Blood–Brain Barrier Disruption And Lesion Localisation In Experimental Autoimmune Encephalomyelitis With Predominant Cerebellar And Brainstem Involvement

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Abstract
The role of the blood–brain barrier (BBB) in determining lesion distribution was assessed in an atypical model of experimental autoimmune encephalomyelitis (EAE) induced in C3H/HeJ mice by immunisation with peptide 190–209 of myelin proteolipid protein, which can result in two distinct types of EAE, each with distinct lesion distribution. Areas of the BBB showing constitutively greater permeability in naïve mice did not correlate with the lesion distribution in EAE. BBB disruption occurred only in sites of inflammatory cell infiltration. Irrespective of the clinical type, the BBB was disrupted in the cerebellum and brainstem. Pertussis toxin had no effect on lesion distribution. Thus, lesion distribution is not influenced solely by BBB permeability.

Keywords
experimental autoimmune encephalomyelitis; multiple sclerosis; blood–brain barrier; cerebellum; brainstem; vascular endothelium

1. Introduction
The blood–brain barrier (BBB) is a specialised selectively permeable barrier which is unique to the vasculature in the central nervous system (CNS). Its role is to protect the CNS from potentially harmful agents in the peripheral circulation and it is largely impermeable to macromolecules, and closely controls the movement of hematogenous cells into the CNS. It has been shown, however, that activated T lymphocytes can freely cross the intact BBB in a random fashion in search of their specific antigen, a process known as immunological surveillance (Hickey, 2001). If these T cells do not encounter their antigen, they exit the CNS without incident. Conversely, if the activated T cells encounter their antigen in association with the appropriate MHC molecule on an antigen-presenting cell within the CNS, a potentially detrimental inflammatory reaction can be elicited, leading to the influx of inflammatory cells and disruption of the BBB. Such is the case in experimental autoimmune encephalomyelitis (EAE), a putative animal model of multiple sclerosis (MS). The mechanisms by which the BBB permeability increases during the disease process in EAE are not completely understood; however, it is thought that it involves a combination of cell mediated injury, and molecular dependent disorganisation of BBB structures, e.g. loss of the tight junction proteins occludin and zonula occludens-1 (Bolton et al., 1998), loss of endothelial barrier antigens (Sternberger et al., 1989), and proteolytic degradation of the extracellular matrix.

It is well accepted that there are areas of the BBB that are not as “tight” as others, i.e. the circumventricular organs and spinal root entry and exit zones (Pedersen et al., 1997 and Ueno et al., 2000). Based on lesion formation in the CNS parenchyma adjacent to these areas, Juhler et al. (1985) suggested that it is via these permeable microvessels that the inflammatory cells and/or antibodies traverse the BBB to initiate CNS inflammation in EAE and MS. Most EAE animal
models are characterised by a clinical course of ascending paralysis. Predisposition of the spinal cord to early damage and the resultant typical caudal to rostral pattern of lesion development in EAE have been suggested to occur due to increased fragility of the BBB in this area (Cross et al., 1993, Juhler et al., 1984 and Namer et al., 1993). Recent findings, however, have challenged this view. Tonra et al. (2001) found that SJL/J mice immunised with peptide 139–151 of myelin proteolipid protein (PLP139–151), which develop an ascending paralysis form of EAE, show increased permeability of the cerebellar BBB to rabbit IgG prior to the onset of clinical signs of EAE and prior to increases in permeability of BBB in the lumbosacral region of the spinal cord. Recent magnetic resonance imaging (MRI) studies have additionally shown that early after the onset of clinical signs of ascending paralysis EAE in Lewis rats immunised with guinea pig myelin basic protein there is increased leakage of gadolinium into the CNS not only in the spinal cord but also in the brainstem and cerebellum (Floris et al., 2004). However, it still remains controversial how BBB damage correlates with the sites of lesion development in EAE and MS and whether BBB breakdown is necessary for the development of inflammatory lesions or whether BBB damage occurs as a result of the presence of inflammatory cell infiltration.

