injection. Histological examination revealed that there were extensive areas of the neocortex with very few Aβ plaques. Those areas of the cortex that were devoid of Aβ plaques contained neurofibrillary tangles, neuropil threads and cerebral amyloid angiopathy (CAA) similar to untreated AD, but lacked plaque-associated dystrophic neurites and astrocyte clusters. These findings were very similar to those obtained in mouse models of AD after Aβ immunotherapies and suggested that the immune response generated against the peptide accelerated Aβ clearance.[12] Essentially the same findings were found in subsequent two autopsy cases.[13,14]

The effects of Aβ immunotherapies on cognitive functions are controversial. Hock et al. followed thirty patients (24 patients received 2 immunizations and 6 received placebo) in Zurich University for 1 year after halt of the AN-1792 trial.[15] Twenty patients generated antibodies against Aβ, as determined by tissue amyloid plaque immunoreactivity assay. AD patients who generated antibodies performed markedly better on the Mini Mental State Examination (MMSE) scores after the immunization compared to control patients. As compared to baseline, patients who generated antibodies against Aβ remained unchanged after 1 year. In contrast, patients in the control group worsened significantly.[15] Later, Gilman et al. reported the results of large-scale phase II-A studies that did not include Hock's data.[16] Of the 300 AN1792 (QS-21)-treated patients, 59 (19.7%) developed the antibody response. Complete analysis of all the treated patients demonstrated that no significant differences were found in 11 of 14 cognitive disability global change score between antibody responder and placebo groups. However, antibody responders had an improvement in the Wechsler Memory Verbal-Delayed (WMVer-D) scale. Furthermore, nine-component composite NTB z-score and all memory composite z-score ($p = 0.033$) indicate less worsening at 12 month from the first analysis. CSF examinations performed in a small number of subjects revealed that CSF tau was decreased in antibody responders ($n = 11$) compared with placebo-given patients ($n = 10$; $p < 0.001$).[16] Recently, long-term (6years) effects of 80 patients in phase I clinical trial, were reported.[17] This report contains both expected and unexpected results. Expectedly, cortical Aβ loads were lowered in treated patients than in the control group. Furthermore, in patients with higher antibody titers showed more extensive Aβ removal. Unexpectedly however, there was no statistically significant evidence for improvement in cognitive function or survival, even in patients with high antibody titers. These results suggest that plaque removal is not enough to halt progressive neurodegeneration in AD.

There are a number of possible mechanisms to explain the differences in the outcome. First, Aβ plaque formation might be necessary to initiate pathological processes, but not to progressive neurodegeneration. This hypothesis explains well why there is a poor correlation between Aβ plaque load and the degree of dementia.[18] Second, plaque removal occurs progressively over a period of months.[19] Therefore, it is easily assumed that the improvement of cognitive functions after Aβ removal by the treatment takes a considerable time. Follow-up period may be too short to elucidate the relationship between Aβ clearance and the cognitive function. Third, it is well known that Aβ oligomers are critical factor to induce synaptic dysfunction in AD patients.[20,21] The degree of oligomeric Aβ removal by the treatment should be estimated, which was not performed in these studies. Fourth, the human clinical studies were performed on aged patients with mild to moderate AD.[8] Obviously, anti-Aβ immunotherapies should be initiated on younger patients with mild signs and symptoms before irreversible AD-related brain damages occur.[22] Taken together, further studies are necessary to evaluate the effects of anti-Aβ immunotherapies for AD patients.

Development of New Aβ Peptide Vaccines

To control harmful T cell responses, new Aβ vaccines are developing after the clinical trials with Aβ peptide vaccines. Nasal vaccination with proteosome-based adjuvant plus glatiramer acetate cleared Aβ plaques in Alzheimer model mice.[23] Fine epitope analysis with overlapping oligomers of the Aβ1-42, sequence identified the 1–15 region as a dominant B cell epitope.[24] A short Aβ peptide (Aβ1-15), which contains antibody epitopes but lacks T-cell reactive sites, induced anti-Aβ antibodies in the absence of T cell responses in wild-type mice[25] and significantly reduced Aβ plaques in APP transgenic mice.[26] Aβ epitope vaccine consists of self-B cell epitope of Aβ and non-self T helper cell epitope produced high titers of anti-Aβ antibodies in mice. In addition, the new approach to develop vaccine using virus-like particles (VLPs) was reported.[27] Due to their virus-like nature, VLPs are very immunogenic and their potential to be effective vaccines have long been recognized.[28] Aβ conjugated to VLPs elicited anti-Aβ antibody responses at low doses and without the use of adjuvants. The flexibility of these virus-based display systems allowed to induce antibodies against short Aβ-derived peptides from the amino- and carboxyl-termini of the peptide.[27]

Recently, other clinical trials using second-generation peptide vaccines were started.[29] Wyeth and Elan Inc., developed ACC-001, which contains Aβ1-7 derivatives. The trial was suspended in April 2008 because a patient in a phase II trial was hospitalized with skin vasculitis. Vasculitis was considered as immune, or allergic, reactions. However, the patient was treated successfully and the trial was restarted 6 weeks later. Novartis Inc., developed CAD106 which consists of carrier Qb coupled with Aβ1-6. Phase I trial was finished without problems and Phase II trial was started in 2007 (all information was obtained from press-release of the indicated companies).

Passive Immunization with Anti-Aβ Antibodies

Passive transfer of anti-Aβ antibodies is an alternative strategy, which is effective as well as active immunization in the mouse model of AD. Peripheral administration of antibodies against Aβ peptide was sufficient to reduce amyloid burden. Despite relatively modest serum levels, passively administrated antibodies were able to enter the central nervous system, decorated plaques and induced clearance of Aβ deposits.[30] Passive immunization is more acceptable than active vaccination because it does not need an adjuvant and does not elicit hazardous cellular responses found in the clinical trial of active immunization. In addition, the dose can be controlled easily. However, administration of anti-Aβ antibodies may cause microhemorrhages in old APP mice[19] and its incidence was significantly increased following 5 months of passive immunization.[31]

Phase II clinical trial of Aβ-specific N-terminal directed humanized monoclonal antibodies, bapineuzumab (AAB-001, Elan and Wyeth), was completed in 2008.[29,32] On July 29, 2008, Elan and Wyeth Corporation reported in the press release that the treatment showed beneficial effects on the Apolipoprotein E4 (ApoE4) no-carrier population. However, vasogenic cerebral edema occurred dose-dependently in 12 out of 124 antibody-treated patients and

10 patients carried the ApoE4 allele. Moreover, eight of 12 patients belonged to the high-dosage group. As was mentioned above, it is also well known that a number of microhemorrhages occurred after administration of anti-Aβ in old APP mice.[31,33] These findings suggest that the abrupt reduction of Aβ deposits in the vessel wall may result in the functional change of the blood brain barrier, leading to these adverse effects. Another humanized monoclonal antibody, LY2062430 (Eli Lilly Co.,), is now in the phase II trial.[32] The favorable outcome is expected for LY2062430 because this antibody directs to the central domain of the Aβ1-42 peptide and does not induce intracerebral hemorrhage or vascular pathology in the experiment.[33] However, there are still some problems to be solved in passive immunization. In vivo production of anti-idiotype antibodies, which may neutralize the effects of anti-Aβ antibodies, must be controlled to maintain the effect of the antibodies. Moreover, a serious disadvantage of passive immunization is the cost of the antibodies. The expense of weekly or monthly monoclonal infusion for decades could be extremely difficult from the economic point of view. Many patients cannot afford expensive medical costs which is estimated over several tens thousands dollars totally.

Development of DNA Vaccines as Effective Drugs in the Next Generation

To compensate the disadvantage of conventional immunotherapies, DNA vaccination has been developed as a new therapy for AD, which is simple, easily modified and can be administered without adjuvants.[34,35] At the injection site, vaccines are taken up by muscle cells and the Aβ peptide-protein complex is produced for a certain period.[36] Translated Aβ complex stimulates immune responses in the host, and induced anti-Aβ antibodies.[37] Importantly, immune responses of the host can be easily manipulated to obtain a Th2 type reaction.[34,38,39]

DNA vaccines with viral vectors. Viral DNA vaccines were developed using adeno-associated virus (AAV) vectors[40,41] or adenovirus vector.[42] The effects of viral DNA vaccines are expected to continue for several months after one administration because the viral vector has the high introduction efficiency. An AAV vaccine developed by Zhang et al. could express CB-Aβ1-42 (Cholea toxin B subunit and Aβ1-42 fusion protein) in vivo. A single administration of the AAV vaccine induced a prolonged (at least 12 month) production of Aβ-specific serum IgG (1:4,096–1:16,384) in model mice and resulted in improved ability of memory and cognition, decreased Aβ depositions and plaque-associated astrocytosis in the brain.[43] Hara et al. constructed AAV vaccines that express for Aβ1-43 or Aβ1-21, and administrated to Tg2536 mice transorally. IgG antibodies were detected in the serum at 4 weeks, and existed in the body for more than 6 months. Quantitative analysis of the brain showed a significant decrease of Aβ burden in all vaccinated mice.[41] Intranasal injection of an adenovirus vector encoding Aβ1-42 and granulocyte/macrophage-colony stimulating factor (GM-CSF) decreased the Aβ load in Tg2536 mice.[42]

Although viral DNA vaccines are effective, it remains to be determined whether viral vectors are really safe. In 1999, an 18-year-old patient with an inherited enzyme deficiency died 4 days after gene therapy with a genetically altered adenovirus vector in Pennsylvania, USA.[44] In France, clinical trials of gene therapy with retrovirus vector to treat X-linked severe combined immunodeficiency disease (SCID) were halted due to the development of leukemia.[45] Furthermore, significantly increased incidence of hepatocellular carcinomas and angiosarcomas was noted in rAAV-mediated gene therapy.[46,47] Thus, these problems should be overcome as soon as possible. In addition, limitations to scale up the AAV vector production severely restrict the commercialization and use of AAV-associated DNA vaccines.[48] Taken together, the clinical application of DNA vaccines using viral vectors seems to be difficult at present.

Non-viral DNA vaccines. Others and we have focused on the use of plasmid vectors for Aβ DNA vaccines. Non-viral DNA vaccines have many advantages over those with virus vectors. Non-viral plasmid DNA vaccines can be easily mass-produced at a low cost[34,35] have no possibility of viral infection or transformation.[49,50] Non-viral DNA vaccine with Aβ fused to mouse interleukin-4 (pAβ 42-IL-4) as a molecular adjuvant generated anti-Aβ antibodies in wild type B6 mice.[51] Aβ DNA vaccine with a secretary signal, tissue-type plasminogen activator (tPA) reduces amyloid plaque in mice mode with simultaneous use of low dose Aβ peptide. However, significant reductions of Aβ deposition were not obtained only with these DNA vaccines.[52] In addition, other groups utilized gene-gun for the delivery of DNA vaccines, which has the advantage over peptide vaccination of higher efficacy in breaking self-tolerance and for inducing beneficial Th2-based immune responses[53,54] to reduce the possible adverse effects related to Th1 adverse responses seen with Aβ1-42 peptide vaccine. Aβ1-42 and Aβ1-16 gene were chemically fused and delivered to APPswe/PS1DE9 transgenic mice with gene-gun.[55]

In our laboratory, three types of Aβ DNA vaccines were prepared using a mammalian expression vector and reduce Aβ plaque in AD model mice without side effects.[56] The first vaccine possessed the core 1–42 sequence inserted into plasmid vector (K-Aβ vaccine). The second one possessed the immunoglobulin leader sequence at the N terminus (IgL-Aβ vaccine) to increases the secretion of the Aβ peptide. Furthermore, human immunoglobulin Fc portion was added to the third vaccine to stabilize the vaccine product (Fc-Aβ vaccine). These leader sequences inserted into the plasmid were important for the Aβ secretory property of the vaccines. Supernatants of cultured cells that had been transfected with IgL-Aβ and Aβ-Fc vaccines contained translated proteins, whereas K-Aβ-transfected cells did not secrete the peptide into the extracellular space. K-Aβ vaccine was less effective in Aβ reduction than the former two and was not used in subsequent experiments. AD model mice received 6 weekly and subsequent biweekly injections of the vaccines. Prophylactic treatment, administration before the appearance of amyloid deposition, with Aβ-Fc vaccine demonstrated that the final reduction rate of Aβ burden in the cerebral cortex at 18 months of age was approximately 39% of untreated groups (Fig. 1). Therapeutic treatment, administration after the appearance of Aβ deposition revealed almost equal Aβ plaque reduction as prophylactic treatment (Figs. 1 and 2). Concerning the excessive immune reaction of our vaccine, T cell activation and proliferation, as measured by {^{3}H}-thymidine incorporation of T cells from vaccinated mice, was negative in both wild type B6 and model mice (Fig. 3). Furthermore, 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. Subsequent analysis revealed that Aβ-monomers and dimers were significantly reduced after DNA vaccination.[57] Recently, to make DNA vaccine more safe and

effective, DNA epitope vaccine was developed, which consists of 3 copies of the self-B cell epitope of Aβ1-11, a non-self T helper cell epitope (PADRA), and macrophage-derived cytokine (MDC/CCL22).[58] It generated high titer of anti-Aβ antibody (200–1,000 μg/ml) after 3–5 times vaccination and reduced Aβ plaques in triple transgenic mice. The Aβ oligomers were significantly reduced and behavioral deficit of model mice were improved in the vaccine mice.

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 at a large amount with standard technology. One can make the vaccines at a low cost and the safety has been established. The primary concern for non-viral DNA vaccines is their potential to integrate into the host cell genome. With an integration assay based on purification of high-molecular-weight genomic DNA away from free plasmid using gel electrophoresis, such that the genomic DNA can be assayed for integrated plasmid using a sensitive PCR method. The assay sensitivity was approximately 1 plasmid copy/μg DNA (representing approximately 150,000 diploid cells).[59] When integration occurred, the frequency was 1–8 integrations per 150,000 diploid cells, which would be below the spontaneous mutation rate.[59] Thus, the risk of mutation due to integration of plasmid DNA vaccines following intramuscular injection is negligible. In addition, if unwanted side effects occur, they can be easily controlled by stopping further administration of the vaccine because their half-life within the body is shorter than viral vaccines.[60] When considering clinical use, AD patients receive vaccines for life long and such advantages are quite important factors for the choice of the treatment. Non-viral plasmid vector is the best choice at present for the DNA vaccine treatment against Alzheimer's disease.

