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Review

Transient decomplementation of mice delays onset of experimental autoimmune encephalitis and impairs MOG-specific T cell response and autoantibody production

Nóra Terényi[a], Nándor Nagy[c], Krisztián Papp[b], József Prechl[b], Imre Oláh[c], Anna Erdei[a,b,*]

[a] Department of Immunology, Eötvös Loránd University, H-1117 Budapest, Hungary
[b] Immunology Research Group of the Hungarian, Academy of Sciences at Eötvös Loránd University, H-1117 Budapest, Hungary
[c] Department of Human Morphology and Developmental Biology, Faculty of Medicine, Semmelweis University, H-1094 Budapest, Hungary

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ABSTRACT

Multiple sclerosis (MS) is the most common inflammatory and demyelinating disease of the central nervous system. In both MS and its animal model experimental autoimmune encephalomyelitis (EAE), it is thought that infiltrating CD4$^+$ T cells initiate an inflammatory process and collect other immune effectors to mediate tissue damage. The pathophysiology of the disease however remains unclear. Here we focus on the role of the complement system in the pathomechanism of EAE, employing mice with transiently depleted complement activity achieved by a single injection of cobra venom factor (CVF) 2 days before the induction of the disease. Our results show that in decomplemented C57BL/6 mice immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55, the onset of the disease is significantly delayed. In SJL/J mice which develop a relapsing-remitting form of EAE after injection with proteolipid protein (PLP) peptide 139–151, the attenuation of both phases could be observed in CVF-treated animals. In C57BL/6 mice the level of MOG specific autoantibodies and their complement activating capacity evaluated on day 21 were found significantly reduced in animals transiently decomplemented before induction of the disease. The *in vitro* response of T cells isolated from the lymph nodes of MOG-immunized animals at the onset of EAE was also investigated. We found that the proliferative capacity of MOG-specific T lymphocytes derived from CVF treated animals is significantly reduced, in agreement with the histology of the spinal cords showing a decreased infiltration of CD4$^+$ T cells in these mice. Our data suggest, that lack of systemic complement at the time of induction of EAE delays the onset and attenuates the course of the disease most probably via diminishing the response of MOG-specific T cells and production of autoantibodies.

1. Introduction

Multiple sclerosis (MS) is the most common inflammatory autoimmune disease of the central nervous system (CNS). Immune response against the oligodendrocytes destroys their product, the myelin sheath. These lesions and the following axonal damage cause very diverse sensual and vegetative symptoms, depending on the site of the immune attack. Experimental autoimmune encephalitis (EAE) is a widely used animal model of MS (Baxter, 2007). It is believed that Th1 type regulatory T cells induce the inflammatory process and activate the effector cells, such as T_c cells, macrophages, resident astrocytes and microglia cells (Martin et al., 1992; Sospedra and Martin, 2005). More recently the possible role of Th17 cells was also described (Bettelli et al., 2007) but still remain some open questions regarding the pathogenesis of the disease (Steinman, 2008).

The complement system is known to play an important role not only in the effector phase, but also in the initiation and regulation of adaptive responses (Pepys, 1974; Kerekes et al., 1998; Heyman, 2000; Carroll, 2004; Hawlisch and Kohl, 2006). The role of complement proteins in the pathogenesis of MS and EAE has been investigated for a long time; the results however are still contradictory. Some investigators using C3 KO mice found, that complement is not required for the development of EAE (Calida et al., 2001) or at least C4 is not essential (Boos et al., 2005). Nevertheless many other investigations have proven that complement has an important role in the course of this disease. These studies were carried out in C3 KO mice (Nataf et al., 2000), with rats decomplemented with cobra venom factor (Linington et al., 1989), with transgenic mice expressing sCrry in the nervous system (Davoust et al., 1999) and by using

Q1 * Corresponding author at: Immunology Research Group of the Hungarian, Academy of Sciences at Eötvös Loránd University, H-1117 Budapest, Hungary.
Tel.: +36 1 381 2175; fax: +36 1 381 2176.
E-mail address: anna.erdei@freemail.hu (A. Erdei).

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sCR1 treated rats (Piddlesden et al., 1994). So far no investigations of EAE have been carried out using cobra venom factor-treated mice. Cobra venom factor (CVF) is widely used for transient decomplementation of mice and rats. This complement activating protein in cobra venom is a C3b-analogue, which is able to bind to factor B and generate a relatively stable C3/C5 convertase. This leads to the activation of the complement cascade in CVF-treated animals and results in the elimination of C3 and C5 from the circulation. Normal levels of C3 will be restored in a week (Vogel et al., 2004).

