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Influenza vaccine and learning in C57BL mice with an acute experimental autoimmune encephalomyelitis

Research Article

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Abstract: Literature data suggest possible link between influenza vaccination and development of autoimmune processes. Therefore, the aim of the study was to investigate the effect of influenza vaccination on spatial learning in mice with experimental autoimmune encephalomyelitis (EAE). EAE was induced in eight-week-old C57BL/6J female mice by subcutaneous immunization (MOG_{35-55} in complete Freund's adjuvant) and Pertussis vaccine injected intraperitoneally. Mice were vaccinated with influenza vaccine three days before MOG immunization. The hippocampal-dependent spatial learning test, Morris Water Maze test (MWM), was performed before and after EAE induction. Significant difference ($P < 0.05$) in the time for completing the Morris Water Maze task was found between mice with mild clinical signs of EAE when compared to other mice. However no significant difference was observed between mice with EAE and mice with EAE that were vaccinated with influenza vaccine. Hippocampal tissue lesions in EAE mice are in correlation with memory impairment. Study shows no influence of influenza vaccine on progression of clinical signs of EAE, spatial learning and memory impairment.

Keywords: *Autoimmunity • EAE • Morris water maze • Spatial learning • Memory impairment*

1. Introduction

Some autoimmune diseases have been associated with vaccinations. It is estimated that influenza vaccination may have an impact on the development of autoimmune diseases such as Guillain-Barré syndrome [1,2]. Conflicting data exist regarding the connection between vaccines and multiple sclerosis [1]. Recent data suggest that peripheral infection with influenza virus elicits a central inflammatory response and impacts hippocampal structure and function, leading to cognitive dysfunction [3]. Cognitive dysfunction in infected mice was related to their failure to update the search strategy for the platform when the platform location changed. Impaired ability to efficiently navigate to the new location was shown by an increase in time and path length to the new platform location of the infected mice compared to controls [3]. However, there is insufficient data regarding influenza vaccination and its impact on hippocampal structure.

Experimental autoimmune encephalomyelitis (EAE), an autoimmune disease directed against myelin protein in the brain, is still the most commonly used animal model of multiple sclerosis (MS) [4,5]. Multiple sclerosis affects motor, sensory as well as behavioral and cognitive functions. Cognitive deficits are considered an early manifestation of the disease in MS patients. Similar memory impairment in EAE-induced mice is shown in many other studies [6-9]. EAE causes deficits in hippocampal-dependent learning and memory sight that is associated with early microglial activation, synaptic loss and neurodegeneration [7]. Studies in humans and animal models with hippocampal damage and lesions have provided evidence that this region of the brain plays a critical role in spatial memory; the part of memory responsible for regulating and encoding information about the surroundings and orientation in space [10,11]. Hippocampal formation is closely related to spatial learning because it consists of cells signaling the position of animal in space. In addition, mechanical or chemical inactivation of the hippocampus and neighboring cortex has shown to

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interfere with the ability of animal to learn a spatial environment [12-14]. Hippocampal pathology in EAE mice accompanied with Morris Water Maze (MWM) training may give useful insights into the basis for learning and memory impairment [7,15-17].

Water maze test investigates how rodents swim to an escape platform that can be hidden (spatial version of the test) or visible (non-spatial version). MWM test involves the task of swimming, learning the platform location and that there are no alternative escape routes. Reward for completing the task is escape from water. The test also measures where the experimental animal is in relation to the target during the course of swimming and its motor functions [15,17,18]. This test is able to dissociate hippocampal function from non-hippocampal function, such as general behavioral performance abilities. It was shown that hippocampus-lesioned rodents are impaired in hidden- but not in visible-platform MWM learning [19]. Cho *et al.* [20] also reported that hippocampus-lesioned C57BL/6J mice could be still tested for contextual fear conditioning even though the hidden-platform acquisition was impaired.

In the present study we investigated the effects of an influenza vaccination and EAE induction on memory loss and motor dysfunctions in C57BL/6J mice using MWM test. Hematoxylin-eosin staining of brain tissue sections was performed in order to detect lesions in the mice brain and to investigate the possible role of influenza vaccination in the progression of motor dysfunctions and memory impairment.

2. Experimental Procedures

2.1 Mice

Female C57BL/6J mice 8 weeks of age were purchased from Military Medical Academy (VMA, Serbia). 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. 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. The study was conducted on 40 mice divided in four groups: EAEVCC (mice immunized with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant (CFA) and vaccinated with inactivated influenza split vaccine; n=10), EAE (mice immunized with MOG in CFA; n=10), VCC (mice vaccinated with inactivated influenza split vaccine; n=10) and IC group (non-treated animals; n=10).