Mechanisms of Aβ Reduction with Vaccine Therapies

Immunotherapies against AD are effective not only in the mouse model[4,7] but also in human clinical trials.[10] However, the mechanisms of Aβ reduction in the brain remain to be elucidated. There are at least three hypotheses. One possible mechanism is operated through microglial phagocytosis.[61,62] When examined in an ex vivo assay with sections of PDAPP mice or AD brain tissue, antibodies against Aβ-peptide triggered microglial cells to clear plaques through Fc receptor-mediated phagocytosis and subsequent peptide degradation.[30] However, recent reports suggest that other receptors also participate to clear Aβ plaques. Immunization of Tg2576 mice crossed with Fc receptor gamma knockout mice were as efficient at clearing plaques as Tg2576 mice alone.[63] Murine microglia bind and internalize fibrillar Aβ microaggregates via the type A scavenger receptor.[64] The second mechanism is direct binding of antibodies leads to dissolution of Aβ peptides.[65,66] Anti-Aβ antibodies arose from mice selectively directed against residues 4–10 of Aβ42, and that these antibodies inhibit both Aβ fibrillogenesis and cytotoxicity without eliciting an inflammatory response.[67] Using in vivo multiphoton microscopy, FITC-labeled $F(ab')_2$ fragments of 3d6 (which lack the Fc region of the antibody) also led to clearance of 45% of the deposits within 3 days, similar to the results obtained with full-length 3d6 antibody.[65] The third mechanism is that blood-circulating antibodies increase the net efflux of Aβ peptides from the brain to the circulation (the peripheral sink hypothesis).[68,69] The monoclonal antibody m266, having a very high affinity for soluble

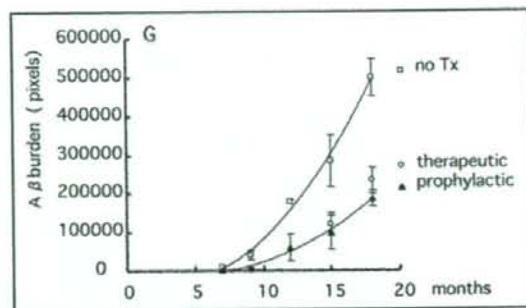

Figure 1. Reduction of Aβ burden in APP23 mice after DNA vaccination. The overall quantitative analysis is depicted. The amyloid deposition was first detected in untreated mice at 7 months of age and rapidly increased after 15 months of age (open squares). Prophylactic administration of Fc-Aβ vaccine 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 were almost same as those of prophylactic administration (open circles). Each group consists of 4–6 mice.

Aβ, may not require entering the brain, which can extract free brain Aβ into blood circulation when given peripherally.[68] Peripheral administration of m266 to PDAPP transgenic mice results in a rapid 1,000-fold increase in plasma Aβ,[68] which can rapidly reverse memory deficits in both an object recognition task and a holeboard learning and memory task.[69]

We examined these possible Aβ reduction mechanisms to determine which the major route of Aβ clearance is in our DNA vaccination system.[57] Immunohistochemical examinations revealed that activated microglia significantly increased after DNA vaccination not only around plaques but also in areas remote from plaques. Furthermore, microglia in treated mice phagocytosed Aβ debris more frequently than in untreated mice. Although microglia had an activated form, they did not produce a significant amount of TNFα. Amyloid plaque immunoreactivity and Aβ concentration in plasma increased slightly in the treated group at 9, but not at 15, months of age. Collectively, these data indicate that phagocytosis of Aβ deposits by microglia play a central role in Aβ reduction after DNA vaccination. There are at least two explanations for these results. First, anti-Aβ antibodies in plasma were mildly elevated after DNA vaccination,[56] compared with active immunization,[4] which is sufficient for Aβ clearance by microglia but not for transition of Aβ from the brain to the blood stream. Second, cerebral amyloid angiopathy may progress, especially in the late stage, and interfere with the perivascular drainage pathway of Aβ.[70] The findings obtained in this study provide useful information for the development of new and more effective DNA vaccines against AD because DNA vaccines are easily reconstructed by adding or changing the sequence in the plasmid vector.

Recently, it is also known that Aβ clearance is augmented by drugs via the antibody-independent and cell-mediated pathways. Nasal vaccination with a proteosome-based adjuvant that is well tolerated in humans plus glatiramer acetate, an FDA-approved synthetic copolymer used for treatment of multiple sclerosis, decreases Aβ plaques in model mice.[23] This effect did not require the presence of anti-Aβ antibodies in the brain because Aβ reduction by the treatment was also observed in B cell-deficient (Ig mu-null) mice. Vaccinated

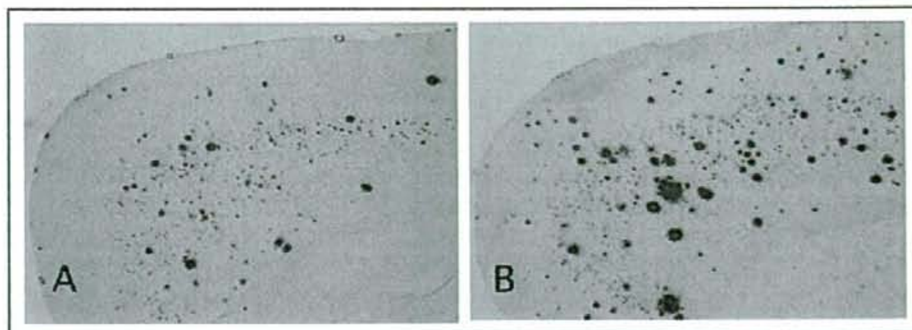

Figure 2. Aβ burden reduction at 18 months of age after therapeutic treatment starting from 12 months. While large Aβ deposits (>100 μm) were observed in the frontal cortex of control mice at 18 months of age (B), significant reduction was observed after 6 month therapeutic administration of the IgL-Aβ vaccine (A). Magnification (A and B), x27.

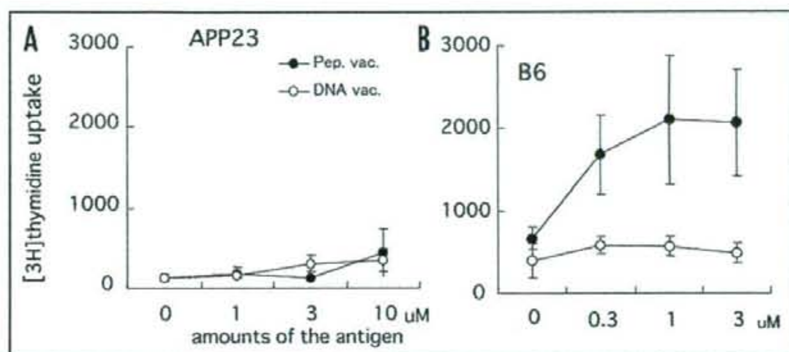

Figure 3. T cell responses in APP23 (A) and B6 (B) mice after immunization with Aβ peptide/CFA or DNA vaccination. Lymphocytes isolated from two strains were incubated with Aβ peptide (0–10 μM) for 3 days. Incorporation of {^{3}H} thymidine was measured by liquid scintillation spectrometry. In APP23 mice, neither T cells from peptide-immunized mice nor those from DNA vaccinated mice were activated in the presence of Aβ1-42 (n = 3) (A). In contrast, Aβ peptide immunization, but not DNA vaccination, induced a significant T cell response in B6 mice (n = 3) (B). All the data are the mean values ± S.D. and the representative results from three different experiments are shown. Figures 1–3 are reproduced with permission from PNAS (ref. 56).

animals developed activated microglia that colocalized with Aβ fibrils, and the extent of microglial activation correlated strongly with the decrease in Aβ fibrils.[23]

Role of Microglia in Aβ Accumulation or Reduction

Microglia are resident brain cells and react with various pathological conditions including autoimmune inflammation, infection and degeneration in the central nervous system. Previously, microglia were thought to be harmful and toxic to neurons in the AD brain as there were sustained inflammatory responses including complement activation;[71] however it becomes consensus that microglia could play a neuroprotective role under a certain circumstance as well as pathogenic role in the disease processes.[72-75] It has been shown that microglia react with Aβ plaques and phagocytose Aβ deposits under various stimulations.[76-79] When activated, microglia express receptors involved in the clearance and phagocytosis of Aβ (e.g., class A scavenger receptor, CD36 and receptor for advanced-glycosylation endproducts).[80,81] Soluble Aβ can be directly bound by microglial receptors such as heparin sulphate proteoglycans (HSPGs),[82] insulin receptors[83] and proteinase inhibitor serpin-enzyme complex receptor (SEC-R),[84] resulting in phagocytosis of soluble Aβ. Activated microglia can degrade Aβ by releasing Aβ degrading enzymes including metalloproteases, insulin-degrading enzyme[55,85] and gelatinize A.[86] In addition, activation of microglia with toll-like receptors (TLRs), a family of pattern-recognition receptors in the innate immune system, markedly boosted ingestion of Aβ in vitro.[87]

Conversely, dysfunction of microglia is related to the progression of AD. A large number of apoptotic microglia are present in AD brain.[88-90] Microglia from old PS1-APP mice showed decrease expression of the Aβ-binding scavenger receptors A (SRA), CD36 and RAGE (receptor for advanced-glycosylation endproducts), and the Aβ-degrading enzymes insulysin, neprilysin, and MMP9, compared with their littermate controls.[91] PS1-APP microglia had a 2.5-fold increase in the proinflammatory cytokines IL-1β (interleukin-1β) and tumor necrosis factor-α (TNFα), suggesting that there is an inverse correlation between cytokine production and Aβ clearance.[91] In addition, microglial accumulation plays a protective role in the early stages of AD by promoting Aβ clearance. The deficiency of CC-chemokine receptor 2 (Ccr2),[92] a chemokine receptor expressed on microglia, which mediates the accumulation of mononuclear phagocytes at the site of inflammation, accelerates early disease progression and markedly impairs microglial accumulation in Tg2576 mice.[93] These findings suggest that Aβ clearance by microglia is essential for the maintenance of normal neural function in the brain.

Effect of Aβ Vaccines in Non-Human Primates

Treatments of non-human primates with DNA vaccines provide useful information as an essential step before clinical trials. As non-human primates have the same Aβ sequence as humans and develop Aβ plaques with aging, certain kinds of monkeys provide

an attractive Alzheimer's disease animal model.[94] Investigators have been employed vervet monkeys for studies on brain injuries[95,96] and AD.[97,98] Lemere et al. immunized five aged vervets (16–25 yr) with Aβ peptide over 10 months and examined plasma and CSF samples longitudinally. By day 42, immunized monkeys generated plasma Aβ antibodies that labeled plaques in the human AD and model mouse brain. The antibody levels peaked at day 100 (1,677 μg/ml in plasma, 2.1 μg/ml in CSF) in the immunized animals and dropped to relatively steady levels by Day 301 (452 mg/ml in plasma, 0.2 mg/ml in CSF). Soluble Aβ levels were elevated approximately 2- to 5-fold in plasma and decreased up to 64% in CSF in immunized vervets. Insoluble Aβ was decreased by 66% in brain homogenates of the immunized animals compared to controls.[99] Since it is difficult to obtain vervet monkeys, rhesus monkeys (Macaca mulatta) were also examined extensively.[100] Senile plaques appear in variable numbers in animals >23 years but were not seen in monkeys <15 years of age.[101] Aβ deposits were observed in the cerebral cortex of all aged animals.[102] Gandy et al. injected aggregated Aβ1-42 to rhesus monkeys (Macaca mulatta). Immunized monkeys developed anti-Aβ titers exceeding 1:1,000, and their plasma Aβ levels were 5–10-fold higher than the plasma Aβ levels observed in monkeys vaccinated with aggregated amylin.[103]

Recently, we evaluated the effects of DNA vaccines on Aβ reduction found in aged rhesus monkeys (15–18 years old) (manuscript in preparation). DNA vaccine and empty vector (control) at a dose of 3 mg were injected 15 times for 6 months (n = 3 in each group). Quantitative analysis revealed a significant reduction of Aβ in treated monkeys without obvious side effects. Previous two studies[99,103] and our examination clearly demonstrated Aβ reduction in monkeys after vaccination. Analysis of oligomeric Aβ and cognitive assessment will be necessary to determine whether such immunotherapies provide beneficial effects on behavioral disorders. Collectively, these findings suggest that DNA vaccines are also effective in Aβ reduction in non-human primates.

Conclusion

Current drug therapies for AD aim at slowing the cognitive decline and ameliorating the affective and behavioral signs associated with disease progression. Based on the results obtained in animal and human clinical studies, Aβ vaccine therapy is a principal strategy for AD treatment. Active peptide immunotherapy and passive immunotherapy have some problems to be solved before clinical application. Newly developed DNA vaccines were effective in reducing Aβ plaque on model mice and aged monkeys. Oligomeric Aβ, which is most neurotoxic among Aβ species, was also decreased in the mouse brain after vaccination. Although preclinical trials are under way, DNA vaccination will be a promising therapy for Alzheimer's disease in the near future.

