specific staining.

2.6. ELISA

The levels of anti-MOG and anti-MBP antibodies were measured using ELISA. Recombinant MOG or purified MBP (10 µg/ml) were coated onto microtiter plates and serially diluted sera from normal and immunized animals were applied. After washing, appropriately diluted horseradish-conjugated anti-rat IgG was applied. The reaction products were then visualized after incubation with the substrate. The absorbance was read at 450 nm.

2.7. Real-time PCR

After residual genomic DNA was removed with DNase (TURBO DNA-free ™, Ambion, Tokyo, Japan), first strand cDNA was synthesized from 1 µg of total RNA using random hexamers and ReverTra Ace (TOYOBO). SYBR Green real-time PCR reactions were performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA) in a total volume of 25 µl using the SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). Each PCR was performed in duplicate under the following thermocycler conditions: stage 1, 95 °C for 10 min for one cycle and stage 2, 95 °C for 15 s and 58 °C for 1 min for 50 cycles. All primers, except for 18 S rRNA, were designed on an intron–exon junction to prevent the coamplification of genomic DNA. The relative quantification of mRNA was performed using the standard curve method. Cytokine mRNA was normalized to 18 S RNA for each sample (Bas et al., 2004). The absence of nonspecific amplification was confirmed by the dissociation curve analysis.

2.8. Statistical analysis

Data were analyzed by Student's *t* test or Mann–Whitney's *U*-test. *P*-value less than 0.05 was considered as statistically significant.

3. Results

3.1. Clinical course and pathology of acute, biphasic and chronic EAE

In the present study, we induced acute, biphasic and chronic EAE by the immunization protocols as reported in detail previously (Kim et al., 1998; Matsumoto et al., 2005; Sakuma et al., 2004). The representative clinical courses of individual rats with acute, biphasic and chronic EAE are illustrated in Fig. 1A, C and E, respectively, and all the results are summarized in Table 1. Acute EAE was induced in LEW rats by immunization with MBP. As shown in Fig. 1A and Table 1 (Group B), rats developed acute monophasic EAE with the onset of day 12.3±0.5 and the maximal clinical score was 2.9±0.2. Immunization of DA rats with purified myelin-induced biphasic EAE with the fist peak on day 9.3±0.8 with the maximal clinical core of 4, followed by the relapse on day 17.8±0.8 with the clinical score of 3.0±0.4 (Fig. 1C and Table 1, Group D). These two types of EAE showed relatively uniform clinical courses and did not show any further relapse by day 60 (unpublished observations). In contrast, LEW.1AV1 rats immunized with MOG developed RR or SP form of chronic EAE as shown in a previous study (Sakuma et al., 2004). In Fig. 1E, SP EAE is shown.

3.2. 7ND treatment modulates biphasic and chronic, but not acute, EAE

7ND (100 µg), a dominant inhibitor of MCP-1/CCL2 (Zhang et al., 1994), was injected intramuscularly three times a week from day 0 to day 21 to rats that had been immunized for acute, biphasic and chronic EAE. Control rats received an empty vector. The representative clinical courses of treated animals are shown in Fig. 1B, D and F and all the data are summarized in Table 1. Interestingly, 7ND modulated the clinical course of three types of EAE very differently. Acute EAE was influenced very slightly by 7ND treatment (Fig. 1A and B). All the treated rats developed severe EAE with a maximal clinical score of 2.4±0.7 (Table 1, Group A) and there was no significant difference between the treated and control groups. In contrast, biphasic EAE was modulated characteristically. As depicted in Fig. 1C and D and summarized in Table 1, Groups C and D, the clinical score during the first attack was slightly suppressed compared with that of the control rats but all the rats developed severe EAE. Interestingly,

Table 1
Summary of clinical courses of acute, biphasic and chronic EAE of rats treated with 7ND

Group	Ag	Strain	Treatment		Incidence	Onset	Max. clininical sign
A	MBP	LEW	7ND		7/7	13.3±0.9	2.4±0.7
B			Control		3/3	12.3±0.5	2.9±0.2
C	Myelin	DA	7ND	1st attack	4/4	10.8±0.4	3.0±0.7
				Relapse	0/4	(−)	(−)
D			Control	1st attack	4/4	9.3±0.8	4
				Relapse	4/4	17.8±0.8	3.0±0.4
E	MOG	LEW.1AV1	7ND		1/5	36	2.5
F			Control		5/5	27.8±3.7	3.9±0.9

LEW, DA and LEW.1AV1 rats were immunized with MBP, myelin and MOG, respectively, and observed daily for clinical signs. Observation periods were 21–23 days for acute EAE, 31–34 days for biphasic EAE and 37–40 days for chronic EAE. Rats listed in Tables 2 and 3 are not included in this table.

the relapse of the disease was completely suppressed by the treatment (Table 1, Group C vs. D). Finally, chronic EAE induced by immunization with MOG was completely suppressed in 4 out of 5 rats by the treatment (Fig. 1F and Table 1, Group E).