The role of autoantibodies in MS is still controversial; yet one of the most important diagnostic hallmarks is the presence of oligoclonal Igs and plasma cells in cerebrospinal fluid (CFS). There is a growing number of evidence supporting the view that B cells and antibodies play a role in the pathogenesis of MS (Corcione et al., 2004; Urich et al., 2006). As experiments with B cell depleted animals show less clear results, the role of Igs seems to strongly depend on the experimental system (Lyons et al., 1999).

In the present study our aim has been to reveal how lack of complement at the initiation of EAE influences the onset and course of the disease. As it is known for long that complement is involved in both T-dependent and T-independent antibody responses, we compared the amount of MOG-specific antibodies in normal and CVF-treated mice, after induction of EAE. In addition to the levels of these autoantibodies, their complement activating ability was also assessed, using an antibody-complement (AbC) array system, developed recently at our department (Papp et al., 2007; Papp et al., 2008). Finally, the proliferative capacity of MOG-specific lymph node T-cells derived from normal and decomplemented animals has also been compared.

2. Methods

2.1. Animals, EAE induction

Female, 6–8 weeks old C57BL/6 and SJL/J mice, purchased from Charles River Laboratories were used. All animal experiments were in accordance with national regulations and were authorized by the ethical committee of the institute. C57BL/6 mice were injected *s.c.* on day 0 with 100 μg of Myelin Oligodendrocyte Glycoprotein 35–55 peptide (MOG, MEVGWYRSPFSRVVHLYRNGK) and SJL/J mice with 100 μg Proteolipid Protein peptide 139–151 (PLP, HSLGKWLGHPDKF) emulsified in CFA containing 4 mg/ml Mycobacterium tuberculosis (Chondrex Inc.). The peptides were synthesized by Gábor Tóth (University of Szeged, Hungary). In addition to the peptides, on days 0 and 2 mice were given *i.p.* 200 ng pertussis toxin (List Biological Laboratories).

Clinical signs of EAE were assessed daily using the following standard scale: 0, no clinical signs; 1, loss of tail tone; 2, incomplete hind limb paralysis; 3, complete hind limb paralysis; 4, moribund; 5, death. Animals with score 4 for more than 2 days were sacrificed. Representative of two to four independent experiments is shown.

2.2. Decomplementation

For the decomplementation of mice Cobra Venom Factor (CVF from Naja naja, Aczon SpA) was used. Mice were injected *i.p.* with 50 μg CVF in physiological saline 2 days before the induction of EAE.

2.3. Histological and immunocytochemical procedures

For cryostat sections the spinal cord and the lymphoid organs were excised, covered with liver for cryoprotection and snap-frozen in liquid nitrogen. Ten-micron-thick frozen sections were collected on poly-L-lysine-coated slides (Sigma–Aldrich), fixed in cold acetone, and air dried. For light microscopy the tissue samples were fixed in buffered 4% glutaraldehyde (Polysciences Inc.) solution for 24 h and postfixed in 1% osmium tetroxide (Polysciences) solution for 2 h. After rehydration in graded ethanol, the tissue samples were embedded in Polybed/Araldite 6500 mixture (Polyscience).

Immunocytochemistry was performed on cryostat sections according to standard techniques. Briefly, after rehydration in PBS, the acetone fixed sections were incubated with primary antibodies for 45 min, followed by biotinylated rabbit anti-rat IgG (Vector Laboratories). The endogenous peroxidase activity was quenched by treatment of the sections with 3% hydrogen peroxide (Sigma–Aldrich) for 10 min. Rigorous washing was followed by incubation of avidin-biotinylated peroxidase complex (Vector, Vectastain Elite ABC kit) at room temperature. The binding sites of the primary antibodies were visualized with 4-chloro-1-naphthol (Sigma–Aldrich). To identify macrophages rat anti-mouse CD11b mAb (clone: M1/70.15) was used. For T-cell staining anti-CD4 mAb (clone: YTS 091.1.2) was used. Appropriate isotype control antibodies were used at the same concentration. All antibodies were purchased from ImmunoTools GmbH.