The aim of the current study was to examine the relationship between the spatial and temporal breakdown of the BBB and lesion localisation using an atypical model of EAE induced by immunisation of C3H/HeJ mice with PLP190–209. The majority of these mice and other H-2^k strains susceptible to this peptide develop an acute monophasic form of EAE, characterised by loss of balance and coordination, which results in the animals rolling in an axial rotatory manner (Greer et al., 1996 and Muller et al., 2000). In this model, lesions are found predominantly in the brainstem and cerebellum, and to a lesser extent in the sacral and lumbar spinal cord (Muller et al., 2000). Regardless of where the lesions occur the cellular composition is similar, being composed of lymphocytes, macrophages and a large number of neutrophils. After initially showing signs of axial rotatory EAE, a percentage of the mice subsequently develop clinical signs of ascending paralysis. Less frequently, mice do not develop axial rotatory EAE, but instead develop typical ascending paralysis EAE (Muller et al., 2000). The mice that develop clinical signs of ascending paralysis develop lesions mainly in the lumbosacral spinal cord. This model is therefore a particularly useful one in which to study BBB breakdown, as two distinct clinical forms with different lesion localisation can occur, thus allowing the questions of temporal and spatial breakdown of the BBB and their relevance to lesion localisation to be addressed. The role of Bordetella pertussis toxin, an agent which is thought to increase vascular permeability via enhancing the activity of vasoactive amines, in determining localisation of lesions in this model is also examined.

2. Materials and methods

2.1. Animals
Female C3H/HeJ mice (6–8 weeks old) were purchased from the Animal Resources Centre (Perth, Australia). Mice were immunised at 8–10 weeks of age. All animals were maintained in accordance with the guidelines of the National Health and Medical Research Council of Australia.

2.2. PLP peptide
PLP peptide 190–209 (SKTSASIGSLCADARMYGVL) was synthesised by Auspep (Melbourne, Australia) according to the human sequence (Hudson et al., 1989) and was >95% pure by high performance liquid chromatography and mass spectral analysis. This peptide is moderately hydrophobic and was dissolved at a concentration of 5 mg/mL in 0.2 M acetic acid prior to dilution in phosphate-buffered saline (PBS).

2.3. Active induction of EAE
Mice were injected subcutaneously with 100 µg of PLP190–209 peptide and 400 µg Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit) in an emulsion with complete Freund's adjuvant (CFA, Difco). The mice also received 300 ng pertussis toxin (List Biological Laboratories, Campbell, California) intravenously at the same time as the peptide and again 3
days later, except in experiments investigating whether pertussis toxin affects lesion distribution as detailed in Section 3.5.

2.4. Clinical evaluation
Mice were weighed daily and assessed for clinical signs of EAE. Signs of ascending paralysis EAE were scored according to the following criteria: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; 5, moribund state. Signs of axial rotatory EAE were assessed as previously described (Muller et al., 2000); 0, no disease; 1, head turned slightly; 2, head turning more pronounced; 3, inability to walk in a straight line; 4, lying on side; 5, rolling continuously unless supported.

2.5. Horseradish peroxidase (HRP) injection
Horseradish peroxidase (HRP) (0.4 mg/g body weight) diluted in saline was injected intravenously. Ninety minutes later, the mice were perfused for histology (see Section 2.6)

2.6. Histology for HRP visualisation
Animals were anaesthetised and perfused through the aorta with saline followed by 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 and postfixed in a 1:2 dilution of the perfusate. One hundred micrometers transverse serial vibratome sections were cut and collected into 0.1 M phosphate buffer. Processing of the tissue was based on the method described by Ueno et al. (2000). Sections were transferred to 3,3'-diaminobenzidine (DAB) incubation medium with hydrogen peroxide. After visualisation of reaction product was achieved, sections were washed in 0.1 M phosphate buffer, mounted onto poly-L-lysine coated microscope slides and covered. Approximately 45 sections were analysed from the cerebellum and brainstem and approximately 100 sections from the lumbar and sacral spinal cord from each mouse. All sections were analysed using light microscopy for presence of peroxidase staining. The scale for measuring HRP extravasation was: −, no HRP extravasation; +, small amount of HRP visible in areas immediately surrounding inflammatory infiltrate; ++, HRP extravasation extending into the parenchyma and staining resident cells in areas immediately adjacent to the inflammatory infiltrate; ++, majority of section stained with HRP; ++++, entire section stained with HRP. Inflammation was assessed as follows: −, no inflammation; +, mild inflammation, 1–3 lesions/high power field (hpf); ++, moderate inflammation, 4–6 lesions/hpf; +++, extensive inflammation, 7–10 lesions/hpf; ++++, confluent inflammation. hpf=×20 magnification.

