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Cell Injury, Repair, Aging and Apoptosis

Deleterious Role of IFNγ in a Toxic Model of Central Nervous System Demyelination

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Interferon-γ (IFNγ) is a pleiotropic cytokine that plays an important role in many inflammatory processes, including autoimmune diseases such as multiple sclerosis (MS). Demyelination is a hallmark of MS and a prominent pathological feature of several other inflammatory diseases of the central nervous system, including experimental autoimmune encephalomyelitis, an animal model of MS. Accordingly, in this study we followed the effect of IFNγ in the demyelination and remyelination process by using an experimental autoimmune encephalomyelitis model of demyelination/remyelination after exposure of mice to the neurotoxic agent cuprizone. We show that demyelination in response to cuprizone is delayed in mice lacking the binding chain of IFNγ receptor. In addition, IFNγR$^{-/-}$ mice exhibited an accelerated remyelination process after cuprizone was removed from the diet. Our results also indicate that the levels of IFNγ were able to modulate the microglia/macrophage recruitment to the demyelinating areas. Moreover, the accelerated regenerative response showed by the IFNγR$^{-/-}$ mice was associated with a more efficient recruitment of oligodendrocyte precursor cells in the demyelinated areas. In conclusion, this study suggests that IFNγ regulates the development and resolution of the demyelinating syndrome and may be associated with toxic effects on both mature oligodendrocytes and oligodendrocyte precursor cells. ***(Am J Pathol 2006, 168:1464–1473; DOI: 10.2353/ajpath.2006.050799)***

Details of the process of demyelination and remyelination in the central nervous system (CNS) have in the past been somewhat difficult to ascertain because most experimental models of demyelination/remyelination exhibit variability from animal to animal in the severity, localization of lesion site, or time course of the pathophysiology. Some 30 years ago, Blakemore[1] described demyelination restricted to the corpus callosum and superior cerebellar peduncle in mice induced by feeding of the copper chelator cuprizone. Recently, this model has been revived and has been shown to be a very reproducible model of demyelination and remyelination.[2–6] When 8-week-old C57Bl/6 mice are fed with 0.2% cuprizone, mature oligodendroglia is specifically insulted and dies. Oligodendrocyte death is closely followed by recruitment and activation of microglia as well as peripheral macrophages,[2] leading to phagocytosis of myelin. Interestingly, this inflammation is reported to occur in the absence of T lymphocytes and in the presence of an intact blood-brain-barrier.[2] When the cuprizone challenge is terminated, an almost complete remyelination takes place in a matter of weeks. The molecular basis of this process of demyelination/remyelination is not understood, but a recent study has found that cuprizone feeding induces an alteration in metal homeostasis in the brain, which may affect the normal function of several enzymatic systems.[7]

To date, several studies have demonstrated that cytokines play a role in the cuprizone model of de/remyelination. Thus, Arnett et al[8] described a delay in remyelination in mice lacking tumor necrosis factor α (TNF-α), which correlated with a reduction in the pool of proliferating oligodendrocyte precursors. Further study revealed that it was TNF-α working through TNF receptor 2 that was critical to oligodendrocyte regeneration. Interferon-γ (IFNγ) has also been examined in this model by expressing it ectopically at low levels in oligodendrocytes under the control of the myelin basic protein promoter.[9] Such

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animals, surprisingly, did not display any evidence of demyelination when fed cuprizone, nor did they show signs of oligodendroglial death, astrogliosis, or microglial activation, which are typically seen in this model.

A caveat, however, with respect to the *IFNγ* transgenics used should be given. There was a direct association between transgene expression and primary demyelination, which was accompanied by clinical abnormalities consistent with CNS disorders. Additionally, multiple hallmarks of immune-mediated CNS disease were observed, including up-regulation of major histocompatibility complex (MHC) molecules, gliosis, and lymphocytic infiltration.[10] In another study using similar transgenic animals, it was found that such mice also had dramatically less CNS myelin than the control animals and showed reactive gliosis and increased macrophage/microglial activation.[11] Both of these studies indicate that the presence of IFNγ in the CNS disrupts the developing nervous system and results in a quite abnormal phenotype.