2.4. Bone marrow derived dendritic cells (BMDC)

Six-week-old female C57BL/6 mice were sacrificed and their femurs were removed. Bone marrow was washed out with 5 ml of RPMI medium. Red blood cells were depleted by ammonium chloride treatment for 1 min at room temperature. Cells were cultured at 10^6/ml in RPMI medium, containing 10% of X63 cell (kindly provided by Dr. A. Rolink, University of Basel, Switzerland) supernatant, as the source of GM-CSF. Cytokine was added on every second day and BMDC were used as antigen-presenting cells (APC) on day 7.

2.5. T cell proliferation assays

Cells were isolated from the draining lymph nodes of mice injected with MOG peptide after 15 or 39 days of induction, as indicated. For the FACS-analysis lymph node cells were stained with CFSE (Molecular Probes). As APC spleen cells or BMDC were used. The ratio of APC and lymphocytes was 1:10. MOG 35–55 peptide was used at a concentration of 50 μg/ml. Cells were cultured in RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05% 2-mercaptoethanol (Sigma–Aldrich). Proliferation was assessed either by FACS (BD LSR II), or by measuring the incorporation of ^{3}H thymidin, as indicated. In the case of CFSE-labeled cells data were analyzed by FCS Express.

2.6. Antigen array

For testing the antibody response a protein array system was used. PLP 139–151 and MOG 35–55 peptides (both 5 mg/ml), myelin basic protein (MBP, 1 mg/ml) and MOG (0.6 mg/ml; kindly provided by Dr. H. Wekerle, Martinsried, Germany) were used. Fourfold dilution series were applied in triplicates, using Calligrapher miniarrayer (BioRad), and samples were printed onto home-made nitrocellulose coated glass slides. All materials were diluted in PBS-azide. The generation of microarray data is described elsewhere in detail (Papp et al., 2007; Papp et al., 2008). Briefly, dried arrays were rinsed for 15 min in PBS just before use, and then incubated with sera of mice in a humidified chamber at 37 °C degrees for 60 min. The reaction was terminated by washing the array with 0.05% Tween 20 containing PBS. Slides were then incubated in a mixture of detecting antibodies with gentle agitation for 30 min at room temperature in the dark. FITC conjugated goat anti-mouse C3 $F(ab)_2$ and Alexa Fluor 647 labeled goat anti-mouse IgG (H + L) (Molecular Probes) were diluted in 5% BSA, 0.05% Tween 20 containing PBS for 10,000 and 5000 times, respectively. Following washing and drying, slides were scanned on a Typhoon Trio + Imager (Amersham Bioscience) following standard protocols. Data were analyzed with ImageQuantTL (Amersham Bioscience) software. Fluorescence

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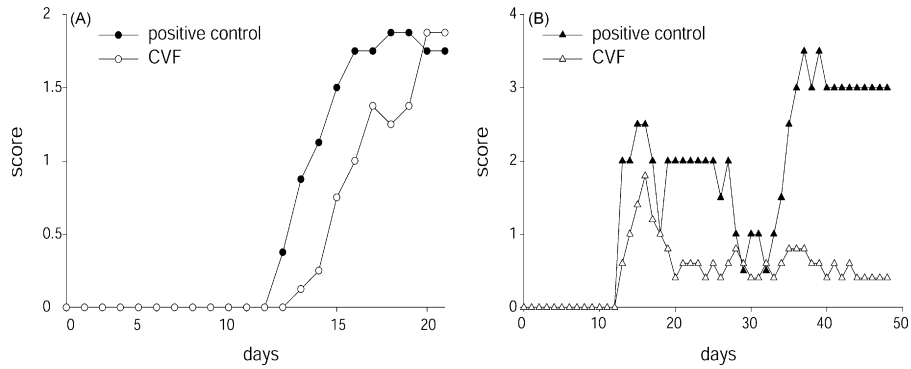

Fig. 1. Effect of a single dose of CVF (50 μg) injected *i.p.* prior to the induction of EAE. Panel A: In C57BL/6 mice EAE was induced with 100 μg MOG 35–55 peptide. Panel B: In SJL/J mice EAE was induced with 100 μg PLP 139–151 peptide. Results shown are the daily mean scores of the CVF-treated (open symbols) and control (filled symbols) animals.

intensity data were normalized, both for IgG and C3, to yield identical pLA derived values.

