RESEARCH ARTICLE

Abnormal Endothelial Tight Junctions in Active Lesions and Normal-appearing White Matter in Multiple Sclerosis

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Blood-brain barrier (BBB) breakdown, demonstrable in vivo by enhanced MRI is characteristic of new and expanding inflammatory lesions in relapsing-remitting and chronic progressive multiple sclerosis (MS). Subtle leakage may also occur in primary progressive MS. However, the anatomical route(s) of BBB leakage have not been demonstrated. We investigated the possible involvement of inter-endothelial tight junctions (TJ) by examining the expression of TJ proteins (occludin and ZO-1) in blood vessels in active MS lesions from 8 cases of MS and in normal-appearing white (NAWM) matter from 6 cases. Blood vessels (10-50 per frozen section) were scanned using confocal laser scanning microscopy to acquire datasets for analysis. TJ abnormalities manifested as beading, interruption, absence or diffuse cytoplasmic localization of fluorescence, or separation of junctions (putative opening) were frequent (affecting 40% of vessels) in oil-red-O-positive active plaques but less frequent in NAWM (15%), and in normal (<2%) and neurological controls (6%). Putatively "open" junctions were seen in vessels in active lesions and in microscopically inflamed vessels in NAWM. Dual fluorescence revealed abnormal TJs in vessels with pre-mortem serum protein leakage. Abnormal or open TJs, associated with inflammation may contribute to BBB leakage in enhancing MRI lesions and may also be involved in subtle leakage in non-enhancing focal and diffuse lesions in NAWM. BBB disruption due to tight junctional pathology should be regarded as a significant form of tissue injury in MS, alongside demyelination and axonopathy.

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system characterized by the irregular development through time of multiple focal lesions throughout the neuraxis, but with a predilection for optic nerves, periventricular white matter, brain stem and spinal cord (2, 37, 38). Current views of the pathology of MS paint a complex picture in which marked lesional heterogeneity is postulated to reflect the action of multiple pathogenic mechanisms whose involvement and tempo may vary with time and differ between cases (23, 37, 38, 59). Ruddick et al suggest that following an early inflammatory phase characterised by gadolinium-enhancing brain lesions and responsiveness to anti-inflammatory drugs, a more entrenched autoimmunity develops and the disease progressively becomes more degenerative in character (59). However, the seeds of this secondary progression are sown during the early inflammatory phase, when cumulative, largely irreversible injury to both the oligomyelin unit and the axons first occurs (54, 59, 62, 65). Other scenarios have been postulated to account for the imaging and pathological findings (37). Thus, the possibility has been raised that in certain cases, pre-plaque pathology in the form of "subtle, progressive alterations in tissue integrity" (70) or demyelination (20, 43) may precede inflammation and BBB leakage

A phase of gross focal disruption of the BBB, associated with inflammation has indeed consistently been demonstrated in vivo in acute and chronic active MS lesions using gadolinium-enhanced magnetic resonance imaging (GdGTPA MRI) (21, 27, 42, 44, 55, 63). In addition there is evidence of demyelination occurring during the initial phase of gadolinium enhancement, as indicated by mobile lipid peaks representing myelin breakdown products, on short transfer echo proton MR spectroscopy (14). Correlative MRI-pathological studies of acute lesions have revealed these focally enhanced areas to be sites of fresh lesions showing intense inflammation, oedema, perivascular cuffing, and in some cases parenchymal mononuclear cell infiltration (5, 11, 16, 26, 52). Furthermore, there is pathological and specialized MRI evidence of a persisting lower amplitude leakage in both chronic active and chronic inactive lesions, which is not detectable by routine enhanced MRI scanning (12, 55). A recent quantitative

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MRI study (61) has confirmed the presence of a subtle BBB leak in such "visibly non-enhancing" focal lesions, and in agreement with other studies (18, 40, 70, 71), suggests that there may also be a subtle widespread BBB disturbance in MS normal-appearing white matter (NAWM). In the study by Silver et al, this was most marked in progressive MS, and in particular, primary progressive MS (60). If confirmed, such findings support the hypothesis that disturbance of the BBB "is an invariable and perhaps obligatory event in the development and expansion of lesions," (32) and of non-focal pathological abnormalities in MS (3), not only in relapsing-remitting and chronic progressive forms but also in primary progressive MS (12, 55, 61).

Although numerous enhanced MRI studies have clearly revealed the fluctuating pattern of vasocentric inflammation in MS, the anatomical pathology and physiological mechanism(s) of the increased barrier permeability has not yet been clearly demonstrated. Previous pathological studies confined largely to chronic inactive plaques have demonstrated evidence of slight serum protein leakage and changes in the ultrastructure of endothelial cells, consistent with alterations in transendothelial vesicular transport (12, 31). Pathological studies of acute lesions are more limited but have shown leakage of plasma proteins (20), and in one report, electron microscopic evidence of an open interendothelial tight junction (TJ) (9). TJs, disposed as a series of continuous (zonular) junctions between adjacent endothelial cells in the lining of the cerebral vascular walls, serve to maintain the restrictive permeability of the BBB (30). In this important role they function as both barriers to occlude the paracellular cleft between adjacent cells and as fences to maintain the apico-basolateral polarity of each endothelial cell (17, 25, 68).

In view of these previous MRI and pathological findings in MS, and as there there is increasing evidence of the disruption of BBB tight junctions (TJ) in other inflammatory and infective CNS diseases (7, 8, 10, 13, 36), we designed the present semi-quantitative study primarily to investigate the condition of the TJ proteins occludin and zonula occludens-1 (ZO-1) (65) in active (34, 60, 69) MS lesions and in MS NAWM. For this study we used robust immunofluorescence and confocal microscopic protocols, optimised for snap-frozen autopsy tissues.

Materials and Methods

Tissues and histology. Case selection was retrospective on the basis of a confirmed clinical and neuropathological diagnosis of MS and availability of suitable snap-frozen tissue samples containing either active lesions or NAWM. Preliminary "oil-red O" (ORO) screening of blocks from 21 MS autopsy cases resulted in the identification of 8 cases which contained plaques with oil-red O-positive areas, indicating active disease (20, 34, 60, 69). In 2 of these 8 cases, formalin-fixed, paraffin-embedded tissue blocks were available from areas adjacent to the blocks used in the frozen section study. Sections (7 μm) from these paraffin blocks were immunostained for HLA-DR using diaminobenzidine as peroxidase substrate as described, then washed and stained for myelin by standard luxol fast blue as described previously (45).

The study of cases with active disease incorporated autopsy CNS tissue samples (45 blocks) from 8 active MS cases (Table 1). Control white matter for this study was obtained from 2 normal (road traffic accident) and 5 other neurological disease (OND) controls (1 head injury, 2 sub-acute sclerosing panencephalitis (SSPE), 1 metastatic carcinoma and 1 transverse myelitis). In addition, following completion of the study of "active" lesions a further set of 17 blocks (designated "NAWM") in which neither plaques nor ORO staining could be detected were identified from 6 MS cases (Table 1), including 4 not used in the study of active lesions.

