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Research Report

Role of complement component C5 in cerebral ischemia/reperfusion injury

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C1-INH, C1-inhibitor
CVF, cobra venom factor
MCAO, middle cerebral artery occlusion
sCR1, soluble complement receptor 1
ZAS, zymosan-activated rat serum

ABSTRACT

We evaluated the role of complement component C5 during the course of cerebral ischemic reperfusion injury in a rat model of middle cerebral artery occlusion (MCAO). Systemic C5 inhibition was achieved with an anti-C5 monoclonal antibody, which significantly prevented the deterioration of the motor functions by reducing cerebral lesion and edema. Our results show that activated C5 complement components played an important role in cerebral tissue inflammation resulting from ischemia/reperfusion injury.

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1. Introduction

Cerebral stroke continues to be a leading cause of death and disability worldwide due to the limited efficacy of current therapy (Fisher, 2002). There is a great need for curative treatments, but also for good prevention protocols for populations at high stroke risk (Wolf et al., 1999). A better understanding of the pathogenesis of cerebral tissue inflammation, which occurs after an ischemia/reperfusion insult, may contribute to the development of new prophylactic and

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therapeutic treatments. The complement system is a key component of innate immunity that plays a major role in host defense. However, some of the activated components of the complement cascade are also known to be potent proinflammatory mediators and have been reported to participate in ischemia/reperfusion injury (Bhole and Stahl, 2003; Seelen et al., 2005). Complement can be activated through the classical, the alternative and the lectin pathways (Seelen et al., 2005). The classical and lectin pathways are initiated by the deposition of antibody and serum lectins such as mannose-binding lectin (MBL), respectively. Both generate membrane-bound C4b, C3b and the complex C5b-9 in this order, as well as producing anaphylatoxins C4a, C3a and C5a. The alternative pathway starts by the direct deposition of C3b, which initiates a proteolytic cascade that converges with the other complement pathways at the C5 cleavage (Seelen et al., 2005). C3b is also known for its ability to opsonize immune complexes and microbials (Carroll, 1998). In fact, the components C1q, C4b, C3b and breakdown products are ligands for several complement receptors expressed on immune cells and promote immune functions such as B cell activation and phagocytosis (Carroll, 1998; Seelen et al., 2005). Lastly, C5a recruits and activates leukocytes, whereas the membrane-attack complex (C5b-9) leads to cell lysis and death (Seelen et al., 2005). Thus, all three pathways involve the activation of C3 and C5 complement components, which are considered to be attractive targets for blocking complement activation (Bhole and Stahl, 2003).

Recent studies have pointed out a role of complement activation in cerebral ischemia/reperfusion injury. Focal deposition of C5b-9 has been detected in the brain infarcted areas of stroke patients (Lindsberg et al., 1996). Furthermore, transitory systemic complement activation follows stroke in humans (Pedersen et al., 2004). However, it is not well understood how activated complement components contribute to cerebral tissue inflammation during ischemia/reperfusion injury and what is the role of the different complement proteins. Complement inhibition has shown success in several small animal models of cerebral ischemia (Huang et al., 1999; Heimann et al., 1999; De Simoni et al., 2003; Akita et al., 2003). The use of soluble complement receptor 1 (sCR1) led to a reduction in infarct volume and neutrophil accumulation after 45-min MCAO in mice (Huang et al., 1999). A neuroprotective effect of C1-inhibitor (C1-INH) was also observed in various rodent models of focal cerebral ischemia (Heimann et al., 1999; De Simoni et al., 2003; Akita et al., 2003). Akita et al. (2003) suggested that this effect is due to diminished recruitment of inflammatory cells to the ischemic area. Finally, cobra venom factor (CVF) administered 24 h prior to MCAO also reduced dramatically cerebral infarct volume in rats (Figueroa et al., 2005). Thus, these studies support a role of complement activation in brain infarction. Nevertheless, these early studies used reagents that were not specific inhibitors of a single complement component and may potentially compromise the opsonizing and host defense functions of the complement cascade. The complement receptor 1 and its soluble form sCR1 bind different components of the complement cascade including C1q, MBL, C4b and C3b (Liszewski and Atkinson, 1993; Huang et al., 1999; Ghiran et al., 2000). C1-INH also inhibits components from the three complement pathways (C1r, C1s, MBL-associated serine proteinases-1 and -2, C3b), in addition to proteases of the contact system of kinin generation, coagulation factors and selectins (Carroll, 1998; Jiang et al., 2001; Cai and Davis, 2003). Finally, CVF depletes all complement components downstream of C3 (Figueroa et al., 2005).

We have hypothesized that activated complement components, in particular C5a and C5b-9, are critically involved in the development of cerebral inflammation during ischemia/reperfusion injury. In this study, we set up to elucidate the role of activated C5 components in a rat MCAO model. To this end, we used a monoclonal antibody specifically blocking the cleavage of C5 into C5a and C5b-9, which prevents the generation of these potent proinflammatory mediators. The impact of systemic C5 inhibition on the development of cerebral inflammation after MCAO is presented and discussed.

2. Results

2.1. *Systemic inhibition of complement component C5*

To assess the role of C5 in a rat MCAO model, we used the anti-rat C5 mAb 18A10.62, which prevents C5 cleavage and the generation of C5a and C5b-9 (Vakeva et al., 1998). To further characterize the antibody function, we evaluated the ability of 18A10.62 to bind directly to either C5b or C5a once C5 is cleaved. We observed that 18A10.62 did not bind C5b after inducing complement activation on ELISA plates (another anti-C5 antibody 13E8.15 did, data not shown). Moreover, it did not inhibit C5a directly either. This effect was determined in vitro in a functional assay that measured the ability of 18A10.62 to prevent C5a-induced migration of rat neutrophils after exposure to zymosan-activated rat serum (Fig. 1A). The anti-C5 mAb 18A10.62 effectively averted neutrophil migration when added to the serum prior to zymosan, but did not inhibit it when added afterwards (Fig. 1A).

