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PERTUSSIS VACCINE-INDUCED EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN MICE

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Abstract

Background: A small dose of the *Bordetella pertussis* vaccine is used as an adjuvant for the induction of experimental autoimmune encephalomyelitis (EAE) in mice. The effects of two doses of the *Pertussis* vaccine on clinical signs, antibody titers, and the expression of CD4 and MHC molecules in brain tissue sections of mice with EAE were examined. Methodology: EAE was induced by spinal cord homogenate in Complete Freund adjuvant (CFA) in 30 of 40 C57BL/6 mice divided in groups: EAE mice with a small adjuvant dose of the *Pertussis* vaccine (EAE-1), EAE mice with a human dose of the *Pertussis* vaccine (EAE-2), EAE mice (EAE-3). Results: None of the mice from the EAE groups progressed to severe EAE. Five mice from the EAE-2 group were found dead on the 13th day post-immunization. A significant increase of anti-MOG (myelin oligodendrocyte glycoprotein) antibodies was detected in mice with EAE compared to non-treated mice. Myelin loss and brain tissue lesions were observed in EAE-1 and EAE-2 mice compared to EAE-3 and non-treated mice. A high expression of MHC-II and a mild expression of MHC-I was detected in the brains of mice with EAE. No expressions were detected in intact brains. Scattered CD4-positive cells were detected in the brains of EAE-1 and EAE-2 mice compared to EAE-3 and non-treated mice. Conclusion: A small dose of the *Bordetella pertussis* vaccine could maintain the developed clinical signs and histological changes in mice with EAE, while higher doses led to additional adverse effects. The expression of CD4 and MHC class I and II molecules, as well as an increase in anti-MOG antibodies could be used as markers capable of monitoring the development and progression of EAE.

Keywords

• Autoimmunity • EAE • MHC molecules • Pertussis vaccine

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Introduction

Human vaccines can induce autoimmunity when administered with autoantigens, exacerbate autoimmunity when given alone, and can even induce autoimmunity when administered without autoantigens [1,2]. The *Bordetella pertussis* bacterium produces pertussis toxin, which represents its main virulence factor. However, the pertussis vaccine has been shown to be an adjuvant for the induction of a number of autoimmune diseases. In the animal model of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), the *Bordetella pertussis* vaccine and pertussis toxin are used to increase disease incidence and severity when administered simultaneously with the autoimmune challenge [3-5]. It is suggested that the pro-inflammatory activity of *Bordetella pertussis* is mainly attributed to an increased permeabilization of the blood-brain barrier leading to an influx of immune cells into the CNS [6].

EAE can be induced in susceptible animals by active immunization with myelin antigens with or without addition of the pertussis vaccine [4,7-9].

Active immunization induces a primarily $CD4^+$ T cell-dependent immune response, leading to CNS demyelination and the appearance of clinical signs. Inflammatory infiltrates composed of macrophages/microglial cells, T cells, and B cells are found in lesions. Activation of myelin specific $CD4^+$ T cells allows them to cross the blood-brain barrier more efficiently than naïve T cells [10,11].

The altered expression of MHC class I and/or II antigens has been associated with a variety of disorders with neurological symptoms, including MS and EAE. It is suggested that MHC class I has a role in the normal development of the CNS, as well as the immune response in EAE [12], while the expression of MHC class II molecule acts as a stimulus for the activation of $CD4^+$ T cells [13]. MHC class II antigen expression is always increased in EAE, and MHC class II-positive cells are abundant in and around active EAE lesions [13,14].

Humoral immunity plays a major role in disease pathogenesis. Some studies reported a pathogenic role of autoantibodies directed against one or several myelin proteins, while other studies suggest that natural autoantibodies could mediate the repair to myelin membranes (i.e. remyelination/axon protection) [15] indicating that the detection of antibodies may be useful in assessing the clinical course of the disease. Even though the presence of the antibody response within the CNS in MS patients is well established, the pathogenic role of antibodies still remains obscure and their antigenic targets have yet to be determined [16,17].

In the present study, the effects of two different doses of the *Bordetella pertussis* vaccine on the regulation of EAE were examined in C57BL/6 mice through the monitoring of EAE clinical signs, antibody titers and the expression of CD4 and MHC molecules in the brain tissue of the mice.