2.2 Vaccination

Influenza split virion inactivated vaccine (Vaxigrip, Sanofi Pasteur S.A., France) was used in this study. Influenza vaccine is standardized to include 15 µg of hemagglutinin (HA) of three strains suspended in saline solution. Three days before EAE induction, mice from EAEVCC and VCC groups were vaccinated intramuscularly (*i.m.*) in the gluteal muscle with 50 µl of inactivated influenza split vaccine (Vaxigrip, Sanofi-Pasteur).

2.3 EAE induction

The procedure used in the study was a modification of that used by Kuerten *et al.* [21]. Eight-week-old female C57BL/6J mice were each injected subcutaneously with 100 µg of myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}, Sigma Aldrich) in complete Freund's adjuvant (CFA) containing 1 mg mL^{-1} of heat-killed and dried *Mycobacterium tuberculosis* H37Ra (Sigma Aldrich) on the right side of the flank. 150 µl of 10^9 heat-killed *Bordetella pertussis* vaccine (British Reference reagent 88/522; National Institute for Biological Standards and Control, Potters Bar, Hertsfordshire, UK) was given *i.p.* on the day of immunization and 48 h later [22]. 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 intermediate in increments of 0.5. Animals were sacrificed on day 24 post immunization.

2.4 Morris Water Maze Testing

Spatial learning and memory were assessed using the standard Morris Water Maze (MWM), consisting of a circular tank (d=150 cm; temperature at 24-26°C) filled with opaque water and a hidden platform in a fixed spot. The water was made opaque by adding 100 mL of non-toxic white paint. Platform used in testing was 10 cm in diameter and submerged 1 cm below the water surface. Mice were released from one of the four start locations (N, S, E, W) facing the wall of the pool. The pool was positioned in a room with external cues visible to the swimming animal. The path, time, and motor function during their search for the platform were recorded. When the platform was found, the mouse remained on it for 10 s. If the platform was not found in 120 s, the mouse was gently guided to the platform and then remained on it for 10 s. After each trial the mouse was returned to its heated home cage. Each mouse was given two trials a day.

2.5 Statistical evaluation

Differences in the time required to find the hidden platform in MWM were evaluated by ANOVA (SPSS Statistics 20,

United States). Post-hoc LSD test was used to compare differences between groups. A probability of $P<0.05$ was considered significant. Influence of EAE induction and influenza vaccination on latency in MWM test was investigated by two-way ANOVA (ANOVA: two factor without replication).

2.6 Tissue collection and histology

All mice were deeply anaesthetized and transcardially perfused with saline followed by 4% PFA in 0.1 mol L^{-1} phosphate buffer twenty-four days post immunization. Brain tissue samples were immersed in OCT embedding (Kilik, Bio Optica, Italy) compound, frozen in liquid nitrogen and stored until use at -80°C. A representative part of the frozen tissues was sectioned into 5 µm thick sections using a cryomicrotome (Jung-Reichert Cryocut E; Cambridge Instruments, Heidelberg, Germany). Frozen sections from each experimental group were stained with hematoxylin and eosin and the tissue morphology was examined.

2.7 Tissue analysis

Brain sections were analyzed by capturing images of sections using a BH2 research microscope (Olympus Optical Co. LTD. Tokyo, Japan) equipped with Color View III digital camera (Olympus). Analysis Docu software (Olympus) was used to acquire images. All images were taken using a 10x objective.

3. Results

3.1 Morris Water Maze test

Mice were examined daily for weight loss and clinical signs of experimental autoimmune encephalomyelitis (EAE). It was found that twenty-four days post immunization, all the experimental mice gained weight, on average 6.6% (Table 1).

Clinical signs, a weakened tail (score 0.5), appeared on the 13th day post immunization in 10 of 20 mice with EAE (6 of 10 mice from EAEVCC group and 4 of 10 mice from EAE group). In both EAE and EAEVCC groups, hind limbs weakness (score 2) after swimming was observed immediately after Morris Water Maze test starting from the 18th day post immunization and lasted for 2 h. Full hind leg paralysis, quadriplegia, or death was not observed in any mice.

Over a number of trials in MWM test, animals learn that the reward for reaching the submerged platform means an escape from the pool. With time, the escape latency decreased (Figure 1). Two trials were necessary for mice to learn to swim in the quadrant with the platform. After 8 training days all the mice swam directly to the platform. Swimming for longer than 120 s was rare and observed in 2 of the 40 trained mice for two trials.

