Administration of the soluble complement inhibitor, Crry-Ig, reduces inflammation and aquaporin 4 expression in lupus cerebritis

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Abstract

Changes in brain water and cerebral volume can lead to brain edema that may be one of the underlying causes of death in many neurological diseases. Cerebral water content is regulated by aquaporin 4 (AQ4) present in astrocytic end feet and around blood vessels. In systemic lupus erythematosus (SLE), magnetic resonance imaging (MRI) studies of the brain have demonstrated lesions with the prominent appearance of edema. Activation of complement may play a significant role in the pathogenesis of lupus cerebritis by causing inflammation that can lead to edema. In this study, the well-established MRL/lpr lupus mouse model was used to evaluate the role of complement in lupus cerebritis. IgG and C1q colocalized in perivascular deposits indicating that the blood–brain barrier was compromised. Both RNA and protein expressions of AQ4 were significantly increased in brains of MRL/lpr mice. Chronic administration of the soluble complement inhibitor, Crry-Ig, reduced inflammation as measured by decreased accumulation of IgG. In contrast to control MRL/lpr mice, AQ4 expression in complement inhibited MRL/lpr mice was not changed relative to untreated congenic controls. These results illustrate that complement activation in brains of lupus mice leads to enhanced AQ4 expression and inflammation. It is conceivable that increased AQ4 expression results in cerebral edema and hence complement inhibition may provide a new therapeutic option in inflammatory cerebral disorders such as lupus cerebritis.

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Keywords: Crry-Ig; Inflammation; Aquaporin 4 expression; Lupus cerebritis

1. Introduction

Brain water content and volume are tightly regulated to assure the normal functioning of the central nervous system (CNS). The brain is encased in the rigid cranium and is highly sensitive to any increase in intracranial pressure. Alterations in cerebral water homeostasis and distribution may lead to neuronal and glial swelling known as cytotoxic brain edema, due to accumulation of intracellular water. Cerebral edema plays a significant role in the morbidity and death associated with many common neurological disorders such as systemic lupus erythematosus (SLE) and multiple sclerosis.

The complement cascade is involved in the development of brain pathology in diseases such as SLE and injury [1]. Evidence from experimental, clinical, and in vitro studies highlight an important role for the complement system in contributing to inflammation within the injured brain [2–5]. Staining for complement components C1q, C3b, C3d and the membrane attack complex (MAC) were increased in resected tissue from patients with intracranial hypertension [6,7]. These studies suggest that complement activation may play an important role in the development of secondary brain damage such as edema. This is substantiated by the studies in which complement inhibition with \textit{N}-acetylheparin and complement depletion with cobra venom factor reduced brain edema after experimental intracerebral hemorrhage [8].

Each member of the aquaporin (AQ) family has a unique tissue distribution. In brain, AQ1 is present in the apical plasma membranes of cells of the choroid plexus in the ventricles, where it has been suggested to participate in the secretion of cerebrospinal fluid. AQ4 is a highly conserved water channel protein present in the plasma membranes of ependymal cells and astrocytes. AQ4 was shown to have a role in the formation of brain edema and brain water homeostasis [9].
CNS involvement is an often-devastating manifestation of SLE. Transient hypodensities have been noted on computerized tomography (CT) scans of SLE patients. Magnetic resonance imaging (MRI) scans of SLE patients reveal vasculitis, arterial spasm and increased vascular permeability, all of which eventually can lead to cerebral edema [10]. Here we studied the well-established lupus mouse model MRL/lpr. This strain is on the autoimmune MRL/MpJ background and contains the lpr gene in homozygosity leading to deficiency of the apoptosis-promoting Fas (CD95) protein [11,12].

Cry is a widely distributed membrane-bound intrinsic complement inhibitor [14,15]. It has cofactor activity for the factor I-mediated cleavage of C3b and can cleave mouse C4b in the presence of factor I [16]. Cry exhibits decay-accelerating activity for both the classical and alternative pathways and therefore is a potent homologous mouse complement inhibitor. Cry-Ig is a chimeric form of Cry in which the non-complement fixing mouse IgG1 Fc region was bound to the to the functional domain of mouse Cry. The presence of Ig prolongs the half-life making this a potent in vivo complement inhibitor. In this study we examined the effect of complement inhibition with Cry-Ig [13] in MRL/lpr mice. Two major findings were that complement activation led to inflammation and a breach in the integrity of the blood–brain barrier, which, in turn, led to heightened expression of AQ4 at both the mRNA and protein level. These alterations were restored to normal by complement inhibition.