3.3. Pathological changes in treated animals

As biphasic and chronic, but not acute, EAE was affected by 7ND treatment, we tried to characterize pathology of biphasic and chronic EAE in treated animals and compared the results with those obtained from control rats. At the first attack of biphasic EAE, there was marked macrophage infiltration in control rats (Fig. 2A). In 7ND-treated rats at the same period, the number of infiltrating macrophages was reduced but some were present in the lesion (indicated by arrow heads in Fig. 2B). During the relapse of biphasic EAE, many macrophages were detected in the control rats and some of them showed the feature of "foamy macrophages" (arrows in Fig. 2C). 7ND treatment
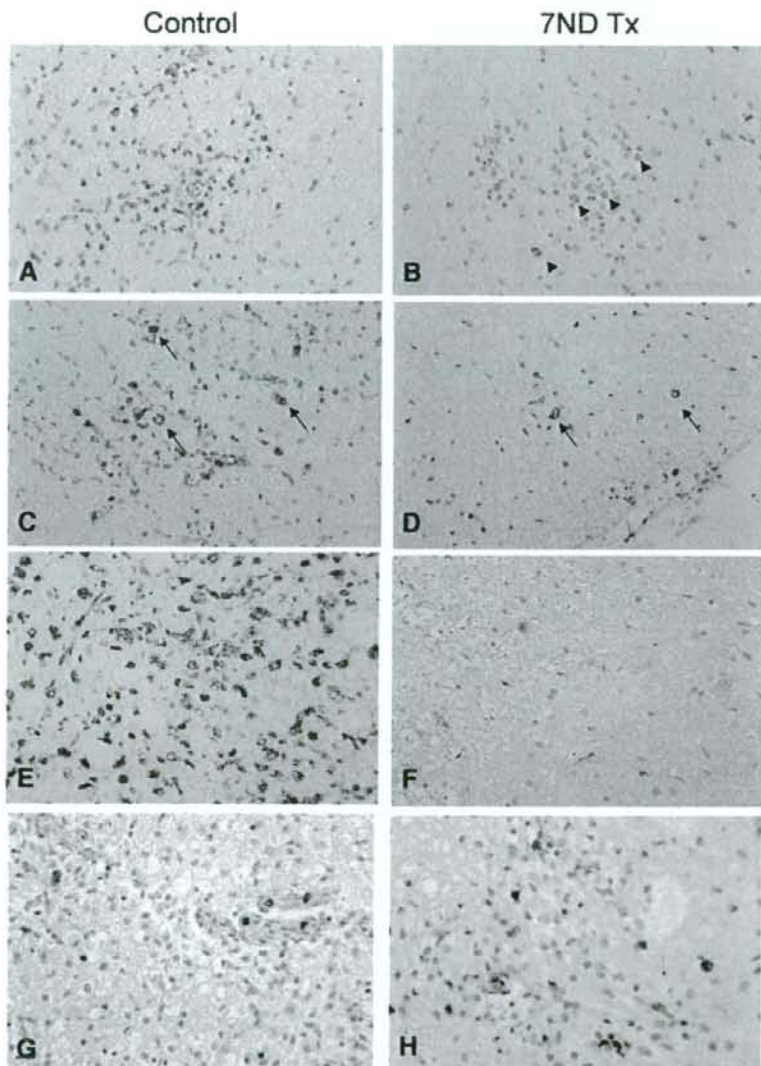

Fig. 2. Immunohistochemical staining of macrophages (A–F) and T cells (G, H) in the spinal cords of the control (A, C, E and G) and 7ND-treated (B, D, F and H) rats. At the first attack of biphasic EAE, there is marked macrophage infiltration in control rats (A). In treated rats with clinical signs at the first attack, the number of infiltrating macrophages is reduced but some are present in the lesion (arrow heads in B). During the relapse of biphasic EAE, many macrophages are detectable in control rats and some of them show the feature of "foamy macrophages" (arrows in C). 7ND treatment reduced the number of inflammatory lesions with macrophages during the same period (arrows in D). In chronic EAE, dense macrophage infiltration found in control rats (E) is completely absent in treated rats (F). In T cell staining, the distribution and density of T cells were not significantly different between the control (G) and treated (H) groups in biphasic EAE (the first attack, histological grade 3). A–F, ED1 staining; G & H, W3/13 staining, ×120.