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Experimental procedures

Mice

Female C57BL/6 mice, 6 weeks old, were purchased from Military Medical Academy (VMA, Belgrade, Serbia). The protocols for animal experiments were conducted in accordance with the guidelines (86/609/EEC) of the European Community Council Directives and the Serbian Laboratory Animal Science Association (SLASA). Mice were kept under standard laboratory conditions (room temperature 21±1°C, humidity 30%, 12/12 h light/dark cycle) and food and tap water *ad libitum*.

The study was performed on 40 mice divided into four groups, each containing 10 mice: mice with EAE and an adjuvant dose of the *Bordetella pertussis* vaccine (EAE-1); mice with EAE and a human equivalent dose of the *Bordetella pertussis* vaccine (EAE-2); mice with EAE (EAE-3) and non-treated mice (internal control, IC).

EAE induction

Mice were immunized with mouse spinal cord homogenate (MSCH) in Complete Freund adjuvant (CFA) according to the modified method of Frei *et al.* [7]. On day 0, each mouse received 0.3 mL of a mixture containing 5.0 mg MSCH in 0.15 ml PBS (pH 7.4) and 0.15 mL of CFA containing 1 $mgmL^{-1}$ of heat-killed and dried *Mycobacterium tuberculosis* H37Ra (Sigma Aldrich, St. Louis, MO, USA). The inoculum of 0.3 mL was injected at 100 µL in each of three spots on the back (one anterior, two posterior on each side). On the day of immunization and 2 days later, 10^{10} of the *Bordetella pertussis* vaccine (British Reference reagent 88/522; National Institute for Biological Standards and Control, Potters Bar, Hertsfordshire, UK) in 0.3 mL PBS was injected intraperitoneally to mice from the EAE-1 group. Mice from the EAE-2 group were injected intraperitoneally with 1/5 of human dose of pertussis vaccine (4×10^{10}) in 0.3 mL of PBS [18].

Clinical assessment of EAE was performed daily according to the following criteria: (0) no disease; (1) floppy tail; (2) hind leg weakness; (3) full hind leg paralysis; (4) quadriplegia; (5) death. Mice that were in between the clear-cut gradations of clinical signs were scored as intermediate in increments of 0.5. Mice were sacrificed on day 24 post-immunization.

ELISA assay

Serum samples were collected by cardiac puncture from the anesthetized animals, obtained 24 days post-immunization, and tested for the presence of anti-MOG (myelin oligodendrocyte glycoprotein) antibodies using ELISA assay (cat. no. 163913-87-9, Sigma-Aldrich, St. Louis, MO, USA).

The procedure used was a modification of that used by Voller *et al.* [19]. Flat-bottomed tissue culture plates (NUNC, Roskilde, Denmark) were coated with 50 µl/well MOG_{35-55} antigen in concentration 5 µg/ml in carbonate buffer and incubated overnight at 4°C. The plates were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, 200 µl/well) for 1 h at 37°C. Following blocking, the plates were washed four times with washing buffer (0.05% Tween 20 in PBS; 200 µl/well). In the next step, the serum samples were added to each plate (50 µl/well of a 1:50 serum dilution). The samples were made in PBS containing 1% BSA, applied in duplicate and incubated for 1 h at room temperature (RT). After three washings, 50 µl/well of horseradish peroxidase (HRP) conjugated rabbit anti-rat polyclonal IgG (DAKO, Glostrup, Denmark; dilution 1:100) were allowed to react with the bound antibodies. Reaction mixtures were incubated for 1 h at RT and the wells were then washed three times with washing buffer. Substrate solution (50 µl/well), prepared by adding 12 µL of 12% H_2O_2 to 10 mL of freshly prepared 1 $mgmL^{-1}$ *o*-phenylenediamine (Sigma Aldrich, St. Louis, MO, USA) in citrate-phosphate buffer (pH 5), was added to each well. The reaction was stopped after 15 min by the addition of 50 µl/well of 2 M sulfuric acid. The absorbance value was measured on Multiscan Ascent (Labsystems, Thermo Fisher Scientific, Waltham, MA, USA) at 492/620 nm.

Statistical evaluation

The differences of antibody titers were evaluated by ANOVA (SPSS Statistics version 20, SPSS Inc., Chicago, IL, USA). The differences among the groups were compared with a *post-hoc* Scheffé´s test. A probability of $p < 0.05$ was considered significant.