2.7. Histological analysis of sections from mice with active EAE not receiving pertussis toxin
At the peak of disease these animals were perfused through the aorta with periodate-lysine-paraformaldehyde. The brainstem, cerebellum and spinal cord were removed and 15 µm transverse frozen sections were cut and stained with hematoxylin for lesion visualisation.

3. Results
3.1. The cerebellum is the initial site of increased BBB permeability in actively induced axial rotatory EAE
Ten C3H/HeJ mice were immunised with PLP190–209 to induce EAE. At the peak of clinical signs or 3 days following the onset of clinical signs, mice were given an intravenous injection of HRP and were perfused 90 min later. The clinical scores for these mice are shown in Table 1. Five of the mice displayed only axial rotatory signs of EAE, 3 mice initially developed axial rotation and subsequently developed ascending paralysis, and 2 of the mice developed only ascending paralysis.
Tissue was processed and analysed to detect HRP extravasation and inflammation. The presence of inflammation within the CNS was identified by positive peroxidase staining within subsets of inflammatory cells following the DAB reaction. Inflammatory lesions were located primarily in the perivascular regions of the white matter of the cerebellum, the cranial nerves within the brainstem and to a lesser extent of the white matter of the spinal cord. Neither inflammation nor HRP extravasation was observed in the CNS rostral to the brainstem. HRP staining in the parenchyma did occur in the absence of clinical signs but was not found in any areas of the CNS that were not affected by inflammation. The intensity of staining was generally proportional to the severity of inflammation (Fig. 1).

### Table 1
Clinical and histopathological scores of C3H/HeJ mice immunised with PLP190–209

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Axial rotatory score</th>
<th>Ascending paralysis score</th>
<th>Day of onset post-immunisation</th>
<th>Day of neurological signs on which perfused*</th>
<th>Inflammation</th>
<th>HRP extravasation</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Cerebellum/brainstem</td>
<td>Spinal cord</td>
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<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>16</td>
<td>3</td>
<td>+++</td>
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<tr>
<td>2</td>
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<td>0</td>
<td>16</td>
<td>3</td>
<td>+++</td>
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<tr>
<td>3</td>
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<td>0</td>
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<td>3</td>
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<tr>
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<td>0</td>
<td>19</td>
<td>2</td>
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<td>0</td>
<td>24</td>
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<tr>
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</table>

*Day 1=1st day of neurological signs.

Fig. 1. HRP staining in the cerebellar parenchyma in a mouse with active EAE (×2.5). V, fourth ventricle; Cb, cerebellum; →, perivascular HRP extravasation in areas of inflammation; ←HRP staining in white matter of the cerebellum.

In the cerebellum and brainstem, HRP staining was most intense perivascularly, coinciding with the presence of inflammatory infiltration, but was also present in neuronal cell bodies in the surrounding parenchyma and appeared to follow the white matter tracts. Inflammatory cells were often seen adhering to the lumina of the blood vessels in areas where HRP extravasation and cellular infiltration, including red blood cells, were evident in the parenchyma. In the spinal cord the inflammation was primarily submeningeal and disseminated from the surface of the spinal cord and tended to be more severe in the root entry and exit zones and in the dorsal and ventral columns. HRP staining was diffuse; however, there was a concentration of...
staining around the central canal (Fig. 2A). HRP staining was particularly apparent in areas affected by inflammation (Fig. 2B).

Fig. 2. Spinal cord of mouse with active EAE (×20). (A) , HRP staining is concentrated around the central canal; , inflammatory cells. (B) , massive inflammation and HRP extravasation in the sacral spinal cord.