The 18A10.62 dosing regime (described in the Experimental Procedure) was established following pharmacodynamic experiments in male Wistar rats. We based our anti-C5 mAb treatment on the presented pharmacodynamic experiment (Fig. 1B), which comprised an i.v. injection of 20 mg/kg 18A10.62 at time 0 and 2 i.p. injections (10 mg/kg) given 6 and 25 h later. This protocol attained more than 50% reduction in serum C5 activity as assessed by C5b-9-mediated hemolytic assay. We determined a 60% inhibition at 2 h and 12 h after initial i.v. administration of 18A10.62 and around half the activity at the other time points assessed. In the MCAO experiments, C5 inhibition was started at two different time points relative to the initiation of MCAO: 1 h prior to MCAO (pretreatment experiment) and 5 min after (posttreatment experiment). Note that we did not bleed the rats subjected to MCAO during the study, but we conducted a parallel treatment in healthy rats and determined serum C5 activity daily that confirmed comparable C5 inhibition (data not shown).

2.2. *Changes of physiological parameters after MCAO*

During the MCAO experiments, we monitored changes in body weight and rectal temperature and observed no

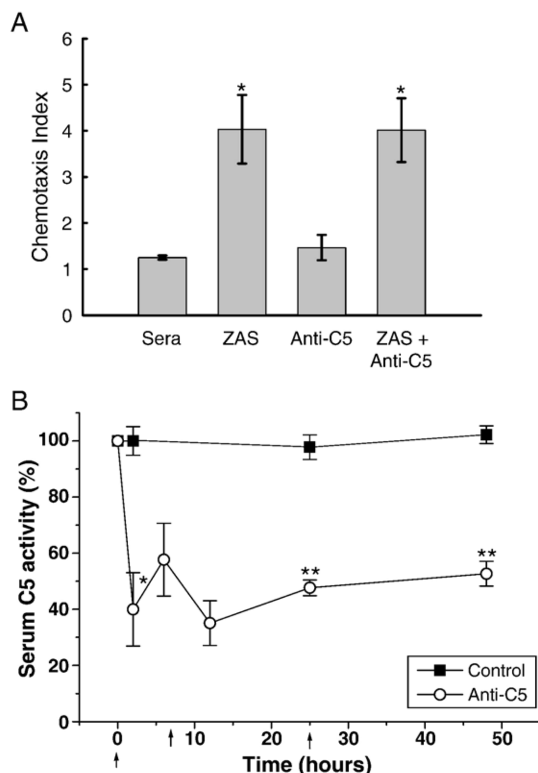

Fig. 1 – Anti-C5 blocking activity by the mAb 18A10.62. (A) Chemotaxis assay showing that 18A10.62 averted rat neutrophil migration when added to the serum prior to zymosan (anti-C5) but did not inhibit it when added to ZAS (ZAS + anti-C5). Results represent the mean ± SE of 8 experiments. Significant differences were observed in the ZAS treatment alone and the ZAS + anti-C5 compared to the unmarked groups "sera" and "anti-C5" (*$P < 0.05$). (B) Pharmacodynamic study showing inhibition of complement component C5 in Wistar rats. This experiment comprised an i.v. injection of 20 mg/kg 18A10.62 at time 0 and 2 i.p. injections (10 mg/kg) given 6 and 25 h later (just after blood sampling). The injection time points are indicated with arrows. Blood was collected before 18A10.62 administration to obtain pretreatment values and at 2, 6, 12, 25 and 48 h after the first injection ($n = 2$). Blood samples were also collected at 2, 25 and 48 h after the first injection in rats receiving irrelevant antibody HFN 7.1 ($n = 3$). C5 activity was reduced in serum of 18A10.62-treated rats as assessed with hemolytic assay and was significantly different from that corresponding to the control cohort (*$P < 0.05$, **$P < 0.005$). These observations reproduced in the 2 experiments conducted simultaneously to the MCAO.

significant differences between the various cohorts (Table 1). Body weight was reduced around 5–7% in the day after surgery in all groups, although there was a trend to less body weight loss in the anti-C5 mAb-treated groups (Table 1). No significant hypothermia or hyperthermia, which may influence infarct size (Chen et al., 1992), was observed due to the control of body temperature during anesthesia, surgery and recovery. Rectal temperature was slightly decreased after MCAO surgery and increased around 1 °C after starting reperfusion in all cases. Blood glucose was also determined of various rats treated with either irrelevant or anti-C5 mAb (collected at the 72-h harvest) and observed no effect of 18A10.62 treatment (158.6 ± 13.1 vs. 161.1 ± 11.2 controls, $n = 11$ in each group).

2.3. *Changes of neurological functions after MCAO*

The neuromotor function was evaluated in the pretreatment and posttreatment experiments. In both experiments, control rats show a similar pattern, starting with a mean score of 2 (corresponding to circling to the left) 1 h after MCAO (before reperfusion). The control rats condition worsened during reperfusion in the following 24–34 h and returned to the score of 2 by day 3. Pretreatment with anti-C5 antibody significantly reduced the neuromotor deficit score at 24 and 72 h after MCAO compared to the control group (Fig. 2A). The significant difference at 24 h was due in part to less mortality in the anti-C5 mAb-treated cohort (15.4%) relative to controls (30.8%). However, differences in mortality alone did not reach significance and the mortality peak was similar for all groups, around 30 h post-MCAO (Table 2). On the contrary, differences at 72 h simply reflected the improvement in neuromotor functions because no mortality was recorded at this time. The anti-C5 pretreated group showed at day 3 a mean score of 1, which corresponded to a failure to extend the left forepaw (usually less severe than at 1 h post-MCAO) and straight walking. Initiating C5 inhibition after MCAO also had a beneficial effect on the neuromotor functions and diminished the mean score at 48 and 72 h (Fig. 2B). Consistent with the comparable score at 24 h, no clear difference in mortality was observed in the posttreatment experiment (Table 2). In contrast, the majority of anti-C5 posttreated rats showed straight walking at 72 h as in the anti-C5 pretreated cohort.