2. Materials and methods

2.1. Mice

Male MRL/lpr mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Age-matched unmanipulated MRL/+ mice were studied in parallel. Here we studied 9 mice that had been injected intraperitoneally with 3-mg Cry-Ig every other day [13] and 10 controls that received saline at the same schedule from 12 to 24 weeks of age, at which age they were sacrificed. An additional control group of MRL/lpr mice was treated with normal mouse IgG purified from normal mouse plasma by protein G chromatography (Amersham-Pharmacia-Biotech, Uppsala, Sweden) instead of Cry-Ig. All work with mice was approved by the University of Chicago Animal Care and Use Committee and was performed in accord with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Measurements from tissue

Brains were isolated from animals sacrificed at 24 weeks of age. Cerebellum and brain stem were discarded. The anterior region of the cerebral cortex was snap frozen for immunofluorescence (IF) microscopy, and the rest was processed for RNA isolation.

2.3. Tissue processing

Cryostat sections (4 μm) were processed for direct IF microscopy using FITC- or TRITC-conjugated mouse IgG (Cappel Laboratories, Durham, NC) antibody and indirect IF with rat AQ4 antibody (Chemicon International, Temecula, CA) followed by FITC-conjugated rabbit anti-rat IgG. They were scored from 0.5 to 4 where 0.5 was negligible staining and 4 very intense staining. Anti-mouse C1q antibody was a kind gift from Dr Andrea Tenner (University of California, Irvine). C1q was detected using FITC-labeled anti-rabbit IgG.

2.4. DNA microarray analysis

RNA was isolated from the brains of 24-week-old MRL/lpr mice, with and without Cry-Ig treatment, along with the MRL/+ strain control using TRIzol reagent (Life Technologies, Grand Island, NY). The integrity of all RNA was assessed by an Agilent Technologies 2100 Bioanalyzer (Palo Alto, CA) and determined to be of excellent quality. Five-microgram RNA was used as the template for the first strand cDNA synthesis in a reaction primed with oligo(dT) containing a T7 RNA polymerase promoter sequence. The second cDNA strand was synthesized using *E. coli* DNA polymerase 1 and ligase and was then used as template to make biotinylated RNA probe by in vitro transcription. The RNA probes were hybridized to MG-U74Av2 arrays (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions. The hybridized arrays were scanned using a Genearray Scanner (Agilent Technologies).

Data mining was done using both Affymetrix and GeneSpring software (Silicon Genetics, CA). For Affymetrix software, global scaling was done to a target intensity of 500. Single array analysis was done to ascertain that the arrays were of good and comparable quality. The parameters included expression of glyceraldehyde phosphate dehydrogenase (GAPDH) and its 3′/5′ signal ratio, hybridization efficiency using the ratio of the expression of the spiked bacterial genes *bioB, bioC, bioD* and *cre* as well as the scaling factors. Comparative analysis was done using the statistical algorithms of MAS v.5.0. Standard GeneSpring analysis involved importing Affymetrix signal data, which were then normalized per chip and then per gene such that the median value of each gene was 1.0. Restrictions were done to eliminate genes scored as absent and to select for genes that showed at least 1.3-fold increase in saline-treated MRL/lpr brains compared to those from MRL/+ control mice.

2.5. Quantitative RT-PCR in lupus mice

To remove all traces of genomic DNA from brain RNA isolated using TriZol reagent, samples were treated with Rnase-free RQ1 DNase (1 U/μg RNA; Promega, Madison, WI) in 10-μl reaction buffer (final concentration, 40 mM Tris–HCl, 10 mM MgSO₄, and 1 mM CaCl₂, pH 8) at 37 °C.
for 30 min. This was followed by addition of 1-μl 20 mM EGTA (pH 8) to stop the reaction and incubation at 65 °C for 10 min to inactivate DNase. cDNA was generated from RNA using random hexamers as primers with the Super-Script first-strand synthesis kit (Life Technologies), according to the manufacturer’s instructions. Real-time PCR was performed using a Smart Cycler (Cepheid, Sunnyvale, CA). The probes were labeled at the 5’ end with the reporter dye molecule FAM (6-carboxyfluorescein; emission max 518 nm) and at the 3’ end with the quencher dye molecule TAMRA (6-carboxytetramethyl-rhodamine; emission max 582 nm). Each reaction was conducted in a total volume of 25 μl with 1-μl TaqMan Master Mix (PE Applied Biosystems, Foster City, CA), 3-μl sample or standard cDNA, primers at 200 nM each, and probe at 100 nM. PCR was conducted with a hot start at 95 °C (5 min), followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. For each

Fig. 1. Complement-dependent compromise of the blood–brain barrier in murine lupus. Perivascular IgG (shown by arrows) staining seen in brains of MRL/lpr mice was prevented by complement inhibition. MRL/+ (A), MRL/ lpr mice treated with saline (B) or Crry-Ig (C).

