

CD59 Efficiently Protects Human NT2-N Neurons Against Complement-mediated Damage

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Abstract

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The complement regulatory protein CD59 controls cell survival by the inhibition of C5b-9 formation on the cell membrane. Loss of CD59 increases the susceptibility of cells to complement-mediated damage and lysis. Deposition of IgM can induce complement activation with subsequent cell death. We have previously demonstrated the presence of CD59 on human NT2-N neurons. In this study, we investigated the functional role of CD59 for NT2-N cell survival after IgM-mediated complement activation. Complement activation was induced on NT2-N neurons with human serum following incubation with the IgM monoclonal antibody A2B5 reacting with a neuronal cell membrane epitope. Deposition of C1q and C5b-9 was detected on the cell membrane and sC5b-9 in the culture supernatant. Specific inhibition of complement was obtained by the C3 inhibitor compstatin, and by anti-C5/C5a MoAb. CD59 was blocked by the MoAb BRIC 229. Membrane damage of propidium iodide-stained NT2-N cells was confirmed by immunofluorescence microscopy and degeneration of neuronal processes was shown with crystal violet staining. A2B5, but not the irrelevant control IgM antibody, induced complement activation on NT2-N neurons after incubation with a human serum, as detected by the deposition of C1q. A marked membrane deposition of C5b-9 on NT2-N neurons with accompanying cell death and axonal degeneration was found after the blocking of CD59 with MoAb BRIC 229 but not with an isotype-matched control antibody. Compstatin and anti-C5 monoclonal antibodies which blocked C5 activation efficiently inhibited complement activation. In conclusion, CD59 is essential for protecting human NT2-N neurons against complement-mediated damage, which is known to occur in a number of clinical conditions including stroke.

Introduction

One of the crucial effects of complement activation is the formation of the terminal C5b-9 complement complex (TCC), which occurs in two forms: either inserted as C5b-9 in a membrane (the membrane attack complex) or formed in the fluid phase as the soluble, inactive sC5b-9, which is used as an indicator of complement activation. The insertion of C5b-9 into a cell membrane creates a pore and leads to either sub-lytic effects of cell activation or to membrane damage and cell death. C5b-9 is suggested to play a significant role in neuro-inflammation as a result of the clearance of apoptotic debris, phagocytosis and neuronal cell lysis [1, 2]. Furthermore, C5b-9 deposition was shown in the brain parenchyma after stroke [3],

and an increase of sC5b-9 was found in the systemic circulation in patients with cerebral infarction [4]. Experimental studies support the terminal sequence of complement as important for the pathogenesis of stroke, as inhibition of C5 resulted in 40% reduction of infarction volume and 65% reduction of brain edema [5].

CD59 is known to protect cells against complement-mediated lysis by its ability to inhibit C5b-9 insertion at the stage of C8 and C9. Co-localization of beta-amyloid peptide and C5b-9 was found in human Alzheimer's brain where this peptide induced neuronal cell death following treatment with anti-CD59 [6]. The results of this study suggest that beta-amyloid peptide activates complement to induce C5b-9, and a deficiency of endogenous CD59 may increase the vulnerability of neurons to complement-

mediated cytotoxicity. In general, loss of complement regulators causes susceptibility of neurons to complement-mediated cell lysis and neuronal death [2, 7, 8].

IgM and complement have been suggested to play an important role in the pathogenesis of ischaemia-reperfusion injury (IRI) [9]. Furthermore, IgM which is traditionally related to classical pathway activation, has in addition recently been shown to activate the lectin pathway [10]. Whereas the mechanism of complement activation in stroke is largely unknown, a focal axonal damage in CNS was demonstrated in the complement- and IgM-mediated rat model of multiple sclerosis [11].

In the present study, we used the human NT2-N cell line which is phenotypically very similar to neurons [12–14] and already a well-established surrogate for human neurons [15–17]. In a recent study, we demonstrated substantial amounts of CD59 on NT2-N neurons [18]. In the present study, we investigated the functional role of CD59 for these cells undergoing complement attack with an anti-neuronal IgM antibody as a trigger of activation.

Materials and methods

Human serum. Human serum was collected from 10 AB-positive healthy volunteers, pooled, aliquoted, frozen and stored at -70°C . Heat-inactivated serum was obtained by incubating the serum in a water bath at 56°C for 30 min.