References

1. Citron M. Alzheimer's disease: treatments in discovery and development. Nat Neurosci 2002; 5:1055-7.
2. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci 1991; 12:383-8.
3. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. Science 1992; 256:184-5.
4. Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 1999; 400:173-7.
5. Janus C, D'Amelio S, Amitay O, Chishti MA, Strome R, Fraser P, Carlson GA, Roder JC, St. George-Hyslop P, Westaway D. Spatial learning in transgenic mice expressing human presenilin 1 (PS1) transgenes. Neurobiol Aging 2000; 21:541-9.
6. Morgan D, Diamond DM, Gottschall PE, Ugen KE, Dickey C, Hardy J, Duff K, Jantzen P, DiCarlo G, Wilcock D, Connor K, Hatcher J, Hope C, Gordon M, Arendash GW. A beta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. Nature 2000; 408:982-5.
7. Janus C, Pearson J, McLaurin J, Mathews PM, Jiang Y, Schmidt SD, Chishti MA, Horne P, Heslin D, French J, Mount HT, Nixon RA, Mercken M, Bergeron C, Fraser PE, St. George-Hyslop P, Westaway D. A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature 2000; 408:979-82.
8. Bayer AJ, Bullock R, Jones RW, Wilkinson D, Paterson KR, Jenkins L, Millais SB, Donoghue S. Evaluation of the safety and immunogenicity of synthetic Abeta42 (AN1792) in patients with AD. Neurology 2005; 64:94-101.
9. Orgogozo JM, Gilman S, Dartigues JF, Laurent B, Puel M, Kirby LC, Jouanny P, Dubois B, Eisner L, Flitman S, Michel BF, Boada M, Frank A, Hock C. Subacute meningoencephalitis in a subset of patients with AD after Abeta42 immunization. Neurology 2003; 61:46-54.
10. Nicoll JA, Wilkinson D, Holmes C, Steart P, Markham H, Weller RO. Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. Nat Med 2003; 9:448-52.
11. Pride M, Seubert P, Grundman M, Hagen M, Eldridge J, Black RS. Progress in the active immunotherapeutic approach to Alzheimer's disease: clinical investigations into AN1792-associated meningoencephalitis. Neurodegener Dis 2008; 5:194-6.
12. Bacskai BJ, Kajdasz ST, Christie RH, Carter C, Games D, Seubert P, Schenk D, BT H. Imaging of amyloid-beta deposits in brains of living mice permits direct observation of clearance of plaques with immunotherapy. Nat Med 2001; 7:369-72.
13. Ferrer I, Boada Rovira M, Sanchez Guerra ML, Rey MJ, Costa-Jussa F. Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. Brain Pathol 2004; 14:11-20.
14. Masliah E, Hansen L, Adame A, Crews L, Bard F, Lee C, Seubert P, Games D, Kirby L, Schenk D. Abeta vaccination effects on plaque pathology in the absence of encephalitis in Alzheimer disease. Neurology 2005; 64:129-31.
15. Hock C, Konietzko U, Streffer JR, Tracy J, Signorell A, Muller-Tillmanns B, Lemke U, Henke K, Moritz E, Garcia E, Wollmer MA, Umbricht D, de Quervain DJ, Hofmann M, Maddalena A, Papassotiropoulos A, Nitsch RM. Antibodies against beta-amyloid slow cognitive decline in Alzheimer's disease. Neuron 2003; 38:547-54.
16. Gilman S, Koller M, Black RS, Jenkins L, Griffith SG, Fox NC, Eisner L, Kirby L, Rovira MB, Forette F, Orgogozo JM. Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial. Neurology 2005; 64:1553-62.
17. Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, Bayer A, Jones RW, Bullock R, Love S, Neal JW, Zotova E, Nicoll JA. Long-term effects of Abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. Lancet 2008; 372:216-23.
18. Davis DG, Schmitt FA, Wekstein DR, Markesbery WR. Alzheimer neuropathologic alterations in aged cognitively normal subjects. J Neuropathol Exp Neurol 1999; 58:376-88.
19. Wilcock DM, Rojiani A, Rosenthal A, Subbarao S, Freeman MJ, Gordon MN, Morgan D. Passive immunotherapy against Abeta in aged APP-transgenic mice reverses cognitive deficits and depletes parenchymal amyloid deposits in spite of increased vascular amyloid and microhemorrhage. J Neuroinflammation 2004; 1:24.
20. Patton RL, Kalback WM, Esh CL, Kokjohn TA, Van Vickle GD, Luehrs DC, Kuo YM, Lopez J, Brune D, Ferrer I, Masliah E, Newel AJ, Beach TG, Castano EM, Roher AE. Amyloid-beta peptide remnants in AN-1792-immunized Alzheimer's disease patients: a biochemical analysis. Am J Pathol 2006; 169:1048-63.
21. Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 2007; 8:101-12.
22. Boche D, Nicoll JA. The role of the immune system in clearance of Abeta from the brain. Brain Pathol 2008; 18:267-78.
23. 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; 115:2423-33.
24. Cribbs DH, Ghochikyan A, Vasilevko V, Tran M, Petrushina I, Sadzikava N, Babikyan D, Kesslak P, Kieber-Emmons T, Cotman CW, Agadjanyan MG. Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. Int Immunol 2003; 15:505-14.
25. Maier M, Seabrook TJ, Lemere CA. Developing novel immunogens for an effective, safe Alzheimer's disease vaccine. Neurodegener Dis 2005; 2:267-72.
26. Maier M, Seabrook TJ, Lazo ND, Jiang L, Das P, Janus C, Lemere CA. 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; 26:4717-28.
27. Chackerian B, Rangel M, Hunter Z, Peabody DS. Virus and virus-like particle-based immunogens for Alzheimer's disease induce antibody responses against amyloid-beta without concomitant T cell responses. Vaccine 2006; 24:6321-31.
28. Jennings GT, Bachmann MF. The coming of age of virus-like particle vaccines. Biol Chem 2008; 389:521-36.
29. Masters CL, Beyreuther K. Alzheimer's centennial legacy: prospects for rational therapeutic intervention targeting the Abeta amyloid pathway. Brain 2006; 129:2823-39.

30. Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Lieberburg I, Motter R, Nguyen M, Soriano F, Vasquez N, Weiss K, Welch B, Seubert P, Schenk D, Yednock T. 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.
31. Pfeifer M, Boncristiano S, Bondolfi L, Stalder A, Deller T, Staufenbiel M, Mathews PM, Jucker M. Cerebral hemorrhage after passive anti-Abeta immunotherapy. Science 2002; 298:1379.
32. Brody DL, Holtzman DM. Active and passive immunotherapy for neurodegenerative disorders. Annu Rev Neurosci 2008; 31:175-93.
33. Racke MM, Boone LI, Hepburn DL, Parsadainian M, Bryan MT, Ness DK, Piroozi KS, Jordan WH, Brown DD, Hoffman WP, Holtzman DM, Bales KR, Gitter BD, May PC, Paul SM, DeMattos RB. Exacerbation of cerebral amyloid angiopathy-associated microhemorrhage in amyloid precursor protein transgenic mice by immunotherapy is dependent on antibody recognition of deposited forms of amyloid beta. J Neurosci 2005; 25:629-36.
34. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. Nature 1992; 356:152-4.
35. Barry MA, Lai WC, Johnston SA. Protection against mycoplasma infection using expression-library immunization. Nature 1995; 377:632-5.
36. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. Science 1990; 247:1465-8.
37. Wang B, Ugen KE, Srikantan V, Agadjanyan MG, Dang K, Refaeli Y, Sato AI, Boyer J, Williams WV, Weiner DB. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc Natl Acad Sci USA 1993; 90:4156-60.
38. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259:1745-9.
39. Hoffman SL, Doolan DL, Sedegah M, Gramzinski R, Wang H, Gowda K, Hobart P, Margalith M, Norman J, Hedstrom RC. Nucleic acid malaria vaccines. Current status and potential. Ann N Y Acad Sci 1995; 772:88-94.
40. Zhang Z, Yu CX. Effect of melatonin on learning and memory impairment induced by aluminum chloride and its mechanism. Yao Xue Xue Bao 2002; 37:682-6.
41. Hara H, Monsonego A, Yuasa K, Adachi K, Xiao X, Takeda S, Takahashi K, Weiner HL, Tabira T. Development of a safe oral Abeta vaccine using recombinant adeno-associated virus vector for Alzheimer's disease. J Alzheimers Dis 2004; 6:483-8.
42. Kim HD, Kong FK, Cao Y, Lewis TL, Kim H, Tang DC, Fukuchi K. Immunization of Alzheimer model mice with adenovirus vectors encoding amyloid beta-protein and GM-CSF reduces amyloid load in the brain. Neurosci Lett 2004; 370:218-23.
43. Zhang J, Wu X, Qin C, Qi J, Ma S, Zhang H, Kong Q, Chen D, Ba D, He W. 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.
44. Marshall E. Gene therapy. Second child in French trial is found to have leukemia. Science 2003; 299:320.
45. Check E. Gene therapy put on hold as third child develops cancer. Nature 2005; 433:561.
46. Donsante A, Vogler C, Muzyczka N, Crawford JM, Barker J, Flotte T, Campbell-Thompson M, Daly T, Sands MS. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. Gene Ther 2001; 8:1343-6.
47. Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, Sands MS. AAV vector integration sites in mouse hepatocellular carcinoma. Science 2007; 317:477.
48. Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum Gene Ther 2002; 13:1935-43.
49. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Hum Gene Ther 2001; 12:861-70.
50. Nishikawa M, Hashida M. Nonviral approaches satisfying various requirements for effective in vivo gene therapy. Biol Pharm Bull 2002; 25:275-83.
51. Ghochikyan A, Vasilevko V, Petrushina I, Movsesyan N, Babikyan D, Tian W, Sadzikava N, Ross TM, Head E, Cribbs DH, Agadjanyan MG. 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.
52. Schiltz JG, Salzer U, Mohajeri MH, Franke D, Heinrich J, Pavlovic J, Wollmer MA, Nitsch RM, Moelling K. 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.
53. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. J Immunol 1997; 158:2278-84.
54. Weiss R, Scheiblhofer S, Freund J, Ferreira F, Livey I, Thalhamer J. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. Vaccine 2002; 20:3148-54.
55. Qu B, Boyer PJ, Johnston SA, Hynan LS, Rosenberg RN. Abeta42 gene vaccination reduces brain amyloid plaque burden in transgenic mice. J Neurol Sci 2006; 244:151-8.
56. Okura Y, Miyakoshi A, Kohyama K, Park IK, Staufenbiel M, Matsumoto Y. Nonviral Abeta DNA vaccine therapy against Alzheimer's disease: long-term effects and safety. Proc Natl Acad Sci USA 2006; 103:9619-24.
57. Okura Y, Kohyama K, Park IK, Matsumoto Y. Nonviral DNA vaccination augments microglial phagocytosis of beta-amyloid deposits as a major clearance pathway in an Alzheimer disease mouse model. J Neuropathol Exp Neurol 2008; 67:1063-71.
58. Movsesyan N, Ghochikyan A, Mkrtichyan M, Petrushina I, Davtyan H, Olkhanud PB, Head E, Biragyn A, Cribbs DH, Agadjanyan MG. Reducing AD-like pathology in 3xTg-AD mouse model by DNA epitope vaccine—a novel immunotherapeutic strategy. PLoS ONE 2008; 3:2124.
59. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG, 2nd, Harper LB, Beare CM, Bagdon WJ, Nichols WW. Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. Intervirology 2000; 43:258-72.
60. Song YK, Liu F, Chu S, Liu D. Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. Hum Gene Ther 1997; 8:1585-94.
61. Bard F, Barbour R, Cannon C, Carretto R, Fox M, Games D, Guido T, Hoenow K, Hu K, Johnson-Wood K, Khan K, Kholodenko D, Lee C, Lee M, Motter R, Nguyen M, Reed A, Schenk D, Tang P, Vasquez N, Seubert P, Yednock T. Epitope and isotype specificities of antibodies to beta-amyloid peptide for protection against Alzheimer's disease-like neuropathology. Proc Natl Acad Sci USA 2003; 100:2023-8.
62. Wilcock DM, Munireddy SK, Rosenthal A, Ugen KE, Gordon MN, Morgan D. Microglial activation facilitates Abeta plaque removal following intracranial anti-Abeta antibody administration. Neurobiol Dis 2004; 15:11-20.
63. Das P, Howard V, Loosbrock N, Dickson D, Murphy MP, Golde TE. Amyloid-beta immunization effectively reduces amyloid deposition in FcRgamma$^{-/-}$ knock-out mice. J Neurosci 2003; 23:8532-8.
64. Brazil MI, Chung H, Maxfield FR. Effects of incorporation of immunoglobulin G and complement component C1q on uptake and degradation of Alzheimer's disease amyloid fibrils by microglia. J Biol Chem 2000; 275:16941-7.
65. Bacskai BJ, Kajdasz ST, McLellan ME, Games D, Seubert P, Schenk D, Hyman BT. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta in vivo by immunotherapy. J Neurosci 2002; 22:7873-8.
66. Solomon B, Koppel R, Frankel D, Hanan-Aharon E. Disaggregation of Alzheimer beta-amyloid by site-directed mAb. Proc Natl Acad Sci USA 1997; 94:4109-12.
67. McLaurin J, Cecal R, Kierstead ME, Tian X, Phinney AL, Manea M, French JE, Lambermon MH, Darabie AA, Brown ME, Janus C, Chishti MA, Horne P, Westaway D, Fraser PE, Mount HT, Przybylski M, St. George-Hyslop P. Therapeutically effective antibodies against amyloid-beta peptide target amyloid-beta residues 4–10 and inhibit cytotoxicity and fibrillogenesis. Nat Med 2002; 8:1263-9.
68. DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. 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; 98:8850-5.
69. Dodart JC, Bales KR, Gannon KS, Greene SJ, DeMattos RB, Mathis C, DeLong CA, Wu S, Wu X, Holtzman DM, Paul SM. Immunization reverses memory deficits without reducing brain Abeta burden in Alzheimer's disease model. Nat Neurosci 2002; 5:452-7.
70. Weller RO, Massey A, Newman TA, Hutchings M, Kuo YM, Roher AE. Cerebral amyloid angiopathy: amyloid beta accumulates in putative interstitial fluid drainage pathways in Alzheimer's disease. Am J Pathol 1998; 153:725-33.
71. McGeer PL, McGeer EG. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 1995; 21:195-218.
72. Town T, Tan J, Sansone N, Obregon D, Klein T, Mullan M. Characterization of murine immunoglobulin G antibodies against human amyloid-beta1-42. Neurosci Lett 2001; 307:101-4.
73. Schenk DB, Yednock T. The role of microglia in Alzheimer's disease: friend or foe? Neurobiol Aging 2002; 23:677-9.
74. Morgan D, Gordon MN, Tan J, Wilcock D, Rojiani AM. Dynamic complexity of the microglial activation response in transgenic models of amyloid deposition: implications for Alzheimer therapeutics. J Neuropathol Exp Neurol 2005; 64:743-53.
75. Walker DG, Lue LF. Investigations with cultured human microglia on pathogenic mechanisms of Alzheimer's disease and other neurodegenerative diseases. J Neurosci Res 2005; 81:412-25.
76. Wisniewski HM, Barcikowska M, Kida E. Phagocytosis of beta/A4 amyloid fibrils of the neuritic neocortical plaques. Acta Neuropathol (Berl) 1991; 81:588-90.
77. Rogers J, Strohmeyer R, Kovelowski CJ, Li R. Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. Glia 2002; 40:260-9.
78. Herber DL, Roth LM, Wilson D, Wilson N, Mason JE, Morgan D, Gordon MN. Time-dependent reduction in Abeta levels after intracranial LPS administration in APP transgenic mice. Exp Neurol 2004; 190:245-53.
79. Akiyama H, McGeer PL. Specificity of mechanisms for plaque removal after A beta immunotherapy for Alzheimer disease. Nat Med 2004; 10:117-8.
80. Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli A, Nawroth P, Stern D, Schmidt AM. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature 1996; 382:685-91.
81. El Khoury J, Hickman SE, Thomas CA, Loike JD, Silverstein SC. Microglia, scavenger receptors, and the pathogenesis of Alzheimer's disease. Neurobiol Aging 1998; 19:81-4.
82. Giulian D, Haverkamp LJ, Yu J, Karshin W, Tom D, Li J, Kazanskaia A, Kirkpatrick J, Roher AE. The HHQK domain of beta-amyloid provides a structural basis for the immunopathology of Alzheimer's disease. J Biol Chem 1998; 273:29719-26.
83. Xie L, Helmerhorst E, Taddei K, Plewright B, Van Bronswijk W, Martins R. Alzheimer's beta-amyloid peptides compete for insulin binding to the insulin receptor. J Neurosci 2002; 22:221.

