Nervous immunity: A study on the role of complement system in neuronal degeneration and regeneration
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Chapter 2

Macrophage depletion delays myelin clearance after peripheral nerve injury

M. Sta, V. Ramaglia, K. Fluiter, J.M.B.V. de Jong and F.Baas
Abstract

Background: Deficiency for complement component C6 results in slower Wallerian degeneration (WD) and rapid nerve regeneration after crush injury in rats. C6 is essential for the formation of the membrane attack complex. C6 deficient rats are unable to form this complex, but retain upstream complement effector functions. Though capable to attract macrophages to the damage site, C6 deficient animals show significantly lower amounts of activated macrophages in the damaged nerve compared to WT animals.

Hypothesis: The lowered amount of activated macrophages in injured peripheral nerves is responsible for the delayed WD in C6⁻/⁻ rats.

Method: WT animals were depleted of circulating macrophages by treatment with clodronate-containing liposomes before and after the sciatic nerve crush injury. PBS-containing liposomes and PBS were used as controls.

Results: Macrophage-depleted animals showed delayed myelin clearance, but the extent of axonal damage was unchanged compared to animals treated with PBS or liposomes only. In addition, depletion of macrophages reduced the amount of complement deposition in the injured nerves.

Conclusion: Activated macrophages in the nerve are responsible for rapid myelin clearance during WD and increase complement activation in the injured nerve. However, macrophage depletion did not delay axonal degeneration during WD.
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Introduction

After injury, peripheral nerves undergo Wallerian degeneration (WD), a process in which the nerve degenerates completely from the first node of Ranvier proximal of the damage site, all the way to the distal end (Waller, 1850). During WD the nerve undergoes profound changes: axons degenerate, myelin is removed and Schwann cells dedifferentiate. Furthermore, macrophages are attracted to the nerve and activated to remove debris (Hirata and Kawabuchi, 2002). The dedifferentiated Schwann cells will align inside the endoneurial tube, where they provide a framework along which new axon sprouts can grow (Chen et al., 2005). Eventually, Schwann cells proliferate, engage in a one to one relationship with an axon and start the remyelination process.

The complement system, part of innate immunity, is activated during WD (Alexander et al., 2008; Bonifati and Kishore, 2007; de Jonge et al., 2004; Morgan et al., 1997; Ramaglia and Baas, 2009). The complement system is a cascade of self-cleaving proteases. The cleaved complement proteins have several effector functions (for a full description see (Ricklin et al., 2010)); they opsonize damaged structures (e.g. C3b), attract phagocytes (e.g. C3a and C5a), and form the membrane attack complex (MAC), a pore-forming structure, also known as the terminal complement complex or C5b-9. It is thought that activation of the complement system facilitates efficient clearance of the debris in the degenerating nerve.

We previously showed that in a rat sciatic nerve crush injury model WD was delayed when complement activation or MAC formation was prevented (Ramaglia et al., 2007; Ramaglia et al., 2008). Deletion or inhibition of complement protected the injured nerve from loss of myelin and axonal damage. In addition, the number of macrophages in the damaged nerves was reduced and the macrophages were not activated as measured by the absence of the cd11b/cd18 surface marker. Despite the delayed clearance of myelin debris, the complement inhibited or C6 deficient animals showed fast regeneration (Ramaglia et al., 2009); a surprising finding, in view of the notorious inhibitory properties of myelin debris on axonal outgrowth (Shen et al., 1998).

Furthermore, the morphology of the regenerated nerves in the complement inhibited or deficient animals was improved, showing more large caliber fibers and better myelination of the nerve fibres (Ramaglia et al., 2009).

The mechanism that is responsible for the improved regeneration of injured nerves in these complement inhibited or deficient animals is not well understood. Possible explanations for the observed improved regeneration are: 1) complement-mediated damage to the nerves is reduced or 2) macrophages-mediated damage to the nerves is reduced in the complement deficient animals.

