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Early cellular events in multiple sclerosis Intimations of an extrinsic myelinolytic antigen

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Abstract

In a previous immunohistological study of tissues from unusually early cases of MS cluster analysis revealed a progression of demyelination through five distinct stages [Gay F, Drye T, Dick G, et al. The application of multifactorial cluster analysis in the staging of plaques in early multiple sclerosis. Identification and characterization of the primary demyelinating lesion. Brain 1997;120:1461–83]. Tissues from six of the earliest cases in this series contained regions of normal appearing white and grey matter in which well developed inflammatory events, concentrated in perivascular spaces, were found to extend locally into the perivascular parenchyma to envelop ostensibly intact myelin sheaths. The beginnings of myelin sheath lysis and phagocytosis were subsequently detected within these lesions and similar foci were found in subpial and in subependymal tissues. They were characterised by a spreading HLA Class II antigen expression on microglia, and by the presence of co-locating C3 complement–IgG complexes on capillary basement membranes, on microglial cell membranes and within the cytoplasm of large bodied activated astrocytes. Parenchymal lesions contained significantly few CD4+ T cells and showed no evidence of capillary leakage of plasma proteins. Despite the presence of complexed immunoglobulin and complement, opsonization of the myelin sheath could not be demonstrated. These observations point to the presence in early MS of a diffusing, complement-fixing, myelinolytic antigen, processed mainly within the Virchow-Robin spaces and distributed in the cerebrospinal and extracellular fluid compartments of the central nervous system.

Keywords: Multiple sclerosis; Myelinolysis; Immune complexes

1. Introduction

In the course of a histological and immunocytological study of frozen and paraffin embedded tissues from 13 exceptionally early cases of multiple sclerosis [1], 6 of the earliest cases had foci in which normal appearing myelinated tissue contained well developed cellular and humoral inflammatory processes often at some distance from demyelinating plaques. These parenchymal foci were later found to contain evidence of the beginnings of myelin sheath lysis, associated with HLA Class II+ microglia containing myelin inclusions. Some foci were located in perivascular parenchyma where the Virchow-Robin (VR) spaces were dilated with inflammatory cell cuffing, but they were also found around vessels including arterioles, where cellular cuffing was absent or minimal. The majority, and the most extensive, occurred in oedematous subependymal and subpial white and grey matter.

Astrocyte activation [2] and VR cuffing [3] have been recorded in normal appearing white matter, and more recently, HLA Class II+ foci [4] have been described which may represent primordial plaques in early acute MS tissues. Data from magnetic resonance diffusion imaging [5] and spectroscopy [6] support these histological observations suggesting the presence of a subtle progressive myelinolysis in normal appearing brain.

The present study describes the cellular and humoral characteristics of ‘pre-demyelinating’ lesions which argue for the presence in early multiple sclerosis of a diffusing myelinolytic antigen which may be distributed in the cerebrospinal and extracellular fluids of the CNS.

Abbreviations: AEC, 3-amino-9 ethylcarbazole; DAB, diaminobenzadine; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; NBT, nitro-blue tetrazolium; TRITC, tetrarhodamine isothiocyanate

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Table 1
Autopsy cases: acute MS and controls

	Autopsy number	Age (years) sex	Clinical diagnosis	Total duration	Terminal duration	Histological diagnosis
Cases						
1	P72/479	45 M	Acute MS	2 Weeks	2 Weeks	Acute brainstem MS
2	W 69	26 F	Acute MS	4 Months	2 Months	Fulminating MS
3	B561	51 F	Acute MS	2 Years	1 Week	Acute MS
4	B285	35 M	Acute MS	6 Months	1 Week	Acute MS
5	183/86	23 F	Acute MS	5 Weeks	5 Weeks	Acute MS
6	P63/324	44 F	Acute MS	20 Weeks	20 Weeks	Acute MS
Controls						
1	B260B7	33 F	Chronic MS	10 Years	6 Months	Chronic active MS
2	B9055	65 F	Chronic MS	45 Years	2 Months	Chronic inactive MS
3	B8946	61 F	Chronic MS	43 Years	6 Months	Chronic inactive MS
4	458/87	18 F	SSPE	13 Years		Measles SSPE
5	C1087	15 F	Epilepsy			R hemispherectomy
6	C645	20 M	Acute encephalitis	2 Weeks	2 Weeks	Perivenous encephalomyelitis

