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Experimental autoimmune encephalomyelitis in cynomolgus monkeys

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Experimental autoimmune encephalomyelitis was induced in macaques. T cell clones infiltrated into the brain lesion area were compared with those in blood. Intradermal immunization of macaques with brain white matter derived from healthy macaque in combination with pertussis toxin, induced neurological symptoms in two macaques. One died on day 25 after immunization, whereas the other survived. Gross examination of the brain from the dead macaque, showed clear hemorrhagic lesions in the white matter. Hematological analysis showed that drastic T cell response was induced in macaques immunized with white matter, but not in control macaques. Flow cytometric analysis of blood cells from the affected macaques demonstrated an increase of CD4 and CD8 T cell populations expressing the CD69 early activation marker. Single strand conformation polymorphism (SSCP) analysis of T cell receptor beta chain showed T cell clones infiltrated into the brain lesion, which were different from those found in the peripheral blood of the same monkey. The present paper shows that SSCP analysis of TCR is useful in studing clonality of T cells infiltrating into the brain tissue of macaque with EAE.

Key word: EAE in cynomolgus monkeys

Introduction

Experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) in humans, a spontaneous inflammatory demyelinating disease of young adults, results from immunization with central nervous system (CNS) myelin proteins or peptides under Th1 conditions or by the adoptive transfer of CNS Ag-specific CD4+ Th1 cells [1, 21, 22]. The process involveds the death of cellular constituents of the CNS, including oligodendroglia and neurons as well as damage to myelin.

The majority of EAE models have been developed in rodents. However, some groups have developed EAE models in primates. The representative EAE model in primate was developed by Hauser and Genain using a New World primate species, *Callithrix jacchus*, and this model has been continuously refined [6]. By comparison with rodent EAE models, EAE models in primates are more similar to MS as assessed by the chronicity of the relapse, primary inflammatory demyelination, and changes on the magnetic resonance imaging brain scans. However, this model has some limitations. The marmoset is small (adult marmosets weigh between 250 and 500 g), which precludes multiple analyses of blood and cerebrospinal fluid from the animals. Perhaps, the most significant benefit of EAE studies in non-human primates concerns the direct application of reagents that are targeted against human molecule [7]. However, limited immunological reagents that are prepared for analyzing human material can be used in the study of marmoset.

EAE in cynomolgus monkey (*Macaque fasciculrais*) could improve these limitations [12, 15]. In the present study, EAE were induced in cynomolgus monkey by immunizing with brain white matter from cynomolgus monkey. In the macaque that showed clear neurological disorders, clear gross lesions were found in the brain. By analyzing T cell receptor (TCR) transcripts by SSCP, T cell clones infiltrated into the brain lesions were demonstrated, and changes of T cell clonality in the blood were observed.

Materials and Methods

Animals

Four male cynomolgus monkeys were used for this study. Their ages ranged between four and five years old. All monkeys used were bred and reared in conventional indoor breeding facilities in the Tsukuba Primate Center in Japan [8].

Immunization protocol

Homogenate of snap-frozen monkey white matter(100 mg) collected from normal monkey was diluted with PBS (final 2.5 ml) and then emulsified with incomplete Freund

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adjuvant (IFA) (Difco Laboratory, Detroit, MI) supplemented with heat-killed M. tubeculosis H37Ra (10 mg/ml, Difco). Two monkeys (#3 and #4) were immunized with emulsified monkey brain white matter (10 mg/monkey, intradermally) and received an intravenous injection of pertussis toxin (2 ug/ml, 2.5 ml in PBS/ monkey, Seikagaku Kogyo, Tokyo, Japan) on the same day. The other two monkeys (#1 and #2) received CFA and pertussis toxin served as negative controls. Two days later, all of the monkeys were administered with the same amount of pertussis toxin intravenously through the saphenous vein.

The monkeys were checked every other day for the following symptoms; lethargy, anorexia, weight loss, ataxia, tremor, blindness, paraplegia, hemiplegia, quadriplegia, quadriparesis and moribund. When moribund was observed, the monkey was euthanized in accordance with the regulations of Tsukuba Primate Center.

Cell preparation

To monitor immunological changes after immunization, blood was collected on days 0, 3, 6, 13, 20, 27, 34, 41, 48, 54, 62 and 76 following immunization. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (Pharmacia, Milton Keynes, U. K.), washed with PBS, and suspended in PBS supplemented with 1% heat-inactivated fetal calf serum.