All tissue blocks had been obtained fresh at early autopsy (<24 hours for 11 out of the 12 MS cases, see Table 1 for details) and were snap-frozen in liquid nitrogen-cooled isopentane prior to storage in a -70°C freezer. Cryostat sections (12 μm) were cut onto aminopropyltriethoxysilane (APES)-coated glass slides and stained routinely with H&E/ORO for histology and grading of lesion activity. For immunocytochemical procedures, sections were fixed in ice-cold acetone for 10 minutes and then air-dried. Sections from all 62 MS and all 34 control blocks were labelled by indirect immunofluorescence (see below) for HLA-Dr to enable the assessment of microglial activation. Similarly, sections from all active MS and control blocks were labelled for fibrinogen (FITC) to assess on a regional basis, the occurrence of pre-mortem serum protein leakage (20). Additional indirect immunofluorescent antibody labelling and biotinylated lectin labelling was applied to the MS and control sections, as detailed in the following section, to enable assessment of vascular integrity and the state of the inter-endothelial tight junctions. A total of 450 data sets were gathered from the 8 active MS cases using confocal microscopy (see details below) and assessed offline for abnormal TJ protein expression and disposition. Each dataset consisted of the

Case No.	Age/ Sex	Disease Duration (yrs)	Clinical Diagnosis	Death-autopsy interval (h)	No. of blocks studied			Neuropathological Summary
					Active MS	NAWM	Control	
MS1	40/F	17	SPMS	11	3	2		Plaques throughout brain in WM. Most extensive in SC.
MS2	34/M	10	SPMS	1.5	5			Plaques in varying stages of demyelination in cortex, pons and SC.
MS3	58/M	20	SPMS	24	3			Extensive demyelination in cerebral cortex, midbrain and SC.
MS4	35/M	9	SPMS	7.5	7			Extensive demyelination particularly in the regions of the lateral ventricles
MS5	53/F	25	SPMS	19.5	13			Extensive demyelination extending to the cerebellum, SC and deep white matter
MS6	22/F	5mths	acute MS	10	10	5		Active plaques observed in pons
MS7	24/M	17mths	acute MS	3.5	2			Multiple active plaques throughout brain and SC
MS8	54/M	6	SPMS	12	2			Most extensive demyelination in SC
MS9	60/F	25	SPMS	29		1		Chronic plaques in frontal, temporal and parietal lobes and SC.
MS10	62/M	12	SPMS	23		4		Chronic periventricular plaques in an otherwise generally normal white matter
MS11	66/M	26	SPMS	4.5		1		Demyelination concentrated in tempero-parietal and occipital regions.
MS12	69/F	46	SPMS	18		4		Extensive chronic demyelination in brainstem and SC. A few periventricular plaques in WM
ONDC1	45/M	2	transverse myelitis	8.5			5	Primary demyelination of SC
ONDC2	16/M	4	SSPE	8			2	Measles virus present in cortex. Widespread perivascular inflammation
ONDC3	21/M	7	SSPE	10			2	Measles virus abundant in cortex.
ONDC4	18/M	n.a.	Head injury	10			4	Normal histology outside area of contusion
ONDC5	74/F	n.a.	Bronchial Carcinoma	10			8	Normal histology outside area of metastatic tumour
NC1	24/F	n.a.	r.t.a.	14			3	Normal histology
NC2	29/F	n.a.	r.t.a.	4			10	Normal histology

SPMS, chronic secondary progressive phase of relapsing remitting MS: PPMS, primary progressive MS: WM, white matter: SC, spinal cord: SSPE, subacute sclerosing panencephalitis. ONDC, other neurological disease control: NC, normal control: NAWM, normal-appearing white matter: n.a., not applicable r.t.a., road traffic accident

Table 1. Summary of clinical and neuropathological details.

image series obtained on confocal scanning of a single intercepted vessel segment on a section cut from one of the 45 MS blocks from the 8 active MS cases. Recognizable vessels whose profiles were contained within a $\times 40$ field on the confocal microscope were imaged. Larger vessels, which extended beyond the confines of the field were excluded, as were very small fluorescent objects which could not reliably be identified or assessed without further enlargement. Ten such datasets were obtained from a section from each active MS block. In a similar manner, a total of 300 data sets were obtained from the 34 control blocks. For the separate study of TJ abnormality in NAWM, 962 datasets were obtained from 17 blocks (ca 55 data sets per block)

Immunofluorescence. Indirect immunofluorescence was carried out on tissue sections using the following antibodies and dilutions: monoclonal antibodies to HLA-Dr (Novocastra, 1:100), leucocyte common antigen (LCA, Dako, 1:50), ZO-1 (Zymed, 1:25), Laminin (Sigma, 1:1000) and polyclonal antibodies to occludin (Zymed, 1:50) and ZO-1 (Zymed, 1:25). A biotinylated *Ulex europaeus* agglutinin (UEA) (Vector, 1:500) was used as the primary marker for endothelial cells, supplemented in some cases by Factor VIII (polyclonal antibody, Signet, 1:100). Serum protein leakage was assessed using an antibody to fibrinogen conjugated to FITC (Dako, 1:100)

For single-label immunofluorescence, primary antibodies, biotinylated UEA, and fibrinogen-FITC were incubated on sec-

tions overnight at 4°C. Following two 5-minute washes in 10 mM phosphate buffered saline (PBS), pH 7.6, sections were incubated for one hour at 37°C in rabbit anti-mouse Alexa 488 (Molecular probes, 1:500) for monoclonal antibodies or swine anti-rabbit FITC (Dako, 1:50) for polyclonal antibodies. Biotinylated UEA was detected by incubating in Z-Avidin FITC (Zymed, 1:50) for one hour at 37°C. Following further PBS washes, sections were counter stained for thirty seconds using propidium iodide (Sigma, 2 μg/ml), washed in PBS then mounted in citifluor™.

Dual-label immunofluorescence was carried out with polyclonal anti-rabbit occludin or ZO-1 and either HLA-Dr, fibrinogen-FITC, laminin, LCA, or biotinylated UEA following a sequential detection method. Polyclonal antibody was incubated for one hour at 37°C, washed in PBS and detected by incubating in goat anti-rabbit Alexa 568 (Molecular probes, 1:500) for one hour at 37°C. Sections were washed in PBS then incubated in monoclonal antibody for one hour at 37°C, washed in PBS, then detected by incubating in rabbit anti-mouse Alexa 488 (HLA-Dr, LCA, laminin) or Z-avidin FITC (biotinylated UEA) for one hour at 37°C. Sections were finally washed in PBS and mounted in citifluor.