2. Materials and methods

The origins and characteristics of autopsy tissues, control tissues and the immuno-histological methods employed in this study have been described elsewhere [1]. Summaries of tissues and antibodies are given in Tables 1 and 2. Sections were stained with haematoxylin and eosin (H&E). Myelin sheath integrity was assessed using luxol fast blue (LFB) and Oil red O, and polarized light was used to detect birefringent myelin inclusions and myelin sheath disruption [1,7]. Myelin protein lysis was detected using antibody for myelin basic protein neo-antigen [8]. The structural integrity of vessels was assessed using antibodies for laminin, collagen IV and actin, and vascular leakage was assessed using antibodies for fibrinogen and α-2 macroglobulin [7]. Astrocytes were studied using H&E and antibodies for glial fibrillary acidic protein (GFAP). C3 complement, immunoglobulins and immunoglobulin-complement complexes were detected using double FITC-TRITC labelling as previously described [1,7].

Identification of CD4+, CD8+ and gamma–delta T cells, and IgG+ and IgM+ plasma cells was carried out using peroxidase-phosphatase double labelling and concurrent single field analysis in cryostat sections as previously described [1]. Cells of the macrophage lineage were identified using a range of antibodies known to detect cellular activation markers and antibodies for Class II HLA-DR antigens were used to identify activated cells.

3. Results

Table 3 shows the location and characteristics of the foci of parenchymal white and grey matter classified as "pre-

Table 2
Monoclonal and polyclonal antibodies employed

Antibody to	Antigen source	Antibody source	Labels	Second antibody	Source of primary antibody
Complement C3d	Human	Rabbit	FITC	n/a	Dako
IgG	Human	Rabbit F(ab′)2	n/a	Swine anti rabbit-TRITC	Dako
IgM	Human	Rabbit F(ab′)2	n/a	Swine anti rabbit-FITC	Dako
Collagen IV	Human	Mouse mc CIV 22	n/a	Rabbit anti mouse TRITC	Bionuclear
Laminin	Human	Mouse mc CIV 22 Mouse mc	n/a	Rabbit anti mouse FITC	Serotec
Actin	Human	HHF35 I A4	n/a	Rabbit anti mouse TRITC	Dako
Fibrinogen	Human	Rabbit	FITC	n/a	Dako
Alpha2 macroglobulin	Human	Rabbit	FITC	n/a	Dako
Macrophage	Human	Mouse mc Y1/82A	n/a	Rabbit anti mouse TRITC	Davey et al. [15]
Macrophage	Human	Mouse mc EBM 11	n/a	Rabbit anti mouse FITC	Franklin et al. [16]
Macrophage	Human	Mouse mc HAM 56	n/a	Rabbit anti mouse Peroxidase	Dako
CD4+ T cell	Human	Mouse mc MT 310	Biotin	Streptavidin-peroxidase	Dako
CD4+ T cell	Human	Mouse mc Q 4120	n/a	Rabbit anti mouse peroxidase	Dako
CD4+ T cell	Human	Mouse mc T4	n/a	Rabbit anti mouse peroxidase	Dako
CD8+ T cell	Human	Mouse mc RFT8	n/a	Goat anti mouse IgM phosphatase	Prof G Janossy
Gamma–delta T cell	Human	Mouse mc 5.A6E9	n/a	Rabbit anti mouse biotin	Serotec
HLA-DR	Human	Mouse mc	n/a	Rabbit anti mouse peroxidase	Dako
MBP	Human	Clone 10	n/a	Rabbit anti mouse biotin	Groome et al. [8]
GFAP	Human	Mouse mc GF2	n/a	Rabbit anti mouse TRITC	Dako