Complement inhibitors. A total of $25\ \mu\text{M}/\text{ml}$ of the inhibitory cyclic peptide compstatin, Ac-ICV(1-methyl-W) QDWG A HRCT-CONH₂, was used to block complement activation of all three initial activation pathways at the common C3 convertase level [19]. The monoclonal anti-C5/C5a antibody (clone 137–26) blocks C5a by binding to the C5a moiety on C5 without interfering with C5 cleavage [20]. The monoclonal anti-C5 antibody (clone 137–76) binds to C5 and prevents its cleavage, thereby blocking the formation of both C5a and C5b–9 [21].

Cell cultures. NT2-N neurons, used in this study, originated from a human teratocarcinoma-derived cell line – Ntera2/clone D1, which differentiate into post-mitotic neurons (NT2-N neurons) upon treatment with retinoic acid [22, 23]. NT2-N neurons were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, as previously described in these studies.

The cells were grown in 96-well cell culture plates (Costar, Corning, NY, USA) with 2.4×10^5 cells per well. Determined microscopically, the culture consists of 95–98% differentiated neurons.

Experimental model. Supernatants were collected after each step of incubation, centrifuged and immediately frozen at -70°C . The experiments consist of several steps of incubation. The cells were kept in a MiniGalaxy Incu-

bator (RS Biothech, Ayrshire, Scotland) with humidified 5% CO₂ and 1% O₂ at 37°C during all incubations. The cultivated NT2-N neurons in 96-well ELISA plate were first incubated with $25\ \mu\text{g}/\text{ml}$ mouse anti-human CD59 (Clone BRIC229, National Blood Service, Bristol, UK) or an IgG2b isotype control (R&D system, Minneapolis, MN, USA) for 30 min. Then the cells were incubated with $5\ \mu\text{g}/\text{ml}$ of the IgM mouse anti-human neuronal membrane marker A2B5 (Chemicon International, Temecula, CA, USA) or an IgM isotype control (Southern Biotech, Birmingham, AL, USA) for 30 min. Finally, the cells were incubated during 4 h with 50% serum, 50% heat-inactivated serum or 50% serum containing one of the following complement inhibitors: compstatin, MoAb anti-C5/C5a (clone 137–26) or MoAb anti-C5 (clone 137–76).

Cellular enzyme-linked immunosorbent assay. After incubating the NT2-N neurons in 96-well ELISA plates, the cells were fixed with 0.05% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) and washed twice with PBS. They were then incubated with different primary antibodies with the following detection systems: (a) mouse anti-human MoAb C1q (Quidel, San Diego, CA, USA), mouse anti-human MoAb A2B5 (Chemicon International) or mouse anti-human IgM (Southern Biotech) as primary antibodies followed by anti-mouse IgG (Fab)₂, horseradish peroxidase (HRP) (Amersham, Buckinghamshire, UK) or goat anti-mouse IgM-HRP (Southern Biotech); (b) mouse anti-human C5b–9 (clone aE11, Diatec, Oslo, Norway) as primary antibody followed by biotin-conjugated anti-mouse IgG2a (BD Pharmingen, Franklin Lakes, NJ, USA), and finally with Streptavidin HRP (Amersham). In both systems cells were incubated on a shaker for 60 min at 20°C and washed with PBS between all steps. *O*-phenylenediamine (Sigma-Aldrich) in citrate buffer was used as peroxidase substrate solution. The colour reaction was stopped with $100\ \mu\text{l}$ 1 M HCL per well and recorded with an MRX ELISA Reader (Dynatec Laboratories, Alexandria, VA, USA) at 490 nm. The cells were subsequently stained with 0.05% crystal violet in 10 min and a nuclear stain eluted in 33% acetic acid before the reading of optical density (OD) at 540 nm. Experiments were performed in triplicates and OD ratios were corrected for the number of cells present in each well. Isotype-matched antibodies in equal concentrations served as negative controls.

Enzyme-linked immunosorbent assay. The soluble terminal C5b–9 complement complex (sC5b–9) was measured principally as described previously using an ELISA developed in our laboratory [24]. This assay is based on a monoclonal antibody (aE11) specific for a neoepitope, which is exposed when C9 is incorporated into the TCC. The results are given in arbitrary units (AU)/ml, related to a standard of zymosan activated serum, defined to contain 1000 AU/ml. The original assay was slightly

modified, giving a reference range of <1.0 AU/ml. The modification was made in the detection step with the replacement of the polyclonal anti-C5 antibody with a monoclonal biotinylated anti-C6 antibody (clone 9C4, produced in our own laboratory), and subsequent enzyme-linked avidin, giving a substantially lower background than in the original assay [25].