2.4. *Infarct size*

Infarct size was determined in serial coronal sections after ischemia contrast staining. The volume of total cerebral ischemic lesion (comprising penumbra and infarct core) was reduced 40% in the anti-C5 mAb-pretreated group relative to controls (Fig. 3A). Significant differences were also observed between the two pretreated cohorts when comparing the areas of ischemic lesion in the corresponding serial coronal planes (Fig. 3B). In the posttreatment experiment, which included more rats per group, significant differences were also achieved (Figs. 3C and D). However, the reduction in ischemic lesion was less dramatic in this case (about 30%). These findings paralleled our observations of the MCAO lesion in H&E-stained samples (data not shown).

2.5. *Extent of edema*

C5 inhibition had the most dramatic effect on the extent of brain edema after MCAO. The extent of edema was reduced 65% in the anti-C5 mAb-pretreated cohort relative to controls (Fig. 4A). In the posttreatment experiment, anti-C5

Table 1 – Physiological parameters

Treatment	Body weight day −1 (g)	Body weight day 1 (g)	Body temperature pre-MCAO (°C)	Body temperature MCAO (°C)	Body temperature reperfusion (°C)
Pretreatment control Ab	215.5 ± 3.7	201.8 ± 3.9	36.2 ± 0.2	35.4 ± 0.3	37.3 ± 0.2
Pretreatment anti-C5 Ab	215.3 ± 3.2	203.7 ± 4.1	36.2 ± 0.2	35.7 ± 0.2	37.7 ± 0.3
Posttreatment control Ab	231.1 ± 1.8	213.6 ± 3.3	36.5 ± 0.2	34.7 ± 0.2	38.1 ± 0.1
Posttreatment anti-C5 Ab	230.7 ± 1.8	214.9 ± 3.5	36.7 ± 0.2	34.6 ± 0.1	38.2 ± 0.1

Body weight was determined the day before and the day after surgery in all MCAO rats. Rectal temperature was also determined, right before surgery, after initiating MCAO and after initiating reperfusion.

mAb led to a 55% reduction (Fig. 4C). We also followed the extent of edema in coronal sections and observed that the MCAO-induced edema is located in the frontal portion of the cerebrum affecting cortex and striatum (Figs. 4B and D). Thus, differences between the control and anti-C5 mAb-treated cohorts were observed at the frontal coronal layers between 2 and 8 mm from the frontal pole (Figs. 4B and D).

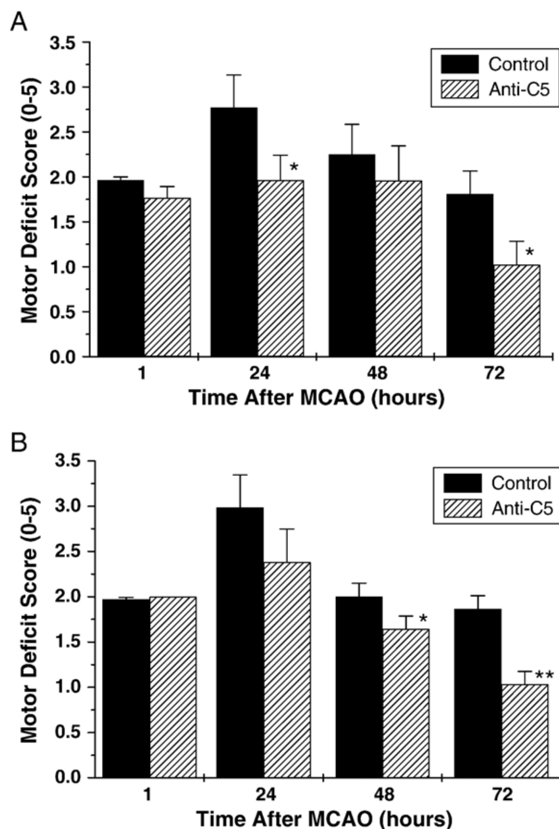

Fig. 2 – Neurological motor deficit. A neurological score was assigned to each rat at 1, 24, 48 and 72 h after MCAO and the mean ± SE is presented for (A) pretreatment experiment (*n* = 13/controls, *n* = 13/anti-C5) and (B) posttreatment experiment (*n* = 16/controls, *n* = 15/anti-C5). We used the standard grading score of 0–5 (0 = normal, 1 = failure to extend left forepaw, 2 = circling to the left, 3 = falling to the left, 4 = depressed consciousness, 5 = death), $^{*}P \leq 0.05$, $^{**}P \leq 0.005$.

2.6. *Histologic examination*

A histological examination of serial cerebrum sections stained with H&E confirmed the infarct lesion in the right cerebral cortex and striatum 3 days after MCAO. Control rats showed a thickened pia mater with extensive immune cellular infiltrate in the penumbra that penetrated the cortex mostly following the perivascular spaces (Fig. 5A). A less intense diffuse infiltrate and some pockets of mononuclear cells were observed in the striatum of control rats (data not shown). Control rats showed a mixed inflammatory infiltrate composed of neutrophils and macrophages, as well as reactive gliosis, degenerated neurons, also known as red neurons (Fig. 5C, insert) and vascular proliferation (Figs. 5A and C). The anti-C5 mAb-pretreated cohort exhibited a similar pattern but had an overall reduction in the amount of immune cellular infiltrate compared to controls (Figs. 5B and D). To evaluate the impact of the anti-C5 mAb pretreatment on the inflammatory cellular infiltrate, we utilized a score system ranging from 0 (no infiltrate) to 4 (massive infiltrate) (Fig. 5E). The mean score of the control group was significantly higher than the mean of the anti-C5

Table 2 – Mortality rate

Treatment	Rats died [a]/ total rats	Percentage mortality (%)	Survival time [b] (hours)
Pretreatment control Ab	4/13	30.8	30.25 ± 3.9
Pretreatment anti-C5 Ab	2/13	15.4	32.3 ± 8.6
Posttreatment control Ab	5/16	31.2	29.4 ± 2.5
Posttreatment anti-C5 Ab	3/15	20	29 ± 0.6

Rats subjected to MCAO were closely monitored during the 3-day study and deaths occurring before harvest were recorded.
[a] Number of rats which died after MCAO before the 72-h harvest.
[b] Survival time since MCAO initiation of rats which died before the 72-h harvest.