Table 3
Characteristics of 26 'pre-demyelinating, pre-plaque' lesions from 6 early acute MS cases

Histological features	Pre-demyelinating lesions ($n = 26$)		
	Perivascular ($n = 12$)		Sub-ependymal ($n = 10$) and sub-pial ($n = 4$)
	Virchow-Robin space	Parenchyma	
Myelin integrity			
MBP neoantigen	neg	+	+
Luxol fast blue inclusions	neg	+	(+)
Birefringent inclusions	neg	(+)	(+)
Oil red O inclusions	neg	neg	neg
Macrophage lineage			
HLA-DR+ microglia	n/a	+	+
HLA-DR+ EBM11/Y182a	++	(+)	+
C3d/IgG+ complexes	+++	+	+
Activated astrocytes C3d/IgG+	n/a	+	++
Lymphocytes			
CD4+ T cells	+++	(+)	(+)
CD8+ T cells	+	+	+
Gamma–delta+ T cells	(+)	(+)	(+)
IgG+ plasma cells	+	(+)	(+)
IgM+ plasma cells	+	(+)	(+)
Vessel integrity			
Collagen IV/laminin fragments	(+)	neg	neg
Fibrinogen leakage	(+)	neg	neg
Parenchymal oedema	n/a	++	++

(+) Occasionally positive.

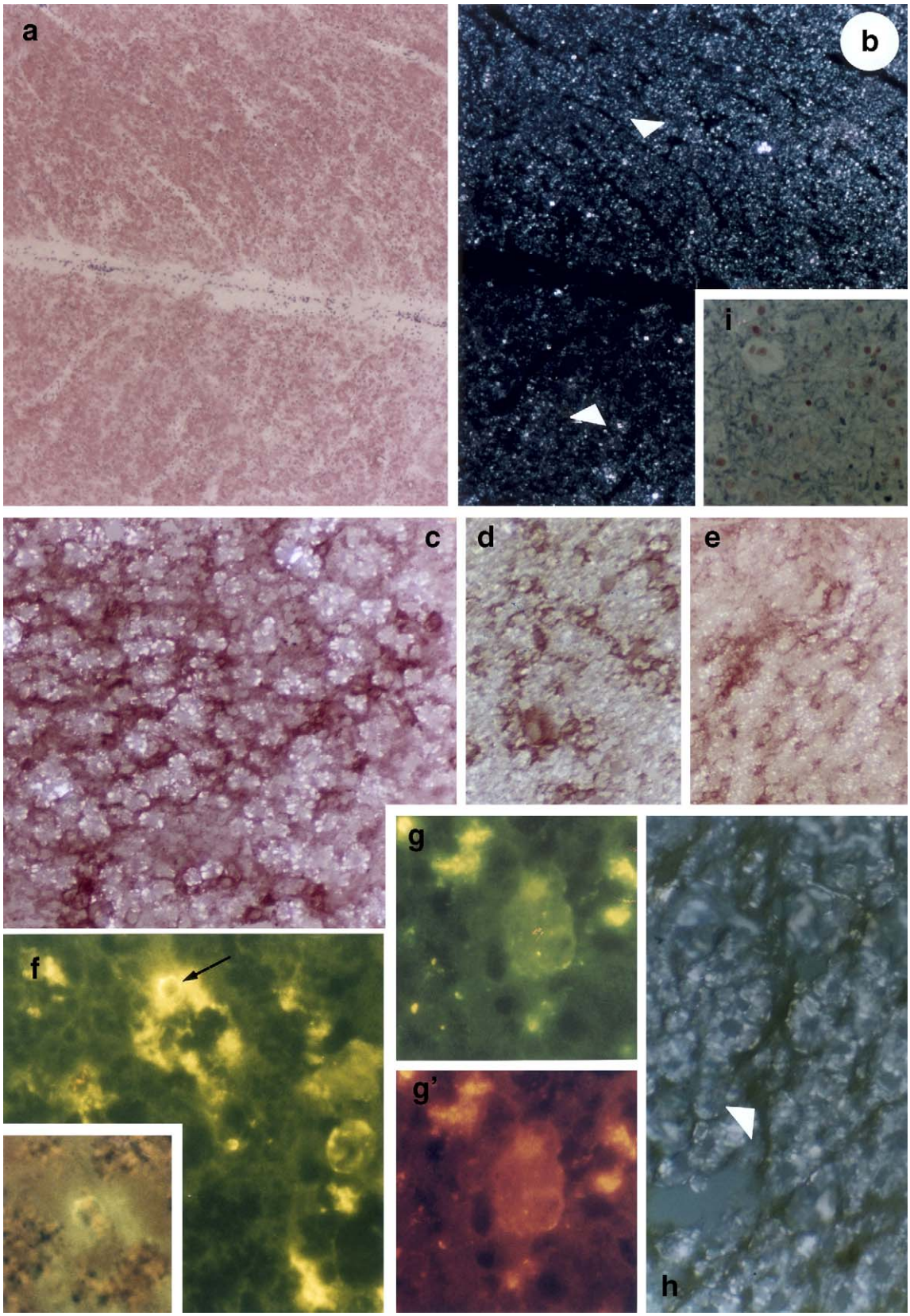
demyelinating." All showed preserved myelin but after more detailed assessment, birefringent (Fig. 1f and h) and LFB+ (Fig. 1i) microglial inclusions, and reactions specific for myelin basic protein neo-antigen (Fig. 1e), indicating peptide fragmentation with Phe-88 as the C-terminal residue, were found to some extent in all. It may be seen in Table 3 that of the 26 foci studied, 14 were subependymal and subpial, and 12 were in parenchyma around cerebral vessels. Of these vessels eight showed dilated VR spaces with marked cellular cuffing (Fig. 2a and b). Four vessels with perivascular parenchymal "pre-demyelinating" lesions, one of which was a cerebral artery with actin positive smooth muscular wall, had little or no inflammatory cuffing.