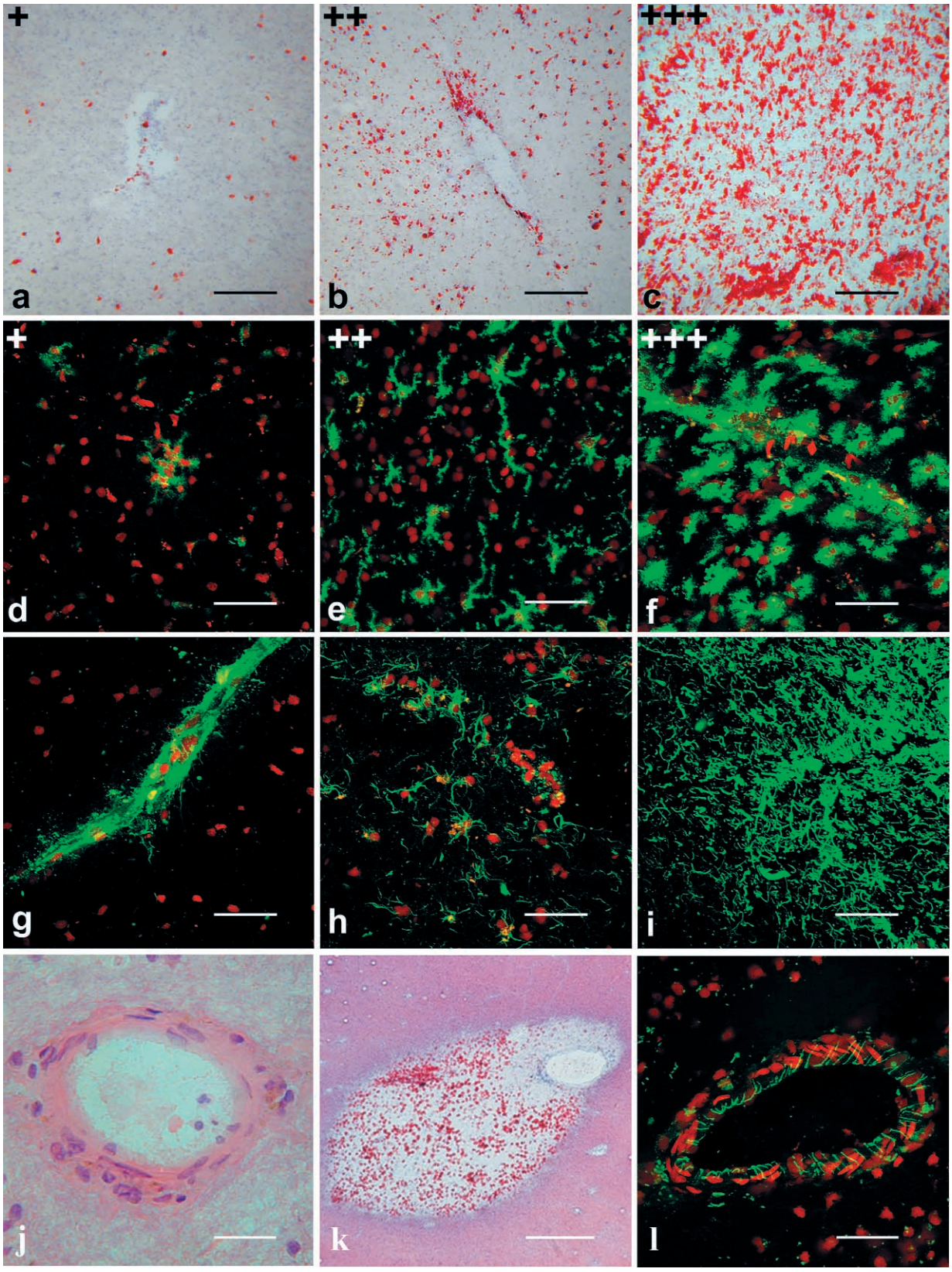
Confocal microscopy. A Leica TCS/NT confocal microscope equipped with a krypton/argon laser was used to examine all sections. Alexa 488 and FITC-labelled secondary antibodies were visualised by excitation at 488nm with a 500-538 band-pass emission filter. Alexa 568-labelled secondary antibodies were imaged by excitation at 568nm with a 568-596 band-pass emission filter. Acquiring 16 levels averaged 4 times through the 12 μm specimens generated data sets from which composite projected images were saved using Adobe PhotoShop 5.0 at 300 dpi without further digital manipulation.

Results

Tissue characterisation and grading. In the 2 MS cases where parallel formalin-fixed blocks were available (case 1, case 3), LFB-positive myelin inclusions were seen within HLA-DR positive foamy macrophages at the white matter/plaque interface (data not shown). In the frozen blocks there was a wide range of reactivity with ORO. Thus, all blocks from the control cases were ORO-negative as were 28 of the MS blocks, comprising 11 from areas of active lesions and all 17 from NAWM. In the "active lesion" areas, 13 MS blocks displayed scattered isolated ORO-positive cells (Figure 1a) while 13 had in addition, one or more foci or clusters of ORO-positive cells (Figure 1b). In 8 blocks, however, ORO-positive cells were abundant throughout the entire section (Figure 1c). Expression of HLA-Dr varied widely in both control and MS blocks. In controls, the blocks from the 2 cases of road traffic accidents, 1 case of metastatic carcinoma and 1 case of transverse myelitis showed only small numbers of HLA-Dr-positive parenchymal microglia. The blocks from the case of head injury and from the 2 SSPE cases showed widespread moderate HLA-Dr positivity either in the parenchyma or in the perivascular infiltrates surrounding blood vessels. In the SSPE cases such HLA-Dr positivity was associated with areas in which there was direct evidence of measles virus (mv) infection (intracellular inclusions and immunoreactivity for mv proteins, data not shown). In 2 active blocks of MS tissue, no HLA-Dr positive cells were detected. Staining of scattered, isolated microglia, or microglial clusters in the parenchyma (Figure 1d) was seen in 8 active MS blocks and 5 MS NAWM blocks. Twenty-one of the active MS blocks and 7 NAWM blocks displayed a diffuse and widespread moderate HLA-Dr positivity, which involved either parenchymal process-bearing cells or cells in the distribution of small perivascular infiltrates (Figure 1e). In a further 14 (active) MS blocks and in 5 NAWM blocks, large HLA-Dr positive cells, corresponding to foamy macrophages seen on H&E staining, were abundant in both perivascular and parenchymal distributions throughout the entire section (Figure 1f).

Extravascular fibrinogen staining was not observed in any of the blocks from control cases except in the case of head injury where it was seen only in the area of recent haemorrhagic contusion, which was excluded from the areas examined for TJ proteins. Neither was it observed in 12 of the blocks containing active lesions. In 33 of the active MS blocks, however, a fibrillary pattern of extravascular fibrinogen staining was present (Figure 1g-i). Most commonly the fibrinogen leakage had a distinct perivascular distribution (Figure 1h), though in a few blocks it was also present more widely throughout the parenchyma (Figure 1i).