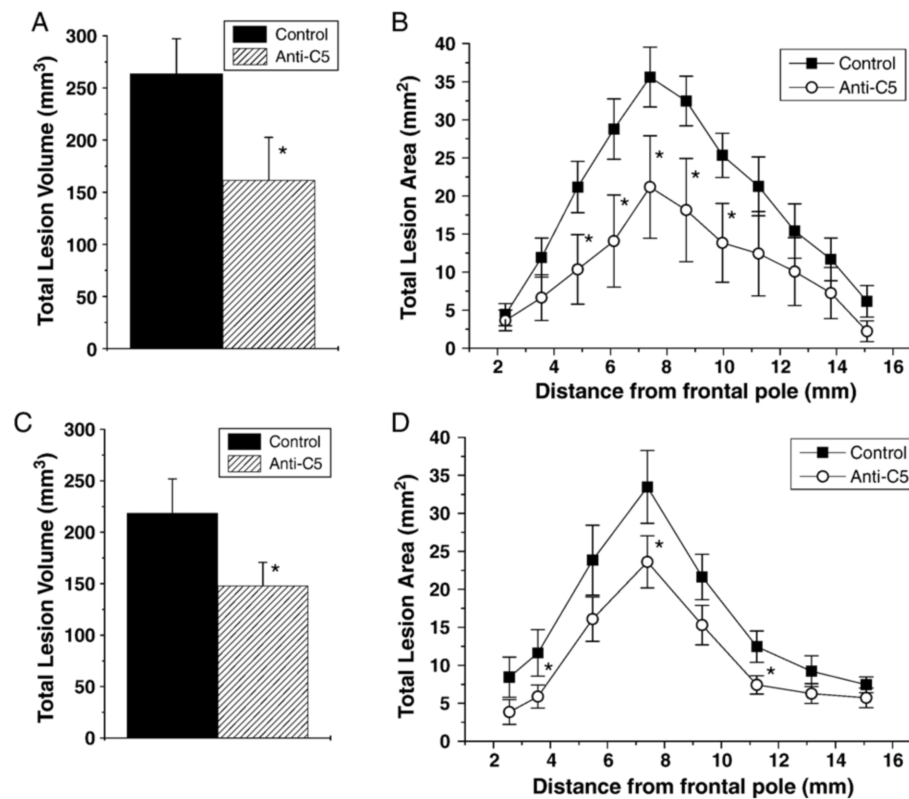

Fig. 3 – Size of cerebral ischemic lesion. It is presented as follows: (A) Total lesion volume in the pretreatment experiment (n = 10/controls, n = 11/anti-C5). (B) Total lesion areas in the pretreatment experiment (n = 8/controls, n = 8/anti-C5). (C) Total lesion volume in the posttreatment experiment (n = 13/controls, n = 13/anti-C5). (D) Total lesion areas in the posttreatment experiment (n = 13/controls, n = 13/anti-C5). $^*P \leq 0.05$.

mAb-pretreated cohort at the 3 different coronal planes analyzed. Moreover, 60% of the anti-C5 mAb-pretreated rats showed an extremely low neutrophilic component. Differences were less apparent in the posttreatment experiment (data not shown).

3. Discussion

This study shows that inhibition of the complement component C5 confers protection from ischemic reperfusion injury in the rat MCAO model. C5-specific inhibition reduced the size of total cerebral ischemic lesion, the extent of edema, the amount of immune cellular infiltrate and the neuromotor deficit. Our results are consistent with the increase in infarction volume observed after administration of the terminal complement component C9 to C9-deficient neonatal rats subjected to common coronary artery ligation and hypoxia (Imm et al., 2002). In fact, sCR1 did not protect in this model (Imm et al., 2002), whereas CVF treatment (which inhibits C3 and downstream complement components) diminished brain tissue damage in MCAO (Figueroa et al., 2005). Therefore, our data are in accordance with our hypothesis that activated C5 components play a critical role in the development of cerebral tissue inflammation after ischemia/reperfusion injury.

Our study indicates that the complement cascade is activated during the ischemia period and that the multiple proinflammatory and harmful effects described for C5a and C5b-9 contribute to the development of severe cerebral tissue inflammation and irreversible tissue damage during the reperfusion period. C5 activation participated in the recruitment of inflammatory cells and promoted vascular permeability as demonstrated by the significant reduction of cerebral inflammation and tissue edema after C5 inhibition. These observations are consistent with C5a being a potent leukocyte chemotaxin and promoter of vascular permeability in different settings, including the blood–brain barrier (Liszewski and Atkinson, 1993; Stahel et al., 1998). Rabbits receiving C5a intracisternally developed meningitis with massive cellular infiltration and brain edema (Stahel et al., 1998). Moreover, C5a mediates local tissue damage by inducing the release of proteases and free radicals by neutrophils and the secretion of proinflammatory cytokines such as TNF-α by monocytes/macrophages (Liszewski and Atkinson, 1993; Stahel et al., 1998). Activation of microglia and astrocytes by C5a causes further neurotoxicity and blood–brain barrier damage after brain trauma (Stahel et al., 1998). In fact, expression of the C5a receptor is upregulated in the affected brain 24 h after MCAO (Barnum et al., 2002), involving endothelium, myeloid cells and reactive astrocytes (Van Beek et al., 2000). Sublytic generation of C5b-9 is also proinflammatory as it is known to induce endothelial cell

