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Dorsal root ganglia cocultured with macrophages: an in vitro model to study experimental demyelination

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Abstract The present investigation introduces an in vitro model to study macrophage properties during demyelination. Rat dorsal root ganglia (DRG) were cultured for obtaining myelinated peripheral nerve fibers. These cultures were exposed to non-resident macrophages. In untreated control cultures, there was no indication of myelin removal by the added macrophages. DRG were exposed to enzymatically generated oxygen radicals using the xanthin/xanthin oxidase or the glucose/glucose oxidase system. Assessment of Schwann cell viability and ultrastructural morphology revealed different patterns of cell cytotoxicity and morphological changes in different experiments. High concentrations caused complete tissue necrosis of the DRG, while low concentrations did not affect either cell viability or ultrastructural morphology. Under intermediate experimental conditions, oxygen radicals caused non-lethal Schwann cell damage leading to Schwann cell retraction and myelin sheath rejection. Myelin lamellae were disrupted and decompacted. These changes were followed by a selective macrophage attack on myelin sheaths, resulting in demyelination. Axons, Schwann cells and sensory ganglion cells survived this attack. The specificity of the oxygen radical effects was tested in experiments using the oxygen radical scavengers catalase and superoxide dismutase. Catalase prevented the described effects on cell morphology and subsequently blocked demyelination by non-resident macrophages.

Key words Dorsal root ganglia · Macrophages In vitro model · Oxygen radicals · Experimental demyelination

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Introduction

Mononuclear cells of the monocyte/macrophage system play a key role in myelin removal during Wallerian degeneration and immune-mediated demyelination [3, 30]. Macrophages phagocytosed myelin independent of a metabolic cell activation induced by interferon-γ [4]. The recognition of degenerating myelin mainly depended on the macrophage complement receptor type 3 and on the presence of serum complement components [6, 7]; cell surface carbohydrates or immunoglobulins were not involved [5, 12]. The role of macrophages in immune-mediated demyelination has been studied in detail during experimental allergic neuritis, an experimental autoimmune disease of the peripheral nervous system [18]. Macrophages were shown to be important effector cells and to interact intensively with T lymphocytes during demyelination [11, 14]. However, the exact mechanisms and sequences of early events during macrophage attack on myelin sheaths have not yet been clarified.

The present study aimed at establishing an in vitro model which contained the same cellular components as the peripheral nervous system and allowed cocultivation with macrophages. Rat dorsal root ganglia (DRG) and nerve fascicles were exposed to rat peritoneal macrophages in vitro to define the conditions under which macrophages attack myelin sheaths and leave Schwann cells and axons intact.

Materials and methods

Preparation and cultivation of dorsal root ganglia

DRG were prepared from newborn Wistar rats as described by Armati et al. [2]. Animals were killed in deep anesthesia. DRG were dissected and collected in Hanks' balanced salt solution (HBSS, Gibco) and incubated for 10 min at 37 °C in a solution containing 0.25 % trypsin (Serva, Heidelberg, Germany) and 0.05 % collagenase type I (Worthington Biochemical Corporation, Freehold, USA). DRG were then suspended in Dulbecco's mini-

mal essential medium (DMEM; Biochrom, Berlin, Germany) containing 10% fetal calf serum (FCS; Biochrom), 200 mM L-glutamine (Biochrom), 100 IU/ml penicillin (Biochrom) and 100 µg/ml streptomycin (Biochrom). DRG were then plated onto poly-L-lysine-coated coverslips and transferred to hydrophobic culture vessels (Petriperm, N. C., Hanau, Germany). Incubation was at 37 °C in a humidified atmosphere containing 5% CO_2.

DRG cultures were maintained for 1 week in DMEM supplemented with 10% Nu-serum (Paesel and Lorei, Frankfurt, Germany), 200 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin as described by Armati et al. [2]. After 1 week, culture medium was changed to DMEM without L-valine, containing 92 mg/l D-valine (Biochrom), 6 mg/ml glucose and 50 µg/ml L-ascorbic acid (Sigma). The medium was supplemented with 10% FCS, 200 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cultures were given this medium weekly until the beginning of the experiments.

Preparation of macrophages

Adult Wistar rats of either sex were used. Peritoneal macrophages were harvested 4 days after intraperitoneal thioglycollate injection (2.9%, NIH, thioglycollate broth; Difco, *nv.* 0257 01) by peritoneal lavage with cold phosphate-buffered saline, pH 7.4. Macrophages were cultured in DMEM containing 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 58 mg/100 ml L-glutamine. Hydrophobic culture vessels were used (Petriperm). Macrophages were tagged with latex beads with defined diameter (Sigma) before their addition to DRG cultures for positive identification of the added cells within the culture.