Histological orientation. Conventional light microscopy, used for orientation purposes and for correlative ORO/confocal studies yielded images (Figure 1j–l) which facilitated neurohistological correlation and neuropathological interpretation.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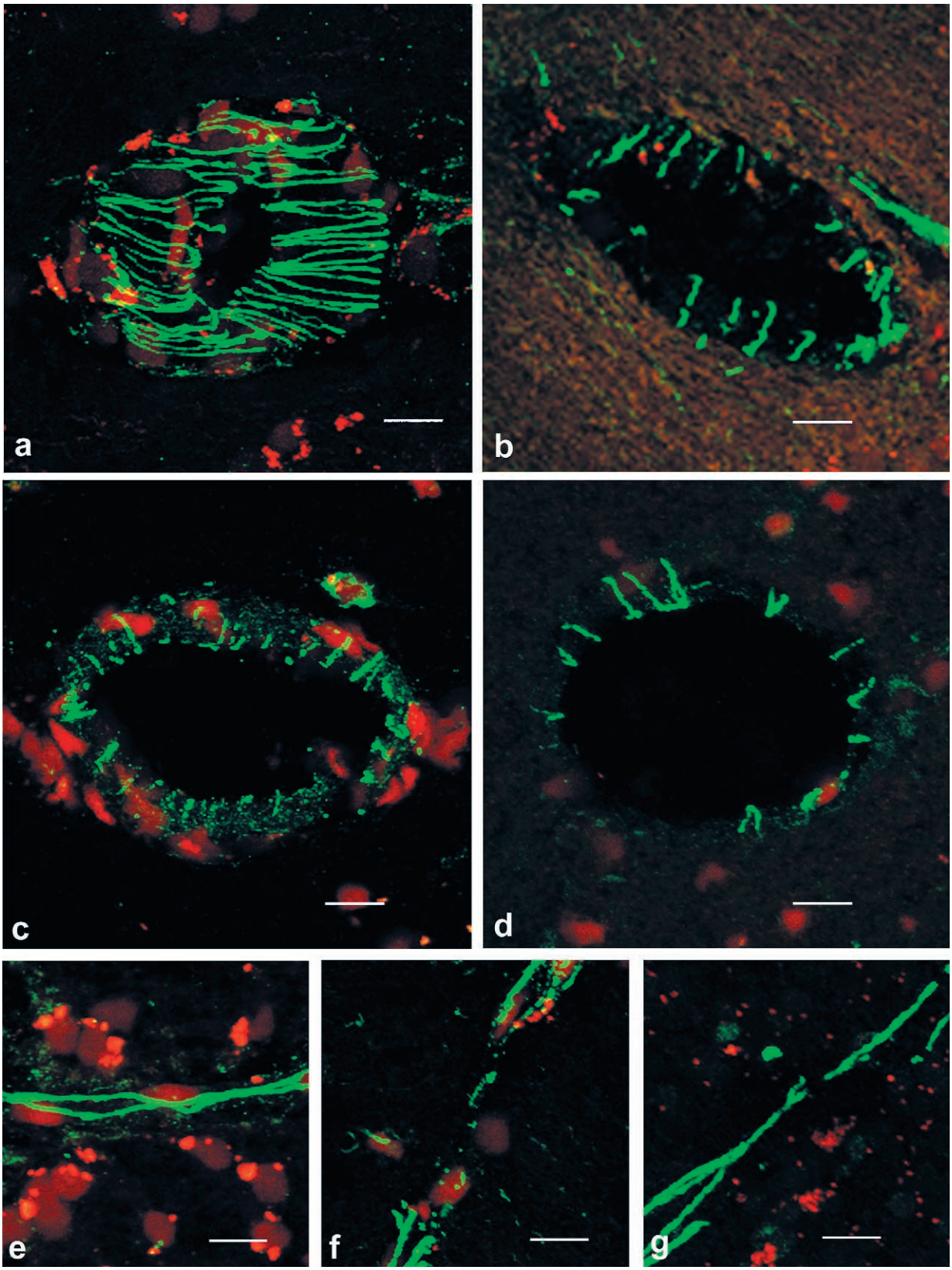
TJ protein expression in normal brain and other neurological diseases (OND). Immunofluorescent marking of normal and OND control CNS tissues with either occludin or ZO-1 resulted in a continuous, linear staining pattern of strong intensity in blood vessels. Our indirect IF technique was optimised for the demonstration of ZO-1 and occludin proteins associated with TJs in endothelial cells and has not been fully tested beyond that use. In the course of this study, therefore, no attempt has been made to record systematically the occurrence of ZO-1 or occludin outside the endothelium. In longitudinal view, fluorescent staining of TJs in vessels was predominantly axial and linear, with occasional anastomoses/bifurcations. In transversely sectioned vessels, however, TJ staining revealed short, radial or near-radial, continuous fluorescent bands (Figure 2a). There was no consistent numerical or spatial relationship between the number of fluorescent bands seen and the number or disposition of nuclei as revealed by the propidium iodide counterstain. Thus, in some vessels, probably in spasm, the number of visible intercellular contacts considerably exceeded the number of nuclei visible (Figure 2a). Such appearances may result from displacement of nuclei to other regions of transversely sectioned vessels in spasm. Only 2% of the blood vessels examined in the normal control brain and 4% of blood vessels in the neurological control brains displayed any abnormal staining or distribution of TJ proteins (Figure 2b).

TJ protein expression in active lesions. For this analysis, abnormality was judged in comparison to the continuous, intense linear expression, and regular disposition observed in normal control brain (Figure 2a) or in normal vessels in MS brain (Figure 2e). The projected confocal images of 10 blood vessels from each section were analysed offline and initially scored, without further quantitation, according to the appearance of the TJ marker as either wholly normal or containing one or more abnormal TJs. The abnormalities recognized in our study include the following: interruption or beading (Figure 2c, 2f); absence (Figure 2c, 2g); separation or opening (Figure 4a); reduced clarity of TJ bands, with a tendency towards more diffuse intra-cytoplasmic staining (Figure 5a). The proportion of blood vessels containing abnormal tight junctions was much greater in tissue from these 8 active cases than in normal, OND, NAWM tissue (Figure 3a), or in chronic inactive MS lesions (unpublished preliminary observations). Abnormal expression of ZO-1 or occludin was shown in 37 to 46% of assessed MS blood vessels, with a trend towards a higher frequency in the lesions with the most ORO. Despite this, vessels with abnormal tight junctions were common even in some of the ORO-negative blocks from these active MS cases.

Abnormality was focal, never affecting all junctions within a single transected vessel. Thus, typically both normal-appearing and abnormal junctions co-exist in varying proportions in the same image, whether of transversely or longitudinally sectioned vessel segments (Figure 2 c, d, f, g). Because of the heterogeneity of size and plane of intercept (ie, shape) of vessel segments comprising our datasets, it was not possible to make a reliable comparative assessment of the severity of TJ disruption within individual vessel segments. All calibres of vessels studied were affected by the abnormalities described above but putatively "open" junctions were seen only in some of the larger vessels identified as veins or venules (Figure 4a) There was no correlation between the length of the death post mortem interval and the frequency or type of abnormalities either in this group of active blocks, in controls or in NAWM (data not shown).

