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Epicutaneous immunization with myelin basic protein protects from the experimental autoimmune encephalomyelitis

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Abstract:
Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) with limited treatment modalities. One of the experimental methods that protect from autoimmune diseases is oral tolerance. However, this method failed to show therapeutic efficacy in clinical trials. In our previous work, we found that epicutaneous (*ec*) immunization with a protein antigen induces a state of profound immunosuppression that inhibits inflammatory response in contact sensitivity (CS), experimental autoimmune encephalomyelitis (EAE) in B10.PL mice that develop chronic form of disease, and also delayed allogeneic skin graft rejection.
In the current work, we showed that *ec* immunization with MBP protects from relapsing and remitting EAE. Protection from the disease correlated with decreased number of mononuclear cells isolated from CNS. Additionally, histological examination showed only a slight mononuclear cell infiltration in spinal cords of mice *ec* immunized with MBP when compared to positive control where animals were *ec* treated with PBS before disease induction.

Key words:
epicutaneous immunization, suppression, myelin basic protein, experimental autoimmune encephalomyelitis

Abbreviations: CFA – Freund's adjuvant, CNS – central nervous system, EAE – experimental autoimmune encephalomyelitis, *ec* – epicutaneous, MBP – myelin basic protein, MS – multiple sclerosis, OVA – ovalbumin, TNP – trinitrophenyl

Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) characterized by the presence of cellular infiltrates, composed primarily of lymphocytes and macrophages, and localized areas of demyelination in the CNS. MS is thought to be initiated by self-reactive $CD4^+$ T cells with specificity for a variety of antigens contained within the myelin sheath [4]. Experimental autoimmune encephalomyelitis (EAE) is a well-characterized animal model that mimics the clinical form of MS, including the presence of cellular infiltrates and demyelination in the CNS.

Treatment modalities for MS are limited, with the most common treatments being steroids or antimitotic drugs acting nonspecifically on the immune system resulting in a general immunosuppression accompanied by many severe side effects [14]. Thus, numerous efforts have been undertaken to develop a treatment method able to specifically control the autoimmune response. One of these methods relied on mucosal deposition of an antigen. It is worth to men-

tion that oral tolerance has been studied in many experimental models, including EAE where it was found that animals fed with myelin basic protein (MBP) were protected from the disease [6, 7]. Other studies showed that orally induced T suppressor (Ts) cells secreted anti-inflammatory cytokines, such as TGF-β, IL-4 and IL-10 [2]. Promising achievements in the field of mucosal tolerance in EAE encouraged clinicians to treat multiple sclerosis patients by feeding them with bovine MBP daily to suppress the disease. In MS patients MBP- and PLP-specific TGF-β secreting Th3-type cells have been observed in the peripheral blood of patients treated orally with bovine myelin preparation and not in patients who received placebo [3]. Despite these promising observations *in vitro,* clinical trial failed to show any therapeutic benefit of bovine MBP feeding beyond the placebo effect [19, 20].

Our previous work, employing B10.PL mice developing chronic form of EAE, showed that application of MBP to the skin prior to the induction of EAE by immunization with MBP protected mice from developing disease. The therapeutic effect was transferable to naive recipients with lymph node cells from MBP-treated mice. These Ts cells were found to be antigen non-specific, as suppression of EAE also occurred when the non-cross-reacting antigens OVA or TNP were *ec* applied [17].

In our current work, we tried to determine if *ec* immunization with MBP could protect from relapsing and remitting form of EAE. Additionally, we found a correlation between clinical disease score and the number of mononuclear cells infiltrating CNS what was determined by isolation of mononuclear cells from CNS and histological examination.

The ease of induction and potent non-Ag specific effect of induced skin Ts cells suggests that this may be a procedure applicable to treatment of autoimmune diseases.

Materials and Methods

Animals

Female SJLxB10.PL (H-2^{u+s}) mice 6–8 weeks old that develop relapsing and remitting disease were from the breeding unit of the Department of Human Developmental Biology, Jagiellonian University, Medical College. Mice were fed autoclaved food, and water. All experiments were conducted according to guidelines of the Animal Use and Care Committee of the Jagiellonian University.

Reagents

Guinea pig myelin basic protein (MBP) and complete Freund's adjuvant (CFA) (Sigma Chemical Co., St Louis MO); pertussis toxin (List Biologicals, Campbell, CA); RPMI 1640; fetal calf serum (FCS) (Life Technologies, Grand Island, NY), percoll (Amersham Biosciences AB, Uppsala, Sweden). Luxol fast blue, Giemsa and Gomori's chrome hematoxylin-phloxin (Sigma Chemical Co., St Louis, MO).