Also, some unusual behavior was observed in EAE induced mice. Six out of ten mice with mild EAE

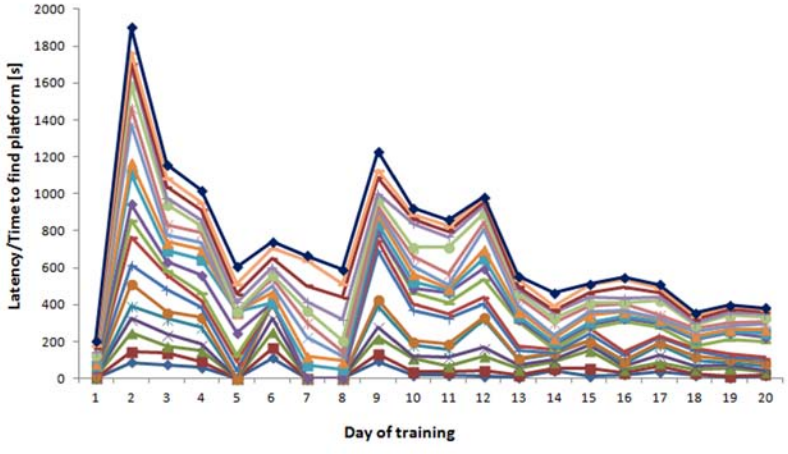

Figure 1. Latency of time per day that mice (n = 40) need for finding the platform. All the forty trained mice learned to locate the hidden platform and their escape latency decreases with ongoing training days. Each line represents the latency of one trained mice. Latency of some mice was identical to others and their plots overlap.

	EAE [g]	EAEVCC [g]	VCC [g]	IC [g]
day 0	19.9±0.6	20.4±1.0	19.8±1.0	19.5±0.8
day 24	21.4±0.5	21.4±0.9	21.3±0.8	20.8±0.9

Table 1. Average weight per group on the day of MOG immunization and 24 days post immunization.

symptoms showed signs of disorientation and anxiety, reacted to every sound with floating paralysis and thigmotaxis (swam along the wall of the pool), swam with the tail out of the water, swam back and forward and around the platform. It was observed that mice with EAE symptoms committed more errors in the trials than other mice (EAE without symptoms, non treated and vaccinated).

Mice with mild clinical signs of EAE needed more time to find the submerged platform ($P<0.05$) compared to other mice (EAE mice without symptoms, influenza vaccinated (VCC) and non-treated (IC)) (Figure 2). No significant difference in the time for finding the hidden platform among all the groups (EAE, EAEVCC, VCC and IC) was observed taking into consideration both, the full time of performing MWM test and the training days after MOG immunization (Figure 3).

3.2 Histology analysis

Hematoxylin-eosin staining of brain tissue sections was performed 24-days post immunization. Histopathological examination showed lesions in the brain parenchyma, especially in the hippocampal region of both EAE groups (Figure 4). No lesions were observed in VCC (influenza vaccinated) and IC (non-treated) mice.

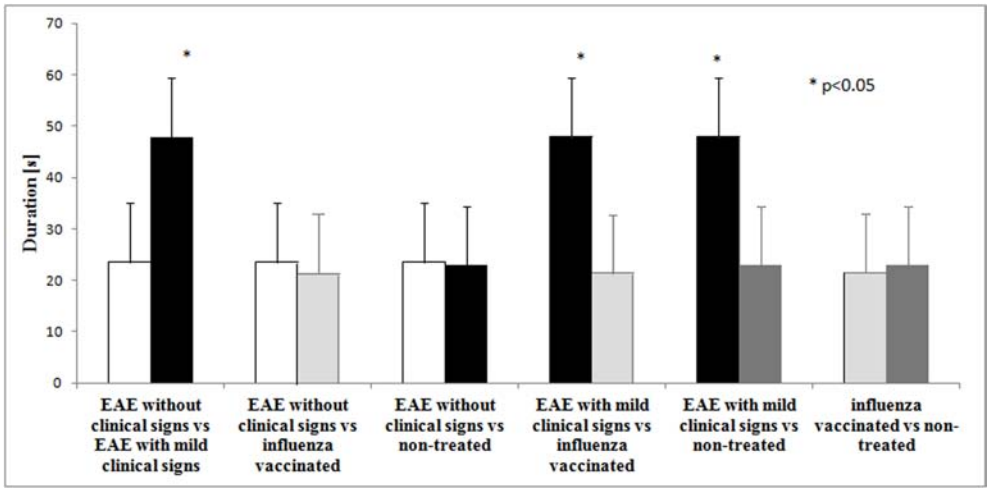

Figure 2. Differences between groups (EAE without clinical signs (n = 10): MOG immunized mice without symptoms (EAE and EAEVCC); EAE with mild clinical signs (n = 10); influenza vaccinated (n = 10): and non-treated (n = 10) were compared by using ANOVA, post-hoc LSD test (SPSS Statistics 20, USA). A probability of $P < 0.05$ or less was considered significant. Significant difference in the time for finding the submerged platform after MOG immunization was seen between mice with mild EAE clinical signs when compared to other mice (EAE without clinical signs, influenza vaccinated and non-treated).