Tissue collection

All mice were deeply anaesthetized and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brain tissue samples were immersed in OCT embedding compound (Killik Bio-Optica S.p.a., Milano, Italy), frozen in liquid nitrogen, and stored until use at -80°C. A representative part of the frozen tissues was processed with a cryo-microtome (Jung-Reichert Cryocut E, Cambridge Instruments GmbH, Heidelberg, Germany) using 5-µm thick sections.

Immunohistochemistry of frozen sections

Thick frozen brain sections (5 µm) were harvested onto superfrost slides (ThermoScientific, Menzel, Braunschweig, Germany), air-dried, and fixed in cold acetone for 10 min at -20°C. The slides were first washed in TBS (Tris buffer saline) and then incubated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity. Followed by a series of washes (three times distilled water), the sections were blocked with 5% normal rabbit serum. Then sections were incubated for 24 h at 4°C in a humidified chamber with a primary antibody to MHC-I (Mouse-anti mouse MHC I antibody, cat. no. 553575, BD Pharmingen, San Diego, USA; dilution 1:20), MHC-II (Rat-anti mouse MHC II antibody, cat. no. MCA2401, AbD Serotec, Bio-Rad Laboratories Inc., Hercules, CA, USA; dilution 1:20) or MOG (anti-MOG D-2 antibody, cat. no. sc-376138, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, dilution 1:20). After three washes with TBS, the sections were incubated with peroxidase-labeled polyclonal rabbit anti-rat IgG antibody (cat. no. P026002, DAKO, Glostrup, Denmark; dilution 1:100) for 1 h at RT, followed by the development of the 3,3'-diaminobenzidine (DAB) substrate for 10 min. Subsequently, all slides were rinsed in distilled water, counterstained with hematoxylin, dehydrated in a graded series of alcohols, and covered with coverslips.

Tissue analysis

The analysis of brain sections was performed by capturing images of sections using a BH2 research microscope (Olympus Optical Co. Ltd, Tokyo, Japan) equipped with a Color View

III digital camera (Olympus). Analysis Docu software (Olympus) was used to acquire images.

Results

Clinical signs of EAE

Mice with EAE were monitored daily beginning from day 7 post-immunization. Mice from the EAE-1 and EAE-2 groups developed a mild form of EAE with partial loss of tail tonicity and mean clinical scores of 0.5. Only one mouse from the EAE-3 group had partial loss of tail tonicity. The clinical signs were observed on the 20th day post-immunization. None of the mice from the EAE-1 and EAE-3 groups progressed to severe EAE. Conversely, problems with hind legs in one half of mice from the EAE-2 group were observed immediately after a repeated dose of the *Bordetella pertussis* vaccine; the mice were turgid and were found dead on the 13th day post-immunization. In mice with EAE that were adjuvanted with the pertussis vaccine, slower walking speeds were noticed, as well as stooped posture in all of the mice.

Anti-MOG antibodies titer

Serological monitoring of anti-MOG antibodies was performed using ELISA test. As shown in Figure 1, mice with EAE exhibited an increase in the level of anti-MOG antibodies. Some titer levels of anti-MOG antibodies were found in the serum samples of unimmunized mice. A significant increase in anti-MOG antibodies was detected in mice with EAE compared to the control group ($p < 0.05$). There was no difference found between mice with EAE (with or without addition of the pertussis vaccine).

The MOG molecule in the brain

The mice were sacrificed 24 hours post-immunization, and immunohistochemical staining of brain sections with MOG was performed. Intact myelin sheaths were seen in the hippocampal region and brain parenchyma of non-treated mice. In mice from the EAE-3 group strong staining of brain parenchyma was found, similar to the control group, while some localized staining of MOG antigen was observed in the hippocampal region (Figure 2).

Interestingly, a more pronounced staining of myelin was seen at the rim of EAE lesions in mice from the EAE-1 and EAE-2 groups. Lesions were mainly observed in the hippocampal region of the brain. In addition, myelin loss was observed in the brain parenchyma.

Expression of MHC I and II molecules

In the present study, the detection of MHC molecules expressed on the cell surface was performed by immunostaining brain tissue sections with anti-MHC class I and anti-MHC II antibodies.