Active EAE induction

Mice were actively immunized with 200 μg of MBP in CFA containing 4 mg/ml of heat-killed *M. tuberculosis* H37Ra *sc* in each internal flank. Pertussis toxin (200 ng) in PBS was injected *iv* at the time of immunization and again 48 h later. Individual animals were assessed daily for clinical signs of EAE and were scored using a scale from 1–5 as follows: 0 – no disease; 1 – limp tail and/or hind limb ataxia; 2 – hind limb paresis; 3 – hind paralysis; 4 – hind and fore limb paralysis and 5 – death.

Isolation of mononuclear cells from CNS

Isolation of leukocytes from CNS tissue was performed on day 21 after immunization with MBP + CFA. Mice were anesthetized with ketamine/xylazine mixture and perfused with cold PBS through the left ventricle. Then brains and spinal cords were removed and pushed through a wire mesh to obtain a cell suspension. Mononuclear cells were isolated from the interface of a 37:70% discontinuous percoll gradient after centrifugation for 20 min at 1800 rpm. The number of leukocytes in CNS tissue of individual mice was assessed using trypan blue staining.

Histology

The spinal cords were fixed by perfusion with 10% paraformaldehyde. Paraffin-embedded material was cut into serial 7 μm sections which were stained with

Tab. 1. EAE disease course in SJLxB10.PL mice *ec* MBP immunized

	PBS	MBP
Number of mice	21	19
Incidence (%)	90	58
Average day of onset ± SE[1]	21.1 ± 1.8	30.0 ± 2.9
Average day 55 disease score ± SE[2]	2.6 ± 0.4	1.4 ± 0.4
Average cumulative disease score ± SE for all mice[3]	97.0 ± 15	34.0 ± 9.0
Average cumulative disease score ± SE for mice with EAE[4]	106.8 ± 14.3	59.1 ± 11.2
Mortality (%)	29	10

[1] p = 0.007, [2] p = 0.02, [3] p = 0.002, [4] p = 0.04. The values are the mean ± SE of all the mice that reached a score of at least 1

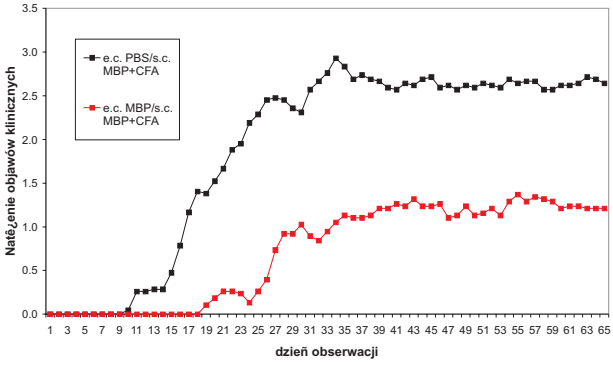

Fig. 1. Epicutaneous immunization with MBP protects from EAE. SJLx B10.PL mice were *ec* immunized with MBP as described in [17] (red squares) or treated with PBS alone (black squares) for one week before active immunization with MBP plus CFA. Individual animals were assessed daily for clinical signs of EAE and were scored. The EAE daily scores of 21 PBS-treated and 19 MBP EC-tolerized SJLxB10.PL mice were averaged

Luxol fast blue [12], Giemsa, or Gomori's chrome hematoxylin-phloxin [10] methods.

Results

Epicutaneous immunization with MBP results in protection from EAE

Our previous studies showed that *ec* immunization with MBP protected B10.PL mice from chronic form of EAE. In the current experiment, we tried to determine if *ec* immunization maneuver could inhibit development of relapsing and remitting EAE in SJLxB10.PL mice. Briefly, cotton gauze soaked with a solution containing MBP was affixed to bare skin on the dorsal side of the mouse. After 7-day exposure, mice were *sc* immunized with MBP in CFA, as described in the methods, to induce EAE. Mice were then monitored daily for clinical signs of disease.

Figure 1 shows that *ec* immunization of SJLxB10.PL mice with MBP before EAE induction results in protection from disease. There was a delay of disease onset by 9 days in MBP *ec* immunized mice when compared to control mice that had been exposed to PBS alone. In addition, disease incidence was also reduced to 58% from 90% and disease mortality was reduced to 10% from 29% in MBP *ec* immunized mice compared to control mice, respectively. Average day 55 disease score in positive control was 2.6 whereas *ec* treatment with MBP reduced it to 1.4 (Tab. 1). In those mice that did develop disease, MBP *ec* immunization resulted in a reduction in disease severity as demonstrated by the significant reduction in mean daily disease score between the MBP *ec* immunized group and controls.

EC immunization with MBP results in decreased mononuclear cell infiltration in CNS

To confirm our *in vivo* findings showing decreased disease severity in *ec* MBP-treated mice, we used two approaches. First, we isolated mononuclear cells from CNS of mice that were *ec* treated with PBS or MBP before EAE induction. Figure 2 shows that *ec* immunization with MBP before EAE induction suppressed mononuclear cell infiltration in CNS when compared to positive control (mice *ec* treated with PBS alone).