84. Boland K, Behrens M, Choi D, Manias K, Perlmutter DH. The serpin-enzyme complex receptor recognizes soluble, nontoxic amyloid-beta peptide but not aggregated, cytotoxic amyloid-beta peptide. J Biol Chem 1996; 271:18032-44.
85. Qiu WQ, Walsh DM, Ye Z, Vekrellis K, Zhang J, Podlisny MB, Rosner MR, Safavi A, Hersh LB, Selkoe DJ. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. J Biol Chem 1998; 273:32730-8.
86. Yamada T, Miyazaki K, Koshikawa N, Takahashi M, Akatsu H, Yamamoto T. Selective localization of gelatinase A, an enzyme degrading beta-amyloid protein, in white matter microglia and in Schwann cells. Acta Neuropathol 1995; 89:199-203.
87. Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K. Role of toll-like receptor signalling in Abeta uptake and clearance. Brain 2006; 129:3006-19.
88. Yang F, Sun X, Beech W, Teter B, Wu S, Sigel J, Vinters HV, Frautschy SA, Cole GM. Antibody to caspase-cleaved actin detects apoptosis in differentiated neuroblastoma and plaque-associated neurons and microglia in Alzheimer's disease. Am J Pathol 1998; 152:379-89.
89. Jellinger KA, Stadelmann CH. The enigma of cell death in neurodegenerative disorders. J Neural Transm Suppl 2000; 21-36.
90. Jellinger KA, Stadelmann C. Mechanisms of cell death in neurodegenerative disorders. J Neural Transm Suppl 2000; 59:95-114.
91. Hickman SE, Allison EK, El Khoury J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci 2008; 28:8354-60.
92. Boddeke EW, Meigel I, Frentzel S, Gourmala NG, Harrison JK, Buttini M, Spleiss O, Gebicke-Harter P. Cultured rat microglia express functional beta-chemokine receptors. J Neuroimmunol 1999; 98:176-84.
93. El Khoury J, Toft M, Hickman SE, Means TK, Terada K, Geula C, Luster AD. Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. Nat Med 2007; 13:432-8.
94. Selkoe DJ, Bell DS, Podlisny MB, Price DL, Cork LC. Conservation of brain amyloid proteins in aged mammals and humans with Alzheimer's disease. Science 1987; 235:873-7.
95. Raleigh MJ, Steklis HD, Ervin FR, Kling AS, McGuire MT. The effects of orbitofrontal lesions on the aggressive behavior of vervet monkeys (Cercopithecus aethiops sabaeus). Exp Neurol 1979; 66:158-68.
96. Raleigh MJ, Brammer GL, McGuire MT, Pollack DB, Yuwiler A. Individual differences in basal cisternal cerebrospinal fluid 5-HIAA and HVA in monkeys. The effects of gender, age, physical characteristics and matrilineal influences. Neuropsychopharmacology 1992; 7:295-304.
97. Liberini P, Pioro EP, Maysinger D, Ervin FR, Cuello AC. Long-term protective effects of human recombinant nerve growth factor and monosialoganglioside GM1 treatment on primate nucleus basalis cholinergic neurons after neocortical infarction. Neuroscience 1993; 53:625-37.
98. Pioro EP, Maysinger D, Ervin FR, Desypris G, Cuello AC. Primate nucleus basalis of Meynert p75NGFR-containing cholinergic neurons are protected from retrograde degeneration by the ganglioside GM1. Neuroscience 1993; 53:49-56.
99. Lemere CA, Beierschmitt A, Iglesias M, Spooner ET, Bloom JK, Leverone JF, Zheng JB, Seabrook TJ, Louard D, Li D, Selkoe DJ, Palmour RM, Ervin FR. Alzheimer's disease abeta vaccine reduces central nervous system abeta levels in a non-human primate, the Caribbean vervet. Am J Pathol 2004; 165:283-97.
100. Uno H, Alsum PB, Dong S, Richardson R, Zimbric ML, Thieme CS, Houser WD. Cerebral amyloid angiopathy and plaques, and visceral amyloidosis in aged macaques. Neurobiol Aging 1996; 17:275-81.
101. Struble RG, Price DL Jr, Cork LC, Price DL. Senile plaques in cortex of aged normal monkeys. Brain Res 1985; 361:267-75.
102. Sani S, Traul D, Klink A, Niaraki N, Gonzalo-Ruiz A, Wu CK, Geula C. Distribution, progression and chemical composition of cortical amyloid-beta deposits in aged rhesus monkeys: similarities to the human. Acta Neuropathol 2003; 105:145-56.
103. Gandy S, DeMattos RB, Lemere CA, Heppner FL, Leverone J, Aguzzi A, Ershler WB, Dai J, Fraser P, St. George Hyslop P, Holtzman DM, Walker LC, Keller ET. Alzheimer's Abeta vaccination of rhesus monkeys (Macaca mulatta). Mech Ageing Dev 2004; 125:149-51.

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Characterization of CD8-positive Macrophages Infiltrating the Central Nervous System of Rats With Chronic Autoimmune Encephalomyelitis

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$CD8^+$ macrophages appear in the central nervous system (CNS) under various pathological conditions such as trauma and ischemia. Furthermore, macrophages expressing CD8 were found in CNS lesions of chronic, but not acute, experimental autoimmune encephalomyelitis (EAE). To further characterize cells with this phenotype, we examined $CD8^+$ macrophages/monocytes in the CNS and peripheral organs during the course of acute and chronic EAE that had been induced by immunization of rats with myelin basic protein and myelin oligodendrocyte glycoprotein, respectively. Counting $CD8^+$ macrophages in CNS lesions revealed that their numbers increased reaching about 60% of total infiltrating macrophages in chronic EAE, while $CD8^+$ macrophages remained less than 5% throughout the course of acute EAE. Unexpectedly, however, higher abundance of $CD8^+$ monocytes/macrophages in the peripheral blood was found in both acute and chronic EAE. Real-time polymerase chain reaction analysis revealed no significant difference in the levels of chemokines and chemokine receptors of blood $CD8^+$ monocytes between acute and chronic EAE. mRNA expression of perforin, a cytotoxic substance, was up-regulated in $CD8^+$ monocytes compared with that of $CD8^-$ monocytes in both acute and chronic EAE. These findings suggest that activated $CD8^+$ macrophages may play a cytotoxic role in chronic EAE lesions and that cells other than $CD8^+$ monocytes/macrophages determined the difference in CNS pathology between acute and chronic EAE. Analysis of $CD8^+$ monocytes/macrophages may provide useful information to permit further dissect the pathomechanisms of multiple sclerosis and to develop effective immunotherapies against autoimmune diseases in the CNS. © 2008 Wiley-Liss, Inc.

Key words: experimental autoimmune encephalomyelitis; chronicity; CD8; macrophage

Multiple sclerosis (MS), which is considered to be an autoimmune disease involving the central nervous system (CNS) (Sospendra and Martin, 2005), is characterized by relapse and remission of the disease (Lublin and Reingold, 1996). In about half of all MS patients, the clinical course changes from relapsing–remitting to secondary progressive MS after 10 years (Noseworthy et al., 2000). However, little is known with regard to the pathomechanism of disease progression. In experimental settings, it is suggested that encephalitogenic T-cell activation is closely associated with disease relapse and progression because inactivation or elimination of pathogenic T cells prevented further relapse (Chen et al., 1998; Shao et al., 2003; Pryce et al., 2005). In contrast, tolerance induction to T cells failed to halt progression of a certain type of experimental autoimmune encephalomyelitis (EAE), suggesting that non-T immune cells, such as macrophages (MΦ), are involved in disease progression (Pryce et al., 2005).

MΦ are major immunocompetent cells that have multiple functions. As reviewed by Duffield (2003), MΦ in inflammatory lesions either promote immune-mediated damage or act as tissue-healing cells, depending on their subtypes, the stage of inflammation, and other undetermined factors. In vitro studies demonstrated that MΦ phagocytose degraded myelin proteins such as myelin basic protein (MBP), proteolipid protein, and myelin oligodendrocyte glycoprotein (MOG) and released tumor necrosis factor alpha (TNF-α) and nitric oxide, which may further contribute to the overall process of demyelination during MS and EAE (van der Laan et al., 1996; van der Goes et al., 2005). Recently, it was also reported that a large number of $CD8^+$ MΦ were found in the spinal cord of rats with chronic, but not acute, EAE (Schroeter et al., 2003), suggesting that $CD8^+$ MΦ play a role in the progression of EAE. Importantly, $CD8^+$ monocytes (MO)/MΦ were found in the peripheral blood of healthy human subjects and in the target

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organ of patients with autoimmune disease (Nakamura et al., 2004; Baba et al., 2006).

In the present study, we tried to elucidate the pathomechanisms of the disease progression of chronic EAE by examining the role of $CD8^+$ MΦ that infiltrate CNS lesions. For this purpose, we first performed quantitative and longitudinal examinations of $CD8^+$ MΦ in injured spinal cord tissues. Then we measured the abundance and the kinetics of this MΦ subpopulation in the peripheral blood and lymphoid organs during EAE. Finally, we determined the levels of chemokine, chemokine receptor, and MΦ-associated factor mRNA of sorted $CD8^+$ MO in the peripheral organs in comparison with those of the $CD8^-$ counterpart. The present analysis revealed several interesting features of $CD8^+$ MΦ and strongly suggested that infiltration of $CD8^+$ MΦ into the CNS is closely associated with the maintenance of chronic lesions in the CNS.

MATERIALS AND METHODS

Unless otherwise indicated, all reagents and apparatus were obtained in Tokyo, Japan.

Animals

LEW.1AV1 rats ($RT1^{av1}$ in the LEW background gene) were provided by Dr. R. Gold, Department of Neurology, Würzburg University, Germany, and maintained in our animal facility. Lewis (LEW) rats ($RT1^l$ in the LEW background gene) were purchased from Japan SLC Inc. (Shizuoka). Rats were 8–12 weeks of age. All animal experiments were approved by the appropriate institutional committee and were performed in accordance with institutional guidelines.

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 by using primers specific to the corresponding MOG sequence. The polymerase chain reaction (PCR) products were then digested with *Sph*I and *Hin*dIII and subcloned into pQE30 (Qiagen) used for large-scale preparation. Recombinant MOG produced in transformed *Escherichia coli* were isolated under denaturing conditions and purified with Ni-NTA Agarose (Qiagen). Then purified MOG was diluted and refolded in phosphate-buffered saline containing 1 M L-arginine, 2 mM glutathione (reduced form), and 0.2 mM glutathione (oxidized form). The obtained protein contained endotoxins at less than 10 EU/1 mg protein as determined with a Toxinometer ET-2000 (Wako). Guinea pig, bovine, and rat MBP were prepared as described previously (Deibler et al., 1972).

EAE Induction and Clinical Evaluation

In early experiments in the present study, acute EAE was induced in LEW rats by immunization with 100 μg MBP emulsified in an equal volume of complete Freund's adjuvant (CFA) (*Mycobacterium tuberculosis*, 2.5 mg/ml) in the footpads and chronic EAE was induced in LEW.1AV1 rats by immunization with 250μg MOG/CFA (*M. tuberculosis H37RA*, 2.5 mg/ml) in the tail base. Later, we learned that LEW.1AV1 rats developed acute monophasic EAE after immunization with MBP/CFA, and we also analyzed this type of EAE. Pertussis toxin (2μg) was administered intraperitoneally once at a time of MOG immunization. Clinical signs were evaluated daily 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 the tail or moribund conditions was 5.

Histological and Immunohistochemical Examination

The optic nerve, cerebrum, brain stem, and cerebellum and the cervical, thoracic, and lumbar spinal cord were routinely examined for histology of chronic EAE, while acute EAE was mainly evaluated by examination of the lumbar spinal cord. The tissues were fixed in 4% paraformaldehyde and processed for paraffin embedding. Six-micron sections were cut and stained with hematoxylin and eosin (HE) and with Kruever and Barrera's (KB) method (Luxol fast blue plus cresyl violet). Inflammatory lesions were graded using sections stained with HE 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 assessed using sections stained with the KB method and ED1 for MΦ into five categories (grade 1, trace of perivascular or subpial demyelination; grade 2, focal demyelination; grade 3, demyelination involving a quarter of tissues examined, i.e., the spinal tract, brain stem, cerebellar white matter or optic tract; grade 4, massive confluent demyelination involving half of the tissue; grade 5, extensive demyelination involving the entire tissues) according to Storch et al. (1998) with a few modifications.