All foci showed some degree of parenchymal oedema (Figs. 1b and 2b and e), which could be extensive especially in subependyma, and all contained a population of cells with microglial morphology and reactivity to Class II HLA DR antigens (Fig. 1d). These cells forming a network between myelinated sheaths gave weak but significant reactions for the panel of antibodies detecting the macrophage phenotype; these HLA DR+ microglia contained birefringent inclusions positive for LFB and the MBP neo-antigen.

Capillaries and small vessels showed normal collagen IV and laminin-positive basement membranes and, in marked contrast to control active plaques, reactions for fibrinogen and α-2 macroglobulin were confined to the lumen of vessels and were not detected in pericapillary parenchyma.

CD4+ T cells were uniformly scarce, and counts did not significantly exceed the density of these cells in normal CNS controls. The oedematous lesion of the anterior funiculi in

Fig. 1. (a–i) 'Pre-demyelinating', pre-plaque lesions. Early myelin damage and immune complexes on microglia. (a and b) Cryostat section, mid-thoracic cord, showing anterior funiculi and the anterior median fissure. (a) Stained for Oil red O, shows no positive inclusions within an extensive area of oedema and partial myelin lysis on either side of the median fissure, detected in the section using polarised light (arrows, b). The vessels of the median fissure show mild inflammatory cellular cuffing (a). (c) Serial section of (a). Complement C3d-peroxidase, with 50% polarised light. Network of strongly positive microglia envelop myelin sheaths, showing mild myelin disruption (rabbit anti-human C3d + biotinylated anti