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Complement inhibition accelerates regeneration in a model of peripheral nerve injury

Valeria Ramaglia[a], Martijn Rudolf Tannemaat[c], Maryla de Kok[a], Ruud Wolterman[a], Miriam Ann Vigar[d], Rosalind Helen Mary King[e], Bryan Paul Morgan[d], Frank Baas[a,b,*]

[a] Neurogenetics Laboratory, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands
[b] Department of Neurology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands
[c] Laboratory for Neuroregeneration, Netherlands Institute for Neuroscience, an Institute of The Netherlands Academy of Arts and Sciences, Meibergdreef 47, 1105BA Amsterdam, The Netherlands
[d] Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK
[e] Royal Free and University College Medical School, London, UK

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ABSTRACT

Complement (C) activation is a crucial event in peripheral nerve degeneration but its effect on the subsequent regeneration is unknown. Here we show that genetic deficiency of the sixth C component, C6, accelerates axonal regeneration and recovery in a rat model of sciatic nerve injury. Foot-flick test and Sciatic Function Index monitored up to 5 weeks post-injury showed a significant improvement of sensory and motor function in the C6 deficient animals compared to wildtypes. Retrograde tracing experiments showed a significantly higher number of regenerated neurons at 1 week post-injury in C6 deficient rats than wildtypes. Pathology showed improved nerve regeneration in tibials of C6 deficient animals compared to wildtypes. Reconstitution with purified human C6 protein re-established the wildtype phenotype whereas pharmacological inhibition of C activation with soluble C receptor 1 (sCR1) facilitated recovery and improved pathology similarly to C6 deficient animals. We suggest that a destructive C-mediated event during nerve degeneration hampers the subsequent regenerative process. These findings provide a rationale for the testing of anti-complement agents in human nerve injury.
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1. Introduction

Functional recovery of damaged peripheral axons is slow and incomplete (Baker et al., 1994). Insights into the mechanisms of post-traumatic axonal degeneration (Wallerian degeneration, WD) may offer an opportunity to improve the subsequent regenerative process. We have shown that the complement (C) system is highly expressed in healthy peripheral nerve, it is activated after injury and in disease (de Jonge et al., 2004; Ramaglia et al., 2007) and may influence recovery (Ramaglia et al., 2008a).

Activation of the C system occurs via three routes. The classical pathway is initiated via the recognition of a foreign antigen by C1q. Upon binding, C1s and C1r form a complex (C1) with C1q, cleaving C4 and C2 to yield the C3 convertase (Harpel and Cooper, 1975). The lectin pathway is triggered by binding of mannose binding lectin (MBL) to carbohydrates on the pathogen surface which activates the MBL-associated serine protease (MASP) cleaving C4 and C2 (Fujita, 2002). The alternative pathway starts by spontaneous low-rate hydrolysis of C3 generating C3(H_2O) which binds to factor B, permitting cleavage by factor D to form the fluid-phase C3 convertase C3(H_2O)Bb. This enzyme cleaves C3 and deposits C3b on surfaces where, in the absence of C inhibitors such as factor H, it binds and catalyses cleavage of factor B to form surface bound C3 convertase C3bBb. All three pathways converge in the cleavage of C3 and C5. This generates chemoattractants, opsonins and C5b which is the anchor for the assembly of the MAC (Nauta et al., 2004).

We have demonstrated in a nerve crush model that inability to form MAC delays both WD and clearance of damaged myelin and axons (Ramaglia et al., 2007) whereas lack of CD59a, the only membrane bound regulator of MAC, exacerbates WD (Ramaglia et al., 2009). We have also shown that systemic treatment with soluble complement receptor 1 (sCR1), a recombinant soluble form of the human membrane bound regulatory protein CR1 which inhibits all three pathways of C activation by dissociating the C3 convertases and targeting C3b and C4b for degradation (Weisman et al., 1990), protects the nerve from early axon loss after injury, delaying WD (Ramaglia et al., 2008b).