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, sections were incubated with mAb ED1 (×200, purified from the hybridoma supernatant) for MΦ for 1 hr at room temperature. In the CNS, ED1 stains fine processes of microglia, whereas the whole cell bodies of MO/MΦ are positive for ED1 (our unpublished observation). Therefore, it can be said that ED1 stains mainly MΦ in diseased spinal cords. After washing, sections were incubated with biotinylated anti-mouse or rabbit IgG (×200, Vector) followed by the horseradish peroxidase–labeled Vectstain Elite ABC Kit (Vector). Horseradish peroxidase binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide. All the procedures were performed at room temperature. To confirm the specificity of staining, primary antibodies were omitted or replaced with normal mouse IgG. The controls did not show any specific staining.

Double immunofluorescence staining was performed as follows. Frozen sections were air-dried and fixed in ether. After washing, the sections were reacted with OX8 (×200, anti-CD8 mAb), followed by incubation with Cy3-labeled anti-mouse IgG (×50, Amersham). The free binding sites of

the secondary antibody were blocked with normal mouse serum and then biotinylated ED1 was applied. Finally, the sections were incubated with Alexa 488-conjugated streptavidin (×100, Molecular Probe). Morphological observations and quantitative analysis were made by microphotographs taken under a confocal microscope (Leica).

Flow Cytometric Analysis

Leukocytes in the spleen, lymph node (LN), blood (peripheral blood leukocytes, PBL) and spinal cord were collected and stained with unlabeled OX35 (×200, anti-CD4) followed by phycoerythrin (PE)-conjugated anti-mouse IgG (×50, Vector). To saturate free binding sites of the secondary antibody, cells were incubated with normal mouse serum. Then FITC-OX42 (×20, anti-MO/MΦ mAb) and biotinylated OX8 was applied in the second step. Finally, the cells were incubated with PerCP-streptavidin (×50). During incubation (15 min each), samples were kept on ice. Ten thousand cells were analyzed in each sample by FACScan (BD Bioscience). We previously confirmed with double immunofluorescence staining that more than 95% of MO/MΦ in the lymphoid organs are positive for ED1 and OX42 (data not shown).

For the isolation of infiltrating leukocytes in the CNS, rats with or without clinical signs of EAE were killed under ether anesthesia. The spinal cords were removed and minced with scissors and treated with 0.015% collagenase/dispase (Boeringer Mannheim) and 0.015% trypsin (Sigma) for 15 min at 37°C. After passing through a stainless mesh screen, two volumes of the cell suspension were mixed with one volume of 90% Percoll solution and centrifuged at 15,000 rpm for 20 min. By means of this procedure, a myelin cake floated on the top and the infiltrating inflammatory cells were contained in the pellet. The pellet was harvested and used for analysis.

Isolation of CD8$^+$ MO/MΦ

CD8$^+$ MO/MΦ were isolated with an AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. In brief, PBL, spleen and LN cells were taken from naive or immunized rats by the density gradient methods and T, B and dendritic cells were removed by negative selection with an antibody cocktail including R73 (anti–T cells), OX33 (anti–B cells) and OX62 (anti-dendritic cells) followed by anti-mouse IgG-Microbeads (Miltenyi Biotec). Then CD4 and CD8 cell populations were negatively selected with OX8 (anti-CD8) and OX35 (anti-CD4), respectively. Finally, bead-free cells were positively selected with OX42 (anti-MO/MΦ). The purity of each population was >90%.

Real-time PCR

Total RNA was extracted from the indicated tissues with an RNAqueous Kit (Ambion) and cDNA was then synthesized by reverse transcription with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). SYBR Green real-time PCR reactions were performed on an ABI PRISM 7500 sequence detection system (Applied Biosystems) in a total volume of 25 µl with the SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). Each PCR was performed in duplicate with the following thermocycler conditions: stage 1, 95°C for 10 min for one cycle and stage 2, 95°C for 15 sec and 58°C for 1 min for 50 cycles. All primers were designed on an intron-exon junction to prevent coamplification of genomic DNA and their sequences were shown in previous reports (Matsumoto et al., 2004, 2005). Relative quantification of mRNA was performed by the standard curve method. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as internal control. The absence of nonspecific amplification was confirmed by dissociation curve analysis.

Statistical Analysis

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

RESULTS

Clinical and Pathological Features of Acute and Chronic EAE

To characterize CD8$^+$ MΦ that appear in EAE lesions in the CNS, we induced acute EAE in LEW and LEW.1AV1 rats and chronic EAE in LEW.1AV1 rats. Figure 1 summarizes the clinical and pathological features of acute and chronic EAE. The clinical course of acute EAE was monophasic and the onset (day 10), peak (day 14), and recovery (by day 18) were relatively constant (Fig. 1A). This time course was almost identical in acute EAE induced in LEW and LEW.1AV1 rats (data not shown). In contrast, chronic EAE showed a variety of clinical courses including relapsing–remitting and secondary progressive forms as shown in our previous study (Sakuma et al., 2004). The rat depicted in Figure 1B developed clinical signs on postimmunization (PI) day 30 with remission around PI day 42, and died during severe relapse.

Routine pathology with HE and KB staining revealed that acute EAE lesions consisted mainly of small mononuclear leukocytes (Fig. 1C) with minimal demyelination (Fig. 1E), whereas there was dense and extensive "foamy macrophage" infiltration (Fig. 1D) with marked demyelination (Fig. 1F) in chronic EAE lesions. Consistent with these findings, MΦ staining with ED1 mAb revealed localized perivascular MΦ infiltration in acute EAE (Fig. 1G) and diffuse MΦ infiltration in chronic EAE (Fig. 1H).

A Large Number of CD8$^+$ MΦ Infiltrate CNS Lesions in Chronic, but Not in Acute, EAE

We then examined the presence or absence of MΦ expressing CD8 in CNS lesions at various time points of acute and chronic EAE. Acute EAE was examined on days 9 (preclinical, Fig. 2A–C), 14 (peak, Fig. 2D–F), and 21 (recovery, Fig. 2G–I). As clearly shown, the vast majority of ED1$^+$ MΦ did not express the CD8 molecule in acute EAE. Only two cells, indicated by arrows in Figure 1C and Figure 1I, were doubly labeled.

As reported previously (Schroeter et al., 2003) and confirmed here, CD8$^+$ MΦ were present in chronic

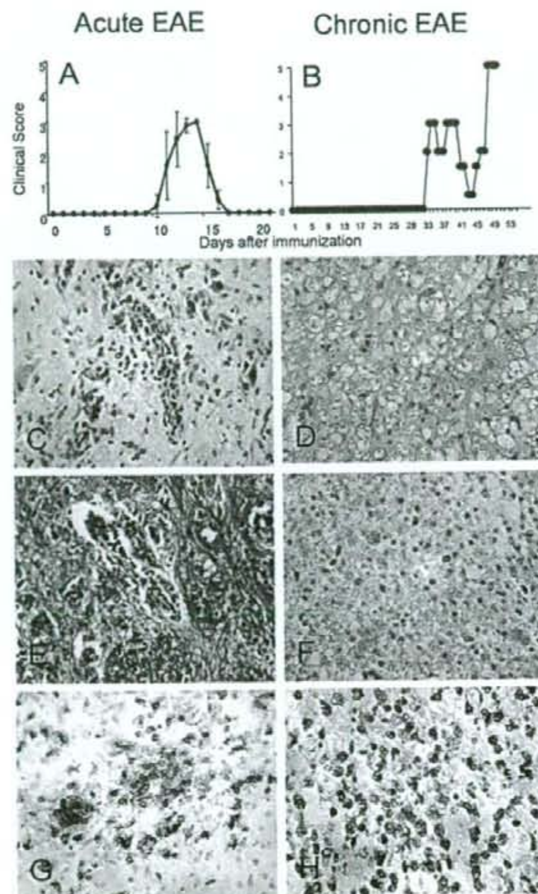


Fig. 1. Clinical and pathological features of acute and chronic EAE. LEW rats that had been immunized with MBP developed acute and monophasic EAE with a relatively constant onset and recovery (**A**). The mean clinical scores ± SD of 9 rats are shown. In contrast, chronic EAE induced by the immunization of LEW.1AV1 rats with MOG showed a variety of clinical courses including relapsing–remitting and secondary progressive EAE. The rat depicted in (**B**) developed clinical signs on PI day 30 with remission around PI day 42, and died during severe relapse. Note that the timescale in (B) is different from that in (A). Routine pathology with HE and KB staining revealed that acute EAE lesions (PI day 14) in the spinal cord consist mainly of small mononuclear leukocytes (**C**) with minimal demyelination (**E**), whereas there is dense and extensive "foamy" MΦ infiltration (**D**) with marked demyelination (**F**) in chronic EAE lesions (PI day 40) in the spinal cord. Consistent with these findings, MΦ staining with ED1 mAb revealed localized perivascular MΦ infiltration in acute EAE (**G**) and diffuse MΦ infiltration in chronic EAE (**H**). C and D, HE staining; E and F, KB staining; G and H, ED1 staining. C–H, original magnification, 120×.

EAE lesions in the spinal cord (Fig. 3). In addition to the previous study, we found an interesting finding by longitudinal examinations on days 14 (early stage, Fig. 3A–C), 20 (intermediate stage, Fig. 3D–F), and 40 (peak stage, Fig. 3G–I). On day 14, only a small number of double-positive cells were detected. Cells in yellow in Figure 3C were difficult to judge whether they were double-positive cells or simple overlap of two types of cells. However, on day 20, a definite number of MΦ expressed CD8 (Fig. 3F) and, on PI day 40, the majority of ED1$^+$ MΦ were also positive for CD8 (Fig. 3I).

Quantitative Analysis of CD8$^+$ED1$^+$ MΦ Infiltrating CNS Lesions in Acute and Chronic EAE

The above findings prompted us to quantitate CD8$^+$ MΦ in CNS lesions at various time points during acute and chronic EAE. As shown in Figure 4A, CD8$^+$ MΦ in acute EAE lesions (hatched bars) accounted for less than 5% throughout the course of the disease. In sharp contrast, approximately 20% of MΦ in chronic EAE lesions expressed CD8 in the early stage (solid bar on day 14). CD8$^+$ MΦ peaked on PI day 40 (58.8 ± 21.3%) and remained at a high level even on PI day 58. These findings indicate that CD8$^+$ MΦ infiltration in CNS lesions is a characteristic pathological feature of chronic EAE and that the number reaches the maximal level at the peak of chronic EAE (around PI day 40).

During analysis, we found that immunization of LEW.1AV1 rats with MBP elicited acute monophasic EAE and its clinical course was essentially the same as that found in acute EAE in LEW rats (data not shown). With spinal cord sections taken from rats with chronic EAE (LEW.1AV1-MOG in Fig. 4B) and with acute EAE (LEW.1AV1-MBP and LEW-MBP in Fig. 4B), we counted CD8$^+$ and CD8$^-$ MΦ at the indicated time points of disease. In chronic EAE, the proportion and absolute number of CD8$^+$ MΦ increased at the peak stage (day 40) compared with the early stage (day 14). The majority of MΦ forming cluster lesions expressed CD8 ("clustered" in Fig. 4B). The proportion of CD8$^+$ MΦ that did not form a cluster lesion ("nonclustered" in Fig. 4B) was relatively low compared with clustered MΦ. By contrast, in acute EAE, the proportion of CD8$^+$ MΦ in the CNS was very small (LEW-MBP). Interestingly, in LEW.1AV1 rats with acute EAE (LEW.1AV1-MBP), the proportion of CD8$^+$ MΦ was very low, as in LEW rats. These findings strongly suggest that CD8$^+$ MΦ infiltration into the CNS is closely associated with the clinical course of EAE and the used antigen but not with the rat strain.

Flow Cytometric Analysis of CD8$^+$ MΦ in the Lymphoid Organ and Peripheral Blood of Rats With Acute and Chronic EAE

Although it is generally believed that CD8 expression on MΦ occurs in CNS lesions (Popovich et al., 2003), there is no definite information regarding this

Fig. 2. Identification of $CD8^+$ MΦ in acute EAE lesions in the spinal cord. Double immunofluorescence staining for CD8 with OX8 mAb and for MΦ with ED1 mAb was performed on days 9 (**A–C**), 14 (**D–F**), and 21 (**G–I**). The majority of MΦ at all the stages examined (A,D,G) are negative for CD8 (B,E,H). Only a few cells indicated by arrows in (C) and (I) are stained positively for CD8 and EDI. Original magnification, A–I, ×200.