All five mice that exhibited only axial rotatory clinical signs showed inflammation and HRP extravasation in the cerebellum and brainstem. In three of these mice there was subclinical inflammation in the spinal cord (Table 1, Fig. 3), but only one of these three showed HRP extravasation in the spinal cord. In the three animals that developed both axial rotation and ascending paralysis, inflammation and HRP extravasation were present in the cerebellum, brainstem and spinal cord. In the two animals that exhibited only ascending paralysis (Mice 9 and 10 in Table 1), inflammation and HRP extravasation were present in the spinal cord, and subclinical inflammation and HRP extravasation occurred in the cerebellum and brainstem (Fig. 4).
3.2. Normal BBB permeability does not account for lesion distribution in C3H/HeJ mice

Three normal unimmunised C3H/HeJ littermates of mice immunised for active induction of EAE received an intravenous injection of HRP on the same day as their littermates with EAE and were analysed as for the EAE mice. No obvious leakage of HRP into the CNS was observed in the areas that corresponded to the pattern of lesion distribution in our EAE model (i.e. brainstem, cerebellum and spinal cord) (Fig. 5). Red blood cells were visible within the blood vessels due to the presence of endogenous peroxidase. The circumventricular organs (areas adjacent to the ventricles), i.e. area postrema, median eminence and the subfornical organ were the only areas that showed increased permeability indicated by low level HRP staining (Fig. 6).
3.3. Parenchymal HRP staining is not due to endogenous peroxidase activity in the CNS

One potential problem associated with using HRP extravasation as a marker of BBB permeability in EAE studies is that red blood cells, neutrophils, eosinophils and macrophages produce a positive reaction product in tissue sections treated with DAB, due to the presence of endogenous peroxidase (O’Brien, 2000 and Sugiyama et al., 2001). To investigate whether the HRP staining seen in the mice with EAE was due to endogenous peroxidase activity or was definitely due to increased BBB permeability, 4 C3H/HeJ mice at the peak of clinical signs of EAE were perfused without prior injection of HRP and processed as detailed in Section 2.6. Two of these mice developed only axial rotatory clinical signs (scores of 4 and 5) and two developed initial severe axial rotation (scores of 5), followed by mild clinical signs of ascending paralysis (limp tails). Red blood cells and inflammatory cells were visualised using the DAB substrate via the reaction with the endogenous peroxidase present in these cells, with the formation of a brown reaction product similar to that produced by HRP. As expected, there was no evidence of HRP staining in the parenchyma, except within the infiltrating hematogenous cells themselves. The endogenous peroxidase reaction product showed that the localisation of inflammatory cells correlated well with the type and severity of clinical signs (Fig. 7A and B).
3.4. BBB permeability does not increase when there is no clinical or histological evidence of disease

Three mice were immunised for active induction of EAE and were injected with HRP and perfused prior to the development of clinical signs of EAE (Day 15 post immunization (PI)). There was no evidence of cellular infiltration, no endogenous peroxidase activity (apart from in red blood cells within vessels) and no HRP staining visualised in the CNS parenchyma of these animals (Fig. 8).
3.5. *B. pertussis* toxin on its own has no effect on BBB permeability and does not determine lesion distribution in C3H/HeJ mice

Pertussis toxin has been used for many years to enhance the development of EAE in mice, and it has traditionally been believed that it increases BBB permeability (Linthicum et al., 1982), although more recent studies suggest it acts via other mechanisms (Blankenhorn et al., 2000). To determine the effect of pertussis toxin on the BBB in the C3H/HeJ model, 4 non-immunised mice were injected with 300 ng pertussis toxin intravenously on day 0 and day 3, and then injected with HRP and perfused within the same time frame as that used for mice in which EAE was induced. As expected, the mice injected with pertussis toxin alone did not develop any clinical signs of EAE. Histologically, these mice showed the same results as for normal non-immunised animals, with red blood cells within vessels being the only site of peroxidase activity within the CNS.

To ensure that pertussis toxin did not effect lesion distribution, 10 animals were immunised for induction of EAE but did not receive injections of pertussis toxin. Four out of the 10 animals developed clinical signs of EAE, with 2 animals developing axial rotation and 2 developing ascending paralysis. Histological analysis showed that the pattern of lesion distribution correlated closely with the clinical signs and was similar to that in those animals with EAE that did receive pertussis toxin.