Here, we re-examine the role of IFNγ in cuprizone-induced demyelination using IFNγR$^{-/-}$ mice and report discordant results with respect to previous work. We show that demyelination associated with cuprizone feeding is delayed in IFNγR$^{-/-}$ mice compared with wild-type controls. Moreover, when cuprizone was removed from the diet, an earlier regenerative response that resulted in an accelerated remyelination was observed in the corpus callosum of IFNγR$^{-/-}$ mice, likely due to the enhanced recruitment of new oligodendrocytes in the demyelinated areas. Taken together, our results suggest a deleterious role rather than a protective role for IFNγ in cuprizone-induced demyelination.

Materials and Methods

Animals

129/Sv, H2^{b} mice of either sex, homozygous for the null mutations of the ligand-binding chain of the IFNγ receptor (IFNγR$^{-/-}$), were obtained from Dr. Michel Aguet (University of Zurich, Zurich, Switzerland). Disruption of the *IFNγR* gene was verified using polymerase chain reaction (PCR) as described previously.[12] The IFNγR$^{-/-}$ mice were crossed with C57Bl/6, and the offspring were screened by PCR for the presence of the null mutation. IFNγR-null mice were then backcrossed for more than 10 generations to the C57Bl/6 parental strain and then intercrossed to homogeneity for IFNγR$^{-/-}$. Mice were maintained in pathogen-free conditions and used between the ages of 8 and 14 weeks. All animal experimentation was approved by the Animal Experimentation Ethics Committee of the Australian National University.

Induction of Demyelination and Remyelination

To induce demyelination, male C57Bl/6 mice were fed 0.25% cuprizone mixed with ground chow. After 6 weeks of feeding, normal food was restored for 4 more weeks. Animals were provided with cuprizone-containing food and normal ground food *ad libitum*. Food levels were checked daily.

Histology, Immunohistochemistry, and Lectin Histochemistry

For histological examination, mice were killed and perfused intracardially with 20 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed and fixed in 4% paraformaldehyde for an additional 3 to 7 days. Brains were placed in a David Knopf brain blocker and trimmed at a level 2 mm anterior to bregma. Serial coronal sections were examined between levels 1 to −1 mm bregma as defined in the mouse brain atlas of Franklin and Paxinos.[13]

Demyelination was evaluated in 5-μm paraffin sections of the corpus callosum using Luxol fast blue with periodic acid-Schiff reaction. Demyelination score was evaluated by two independent readers using a 5-point scale, ranging from 0 (no demyelination) to 5 (total demyelination of corpus callosum). For each mouse, two serial sections at three different levels, around 250 μm apart, between 1 to −1 mm bregma following the mouse brain atlas of Franklin and Paxinos were examined.[13] Results were expressed as average of at least five mice per group.

Paraffin-embedded sections were stained for the pi isoform of glutathione *S*-transferase (GST-pi, a marker of mature oligodendrocytes),[14,15] *Ricinus communis* agglutinin 1 (RCA-1, a marker for macrophages and microglia),[16,17] and glial fibrillary acidic protein (GFAP, a marker for astrocytes). Briefly, for GST-pi and GFAP staining, antigen retrieval was performed by boiling sections in citrate buffer, pH 6.0, for 30 minutes.[18] Sections were incubated with 0.3% of H_2O_2 in methanol for 30 minutes at room temperature (RT) for quenching endogenous peroxidase activity, then blocked with 5% bovine serum albumin/PBS for 1 hour at RT, and incubated with 1/500 and 1/1000 of rabbit anti-GFAP antibody and rabbit anti-GST-pi antibody (Chemicon, Temecula, CA), respectively. After rinsing, the sections were incubated with goat anti-rabbit IgG-horseradish peroxidase (Chemicon) and with AEC substrate pack (Innogenex, San Ramon, CA). For lectin reactivity, antigen retrieval was performed by incubating with proteinase K (MBI Fermentas, Burlington, Ontario, Canada) for 2 minutes at 43°C. Sections were then incubated with biotinylated RCA-1 (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After rinsing, they were incubated for 1 hour with streptavidin-horseradish peroxidase and with AEC substrate (Innogenex).

For staining of the oligodendrocyte precursors, mice were euthanized and perfused intracardially with cold PBS for 15 minutes. Brains were removed and trimmed as with the paraformaldehyde perfused brains. They were then frozen in cold isopentane on dry ice and cut or stored at −70°C. Ten-millimeter sections were fixed with cold acetone for 30 minutes, and endogenous peroxidase activity was quenched with 0.3% of H_2O_2 in methanol. Sections were incubated with rabbit anti-NG2 (Chemicon) at dilution 1/400 overnight at 4°C. As sec-