Delayed clearance of neuronal debris is considered a prerequisite for successful regeneration (Schafer et al., 1996), therefore C activation in the injured nerve is a “double-edged sword”, which

* Corresponding author at: Academic Medical Center, Neurogenetics Laboratory, Meibergdreef 9, 1105 AZ Amsterdam Zuidoost, The Netherlands.
Tel.: +31 20 5663846; fax: +31 20 5669312.
E-mail address: f.baas@amc.uva.nl (F. Baas).

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could either delay or accelerate subsequent nerve regeneration and recovery. C could inhibit or delay recovery by perpetuating or exacerbating nerve injury directly via the MAC or indirectly by recruiting and activating macrophages. C could promote or accelerate recovery by enhancing early macrophage clearance of neuronal debris and stimulating secretion of anti-inflammatory cytokines.

Here we have tested the effects of C activation on nerve regeneration after acute crush injury in two complementary ways: first by examining the effects of genetic C6 deficiency and second by systemic inhibition of C activation with sCR1.

2. Materials and methods

2.1. Animals

This study was approved by the Academic Medical Center Animal Ethics Committee and complies with the guidelines for the care of experimental animals. Male 12 weeks old PVG/c rats (wildtype) were obtained from Harlan (UK) and PVG/c^{-} (C6$^{-/-}$) rats were bred in our facility. The animals weighed between 200 and 250 g and were allowed to acclimatize for at least 2 weeks before the beginning of the study. Animals were kept in the same animal facility during the entire course of the experiment and monitored for microbiological status according to the FELASA recommendations. Animals were housed in pairs in plastic cages. They were given rat chow and water *ad libitum* and kept at a room temperature of 20 °C on a 12 h:12 h light:dark cycle.

2.2. Genotyping of PVG/c- (C6$^{-/-}$) rats

The C6$^{-/-}$ rats carry a deletion of 31 basepairs (bp) in the C6 gene (Bhole and Stahl, 2004). Genotyping was performed as described (Ramaglia et al., 2007).

2.3. Administration of human C6 for reconstitution studies

C6 was purified from human serum. It was administered i.v. in eight C6$^{-/-}$ rats (hereafter referred to as C6^{+} rats) at a dose of 4 mg/kg/day in PBS one day before the crush injury (day −1) and every day thereafter for 1 week (days 0, 1, 2, 3, 4, 5, and 6) (see schedule in Supplementary Fig. 1). Eight wildtype and eight C6$^{-/-}$ rats were treated with equal volumes of vehicle (PBS) alone.

2.4. Administration of sCR1 for inhibition studies

Recombinant soluble C receptor 1 (sCR1) was obtained as previously described (Piddlesden et al., 1994). sCR1 was administered i.p. in six rats at a dose of 15 mg/kg/day. Six rats were treated with equal volumes of vehicle (PBS) alone. The treatment was given one day before the crush injury (day −1) and every day thereafter for 1 week (days 0, 1, 2, 3, 4, 5, and 6) (see schedule in Supplementary Fig. 1).

2.5. Hemolytic assay and ELISA

Blood samples from wildtype PBS-treated, C6$^{-/-}$ PBS-treated, C6^{+} and sCR1-treated rats were collected from the tail vein one day before the crush injury (day −1) and every subsequent day until 1 week post-injury (days 0, 1, 2, 3, 4, 5, 6, and 7) (see schedule in Supplementary Fig. 1). All samples were collected immediately before the next treatment injection. Plasma was separated and stored at −80 °C. C6 activity and sCR1 inhibitory effect were assayed by standard C hemolytic assay (%CH_{50}, the reciprocal of the dilution of serum to lyse 50% of antibody coated sheep red blood cells) (Morgan, 2000). Plasma levels of sCR1 were measured by ELISA assay, as previously described (Mulligan et al., 1992), using serial dilutions assayed in triplicates.