Treatment of cell cultures

DRG were exposed to enzymatically generated oxygen radicals before macrophages were added. Xanthine/xanthine oxidase (X/XO; Sigma) was used as a superoxide radical (O_2^-)-generating system, glucose/glucose oxidase (G/GO; Sigma) as a H_2O_2 radical-generating system [23, 29] in the following concentrations: X: 100 and 150 µM; XO: 0.1 and 0.25 U/ml; G: 6 mg/ml (as in culture medium); GO: 0.01, 0.1 and 1 U/ml. DRG cultures were exposed for 4 h. Cultures exposed to X, XO or G alone served as controls. Experiments were repeated four times.

At the end of the incubation period, cytotoxicity to Schwann cells in DRG cultures was evaluated by incubation with propidium iodide, an accepted indicator for cell cytotoxicity [27]. Schwann cells were identified by their typical morphological appearance [1] and by their positive reaction with the S-100 antibody as described below. A minimum of 100 Schwann cells per culture dish was examined in each experiment.

Latex-tagged macrophages were added to such treated DRG cultures either immediately or 3 days after exposure to oxygen radicals. Macrophages were cocultured with treated and untreated DRG cultures for 3–5 days.

In further experiments, DRG were incubated with oxygen radical scavengers in addition to the above described oxygen radical-generating systems. Superoxide dismutase (SOD; Sigma) is a scavenger for O_2-anions, catalase (Sigma) for H_2O_2. The following concentrations were used: SOD: 100 and 500 U/ml; catalase: 100 U/ml. At the end of these experiments, cell cytotoxicty evaluation and macrophage cocultivation were the same as described above.

Immunofluorescence

For identification of Schwann cells, DRG were incubated with a polyclonal anti-S-100 antibody (Dako) in a concentration of 1:100, applied for 2 h. Secondary antibody was a FITC-conjugated anti-rabbit Ig. Immunofluorescence-stained cultures were examined with a Zeiss fluorescence microscope.

Specimen preparation

In all experiments, tissue was fixed immediately in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in araldite. Semithin sections (1 µm) were stained with toluidine blue. Thin sections, contrasted with uranyl acetate and lead citrate, were examined with a Zeiss EM 10B electron microscope.

Results

The in vitro model

DRG were cultured according to the experimental regimen described above. The composition of cell culture media allowed formation of minifascicles with axonal sprouting, Schwann cell proliferation and efficient myelin formation in vitro. Fibroblast proliferation was inhibited, as was also observed by Armati et al. [2]. Myelin formation occurred after 3–4 weeks; myelin sheath thickness ranged up to 50 lamellae. The usual 1:1 Schwann cell/axon relationship was observed. DRG cultures were maintained in vitro up to 3–4 months. The described culture system produced a reliable and reproducible in vitro model to obtain myelinated nerve fibers containing the same cellular components as the peripheral nervous system (except vascular components), including axons, myelin sheaths and Schwann cells (Fig. 1A).

The DRG culture model allowed cocultivation with peritoneal macrophages. Phagocytic cells were tagged with latex beads before their addition to the DRG to identify them as non-resident cells. In untreated control cultures, these phagocytic cells were found between the myelinated and non-myelinated nerve fibers. Latex beads were mostly seen in electron-dense cytoplasmic lysosomes. There was no indication of a macrophage attack on myelin sheaths or active myelin uptake by the added, non-resident phagocytic cells (Fig. 1B).

Effect of oxygen radicals

DRG cultures were exposed to oxygen radical-generating systems for 4 h, as described above. Subsequently, cell viability and ultrastructure were examined. Three quite different patterns of cell cytotoxicity and morphological changes of the DRG's cellular components were found, all of which were concentration-dependent.

The following concentrations of X/XO and G/GO had no an effect on cell viability or on the ultrastructure of cultivated DRG cells: 100 µM X-0.1 U/ml XO; and 6 mg/ml G-0.01 U/ml GO. These cultures were comparable to untreated controls as described above. Cellular elements showed a regular ultrastructure without indication of cellular, axonal or myelin damage. Myelin sheaths were encompassed by Schwann cell cytoplasm. Subsequent addition of latex-tagged macrophages did

not lead to active myelin removal. Macrophages were seen within the DRG cultures without indication of an attack on myelin sheaths and/or Schwann cells.