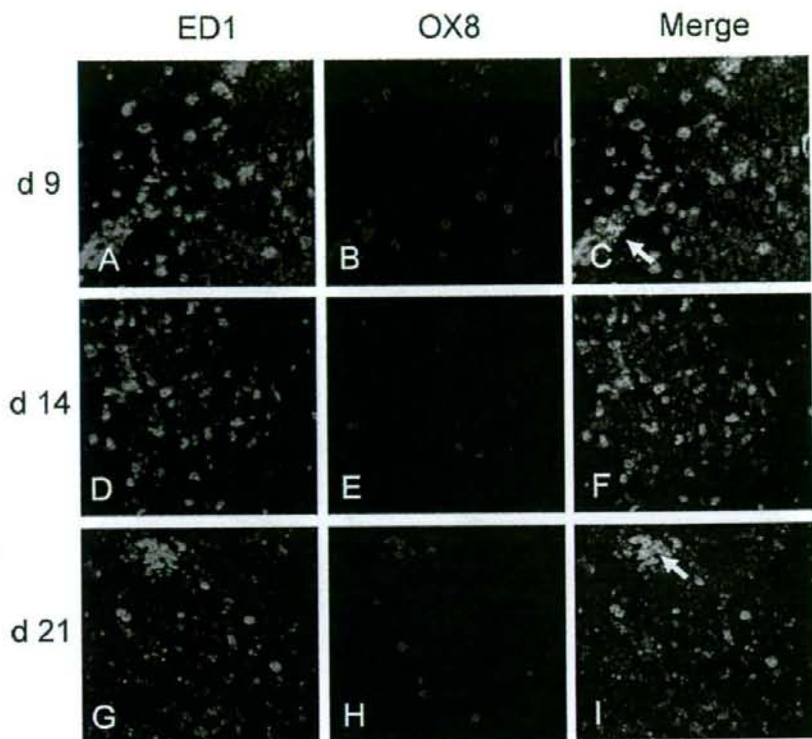
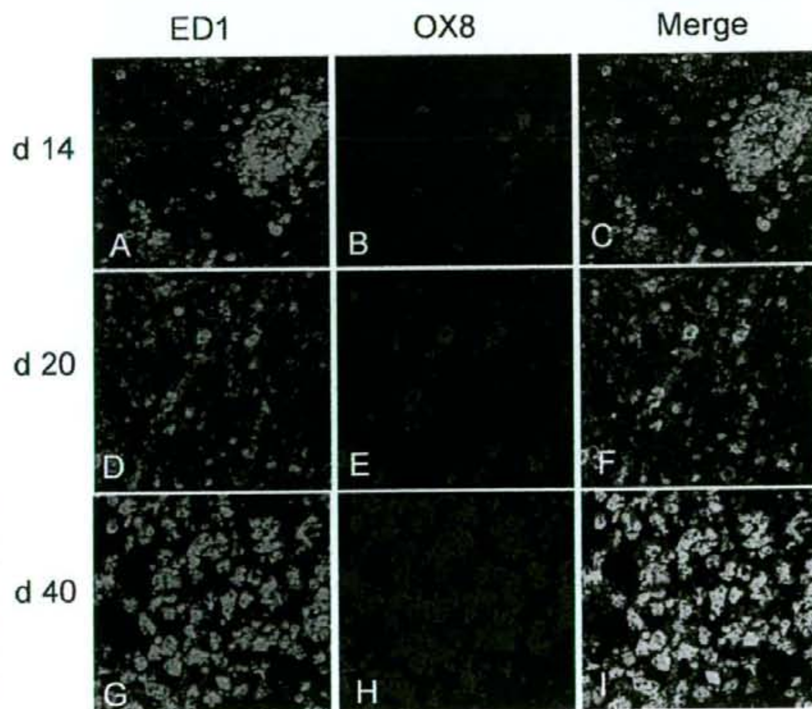


Fig. 3. Longitudinal examination of $CD8^+$ MΦ in chronic EAE lesions in the spinal cord. Double immunofluorescence staining for CD8 with OX8 mAb and for MΦ with ED1 mAb was performed on days 14 (**A–C**), 20 (**D–F**), and 40 (**G–I**). On day 14, only a small number of double-positive cells were detected. However, on day 20, a definite number of MΦ expressed CD8 (**F**) and, on day 40, the majority of $ED1^+$ MΦ were also positive for CD8 (**I**). Original magnification, A–I, ×200.



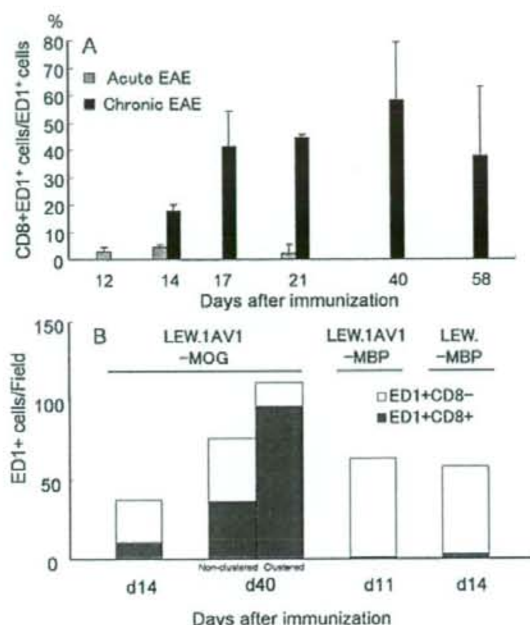


Fig. 4. (**A**) Quantitation of $CD8^+$ MΦ in acute (hatched bars) and chronic (solid bars) EAE lesions. At least three fields containing MΦ infiltration were taken as confocal images and all $ED1^+$ and $ED1^+OX8^+$ MΦ were counted and expressed as percentages ($OX8^+$ MΦ/total MΦ) ± SD. $CD8^+$ MΦ in acute EAE lesions (hatched bars) accounted for less than 5% throughout the course of the disease. In sharp contrast, approximately 20% of MΦ in chronic EAE lesions expressed CD8 in the early stage (solid bar on day 14). $CD8^+$ MΦ peaked on day 40 (58.8 ± 21.3%) and remained at a high level even on day 58. There was significant difference in the percentage of $CD8^+$ MΦ between acute and chronic EAE on days 14 and 21 ($P < 0.01$). (**B**) Comparison of the numbers of $CD8^+$ (solid bars) and $CD8^-$ (open bars) MΦ in the spinal cord at the peak of chronic (LEW.1AV1-MOG) and acute (LEW.1AV1-MBP and LEW-MBP) EAE. In chronic EAE, the proportion and absolute number of $CD8^+$ MΦ increased at the peak stage (day 40) compared with the early stage (day 14). The majority of infiltrating MΦ formed cluster lesions expressed CD8 (clustered). In contrast, the proportion of $CD8^+$ MΦ in the acute EAE lesions is very low (LEW.1AV1-MBP and LEW-MBP). The mean values ± SD for $ED1^+$ CD8- cells are 26.8 ± 7.3 (on day 14 of LEW.1AV1-MOG), 47.8 ± 17.3 (nonclustered on day 40 of LEW.1AV1-MOG), 15.3 ± 11.5 (clustered on day 40 of LEW.1AV1-MOG), 62.0 ± 29.4 (on day 11 of LEW.1AV1-MBP) and 54.5 ± 15.8 (on day 11 of LEW-MBP). At each time point, a total of four to six fields from three rats were photographed, and the mean values of the positive cells are shown.

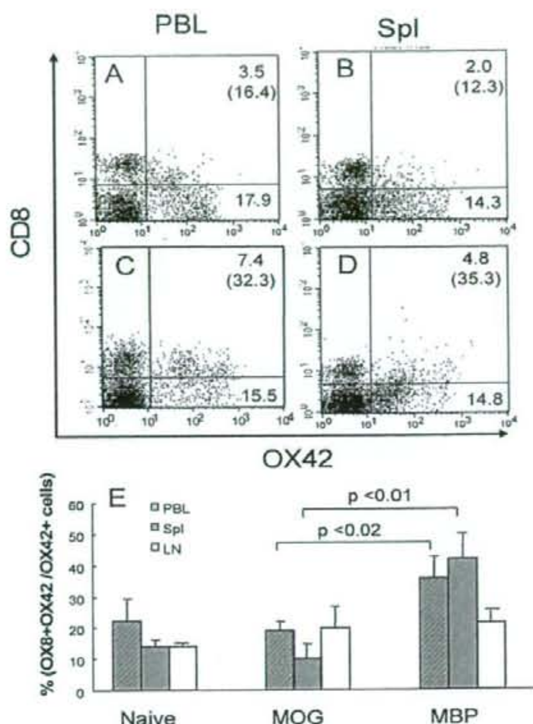
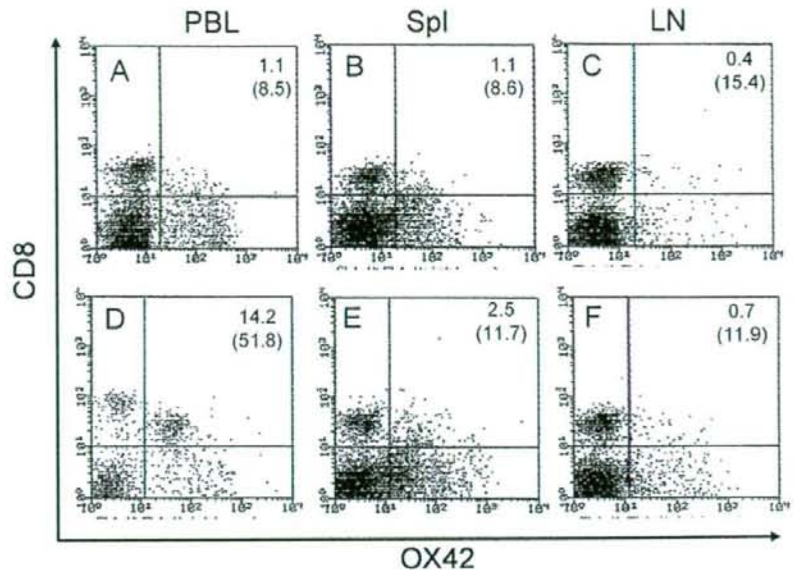


Fig. 5. **A–D:** Representative profiles of $CD8^+$ MO/MΦ in PBL (A,C) and the spleen (B,D) of MOG-immunized (A,B) (day 13) and MBP-immunized (C,D) (day 12) LEW.1AV1 rats. The numbers in parentheses are percentages of $OX8^+$ cells in total $OX42^+$ cells. **E:** In naive and MOG-immunized rats, the proportions of $CD8^+$ MO/MΦ were approximately 15–20% in PBL, spleens, and inguinal LNs. In sharp contrast, $CD8^+$ MO/MΦ in PBL and spleens of MBP-immunized rats accounted for 35% and 40%, respectively. Three rats were examined in each group, and the percentages of $OX8^+OX42^+$ cells in total $OX42^+$ cells (mean value ± SD) are shown.

issue. In order to analyze the nature of $CD8^+$ MΦ in more detail, we examined the peripheral blood, spleen, and LNs of naive, MOG-immunized and MBP-immunized LEW.1AV1 rats at the preclinical stage. In order to surface-label MΦ for flow cytometric analysis, we used OX42 for MO/MΦ instead of ED1 and OX8 for CD8. We previously confirmed by immunohistochemistry that the majority of MΦ in the lymphoid organs are double-labeled with ED1 and OX42 (data not shown). As mentioned above, immunization of LEW.1AV1 rats with MOG induced chronic EAE, whereas immunization of the same strain with MBP resulted in acute EAE. In the CNS lesions, the proportion of $CD8^+$ MΦ was high in chronic EAE and very low in acute EAE (Fig. 4B).

In lymphoid organs, a different finding was obtained. As shown in Figure 5, the proportions of $CD8^+$ MO/MΦ in naive control (Fig. 5E) and MOG-immunized (Fig. 5A,B,E) rats were approximately 15–20% in all organs examined ("Naive" and "MOG" in Fig. 5E). In sharp contrast, $CD8^+$ MO/MΦ in PBL and spleens of MBP-immunized rats (Fig. 5C–E) accounted for about 35% and 40%, respectively ("MBP" in Fig. 5E). These findings suggest that MBP immunization induces a sufficient number of



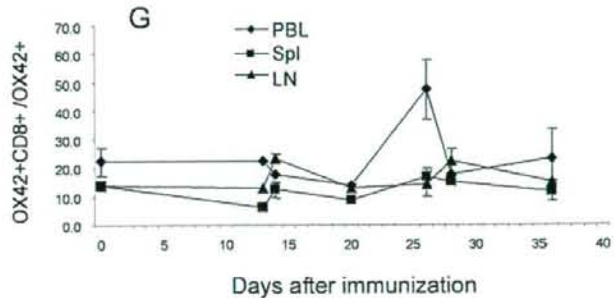


Fig. 6. **A–F:** Flow cytometric analysis of $CD8^+$ MO/MΦ in the blood, spleen, and LN of normal (**A–C**) and chronic EAE (**D–F**) LEW.1AV1 rats. PBL (**A,D**), spleen (Spl) (**B,E**), and LN (**C,F**) cells were isolated and stained for CD8 with OX8 and for MΦ with OX42. In PBL and spleen of a naive rat (A and B, respectively), $CD8^+$ MΦ accounted for 10–15%, whereas those in LN of naive rats (C) are relatively few. In a rat with chronic EAE, $CD8^+$ MO in PBL increased significantly (D), whereas those in the spleen and LNs were not significantly different from a naive rat (E,F). The numbers in parentheses are percentages of $OX8^+$ cells in total $OX42^+$ cells. **G:** During chronic EAE, the numbers of $CD8^+$ MΦ in all organs remained unchanged in the preclinical stage but $CD8^+$ MΦ abruptly increased in number around the onset of chronic EAE in PBL (D and diamonds in G). Such change was observed only in PBL and the proportion of $CD8^+$ MΦ in other organs remained stable throughout the observation period. At each time point, three to four rats were examined.

$CD8^+$ MO/MΦ in the periphery. However, MOG, but not MBP, immunization may up-regulate factors that recruit $CD8^+$ MO/MΦ into the CNS more efficiently.

We also performed similar analysis with mononuclear cells isolated from the spinal cord during the peak of chronic EAE. Unfortunately however, it was revealed that harvested $CD8^+$ cells were always small in both number and size. On the basis of this finding, we judged that the majority of foamy MΦ, the major inflammatory cell population in spinal cord lesions, were destroyed during the processes of isolation and staining for analysis and decided not to use the obtained data.

Kinetics of $CD8^+$ MΦ in the Lymphoid Organ and Peripheral Blood in Chronic EAE

Flow cytometric analysis that used PBL obtained from rats on day 12–13 revealed that the proportion of $CD8^+$ MO was significantly higher in MBP-immunized rats than in MOG-immunized rats (Fig. 5E). To examine in more detail, we made a longitudinal examination of the numbers of $CD8^+$ MO/MΦ in the periphery during the course of chronic EAE. The representative profiles of naive control rats and rats with EAE are shown in Figure 6A–F and the summary is in Figure 6G. In PBL, spleens, and LNs (Fig. 6A,B,C, respectively) of naive control rats, $CD8^+$ MO/MΦ accounted for about 10–20%. During chronic EAE, the numbers of $CD8^+$ MO/MΦ in all organs remained unchanged at the preclinical stage (Fig. 6G) but $CD8^+$ MO/MΦ in PBL abruptly increased in number around the onset of chronic EAE (Fig. 6D and diamonds in Fig. 6G). Such change was observed only in PBL and the proportions of $CD8^+$ MO/MΦ in the spleen and LN remained stable throughout the observation period. Thus, it was demonstrated that $CD8^+$ MO in PBL was equally up-regulated in both MBP- and MOG-immunized rats but

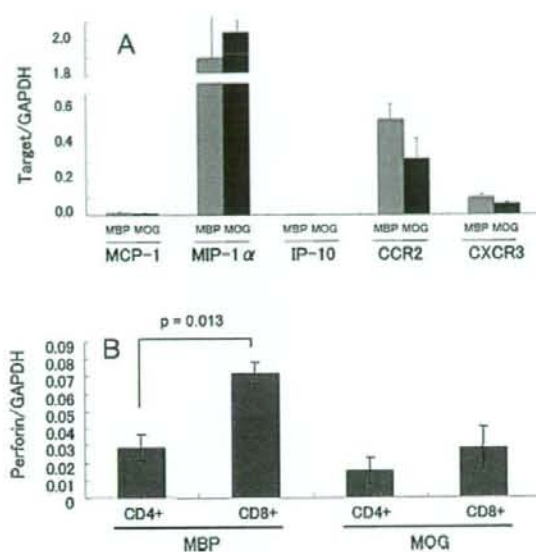


Fig. 7. Real-time PCR analysis of chemokine, chemokine receptor (**A**) and perforin (**B**) mRNA of peripheral blood MO. PBL were taken from MBP-immunized (day 12–13, grade 2.5–3, $n = 4$) and MOG-immunized (day 13–20, grade 2.5–3, $n = 4$) rats with clinical EAE. Then $CD4^+$ and $CD8^+$ MO were purified with AutoMACS. mRNA levels of the indicated molecules were determined by real-time PCR. The mRNA levels of chemokines and chemokine receptors of $CD8^+$ MO (A) and of perforin of $CD4^+$ and $CD8^+$ MO isolated from MBP- and MOG-immunized rats are shown. GAPDH was used as internal control.

that $CD8^+$ MO/MΦ infiltrated the CNS mainly in chronic EAE.