Qualitative analysis of MHC-II expression in brain sections showed rare MHC-II positive cells in the brain parenchyma and hippocampal regions of non-treated mice. Numerous strongly stained MHC-II positive cells were observed in the hippocampus and brain parenchyma of mice with EAE. In the hippocampal region of mice with EAE (with or without addition of the pertussis vaccine) as well as in non-treated mice, MHC-II positive cells with different staining intensities appeared in order as presented in Figure 2. Strong staining was shown with similar intensity in adjuvant-induced EAE (EAE-1) and EAE mice with a human dose of the pertussis vaccine (EAE-2), while mild expression was observed in the brains of EAE mice without the addition of pertussis vaccine (EAE-3).

On the other hand, tissue mildly positive for the MHC-I molecule and scattered cells was observed in the hippocampal region and the brain parenchyma of mice with EAE, and rare positive cells were observed in the brains of non-treated

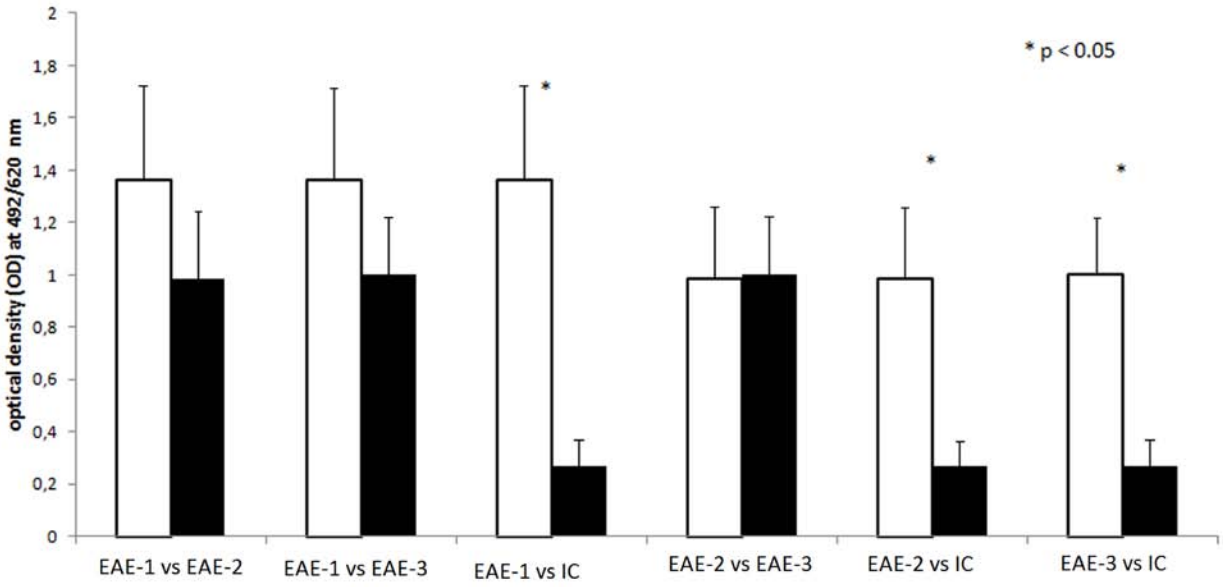

Figure 1. Serum anti-MOG antibodies responses as revealed by ELISA test. Mice were immunized (subcutaneously) with mouse spinal cord homogenate (MSCH) in Complete Freund adjuvant (CFA) with the addition of an adjuvant dose (EAE-1) or a human dose of pertussis vaccine (EAE-2) or without addition of the pertussis vaccine (EAE-3) or non-treated (internal control, IC). The results are expressed as mean values (SD) of optical densities measured at 492/620 nm. The differences between groups were evaluated by Scheffé´s *post-hoc* test and * mark represents statistically significant differences. A probability of $p < 0.05$ was considered significant.

mice (Figure 3). Staining with similar intensity was found in EAE mice with the pertussis vaccine (EAE-1 and EAE-2) injected and in EAE mice without addition of the pertussis vaccine (EAE-3).

Expression of CD4 in the brains of mice with EAE

Detection of the expression of CD4 molecules was performed by fluorescence staining of brain tissue sections with anti-CD4 antibodies in mice with or without addition of the pertussis vaccine.

Small scattered groups of $CD4^+$ cells were detected in the hippocampal region from the EAE-1 and EAE-2 groups. Rare $CD4^+$ cells were observed in mice from the EAE-3 group as well as in non-treated mice (Figure 4).