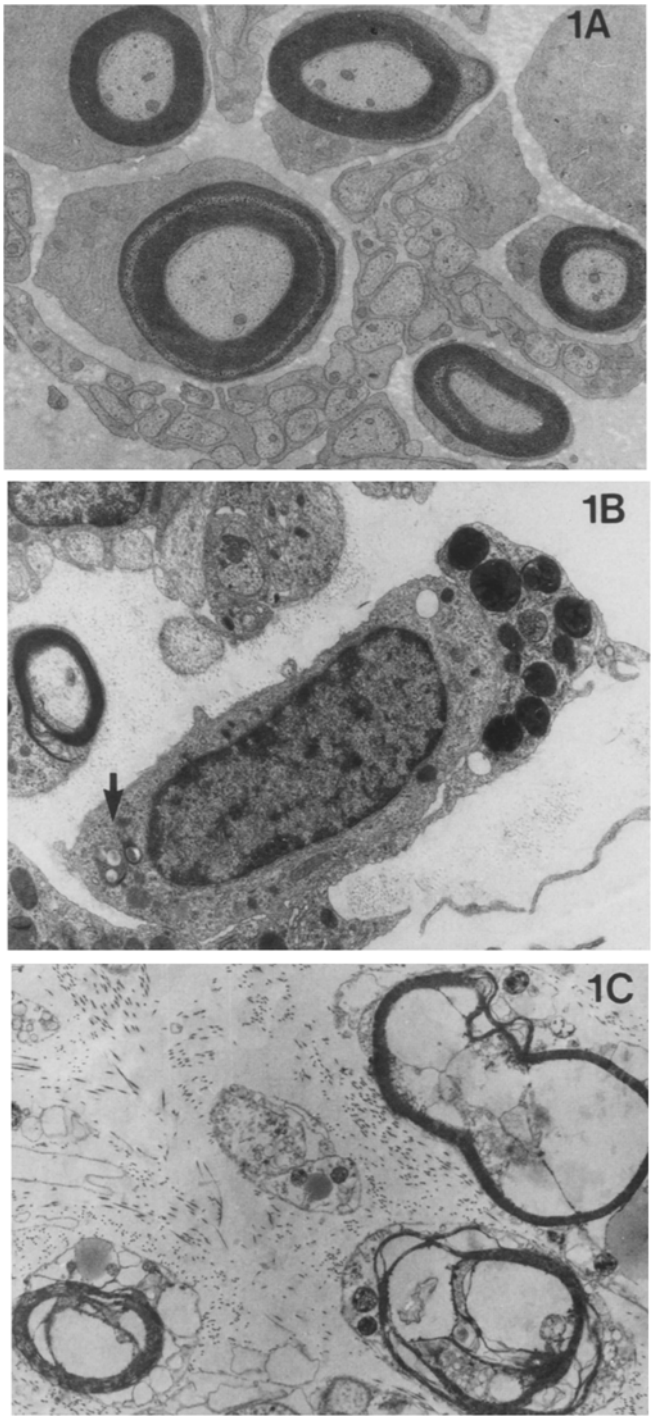
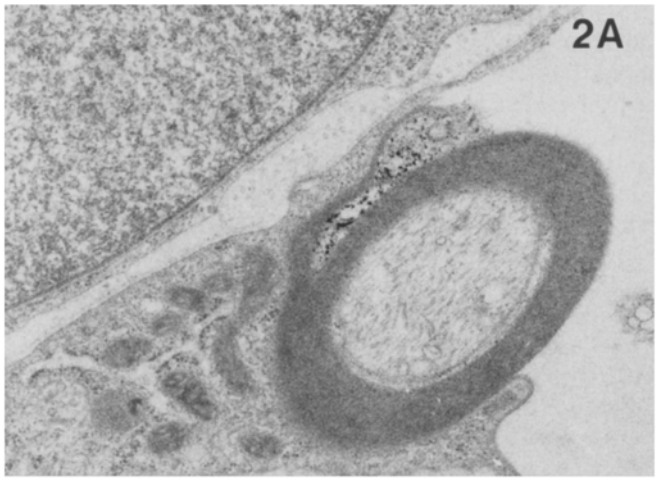