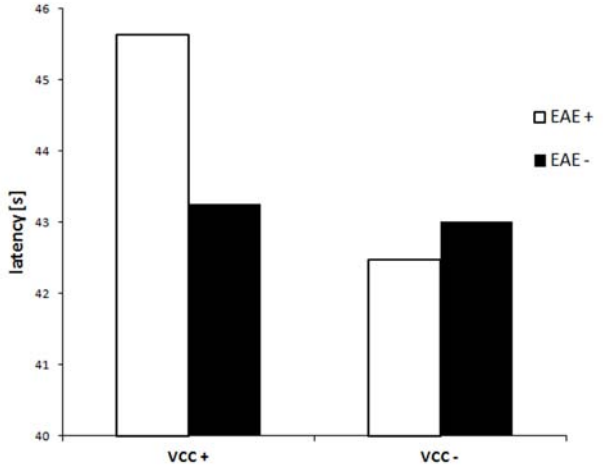

Figure 3. Influence of EAE induction (EAE+/EAE-) and influenza vaccination (VCC+/VCC-) on escape latency in MWM test was investigated by ANOVA: two factor without replication. No significant difference in the time for finding the hidden platform among all the groups (n = 40) [EAE (n = 10), EAEVCC (n = 10), VCC (n = 10) and IC (n = 10)] was observed taking into consideration both, the full time of performing MWM test and the training days after MOG immunization.

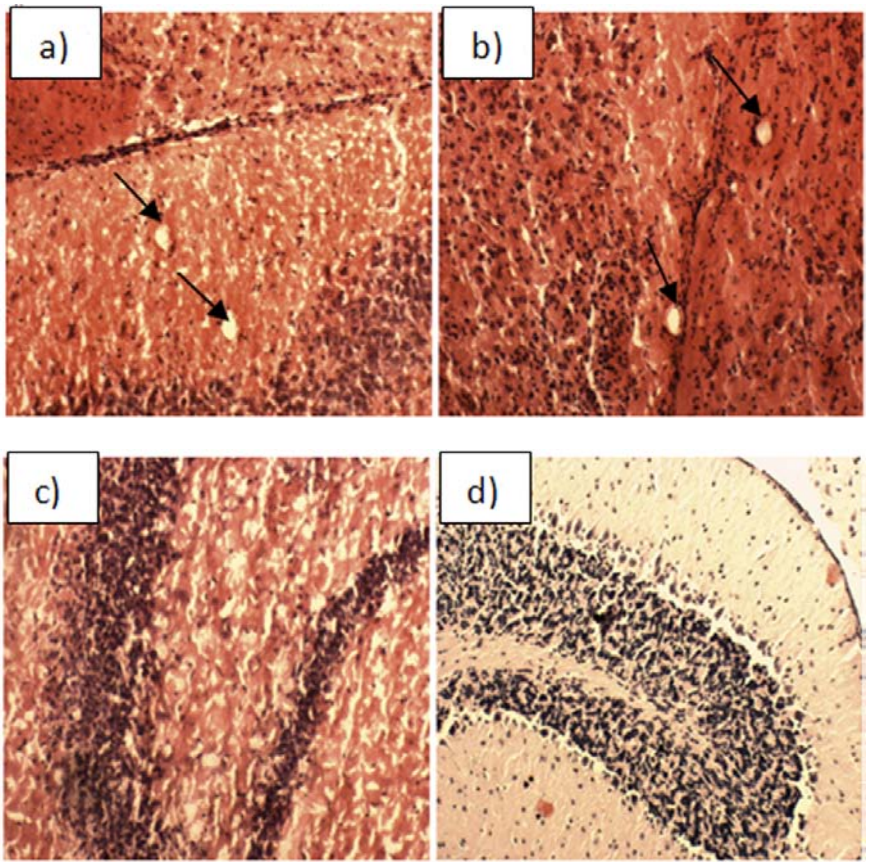

Figure 4. Hematoxylin-eosin staining of the hippocampal region of the brain. Brain sections of 10 mice from each group were analyzed and the results obtained from each group were similar. Representative sections of the brain from each group analyzed are shown. Lesions are indicated by arrows in a) EAEVCC, EAE and influenza vaccinated mice and b) EAE mice. No lesions in c) VCC, influenza vaccinated mice and d) IC, non-treated mice brain sections were observed.