Antibodies and FACS

FITC-labeled anti-CD3 monoclonal antibody (mAb) (clone FN18, Biosource, Camarillo, CA), PE-labeled anti-CD4 mAb (clone NU-TH/1, Nichirei, Tokyo, Japan), PE- or phycoerythrin-Cy5 (Cy5)-labeled anti-CD8 mAb(clone Leu-2a, Becton Dickinson, Mountain View, CA), FITC-labeled anti-CD69 mAb (PharMingen, San Diego, CA), FITC or PE-labeled anti-CD20 mAb, FITC or PE-labeled anti-CD16 mAb (Becton Dickinson) and isotype matched control mAbs (Becton Dickinson) were used.

Reverse transcriptase polymerase chain reaction (RT-PCR) and SSCP

RT-PCR and SSCP were performed as previously described [13, 14]. mRNA from fresh PBMC (1×10^6 cells) and brain tissue (100 mg) obtained from regions with gross lesion were prepared using a QuickPrep (micro) mRNA Purification kit (Pharmacia Biotech, Sweden). The cDNA was synthesized from mRNA using a First-strand cDNA synthesis kit (Pharmacia Biotech). PCR was performed in a 50 ul reaction (30sec at 94°C, 30sec at 60°C, 1 min at 72°C, 35 cycles) containing 5 ul of 10 × Ex Taq Buffer (Takara, Shiga, Japan), 4 ul of dNTPs (Takara), 0.5 ul (2.5 U) of Ex Taq DNA polymerase (Takara) and 3 ul of 10 uM of sense and antisense primers for each of the TCR chains. The primers for each of the TCR family in monkey were described previously [14]. The amplified products for each V (3 ul) were diluted (1 : 1) with denaturing solution containing 95% formamide, 10 mM ethylenediaminetetraacetic acid, 0.1% bromophenol blue and 0.1% xylene cyanol, and heat-denatured at 94°C for 3 min. The product was then loaded (4 ul) in a non-denaturing 4% polyacrylamide gel containing 10% glycerol. After electrophoresis, the DNA was transferred on a nylon membrane (Biodyne A, PAL, Washington, NY), and hybridized with a biotinylated Cβ-specific internal probe after fixation with ultraviolet light (50 mJ/ cm^2). The DNA was visualized using a chemiluminescent substrate system (Phototope Detection kit, New England Biolabs, Beverly, MA).

Results

Clinical observation

Two weeks following immunization, all four monkeys appeared normal on neurological examinations. However, one monkey (#4) which was immunized with brain white matter, showed signs of anorexia, weight loss on day 16, and blindness and quadriparesis on day 20. Finally, the monkey became moribund and was euthanized on day 25. The other monkey immunized with brain tissue (#3) also showed neurological symptoms such as lethargy, anorexia, tremor, and blindness on day 20. However, after day 25, the monkeys neurological symptoms were improved. Both monkeys (#1 and #2) which were immunized with CFA alone, were asymptomatic.

One monkey which had become moribund, was euthanized and the brain was examined grossly. As shown in Figure 1, there was a considerable number of red spots in the white matter, indicative of inflammation in the white matter.

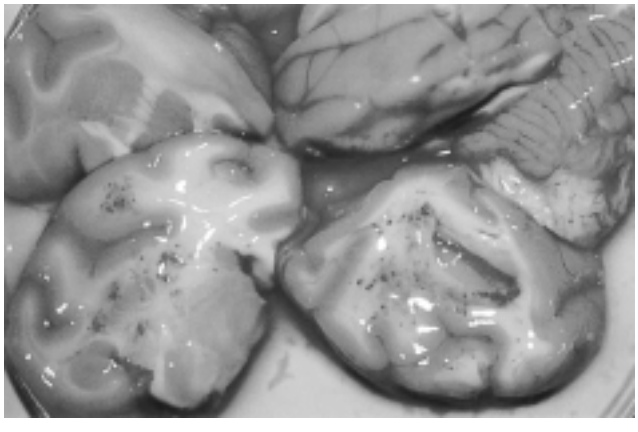

Fig. 1. Immunization with brain white matter in macaque monkeys induces inflammation specifically in brain white matter. Brain sections from the monkey which became moribund and was euthanized.

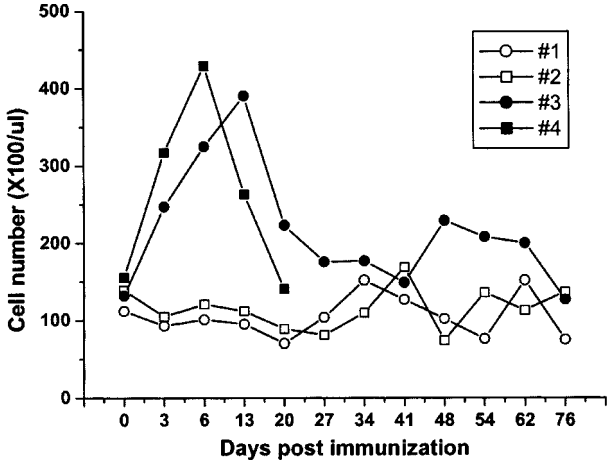

Fig. 2. Changes of leukocyte count after immunization with brain white matter.