Quantitative Analysis of Chemokines and Other Bioactive Substances by Real-time PCR

Quantitation of mRNA encoding chemokines and other bioactive substances such as perforin, granzyme B, and Fas-L was performed by using sorted cell populations of PBL. Figure 7A shows levels of chemokines and chemokine receptors in $CD8^+$ MΦ in the peripheral blood taken from MBP-immunized and MOG-immunized rats at the peak of acute and chronic EAE. Analysis revealed no significant difference between the two groups. We also examined several bioactive substances related to the cytotoxicity of $CD8^+$ MΦ and compared them with those of $CD4^+$ MΦ. As shown in Figure 7B, the perforin mRNA level was significantly higher in the CD8 population than in the CD4 population in MBP-immunized rats ($P = 0.013$), and a similar tendency was noted in MOG-immunized rats. Essentially the same results were obtained with regard to granzyme B (data not shown). However, there was no significant difference between MBP-immunized and MOG-immunized rats.

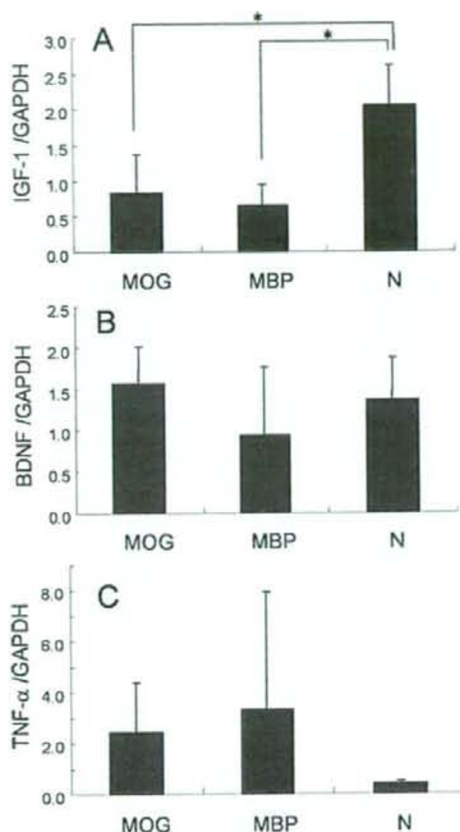


Fig. 8. Real-time PCR analysis of IGF-1 (**A**), BDNF (**B**), and TNF-α (**C**) mRNA of naive control (N) and inflamed (MOG and MBP) spinal cords. LEW.1AV1 rats were immunized with MBP or MOG, and spinal cords were taken from MBP-immunized (PI day 11, grade 4–5, $n = 3$) and MOG-immunized (day 14–20, grade 4–5, $n = 4$) rats with clinical EAE. Then mRNA levels of the indicated molecules were determined by real-time PCR. GAPDH was used as internal control. $^*P < 0.05$. There was no significant difference in TNF-α levels among three groups.

To learn the role of infiltrating MΦ in acute and chronic EAE lesions in the spinal cord, we also determined mRNA levels of tissue repair-promoting (insulin-like growth factor [IGF]-1 and brain-derived neurotrophic factor [BDNF]) and neurotoxic (TNF-α) substances (Fig. 8). IGF-1 was significantly down-regulated in both acute (MBP in Fig. 8A) and chronic (MOG in Fig. 8A) EAE lesions than in control spinal cord tissue. There was no significant difference in BDNF mRNA level among the three groups (Fig. 8B). In contrast, one of major neurotoxic substances, TNF-α, were up-regulated in both acute and chronic EAE lesions. Importantly, there was no significant difference in the levels of IGF-1, BDNF, and TNF-α between acute and chronic EAE

lesions, suggesting that it is less likely that $CD8^+$ MΦ found in chronic EAE lesions play a role in lesion repair.

DISCUSSION

It has recently become known that hematogenous MΦ bearing a unique phenotype, i.e., $CD8^+$ MΦ, infiltrate the CNS under various pathological conditions such as EAE (Schroeter et al., 2003), spinal cord injury (Popovich et al., 2003), focal ischemia (Jander et al., 1998; Schroeter et al., 2001), and Wallerian degeneration (Jander et al., 2001). Although the role of $CD8^+$ MΦ in CNS lesions is largely unknown, there are common pathological conditions. Furthermore, Schroeter et al. (2003) demonstrated that in chronic EAE, there are a large number of $CD8^+$ MΦ in the highly demyelinating lesions, whereas only small lymphocyte-like $CD8^+$ cells were found in acute EAE lesions. These findings strongly suggest that the presence of severely damaged CNS tissues is closely linked with the appearance of $CD8^+$ MΦ.

In the present study, we have extended previous studies and obtained several intriguing findings. By means of acute and chronic EAE systems, we first made longitudinal examinations of the distribution and number of $CD8^+$ MΦ in the spinal cord. Consequently, we found that $CD8^+$ MΦ in chronic EAE lesions increased in number and peaked on PI day 40. At this time point and thereafter, $CD8^+$ MΦ accounted for about 60% of total MΦ. Contrary to the previous study (Schroeter et al., 2003), a small but definite number of MΦ bearing CD8 were present in acute EAE lesions and accounted for about 5% throughout the disease course.

The kinetic study of $CD8^+$ MO/MΦ in the peripheral blood and lymphoid organ during chronic EAE revealed that there was a transient but robust increase of $CD8^+$ MO in the peripheral blood on day 26 when chronic EAE was in the early stage. This suggests that at least a part of MO acquire CD8 molecules outside the CNS and infiltrate the target organ. Although it was previously presumed that the CD8 expression by MΦ takes place in the CNS on the basis of the findings obtained by the treatment experiment with OX8 (Schroeter et al., 2001; Popovich et al., 2003), the present study clearly showed that MO expressing CD8 molecules increase in number in the blood in the early stage of chronic EAE and move into the CNS with EAE lesions. However, this finding does not exclude the possibility of CD8 molecule acquisition by MΦ in CNS lesions.

We also analyzed the numbers and percentages of $CD8^+$ MO/MΦ in the lymphoid organ and CNS with EAE lesions induced by immunization of LEW and LEW.1AV1 with MBP (acute EAE) and of LEW.1AV1 with MOG (chronic EAE). As a result, we obtained two unexpected findings. First, in the CNS lesion of LEW.1AV1 rats with acute EAE elicited by immunization with MBP, the number of infiltrating $CD8^+$ MΦ was very small compared with chronic EAE induced in the same strain. This finding clearly indicates that the appearance of $CD8^+$ MΦ in the CNS is closely associated with the type of EAE and is totally dependent on the encephalitogenic antigen used for EAE induction. Second and most unexpectedly, there were a large number of $CD8^+$ MO/MΦ in the blood of LEW.1AV1 rats with acute EAE. This suggests that, in LEW.1AV1 rats, the factors that promote the migration of $CD8^+$ MO/MΦ from the blood to the inflamed CNS are induced by MOG, but not MBP, immunization. The quantitation of chemokine and chemokine receptor mRNA by real-time PCR in the peripheral blood using sorted cell populations revealed no significant difference in the MO/MΦ phenotype between MBP-immunized and MOG-immunized rats. This finding suggests two possibilities. First, cytokines and chemokines that were not examined in the present study play a role in determining the kinetics of $CD8^+$ MO/MΦ. Second, factors produced by non-MO/MΦ, such as T and dendritic cells, are critical for the migration of $CD8^+$ MO/MΦ. In this regard, we are currently investigating more than 30 cytokines and chemokines at both protein and mRNA levels by multiplexed flow cytometric analysis.

The role of $CD8^+$ MO/MΦ is poorly understood. By means of alveolar MΦ expressing CD8, it was demonstrated that these MΦ produce TNF, IL-1β and nitric oxide after stimulation through the CD8 molecule (Hirje et al., 1998; Lin et al., 2000). Moreover, $CD8^+$ MΦ were reported to secrete perforin and granzyme B and to kill tumor cells (Baba et al., 2006, 2008). We also evaluated the function of $CD8^+$ MΦ by examining tissue repair-promoting substances such as IGF-1 and BDNF. However, we were unable to obtain the results suggesting such role. Collectively, it is possible that $CD8^+$ MΦ show strong cytotoxic effects in the CNS of rats with chronic EAE. Another important issue is related to the presence or absence of the $CD8^+$ MO/MΦ population in humans. Two groups clearly demonstrated that $CD8^+$ MO/MΦ are present in PBL of healthy subjects (Baba et al., 2006) and that a substantial number of cells with this phenotype were found in the thyroid gland of patients with autoimmune thyroid disease (Nakamura et al., 2004). Therefore, analysis of MO/MΦ with this subtype provides useful information with regard to elucidation of the pathomechanisms of MS.

In summary, we characterized the nature and kinetics of $CD8^+$ MO/MΦ during the course of acute and chronic EAE. Although $CD8^+$ MO/MΦ increased in number in the peripheral organs of rats with both acute and chronic EAE, cells with this subtype infiltrated the CNS mainly in chronic EAE. Targeting of these cells and/or the processes of their migration from the peripheral blood to the CNS could be effective immunotherapies against autoimmune diseases in the CNS.

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REFERENCES

Baba T, Ishizu A, Iwasaki S, Suzuki A, Tomaru U, Ikeda H, Yoshiki T, Kasahara M. 2006. $CD4^{+}/CD8^{+}$ macrophages infiltrating at inflammatory sites: a population of monocytes/macrophages with a cytotoxic phenotype. Blood 107:2004–2012.

Baba T, Iwasaki S, Maruoka T, Suzuki A, Tomaru U, Ikeda H, Yoshiki T, Kasahara M, Ishizu A. 2008. Rat $CD4^{+}CD8^{+}$ macrophages kill tumor cells through an NKG2D- and granzyme/perforin-dependent mechanism. J Immunol 180:2999–3006.

Chen Y, Hancock WW, Marks R, Gonnella P, Weiner HL. 1998. Mechanism of recovery from experimental autoimmune encephalomyelitis: T cell depletion and immune deviation in myelin basic protein T cell receptor transgenic mice. J Neuroimmunol 82:149–159.

Deibler GE, Martenson RE, Kies MW. 1972. Large scale preparation of myelin basic protein from central nervous tissue of several mammalian species. Preparat Biochem 2:139–165.

Duffield JS. 2003. The inflammatory macrophage:a story of Jekyll and Hyde. Clin Sci 104:27–38.

Hirje N, Lin T, Bissonnette E, Belosevic M, Befus AD. 1998. Mechanisms of macrophage stimulation through CD8: macrophage $CD8^{+}$ and $CD8^{+}$ induce nitric oxide production and associated killing of the paracyte *Leishmania major*. J Immunol 160:6004–6011.

Jander S, Schroeter M, D'Urso D, Gillen C, Witte OW, Stoll G. 1998. Focal ischaemia of the rat brain elicits an unusual inflammatory response: early appearance of $CD8^{+}$ macrohpages/microglia. Eur J Neurosci 10:680–688.

Jander S, Lausberg F, Stoll G. 2001. Differential recruitment of $CD8^{+}$ macrophages during Wallerian degeneration in the peripheral and central nervous system. Brain Pathol1127–1138.

Lin T, Hirji N, Stenton GR, Gilchrist M, Grill BJ, Schreiber AD, Befus AD. 2000. Activation of macrophage CD8: pharmacological studies of TNF and IL-1β production. J Immunol 164:1783–1792.

Lublin FD, Reingold SC. 1996. Defining the clinical course of multiple sclerosis: results of an international survey. Neurology 46:907–911.

Matsumoto Y, Fujiwara M. 1987. The immunopathology of adoptively transferred experimental allergic encephalomyelitis (EAE) in Lewis rats. Part 1. Immunohistochemical examination of developing lesion of EAE. J Neurol Sci 77:35–47.

Matsumoto Y, Tsukada Y, Miyakoshi A, Sakuma H, Kohyama K. 2004. C protein-induced myocarditis and subsequent dilated cardiomyopathy: rescue from death and prevention of dilated cardiomyopathy by chemokine receptor DNA therapy. J Immunol 173:3535–3541.

Matsumoto Y, Sakuma H, Miyakoshi A, Tsukada Y, Kohyama K, Park I, Naoyuki T. 2005. Characterization of relapsing autoimmune encephalomyelitis and its treatment with decoy chemokine receptor gene. J Neuroimmunol 170:49–61.

Nakamura Y, Watanabe M, Matsuzuka F, lMaruoka H, Miyauchi A, Iwatani Y. 2004. Intrathyroidal $CD4^{+}$ T lymphocytes express high levels of Fas and $CD4^{+}CD8^{+}$ macrophages/dendritic cells express Fas ligand in autoimmune thyroid disease. Thyroid 14:819–824.

Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. 2000. Multiple sclerosis. N Engl J Med 343:938–952.

Ohmori K, Hong Y, Fujiwara M, Matsumoto Y. 1992. In situ demonstration of proliferating cells in the rat central nervous system during experimental autoimmune encephalomyelitis. Evidence suggesting that most infiltrating T cells do not proliferate in the target organ. Lab Invest 66:54–62.

