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Postvaccination Accumulation of the Influenza Virus Antigen in the Rat Choroid Plexus

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We examined the accessibility of influenza virus and diphtheria-tetanus toxin (DiTe) antigens to the choroid plexus (CP) within the postvaccination period and the expression of CD11b molecules (by immunohistochemistry). Eighteen Dark Agouti (DA) rats were divided into three groups: (i) animals administered with influenza vaccine (Flu), (ii) animals administered with DiTe vaccine (DiTe), and (iii) nontreated (Contr) animals. The serum antibody titers following influenza and diphtheria-tetanus vaccination were detected by the ELISA test. Immunohistochemical staining revealed a great number of viral antigen-positive and CD11b-positive brain cells in Flu rats compared to a very small number of the respective cells in DiTe animals and no staining in the Contr group. DiTe- and Flu-rats showed significant increases in the serum anti-tetanus toxoid and anti-influenza virus antibody levels compared to those in the Contr group. The results obtained attract attention towards the dynamic role of the CP in the immunosurveillance of the CNS. Based on the viral antigen deposits accumulated in the CP, it has been proposed that the latter can play an active role in modulation of the immune response after influenza vaccine immunization.

Keywords: vaccination, influenza, diphtheria toxin, choroid plexus, CD11b.

INTRODUCTION

Vaccination against influenza virus remains the most effective method to prevent the disease, and inactivated influenza virus vaccine has been used for decades to prevent this infection and its complications. The involvement of the CNS during influenza infections and influenza vaccination in humans is still unresolved, although there is little doubt that encephalopathy does occur in some patients with influenza [1]. Also, influenza vaccination has been linked to autoimmune diseases and autoimmune adverse events relating to neurological diseases [2]. Many studies in humans and on animal models examined the health outcome and related effects of influenza vaccination on multiple sclerosis; it was shown that there is no significant risk for multiple sclerosis postvaccination [3-6].

Vaccine-induced immune effectors are, essentially, antibodies capable of binding specifically to a toxin or a pathogen. Most antigens and vaccines trigger both B and T cell responses, although they are considered to be poor stimulators of cell-mediated immunity [7]. After vaccination, a significant amount of specific antibodies has been found in the peripheral circulation but not in the CNS. Despite the growing interest in the pathways of interaction between the brain and the peripheral immune system, the precise mechanism by which the latter transmits signals to the brain remains unknown. There is, however, preliminary evidence suggesting that the choroid plexus (CP) senses inflammation in the periphery and conveys inflammation-induced signals to the brain via the cerebrospinal fluid (CSF). Preliminary data suggested more specifically that acute inflammation leads genes in the choroid plexus to modify their production of proteins involved in the immune inflammatory process [8]. The blood-brain barrier (BBB) is a dynamic interface of the CNS that separates the brain from the circulatory system. The BBB endothelial cells restrict the diffusion of microscopic objects (e.g., bacteria) and large or hydrophilic molecules into the CSF, while allowing

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the diffusion of small hydrophobic molecules (O_2, CO_2, or hormones). Only about 0.1% of antibodies have been estimated to cross the BBB [9].

The CP is a leaf-like highly vascular structure, which sits inside the ventricles, between the blood and CSF, and its main function is the production of the latter [10, 11]. A dominant role of these barriers is to regulate the crossing of immune cells, proteins, and other nutrients from the blood to the brain and to shield the CNS against circulating neurotoxic pathogens and toxins [12]. Findings about the pathway of communication between the immune system and CP indicated that this plexus acts as an "immunosensor" to a peripheral inflammatory stimulus and plays a crucial role as a mediator of the interactions between the CNS and the peripheral immune system [13]. It is thought that the CP is more responsive to systemic immune activation than the brain itself [14]. It has been also suggested that CP cells might play a role in dissemination of infectious agents or even function like a reservoir of antigens [15–17].

The myeloid cell lineage plays a crucial role in the continuous monitoring and development of the immune responses at the CNS level; this lineage comprises two main subtypes; (i) resident microglia distributed throughout the brain parenchyma, and (ii) perivascular macrophages located in the brain capillaries of the basal lamina and the CP [18]. Perivascular macrophages and microglia, even though immunophenotypically distinctive from one another, do share surface markers, (like CD11b), and are considered primary cell types productively infected in the CNS [19]. It is thought that CD11b plays certain roles in cellular trafficking, migration, co-stimulation, synapse formation, and phagocytosis [20]. CD11b-positive cells may represent a brain intrinsic mechanism of cellular responses in the CNS [21].