In this study we addressed the role of infiltrating macrophages during post-traumatic WD. Therefore, we depleted complement sufficient rats of hematogenous macrophages 24 hours prior to and 18 hours after crush injury of the sciatic nerve by intravenous administration of clodronate containing liposomes. Nerve degeneration was studied by quantifying the amount of myelin and axonal damage, complement activation and macrophages. To exclude effects from liposomes, we included control groups, which received either PBS-containing liposomes or PBS alone. Furthermore, the spleen and lymph nodes were analyzed for the presence of macrophages in order to determine the effect of clodronate on peripheral macrophages.
These experiments allowed us to determine the role of macrophages in post-traumatic sciatic nerve degeneration. Our findings suggest that macrophages are responsible for rapid myelin clearance, but macrophage depletion does not alter axonal damage and clearance.

Materials and methods

In vivo depletion of macrophages:

The experiment was approved by the Animal Ethical Committee of the AMC (DNL1000 – 100729). 24 Wild type PVG rats (HSD, Germany) were injected intravenously with 2ml of the treatment solution. For depletion of macrophages a solution of liposomes containing dichloromethylene diphenylphosphate (also known as clodronate) (ClodLip b.v., clodronatelposomes.com, the Netherlands) was used (9 rats), as a control for the liposomes a PBS liposome solution (ClodLip b.v.) was used (9 rats). Six rats, treated with PBS only were included as placebo group. Intravenous injections were given twice; 24 hours (hrs) before and 18 hrs. after crush injury. Crush injury: the sciatic nerve of the right hind leg was crushed under isoflurane anesthesia and temgesic analgesia, by crushing for 3 times 10 seconds (Ramaglia et al., 2007). Animals were housed in filter top perspex cages with food and water ad libitum. To determine the extent of nerve degeneration, 3 animals per group were killed at 3 days (d) after crush; left and right sciatic nerves, spleen and lymph nodes from the groin and thorax were processed for paraffin embedding. The effectivity of the treatment was assessed by immunohistochemical analysis for CD68+ cells which confirmed reduction of macrophages in spleen and lymph nodes. Immunohistochemical analysis was done to verify whether the depletion of macrophages with the clodronate liposomes was successful.

Immunohistochemistry

Paraffin embedded tissue was processed. Sections of 6µm for nerve, 5µm for lymph node and 3µm for spleen were mounted on Superfrost slides and kept to dry overnight at 37°C.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Dilution</th>
<th>Art. no/ company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-CD68 (ED1)</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
<td>AbD Serotec (Oxford, United Kingdom)</td>
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<tr>
<td>MBP</td>
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<td>Phosphorylated neurofilament (SMI31 clone)</td>
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<td>1:10000</td>
<td>Covance Inc. (Rotterdam, the Netherlands)</td>
</tr>
<tr>
<td>C9</td>
<td>Rabbit polyclonal</td>
<td>1:300</td>
<td>B.P. Morgan (Cardiff, UK)</td>
</tr>
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Table 1. Primary antibodies used for immunohistochemistry.

Sections were deparaffinized and endogenous peroxidase activity was blocked using methanol/0.3% H2O2. Slides were washed and antigens were retrieved by heat in Tris/EDTA buffer [100 mM Tris, 10mM EDTA pH 6.5] for 13 minutes ('') in microwave. Antigen was targeted with primary antibodies (see table 1) for 90’ at room temperature (RT). The primary antibody was detected by incubation with a secondary antibody, either biotinylated (DAKO, Glostrup, Denmark) or directly fluorescent labeled (Invitrogen, Carlsbad, USA), for 30’ at RT. In case of a biotinylated secondary antibody, slides were washed and incubated with streptavidin-HRP (DAKO) for 20’ at RT. Detection was done by incubating the slides with 0.05% 3-aminophenyl-ethylcarbazole in acetate buffer (pH 5) for 5 minutes, followed by a counterstain with hematoxylin for 30 seconds. Slides were mounted in Kaiser’s glycerin gelatin (Merck, Darmstadt, Germany). In case of a directly fluorescent labeled secondary antibody, slides were
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counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, St Louis, USA) and enclosed in Vectashield Hard Set mounting medium (Vector Laboratories Inc., Burlingame, USA).