Hematological observation

A considerable increase of total leukocyte count was observed in monkeys (#3 and #4) immunized with white matter in CFA, but not in monkeys (#1 and #2) immunized with CFA alone (Figure 2). This increase in leukocyte count was observed as early as day 3 post-immunization, and reached peak between day 6 and day 13. However, it had returned to normal range by day 27. In terms of the other values analyzed, for instance, RBC, Hemoglobin and hematocrit remained unchanged during the observation period (data not shown).

Flow cytometric analysis

Flow cytometric analysis of PBMC confirmed that the CD3+ T cell population predominated in immunized monkey with macaque white matter (Figure 3, A). One monkey (#3) which recovered from neurologic symptoms, showed a increase in the percentage of CD3+ T cells in PBMC within 3 days of immunization. The increased level of CD3+ T cells remained elevated for 4 weeks. The other monkey (#4) which was euthanized on day 25, also showed an increase in CD3+ T cells. Further investigation of the elevated CD3+ T cells revealed that they accompanied with the expression of CD69 (Figure 3, B and C). Whereas CD3+ T cells from the two monkeys (#1 and #2) which had CFA alone, did not.

Although the proportion of CD16+ NK cells in PBMC decreased in both monkeys (#3 and #4), this decrease was thought to be the reflection of an increase in total leukocyte number (data not shown), indicating the NK cells were not the major responding cells during early immunization.

An increased proportion of CD20+ B cells was also observed in both monkeys (#3 and #4) (Figure 3 D). The increase in B cell proportion remained elevated for the first 3 weeks.

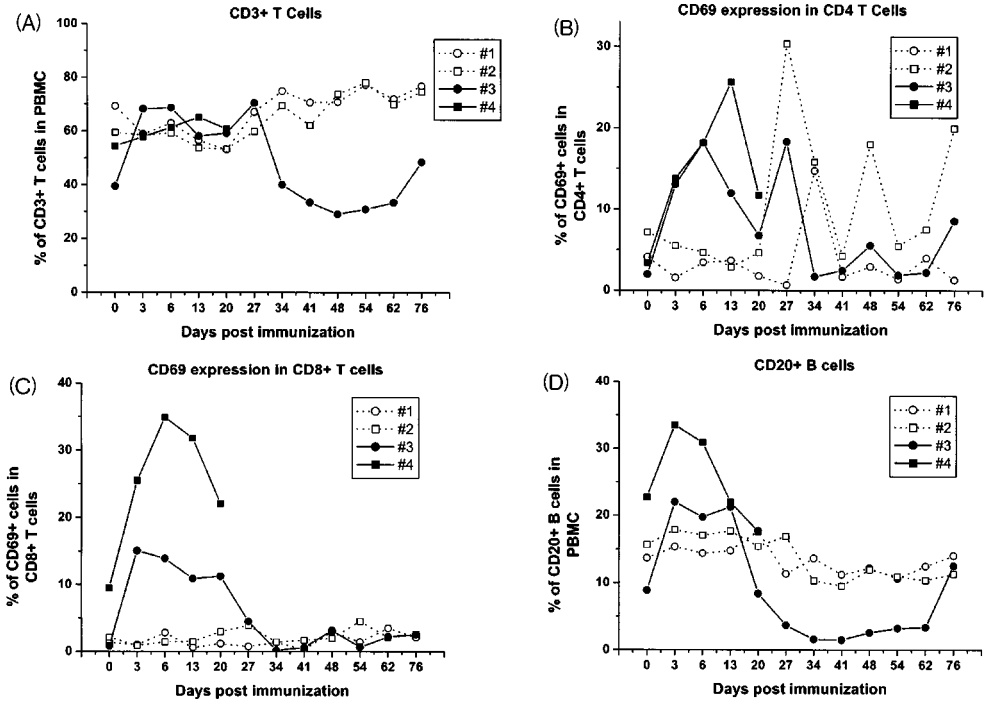

Fig. 3. CD4+ T cells were the main responders after immunizing monkeys with monkey brain white mater. A, changes in the percentage of CD3+ T cells in PBMC. B, changes in the proportion of CD69+ cells among CD4+ T cells. C, changes in the proportion of CD69+ cells among CD8+ T cells. D, changes of the percentage of CD20+ B cells in PBMC.