rabbit + streptavidin-HRP-AEC). (d) Serial section of (a). Microglia, HLA Class II+. Mouse monoclonal to human HLA Class II antigens + rabbit anti-mouse peroxidase-AEC. 50% polarised light. (e) Serial section of (a). Myelin basic protein neo-antigen. Positive reaction on sheath surfaces and on microglia. Mouse monoclonal, clone 10 (see ref [8]), +rabbit anti-mouse-biotin + streptavidin-HRP-AEC. (f and inset) Serial section of (a). Microglia on lytic myelin sheaths, strongly positive for complement C3d-FITC. *Inset* (oil immersion): addition of polarised light identifies a birefringent myelin inclusion enclosed within a C3d+ vacuole membrane (arrow). Complement C3d-FITC. (g and g′) Cryostat section; normal appearing myelinated white matter. Pericapillary microglia with co-locating complement (g) and immunoglobulin G (g′). Complement C3d-FITC with immunoglobulin IgG–TRITC. (h) Cryostat section; spinal cord lesion, with HLA DR+ microglia between myelinated axons (arrows). Myelin sheath surfaces showing early lysis and fragmentation. Polarised light. (i) Luxol fast blue positive inclusions in central grey matter. Paraffin section.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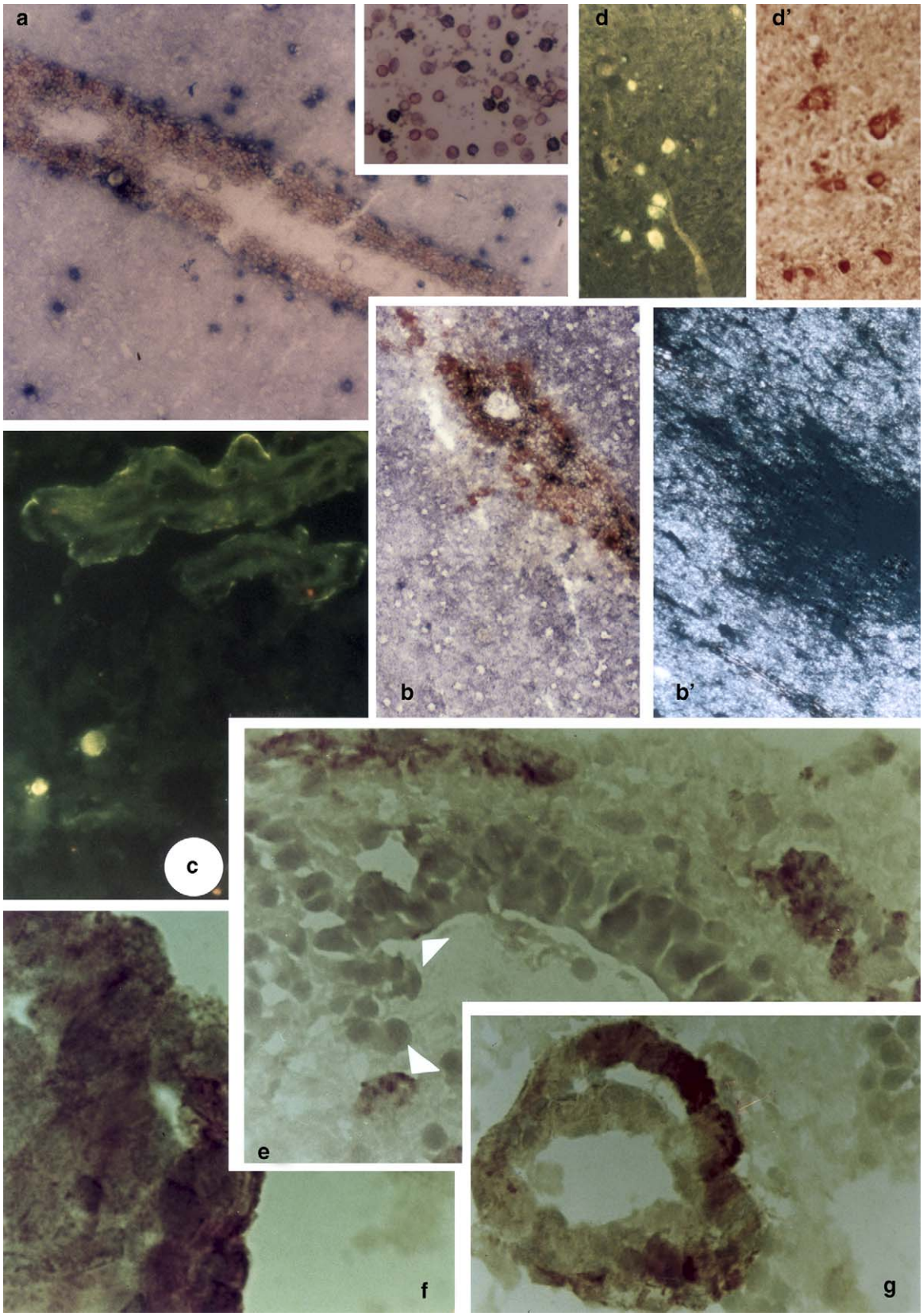
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Fig. 1b, contained a total of four CD4+ T cells. Fig. 2b shows a dilated spinal cord vessel with oedematous perivascular partial demyelination in which CD4+ and CD8+ cells are almost entirely confined to the VR space. CD8+ cells were rather more frequent in the parenchyma (Fig. 2a) and their numbers were significantly raised. IgG+ and IgM+ plasma cells were occasionally found in the parenchyma.