Discussion

In this study, the effects of two different *Bordetella pertussis* vaccine doses were examined through the monitoring of EAE clinical signs, antibody titers, and the expression of CD4 and MHC molecules in brain tissue sections.

The clinical signs were observed on the 20th day post-immunization. The dynamics of the development of clinical signs in mice differ from those in rats, showing the clinical manifestation around 20 days post-immunization [20]. Partial tail tonicity loss was observed in EAE mice whom the addition of a small dose (concentration of 10^{10} *Bordetella pertussis* microorganisms) of the pertussis vaccine was given. The development of a mild form of EAE is a characteristic of C57BL/6 mice that had been immunized with mouse spinal cord homogenate [7], which is consistent with the clinical signs observed in the present study. Different doses of the pertussis vaccine are able to increase the effects of spinal cord homogenate in the development of EAE, indicating that the pertussis vaccine addition plays an important

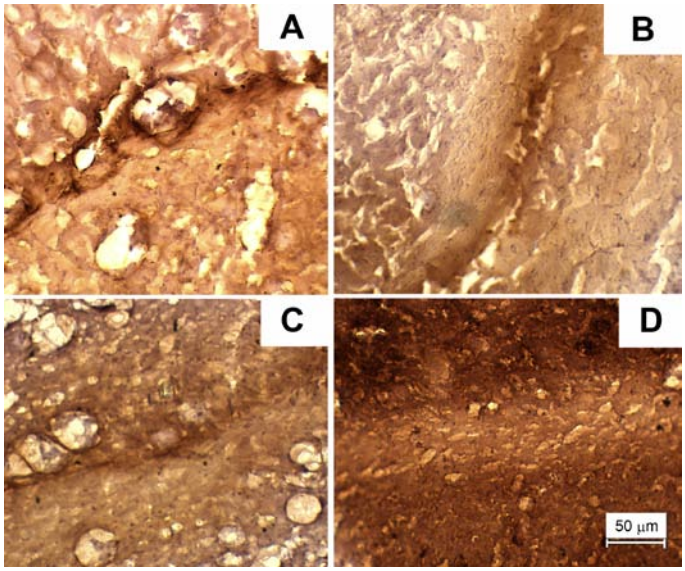

Figure 2. MOG staining in the hippocampal region of brain tissue of a) EAE-induced mice with an adjuvant dose of the pertussis vaccine, b) EAE-induced mice with a human dose of the pertussis vaccine, c) EAE-induced mice without pertussis vaccine and d) non-treated mice. Scale bar = 50 µm.