Popovich PG, van Rooijen N, Hickey WF, Preidis G, McGaughy V. 2003. Hematogenous macrophages express CD8 and distribute to regions of lesion cavitation after spinal cord injury. Exp Neurol 182: 275–287.

Pryce G, O'Neill JK, Croxford JL, Amor S, Hankey DJ, East E, Giovannoni G, Baker D. 2005. Autoimmune tolerance eliminates relapse but fail to halt progression in a model of multiple sclerosis. J Neuroimmunol 2005:41–52.

Sakuma H, Kohyama K, Park IK, Miyakoshi A, Tanuma N, Matsumoto Y. 2004. Clinicopathological study of a myelin oligodendrocyte glycoprotein-induced demyelinating disease in LEW.1AV1 rats. Brain 127:2201–2213.

Schroeter M, Jander S, Huitinga I, Stoll G. 2001. $CD8^{+}$ phagocytes in focal ischemia of the rat brain: predominant origin from hematogenous macrophages and targeting to area of pannecrosis. Acta Neuropathol 101:440–448.

Schroeter M, Stoll G, Weissert R, Hartung HP, Lassmann H, Jander S. 2003. $CD8^{+}$ phagocyte recruitment in rat experimental autoimmune encephalomyelitis: association with inflammatory tissue destruction. Am J Pathol 163:1517–1524.

Shao H, Lei S, Sun SL, Kaplan HJ, Sun D. 2003. Conversion of monophasic to reccurent autoimmune disease by autoreactive T cell subsets. J Immunol 171:5624–5630.

Sospendra M, Martin R. 2005. Immunology of multiple sclerosis. Annu Rev Immunol 23:683–747.

Storch MK, Stefferl A, Brehm U, Weissert R, Wallstrom E, Kerschensteiner M, Olsson T, Linington C, Lassmann H. 1998. Autoimmunity to myelin oligodendrocyte glycoprotein in rats mimics the spectrum of multiple sclerosis pathology. Brain Pathol 8:681–694.

van der Goes A, Boorsma W, Hoekstra K, Montagne L, de Groot CJ, Dijkstra CD. 2005. Determination of the sequential degradation of myelin proteins by macrophages. J Neuroimmunol 161:12–20.

van der Laan LJ, Ruuls SR, Weber KS, Lodder IJ, Dopp EA, Dijkstra CD. 1996. Macrophage phagocytosis of myelin in vitro determined by flow cytometry: phagocytosis is mediated by CR3 and induces production of tumor necrosis factor-alpha and nitric oxide. J Neuroimmunol 70:145–152.

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総説

アルツハイマー病に対する新ワクチン療法
──現状とわれわれの試み

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Novel Vaccine Therapy for Alzheimer's Disease
──Recent Progress and Our Approach

Yoshio Okura*, Yoh Matsumoto*

Abstract

Alzheimer's disease (AD) is the most common cause of dementia with very few drugs available for its treatment. In 1999, Schenk et al reported that Aβ 1-42 peptide vaccination in AD model mice causes the reduction in Aβ deposits. Thereafter, a vaccine therapy was developed for the curative treatment of AD. Clinical trials of active vaccination for AD patients were halted due to the development of meningoencephalitis in some patients; however, vaccine therapy is thought to be effective based on the clinical and pathological findings of the vaccinated patients. Based on this information, active and passive vaccines have been developed, some of which are now urdergoing clinical trials in Europe and USA. However, there are still some problems for general application of such drugs for AD patients. Recently, we developed nonviral DNA vaccines and used them to obtain a substantial Aβ reduction in AD model mice without any side effects. In this article, we will review conventional vaccine therapies and introduce our non-viral DNA vaccine therapy. Finally, we will present data regarding the mechanisms of Aβ reduction after DNA vaccination. DNA vaccination for AD may open up new avenues in vaccine therapy for the treatment of AD.

Key words : Alzheimer's disease, nonviral DNA vaccine, Aβ reduction, microglial activation, phagocytosis by microglia

はじめに

アルツハイマー病は今から100年前，ドイツの精神医学者 Alois Alzheimer により最初に報告された神経疾患である。認知障害(記憶障害，見当識障害，学習の障害，注意の障害，空間認知機能，問題解決能力の障害など)を主症状として中年期以降に多発し，世界中で1,200万人を超える患者が存在すると考えられている[1]。発症後数年の経過を経て徐々に症状は進行し，重度になると摂食や着替え，意思疎通なども不可能となり，数年から十数年で寝たきりになり死に至る。経過中に被害妄想，幻覚や暴言・暴力・徘徊・不潔行為などの問題行動が出現することが多く，患者本人ばかりか家族や介護者を含めて大きな社会問題となっている。

I．アミロイド仮説

アルツハイマー病は，肉眼病理的に高次機能をつかさどる前頭葉，前頭葉連合野や側頭葉，海馬領域の中等度から高度の脳萎縮によって特徴付けられ，大脳皮質や海馬の萎縮を反映して脳室は拡大する。顕微鏡レベルでは，

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Table 1 Viral vs Nonviral DNA vaccines

	Nonviral	Viral
Advantage	· **Safe** · **Mass-producible** · **Low cost**	· Inducible to particular cells · High induction efficiency
Disadvantage	· Low induction efficacy	· **Possibilities of tumorigenecity or leukemia** · **Possibilities of adverse effects (immunogenicity, cytokine storms)** · **Difficulty in mass production**

老人斑，神経原線維変化(neurofibrillary tangle)，神経細胞脱落の3つの特徴がある。アミロイドの蓄積がタウや神経細胞の変化に先行することは多くの研究により報告され，非認知症老人やダウン症の剖検脳でも観察されている。近年，アミロイドの沈着がこの病態の最上流に位置しアミロイドの蓄積を防止できるならば，その後に起きる事象，すなわち，神経細胞内のタウの蓄積，神経細胞の脱落などをある程度防ぐことができるとする「アミロイド仮説」が広く受け入れられるようになった[2]。

II．ワクチン療法の開発　能動免疫療法

アミロイド仮説をもとに，1999年Schenkらによりアルツハイマー病の根治的治療法としてAβペプチド・ワクチン療法が提唱された[3]。彼らは，体外からAβペプチドを免疫賦活剤（アジュバンド）とともに投与し，体内で抗Aβ抗体の産生を誘導し，脳内のAβ蓄積が減少することをアルツハイマーモデルマウスで証明した。その後，脳内の病理学的所見のみならず学習記憶能力もワクチン投与により改善されることが明らかになり[4,5]，欧米においてAβワクチン（Aβ1-42アジュバンド）のヒト臨床試験が開始されることとなった。Elan社にて製造された合成Aβ42製剤であるBetabloc (AN-1792)を用いた第I相試験（安全性試験）では，約100例の軽度から中等度のアルツハイマー病の患者に投与された。この試験は重要な副作用もなく終了し，投与された患者の血清中に抗Aβ抗体が生体内で合成されたことが確認された[6]。第I相試験に引き続きアメリカとヨーロッパの30の施設において軽度から中等度のアルツハイマー患者に対して第II相試験（二重盲検試験）が行われた。しかし実薬投与を受けた298例のうち18例（6％）に髄膜脳炎が発症し，障害を残す重症症例も出たため，2002年1月に治験自体が中止された[7]。ワクチンの免疫活性化作用によりTリンパ球などの組織障害性細胞性免疫が惹起され，一部の患者でAβに反応するTh1型CD4陽性細胞が脳に浸潤し，アレルギー性実質的脳脊髄炎のような髄膜脳炎を引き起こしたのではないかと推察された。

臨床試験は不満足な結果に終わったものの，その後の検索によってワクチン治療が有益であるという重要な所見が示された。2003年，ワクチン投与を受けた患者の最初の剖検例が報告された[8]。症例は72歳の女性で5年間の緩徐進行性の記憶障害の経過があり，AN-1792 (pre-aggregated Aβ 42; 50 μg)を5回投与された後，2001年5月から脳炎症状が出現した。投薬は直ちに中止され脳炎の治療が行われたものの，寝たきりとなり20カ月後の2002年2月に肺梗塞のため死亡した。脳病理はBraak stage V-VIのアルツハイマー病の所見であった。脳炎の所見として髄膜，髄膜血管周囲および大脳皮質へのT細胞とマクロファージの浸潤が認められ，大脳白質には髄鞘線維の減少が認められた。しかしその一方，新皮質では老人斑が消失し，それに伴うアストロサイトの増殖や変性軸索も消えていた。老人斑が消失している部位ではAβ分解産物を貪食したミクログリアの像も認められ，この所見からワクチンがヒトのアルツハイマー病においても老人斑の減少効果があると推測された。さらにその後，Gilmanらは臨床試験の抗Aβ抗体の抗体価と高次機能の改善について最終報告を行った[9]。ワクチン投与300名中59人が抗体陽性であり，陽性群では各種高次機能試験のうちneuropsychological test battery (NTB)で有意の改善を認めた。これらの事実は，能動免疫療法により一部患者に脳内炎症症状が出現したものの，ワクチンによるAβ減少効果がヒトアルツハイマー病症例においても認められることを示すものであり，過剰な免疫反応を抑制することができれば，ヒトにおいてもワクチン療法が有効である可能性が高いことを示唆している。

臨床治験後，脳炎を惹起しないようなワクチン薬剤を作ることを目標として薬剤開発が行われている。Aβペ

プチドはそのC（カルボキシル）末端側フラグメントが主としてTh1反応を誘導し，N（アミノ）末端側フラグメントがTh2反応を誘導することがわかっている[10]。そこで，Elan社はAβのN末端側フラグメントをキャリア蛋白質に結合させたワクチンを開発し，このワクチンを用いた治験を開始している[11]。そのほか，キャリア蛋白質を使用しないN末端フラグメントワクチンとしてAβ1-15ペプチド2本をリジン残基でつないだものが作られ，アルツハイマー病モデルマウスでその有効性が確認されている[12]。Th1反応を起こしにくいとされる粘膜免疫反応を使って，抗体産生を誘導するワクチンも開発されている[13]。しかし，能動免疫療法はアジュバントを必要とするために，脳炎などの副作用を完全に克服することは現時点で難しいと考えられる。

III．受動免疫療法

抗Aβ抗体を直接体内に投与する受動免疫療法は，能動免疫療法に代わりうる方法であり，早い段階から能動免疫療法と並行して開発が進められている。末梢から抗Aβ抗体を投与することにより，アミロイド斑の減少が認められており，さほど高濃度でなくとも脳内に入りアミロイド斑の減少効果があることがわかっている[14,15]。最近の研究ではAβ蛋白のN末端が抗体認識部位として重要であることが示され，これは凝集したAβのN末端が突出し，抗体が認識しやすいからであると考えられている。受動免疫療法の最大の利点は免疫賦活剤を使用しないことであり，そのため能動免疫療法のヒト臨床試験で問題となった過剰な細胞免疫が誘発されにくい。既に欧米においてElan-Wyeth社のヒト化モノクローナル抗体（AAB-001）のヒト臨床試験が第III相試験まで進行している。国内においても順天堂医院において治験参加者が募集されており，近日，第I相試験が予定されている。

しかし受動免疫療法には，投与された抗Aβ抗体はアミロイド斑のみならずアミロイドが沈着している血管にも結合して血管壁の脆弱化を促進し，出血をきたすことが報告されていること[16]，投与された抗体に対して抗体が産生され（抗イディオタイプ抗体）複数回の投与により効果が減弱，ないしは消失する可能性があることなどクリアしなければならない課題も多い。さらに，受動免疫療法は抗ヒト型抗体の製作費用が高いために，治療費が莫大な額になることが予想されており，それにより治療が不可能となるケースが出るであろうと考えられている。

IV．次世代に向けた新しいワクチン療法 ―DNAワクチンの開発

これらに代わるアルツハイマー病の新ワクチン治療として，開発されたのがDNAワクチン療法である。DNAワクチンは遺伝子を運ぶベクター（プラスミドやウイルス）にAβ蛋白を作らせる遺伝子を組み込んで投与し，ワクチンを取り込んだ細胞にAβ蛋白を作らせる。産生されたAβは免疫系を刺激し，抗Aβ抗体を誘導する（Fig. 1）。DNAワクチンは，投与後で長時間体内にとどまり，コードされたペプチドを緩徐に作り続けるために過剰な免疫反応を避けることが可能で，単純な構造であるため簡単に改良することができる。[17,18]。さらに，宿主に誘導される免疫反応はTh2型であるという利点がある[17,19,20]。

いくつかのグループから，アデノウイルスベクター[21]，アデノ随伴ウイルスベクター（AAV）[22,23]などのウイルス性ベクター用いてワクチンを開発した報告がなされた。Zhangらは，AAVワクチンを1回のみ投与することにより長期間抗Aβ抗体がアルツハイマーモデルマウスに誘導され，脳内のAβ沈着が減少し，記憶認知能力が改善することを報告した[22]。さらにKimらは，アデノウイルスベクターに組み込んだGM-CSFおよび11 tandem repeats of Aβ 1-6を同時投与することにより，高い抗体価が得られたことを報告した[21]。しかしながら，アデノウイルスやアデノ随伴ウイルスは悪性腫瘍を誘発する可能性が完全に否定することができない（後述）[24,25]。また，AAVベクターを患者治療に用いる量までにスケールアップするのは非常に困難で，商業利用できるレベルではないこと[26]などから，ウイルス性ベクターを用いたDNAワクチンを臨床応用することは，現在のところ障害が大きい。

一方，非ウイルス性のプラスミドベクターを用いてDNAワクチンを作製した報告も認められる。プラスミドベクターによる非ウイルス性DNAワクチンは低いコストにより大量生産が可能であり[17,18]，ウイルス感染や形質転換の危険性がない[27,28]などの利点がある。Ghochikyanらは，非ウイルス性プラスミドベクター中にAβ1-42とTh-2 cytokineであるIL-4 sequenceを導入し，wild type B6マウスに抗Aβ抗体を誘導した[29]。Schiltzらは分泌シグナルである組織プラスミノーゲンアクチベーターの遺伝子を含むDNAワクチンを低用量のAβ蛋白と同時投与することにより，アルツハイマーモデルマウスの脳内Aβの減少に成功した。しかしDNAワク