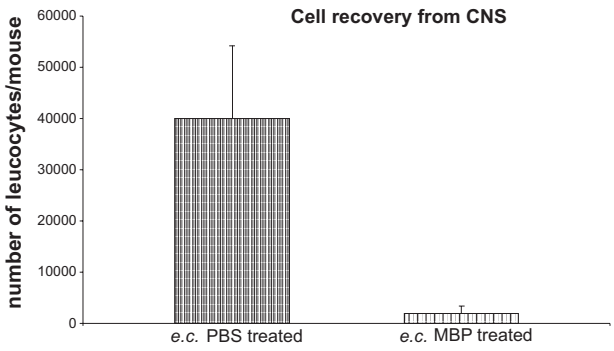

Fig. 2. Epicutaneous immunization with MBP inhibits mononuclear cell infiltration in CNS. SJLxB10.PL mice were EC treated with PBS (black bar) or MBP (white bar) before immunization with MBP + CFA. On day 21 after immunization, mononuclear cells were isolated from CNS and the number of leukocytes was estimated

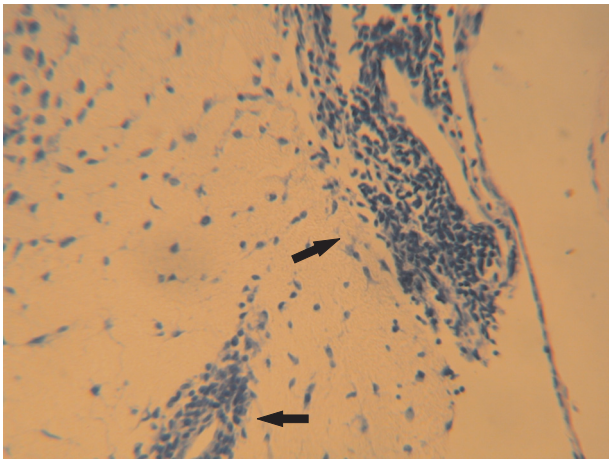

Fig. 3. Fragment of spinal cord of an EAE mouse. Arrows indicate massive leukocyte infiltrations. Giemsa staining. Original magnification x 160

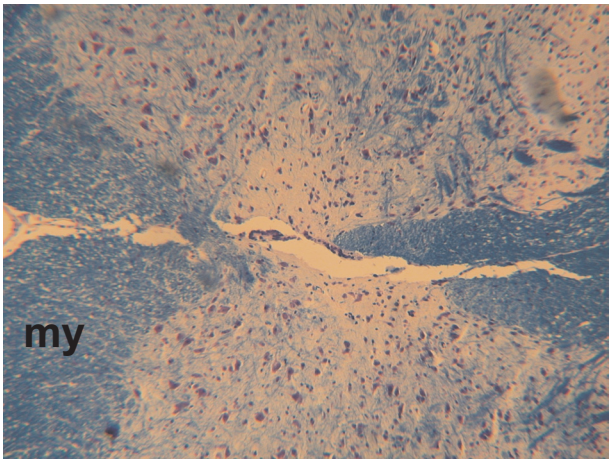

Fig. 5. Fragment of spinal cord of a treated mouse. No demyelinization is present. my – myelinated fibers in cross-section. Luxol fast blue staining. Original magnification x 160

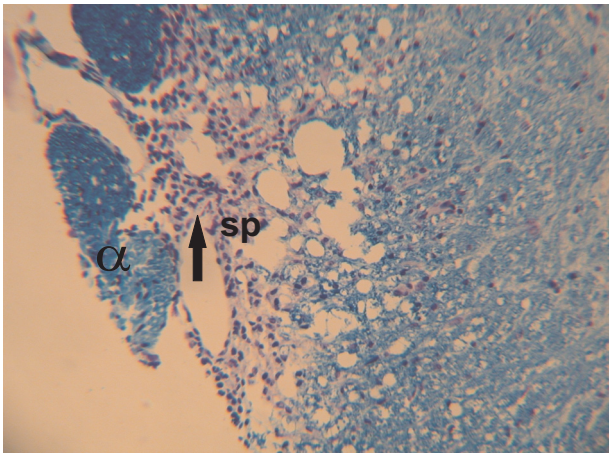

Fig. 4. Fragment of spinal cord of an EAE mouse. d – demyelinated fragment, sp – sponginess, arrow – leukocyte infiltration. Luxol fast blue staining. Original magnification x 160