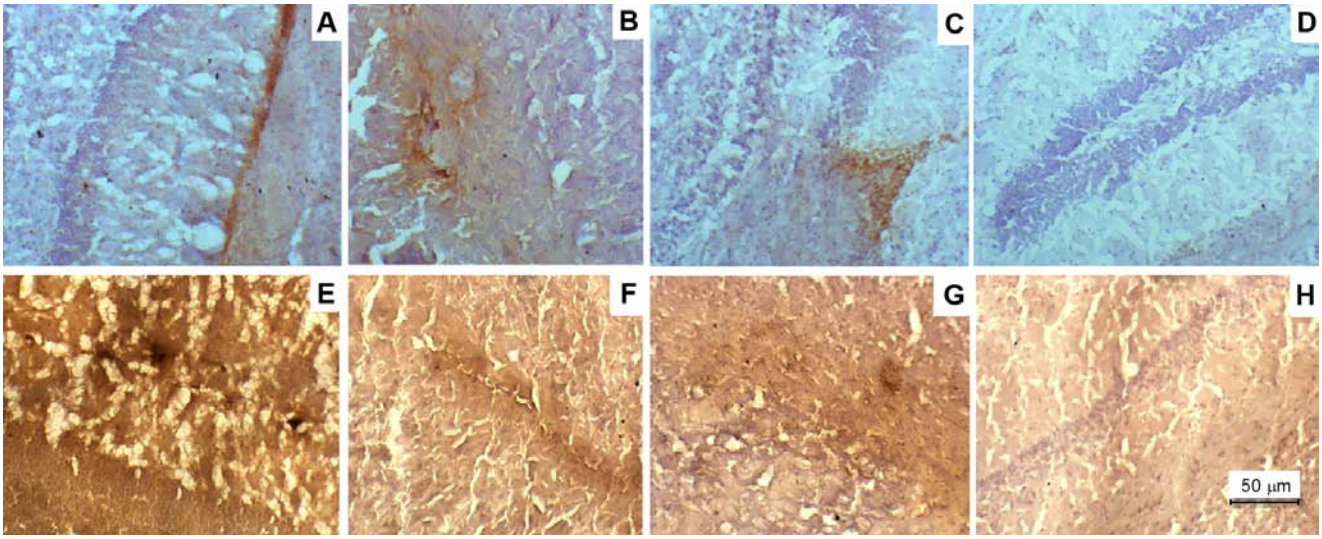

Figure 3. Expression of MHC II molecule in hippocampal region of a) EAE-induced mice with an adjuvant dose of the pertussis vaccine, b) EAE-induced mice with a human dose of the pertussis vaccine, c) EAE-induced mice without addition of the pertussis vaccine and d) non-treated mice. Expression of MHC I molecule in hippocampal region of e) EAE-induced mice with an adjuvant dose of the pertussis vaccine, f) EAE-induced mice with a human dose of the pertussis vaccine, g) EAE-induced mice without addition of the pertussis vaccine and h) non-treated mice. Scale bar = 50 µm.

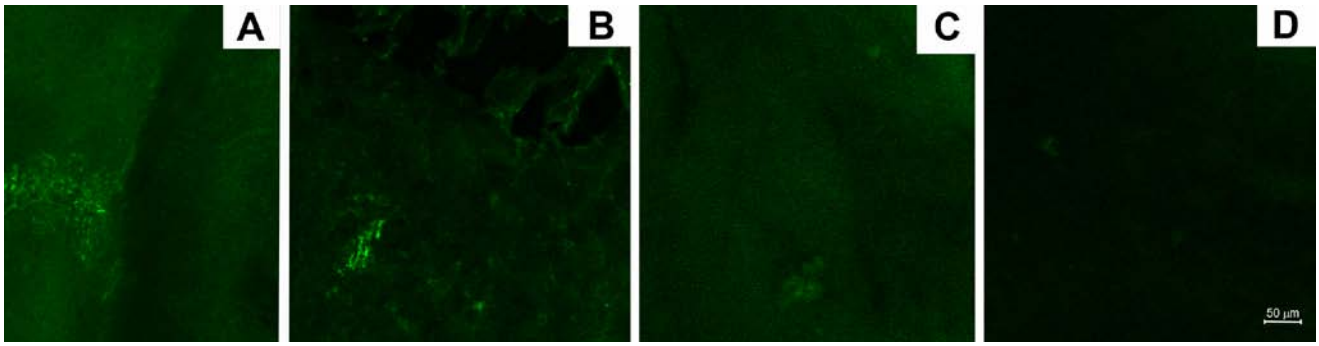

Figure 4. Immunofluorescence staining of anti-CD4 antibody for confocal microscopy as described in Materials and methods. Expression of the CD4 molecule in the hippocampus of a) EAE-induced mice with an adjuvant dose of the pertussis vaccine, b) EAE-induced mice with a human dose of the pertussis vaccine, c) EAE-induced mice without addition of the pertussis vaccine and d) non-treated mice. Scale bar = 50 µm.

role in sustaining the developed clinical signs of EAE.

Dose of 4 x 10^{10} heat-killed pertussis organisms may cause an increase in brain vascular permeability in normal mice [21]. High doses of pertussis organisms injected using the *B. pertussis* vaccine contain a sufficient amount of pertussis toxin that leads to the development of encephalopathy and death in mice [22]. That might be a reason for the serious adverse effects leading to death in mice with EAE treated with a human dose equivalent of pertussis vaccine; the synergistic effect of autoantigens and the high-dose of pertussis vaccine may also play a role. The onset of encephalopathy largely falling between the 10th and 13th days post-immunization [23] was in accordance with the findings in the present study.

Although mice were immunized with spinal cord homogenate containing different kinds of myelin proteins, in the present study we have performed the detection of anti-MOG_{35-55} antibody. Some literature data indicate that activation of anti-MOG antibodies could contribute to EAE pathogenesis [16,24] by increasing the migration of macrophages and the activation of microglia in MS lesions [24]. Relatively high titer of anti-MOG_{35-55} antibodies was found in serum samples of mice with EAE (with or without addition of the pertussis vaccine). According to the literature data [16], anti-MOG antibodies can be involved in the activation of antigen-presenting cells in central nervous system (CNS) lesions. This is consistent with our results, which demonstrated an increase in the expression of MHC molecules in the brain tissue of mice with EAE. Additionally, some titer levels of anti-MOG antibodies were found in the serum samples of unimmunized mice indicating susceptibility to the induction of autoimmune diseases [25].