greatly reduced the number of inflammatory lesions during this period (Fig. 2D). Table 2 summarizes the histological severities of all the treated and control rats with biphasic EAE. At the first attack, the mean histological scores of the spinal cord of treated and control rats were 1.6 ± 1.0 and 2.7 ± 0.8, respectively, and treated rats showed a significantly milder pathology ($p=0.015$). In addition, in treated rats, severe lesions were limited to one or two segments of the spinal cord, while almost the entire spinal cord was involved in control rats (1° in Table 2). During the relapse, treated rats except one case showed no or minimal pathology compared with control rats (2° in Table 2). Infiltration in biphasic EAE was further evaluated by counting ED1-positive cells under high magnification and expressed as % ED1-positive cells (ED1-positive cells/ED1-positive plus ED1-negative cells × 100). The results are shown in Table 3. In control rats, macrophages accounted for $50.2\pm5.0\%$ during the first attack. During the relapse, the percentage increased slightly but significantly ($p=0.013$). In 7ND-treated rats, macrophage infiltration was significantly inhibited during the first attack and relapse ($p=0.36$ and $p=0.00001$, respectively). The inhibition was more marked during the relapse than during the first attack. These findings were correlated well with the clinical course.

In chronic EAE, dense macrophage infiltration found in control rats (Fig. 2E) was completely absent in treated rats (Fig. 2F). Two of three treated rats were completely normal histologically and one rat without clinical signs showed moderate pathology (Table 4). In contrast, control rats showed moderate to severe inflammation and/or demyelination at least in one segment of the spinal cord (Table 4).

We also examined the distribution and density of infiltrating T cells in the spinal cord of treated and control rats during

Table 2
Pathology of the CNS of DA rats with myelin-induced EAE after 7ND treatment

	Tx	Sampling	Clinical score[a]	Opt[b]	Cbr	BS/Cbll	C	Th	L
1°	7ND	d12	4	0	0	0	3	2	1
	7ND	d12	3.5	0	0	2	0	3	2
	7ND	d12	3	0	0	1	1	1	1
	7ND	d11	4	0	0	2.5	3.5	2	0
	Control	d11	4	1	0	2	3	3.5	3
	Control	d11	4	0	0	2	2.5	3.5	1
	Control	d12	3	0	0	1	2	3	3
2°	7ND	d19	0 (3)[a]	n.e.[c]	0	0	0	0	0
	7ND	d19	0 (3.5)	1	0	1	0	0	1
	7ND	d19	3 (4)	0	0	0	3	3	0
	7ND	d19	0 (3.5)	0	0	0	1	0	0
	Control	d20	3 (3)	0	n.e.	n.e.	3	3.5	3
	Control	d20	3 (4)	0	n.e.	n.e.	2.5	3.5	1
	Control	d22	2.5 (4.5)	0	0	1/3.5[d]	0	2	0.5
	Control	d22	2 (4)	0	0	1.5	3	2.5	2

Rats listed in Table 1 are not included in this table.

[a] Clinical score at time of sampling. The number in the parenthesis indicates the maximal clinical score throughout the observation periods.

[b] Opt, optic nerve; Cbr, cerebrum; BS/Cbll, brain stem/cerebellum; C, cervical spinal cord; Th, thoracic spinal cord; L, lumbar spinal cord.

[c] n.e.: not examined.

[d] 1/3.5 indicates inflammation score/demyelination score. All other scores indicate inflammation score.

Table 3
The density of infiltrating macrophages in the spinal cord of 7ND-treated and control rats with biphasic EAE

	1st attack	Relapse
7ND	30.2 ± 14.6[a]	12.3 ± 7.4[a]
Control	50.2 ± 5.0[a]	61.9 ± 5.6[a]

Macrophage infiltration was evaluated by counting ED1-positive cells under high magnification ($n=6$ per group) and expressed as % ED1-positive cells (ED1-positive cells/ED1-positive and negative cells × 100). The mean % ± SD are shown.