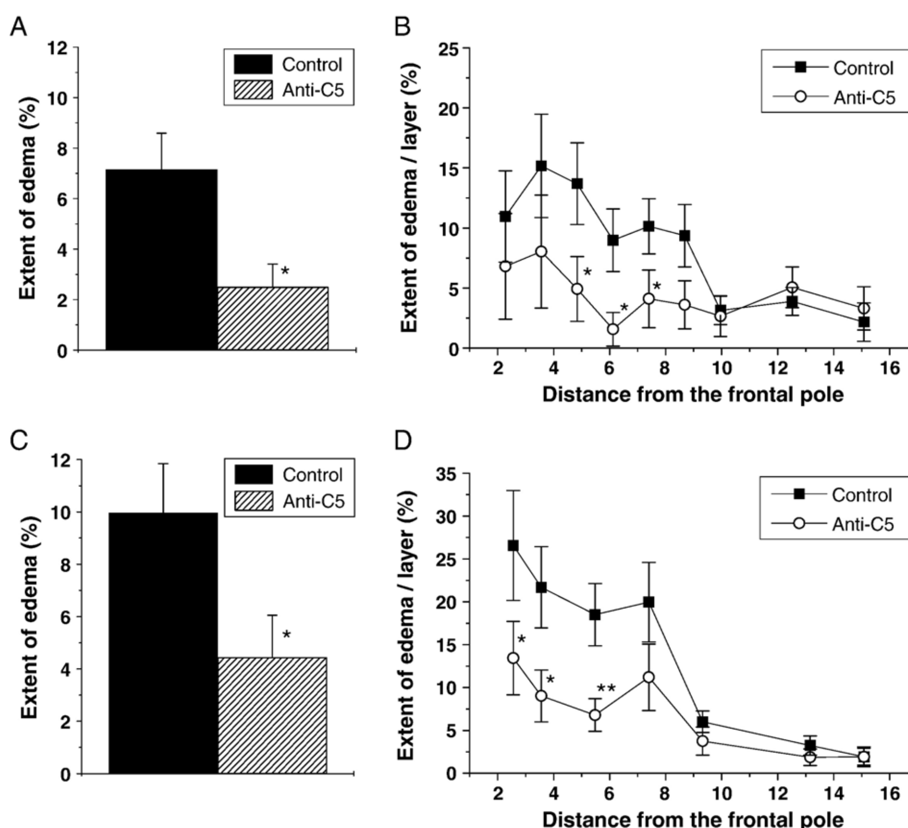

Fig. 4 – Extent of edema. It is presented as follows: (A) percentage extent of edema in the pretreatment experiment ($n = 9$/controls, $n = 11$/anti-C5). (B) Extent of edema at specific coronal planes in the pretreatment experiment ($n = 7$/controls, $n = 8$/anti-C5). (C) Percentage extent of edema in the posttreatment experiment ($n = 12$/controls, $n = 12$/anti-C5). (D) Extent of edema at specific coronal planes in the posttreatment experiment ($n = 12$/controls, $n = 12$/anti-C5). $^{*}P \le 0.05$, $^{**}P \le 0.005$.

activation with upregulation of adhesion molecules and secretion of cytokines and chemokines (Bhole and Stahl, 2003). Finally, C5b-9 may contribute to tissue injury as the membrane attack complex causes cell lysis and cell death, which is associated with necrosis (Seelen et al., 2005). This is probably a major complement-mediated mechanism of tissue damage in cerebral ischemia. Thus, inhibiting the generation of the two potent mediators C5a and C5b-9 may result in the reduction of the lesion and the extent of edema and the subsequent improvement of neuromotor deficit and mortality.

Pre-ischemic C5 inhibition (pretreatment experiment) led to the most favorable outcome compared to treatment after MCAO. Together with the marked reduction in infarct volume, this suggested the importance of C5 activation during ischemia. Moreover, local activation of the complement cascade also resulted in the recruitment of inflammatory cells and release of multiple inflammation mediators during the reperfusion period (at which we continued to treat) with subsequent irreversible neural cell damage (larger lesion in the controls). In agreement with a deleterious role of complement activation during reperfusion is the fact that SC5b-9 is increased in serum of patients at 3 and 7 days after ischemic stroke (Pedersen et al., 2004). Furthermore, C5a receptor expression is upregulated from 12 h to 3 days after MCAO (Van Beek et al., 2000; Barnum et al., 2002). The pharmacodynamics of systemic C5 inhibition may have nevertheless contributed to the reduced efficacy in the posttreatment cohort, as systemic C5 inhibition was achieved approximately within 1 h after initial i.v. administration and cerebral C5 inhibition may be achieved at an even later time point. In other models of organ ischemia/reperfusion injury, C5-specific inhibition had a similar or more dramatic impact (Vakeva et al., 1998; De Vries et al., 2003). C5 inhibition reduced the infarct size in rats subjected to 4-h myocardial ischemia without reperfusion (Vakeva et al., 1998), whereas inhibition of C5 just before reperfusion also prevented inflammation and renal dysfunction in a renal model of ischemia/reperfusion (De Vries et al., 2003). Differences between these models are likely due to multiple factors, including the antibody specificity and the animal species or strain. Moreover, there are unique characteristics in the MCAO model that may influence our results such as sensitivity to ischemia by neuron cells and the presence of the blood–brain barrier. Further studies with radioisotope-labeled anti-C5 mAb may address the kinetics of cerebral C5 inhibition.

Results from this work encourage further research in understanding the role of activated complement components in cerebral ischemia/reperfusion injury. Our results from the posttreatment experiment suggest that other inflammatory mediators are likely to play an important