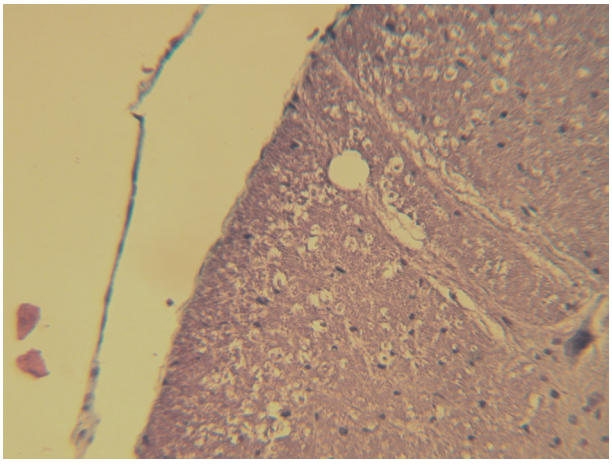

Fig. 6. Fragment of spinal cord of a treated mouse. No infiltration of leukocytes or other pathological changes are evident. Gomori's chrome hematoxylin-phloxin staining. Original magnification x 160

To confirm that finding, we conducted histological examination of spinal cords. Figures 3 and 4 show fragments of the spinal cord of animals *ec* treated with PBS before disease induction. In this group of mice, perivascular leukocyte infiltration at meningeal and parenchymal blood vessels' venules and sponginess of the tissue and partial demyelinization with leukocyte infiltration were present, respectively. Figures 5 and 6 show spinal cords of mice *ec* treated with MBP before EAE induction. In both figures, undamaged myelinated tracts and no discernible pathology can be observed.

Discussion

MS is a chronic inflammatory disease of the CNS caused by infiltrating auto-reactive CD4+ cells and activated macrophages [4].

There are many therapeutic approaches to treat MS. However, in clinical practice, commonly used immunosuppressive drugs e.g. steroids act non-specifically on the immune system of the host which results in profound immunosuppression and many other side effects. For many years there have been numerous ef-

forts to find an effective method that would allow to control unwanted immune response specifically. There are many methods that help to treat autoimmune diseases including MS.

One of these methods relies on mucosal deposition of an antigen. In EAE it was shown that animals fed with MBP were protected from the disease [1]. Unfortunately, clinical trial failed to show any clinical benefit of bovine MBP feeding beyond the placebo effect [15].

For many years the skin has been considered an organ where strong T cell-mediated immune responses such as contact sensitivity could easily be induced. However, the skin as a site for induction of tolerance has received limited attention [13].

Wang et al. [18] showed that *ec* application of protein antigens like OVA induces the ability to elicit allergic dermatitis accompanied with appearance of IL-4 secreting Th2 cells. It is well known that cytokines released by Th2 lymphocytes are able to inhibit Th1-mediated immune responses [9]. The data showing that *ec* application of protein antigen results in the induction of T cells secreting Th2 cytokines led us to speculate that skin immunization with protein antigen before induction of Th1-mediated immune response might cause its suppression. Our work in CS system showed that *ec* immunization with protein antigen before hapten application caused strong peripheral suppression of Th1 mediated immune response [11, 16]. Our other study showed that *ec* immunization with MBP before EAE induction decreased disease severity [17]. The therapeutic effect of *ec* immunization was transferable with TCRαβ+ CD4+ CD8+ double positive Ts cells. These Ts cells were found to be antigen non-specific and to inhibit inflammatory response *via* released TGF-β [17]. All the previous experiments were done employing B10.PL mice that develop a chronic form of EAE.

In the current study, we determined if *ec* immunization with soluble protein antigen MBP spread over a gauze patch could protect from relapsing and remitting form of EAE. To answer this question, we employed SJLxB10.PL mice that develop relapsing and remitting EAE. Data presented in Figure 1 clearly show that *ec* immunization with MBP results in significant reduction of disease severity and also delays onset of EAE. Maneuver of *ec* immunization with MBP also resulted in reduced mortality and disease incidence as shown in Table 1. These data may suggest that *ec* immunization with MBP could be used to treat not only chronic but relapsing and remitting form of the disease, as well. In further experiment, we attempted to determine if *ec* immunization with MBP interferes with mononuclear cell infiltration in CNS accompanying EAE. Data presented in Figure 2 clearly show that *ec* immunization with MBP before EAE induction results in almost complete inhibition of leukocyte infiltration in CNS on day 21 after immunization with MBP plus CFA when compared to positive control. These data may suggest that the observed amelioration of the disease by skin patching could be caused by inhibition of CNS infiltration by mononuclear cells and lack of demyelinization typical of full-blown EAE.

Induction of tolerance *via ec* immunization with protein antigen could be used to inhibit cell-mediated immune responses as we found in CS system, allogeneic skin graft rejection model and animal model of MS [8, 11, 16, 17]. This method is potentially useful to treat different inflammatory diseases. The noninvasive immunization *via ec* application of protein antigen that induces Ts cells inhibiting inflammatory response, becomes an attractive therapeutic method for different clinical situations.

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