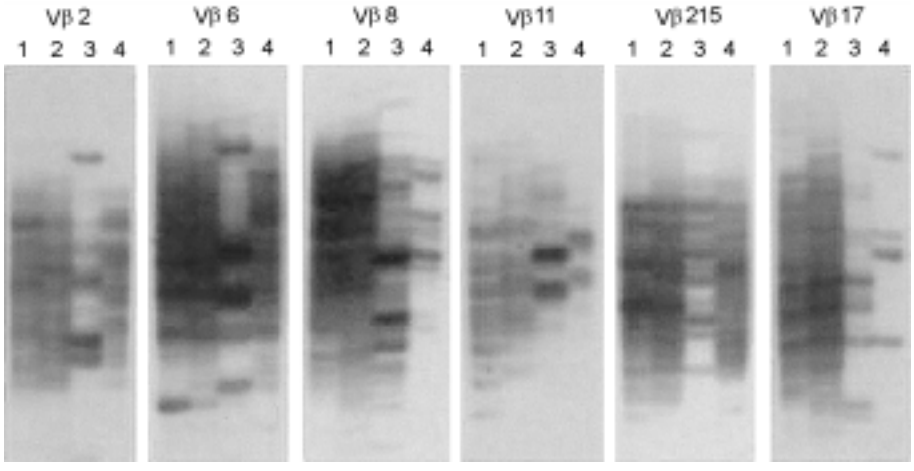

Fig. 4. Detection and comparison of T cell clones by SSCP analysis of the mRNA transcripts extracted from PBMC and inflammatory brain region. Representative TCR V beta families are shown. Lane 1, PBMC on day 0; lane 2, PBMC on day 20; lane 3, brain after euthanization; lane 4, PBMC on day 25.

SSCP analysis of TCR beta chain

SSCP analysis was performed on the TCR beta chain. The RT-PCR product for TCR was designed to include the CDR3 region, which is the most important region for antigen recognition. PBMC obtained on days 0, 20 and 25 post-immunization from #4 monkey, were used for SSCP analysis. The SSCP patterns from the PBMC were compared with those of brain tissue (Fig. 4). Representative SSCP results for V beta 2, 6, 8, 11, 15 and 17 families among the 24 TCR beta chains analyzed are shown. In almost all V beta families, changes in clonality were observed between day 0 and day 21. In general, clones detected on day 0, diminished on day 20 or day 25, as seen in V beta 2 and 15. Importantly, many T cell clones found in the brain white matter were not detected in peripheral blood.

Discussion

In the present study, the induction of EAE in cynomolgus monkeys by immunizing with monkey brain white matter with CFA in combination with pertussis toxin is described. Clear red spots were found in the brain white matter but not in gray matter, indicating an acute inflammatory response. The main responding cells after immunization were CD3+ T cells. Furthermore, SSCP analysis of the TCR beta chain suggested that those infiltrating T cells were unique T cell clones which were not present in the blood.

Most models for EAE have been established with rodents [3, 11, 16]. Rodent models have some benefits, such as homogenous responses to stimulation, because of genetic control [2], and convenience for performance etc. However, there are also some disadvantages, for example, their relatively small size may be a limiting factor during analysis, and their different immune systems might make extrapolation to human difficult.

Some groups have developed EAE using marmosets [5, 6]. However, marmosets are small and many of the reagents used for humans fail to work in marmosets. Some groups have also described the macaque model for [12, 15], though they did not used macaque monkeys brain tissue. Using the macaque monkeys EAE model, we realized several advantages. An adult macaque weighs over several kilograms. Therefore, several milliliters of cerebrospinal fluid are available for analysis without autopsy, and multiple blood drawing is possible. Moreover, most reagents, such as monoclonal antibodies prepared for human are useful [10, 18, 19, 20], and most importantly, macaque monkeys are very similar to human, genetically and phylogenetically, enabling extrapolation into human.

In the macaque EAE model described here, symptoms observed in human MS, including blindness and quadriparesis were observed. In human, relapsing-remitting multiple sclerosis phases are repeated over time [4, 9]. Recovery from sickness showing neurological symptoms in one monkey may implies the possibility of the cycle. However, to know the exact disease course of the present macaque EAE models, further long-term studies are required.

The CD3+ T cell population expanded in the monkey immunized with brain white matter as shown by flow cytometry. This is consistent with our present knowledge on MS in human [17]. CD4+ T cell involvement in the immunized monkeys was also confirmed by their CD69 expression which is an early marker for activated T cells.

The reduced percentage of CD16+ NK cells in monkeys with EAE, may be attributed to the increase in total leukocyte count. It is a possible that the decrease of NK cells was associated with EAE induction as seen in rodents

[23].

The SSCP band patterns from the PBMC also indicated drastic T cell response, resulting in a disturbance of bands present before the immunization. Although some T cell clones observed in brain tissue by SSCP analysis were observed in PBMC, most T cells clones found in the lesions were not detected in PBMC, indicating the specific recruitment of pathogenic T cell clones into brain white matter area.

The present paper shows that SSCP analysis of TCR is useful in studing clonality of T cells infiltrating into the brain tissue of macaque with EAE.

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