Quantification of sections

To determine the amount of CD68 positive cells per area, we stained and analyzed 5 non-consecutive sections, with a minimum interspace of 30µm to avoid double counting. CD68+ cells that contained a nucleus in the same plane were counted manually per nerve section. The area analyzed was measured in square microns using ImagePro Plus 7.0 “measurement” tool. Per section, the total amount of CD68+ cells per mm² was calculated [total count of CD68+ cells per section/ area of the section]. Per nerve, all CD68/mm² were averaged and used for statistical analysis.

Quantification of C9 positivity: Low magnification pictures of sections stained with the C9 antibody were processed in ImagePro, using the measurement setting “percentage positive per area”. The nerve area was encircled and the color, which identified C9 positivity, was assigned. The percentage of positive area was calculated and exported to Excel for statistical analysis.

Statistics

For statistical analysis on macrophage count we performed a one way ANOVA with Bonferroni correction for multiple testing, assuming normal distribution. Changes were considered statistically significant if P < 0.05. To calculate the correlation between the amount of C9 deposition and the amount of CD68+ macrophages we used the Pearson’s Rho correlation coefficient.

Results

Clodronate liposome treatment depletes macrophages in peripheral lymphoid organs

Animals received intravenous injections of clodronate-containing liposomes, PBS-containing liposomes or PBS alone 1 day prior to the crush and 18 hours after the crush. Nerves were harvested at 3 days post-injury (d PI). The efficiency of the macrophage depletion was assessed by the reduction of macrophages in spleen and lymph nodes. The immunohistochemical assessment of spleen (Fig. 1) and lymph nodes (not shown) showed macrophage depletion in clodronate treated animals was successful. The amount of observed CD68+ cells is much lower in clodronate treated animals (1C) compared to PBS (1A) and PBS liposome (1B) treated animals. A similar decrease of CD68+ cells was observed in the lymph nodes (not shown).

Clodronate liposome treatment decreases influx of blood-derived macrophages in the injured nerve

To determine the amount of infiltrated and phagocytic (activated) macrophages inside the injured peripheral nerve at 5 mm distal of the crush site, we stained for macrophages and myelin (Fig. 2). PBS only treated animals showed enlarged and foamy macrophages with inclusions of myelin positive debris, as shown by the colocalization of the CD68 and the MBP signals, indicating phagocytosis of myelin by active macrophages (arrows, 2B). Similar amounts of CD68+ cells and signs of phagocytosis (arrows, 2C) were observed in animals treated with PBS.
In contrast to the two control groups, animals treated with clodronate liposomes showed reduced amount of CD68\(^+\) cells with a small and quiescent morphology, lacking signs of phagocytosis (arrow: Fig. 2D).

Figure 1: CD68 in spleen sections at 3 d PI. A) WT spleen. B) PBS liposome spleen and C) Clodronate liposome spleen. A-B) show CD68\(^+\) cells throughout the section. Spleens from animals treated with clodronate liposomes showed a dramatic decrease in nuclei, and few CD68\(^+\) cells.

Figure 2: Cross sections of sciatic nerves stained for myelin (MBP; Green) and macrophages (CD68; Red) 5mm distal of crush injury site. A) Uninjured nerve, does not show any CD68 staining and shows normal myelin; B) Injured nerve of PBS treated animals, many CD68 positive (\(^+\)) cells are found, containing myelin (arrows); C) Injured nerve of PBS liposome treated animal, shows CD68\(^+\) cells containing myelin (arrows). The amount of damaged myelin is consistent with the quantification shown in figure 2 (B > C > D); D) Injured nerve of clodronate liposome treated animal, some CD68\(^+\) cells are found, but much smaller in size than those found in B and C. Additionally, the CD68\(^+\) cells in the clodronate liposome treated animals did not contain myelin (arrow).