2.7. Statistical analysis

Daily average scores of diseased animals were compared using Mann–Whitney *U*-test. Differences in the day of onset of disease were analyzed by one-way ANOVA. Statistical significance of the effect of CVF treatment on *in vitro* restimulation assays was calculated by χ^2 test. Null hypotheses were rejected when p was less then 0.05.

3. Results

3.1. Decomplementation delays onset and severity of EAE

CVF treatment is known to induce transient depletion of serum complement in laboratory animals resulting in reduced levels of component C3 (Vogel et al., 2004). To see how low complement levels influence onset and severity of EAE, we induced the disease 2 days after CVF-treatment. As the course of the disease is different in C57BL/6 and SJL/J mice (Mendel et al., 1995; Lindsey, 1996), the experiments were carried out using both strains. In C57BL/6 mice the disease is monophasic and can be readily induced by MOG or MOG-derived peptides, while in the SJL/J strain PLP is used to initiate EAE, and the disease manifests in relapsing and remitting phases.

As shown in Fig. 1A in C57BL/6 mice the onset of the disease was significantly delayed in the CVF treated group, and the average score was significantly lower on days 13 and 14. In SJL/J mice complement depletion slightly decreased the severity of the disease in the early phase and scores in the relapsing phase were strongly reduced (Fig. 1B). In these animals the onset of the disease however, was not altered by CVF-treatment. It also has to be mentioned, that there was no significant difference in the incidence of the EAE when CVF treated and the untreated mice were compared, moreover the phenotype of the disease did not change either.

3.2. Histology of the spinal cord; infiltration of CD4+ T cells is slightly reduced

As transient decomplementation of mice with CVF results in characteristic changes in the onset and course of EAE (Fig. 1), next we investigated if histopathological differences can also be observed in these animals. We found, that semithin sections of CVF-treated animals are quite similar to those of mice with normal complement activity and harmonize with the clinical symptoms (Fig. 2A and D). In the spinal cord axon degeneration and consequently demyelinization occurs in the white matter. No histological identifiable changes are seen in the gray matter and the ventral root of spinal nerves. Generally, the anterior and lateral fascicules of the spinal cord's white matter are more deeply involved in the histological changes than the posterior one. The degeneration of the axons and demyelinization can be seen on the surface area of the spinal cord while the white matter close to the gray one is almost untouched (Fig. 2A and D). In the marginal zone of the fascicules, lateral and anterior, the majority of the axons disappeared unlike the deeper part of the fascicule, where the cross-section of the axons and myelin sheaths show up a round or oval-shaped heavily-stained rings (Fig. 2A and D). Immunohistochemical staining shows a slightly decreased number of infiltrating $CD4^+$ T cells in samples derived from decomplemented animals (Fig. 2B and E), while the amount of CD11b positive macrophages was quite similar in the area involved in the pathological changes (Fig. 2C and F). $CD8^+$ T cells, granulocytes and $CD45R^+$ B lymphocytes also immigrate into the spinal cord (data not shown).

3.3. Reduced proliferation of MOG-specific T cells derived from CVF-treated diseased animals

In the pathology of EAE and MS, T cells are known to play a central role (Sospedra and Martin, 2005). As others as well as we (Fig. 1) have found that complement might be involved in the course of this disease, moreover our histochemical results show that decomplementation slightly reduces infiltration of T cells (Fig. 2B and E) next we set out to investigate whether the response of MOG-specific T cells is also affected by CVF-treatment. We set up an *in vitro* antigen presentation assay to compare the activation of T cells derived from decomplemented and normal mice after EAE induction. As shown in Fig. 3A, T cells prepared from CVF-treated animals have a significantly reduced proliferative response to MOG than lymphocytes derived from mice with normal complement activity. In this set of experiments lymphocytes were isolated on day 15 after induction of EAE. Fig. 3B shows that this difference still exists when lymphocytes are isolated 39 days after initiation of the disease. These data clearly demonstrate that low complement levels at the time of the induction of EAE strongly influence the proliferative response of the autoantigen specific T cells. This finding is in accordance with earlier reports describing impairment of T-cell functions in complement-depleted animals (Suresh et al., 2003; Strainic et al., 2008).