In this paper, we focus on the alertness of the brain of robust rats to the influenza virus and diphtheria-tetanus (DiTe) antigens postvaccination. DiTe has been practiced for mass immunization worldwide, and it has not been known for its neurotropism; we compared its effect with the effect of influenza vaccine on the immune response in the CP.

METHODS

Animals. The experiments were carried out on 10-month-old male Dark Agouti (DA) rats. The animals were kept under standard laboratory conditions (room temperature 21 ± 1°C, humidity 30%, and 12/12 h light/dark cycle) with food and tap water *ad libitum*.

Vaccination. DiTe vaccine (Ditevaksal-T, Institute of Torlak, Serbia) and influenza split virion inactivated vaccine (Vaxigrip, Sanofi Pasteur S.A., France) were used in this study. Influenza vaccine was standardized to include 15 μg of hemagglutinin (HA) of three strains suspended in saline solution. Rats were vaccinated intramuscularly (i.m.) with DiTe or influenza vaccine (0.2 ml/rat, via the *quadriceps* muscle). The study was conducted on 18 rats divided into three groups, (i) Flu (animals administered with influenza vaccine; $n = 6$), (ii) DiTe (animals administered with DiTe vaccine; $n = 6$), and C group (nontreated animals; $n = 6$). The animals in the DiTe group received a repeated dose of DiTe vaccine with a 4-week interval.

ELISA. Serum samples were collected by cardiac puncture from the anesthetized animals eight weeks after vaccination; the rats were tested for the presence of anti-tetanus toxoid and anti-influenza antibodies using ELISA. The procedure used was a modification of that used by Voller et al. [22]. Ninety-six-well flat-bottomed tissue culture plates (NUNC, Roskilde, Denmark) were coated with tetanus toxoid antigen or influenza antigen at concentrations of 1.0 and 2.5 μg/ml in PBS, respectively. Non-specific binding was blocked with 1% bovine serum albumin (BSA) in PBS. After blocking, the plates were washed with the buffer (0.05% Tween-20 in PBS); then the primary antisera were added to each plate (1:200 serum dilution of DiTe-immunized rats or 1:100 serum dilution of Flu-immunized rats) and incubated for 1 h at room temperature. After washings, horseradish peroxidase (HRP)-conjugated rabbit anti-rat polyclonal IgG (DAKO, Denmark; dilution 1:1000) was allowed to react with the bound antibodies. Reaction mixtures were incubated for 1 h at room temperature, and the wells were washed. Peroxidase substrate solution containing 1 μg/ml o-phenylenediamine; (Sigma Aldrich, USA) and 0.01% H_2O_2 in citrate-phosphate buffer pH 5.0 was added; after 15 min, the reaction was stopped by the addition of 2.0 M sulfuric acid. The absorbance value was measured on Multiscan Ascent (Labsystems, Thermo Fisher Scientific, USA) at 492/620 nm.

The difference of antibody titers between vaccinated and nontreated animals was evaluated by ANOVA (Origin 8.0, OriginLab Corporation, USA).

Differences with $P = 0.05$ or less were considered significant.

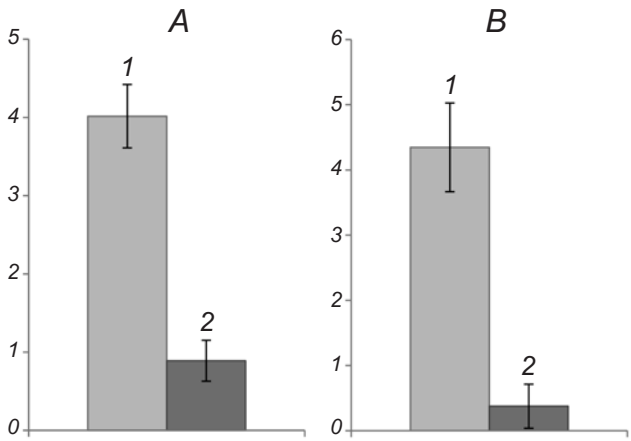
Brain Tissue Samples. All rats were deeply anesthetized and then transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brain tissue samples were immersed in an OCT embedding compound (Kilik, Bio Optica, Italy), frozen in liquid nitrogen, and stored until use at –80°C. A representative part of the frozen tissues was processed with a cryomicrotome (Jung-Reichert Cryocut E; Cambridge Instruments, Germany) in 5-µm-thick sections.

