Activated non-neural specific T cells open the blood–brain barrier to circulating antibodies


Summary

Previous studies have shown that activated T cells can successfully cross endothelial barriers and will accumulate in tissue which contains their specific antigen. Myelin specific T cells (e.g. myelin basic protein specific) are recognized to play an important role in the induction of experimental autoimmune demyelinating disease of the CNS and have been shown to induce blood–brain barrier breakdown effectively. In this study we injected T cells reactive to a non-neural antigen (ovalbumin) systemically into Lewis rats and caused them to accumulate in the thoracic dorsal column by a prior injection of ovalbumin. Selected rats were given systemic demyelinating antibody, antimyelin oligodendrocyte antibody (anti-MOG antibody), to provide evidence of permeability changes to the blood–brain barrier. These animals were compared with control rats given systemic anti-P0 monoclonal antibody and to other rats given a direct micro-injection (3 µl) of anti-MOG antibody into the thoracic dorsal column. All animals were monitored by serial neurophysiological studies and by histological examination. Direct anti-MOG antibody injection produced a focal block in conduction at the injection site and a large circumscribed area of primary demyelination with axonal preservation within the dorsal column. An even more profound conduction block and more extensive plaque-like region of demyelination were seen in animals given antigen, activated T cells and systemic antibody. However, animals given antigen and T cells without relevant antibody did not show conduction impairment or demyelination, except when very large numbers of T cells were given; such rats developed severe irreversible axonal damage. This study demonstrates the blood–brain barrier is disrupted by activated T cells of non-neural specificity and allows large plaque-like regions of demyelination to form in the presence of circulating antimyelin antibody. The relevance of this finding to multiple sclerosis is discussed.

Keywords: blood–brain barrier; demyelination; activated T cell; myelin oligodendrocyte glycoprotein; conduction block

Abbreviations: BBB = blood–brain barrier; EAE = experimental allergic encephalomyelitis; i.p. = intraperitoneal; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein; PLP = proteolipid protein; SEP = sensory evoked potential

Introduction

Breakdown of the blood–brain barrier (BBB) is a crucial event in multiple sclerosis pathogenesis. Studies using MRI have shown that the first detectable change in new lesion formation or extension of old lesions is breakdown of the BBB (Miller et al., 1988; Kermode et al., 1990). The mechanisms involved in BBB breakdown have yet to be defined but some insights have resulted from studies in animal models, particularly experimental allergic encephalomyelitis (EAE) (Claudio et al., 1989). The use of gadolinium enhanced MRI studies in EAE animals has shown that BBB breakdown is necessary for the induction of disease (Hawkins et al., 1990).

An important role for activated T cells in BBB breakdown was clearly demonstrated in functional studies by Linington et al. (Linington et al., 1988) in which myelin basic protein (MBP)-activated T cells and antibody to myelin oligodendrocyte glycoprotein (MOG) were co-administered systemically to Lewis rats. Marked augmentation of demyelination was demonstrated in these animals compared with those given T cells alone, but no pathological changes resulted in animals given only anti-MOG antibody. Clearly BBB breakdown, associated with activated MBP T cells, permitted entry of anti-MOG antibody into the CNS. Histological studies by Lossinsky et al. (Lossinsky et al., 1989) and Claudio et al. (Claudio et al., 1990) in EAE have shown serum leakage into the brain associated with the presence of inflammatory cells and an increase in vesicular transport in BBB endothelial cells.
In more recent experiments Kojima et al. (Kojima et al., 1994) made the interesting observation that BBB opening could be achieved by T cells activated to CNS antigens not associated with the myelin sheath, i.e. the astroglial calcium binding protein S100β.

In this study we have addressed the question as to whether activated T cells of non-neural specificity could equally cause BBB breakdown, and hence permit potentially demyelinating antibody access to the CNS. In the lesions of multiple sclerosis, perivascular inflammatory cell infiltrates are commonly found and are presumed to play an important role in BBB breakdown; however, there is no evidence that these cells show specific reactivity to any particular CNS antigen such as MBP or proteolipid protein (PLP). The demonstration of an association between multiple sclerosis attacks and viral infections (Sibley, 1985; Anderson et al., 1993) suggests that other antigens such as those on infective organisms including viruses should be considered as potential T-cell targets. In this study, therefore, we have caused T cells of non-neural reactivity to accumulate within a predetermined area of the CNS. Breakdown of the BBB has been shown by the effects of systemically administered anti-MOG antibody leaking into the CNS.