Immunofluorescence microscopy. At the end of the experiments, the cells were incubated with 10 $\mu\text{g}/100\ \mu\text{l}$ per well propidium iodide (Sigma-Aldrich) for 10 min at 37 °C and analysed in the inverted Zeiss fluorescence microscope (Zeiss, Altlussheim, Germany) with a rhodamine filter. PI binds to double-stranded DNA and can only cross the plasma membrane of non-viable cells. The Zeiss Axio Camera (Zeiss) with the computer software MetaSystem ISIS 4.4.25, In Situ Imaging, was used to acquire pictures.

Results

IgM-mediated complement activation on NT2-N neurons

Incubation of the NTN-2 neurons with the IgM antibody A2B5, reacting with a human neuronal marker, led to a significant IgM deposition compared with the control IgM antibody (Fig. 1, left panel). Substantial C1q deposition on the cells exterior was observed following incubation with normal human serum, which was not seen using the control antibody (Fig. 1 right panel). The

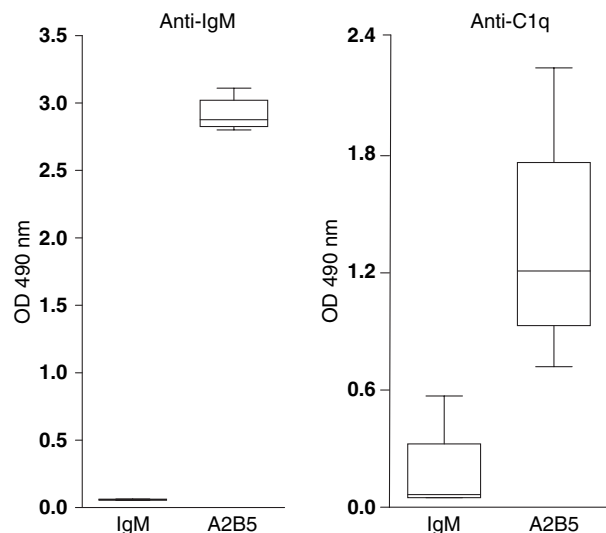


Figure 1 Expression of the neuronal membrane marker A2B5 and IgM-mediated complement activation on NT2-N neurons. Left panel: the deposition of IgM MoAb A2B5 (right) on NT2-N neurons compared with an IgM control MoAb (left) ($n = 6$). Right panel: C1q deposition on NT2-N neurons after incubation with MoAb A2B5 (right) or a control IgM (left) followed by 50% normal human serum ($n = 4$). Data are shown as box and whiskers plots with medians, and lower and upper quartiles.

data indicate that the IgM antibody A2B5 was able to induce specific *in situ* complement activation on the membrane of NT2-N cells.

Terminal complement activation on NT2-N neurons

A modest deposition of C5b-9 on the NT2-N neurons was observed using the IgM A2B5 antibody followed by human serum, whereas the control IgM antibody showed values identical to the medium background (Fig. 2). Incubation with the anti-CD59 neutralizing antibody, prior to the addition of A2B5 antibody and serum, substantially increased the deposition of C5b-9 (Fig. 2, left panel). Heat inactivation of the serum completely abolished the C5b-9 deposition on NT2-N neurons (Fig. 2, left panel).

Terminal complement activation in the NT2-N neurons culture supernatant

Consistent with the spontaneous complement activation occurring in serum in the fluid phase, a modest formation of the sC5b-9 was observed when control antibodies and serum was added (Fig. 2, right panel). A marked increase was, however, seen when MoAb A2B5 was supplied. The increase was substantially enhanced when neutralizing anti-CD59 was added. The formation of sC5b-9 was completely abolished when the serum was heat inactivated (Fig. 2, right panel).

The effect of specific complement inhibition on C5b-9 deposition on NT2-N neurons

When the complement activation was induced by MoAb A2B5, the deposition of C5b-9 on NT2-N neurons was markedly reduced by using the specific C3 inhibitor compstatin (Fig. 3, left panel). The C5b-9 deposition was completely abolished by the MoAb 137-76 which blocks the cleavage of C5 (Fig. 4). By contrast, MoAb 137-26, which neutralizes C5a, but does not prevent C5b-9 formation, had no blocking effect on the deposition of C5b-9 (Fig. 3, left panel).