Figure 1. (Opposing page) Digital photographic (**a-c**, **j**, **k**) and confocal microscopic immunofluorescent images (**d-i**, **l**) from snap-frozen, cryostat sections of MS tissue.
a. ORO grade +: neutral lipids are present but sparse in this active MS lesion. ORO, bar = 200 μm
b. ORO grade ++: frequent neutral-lipid positive cells in an active MS lesion. ORO, bar = 200 μm
c. ORO grade +++: abundant neutral lipid positive cells in an active MS lesion. ORO, bar = 200 μm
d. HLA-DR grade +: Single cluster of microglial cells within an active MS lesion. HLA-DR (FITC) / propidium iodide, bar = 50 μm
e. HLA-DR grade ++: Numerous positive, branching microglial cells throughout an active MS lesion . HLA-Dr (FITC) / propidium iodide, bar = 50 μm
f. HLA-DR grade +++: Abundant, plump macrophages within a perivascular cuff and surrounding parenchyma of an active MS lesion .. HLA-Dr (FITC) / propidium iodide, bar = 50 μm
g-i. Variation of serum protein leakage within MS lesions. All are fibrinogen (FITC) / propidium iodide. (g) Slight perivascular leakage, though still largely confined to the vessel and not spreading far into the parenchyma. (h) moderate perivascular leakage in a fibrillary pattern (i) severe and widespread leakage. bars = 50 μm
j-k. Conventional light microscopy of vessels representative of those at the upper limits of size used in the confocal microscopic study. **j**. H & E; **k.** ORO / Haematoxylin, bars = 50, (**j**), 200 μm (**k**)
l. The vessel in k, adjacent to an intense focus of lipid phagocytes, is shown in a parallel section after staining to reveal tight junctional proteins and nuclei. Occludin / propidium iodide, bar = 50 μm.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TJ proteins and microglial activation in active MS lesions. Comparison of areas with different grades for HLA-Dr showed that blocks with the most microglial activation also had (marginally) the highest frequency of vessels with abnormal TJs (Figure 3b), though even the HLA-Dr-negative areas had significant levels of TJ abnormality. Dual labelling for occludin and HLA-Dr provided clear evidence for abnormality coincident with high HLA-Dr positivity on the numerous foamy macrophages, which were so evident in the vessel wall and throughout the surrounding parenchyma (Figure 4c).

TJ protein expression and microglial activation in NAWM. The proportion of junctions in NAWM that were clearly abnormal (15%), while greater than in controls was considerably less than the number found in active lesions. Thus, 85% of the NAWM vessels examined had no definite abnormality (Figure 3c). All sections of NAWM examined showed microglial activation and there was no evidence of any correlation between the extent of abnormality and the degree of microglial activation.

TJ proteins and lymphocytic infiltration in active lesions and in normal appearing white matter (NAWM). Lymphocytic infiltration as detected by immunofluorescence for LCA was observed in many sections. LCA-positive cells were sequestered to the perivascular cuff and only very rarely seen in the surrounding parenchyma. Dual immunofluorescence for LCA and occludin showed TJ abnormality in the presence of infiltrating lymphocytes (Figure 4d). Evidence of TJ abnormality associated with leucocyte infiltration was also apparent in a dual-fluorescent (ZO-1 and LCA) confocal image from an MS NAWM block examined earlier, in advance of the major quantitative study of TJs (Figure 4b). Such junctions, similar to the "open" junctions found in the active MS blocks were not seen in controls. They showed an apparent separation of the normal TJ band into 2 parallel bands each with a diminished TJ protein signal.

TJ proteins and serum protein leakage. Serum protein leakage as detected by anti-fibrinogen FITC was observed in 70% of the MS blocks examined and in none of the normal or OND control cases. In contrast to the intra-vascular localization of fibrinogen-FITC fluorescence in controls, fibrinogen staining in the active MS cases was often extravascular and disposed in a fibrillar pattern (Figure 1g). There was no correlation between the extent of such staining and the length of the death-post mortem interval (data not shown). Dual-labelling, where applied, enabled the detection of TJ abnormalities in such vessels. These comprised a loss of TJ band clarity with increased cytoplasmic staining (Fig 5a). No significant difference in the appearances of the TJ proteins was attributable to the use of different fluorophores necessary for dual labelling.

Blood vessel proteins. Uniform and continuous expression of markers for endothelium (UEA, factor VIII), and blood vessel wall (laminin) was observed in sections from all MS and control blocks even in vessels in which double labelling revealed abnormal TJ expression (Figure 5b).

Discussion

Immuno-labelling and confocal laser scanning microscopy together provide a reliable method for the demonstration of inter-endothelial junctions in blood vessels in the CNS. The unfamiliarity to most neurohistologists of the high magnification confocal images generated causes some difficulty in interpretation, as might be expected with a novel technique. We have sought to minimise this by fully documenting the range of appearances seen, by devising a descriptive terminology and through the use of semi-quantitative scoring systems that reduce subjectivity. We have also used parallel conventional microscopy and parallel section multiple

Figure 2. (Opposing page) All images are confocal microscopic images from snap-frozen, cryostat sections.
a. "Continuous, linear and intense" appearance of the interendothelial TJ protein ZO-1 as viewed in a section of a cerebral blood vessel, in spasm, from a normal control brain. ZO-1 (FITC) / propidium iodide, bar = 20 μm
b. Abnormality and partial absence of TJ protein expression in a blood vessel from outside the area of contusion in a case of closed head injury. Such abnormalities accounted for only 10% of the vessels examined. ZO-1 (FITC) / propidium iodide, bar = 20 μm
c. Beaded and discontinuous abnormal TJ protein expression in active MS lesion. occludin (FITC) / propidium iodide bar = 20 μm
d. Abnormal TJ protein expression in an active MS lesion, affects more than 50% of the vessel circumference. ZO-1 (FITC) / propidium iodide, bar = 20 μm
e. Continuous bright linear fluorescence marking tight junctions in a normal small vessel, MS tissue. ZO-1 (FITC} / propidium iodide, bar = 20 μm
f and g. Beaded and interrupted fluorescence characterize abnormal capillaries in active MS lesions. ZO-1 (FITC} / propidium iodide, bar = 20 μm