Methods

Experimental design

Ovalbumin was injected into the dorsal column of 48 inbred Lewis J C rats, which were arranged in groups of six, matched for age, weight and sex. On the same day, 36 of these were given systemic intraperitoneal (i.p.) injections of ovalbumin reactive T cells. Twelve of these animals were given systemic anti-MOG antibody in RPMI (Roswell Park Memorial Institute)-based hybridoma cell medium on the day of injection and for the two subsequent days. Another 12 control animals received monoclonal antibody to P0 protein (i.p.) of the same isotype as the anti-MOG antibody, IgG1, while a third group of 12 received medium (i.p.) only. The remaining 12 of the 48 rats were not given ovalbumin responsive T cells but did receive systemic anti-MOG antibody in RPMI based medium to test BBB security after possible dorsal column injection trauma.

Conduction through the dorsal column including the injection site was measured before and thence serially after the ovalbumin injection by recording centrally stimulated antidromic somatosensory evoked potentials (SEPs) from the base of the rat’s tail. Half of all rats were sacrificed for histological examination at different time points throughout the study.

To test the activity of the anti-MOG antibody solution, three groups of 10 rats were given direct intraspinal injections of anti-MOG antibody, anti-P0 antibody or medium alone. These animals were also followed by serial electrophysiological and histological studies.

All procedures were in accordance with and approved by the Animal Care and Ethics Committee of the University of Sydney, Sydney, Australia.

Ovalbumin T-cell line

Ovalbumin reactive CD4⁺ T-cell lines were prepared as previously described (Pollard et al., 1995; Spies et al., 1995). In brief 12–14 week old Lewis J C rats were inoculated in the hind foot pads with 0.1 ml of an emulsion containing ovalbumin (30 mg/ml) in complete Freund’s adjuvant. Nine to eleven days later the draining lymph nodes were removed and a lymph node cell suspension prepared which was then incubated for 3 days in medium containing ovalbumin 40 μg/ml. Lymphoblasts were separated on a Ficoll gradient, resuspended in culture medium containing T-cell growth factor for 5–7 days, and following a further separation were restimulated with ovalbumin for 3–4 days. A stable T-cell line was generated by alternating antigen dependent activation episodes with T-cell growth factor driven propagation phases. T cells were harvested, counted and suspended in RPMI 1640 medium for immediate injection into recipient rats.

Antibodies and reagents

IgG anti-MOG antibody was obtained from the supernatant of a mouse hybridoma cell line (8–18C5) (Linington et al., 1984). Cells were thawed at 37°C and 5 × 10⁵ cells/ml were raised in RPMI 1640 medium containing 10–15% inactivated foetal calf serum, 1 ml 200 mM glutamine, 1 ml 100 mM sodium pyruvate, 1 ml penicillin/streptomycin (5000 IU/ml and 5 mg/ml), 1 ml Fungizone (amphotericin B 250 μg/ml) and 1 ml 5 mM mercapto-ethanol diluted to a volume of 100 ml with RPMI. After 7–12 days, the cells were spun down and the supernatant removed and frozen. IgG monoclonal antibody to P0 protein was prepared in a similar way and used for control experiments.

Demyelinating capacity of both of these antibodies was tested by injecting 2–3 μl augmented with 20% normal guinea pig serum as a source of complement, directly into the dorsal columns of two groups of 10 Lewis rats, while another 10 were similarly injected with a sample of hybridoma medium also containing normal guinea pig serum. Electrophysiological studies were conducted on all animals and selected animals sacrificed for histological studies.

Intraspinal injections

Mature female inbred Lewis J C rats were injected i.p. with 3 × 10⁶ freshly prepared ovalbumin reactive T cells. Within 4 h each animal was placed under 2% halothane-oxygen anaesthesia and the spinal cord exposed through a burr hole in the tenth thoracic vertebra (T10). A single unilateral intraspinal injection of 5 μl ovalbumin solution (1 mg/ml, Sigma Chemicals) in normal saline was made into the dorsal column using a hand held 30 gauge needle, its bevel facing medially, and attached by a fine vinyl tube to a microsyringe.
as previously described (Harrison and Pollard, 1984). An analgesic (pethidine 1 mg subcutaneous) and antibiotic (oxytetracycline HCl 10 mg intramuscular) were then administered. A 2 ml quantity of the day 8 hybridoma supernatant (anti-MOG antibody, anti-P0 antibody or supernatant alone) was then given (i.p.) and repeated on the following 2 days.