The effect of specific complement inhibition on sC5b-9 formation in the fluid phase

The formation of sC5b-9 in the fluid phase, induced by MoAb A2B5, was efficiently inhibited by compstatin and the C5 blocking antibody 137-76, whereas the C5a neutralizing antibody 137-26 had no effect (Fig. 3, right panel).

Cell death

When CD59 was neutralized and the cells were incubated with the A2B5 antibody and serum, membrane

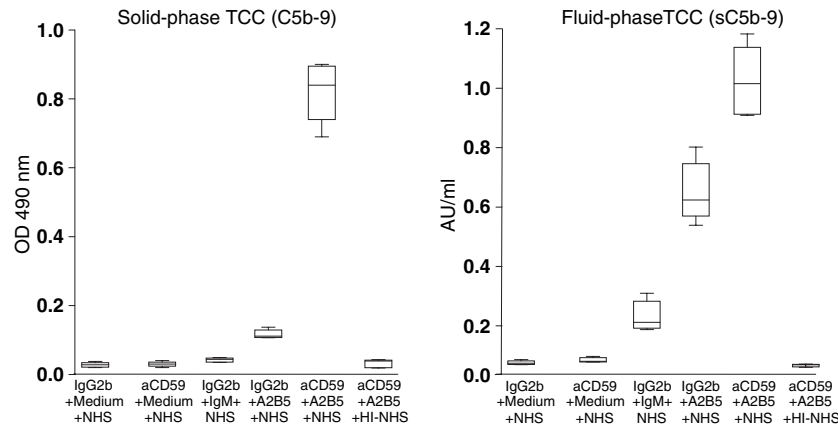


Figure 2 Terminal complement activation by NT2-N neurons. Left panel: substantial deposition of C5b-9 on NT2-N neurons after incubation with the neutralizing anti-CD59 MoAb, followed by MoAb A2B5 and 50% normal human serum (NHS). A modest deposition of C5b-9 was obtained when the cells were incubated with A2B5 and an isotype-matched control antibody (IgG2b) instead of anti-CD59. No significant deposition was seen when an IgM control antibody was used instead of MoAb A2B5 or when the IgM antibodies were replaced by medium. A2B5-induced C5b-9 deposition was completely abolished when heat-inactivated serum (HI-NHS) was used. Data are shown as box and whiskers plots with medians, and lower and upper quartiles ($n = 5$). Right panel: a modest formation of the soluble C5b-9 complex (sC5b-9) in culture supernatants from NT2-N neurons was observed after incubation with control antibodies (IgG2 and IgM) and 50% normal human serum (NHS). sC5b-9 increased markedly after incubation with MoAb A2B5 and further increased when neutralizing anti-CD59 was added. The formation of sC5b-9 was completely abolished when the serum was heat inactivated (HI-NHS). Data are shown as box plots with median and 25 and 75 percentiles ($n = 6$).