3.4. The amount of MOG-specific antibodies and their complement activating capacity is diminished in CVF-treated animals

It is well accepted that lack of complement—particularly the absence of the activation fragments of component C3 and their

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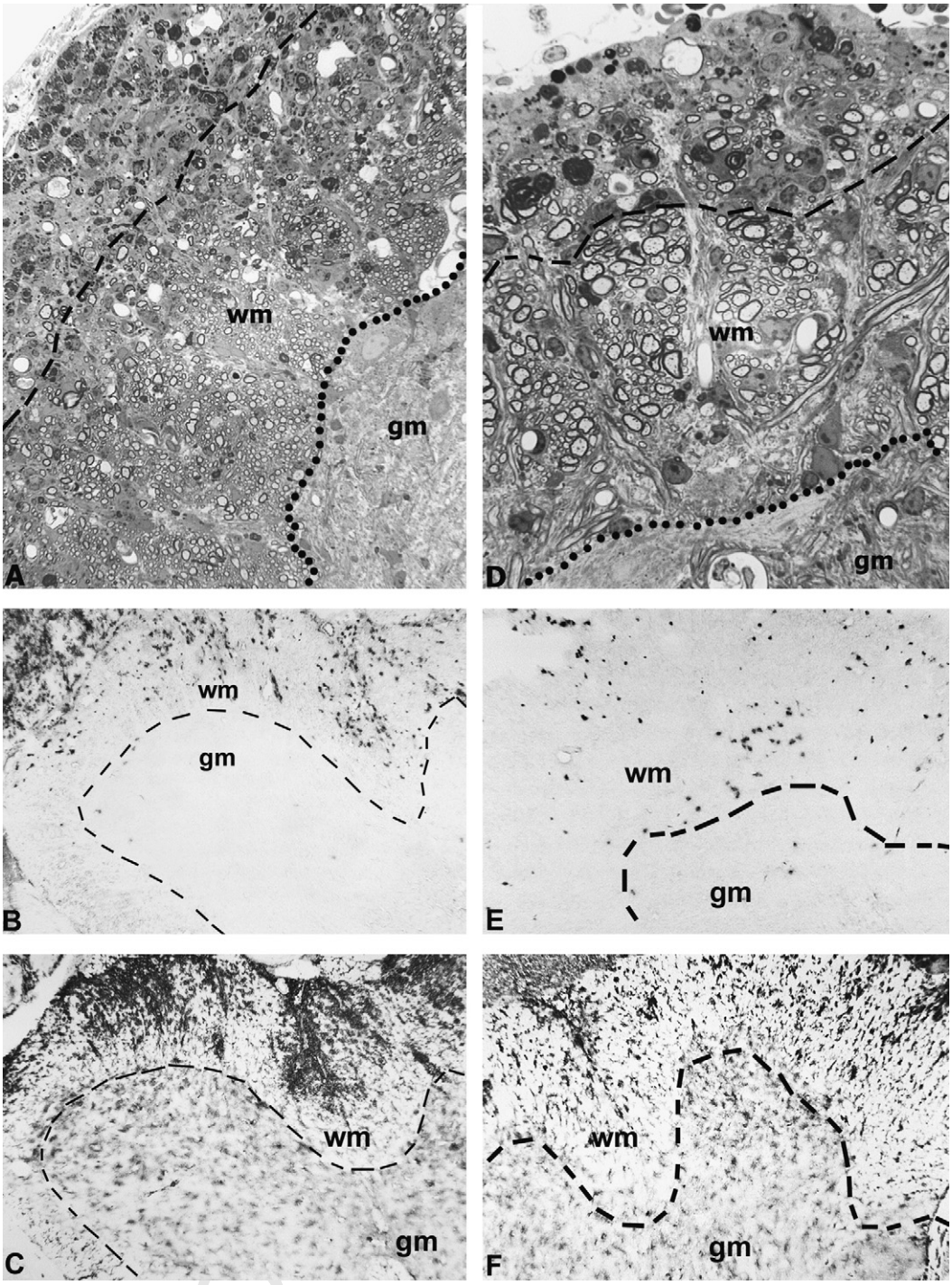

Fig. 2. Histologic analysis of the spinal cord of control (photos A–C) and CVF-treated (photos D–F) C57BL/6 mice. (A) Magnification, 400× and (D) magnification, 200×: semithin sections stained with toluidin blue. Lateral and ventral fascicles are sharply divided for peripheral and deeper zones (shown by dashed line). (B and E) Sections stained with anti-CD4 antibody (magnification: 40×). (C and F) Sections stained with anti-CD11b antibody (magnification: 40×).