Fig. 1 **A** A 3-month-old DRG showing myelinated and non-myelinated nerve fibers with regular ultrastructure. **B** Control DRG culture exposed to non-resident macrophages. A macrophage containing latex beads (*arrow*) in its cytoplasm is seen in the culture without indication of myelin removal. **C** Treatment with 6 mg/ml G-1 U/ml GO. DRG cultures were necrotic with axonal breakdown, disruption of myelin sheaths and Schwann cell necrosis. (*DRG* dorsal root ganglion, *G* glucose, *GO* glucose oxidase) **A** × 9500; **B** × 7900; **C** × 5600

Quite different features of cell cytotoxicity and ultrastructural morphology were seen with the concentrations 150 μM X-0.25 U/ml XO and 6 mg/ml G-1 U/ml GO. In these experiments, the propidium iodide exclusion test showed almost complete loss of Schwann cell and ganglion cell vitality. More than 80% of Schwann cells were destroyed by such treatment. Ultrastructurally, most cellular elements of the DRG cultures showed features characteristic of cell necrosis. Only few surviving Schwann cells were identified by the propidium iodide test or by morphological observations. The Schwann cells showed vacuolar or fatty degeneration of their cytoplasm and their nuclear chromatin was disintegrated. Mitochondria had lost their regular ultrastructure and other catoplasmic organelles were not identifiable. The Schwann cell's basal lamina envelope had lost its continuity. Ganglion cells showed the same morphological features of tissue necrosis. Subsequently, axonal processes degenerated and neurofilaments disintegrated. Myelin sheaths were split and partly compacted or showed complete vesicular disruption (Fig. 1C). Subsequent addition of latex-tagged macrophages caused indiscriminate phagocytosis of all necrotic tissue elements, including the myelin sheaths. A selective phagocytosis of myelin was not observed. Control experiments using 150 μM X or 0.25 U/ml XO alone did not result in tissue necrosis. The cellular elements of the DRG cultures were viable and showed a regular ultrastructure.

The third pattern of cell cytotoxicity and ultrastructural changes was observed with the concentrations 150 μM X-0.1 U/ml and 6 mg/ml G-0.1 U/ml GO. In these experiments, cell viability was not affected, as revealed by the propidium iodide exclusion test. Less than 10% of the Schwann cell population showed loss of viability in repeated experiments. Ultrastructurally, however, Schwann cells demonstrated distinct morphological alterations: their cytoplasm was partly retracted from the myelin sheath (Fig. 2A). The basal lamina's continuity was also partly disrupted. The myelin sheath itself showed disruption of the regular lamellar structure, usually beginning with the outer lamellae. Decompaction and splitting of myelin sheaths was also observed (Fig. 2B). The fine structure of Schwann cells was without indication of cell necrosis. Cell organelles were intact and nuclear chromatin was regular. Few Schwann cells showed cytoplasmic lipid droplets. Thus, Schwann cell retraction from its myelin sheath and subsequent myelin sheath rejection seemed to be an active response of a vital Schwann cell and not a consequence of cellular or axonal breakdown. Ganglion cells and their axons did not show any signs of degeneration. These morphological patterns were reproducible in repeated experiments with the described experimental regimen.

When latex-tagged macrophages were added to DRG cultures treated with 150 μM X-0.1 U/ml XO and 6 mg/ml G-0.1 U/ml GO, this treatment triggered a selective attack by the added macrophages on the mye-