Electrophysiological studies

Rats were anaesthetized with 2% halothane in oxygen. SEPs were induced antidromically by supramaximal stimulation of the dorsal column both caudal (T12/13) and rostral (T8/9) to the T10 injection site, and recorded serially from the coccygeal nerves of the tail (Fig. 1). This technique obviates the need for paralysis and respiratory support to eliminate muscle artefact in the recordings. This tail SEP was identical to that recorded in pilot studies when animals were curarised and ventilated. The tips of 30G stainless steel needle recording electrodes were placed subcutaneously at marked points at the tail base (negative input) and near its tip (as the positive input to a high impedance differential preamplifier). The earth electrode was inserted over the gluteal muscles. Recorded responses were the average of 32 sweeps obtained through an analogue to digital converter (Applied Engineering) by an Apple II microcomputer at a repetition rate of 50/s, each sweep induced by a supramaximal stimulus. The amplitude of the tail SEP was defined as its greatest negativity measured from the base line. Reducing the acquisition rate to 1/s did not alter the mean amplitude of the responses at any point in the time course of the disease under study. The stimulus pulses, of 50 μs duration and from 3.6 to 18.0 V amplitude, were derived from an isolated voltage stimulator (Devices, MIV) triggered by the microcomputer and were delivered through a temporarily placed Teflon-coated stainless-steel needle electrode, its tip located extradurally between the vertebrae. The positive electrode was placed beneath the skin at the T4 vertebral level. The ratio of the tail SEP amplitudes from rostral and caudal stimulation (rostral : caudal ratio) was determined and used as a measure of dorsal column conduction (conduction block). For the purposes of this study, conduction block was taken as a drop of the rostral : caudal ratio of >20%.

For each day of measurement the amplitude ratios from individual animals in each group were averaged and the standard deviation determined. Two-tailed Student’s t test was used for statistical comparison between the groups. A P value of 0.05 or below was considered significant.

Histological studies

Rats were sacrificed at post-ovalbumin injection on days 2, 3, 4, 6, 9, 12 and 45, under deep pentobarbitone anaesthesia and their spinal cords from T6 to L4 quickly removed and immersed in cold 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight. The region about the injection site was divided into three portions 2 mm thick and further fixed in glutaraldehyde followed by 2% osmium tetroxide. Tissue blocks were dehydrated in graded concentrations of ethanol and propylene and then embedded in spurs epoxy resin. Thin sections were stained with toluidine blue for light microscopy. Ultra-thin sections mounted on copper mesh grids were double stained with lead and uranyl acetate for observation with a Philips 201 electron microscope.

Results

Electrophysiological studies

Direct intraspinal antibody injections

The tail recorded SEPs consisted of an initial negativity derived from the higher velocity somatosensory fibres followed by responses from the slower conducting fibres. The amplitude of the tail SEP due to stimulation rostral to the T10 dorsal column site injected with anti-MOG antibody, was shown to have diminished significantly by day 3 post-injection (Fig. 2), compared with tail SEPs induced through intraspinal injections of hybridoma medium alone or hybridoma medium containing anti-P0 antibody. It may also be seen from Fig. 2 that, whereas the tail SEP amplitude obtained from stimulation rostral to the site of injection at day 3 was markedly reduced, from stimulation caudal to the injection site it was undiminished, indicating conduction block only at the injection site. Following direct injection of anti-MOG antibody, a rapid decline in rostral : caudal ratio occurred over the first 3 days post-injection (Fig. 3A); recovery of conduction block commenced on day 10. No further sign of improvement could be seen after 35 days post-injection.

Ovalbumin injected animals

Dose response studies. The following results were obtained using the amounts of ovalbumin, ovalbumin reactive T cells and anti-MOG antibody described in the Methods. Numerous dose response studies had previously been conducted to determine these optimal amounts. When higher T-cell numbers were used, varying degrees of axonal damage

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occurred within injected dorsal columns; severe axonal damage resulted when the T-cell number was doubled. Reducing the anti-MOG antibody dose reduced the degree of demyelination.

**Time course of conduction block.** Animals injected with ovalbumin into the dorsal column and given ovalbumin T cells and anti-MOG antibody systemically showed changes in tail SEP amplitudes very similar to those directly injected intraspinally with anti-MOG antibody. From Fig. 4 it may be seen that following ovalbumin injection for 2 days conduction altered very little from the pre-injection value in either experimental (rostral : caudal ratio from 0.84 ± 0.09 to 0.82 ± 0.06 on day 2) or control rats given systemic anti-\(P_0\) antibody (from 0.83 ± 0.01 to 0.82 ± 0.02 on day 2). Thereafter, a rapid decline in conduction occurred only in animals given systemic anti-MOG antibody.