Fig. 2. C1q expression is colocalized with IgG in lupus brains. IF using TRITC-labeled antibody for IgG staining (A) and FITC-labeled antibody for C1q staining (B) clearly superimpose on one another (C) indicating that the IgG colocalizes with immune complexes in lupus brain.
sample, the number of cycles required to generate a given threshold signal ($C_t$) was recorded. Using a standard curve generated from serial dilutions of brain cDNA, the ratio of AQ4 expression relative to GAPDH expression was calculated for each experimental animal. This was a suitable control (i.e., “housekeeping” gene) as shown by both Affymetrix and GeneSpring data analysis, in which GAPDH gene expression was unchanged among all experimental animals. Primers synthesized by Integrated DNA Technologies (Coralville, IA) and probe by Synthegen (Houston, TX) for GAPDH were:

forward primer: 5′-GGCAATTCAACGCCAGGT-3′;
reverse primer: 5′-AGATGTTGATGGGCTTCCC-3′;
probe: 5′-AACCGGAAGATGGGAAGCTTGTCATC-3′;

The sequences of primers and probe for AQ4 synthesized by Operon technologies (Almaeda, CA) as well as the sequence position in U48398 provided in parentheses were:

forward primer: 5′-AGTGGCCTTTATTAGTATG-3′ (738–757)
reverse primer: 5′-CTTCCCTTCTTTCTTTCTCC-3′ (944–925)
probe: 5′-CTGTCCTGATGTGGAGCTCAAACG-3′ (761–785)

Table 1

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2.6. Statistics

Statistical analyses were performed with Minitab Software (College Park, MD, USA). Data are expressed as mean ± S.E. unless noted otherwise and statistical significance was determined by one-way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

3. Results

3.1. The blood–brain barrier is compromised in lupus brains

Perivascular staining of IgG was seen by IF microscopy in the thalamus of MRL/lpr mice given saline (Fig. 1B), consistent with a compromise in the integrity of the blood–brain barrier as compared to the MRL/+ controls (Fig. 1A). Treatment of MRL/lpr mice with Crry-Ig considerably reduced brain perivascular IgG staining (Fig. 1C), indicating that complement activation was responsible for the breach in integrity of the blood–brain barrier. MRL/lpr mice treated with normal IgG showed equivalent IgG staining compared to mice treated with saline (not shown). C1q staining was similarly increased, colocalizing with the IgG, indicating these were immune complexes (Fig. 2).

3.2. Microarray studies identify complement-dependent alterations in AQ4 expression

Initial studies were done to examine global gene expression in brains of lupus mice as well as to examine which of these might be affected by complement inhibition. Two 24-week-old mice from each of the three conditions were studied, MRL/lpr mice treated with saline or Crry-Ig and unmanipulated MRL/+ mice. RNA from individual mouse brains was hybridized with separate arrays. Microarray studies indicated that AQ4 gene expression was increased in brains of MRL/lpr mice as compared to the MRL/+ strain control (Fig. 3). As there are two AQ4 probe sets on the MG-U74Av2 array (Table 1), data using each probe set is given separately. As can be seen in Fig. 2, AQ4 expression was increased by over twofold using both probes, comparing MRL/lpr mice treated with saline to MRL/+ mice. Treatment with Crry-Ig completely prevented this increase.
in AQ4 expression. The housekeeping gene, GAPDH, remained unaltered in all three conditions with intensities of 1.18, 0.94 and 1.01, respectively.

3.3. Complement inhibition restores up-regulated AQ4 expression to normal in lupus brains

Due to the conservative use of arrays and therefore the small number of animals that were analyzed, we substantiated the microarray data with real-time PCR analysis. As in Fig. 4 showing data from individual animals, AQ4 expression was up-regulated in MRL/lpr mice (0.98 ± 0.38) compared to their controls (0.30 ± 0.15) and this increase was prevented with Crry-Ig treatment (0.20 ± 0.11) (P < 0.01 by analysis of variance). These results substantiated the array data and suggest that complement activation leads to up-regulated AQ4 expression in lupus brains.

The expression of AQ4 protein was significantly increased in MRL/lpr mice (Fig. 5B) as compared to the control MRL/+ strain (Fig. 5A) while complement inhibition with Crry-Ig prevented this increase (Fig. 5C). The staining was seen predominantly around the vessels (arrowhead) and at accumulations of astrocytic end feet (arrow) in the cortex. These results indicate that AQ4 expression is greatly enhanced in lupus mice both at the transcriptional level and at the protein level.

As shown in Fig. 6, there was a close parallel between AQ4 mRNA and protein expression. AQ4 expression was increased in MRL/lpr mice relative to MRL/+ controls, and complement inhibition with Crry-Ig fully prevented this increase.

