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Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice

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Abstract

Axon loss is recognised as a significant contributor to the progression of the disability associated with multiple sclerosis. Although evidence of axon damage is found in areas of chronic demyelination it is more frequently seen in association with acute demyelination. This study compares the incidence of axon degeneration associated with the areas undergoing demyelination in young adult (8–10 weeks) and aged (6–7 months) C57BL/6 mice in cuprizone intoxication; a widely used model of demyelination. The incidence of axon transection, as indicated by the presence of SMI 32 positive axonal spheroids, and evidence of axon loss in the medial corpus callosum, were significantly greater in aged mice, as was the magnitude of the macrophage and astrocyte response to demyelination. Aged C57BL/6 mice are thus more prone to axon degeneration in association with demyelination than young adult mice. A retrospective study indicated that the incidence of axon degeneration was much higher in C57BL/6 mice than in the Swiss albino mice used in the early cuprizone intoxication studies which were fed much higher doses of cuprizone. These results indicate both a genetic and age susceptibility to demyelination-associated axon transection.

Keywords: Multiple sclerosis; Ageing; Remyelination; Oligodendrocyte progenitor cells; Macrophages; Astrocytes

1. Introduction

Axon loss is now recognised as a significant contributor to the progression of the disability associated with multiple sclerosis (Hemmer et al., 2002). Although evidence of axon damage can be found in association with areas of chronic demyelination it is more frequently seen in association with acute demyelination (Ferguson et al., 1997; Trapp et al., 1998; Bitsch et al., 2000; Kornek et al., 2000; Garbern et al., 2002). Axon damage is a striking feature of some immune-mediated forms of experimental demyelination (Rivera-Quinones et al., 1998) and macrophage derived nitric oxide induced uncontrolled entry of Na^+ ions into axons provides a satisfactory mechanism for causing a damaging rise in Ca^{2+} ions within demyelinated axons resulting in focal axon degeneration (Kapoor et al., 2003).

Cuprizone intoxication is being increasingly used as a model of experimental demyelination, with the corpus callosum and the superior cerebellar peduncles the most frequently studied white matter tracts (Matsushima and Morell, 2001). Using these tracts it has been shown that remyelination follows demyelination in both young (Blakemore, 1973a; Ludwin, 1978) and old mice (Blakemore, 1974). Although axon degeneration has been observed in the corpus callosum of young adult mice (Mason et al., 2001; Stidworthy et al., 2003), its presence was not commented on by those who studied demyelination and remyelination using the superior cerebellar peduncles of similar aged but different strains of mice (Blakemore, 1972, 1974; Ludwin, 1978; Johnson and Ludwin, 1981). The extent of axon loss that occurs in the corpus callosum of young adult C57BL/6 mice has been considered to be insignificant, but has never been quantified. In a series of preliminary experiments involving cuprizone intoxication of aged C57BL/6 mice we noticed extensive evidence of axon

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degeneration in the corpus callosum. We therefore conducted a quantitative study to compare the extent of axon degeneration in the corpus callosum axons of young adult and aged C57BL/6 mice and a retrospective examination of material from early cuprizone studies in young and aged Swiss albino mice (Blakemore, 1973a,b, 1974).

2. Material and methods

2.1. Animals

Twelve young adult (8–10 weeks) and 18 aged female (6–7 months) C57BL/6 mice (Harlan UK Limited, Bicester, UK) were fed a diet of ground mouse chow containing 0.2% and 0.4% cuprizone, respectively (Biscyclohexane oxaldihydrazone from Sigma-Aldrich, Poole, UK) for a period of 6 (0.2%) or 7 (0.4%) weeks to induce CNS demyelination. After 6 or 7 weeks of cuprizone intoxication mice were perfused with 4% paraformaldehyde for immunohistochemistry (3 young adult and 6 aged mice) or 4% glutaraldehyde for resin embedding (3 young adult and 4 aged mice). The remaining mice were returned to a normal diet for a further 6 (0.2%) or 7 (0.4%) weeks before being perfused with 4% paraformaldehyde for immunohistochemistry (3 young adult and 4 aged mice) or 4% glutaraldehyde for resin embedding (3 young adult and 4 aged mice). Control C57BL/6 mice were perfused with 4% paraformaldehyde for immunohistochemistry (3 young adult and 4 aged mice) or 4% glutaraldehyde for resin embedding (3 young adult and 4 aged mice) when the first mice were killed.