Immunohistochemical Staining of Brain Sections. Five-µm-thick frozen brain sections were harvested onto superfrost slides (ThermoScientific, Germany), air-dried, and fixed in cold acetone for 10 min at –20°C. The slides were washed in TBS and then incubated with 0.3% H_2O_2 in methanol, to quench the endogenous peroxidase activity. For detection of influenza virus antigens, the sections were blocked with 10% normal rabbit serum and then incubated with 1:50 dilution of serum derived from rats immunized with influenza vaccine (as described above). The formed immune complexes were identified by HRP-conjugated rabbit polyclonal anti-rat IgG antibodies (DAKO, Denmark; 1:1000 dilution in 1% normal rat serum). For tetanus toxoid detection, the sections were blocked with 10% normal rat serum and then incubated with monoclonal mouse biotin-labeled anti-tetanus toxoid antibody [23] (1:1000 dilution in 1% normal rat serum). The reactivity attained at the end was visualized using streptavidin-conjugated HRP (1:1000 dilution). For the analysis of CD11b expression, the sections were blocked with 10% normal rat serum, incubated with monoclonal mouse anti-CD11b antibody (AbD Serotec, Great Britain; 1:50 dilution in 1% normal rat serum), and revealed with HRP-conjugated rabbit polyclonal anti-mouse IgG antibodies (DAKO, Denmark; diluted 1:1000 in 1% normal rat serum). Diaminobenzedine/ H_2O_2 (DAB, BD Pharmingen, USA) was used as a substrate for the horseradish peroxidase enzyme which produces brown staining. Brain sections were stained according to the above-mentioned protocol. The representative images were taken by an Olympus BH2 research microscopy imaging system (Olympus Optical Co. Ltd., Japan) equipped with a Color View III digital camera (Olympus) and analyzed. Analysis Docu (Olympus) was used as image acquisition software. All images were taken under a magnification lens (×10).

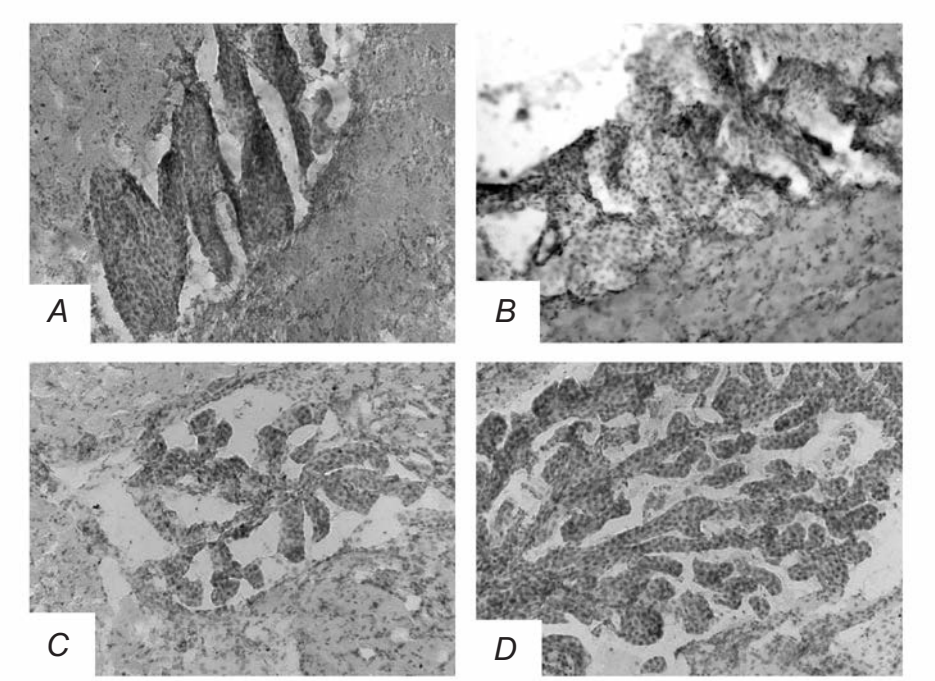
RESULTS

Anti-Influenza and Anti-Tetanus Toxoid Postvaccination Antibodies. Protection after influenza vaccination is primarily mediated by the generation of antibodies against hemagglutinin (HA); thus, the HA titer is often used as a marker for measuring the vaccine efficacy in rats. As shown in Fig. 1A, vaccinated animals exhibited significantly higher levels of anti-influenza antibodies compared to those in the control group ($P < 0.001$). The anti-tetanus toxoid antibody production was measured after the second immunization (Fig. 1B). DiTe-vaccinated rats also showed significantly higher levels of anti-tetanus toxoid antibodies compared to the levels of antibody in nontreated controls ($P < 0.001$).