receptors—results in a significant deterioration of the animals' antibody response (Pepys, 1974; Heyman, 2000). To investigate if this phenomenon can also be observed in our experimental system we compared the level of MOG-specific IgG in the sera of decomplemented and normal mice, 21 days after induction of EAE. In addition to measuring the amount of the autoantibodies, we also assessed their complement-activating capacity, using a recently developed protein-chip system (Papp et al., 2007). As shown in Fig. 4 both the level of MOG-specific antibodies and the consequent complement deposition was significantly lower in the case of sera derived from decomplemented animals. This finding is in line with the clinical manifestation of the disease, proving that the autoantibodies generated during the course of EAE and their complement-activating ability contributes to the pathomechanism. To find out whether the antisera of the diseased animals also react with other myelin-derived proteins than the MOG 35–55 sequence which had been used for the induction of EAE, the whole MOG protein, PLP 139–151 peptide and MBP have also been printed on the protein-chip. No reaction could be detected in the case of any of these, except the whole MOG protein (Fig. 4C). The lack of reaction with the 20 amino

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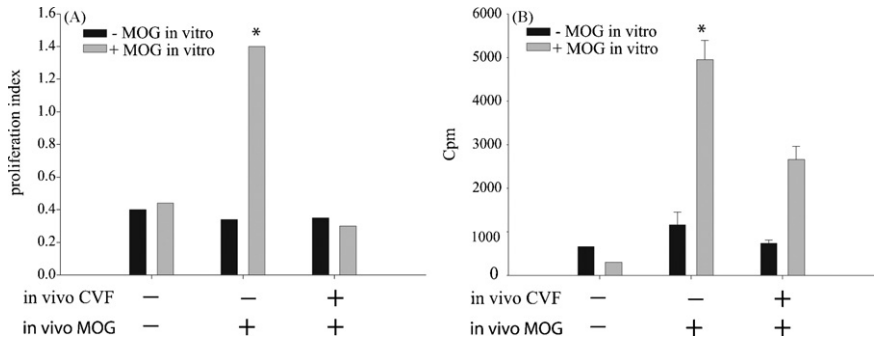

Fig. 3. Proliferation of MOG-specific T-cells derived from CVF-treated and control animals tested on days 15 (panel A) and 39 (panel B) after induction of EAE. Panel A: Lymph node cells from CVF-treated and normal mice, injected with MOG-peptide were isolated on day 15. Lymphocytes were labeled with CFSE and cultured for 3 days in the presence of BMDC and MOG 35–55 peptide. Result of one representative experiment is shown. Panel B: Lymph node cells from CVF-treated and normal mice, injected with MOG-peptide were isolated on day 39. Lymphocytes were cultured for 3 days in the presence of spleen cells and MOG 35–55 peptide followed by pulsing with [H^3] thymidin. Result of one representative experiment is shown ($p < 0.05$).