In experimental animals given anti-MOG antibody, conduction block neared its maximum value by 5 days post-ovalbumin injection at a value of \(0.24 ± 0.12\), which is significantly below \((P < 0.001 \text{ Student’s } t \text{ test})\) the rostral : caudal ratio of \(0.80 ± 0.04\) for control rats (Fig. 4). Recovery in conduction commenced from day 10 post-injection, progressed rapidly for 7 days, then more slowly until day 47 post-injection. No further improvement was detected thereafter up to day 66 post-injection, the maximum value attained being \(0.73 ± 0.08\) (day 47 post-injection), 0.11 below the pre-injection value and 0.09 below the final control group recovery level of \(0.82 ± 0.05\).

In anti-\(P_0\) antibody control animals a minimum conduction of \(0.79\) occurred on days 7 \((± 0.02)\) and 9 \((± 0.05)\). Recovery appeared complete by day 32, at a rostral : caudal ratio of \(0.82 ± 0.07\), showing no tendency to change thereafter.

In control animals lacking ovalbumin responsive T cells but receiving intraspinal ovalbumin injections and systemic anti-MOG antibody, a minimum conduction ratio of \(0.75 ± 0.02\) occurred on day 5 after a pre-injection conduction level of \(0.82 ± 0.02\). The drop in conduction did not differ significantly from that observed in the anti-\(P_0\) antibody control \((P > 0.1)\).
when examined from day 3 to day 12 (Fig. 6). However, in rats significant demyelination and minor degrees of axonal injury, 1 days following adoptive transfer of ovalbumin responsive CD4+ T cells, i.p. injections of anti-MOG antibody hybridoma supernatant, and intraspinal ovalbumin solution (closed circles); compared with responses from control rats similarly treated but receiving anti-P0 antibody hybridoma supernatant (i.p.) (open circles), and control rats receiving intraspinal ovalbumin, anti-MOG antibody (i.p.) but no T cells (open squares). A 2-day delay in onset of conduction loss was followed by a rapid conduction decline to day 5, only in rats given systemic T cells and anti-MOG antibody.

**Histological studies**

**Direct anti-MOG antibody injections**

In all animals given direct intraspinal injection a mild needle tract injury was evident in animals examined at 3–12 days post-injection. No further change was evident in animals injected with hybridoma supernatant with or without anti-P0 antibody.

In animals injected with anti-MOG antibody a localized region of demyelination placed within the dorsal column was evident at days 3–10 (Fig. 3B). Higher power sections show a region of demyelination placed within the dorsal column region of T-cell accumulation. However, in the absence of systemic antibody no more than occasional demyelinated fibres were seen. Rats given $3 \times 10^6$ T cells showed T-cell accumulation, no significant demyelination and minor degrees of axonal injury, when examined from day 3 to day 12 (Fig. 6). However, in rats given systemic anti-MOG antibody in addition to ovalbumin T cells, a large confluent focal area of demyelination was seen in the dorsal column in the region of ovalbumin injection (Fig. 7). Demyelination was first evident at day 3, but well developed at day 6. The area of demyelination produced was more extensive than that obtained by directly injecting anti-MOG antibody intraspinally. From day 3 to day 7 numerous phagocytic cells containing myelin debris were present within the lesions, but by day 9 large confluent areas of naked axons similar to multiple sclerosis plaques were seen (Fig. 7). At this stage the phagocytic cells were largely aligned around draining veins.

**Ovalbumin-injected animals**

All animals injected intraspinally with ovalbumin showed evidence of mild needle tract injury. In rats given intraspinal ovalbumin and systemic anti-MOG antibody without T-cell injections, a sparse inflammatory infiltrate was evident within the spinal cord from days 2–6. In animals given ovalbumin and ovalbumin T cells, marked focal T-cell accumulations within the dorsal column were seen from days 3–7. When larger numbers of T cells were given systemically, destructive lesions occurred within the dorsal column region of T-cell accumulation. However, in the absence of systemic antibody no more than occasional demyelinated fibres were seen. Rats given $3 \times 10^6$ T cells showed T-cell accumulation, no significant demyelination and minor degrees of axonal injury, when examined from day 3 to day 12 (Fig. 6). However, in rats

**Discussion**

Several important conclusions can be drawn from this study. The first is that very small amounts of an antibody, anti-MOG, reactive with an epitope localized on the surface of the oligodendrocyte and myelin sheath (Linnington et al., 1988) can, in the presence of complement, produce large focal areas of primary demyelination. Direct injection of the antibody in the absence of a T-cell response is sufficient to produce such lesions. Electrophysiological studies showed that the conduction failure began within hours of the antibody injection and maximal conduction block was attained by day 3 post-injection. Histological studies showed a close correlation between the occurrence of conduction block and demyelination.