Deposits of Influenza and Tetanus Toxoid Antigens in the CP. In our study, detection of the antigen deposits in the CP of influenza- and DiTe-vaccinated rats was performed by immunostaining with anti-influenza antibodies and anti-tetanus toxoid antibodies, respectively. Intense staining of the CP was detected in Flu-vaccinated rats; very weak and scattered staining was observed in the CP of DiTe-vaccinated rats, and no staining was found in the C group (Fig. 2).



F i g. 1. Serum anti-influenza and anti-tetanus toxoid antibodies-related responses (A and B, respectively) in vaccinated rats. Specific antibodies were detected by ELISA. Rats were vaccinated with influenza split virion inactivated vaccine (Flu, A1), DiTe vaccine (DiTe, B1), or were nontreated (controls, C,2). The results are expressed as means ± s.d. of the measured optical densities.



F i g. 2. Deposits of the influenza and tetanus toxoid antigens in the choroid plexus (CP) of the rat brain, eight weeks after vaccination. Influenza antigen was detected in the CP of rats vaccinated with influenza split virion inactivated vaccine (Flu group; A), and the results are compared with nontreated rats (control, C group; B). Tetanus toxoid antigen was detected in the CP of rats vaccinated with DiTe vaccine (DiTe group; C), and the results are compared with nontreated rats (C group; D).

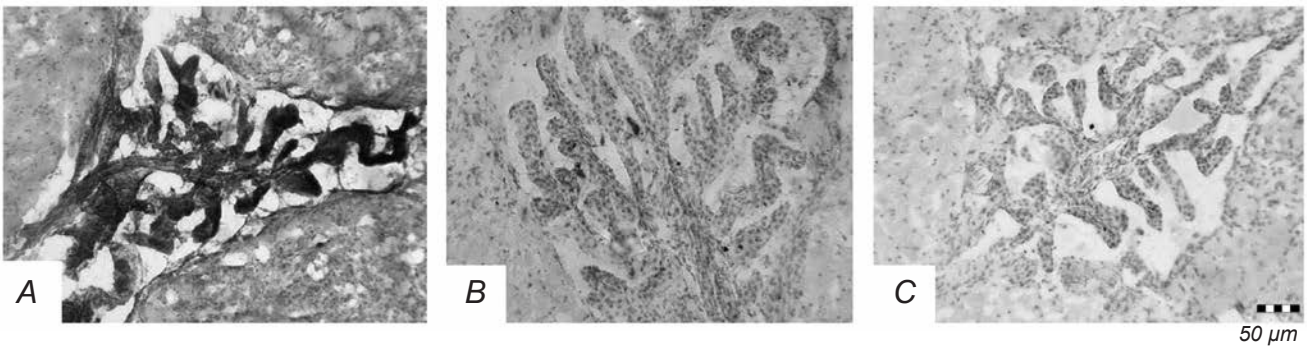
Expression of CD11b in the CP. We investigated the postvaccination effect on the CD11b expression in the rat CP. Numerous strongly stained CD11b-positive cells were observed in the CP of Flu-vaccinated rats (Fig. 3). Only a few CD11b positive cells were detected in the CP of DiTe-vaccinated animals. In the C group of rats, the expression of CD11b-positive cells was not found.

DISCUSSION

Antigen-neutralizing serum antibodies are important in preventing bacterial and viral infections. Anti-tetanus toxoid and anti HA-titers are used as a marker for measuring the vaccine efficacy. In view of the fact that antibodies directed against the binding domain of the toxin strongly correlate with the immune response to this toxin [24, 25], tetanus toxoid is frequently used as an antigen for the detection of antibodies following DiTe vaccination.