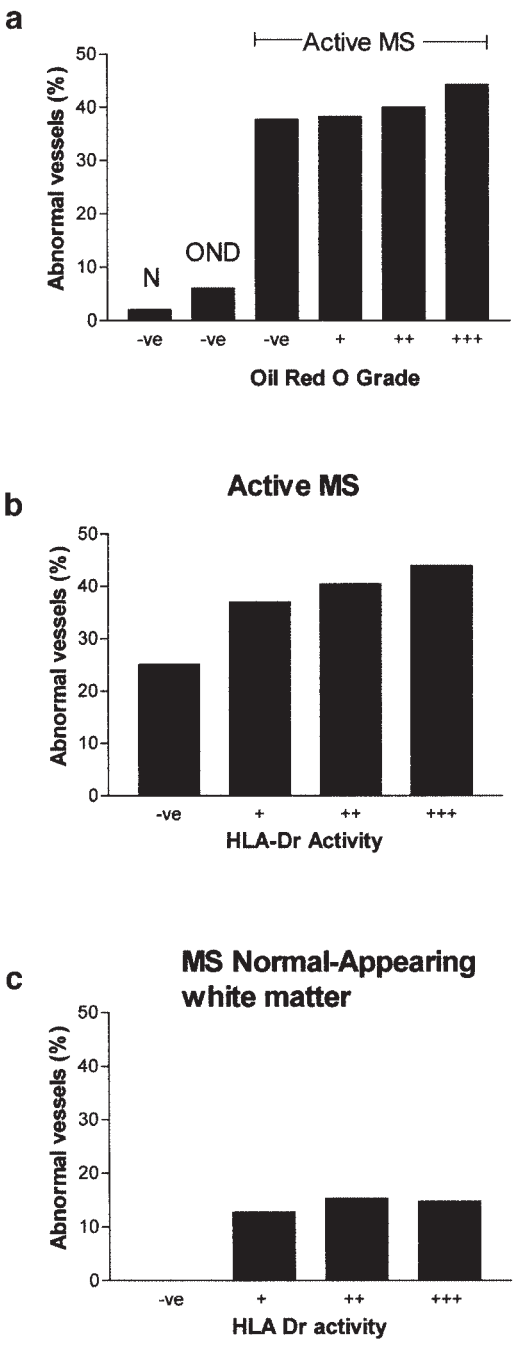

Figure 3. Relationship between TJ disruption and graded characteristics of tissues in active MS lesions and in normal-appearing white matter (NAWM) .
a. Data from normal control (N), other neurological disorders (OND) and MS tissue from cases with active lesions. Percentage of blood vessels examined which showed TJ disruption, assessed across ORO grades.
b. Data from MS active lesions. Percentage of blood vessels examined which showed TJ abnormality, assessed across HLA-Dr activity grades.
c. Data from MS normal-appearing white matter (NAWM). Percentage of blood vessels examined which showed TJ abnormality, assessed across HLA-Dr activity grades.

labelling or dual labelling, wherever possible, as an aid to orientation. Application of this methodology to archived pathological tissues has revealed an increased incidence of abnormal endothelial TJ in active MS lesions. This contrasts with our findings of relatively few abnormal TJ in chronic inactive MS lesions (ongoing study, data not shown) and virtually none in normal CNS and points to a pathological alteration of the paracellular pathway through the BBB. The demonstration of open TJs in active lesions is consistent with Brosnan and Claudio's earlier electron microscopic demonstration of an open inter-endothelial cell junction in an inflamed venule from a patient with acute MS (9). The similar TJ abnormality detected in association with an inflamed vessel in NAWM may point to mechanisms operating in the early inflammatory stages of plaque evolution. It adds to the mounting evidence of biochemical, histological, and imaging abnormalities in the white matter outside plaques, consistent with both inflammatory changes and subtle BBB leakage (3, 18, 40, 61, 71).

The association of TJ damage in MS with perivascular inflammation and with microglial activation suggests possible causal mechanisms. Previous evidence from experimental studies of inflammation indicate that TJ disruption and leakage are common though not inevitable accompaniments to inflammatory infiltration (7, 8). Such changes, directly associated with inflammatory cell infiltration would tend to be associated with veins and venules rather than capillaries in which inflammatory mediators or their products have been implicated in causing leakage (9). Transendothelial migration (diapedesis) of neutrophils in an in vivo rat model led to blood-brain barrier breakdown involving loss of the TJ proteins ZO-1 and occludin and morphological evidence of abnormal junctions by electron microscopy. The authors attributed these changes to the "triggering of signaling cascades which caused junctional disorganization" (7). In post mortem studies of HIV-1 associated dementia, the presence of damaged tight junctions indicated by fragmentation or absence of immunoreactivity for occludin and ZO-1, correlated with the levels of monocyte infiltration (8, 13). The restriction of the "putatively open" junctions to venules in active lesions and microscopically abnormal NAWM and the absence of similar alterations in chronic plaques

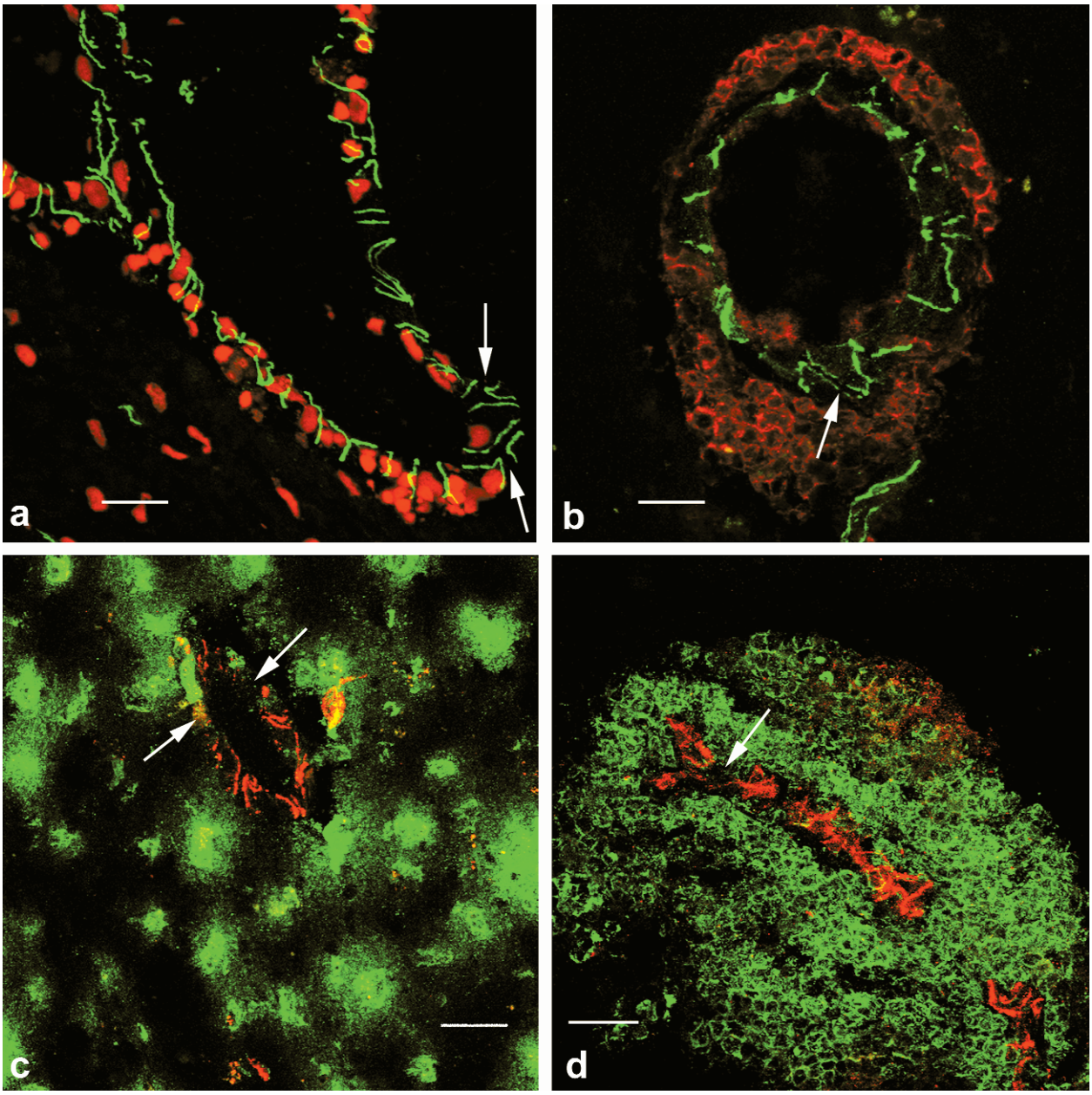

Figure 4. TJ abnormalities in active MS lesions. All images are confocal microscopic images from snap-frozen, cryostat sections
a. "Open" TJs (arrows) in active MS lesion. ZO-1 (FITC) / propidium iodide, bar = 25 μm
b. An open TJ (arrow) in a blood vessel with a perivascular cuff in MS normal-appearing white matter (NAWM). ZO-1 (FITC) / LCA (Cy3), bar = 25 μm
c. Foamy macrophages in the perivascular cuff and surrounding parenchyma of a blood vessel expressing abnormal TJ (arrows). Active MS lesion. HLA-Dr (FITC) / Occludin (Alexa-568), bar = 30 μm
d. A lymphocytic infiltrate cuffs a collapsed vessel which shows abnormal TJs. Active MS lesion. LCA (FITC) / Occludin (Alexa-568), bar = 30 μm

may point to a specific mechanism of reversible junctional disruption restricted to such vessels, and related to the latter's involvement in the diapedesis of inflammatory cells (56).