4. Discussion

Our study shows no effect of influenza split vaccination on memory impairment in EAE induced C57BL/6J mice. Even though serious side effects after influenza vaccination are considered uncommon, the appearance of soreness at the site of the injection is often seen. Also, occasional muscle aching, fever, and feeling unwell is possible [23]. These side effects might play a role in potential motor dysfunctions and therefore might have an impact on the performance of mice in Morris Water Maze (MWM) test. However, side effects were not observed and results obtained indicate that influenza vaccination does not have significant influence on hippocampal-dependent spatial learning.

Immunization of C57BL/6J mice with $MOG_{35–55}$ caused partial loss in tail tonicity in 50% of the experimental animals and hind limb weakness after Morris Water Maze test in all treated mice on the eighteenth day post immunization. Although clinical signs of EAE are very subtle, we found lesions in hippocampal region of the brain that could be associated to memory impairment observed while conducting the MWM test. Our results are consistent with Jones *et al.* [24], who found that pathohistological changes in the brain occur before behavioral/clinical signs of disease. One should keep in mind that scoring of disability in EAE animals could be a subjective evaluation and potentially influenced by observer bias. Also, physical activity has been observed to have a large impact on hippocampal growth and neurogenesis, and therefore on spatial memory [25]. Animal studies have provided a number of evidence for the role of exercise in spatial memory. Rodents undergoing treadmill running on a daily basis show that the time needed to swim to a submerged platform, in Morris Water Maze pool, is decreased at a greater rate compared to the rodents who had not run on a treadmill [25].

Similar to a study performed in the Barnes Maze by Ziehn *et al.* [9] mice with early clinical signs of EAE performed the given task similar to healthy matched controls. Both groups had a significant reduction in the total number of errors committed from the first trial to the last trial. In other words, EAE mice were as good as

age-matched healthy control and influenza vaccinated mice in learning the location of the hidden platform. It is interesting to note that mice with relatively mild EAE signs, from both EAE groups (EAE and EAEVCC), committed more errors in the trials after MOG immunization, than the healthy control mice, influenza vaccinated and EAE mice without clinical signs of the disease. In the present study, prolonged swimming was observed in only two trials in 2 of 40 trained mice, indicating that such behavior during swimming was not common. According to our protocol the cut-off of 120 s was used and measurements above 120 s were censored to avoid possible interference with the statistical analysis of the results obtained. The cut-off of 120 s was set in order to prevent any damage or death to the mice due to prolonged swimming.

It should be emphasized that no significant difference between EAE and EAEVCC mice with clinical signs was found. The results obtained indicate that neurological changes might be present in mice with EAE, also indicating that EAE might cause hippocampal-dependent impairment in learning and memory.

Nowadays, focus is shifting from physical well-being to the potential improvement in mental well-being or maintenance of cognitive function in order to enable a better quality of life [26]. Approximately 50–65% of multiple sclerosis patients experience cognitive deficits [4,27]. Among these reported cognitive deficits, memory dysfunction is especially common [28]. Similar percentage of EAE induced mice had cognitive deficit in this study that is in rank order with data found on humans. Among animals with clinical signs of EAE interindividual behavioral difference was observed, 40% had no changes while 60% had signs of disorientation and anxiety-like behavior. However, it is important to take into account that cognitive performance may be affected by additional stress on experimental animals due to swimming [29]. It is observed that in anxiety- or stress- induced environment mice tend to freeze and postpone active responding rather than search for an escape route [18].

Even though, the initial studies determine that influenza infection induces deficits in spatial learning and memory loss in adult mice at day 7 post infection, conflicting data exist connecting vaccines, multiple sclerosis and other autoimmune illnesses [1,3]. With the results obtained in this study, it can be concluded that influenza split vaccination and cognitive disturbances in mice with an acute experimental autoimmune encephalomyelitis are not related. On the other hand, the appearance of EAE clinical signs might be associated with spatial learning problems. Hippocampal tissue destruction in EAE mice evidently corelates with the cognitive learning and memory impairment. In conclusion, vaccination with influenza vaccine in EAE induced C57BL/6J mice has no influence on the clinical signs of an acute experimental autoimmune encephalomyelitis and hippocampal-dependent spatial learning in Morris Water Maze test.

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