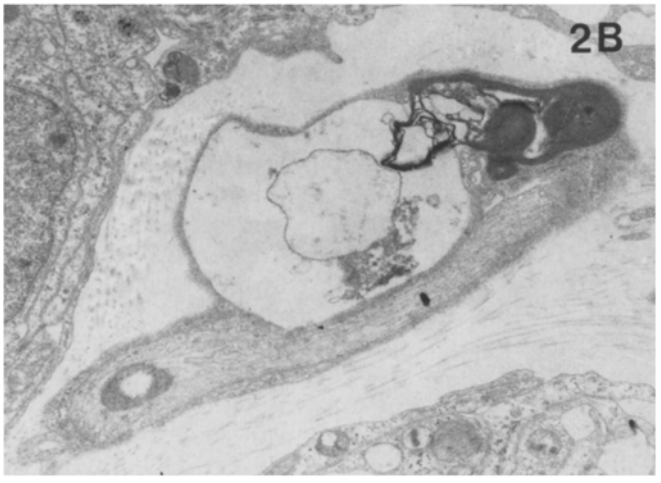
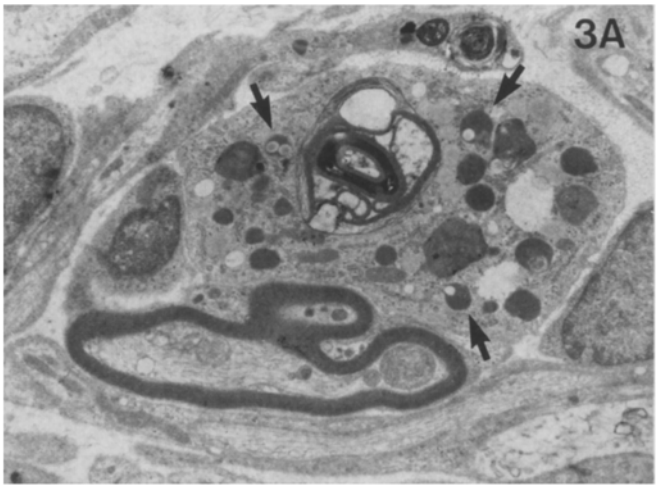


Fig. 2 A, B Treatment of DRG with 150 μM X-0.1 U/ml XO or 6 mg/ml G-0.1 U/ml GO. **A** Schwann cells retracted their cytoplasm from the myelin sheath. **B** Myelin sheath showing splitting and decompaction. (*X* xanthine, *XO* xanthine oxidase) **A** × 19 800;
B × 8550



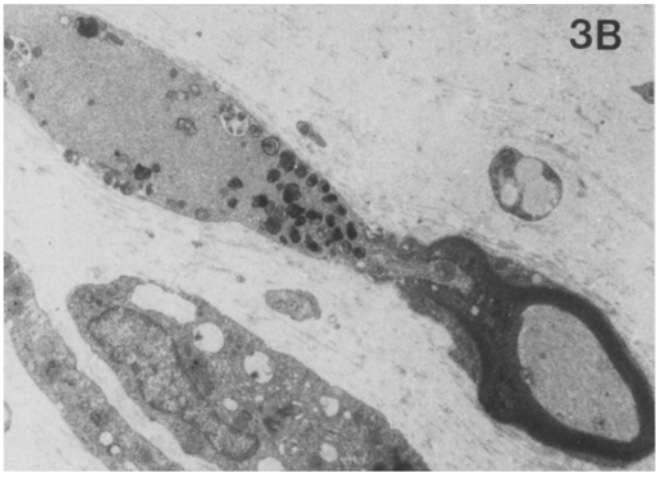


Fig. 3 A, B Treatment of DRG with 150 μM X-0.1 U/ml XO or 6 mg/ml G-0.1 U/ml GO. **A** Phagocytes removed the myelin sheaths and contained latex beads (*arrows*) and myelin debris in their cytoplasm. **B** Demyelinated axon in the DRG culture after oxygen radical treatment and addition of macrophages. **A** × 5600; **B** × 3100

lin sheaths. Schwann cells, ganglion cells and axons were left intact. The most dramatic effect was seen when the non-resident cells were added to the DRG cultures 3 days after oxygen radical treatment. The added macrophages were identified as being non-resident by the presence of latex beads in their cytoplasm. They were found intimately attached to myelinated nerve fibers. Non-resident phagocytic cells removed the myelin sheaths, their cytoplasm containing latex beads as well as myelin particles (Fig. 3A). Such cells were seen between the nerve fibers. Finally, this process resulted in segmental demyelination (Fig. 3B). In repeated experiments, the G/GO system seemed to be more effective in inducing these phenomena than the X/XO system.

Control experiments using 150 μM X or 0.1 U/ml XO alone had no effect on either cell viability or ultrastructural morphology. These cultures were comparable to the untreated controls as described above. Their coculturing with non-resident macrophages did not result in active myelin removal.

Effect of oxygen radical scavengers

DRG cultures were exposed to 6 mg/ml G-0.1 U/ml GO, a H_2O_2 generating system. These cultures were concomitantly treated with the oxygen radical scavengers SOD and catalase. All other experimental conditions were the same as described above. Addition of SOD, a superoxide anion scavenger, did not affect oxygen radical-induced changes. Cell viability and ultrastructural changes were similar to cultures treated with 6 mg/ml G-0.1 U/ml GO alone. Schwann cells retracted from their myelin sheaths and outer myelin lamellae were disrupted. Subsequent addition of latex-tagged, non-resident macrophages caused myelin removal comparable to the previous experiments.