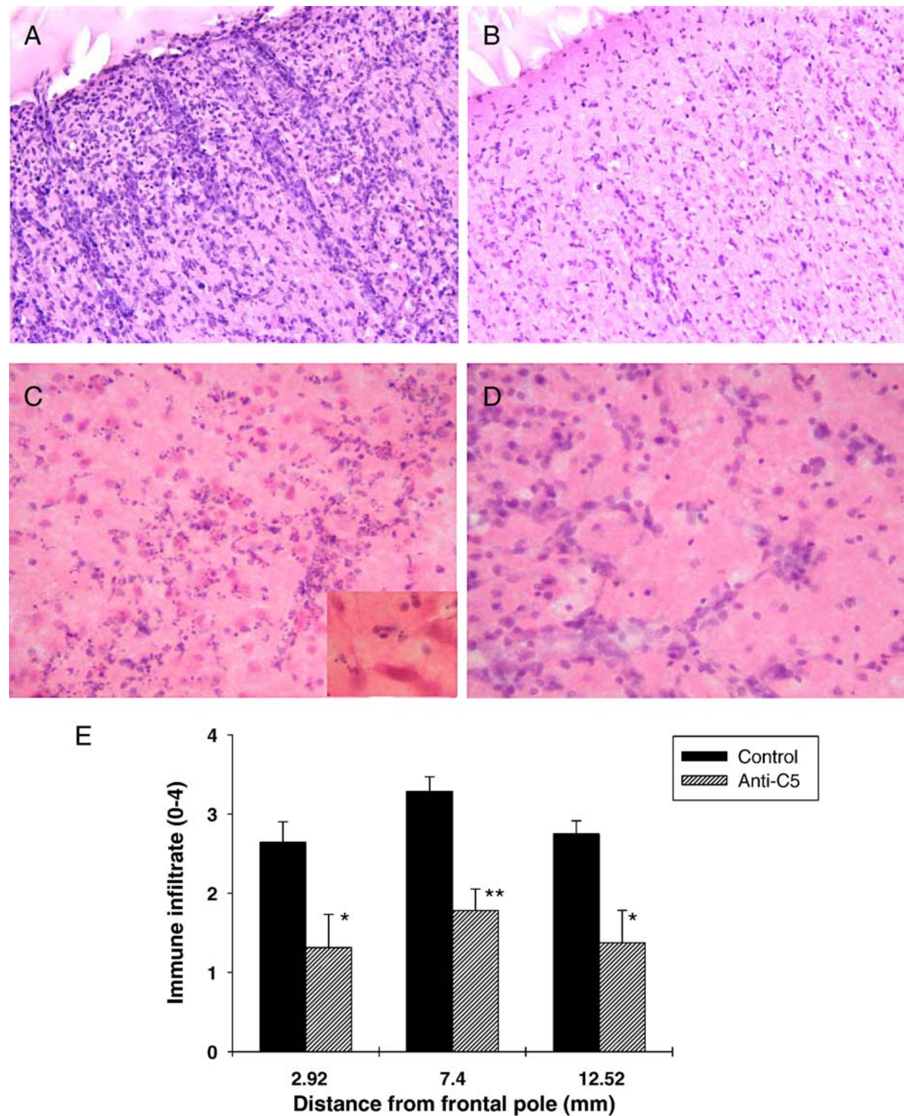

Fig. 5 – Histopathological evaluation. H&E staining of the right hemisphere after MCAO is shown (A–D). (A and C) Cortex of a control pretreated rat. (B and D) Cortex of an anti-C5 mAb-pretreated rat. The original magnification was × 200 (A, B) and × 400 (C, D). (E) Semiquantification of immune cellular infiltrate at 3 different coronal planes. A score paralleling the amount of immune cellular infiltrate was assigned to each sample evaluated and the mean ± SE is presented for the pretreatment experiment (n = 7/controls, n = 8/anti-C5). Score system from 0 to 4 (0 = no infiltrate, 1 = mild infiltrate, 2 = moderate infiltrate, 3 = marked infiltrate, 4 = massive infiltrate), $^{*}P \leq 0.05$, $^{**}P \leq 0.005$.

role in the downstream inflammatory cascade once C5 is activated during ischemia. The interactions of activated complement components with platelets and the clotting cascade deserve special attention. Combinatorial studies such as treatment of anti-C5 mAb with thrombolytics (tPA activates complement) or platelet inhibitors may provide information of the relative importance of activated C5 components versus other key inflammatory processes during cerebral inflammation.

In summary, activated C5 complement components play an important role in cerebral tissue inflammation resulting from ischemia/reperfusion injury. C5 may be an attractive therapeutic target because C5 inhibition preserves the opsonin functions of C3 necessary to fight infection, clear immunocomplexes and maintain self-tolerance (Liszewski and Atkinson, 1993; Carroll, 1998).

4. Experimental procedure

4.1. Antibodies

To inhibit C5 systemically, we used the mouse monoclonal antibody 18A10.62 that blocks rat C5 cleavage (Vakeva et al., 1998). The mouse monoclonal antibody HFN 7.1 (ATCC, Manassas, VA) with specificity for human fibronectin (does not cross-react with the rodent counterparts) was used as irrelevant antibody. HFN7.1 is an IgG1 isotype, whereas

18A10.62 is an IgG2b antibody. The antibodies were produced by ascites. Briefly, hybridoma cells were cultured in DMEM (Mediatech Inc., Herndon, VA)/10% FCS (Hyclone, Logan, UT), washed thoroughly in DPBS (Mediatech Inc.) and injected i.p. (3–5 × 10^6 cells/mouse) into CB17 SCID mice (Jackson Laboratories, Bar Harbor, ME, and Charles River, Malvern, PA). Antibodies were purified from ascites fluid (collected in aseptic conditions) by protein A-Sepharose chromatography (Pharmacia Biotech Inc., Piscataway, NJ) and dialyzed against DPBS. Antibody concentration was determined by spectrophotometry at 280 nm using a coefficient of 0.735. All samples were endotoxin free as tested with a limulus-amebocyte-lysate test (Biowhittaker, Walkersville, MD).

4.2. *Functional assays*

We assessed the ability of 18A10.62 to block C5a in a neutrophil migration assay in which zymosan-activated serum (ZAS) is used to induce chemotaxis. This assay has been previously described in detail (Vakeva et al., 1998). Briefly, 50% rat serum (Sigma, diluted in HBSS) was activated with zymosan (10 mg/ml, Sigma) for 1 h at 37 °C in the presence or absence of 18A10.62 (100 μg/ml). Rat neutrophils were isolated from rat blood with a commercial kit (Cardinal Associates Inc, NM) and loaded in a central well of a gel made of 0.5% agarose and 0.25% gelatin. Chemotactic agents and controls were placed in separate wells equidistant to the central well and migration was assessed after a 2- to 3-h incubation in a humidified chamber at 37 °C.