2.6. Sensory and motor test

All experiments were conducted by the same investigator who was blinded of the genotype and treatment groups (wildtype $n = 8$; C6$^{-/-}$ $n = 8$; C6^{+} $n = 8$; wildtype sCR1-treated $n = 6$; wildtype PBS-treated $n = 14$). Both sensory and motor tests were performed at the same time during the day, every week until 5 weeks post-injury (see schedule in Supplementary Fig. 1). Recovery of sensory function was assessed with the foot-flick test according to De Koning et al. (1986). Briefly, a shock source with a variable current of 0.1–0.5 mA was used. The rats were immobilized, two stimulation electrodes were placed at the same point on the foot sole for every animal and stimulation applied by stepwise increasing the current from 0.1 to 0.5 mA. A response was scored positive when the rat retracted its paw upon stimulation at a given current. The minimum current (mA) needed to elicit a retraction response was recorded. Values are expressed as percentage of normal function and represent the mean ± SEM. An alternative data analysis modified from Van der Zee et al. (2008) was also applied. Briefly, absence of withdrawal response upon stimulation at 0.5 mA was interpreted as no recovery, whereas rats responding with the response withdrawal at any current for 2 consecutive measurements were considered partially or fully recovered. Non-linear regression Boltzmann sigmoidal fit [$y = \text{bottom} + (\text{top} - \text{bottom})/(1 + \exp((\text{V50} - x)/\text{slope}))$ was applied for all analyses (wildtype PBS-treated, $r^2 = 0.99$; C6$^{-/-}$ PBS-treated, $r^2 = 0.99$; C6^{+}, $r^2 = 0.99$; wildtype sCR1-treated, $r^2 = 0.99$). Recovery of motor function was assessed using a standardized walking track analysis and derived sciatic function index (SFI) according to Hare et al. (1992). Briefly, the rats were allowed to walk across a 150 cm long plexiglass platform; their walking pattern was recorded by a camera underneath the platform. The SFI was calculated from the recorded rear footprints using the ImagePro analysis program (Media Cybernetics, The Netherlands). The print length (PL), toe spread (1st to 5th, TS), and intermediary toe spread (2nd to 4th, IT), were recorded from the uninjured normal foot (NPL, NTS, NIT) and the foot on the injured experimental side (EPL, ETS, EIT). The SFI was derived with the formula: $-38.3 \times [(\text{EPL} - \text{NPL})/\text{NPL}] + 109.5 \times [(\text{ETS} - \text{NTS})/\text{NTS}] + 13.3 \times [(\text{EIT} - \text{NIT})/\text{NIT}]$. Where no print was produced by the injured foot, the standard values of EPL = 60 mm, ETS = 6 mm and EIT = 6 mm were used. A higher SFI results from an increase in the print length and toe spreading parameters and indicates reinnervation of the calf and small foot muscles, respectively. The SFI is expressed as mean ± SEM. Non-linear regression sigmoidal fit [$y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(\log \text{EC50} - x)})$ was applied (wildtype PBS-treated, $r^2 = 0.99$; C6$^{-/-}$ PBS-treated, $r^2 = 0.99$; C6^{+}, $r^2 = 0.97$).

2.7. Nerve crush injury

All the surgical procedures were performed aseptically under deep isoflurane anesthesia (2.5 vol% isoflurane, 1 L/min O_2 and 1 L/min N_2O). The left thigh was shaved and the sciatic nerve was exposed via an incision in the upper thigh. The nerve was crushed for three 10 s periods at the level of the sciatic notch using smooth, curved forceps (No.7), resulting in a completely translucent appearance of the crushed area on the nerve. The crush site was marked by a suture through the epineurium which did not constrict the nerve. On the right side, sham surgery was performed which exposed the sciatic nerve but did not disturb it and a marking suture was placed. The muscle and the skin were then closed with sutures. The right leg served as control. Following the crush lesion, the rats were allowed

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