Quantification of the amount of macrophages at 3 d PI, showed a robust and significant (77%, \(P < 0.01\)) reduction in the amount of CD68\(^+\) cells in the nerves of clodronate liposome...
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treated animals (88 ± 39 CD68+ cells/mm²) compared to PBS (387 ± 201 CD68+ cells/mm²) and PBS liposome (290 ± 82 CD68+ cells/mm²) treated controls (fig. 3). PBS loaded liposomes had no significant effect on the amount of activated macrophages in the nerve.

Figure 3: Quantification of the amount of CD68+ cells in 5 non-consecutive sections of sciatic nerves 5mm distal of the crush site. Bars represent absolute count of positive cells divided by the scored surface area of the nerve per treatment condition. One-way ANOVA showed clodronate liposome treated animals had significantly less CD68+ cells per mm² compared to controls (* = p < 0.01). Residual intraneural CD68+ cell count in clodronate liposome treated animals indicates that clodronate liposomes likely did not affect the resident endoneurial macrophage population but depleted blood and lymphoid organs from hematogenous macrophages without crossing the blood-nerve barrier.

Figure 4: Cross sections of sciatic nerves stained for myelin (MBP; Green) and neurofilament (SMI31, Red), 5mm distal of crush injury site at 3 d PI. A: uninjured nerve, same level as injured nerve sections. Normal dense, dotted neurofilament, ensheathed with ring-shaped myelin is visible within the nerve area. B) Injured nerve of PBS treated rat; C) Injured nerve of PBS containing liposome treated rat; D) Injured nerve of clodronate containing liposome treated rat. In all injured nerves similar axonal damage is found, swollen and damaged neurofilament (red arrows). Damaged myelin is observed in all injured nerves (B, C, D: white arrows), but the amount of myelin loss is different; PBS and PBS liposome treated animals show reduced myelin staining compared to clodronate treated animals, indicating that myelin clearance is reduced in clodronate treated animals.
Macrophages are responsible for rapid myelin clearance

To determine the contribution of macrophages to axonal damage and myelin clearance, crushed nerves were analyzed for the presence and morphology of neurofilament, as a marker of axons, and MBP as a marker of myelin, (Fig. 4) in nerve sections at 5 mm distal of the crush at 3 d PI. In PBS (4B) and PBS liposome treated (4C) animals we found a decrease in the amount of myelin staining compared to controls (4A). Myelin that is still found in these nerves is partly collapsed (white arrows Fig. 4). We also observed damaged axons as indicated by the annulated neurofilament staining, consistent with collapsed axonal content (red arrows Fig. 4).

Similar axonal damage was observed in the macrophage depleted animals (4D). However, in macrophage depleted animals less myelin degradation was found in the injured nerves compared to animals treated with PBS or PBS liposomes.

Complement activation depends partly on presence of macrophages

To determine the amount of complement activation in the injured nerves we stained for C9 a marker for the membrane attack complex (MAC) in tissue (Fluit et al., 2014) and we quantified the amount of C9 immunoreactivity per area. All injured nerves showed C9 deposition. The amount of C9 deposition differed between PBS treated compared to macrophage-depleted animals at 3 d PI. Quantification of the C9 staining showed that the amount of C9 deposition was slightly reduced in macrophage depleted animals injured nerves compared to both control groups (Fig. 5). In addition, a trend was observed found between the C9 positivity (Fig. 5) and the amount of activated macrophages in the nerves at 3 d PI (Fig. 3), in which it seems positively correlated, calculation of R² showed a coefficient of 0.96.