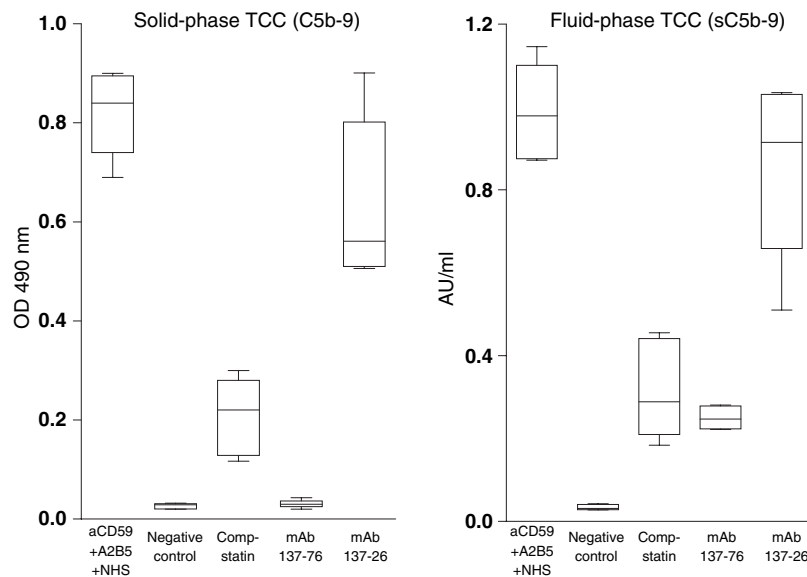


Figure 3 The effect of specific complement inhibition on terminal complement activation by NT2-N neurons. Left panel: the A2B5-induced deposition of C5b-9 on NT2-N neurons was markedly reduced by the specific C3 inhibitor compstatin and was completely abolished by the C5 blocking MoAb 137-76. The MoAb 137-26, neutralizing C5a, but not inhibiting C5b-9 formation, had no effect. NHS, normal human serum. Negative control indicates cells incubated with an isotype MoAb followed by MoAb A2B5 and 50% normal human serum. Data are shown as box and whisker plots with medians and lower and upper quartiles ($n = 5$). Right panel: the A2B5-induced sC5b-9 formation was markedly reduced by the specific C3 inhibitor compstatin and by the C5 blocking MoAb 137-76. The MoAb 137-26, neutralizing C5a, but not inhibiting C5b-9 formation, had no effect. NHS, normal human serum. Negative control indicates cells incubated with an isotype MoAb followed by MoAb A2B5 and 50% normal human serum. Data are shown as box and whiskers plots with medians and lower and upper quartiles ($n = 6$).

integrity was lost and the nuclei stained positive with propidium iodide (Fig. 4A). In comparison, cells incubated with control antibodies were intact (Fig. 4B).

Furthermore, neutralization of CD59 before adding A2B5 and serum led to the degeneration of neuronal processes and lost connection between NT2-N neurons, as

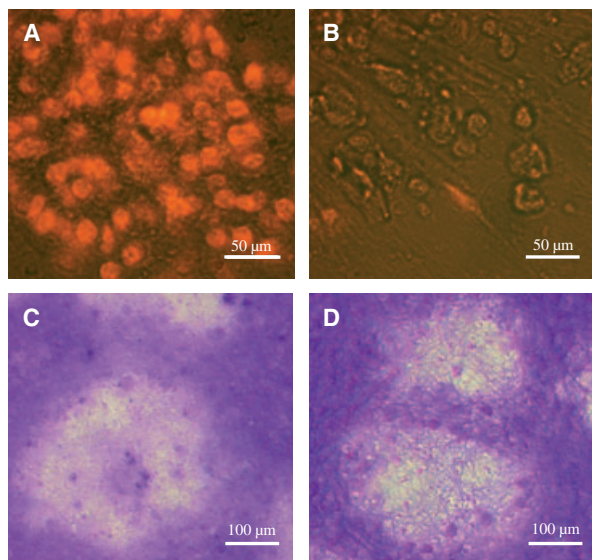


Figure 4 Morphological changes in NT2-N neurons associated with the complement-mediated damage and extensive cell death. The positive staining with propidium iodide indicates NT2-N cell death after incubation with anti-CD59, followed by MoAb A2B5 and 50% normal human serum (A). The same experiment with isotype control instead of anti-CD59 showed viable NT2-N neurons with an intact membrane integrity and neuronal processes after staining with PI (B). Degeneration of neuronal processes and loss of connection between NT2-N cells, demonstrated by staining with 0.05% crystal violet, was observed after incubation with anti-CD59 MoAb followed by MoAb A2B5 and 50% normal human serum (C). The corresponding morphology of control NT2-N neurons (isotype control instead of anti-CD59) was normal (D).

demonstrated by staining with 0.05% crystal violet (Fig. 4C), whereas the neuronal architecture was intact when control antibodies were used (Fig. 4D).

Discussion

In the present study, we demonstrated that the complement regulatory protein CD59, constitutively expressed by human NT2-N neurons, is functionally essential for the protection of these cells against complement attack. CD59 is widely expressed on almost all human cells and prevent damage to 'self' by the inhibition of C5b-9 formation at the C8 and C9 levels, thereby protecting the cells against membrane damage and lysis [26, 27]. Patients with isolated CD59 deficiency have increased susceptibility to erythrocyte lysis. This is similar to patients with paroxysmal nocturnal haemoglobinuria (PNH), who lack both CD55 and CD59, emphasizing CD59 as an essential inhibitor of complement-mediated cell lysis under physiological conditions [28, 29]. Furthermore, CD59 was shown to be important for the protection of host tissue in many other conditions, for example experimental renal IRI [30] and brain axonal injury in a model of multiple sclerosis [31].