[a] Significant differences were noted in all the following combinations. 7ND 1st attack vs. 7ND Relapse ($p=0.036$); 7ND 1st attack vs. Control 1st attack ($p=0.017$); 7ND Relapse vs. Control Relapse ($p=0.00001$); Control 1st attack vs. Control Relapse ($p=0.013$).

the first attack of biphasic EAE and found that there was no significant difference between the two groups (Fig. 2G and H). Using spinal cord sections with inflammatory scores of 2–3, we counted W3/13-positive cells/inflammatory lesion in 7ND-treated and control rats and found that there was no significant difference (9.5 ± 3.1 vs. 9.2 ± 3.2, $p=0.87$). These findings suggest that 7ND treatment minimally interfere with T cell infiltration into the CNS lesions. B cell infiltration was not detected throughout the course of chronic EAE (data not shown).

3.4. Chemokine profiles in 7ND-treateed animals

We performed the quantitative analysis of chemokines in the spinal cord of treated and control rats with biphasic EAE (Fig. 3A and C) or with chronic EAE (Fig. 3B and D) using real-time PCR. In control rats with biphasic EAE, MCP-1/CCL2 was mainly upregulated during the first attack (Day 14 in Fig. 3A), whereas MIP-1α/CCL3 increased during both the first attack and relapse (Days 12 and 19 in Fig. 3C). MCP-1/CCL2 and MIP-1α/CCL3 in the spinal cords of treated rats were suppressed slightly during the first attack and almost completely during the relapse. This finding correlated well with the degree of macrophage infiltration (Table 3).

Table 4
Pathology of the CNS of LEW.1AV1 rats with MOG-induced EAE after 7ND treatment

Tx	Sampling	Clinical score[a]	Opt[b]	Cbr	BS/Cbll	C	Th	L
7ND	d43	0	0	0	0	0	0	0
7ND	d43	0	0	0	0	0	0	0
7ND	d43	0	0	2	2/3[c]	0	0	0/3
Control	d35	0.5 (0.5)	0	1	2	0	0	0
Control	d35	3 (3)	1/4	0	0	0	3/4[c]	3/3[c]
Control	d35	1.5 (2.5)	0	1	1	0	0	1/3

Rats listed in Table 1 are not included in this table.

[a] Clinical score at time of sampling. The number in the parenthesis indicates the maximal clinical score throughout the observation periods.

[b] Opt, optic nerve; Cbr, cerebrum; BS/Cbll, brain stem/cerebellum; C, cervical spinal cord; Th, thoracic spinal cord; L, lumbar spinal cord.

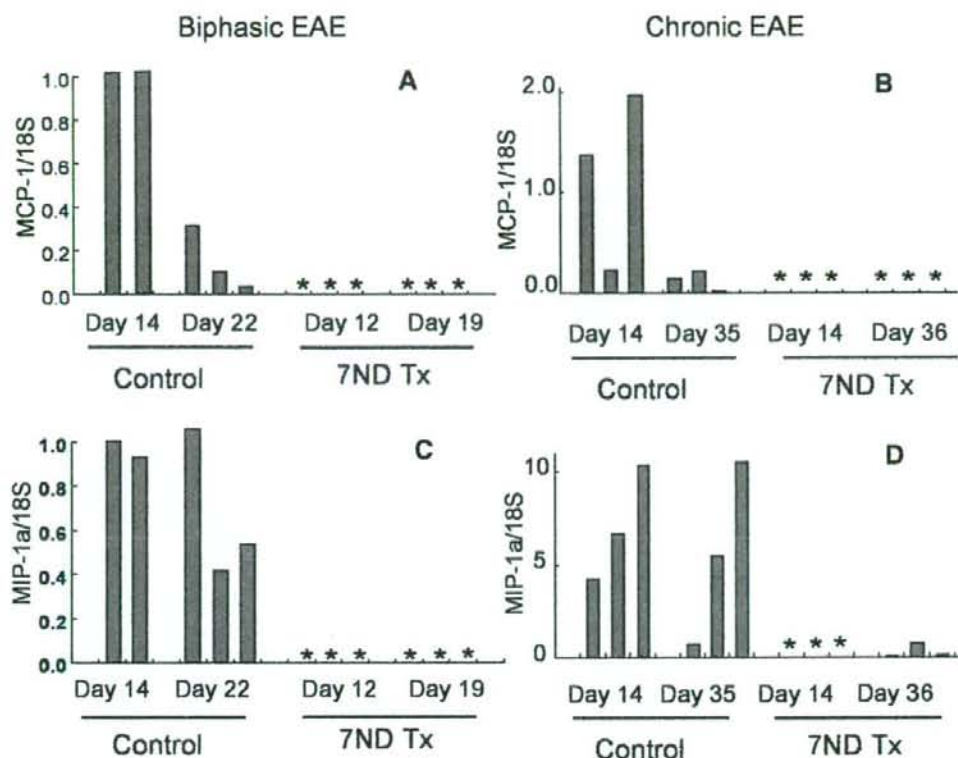[c] 2/3 indicates inflammation score/demyelination score. All other scores indicate inflammation score.