Discussion

Our findings show that macrophages are responsible for rapid myelin clearance in the injured nerves, but do not affect the amount of axonal damage during WD.

Initial activation of the complement system was similar in all animals. All injured nerves showed C9 deposition at 3 d PI. However, the amount of C9 deposition remained low in macrophage depleted animals. More C9 deposition was found in animals treated with PBS. It is
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Known that macrophages themselves are attracted by complement activation and become activated via complement and downstream effects of complement. Previous studies showed that in the absence of C9 (MAC) there was reduced influx and activation of macrophages. We found that in WT animals the amount of C9 deposition positively correlates with the amount of activated macrophages present in the injured nerve at 3 d PI (Fig. 3 and 5). We propose that in addition to being attracted and activated by complement, once activated, macrophages induce more complement activation in the injured nerves. The complement activation can be induced, either directly or indirectly, by exposing more complement activating epitopes.

We found no difference in axonal damage after sciatic nerve injury between all treatment groups. This indicates that axonal damage after peripheral nerve injury is not directly mediated by macrophages.

Previous studies comparing WD in WT and C6\(^{-/-}\) rats showed that the C6\(^{-/-}\) rats had a delay in both axonal and myelin degeneration during WD, as well as a lack of activated macrophages in the nerve 5 mm distal of the crush area at 3 d PI. Comparing the findings of the previous study to the current findings in the macrophage depleted animals we can conclude: the delay in WD observed in the C6\(^{-/-}\) animals is only in part due to the lack of activated macrophages, which are responsible for rapid myelin clearance. The observed delay in axonal degradation in the C6\(^{-/-}\) animals is not caused by the lack of activated macrophages but might be due to the inability to form MAC.

Figure 6: Schematic representation of the events after peripheral nerve damage. Damage induces slow axonal degeneration and triggers Schwann cells to dedifferentiate and proliferate; damage induces complement activation as well. Upstream components of activated complement system attract macrophages and induce influx. Downstream components of activated complement, MAC, induce macrophage activation and rapid axonal degeneration. Activated macrophages are responsible for rapid myelin degradation, myelin clearance and induce more MAC deposition. Dedifferentiated, proliferated Schwann cells can display phagocytic properties, they can degrade and clear myelin too, but this process is slow.

A schematic representation of the events, which occur after damage of the peripheral nerves by the key players, macrophages, complement and Schwann cells, is shown in figure 6. The complement system, specifically MAC, directly attacks the axons, resulting in axonal damage. This is underscored by the differences found in WD between wild type and complement deficient animals (Ramaglia et al., 2007). The difference in myelin clearance between WT and C6\(^{-/-}\) animals previously found is partly dependent on activation of the complement system and especially MAC formation. Activation of the complement system is most likely responsible for the attraction (via the upstream components C3a and C5a) and activation of macrophages (via the MAC). Rapid clearance of myelin is done by macrophages. Additionally, the presence of
activated macrophages results in more complement activation and more axonal damage as consequence of complement activation. The delayed WD observed in C6\(^{-/-}\) rat reported in the study by Ramaglia et al. (2007) might be the result of the absence of activated macrophages, normally responsible for rapid myelin clearance. In the C6\(^{-/-}\) animals other cell types like Schwann cells, must be responsible for myelin clearance (Fernandez-Valle et al., 1995; Gray et al., 2007; Hirata and Kawabuchi, 2002; Kobsar et al., 2006). Contributing to the reported difference in degeneration between WT and C6\(^{-/-}\) animals is that activated macrophages themselves appear to induce more complement activation.

Propagation of damage and degradation in injured sciatic nerves is initiated by complement activation; rapid degradation of axons is mediated by MAC. Inhibition of the complement system at the level of MAC formation, decreasing macrophage influx and inhibition of rapid axonal degradation might therefore be the most powerful tool to inhibit post-traumatic peripheral nerve degeneration.

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Reference List


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