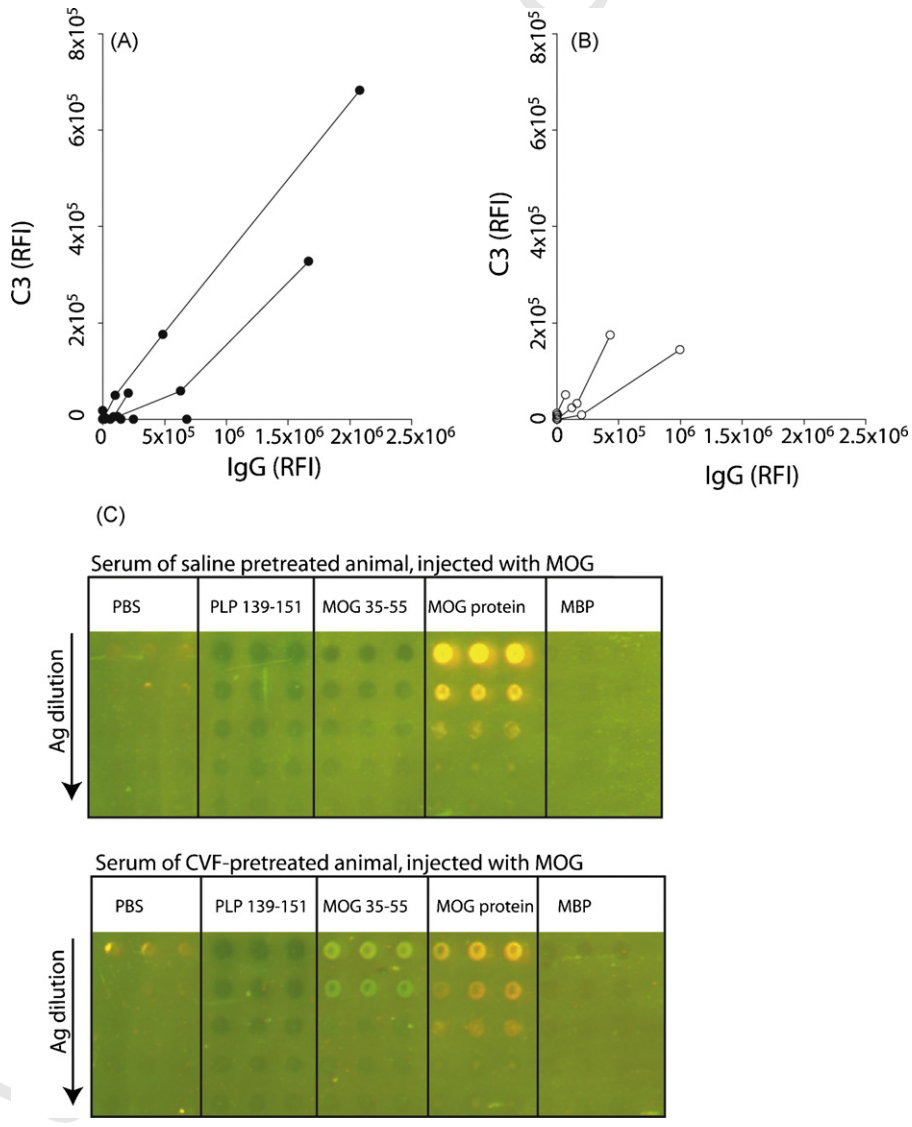

Fig. 4. The level and complement-activating capacity of MOG-specific antibodies in control (panel A) and CVF-treated (panel B) mice on day 21 after induction of EAE. Q3 Antigen-arrays were incubated with the serum of the mice as indicated, and bound IgG and deposited C3 were measured using fluorescently labeled antibodies. The figure shows relative fluorescence intensity of the samples. Panel C: Representative false color image of antigen microarrays incubated in sera of control and CVF-treated mice. IgG deposition: red; C3 deposition: green; merge: yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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acid long MOG-derived peptide is most probably due to steric constraints of the bound sequence.

4. Discussion

Complement proteins have long been implicated in the pathogenesis of both MS and EAE (Oldstone and Dixon, 1968; Levine et al., 1971; Pabst et al., 1971). Although since then several studies have been carried out, the results are contradictory and there are still several unanswered questions. In studies carried out with mice, so far only animals with knocked out complement components were used. Regarding C3 KO mice it has been reported that there is no difference in the severity and onset of EAE as compared to wild type animals (Calida et al., 2001). Another group however, reported that in complement deficient animals the disease is attenuated (Nataf et al., 2000). Investigating active and passive EAE Szalai et al. found reduced severity of the disease in C3 KO mice (Szalai et al., 2007). EAE has also been induced in $C4^{-/-}$ mice (B6) but no significant difference was found when compared to the wild type animals (Boos et al., 2005). Wreeth et al. immunized $C5^{-/-}$ and $C5^{+/+}$ mice with purified guinea pig myelin, and found that C5 KO animals developed less progressive disease (Weerth et al., 2003). ICAM-1 and C3 double KO mice were also investigated, and in this case an enhanced severity of the disease was reported (Smith et al., 2008). Regarding rat model systems, investigating C6 KO animals Tran et al. observed milder signs and a delayed onset (Tran et al., 2002), while Mead et al. found no difference in the clinical course of EAE between C6 sufficient and deficient animals (Mead et al., 2002). All these studies were carried out using KO animals, which may be not be the most adequate model to study the effect of complement in EAE, due to the complexity of the disease and the compensatory mechanisms of the phenotype.

There are a few studies with animals treated with CVF, which results in a transient decrease in systemic complement-activity; however so far no mice have been investigated using this model. Linington et al. used a rat model of EAE and employed CVF just before the expected onset of the disease. They observed the attenuation of active EAE, and concluded that complement-dependent mechanisms are involved both in the clinical course of acute inflammation and in the pathogenesis of antibody-mediated demyelination in EAE (Linington et al., 1989).