Fig. 3. Quantitation of chemokine mRNA. The levels of MCP-1/CCL2 (A and B) and MIP-1α/CCL3 (C and D) mRNA in the spinal cords of treated and control rats with biphasic (A and C) and chronic (B and D) EAE were determined with real-time PCR. Each bar represents a value from individual rats.

MCP-1/CCL2 (Fig. 3B) and MIP-1α/CCL3 (Fig. 3D) mRNA in the spinal cords of treated rats with chronic EAE were suppressed almost completely at the early and late stages of the disease.

3.5. Evaluation of anti-MBP and anti-MOG antibodies in biphasic and chronic EAE

We wished to know whether the therapeutic effects of 7ND administration are mediated by the downregulation of anti-neuroantigen antibodies. For this purpose, we determined the levels of anti-MBP and anti-MOG antibodies of treated and control rats with biphasic and chronic EAE. These antibodies were not detected in naive LEW, DA and LEW.1AV1 rats (data not shown). The results are illustrated in Fig. 4. As clearly shown, during the first attack of biphasic EAE (Days 11–12 in Fig. 4A and C), the levels of both anti-MBP and anti-MOG antibodies in treated animals were almost the same as those in control animals. During the relapse (Days 19–22 in Fig. 4A and C), anti-MOG (Fig. 4C), but not anti-MBP (Fig. 4A), antibodies were significantly lowered in the treated group compared with the control group. In treated and control rats with chronic EAE, the levels of anti-MBP (Fig. 4B) were almost under the detection level. Similarly, anti-MOG antibodies (Fig. 4D) were not affected by 7ND treatment although the disease was completely

suppressed in the majority of rats. These findings suggested that amelioration of chronic EAE occurs without the downregulation of anti-neuroantigen antibodies. In acute EAE, the levels of anti-MBP antibodies remained low in both treated and control rats throughout the course of the disease (data not shown).

4. Discussion

Multiple sclerosis (MS) is thought to be an autoimmune disease characterized by the presence of multiple inflammatory and demyelinating lesions in the CNS (Bar-Or et al., 1999). Notably, there is profound heterogeneity in the clinical course and CNS lesions, suggesting that MS is a disease with heterogeneous pathomechanisms. Through careful observations of CNS tissues with actively demyelinating lesions, Lassmann and colleagues classified MS lesions into 4 patterns (Lassmann, 2004; Lucchinetti et al., 2000). Although their characterization of the MS lesions is highly suggestive of pathogenetic mechanisms of heterogeneous lesion formation in MS, there is limitation for further analysis since these results were obtained by the morphological examinations of autopsy materials.

In the present study, we induced three types of EAE using three antigens and three rat strains and tried to elucidate the pathomechanisms of the lesion formation in MS by examining various types of EAE clinically and pathologically. It should be
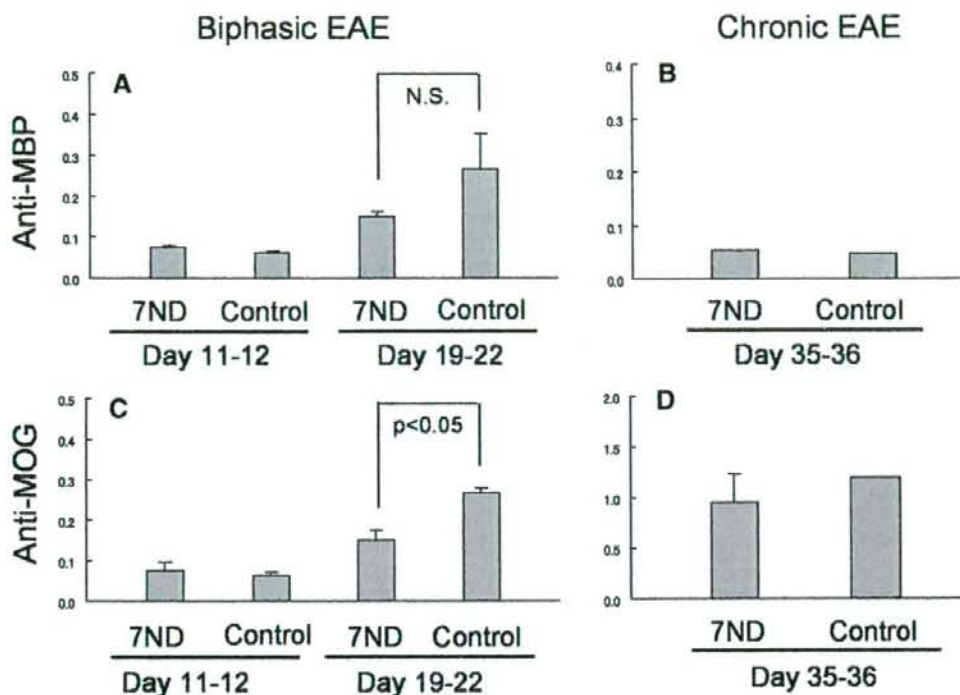
Fig. 4. Anti-MBP and anti-MOG antibodies in sera from rats with biphasic (A and C) and chronic (B and D) EAE. The levels of anti-MBP (A and B) and anti-MOG (C and D) antibodies in sera were determined by ELISA. The sera were collected on days 11–12 post-immunization (PI) during the first attack and days 19–22 PI during relapse of biphasic (A and C) and on days 35–36 and day 43 PI of chronic (B and D) EAE. Each bar represents the mean value (O.D.)±SD of three rats.