2.2. Tissue processing

Following fixation with glutaraldehyde, the brain was sampled from the area between 165 and 195 in the mouse brain atlas (http://www.hms.harvard.edu/research/brain) and trimmed to leave a tissue block that contained the entire corpus callosum. This block was processed into resin and sections cut at 1 μm were stained with alkaline toluidine blue for light microscope examination. Selected areas from some tissue blocks were trimmed, sectioned and examined by electron microscopy.

Following fixation with paraformaldehyde, the whole brain was immersed in 30% sucrose solution (in 0.1 M phosphate buffer) at 4 °C until they sunk, following which they were placed in mounting media (a 50:50 solution of 30% sucrose solution and OCT (RA Lamb Lab supplies, Eastbourne, UK)) for 2 h before rapidly freezing in a cryomould using liquid nitrogen. The blocks were stored at −70 °C until used. Starting at the olfactory bulb 25 μm thick sections were cut at −25 °C with a cryostat until area 165 of the mouse brain atlas (http://www.hms.harvard.edu/research/brain) was reached. From this point on 10 μm thick transverse sections of the brain were cut and mounted onto polylysine coated glass slides (VWR International Ltd, Lutterworth, UK).

2.3. Immunohistochemistry

For immunohistochemistry frozen sections were brought to room temperature, washed in Triton (0.3% in PBS 3 washes for 5 min). Sections for SMI 32 staining were pretreated with 3% hydrogen peroxide in methanol for 30 min and washed. All sections were blocked with 10% normal goat serum (NGS) (Sigma-Aldrich, Poole, UK) in Triton (0.3% in PBS) for 1 h and then incubated overnight at 4 °C with primary antibody diluted in 0.3% Triton in PBS containing 2.5% NGS. The primary antibodies used were monoclonal mouse anti-GFAP [1:200] (glial fibrillary acid protein; Chemicon, Hampshire, UK), polyclonal rabbit anti-NG2 [1:100] (Chemicon Hampshire, UK), monoclonal mouse SMI 31 antibody [1:3000] (Sternberger, CA, USA), monoclonal mouse SMI 32 antibody [1:1000] (Sternberger, CA, USA) and monoclonal rat anti-CD11b [1:400] (Serotec, Oxford, UK). All sections were rinsed and then incubated for 2 h with the relevant biotinylated secondary antibodies [1:200] (anti-rabbit (Vector Laboratories, Peterborough, UK), anti-rat (Vector Laboratories, Peterborough, UK) or anti-mouse (Southern Biotech, AL, USA)). Sections incubated with the anti-NG2, -SMI 32 and -CD11b primary antibodies were rinsed and then incubated with standard ABC reagent (Vector laboratories, Peterborough, UK) for 1 h at room temperature followed by visualisation with diaminobenzidine (DAB, Vector laboratories, Peterborough, UK). Slides were then dehydrated with ethanols and xylene and coverslipped using DPX mounting medium (VWR International Ltd, Lutterworth, UK). Sections incubated with SMI 31 and GFAP antibodies were rinsed after the appropriate biotinylated secondary antibody and incubated for 1 h with a dilution of 1:100 streptavidin-FITC (Serotec, Oxford, UK) in PBS. Sections were rinsed and coverslipped using vectashield with DAPI (Vector Laboratories, Peterborough, UK).

2.4. Quantification of the extent of demyelination

Using resin sections and an image of level 165 in the mouse brain atlas (http://www.hms.harvard.edu/research/brain) the regions showing myelin loss in each animal were delineated and then scanned to yield a figure that recorded the extent of demyelination in young and aged mice (Fig. 1).

2.5. Quantification of the axonal degeneration within the corpus callosum of C57BL/6 mice

Using an eye piece graticule that delineated an area of 250 μm^2 using a ×40 objective, the number of SMI 32 positive axonal spheroids in 4 randomly chosen areas of the callosal radiation of both hemispheres, and the medial corpus callosum, were counted. The values for each area