4.3. *Pharmacodynamic study*

Wistar male rats purchased from Harlan (Indianapolis, IN) were used throughout this study. Several pharmacodynamic experiments were carried out to establish the route and dosing. The i.v. injection of 20 mg/kg 18A10.62 gave the fastest C5 inhibition and was chosen to initiate treatment. This was followed by i.p. injections at 10 mg/kg that were convenient for repeated treatment and sufficient to maintain inhibition. C5 complement activity was determined in rat serum with a hemolytic assay using sensitized chicken red blood cells in the presence of C5-depleted human serum (Vakeva et al., 1998). Serum glucose was also determined in rats treated with HFN 7.1 or 18A10.62 using the blood Glucose Monitor (The Rite Aid Corporation, Harrisburg, PA).

4.4. *MCAO model*

Wistar male rats with body weights ranging between 200 and 240 g were used for the MCAO study. Rats were distributed equally between experimental groups to match their body weight. Anesthesia was applied by inhalation with 3% isoflurane for induction and around 2% for maintenance during surgery with continuous monitoring. During anesthesia and recovery, rats were laid on a heating pad kept at 37 °C to maintain constant body temperature. Right MCAO was attained following the intraluminal suture model (He et al., 2000). MCAO was maintained for 90 min followed by 3 days of reperfusion at which time the animals were sacrificed for brain injury evaluation. Because the anti-C5 mAb 18A10.62 has approximately an 8-h half-life, an initial i.v. injection was selected to achieve rapid systemic C5 inhibition followed by subsequent i.p. injections to maintain significant C5 inhibition during the 3 days of reperfusion. In the pretreatment cohorts, rats were equally dosed with either irrelevant or anti-C5 antibody (n = 13/group) with an initial i.v. injection of 20 mg/kg 1 h prior to MCAO, followed by 7 i.p. injections (10 mg/kg) distributed along the reperfusion time (1 i.p. injection 6 h after the first injection and 3 injections per day in the following two days). The control and anti-C5 mAb treatments were intercalated to avoid bias and the same order was maintained throughout the study. In the posttreatment cohorts, which comprised 16 MCAO rats treated with irrelevant antibody and 15 MCAO rats with anti-C5 antibody, the first i.v. injection (20 mg/kg) was given 5 min after initiating MCAO and the following 7 i.p. injections (10 mg/kg) were given as described for the pretreatment study. Previous experience in our laboratory and in others has shown no major effect of using an irrelevant antibody versus a PBS-control group (data not shown) (Furuya et al., 2001); we therefore did not include this type of control to limit the number of rats utilized. Rectal temperature was measured just before surgery, after MCAO and after initiating reperfusion (always under anesthesia). Body weight was determined the day before and the day after surgery. All experimental rats were closely monitored during the length of the study. The neurological motor deficit was determined daily with a standard grading score of 0 to 5 (0 = normal, 1 = failure to extend left forepaw, 2 = circling to the left, 3 = falling to the left, 4 = depressed consciousness, 5 = death) and the mean was calculated without cumulative mortality (death was accounted only once). Animals dying between 26 and 35 h after MCAO were included in the 24-h time point with the highest score, whereas 2 animals which died after that (at 40 and 41 h post-MCAO) were included in the 48-h score. All experiments were approved by the Institutional Animal Care and Use Committee.

4.5. *Tissue processing and histological analyses*

Animals were deeply anesthetized with 4% isoflurane and fixed by transcardial perfusion (first with DPBS followed by 10% phosphate-buffered formalin). In the pretreatment cohorts, brains were retrieved from the skull following perfusion and immersed in fixative. In the posttreatment study, the skulls were placed in fixative and retrieved 1–2 days after. Some brains were also collected from the rats, which died during the study while we were present. In those few cases, the skull was placed in fixative without perfusion. The brains were sent to NeuroScience Associates (Knoxville, TN) for mounting and staining in the MultiBrain™ block system.

Infarct size was determined after Ischemia Contrast Staining performed by NeuroScience Associates and image analysis using Image-Pro[R] Plus software (Media Cybernetics, Silver Spring, MD). Ischemia contrast staining discerns between ischemic tissue that comprises the infarction core and the penumbra (stains dark to light gray) and normal tissue (stains black). The program was calibrated for size (pixels to mm) and detection of staining intensity prior to the analysis with the same samples to be evaluated. The first image analysis comprised 8 rats HFN 7.1-pretreated and 8 18A10.62-

pretreated rats (1 block) and involved the analysis of 11 coronal sections. It contained one control, which died at 26 h post-MCAO. In the second analysis, we assessed 8 coronal sections for the remaining 32 brain samples (2 blocks). It included one posttreatment control and one 18A10.62-post-treated sample collected before 72 h post-MCAO without perfusion. The lesion volume was calculated in all cases following the same system as previously described (He et al., 2000). The extent of edema was also calculated as a percentage by assessing the difference in volume between the affected and unaffected brain hemispheres as described (Aspey et al., 2000). We did not include the samples that were not perfused for this analysis. We also applied a similar formula to determine the extent of edema at different coronal layers: ((Right area − Left area)/Left area) × 100.

A histologic examination was also conducted of serial cerebrum sections (adjacent to those used for Ischemia Contrast Staining) stained with H&E. A score system was developed to semiquantify the inflammatory cellular infiltrate in samples of the pretreatment study (block 1). Each sample (except the control which died at 26 h) was evaluated at 3 different coronal planes that corresponded to the lesion frontal and caudal edges at 2.92 and 12.52 mm from the frontal pole, respectively, and the center of the lesion (7.4 mm from frontal pole). The scores paralleled the amount of immune cellular infiltrate from 0 to 4 (0 = no infiltrate, 1 = mild infiltrate, 2 = moderate infiltrate, 3 = marked infiltrate, 4 = massive infiltrate). A score was assigned to each sample and the mean was calculated.

4.6. *Statistical analysis*

Values are shown as mean ± SE. Unless stated, differences were assessed using the Student's *t* test and considered statistically significant at $P \leq 0.05$. One-way ANOVA and Dunn's test as a post hoc analysis was used in the chemotaxis experiment. Only in the case of determining the probability of survival in the various cohorts was a log-rank test used.

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REFERENCES

Akita, N., Nakase, H., Kaido, T., Kanemoto, Y., Sakaki, T., 2003. Protective effect of C1 esterase inhibitor on reperfusion injury in the rat middle cerebral artery occlusion model. Neurosurgery 52, 395–401.