Demyelinating antibody given systemically to rats intraspinally injected with ovalbumin/saline, did not penetrate the BBB and gain access to the CNS in amounts sufficient to cause conduction block or produce a demyelinating lesion. The mechanical trauma resulting from injection into the spinal cord by this technique or the mild inflammatory infiltrate that developed at the injection site did not significantly compromise BBB integrity. However, this study demonstrates clearly that in regions of activated T-cell accumulation and only there, focal leakiness of the BBB results, which permits antibody to enter the CNS. In such regions large plaques of primary demyelination may occur, if circulating antimyelin antibody is present. The important contribution of this study is that such leakiness of the BBB may result from the presence of activated T cells of any specificity.

Previous studies have shown that T cells reactive to brain antigens other than those on myelin can cause breakdown of BBB (Weckerle et al., 1994). This is the first demonstration that T cells of non-neural specificity can also cause a leaky BBB. It remains possible that T cells activated against non-neural antigens, i.e. viral or bacterial, may through a process of molecular mimicry recognize a peptide sequence of a CNS antigen on a perivascular antigen presenting cell and accumulate therein, hence causing BBB breakdown. In fact, sequence homologies between certain viral proteins and myelin proteins related to autoimmune encephalomyelitis have been demonstrated (Jahnke et al., 1985). Molecular mimicry is believed to play a role in the major demyelinating disease of
Fig. 5 Photomicrographs of toluidine-blue-stained semi-thin epoxy sections of the lesion site represented in Fig. 3B (dorsal is to the left). (A) Low power micrograph illustrating the full focal demyelinated lesion (L) within the dorsal column. Scale bar = 100 µm. (B) A higher power micrograph from the area of L above showing confluent demyelinated axons and illustrating the very selective demyelinating nature of the lesion. Axonal degeneration was not evident. Scale bar = 20 µm.

the peripheral nervous system, the Guillain–Barré syndrome; in some patients infected with Campylobacter jejuni, antibodies directed against this inciting organism have been shown to cross-react with neural ganglioside (Yuki et al., 1993). Although T cells specific for MBP have been derived from multiple sclerosis brain (Oksenberg et al., 1993) the evidence...
is not strong that MBP is a major autoantigen in multiple sclerosis. Moreover, T cells from multiple sclerosis patients reactive to MBP also react strongly to peptides from a number of common viruses (Wucherpfennig and Strominger, 1995). The specificity(ies) of the perivascular T cell infiltrates characteristic of multiple sclerosis lesions have yet to be determined, but this study shows that BBB breakdown may result from an aggregation of any activated T cells.

This study has not addressed the question of how T cells cause BBB breakdown. Physical penetration of endothelial cells and their basal lamina membrane by T cells as they pass from the vessels to Virchow–Robin spaces may contribute as well as enzymes and cytokines secreted by these cells and others they recruit. There is accumulating evidence that TNF is an important molecule in this process (Spies et al., 1995) and metalloproteinases may also play a role (Rosenberg et al., 1992; Gijbels et al., 1994).

A third important finding of this study is that our model,
Fig. 7 (A) Photomicrograph showing a large area of confluent demyelination containing multiple naked axons (arrow heads) in a rat given intraspinal ovalbumin antigen, ovalbumin T cells and systemic anti-MOG antibody. The entire lesion occupied most of the dorsal column. m = macrophage containing myelin debris. Scale bar = 20 μm. (B) Electron micrograph of an area within A above, showing naked confluent axons of normal appearance. Scale bar = 5 μm.

utilizing an antmyelin antibody and BBB breakdown, produces large confluent areas of demyelination reminiscent of multiple sclerosis lesions (Prineas, 1985). Wekerle (Wekerle, 1994) and others have drawn attention to the fact that several experimental models of multiple sclerosis lack this most fundamental pathological feature of the disease. T-cell
mediated models of inflammatory demyelination in the Lewis rat, both EAE and EAN, show very little demyelination (Linnington et al., 1988; Spies et al., 1995). Extensive demyelination is seen when these models are induced actively using crude antigens presumably since this method induces both T- and B-cell activation (Pollard, 1998). This study has produced further evidence that extensive demyelination, such as is seen in the lesions of multiple sclerosis, involves the participation of antibody. Although there has been considerable interest in the potential for various cytokines released by inflammatory cells to cause demyelination, in the control animals of this study large numbers of activated inflammatory cells accumulated in the injected dorsal column without significant demyelination. Similar findings were described by Wekerle et al. (Wekerle et al., 1994) and Kojima et al. (Kojima et al., 1994) in models using T cells activated to neural antigens. Our results together with those cited above suggest the need for further studies of potential demyelinating antibodies in multiple sclerosis patients.

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References


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