In a previous study, a significant complement-mediated cell lysis was observed when serum was added to NT2-N cells under-expressing CD59, whereas the cells over-expressing CD59 by transfection were well protected [32]. In another study, CD59 was found to be expressed on precursor cells, but absent on differentiated, post-mitotic NT2-N neurons [33]. The latter cells activated complement and were lysed, whereas CD59 incorporation into their membrane led to protection. In contrast to this study, we have with two different MoAb recently demonstrated that NT2-N neurons constitutively express substantial amounts of CD59 on their membrane [18]. The present study confirmed that these cells did not activate complement in the absence of antibodies, but were efficiently lysed by IgM-induced complement activation when CD59 was neutralized. Although all these studies emphasize the importance of CD59 as a protector of the cells against complement attack, the main discrepancy is that our NT2-N neurons constitutively express CD59. The reason for this inconsistency is not clear, but different culture and methodological conditions may explain the various phenotypes of the cells. We argue that the differentiated NT2-N neurons used in the present study have a phenotype close to the human neurons, as CD59 is expressed by human neurons [34, 35]. By contrast, human Purkinje cells have been shown to be heterogeneous with respect to CD59 expression. Therefore, neuronal cells with low CD59 content could be more prone to complement-induced damage [36]. Furthermore, it has been shown that apoptotic neurons lose complement regulatory proteins, including CD59, which causes increased complement activation with subsequent phagocytosis and cell lysis [2].

Complement as a mediator of IRI is now well accepted [37]. However, the role of complement in human ischaemic stroke is not well understood [3, 4]. Animal studies have shown that the inhibition of complement reduce the size of cerebral infraction, but the data are conflicting [5, 38–42]. The role of IgM for complement activation in the brain is uncertain and only few observations are available in the literature [11, 43]. Naturally occurring IgM antibodies have been postulated as a trigger of complement activation in IRI [9, 44]. Although IgM is traditionally known to activate the classical complement pathway, recent data indicate that IgM might activate the lectin pathway during ischaemia and reperfusion [10, 45]. Whether IgM-induced complement activation might play a role in the ischaemic cerebral damage is currently unknown.

Activation of complement by any of three pathways results in the activation of C3 and runs further to C5 activation with a following cleavage into C5a and C5b. C5a is a potent chemoattractant and activates cells through the C5a receptor. C5b subsequently binds C6–C9 and forms the C5b-9 complex, which damages

membrane and subsequent cause lysis of susceptible cells. If the terminal pathway is activated in the fluid phase, a non-lytical soluble sC5b-9 complex will be formed. In the present study, two lines of evidence indicate that the NT2-N neurons died through C5b-9-mediated cell lysis. First, the neutralization of CD59, which prevents the formation of C5b-9, induced lysis. Second, the monoclonal antibody 137-76, which inhibits the cleavage of C5, prevents both C5a and C5b-9 formation [20]. The result of these processes is an efficiently abolished C5b-9 formation on NT2-N neurons, as well as the formation of sC5b-9 in the fluid phase. By contrast, the monoclonal antibody 137-26, known to efficiently inhibit C5a, but not the formation of C5b-9 [20], had no effect. These data indicate that the formation of sC5b-9 in the fluid phase reflects the formation of terminal activation occurring on the cell membrane.

Compstatin is a known complement inhibitor which binds to and prevents the cleavage of C3 [19]. The deposition of C5b-9 on the membrane was less efficiently inhibited by compstatin than by the anti-C5 antibody. One explanation for this may be that the IgM-mediated classical pathway activation on a solid phase is less efficiently inhibited by compstatin than the alternative pathway activation in a fluid phase. Alternatively, it cannot be excluded that a direct activation of C5 may take place without a preceding C3 activation, as has recently been described as a novel mechanism of terminal pathway activation [46].

Mature NT2-N neurons also express the complement regulatory proteins DAF and MCP [18, 33]. These are inhibitors at the level of C3. Although we cannot exclude that these proteins have some protective effects against complement attack, our data strongly support the importance of CD59 as the main inhibitor of complement on NT2-N neurons. Our findings are also consistent with the data obtained in experiments with fetal neurons [8] and with the two human neuroblastoma cell lines IMR32 and SKN-SH [7].

In conclusion, CD59 is an essential regulatory protein for the protection of human NT2-N neurons against complement activation and subsequent cell death induced by IgM binding to the neuron surface. These data have implications for understanding the pathophysiology of tissue damage in human neuronal cells, and should be taken into consideration when therapeutic approaches are developed for the intervention of diseases in CNS where complement is involved, including cerebral stroke.

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Conflict of interest

Michael Fung is employed by Tanox Inc., Houston, TX, USA.

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