Here we studied for the first time how a single injection of CVF influences the onset and course of EAE in mice. Employing this system we were able to investigate the effect of controlled complement depletion, since the animals had reduced systemic complement activity exclusively at the initiation of EAE as normal complement-level is restored in about seven days. Since CVF was given to the mice only once, no antibodies were formed against the protein; moreover this single injection had no effect on the animals' phenotype. We found that lack of complement activity at the time of immunization with MOG-peptide delays the onset of nonrelapsing EAE in C57BL/6 mice (Fig. 1). It has to be pointed out that the transient depletion of systemic complement also exerted a long-term effect, as observed in SJL/J mice, where the disease has remitting and relapsing phases. In these animals CVF-treatment caused a strong inhibition of the scores in the relapsing phase, and the remitting phase was also less severe than in mice with normal complement (Fig. 1). These results clearly show the importance of the intact and active complement system during the initiation of the disease, moreover, point to its role in the pathogenesis of EAE in mice. The possible effect of the anaphylatoxins C3a and C5a released upon administering CVF can be excluded, since EAE was induced two days after the injection of CVF, when systemic complement activation had already subsided.

EAE and MS are considered T cell mediated autoimmune inflammatory diseases of the CNS. It is well accepted that autoreactive T cells stimulated in the peripheral lymph nodes enter the CNS where they are reactivated (Flugel et al., 2001). Recently it has been demonstrated by Stromnes et al that T cells that are specific for different myelin epitopes generate different Th17 to Th1 ratios depending on the functional avidity of interactions between TCR and peptide MHC complexes (Stromnes et al., 2008). We propose that the decreased complement activity at the initiation of EAE also affects antigen-presentation, as we found a significant decrease in the proliferative capacity of MOG-specific T-cells derived from CVF-treated mice. Interestingly, this effect can be observed even 39 days after EAE induction. Our results showing a slightly decreased number of infiltrating $CD4^+$ T cells in the area of the spinal cord involved in the pathological changes are also in line with this observation.

The direct effect of complement on myelin has been shown by several authors. Isolated myelin has been proven to activate the complement cascade via the classical pathway (Vanguri et al., 1982) and human MOG protein have been shown to bind C1q (Johns and Bernard, 1997). Moreover, human oligodendrocytes were found to lack complement-regulatory proteins such as complement receptor type 1 (CR1), membrane cofactor protein (MCP), homologue restriction factor (HRF) and clusterin (Scolding et al., 1998), suggesting that myelin is extremely sensitive for complement-mediated destruction. It is also known that under physiological conditions complement-levels are very low in the CNS; the concentration of C3 and factor B in the cerebrospinal fluid is more than two orders of magnitudes lower than in blood (Stahel et al., 1997). However, when inflammation causes the break-down of the blood brain barrier, complement components as well as Igs enter the CNS. It has also been described that several CNS-resident cells are able to produce complement locally (Morgan and Gasque, 1996). As injection of CVF depletes systemic complement via exhaustion of the components in the treated animal, the possible effect of complement activation also had to be taken into consideration. The results of our histological studies however do not support the direct involvement of CVF-treatment in myelin-destruction, as there was no difference between the histolopathology of the spinal cord of CVF-treated and normal animals.

B cells and antibodies have important role in the pathogenesis of MS, and the presence of oligoclonal antibodies in the CSF is an important criterion in the diagnosis of the disease. In animal models the role of B cells and antibodies is less clear. Using B cell deficient mice in B10.PL background immunized with MBP Ac1–11 peptide no difference was found in the onset and the severity of EAE, but recovery was significantly reduced (Wolf et al., 1996). Another study carried out with C57BL/6 mice and MOG 35–55 peptide or MOG protein showed that B cells are essential when animals were immunized with the whole protein only (Lyons et al., 1999). As the demyelinating potential of antibodies highly depends on their ability to fix complement (Piddlesden et al., 1993), we investigated C3 deposition as well, beside IgG-binding to myelin-derived antigens, using protein chips. Our data clearly show that IgG of transiently decomplemented mice has a significantly reduced MOG-reactivity and complement activating capacity than antibodies of non-treated diseased animals (Fig. 4.), in good agreement with our observation that CVF-treatment attenuates the disease. Our present findings are strongly supported by a recent report of Urich et al., who had clearly demonstrated that autoantibody mediated demyelination in mice depends on complement activation (Urich et al., 2006).

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