Addition of the H_2O_2 scavenger catalase, in contrast, prevented the oxygen radical-induced changes. DRG cultures were comparable to untreated controls. None of the cellular elements showed ultrastructural changes. Myelin sheaths were covered by Schwann cell cytoplasm and basement membranes. Addition of latex-tagged macrophages gave no indication of active and selective myelin removal.

Discussion

Macrophages play an important role as myelin-removing cells during immune-mediated demyelination and during Wallerian degeneration. The mechanisms of macrophage involvement in these processes, however, have not yet been clarified in detail. In the present study, we used cocultures of DRG and peritoneal macrophages to define events leading to a selective macrophage attack on myelin sheaths, while leaving Schwann cells and axons intact.

The sensory neurons of DRG cultures form minifascicles of nerve fibers containing the same types of cellular elements as the peripheral nervous system except for vascular components [22]. Growth and differentiation in vitro are similar to that seen in vivo [8]. The use of special media allows substantial myelination in vitro and long-term maintenance of the cultures [2, 9]. Cocultivation with added macrophages permits the investigation of macrophage-myelin interactions in a defined in vitro situation similar to the in vitro model of Wallerian degeneration described earlier [13]. When using latex-tagged peritoneal macrophages cocultured with DRG, these non-resident cells are easily distinguished from the resident macrophage population known to be present in DRG [26].

The morphological observations made in this in vitro model were quite similar to those seen in vivo during immune-mediated demyelination in the peripheral nervous system, although demyelination was not induced immunologically in our experiments. The presence of mononuclear cells of the monocyte/macrophage system was required to cause demyelination [21]: the Schwann cells lost contact to the myelin sheaths and as in our experiments using non-lethal damage, they survived this event. Myelin removal was attributed to non-resident phagocytes invading the nerves. This is consistent with in vivo observations: macrophages are massively engaged in immune-mediated demyelination during experimental demyelinating diseases as well as in demyelinating neuropathies [10, 30], and their elimination suppresses demyelination [19].

In our model, reactive oxygen radicals were used at carefully controlled conditions for producing selective damage to Schwann cells or myelin, respectively. We found that, while leaving axonal structures intact, exposure to oxygen radicals led to an active retraction of the Schwann cell from its myelin sheath along with disruption of basement membrane continuity and of the regular lamellar structure of the sheath. Such changes – occurring in the absence of macrophage infiltration – are reminiscent of early changes seen in experimental allergic neuritis [25]. Similar morphological patterns with vesicular demyelination could be induced by raised intracellular calcium concentrations [28]. An active retraction of the Schwann cell from its myelin sheath and decompaction of the sheath are also observed in the early phase of Wallerian degeneration [3]. This process, however, involves axonal degeneration, which was not observed in our experiments.

Oxygen radicals belong to a group of partly macrophage-derived, inflammatory mediators shown to be involved in demyelinating disorders of the peripheral and the central nervous system [17]. These include arachidonic acid metabolites, proteases and cytokines [15, 18]. Oxygen radicals and/or other toxic metabolites secreted by macrophages may initiate the chain of events resulting in demyelination. The relatively selective damage to myelin sheaths observed in vitro is consistent with experiments demonstrating that myelin proteins are susceptible to damage by free oxygen radicals [20]. Furthermore, the hypothesis of oxygen radical involvement in demyelination is supported by the fact that oxygen radical scavengers such as SOD and catalase were shown to suppress experimental allergic neuritis [16] in a manner similar to the effect of catalase in our experiments. Conversely, other non-macrophage-derived factors could also play a role: the non-cell-associated myelin damage during EAN was attributed to soluble serum factors [25]. Sera from patients with Guillain-Barré syndrome induced vesicular myelin disruption and Schwann cell damage in rat DRG cultures [24].

The initial changes in Schwann cells or myelin had caused the phagocytic attack by the latex-tagged macrophages in our system. These data indicate that in the in vitro situation, added, non-resident macrophages attack and phagocytose only predamaged myelin. The myelin and/or Schwann cell damage was induced by oxygen radicals in the present study. Other soluble mediators, as mentioned above, might also be involved in this process. Our model, in conclusion, offers a controlled in vitro situation which permits further analysis of the triggering events and the early changes in a living nerve fiber, and of the monocyte/macrophage population during the initiation of a macrophage attack on myelin.

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