In our study, we vaccinated rats with a single dose of split influenza vaccine containing 18 μg HA. The results obtained showed significant increases in the anti-influenza virus antibody levels 8 weeks after vaccination. Hovden et al. [26] pointed out that a dosage higher than 15 μg HA of split vaccine elicited a significant increase in the serum antibody concentration. Our results are, therefore, in accordance with the results obtained by the above authors [26]. It has been noted that the influenza virus-specific IgG responses in wild-type animals can be found as early as 4 weeks and as late as 8 weeks after immunization [27]. After repeated doses of DiTe vaccine, we observed a significant increase in the titer of anti-tetanus toxoid antibodies in vaccinated rats, and this was consistent with the findings of high antibody titers consequent to DiTe vaccination. According to these results, the supplied influenza vaccine and DiTe vaccine provided efficient antibody levels in the peripheral circulation and anticipated protection against viral and bacterial infections.

There is some evidence that viral or bacterial agents injected into the cerebral ventricles rapidly activate immune responses in the brain [28, 17]. According to Matyszak and Perry [16], heat-killed BCG, when injected into the ventricles, generates a type of cell-mediated immune response in the CP, which is comparable to that observed in the skin after subcutaneous BCG injections. Additionally, influenza viral antigen was found in epithelial cells of the CP [29]. The results obtained in our study



F i g. 3. Expression of CD11b molecules in the choroid plexus of rats vaccinated with influenza split virion inactivated vaccine (Flu group; A), rats vaccinated with DiTe vaccine (DiTe group; B), and control animals (no expression of CD11b molecules; C).

showed the presence of large antigen deposits in the CP of rats immunized with influenza vaccine vs. a small number of tetanus toxoid antigens. It was reported that after *Vaccinia* virus vaccination, the respective antigen persists in the brain tissue for weeks [30]. Although the titers of the antibodies against diphtheria-tetanus toxoid and influenza antigens are high, it is known that the immune response of a host to the viral and bacterial vaccines is different [7]. Our knowledge of the mechanisms by which these pathogens invade the brain remains incomplete, essentially because of two reasons, (i) the lack of suitable animal models capable of closely mimicking the human disease, because most of these pathogens are restricted to humans, and (ii) there is no sufficiently adequate *in vitro* model mimicking different components of the blood-CSF barrier [31].

In contrast to the BBB, the CP is structured to interact more vivaciously with the immune system, including mechanisms that reinforce the continuous immunosurveillance and specific responses to diseases and injuries. The epithelium and a large population of macrophages/dendritic cells appear to be major players in these responses due to their capacity for the expression of adhesion molecules, secretion of chemokines, presentation of antigens, and active surveillance [32]. Due to the difficulties encountered in trying to differentiate perivascular macrophages of the CNS from inflammatory macrophages (immigrants from the blood) [19], a sufficiently intense expression of CD11b molecules in the CP after influenza vaccination may also mean that there is recruitment of inflammatory macrophages and consequent induction of the immune response. Our results are consistent with the findings of McMenamin et al. [33] who showed that non-infected cells of the CP do not express CD11b molecules, and also with findings of Takahashi et al. [34] who showed that CD11b+ macrophages are involved in the cellular response after influenza infection.

The paucity of CD11b+ cells in the CP of DiTe-immunized rats, on the one hand, and the opulence of these cells in the CP of influenza-immunized rats, on the other hand, are very prominent. We are, however, unable to formulate a rational explanation for this discrepancy other than that there is a difference between viral and bacterial immune responses of a host itself and the neurotropic effects of influenza virus.

A wide variety of inflammatory diseases temporally associated with the administration of various vaccines has been reported in the literature; a PubMed search from 1979 to 2013 revealed 71 documented cases. The most commonly reported vaccination that was associated with CNS demyelinating diseases included influenza (21 cases) [35]. It is deemed that CD4+ cells exhibiting a Th1 phenotype (IL-2, IFN- and TNF-) are implicated in the sweeping process of neurotropic viruses [36]. Given the fact that a CD11b+ population of CNS macrophages is also located in the CP, it is likely that they are involved in T-cell priming by antigens entering the CNS, as was suggested by Borda et al. [19]. According to the results obtained, the influenza virus antigen deposits presented in the CP could also mean that the brain plays an active role in the immune defense of a host. Therefore, more work is needed to better appreciate the influence of the CP on the development of CNS immune responses. Insights into these mechanisms may provide opportunities to envisage more effective treatments capable of controlling inflammation and repair processes in the CNS. The CNS must act vigilantly at any time to keep the balance between potentially harmful factors and resolving any immunological

response that, if left unabated, can create damage itself [37].

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