4. Discussion

SLE is a multiorgan disease characterized by alterations in the regulation of both cellular and humoral immune responses. The MRL/lpr mouse is a murine model that has the right genetic background coupled with Fas deficiency that results in the expression of disease manifestations similar to human SLE. Disease develops gradually during life beginning at 8 weeks of age when there are elevated levels of serum IgM. By 12 to 16 weeks of age, these mice begin to produce autoantibodies. Finally, by 24 weeks, they develop full-blown disease with proliferative immune complex-mediated glomerulonephritis, vasculitis, arthritis, and massive lymphadenopathy [17]. In this study we examined genes that may be relevant in lupus cerebritis by comparing gene expression in MRL/lpr to control MRL/+ brains. Further, we determined the role of complement inhibition in these animals.

Consistent with previous studies [18], we found perivascular leakage of IgG and C1q presumably reflective of intact immune complexes in MRL/lpr mice while the congenic MRL/+ mice had none. Such IgG deposition seen in the brains of lupus mice is analogous to the IgG deposits seen in the choroid plexus of patients with SLE [19]. The presence of immune complexes can be taken as evidence that the blood–brain barrier is compromised in lupus, and this was completely prevented by treatment with Crry-Ig. It seems likely that deposition of immune complexes leads to complement-dependent inflammation in the brain vessels of MRL/lpr mice, and this is prevented through complement inhibition.

Intra- and extracellular water exchange occurs by water channels within the brain that appear to contribute to excessive water accumulation in different pathological conditions [20–22]. Previous immunohistochemical studies have shown that in rats, AQ4 is the most important water channel protein, and is localized on ependymal cells and glial membranes that line blood vessels and ventricles in the brain at the interface of brain, blood and CSF [23–26]. AQ4 was shown to have the same pattern of mainly astrocytic expression in the cerebellum as other areas of the brain. The time course and pattern of AQ4 expression during development suggest that it plays an important role in brain water metabolism during the second week of development [27]. The strategic localization of AQ4 and its high water permeability [28] enable it to function as efficient water-selective transporting protein necessary for health but deregulated in certain pathologic conditions. For instance, following permanent focal cerebral ischemia, an increase in AQ4 mRNA expression paralleled edema formation [29]. Similarly, knocking out AQ4 expression significantly reduced brain edema formation and improved the neurological outcome following permanent middle cerebral artery occlusion in mice [30].

In our study, AQ4 was expressed in the brain cortex of MRL/lpr mice around microvessels and at accumulations of astrocytic end-feet. Both RNA and protein expression were increased in the brains of lupus mice. This is a significant finding in the pathology of CNS lupus as this AQ4 increase could alter CSF synthesis and absorption, fluid transport...
across the vascular endothelium, and lastly, cell volume regulation [31]. In humans, 50% of SLE patients that had lesions by MRI had vasogenic edema that may cause hypertensive encephalopathy [32]. These lesions may be reversible and caused by edema around small vessels [33]. MRIs of lupus patients with acute diffuse neurologic manifestations such as seizures, psychosis and coma demonstrated a longer spin-spin relaxation time of the gray matter suggestive of the presence of acute cerebral edema compared to other SLE patients [31].

Various mouse strains that develop a lupus-like disease are studied to obtain insight into the etiology and pathology of SLE. These mouse models share many features of the human disease, including glomerulonephritis and antibodies to DNA and other autoantigens, but differ in that once initiated the murine disease is progressive while human SLE is characterized by a fluctuating course of flares and remissions [33].

In CNS-SLE there was an increase of CSF index values for C3 and C4, indicating an important role for the complement cascade in this disease [34]. To study the effect of complement inhibition on murine lupus cerebritis, we treated MRL/lpr mice with Crry-Ig from 12 weeks at the onset of autoimmune disease to 24 weeks when they had full-blown disease. This treatment completely prevented the increase of AQ4 mRNA and protein expression in lupus brains. Circumstantial evidence supporting our findings is that systemic complement inhibition by treatment with cobra venom factor after intracerebral hemorrhage was shown to attenuate brain edema and a positive correlation was demonstrated between AQ4 and brain edema in focal ischemia [8,35].

Our study is novel in that we used the complement inhibitor Crry-Ig, thereby allowing chronic complement inhibition not possible to-date, which prevented the increased expression of AQ4 at both transcriptional and protein levels in brains of lupus mice. It seems likely that the elevated AQ4 protein expression in lupus mice is of pathologic relevance, though this will require future studies to determine the role of cerebral edema and AQ4 in this disease model. These results strongly suggest that complement may play a significant role in brain edema by regulating AQ4 expression, which is relevant in diseases that have inflammation and altered water regulation.

Acknowledgements

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