4. Discussion

This study demonstrates that although the BBB in the cerebellum and brainstem of normal non-immunised C3H/HeJ mice does not show increased permeability to HRP compared to other regions of the CNS, these areas are the major sites of lesion formation and BBB disruption in mice in which EAE has been induced. This cerebellar and brainstem inflammation and BBB disruption occur not only in those animals that display axial rotatory clinical signs of EAE, but also in those that display clinical signs of ascending paralysis. However, animals that exhibit only signs of axial rotation rarely showed evidence of BBB disruption in the spinal cord, and little evidence of inflammatory cell infiltration in the spinal cord. Thus, irrespective of the clinical form of EAE, the cerebellum and brainstem are sites of BBB disruption.
The occurrence of lesions and increased vascular permeability in the cerebellum and brainstem in EAE has not been widely reported or discussed in the past. This may be due to the fact that scoring systems in EAE do not generally take into account vestibular disturbances or to the belief that the inflammation in the spinal cord is the major clinical concern. In the Lewis rat, lesions occur in the spinal cord, but not the cerebellum, during the first attack of EAE, whereas when EAE is reinduced lesions also occur in the cerebellum (Gordon et al., 2001). Recently, several papers using MRI techniques have reported that the permeability of the BBB in the brainstem and cerebellum increases early in the pathogenesis of ascending paralysis EAE (Floris et al., 2004, Rausch et al., 2003 and Seeldrayers et al., 1993). Tonra et al. (2001) found that, in SJL/J mice with PLP139–151-induced EAE, which develop ascending paralysis, disruption of the cerebellar BBB occurs prior to the disruption of the BBB in the spinal cord and the onset of clinical signs. These findings, together with the results from the current study, strongly suggest that, at least in some strains of mice, the cerebellum and possibly the brainstem have increased susceptibility to cellular infiltration and BBB disruption in EAE.

There are many ideas about the temporal features of BBB breakdown in EAE, with various reports of increases in permeability of the BBB either prior to the onset of clinical signs (Koh et al., 1993), at the time of onset of clinical signs (Floris et al., 2004 and Leibowitz and Kennedy, 1972), prior to the onset of inflammation (Floris et al., 2004), at the initial stage of cellular infiltration (Koh et al., 1993), or following cellular infiltration (Rausch et al., 2003). Juhler et al. (1985) suggested that the permeability of the BBB increases in different areas of the CNS at different times, reporting that the BBB in the lumbar spinal cord increased in permeability 1 day before the onset of clinical signs, whereas in other areas of the CNS it was found to coincide with the onset of clinical signs. In the EAE model used in the current study, we have shown that clinical signs do not occur before BBB disruption. Additionally, the presence of inflammatory cells within the spinal cord in the absence of HRP staining in the parenchyma indicates that inflammatory cells have entered the CNS prior to the disruption of the BBB. There is mounting evidence to suggest that inflammatory cells, particularly neutrophils, can migrate across the vascular endothelium via a transcellular route rather than a paracellular route without damaging either the integrity of the BBB or “loosening” the tight junctions (Engelhardt and Wolberg, 2004). Therefore, once sufficient numbers of cells have migrated into the CNS, the BBB may then be disrupted due to the action of proinflammatory mediators released by these cells. These mediators have the capacity to affect the structural organisation of the BBB and transendothelial transport, thus increasing vascular permeability. This was also suggested by Claudio et al. (1990) who found that, in areas of extensive inflammation, the interendothelial space and the permeability at the interendothelial junction was increased, indicating that inflammatory cells may regulate permeability of the tight junctions.

The current study also demonstrates that *B. pertussis* toxin on its own has no effect on BBB permeability, and does not affect the distribution of lesions in actively induced EAE in C3H/HeJ mice, although it did increase the incidence of disease. This was not completely unexpected, as C3H/HeJ mice are one of the few strains of mice that are not susceptible to vasoactive amine sensitization elicited by histamine (VAASH) (Ma et al., 2002), the vascular permeabilisation properties of which are thought to be enhanced by *B. pertussis* toxin. Our results support suggestions that pertussis toxin works by mechanisms other than increasing BBB permeability.

We have shown that in C3H/HeJ mice certain areas of the CNS, particularly the cerebellum and brainstem, are more susceptible to inflammatory cell infiltration and BBB disruption than others, and that inflammatory cells accumulate in the CNS parenchyma before BBB breakdown. Based on these findings, it is suggested that, in future, research into preventive therapies for MS should consider the factors contributing to the susceptibility of certain areas of the CNS to inflammatory insult. Such factors may include increased concentration of target antigen, increased availability of antigen-presenting cells, increased cytokine/chemokine production and increased adhesion molecule expression in the CNS.
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References