noted that such variations are unlikely to influence the results. This was because we only analyzed the role of T cells and macrophages in the lesion formation of each EAE and did not analyze variation-dependent factors such as the antigen specificity of T cells from three strains. For the analysis, we employed decoy chemokine, 7ND, which is a dominant inhibitor of MCP-1/CCL2. 7ND was reported to be a dominant negative inhibitor of MCP-1/CCL2 and not a competitive inhibitor for receptor binding because 7ND inhibited the non-cross-linked, but not the cross-liked, active form of MCP-1/CCL2 (Zhang and Rollins, 1995). As it was later demonstrated that glycosaminoglycan binding and oligomerization are essential for the in vivo activity of MCP-1/CCL2 (Lau et al., 2004; Proudfoot et al., 2003), 7ND may interfere with these processes. 7ND has been used, not only for in vitro studies, but also for the treatment of animal disease models including vascular diseases (Egashira, 2003; Egashira et al., 2000), arthritis (Gong et al., 1997) and pulmonary hypertension (Ikeda et al., 2002). To our knowledge, this is the first report showing that 7ND treatment is effective in preventing some subtypes of EAE. It is very interesting to note that 7ND treatment suppressed the relapse of biphasic EAE and chronic EAE, but not acute EAE and the first attack of biphasic EAE. It was previously reported that depletion of macrophages by liposomes containing dichloromethylene diphosphonate (Cl₂MDP) markedly suppressed the development of MBP-induced acute EAE in rats and mice (Huitinga et al., 1990; Tran et al., 1998). We have confirmed that Cl₂MDP, which was produced and administered

exactly the same as that in the previous reports, suppressed acute EAE in Lewis rats (unpublished observation). The major difference in the immune conditions between Cl₂MDP and 7ND treatment was that 7ND treatment only inhibited macrophage migration and may minimally influence T cell functions, while liposome treatment would downregulate bioactive substances secreted by macrophages. This may be attributable to the difference in the outcome. Immunohistochemical examinations revealed that infiltrating macrophages in biphasic EAE lesions were substantially reduced in number by 7ND treatment and that chronic EAE lesions were completely normalized histologically. In contrast, the distribution and density of T cells in the lesion at the first attack of biphasic EAE were not different between the treated and control groups (Fig. 2) indicating that 7ND treatment does not interfere with T cell infiltration. These findings strongly suggest that the major effector cells are macrophages in the relapse of biphasic EAE and chronic EAE. With regard to the relapse of biphasic EAE, we previously reported that MCP-1/CCL2 was upregulated at this stage (Jee et al., 2002) and Kennedy et al. showed that administration of anti-MCP-1/CCL2 antibodies suppressed the relapse of mouse biphasic EAE (Kennedy et al., 1998). In addition, MCP-1/CCL-2-deficient mice developed significantly mild EAE (Huang et al., 2001). In the present study, we have demonstrated that anti-macrophage migration treatment by 7ND suppressed the relapse of biphasic EAE, indicating that MCP-1/CCL2 plays a key role of EAE relapse. It is less likely that 7ND administration induces the antibody