Leaving aside the issue of inflammatory cell infiltration per se, the coincidence of the greatest number of abnormal junctions with the highest levels of microglial activation (ie, HLA Dr up-regulation) in the present

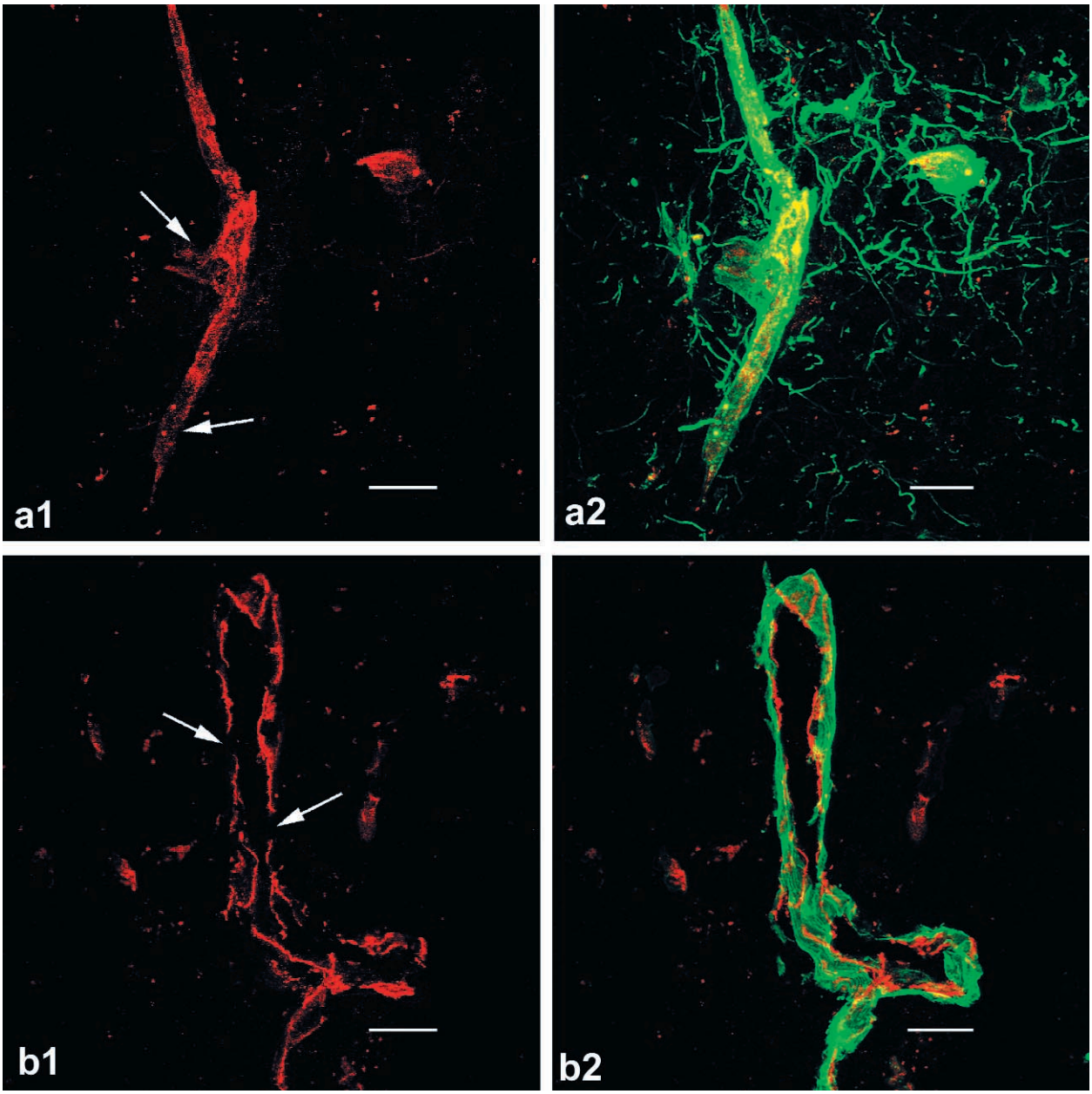

Figure 5. TJ abnormalities in active MS lesions. Dual-labelled immunofluorescence confocal microscopy
a1. Red channel alone, reveals TJs showing loss of intensity, beading and discontinuity (arrows), with increased, diffuse cytoplasmic staining . Occludin (Alexa-568), bar = 25 μm.
a2. Both channels together, shows serum protein leakage from the same vessel, revealed by presence of fibrillar extravascular fibrinogen staining. Occludin (Alexa-568), Fibrinogen (FITC), bar = 25 μm
b1. Red channel alone shows small focal discontinuities in TJs(arrows). Occludin (Alexa-568), bar = 25 μm.
b2. Both channels together shows intact basal lamina of same blood vessel. Occludin (Alexa-568), Laminin (FITC), bar = 25 μm

study of active MS suggests that some of the TJ abnormalities may result from the pathophysiological action of cytokines, matrix metalloproteinases, and other immune effectors present in the disordered milieu of the MS lesion (28, 72). Microglia and their infiltrating precursors the monocytes have both been shown to interact with elements of the blood-brain barrier in HIVE, adversely affecting TJs (8, 13, 33). Correlation between fragmentation and decreased immunoreactivity for ZO-1 and occludin and the accumulation of perivascular

macrophages has also been found in experimental simian immunodeficiency virus encephalitis (36). The authors of that study were unable to ascertain whether such changes precede macrophage accumulation or are secondary to the chronic presence of macrophages around cerebral vessels. Recent data suggests that activated microglia exert a powerful neurodegenerative influence in HIVE (33). Microglial activation, frequent and widespread in our NAWM tissue samples was indeed accompanied by a degree of TJ abnormality, though less than that found in active lesions.