MOG is a component of CNS myelin. Its localization on the outermost surface of myelin sheaths makes it accessible for antibodies from the extracellular space [26]. In this study, the pronounced staining of myelin was observed at the rim of EAE lesions in mice from the EAE-1 and EAE-2 groups. The results obtained indicate the potential myelin loss in the hippocampal region and brain parenchyma due to antibody-dependent demyelination. Literature data suggested that anti-MOG antibodies could be involved in the induction of demyelination [27,28], which supports a pathogenic role of the antibodies in EAE [16,24]. This view is consistent with the fact that active lesions, defined by the ongoing myelin destruction, are infiltrated by macrophages and activated microglial cells.

We detected scattered $CD4^+$ cells in the brain tissue of mice from the EAE-1 and EAE-2 groups (both injected with the pertussis vaccine), while rare positive cells were observed in the brain tissue of mice with EAE with no pertussis vaccine injected. Almolda *et al.* suggested that CD4 expression in T cells and in the population of microglia/macrophages was in close relationship with the clinical signs [29]. During the inductive phase, at a score 0.5 of the clinical signs, only perivascular $CD4^+$ cells were found and from a score of 1, a population of small round $CD4^+$ cells appeared in the vicinity of blood vessels. An increased number of $CD4^+$ cells was observed in mice with EAE that were adjuvanted with the pertussis vaccine, which was consistent with the literature data [29-31]. Expression of MHC class I and class II molecules in the brain tissue of EAE mice was detected regardless of the presence or absence of the pertussis vaccine. The appearance of the clinical signs of EAE was in correlation with the expression of MHC molecules, which is consistent with previous findings [32,33]. According to the results obtained, the effects of the pertussis vaccine on the expression of MHC-I molecules in EAE mice were not detected because a similar staining intensity of brain tissues was found across all EAE groups. However, expression of MHC II molecules differed among mice from different EAE groups, so that EAE mice that were injected with the pertussis vaccine showed strong expression and vigorous proliferation of MHC II positive cells in the hippocampus, while mice with EAE that did not receive the pertussis vaccine showed mild expression of MHC II molecules indicating that pertussis vaccine plays a significant role in activation of the immune response in EAE. The obtained results show an increase in MHC I positive cells in the hippocampal region and brain parenchyma of MSCH-induced EAE in all mice. Moreover, they also show that MHC I expression is not dependent on the pertussis vaccine.

Hoftberger *et al.* [34] showed that both class I and II MHC molecules are highly expressed in active demyelinating lesions of MS compared to intact controls. The available data indicate that in lesions all cells of the CNS are potential targets for MHC I restricted cytotoxic T-cells that may play a role in the destruction of myelin and axons in lesions [34].

The expression of MHC antigens is dependent upon the type of tissue injury, the stage and activity of the lesions, and the genetic background [35-37]. In EAE microglia proliferate vigorously and show a strong expression of MHC-I and II antigens [32], which might, in this case, suggest that MHC molecules found in the brain tissue were mainly expressed on microglia. Knowing that the expression of MHC molecules on microglia is in close relation with the development of the EAE clinical signs, we found our results to be consistent with previous findings [13,32].

Conclusion

In general, the induction of EAE in C57BL/6 mice with spinal cord homogenate causes a mild clinical score of disease. Our findings indicate that additional use of a small dose of the pertussis vaccine could maintain the developed clinical signs and histological changes in mice with EAE. While a concentration of 10^{10} *Bordetella pertussis* microorganisms acts as an adjuvant in mice with EAE, a concentration of 4 x 10^{10} *Bordetella pertussis* microorganisms leads to the development of encephalopathy and death. There is a positive correlation between the expression of CD4, MHC class I and II molecules, anti-MOG antibody titer, and the observed clinical signs of EAE. These molecules could therefore be used as markers capable of monitoring the development and progression of EAE.

Acknowledgment

The authors declare no conflict of interest.

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