Aspey, B.S., Taylor, F.L., Terruli, M., Harrison, M.J., 2000. Temporary middle cerebral artery occlusion in the rat: consistent protocol for a model of stroke and reperfusion. Neuropathol. Appl. Neurobiol. 26, 232–242.

Barnum, S.R., Ames, R.S., Maycox, P.R., Hadingham, S.J., Meakin, J., Harrison, D., Parsons, A.A., 2002. Expression of the complement C3a and C5a receptors after permanent focal ischemia: an alternative interpretation. Glia 38, 169–173.

Bhole, D., Stahl, G.L., 2003. Therapeutic potential of targeting the complement cascade in critical care medicine. Crit. Care Med. 31, S97–S104.

Cai, S., Davis III, A.E., 2003. Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothelial-leukocyte adhesion. J. Immunol. 171, 4786–4791.

Carroll, M.C., 1998. The role of complement and complement receptors in induction and regulation of immunity. Annu. Rev. Immunol. 16, 545–568.

Chen, H., Chopp, M., Zhang, Z.G., Garcia, J.H., 1992. The effect of hypothermia on transient middle cerebral artery occlusion in the rat. J. Cereb. Blood Flow Metab. 12, 621–628.

De Simoni, M.G., Storini, C., Barba, M., Catapano, L., Arabia, A.M., Rossi, E., Bergamaschini, L., 2003. Neuroprotection by complement (C1) inhibitor in mouse transient brain ischemia. J. Cereb. Blood Flow Metab. 23, 232–239.

De Vries, B., Matthijsen, R.A., Wolfs, T.G., Van Bijnen, A.A., Heeringa, P., Buurman, W.A., 2003. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. Transplantation 75, 375–382.

Figueroa, E., Gordon, L.E., Feldhoff, P.W., Lassiter, H.A., 2005. The administration of cobra venom factor reduces post-ischemic cerebral injury in adult and neonatal rats. Neurosci. Lett. 380, 48–53.

Fisher, M., 2002. Developing therapy for acute ischemic stroke. Therapie 57, 564–568.

Furuya, K., Takeda, H., Azhar, S., McCarron, R.M., Chen, Y., Ruetzler, C.A., Wolcott, K.M., DeGraba, T.J., Rothlein, R., Hugli, T.E., del Zoppo, G.J., Hallenbeck, J.M., 2001. Examination of several potential mechanisms for the negative outcome in a clinical stroke trial of enlimomab, a murine anti-human intercellular adhesion molecule-1 antibody: a bedside-to-bench study. Stroke 32, 2665–2674.

Ghiran, I., Barbashov, S.F., Klickstein, L.B., Tas, S.W., Jensenius, J.C., Nicholson-Weller, A., 2000. Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J. Exp. Med. 192, 1797–1808.

He, Z., Yang, S.H., Naritomi, H., Yamawaki, T., Liu, Q., King, M.A., Day, A.L., Simpkins, J.W., 2000. Definition of the anterior choroidal artery territory in rats using intraluminal occluding technique. J. Neurol. Sci. 182, 16–28.

Heimann, A., Takeshima, T., Horstick, G., Kempski, O., 1999. C1-esterase inhibitor reduces infarct volume after cortical vein occlusion. Brain Res. 838, 210–213.

Huang, J., Kim, L.J., Mealey, R., Marsh Jr., H.C., Zhang, Y., Tenner, A. J., Connolly Jr., E.S., Pinsky, D.J., 1999. Neuronal protection in stroke by an sLex-glycosylated complement inhibitory protein. Science 285, 595–599.

Imm, M.D., Feldhoff, P.W., Feldhoff, R.C., Lassiter, H.A., 2002. The administration of complement component C9 augments post-ischemic cerebral infarction volume in neonatal rats. Neurosci. Lett. 325, 175–178.

Jiang, H., Wagner, E., Zhang, H., Frank, M.M., 2001. Complement 1 inhibitor is a regulator of the alternative complement pathway. J. Exp. Med. 194, 1609–1616.

Lindsberg, P.J., Ohman, J., Lehto, T., Karjalainen-Lindsberg, M.L., Paetau, A., Wuorimaa, T., Carpen, O., Kaste, M., Meri, S., 1996. Complement activation in the central nervous system following blood–brain barrier damage in man. Ann. Neurol. 40, 587–596.

Liszewski, M.K., Atkinson, J., 1993. The complement system. In: Paul, W.E. (Ed.), Fundamental Immunology. Raven Press, New York, pp. 917–939.

Pedersen, E.D., Waje-Andreassen, U., Vedeler, C.A., Aamodt, G., Mollnes, T.E., 2004. Systemic complement activation following human acute ischemic stroke. Clin. Exp. Immunol. 137, 117–122.
Seelen, M.A., Roos, A., Daha, M.R., 2005. Role of complement in innate and autoimmunity. J. Nephrol. 18, 642–653.
Stahel, P.F., Morganti-Kossmann, M.C., Kossmann, T., 1998. The role of the complement system in traumatic brain injury. Brain Res. Rev. 27, 243–256.
Vakeva, A.P., Agah, A., Rollins, S.A., Matis, L.A., Li, L., Stahl, G.L., 1998. Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: role of the terminal complement components and inhibition by anti-C5 therapy. Circulation 97, 2259–2267.
Van Beek, J., Bernaudin, M., Petit, E., Gasque, P., Nouvelot, A., MacKenzie, E.T., Fontaine, M., 2000. Expression of receptors for complement anaphylatoxins C3a and C5a following permanent focal cerebral ischemia in the mouse. Exp. Neurol. 161, 373–382.
Wolf, P.A., Clagett, G.P., Easton, J.D., Goldstein, L.B., Gorelick, P.B., Kelly-Hayes, M., Sacco, R.L., Whisnant, J.P., 1999. Preventing ischemic stroke in patients with prior stroke and transient ischemic attack: a statement for healthcare professionals from the Stroke Council of the American Heart Association. Stroke 30, 1991–1994.