Pre-mortem BBB leakage is clearly a feature of active lesions as indicated by our detection of fibrinogen in a fibrillary pattern outside the vessels in over 70% of such blocks. Such staining was not seen in any of our control cases and within the MS cases its occurrence was unrelated to the length of the death-post-mortem interval (data not shown). Association of such leakage with areas of microglial activation is consistent with our finding of increased incidence of TJ abnormality in the same areas. Confocal microscopic evidence of TJ abnormality was also detectable beyond these areas of microglial activation, though because of the limited histological information available from our frozen section samples we were unable to determine whether this represented a plaque interior or periplaque. In studies of HIV-1 encephalitis a close correlation has been found between the occurrence of diminished or absent TJ protein expression and serum protein extravasation, as indicated by both perivascular and diffuse fibrinogen leakage and diffuse astrocytosis (13). As our grading of the degree of fibrinogen leakage was block-based rather than blood vessel-based and we had only few dual-labelled sections, we were unable to address this correlation here though it will be important to do so in any future studies.

Disruption of TJs (ranging from altered TJ protein expression to "opening") as shown here is one of 2 major pathological changes which have been associated with leaky BBBs in disease and which may have a bearing on the route of leakage (4, 50, 66). Thus, while disruption of TJs as demonstrated by alterations in ZO-1 and occludin immunoreactivity in HIVE and malaria (10, 13) or by ultrastructural morphometric changes in inter-endothelial clefts and absence of elevated vesicular profiles in Alzheimer's disease (64) point to the probable involvement of the paracellular route, vesicular transport as demonstrated in chronic relapsing experimental allergic encephalomyelitis (EAE) in guinea pigs (22) and in chronic progressive MS (12) points to a transcellular one. It should be noted that the methodology best suited to demonstrate these two types of pathology is usually quite different, and has so far precluded the simultaneous collection of reliable data on both aspects in either EAE or MS. Although abnormal TJs were not demonstrated in either of the latter studies of the vesicular route in chronic disease, their presence at low frequency in chronic MS lesions cannot be excluded since routine TEM is not ideally suited to detection of scattered and infrequent pathological alteration. Indeed, preliminary CSLM study of chronic inactive lesions in our laboratory (data not shown) has revealed the occurrence of such changes. Ideally a combination of technical approaches is desirable (35) though this may only be fully achieved in experimental studies. Such an approach has recently been applied outside the CNS, to explore the possible role of small inter-endothelial gaps (of 0.1-3 μm between cells normally joined by TJs), as possible paracellular pathways for plasma leakage in inflammation (41). Gap formation would almost certainly involve alterations in the expression of TJ proteins, and it is possible that what we have described in active MS lesions and NAWM points to the formation of such gaps in this inflammatory disease.

Confocal microscopy and sensitive immunofluorescent immunocytochemistry are now clearly the methods of choice for the demonstration of TJ abnormalities in disease (7, 13, 51). Not only can they sensitively detect some forms of "open" junctions, but also with appropriately chosen antibodies, they can give additional insights into the range of disruptive molecular changes which may affect TJs. Routine transmission electron microscopy (TEM) on ultrathin sections has with one exception (9) revealed only "normal" interendothelial junctions in MS. Although such methodology is capable of detecting abnormalities in tight junctions, assessment of scattered focal changes as revealed in the present CSLM study probably requires either detailed ultrastructural morphometry on well-fixed material or immuno-electron microscopy (64,47). Indeed, the first of these methods when applied to biopsy tissues from patients with Alzheimer's disease detected abnormalities in inter-endothelial junctions that suggested "leakiness" of the blood-brain barrier (64). Immuno-electron microscopy has also recently been used successfully to supplement light microscopic immunohistochemistry in studies of TJ proteins in the blood-retinal barrier in whole-mount preparations of blood vessels in the rabbit (47). Clearly, both these high-resolution methods are very highly sensitive, and have the potential to yield further insights if applied selectively to MS, though the availability of suitable tissue would be a limiting factor.

However, where quantitative data is sought on the incidence or distribution of abnormality in a large number of vascular profiles, and where the disruption of TJs is focal the comparative sampling inefficiency of TEM may be a limitation. For this reason, the potential role of paracellular change in contributing to BBB leak may previously have been underestimated.

The use of ZO-1 as a marker for the cytoplasmic link between integral membrane proteins and the cytoskeleton in endothelial TJs is standard practice (7, 8, 10, 13, 36) despite its wider involvement in other junctions and in non-epithelial cells (24, 67). Moreover, it is a responsive marker of TJ dysfunction. Thus interferon-γ (IFN-γ) -induced loss of ZO-1 from epithelial tight junctions in vitro correlates with altered localization of occludin, perturbation of apical actin, disorganization of the tight junctions, and an increase in paracellular permeability (73). Occludin is more specific to the TJ than ZO-1 (17, 19), though like ZO1 it also has been detected outside of TJs. Thus, in vitro studies have demonstrated occludin expression by indirect immunofluoresence in activated T-lymphocytes (1), and in cultured astrocytes (in which expression decreased with differentiation) and neurons (5). Despite this, occludin has been successfully used as the integral membrane protein marker of choice for TJs in the majority of pathological studies of the CNS BBB (7, 10, 13, 36, 51). Recognition of an even closer association between the presence of the claudins 1 and 5 and endothelial TJ integrity has recently emerged (35, 48, 49, 68), but it remains to be seen whether antibodies directed at these proteins, as they become more widely available, will provide different, more reliable or more comprehensive data in the range of human disorders previously examined with antibodies to occludin. Our present approach, comparable with the majority of the recently published pathological literature on TJ in disease has clearly enabled detection of TJ abnormalities in MS, pointing to a possible paracellular route of BBB leakage.

The results of this study underline the dramatic nature of the events that occur at the blood-brain barrier during the earliest stages of the pathogenesis of the individual MS lesion (9). While the systemic or local triggers or other antecedents of such events have not been identified, there is speculation and some circumstantial evidence that viral or other infection may sometimes be involved (4, 29,39). The spectrum of changes resulting from the far-reaching immunological up-regulation of the cerebral endothelium associated with leukocyte infiltration ranges from physiological adaptation (15) to frank damage (46). BBB disruption, resulting in changes to both paracellular and transendothelial permeability should be regarded as one of three distinct and important forms of tissue injury in MS, alongside demyelination and axonal injury. As such, its characteristics are that in its most severe form it occurs early and transiently and its extent and duration can be reduced by some existing therapies, particularly corticosteroids (57, 58). However responsiveness to corticosteroids declines over time (53) and it has not proved possible to entirely block recurrent leakage as lesions re-activate or as new lesions arise. Some of the consequences of this may be very serious, as indicated in Simon's recently stated hypothesis, " That the enhancing lesions are a measure of the initial stages of several forms of damage that will ultimately lead to or be associated with permanent disability or damage" (62). In support of this, he presents evidence of a cardinal role for inflammatory/BBB changes in the generation of axonal injury and cerebral atrophy in MS. Similarly, a correlation between gadolinium enhancement, leukocyte infiltration, and myelin breakdown has long been recognized (32) and may account for much of the demyelination which occurs in MS. The above findings suggest potential long-term benefits from use of current drugs, interferon-β-1a, interferon-β-1b and glatiramer acetate, all of which to varying degrees reduce the number of active, gadolinuium-enhancing lesions on MRI in active relapsing remitting MS (53). In conclusion, although the pathological consequences of TJ disruption cannot be viewed in isolation from other aspects of lesion pathogenesis, suppression or reversal of this process may yet be a specific and worthwhile goal of emerging anti-inflammatory therapy.

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