The most striking feature of the pre-demyelinating parenchyma was the reaction for co-locating C3d–IgG immune complexes on microglial cells (Fig. 1c, f and g). Activated plump astrocytes, particularly in subependymal lesions were also strongly reactive for C3d–IgG complexes (Fig. 2c and d). Complexes were also scattered on the glia limitans of vessels (Fig. 2c, f and g), and occasionally on and between ependymal epithelial cells (Fig. 2e).

The cellular composition of the dilated VR spaces of vessels within 'pre-demyelinating' foci (Table 3, column 1) are strikingly different from those in the adjacent parenchyma (column 2). First, degraded myelin products were not detected in macrophages, which were invariably strongly HLA-DR+ and positive for all pan-macrophage markers, but were readily detectable in vascular cuffs in control plaques. Secondly, both CD4+ and CD8+ T cells were frequently major constituents but were almost entirely confined within the VR space by the glia limitans (Fig. 2a and b). Thirdly, in most instances small amounts of fibrinogen were detected suggesting the beginnings of plasma protein leakage, a feature absent in the adjacent parenchyma. Reactions for C3d–IgG were very strong on macrophage surfaces and within cytoplasmic vesicles and were distributed along the course of the glia limitans (Fig. 2c, f and g).

4. Discussion

The location and characteristics of the perivascular, subependymal and subpial parenchymal lesions described here in six cases of early MS suggest that these lesions are primordial plaques. If this is the case, the primary mechanisms of demyelination in MS, which have been so elusive, should be operating within these early foci, and may be identified here. Three features characterising these lesions were unexpected. First and most notably, was the paucity of T cells in the tissues in general, and CD4+ cells in particular. This confirms the recent findings of Barnett and Prineas in similar clinically early MS tissues [9] and argues against a primary role for CD4+ cells in the initiation of demyelination. However, the concentration of T cells with activated macrophages, plasma cells and immune complexes in the VR spaces strongly points to the establishment of full blown immune activity in this location. The identity of the complement fixing antigen here is clearly the critical question.

Secondly, these primary lesions showed little or no evidence of the plasma protein and cellular leakage which is a consistent feature of acute plaques [7]. The initiation of demyelination is widely believed, largely on the basis of MRI data, to be closely related to, if not dependent on a breach in the blood-brain barrier [10]. Barrier leakage appears not to be necessary to primary lesion development.

Thirdly, despite the presence in the parenchyma of activated complement and associated immunoglobulin, there was no evidence in these lesions for the opsonization of the myelin sheath or of myelin fragments, or of any other identifiable parenchymal targets. This would counter the proposition [10] that myelin or other CNS antigens are the primary target of an antibody-mediated immune attack.

The proposition that demyelination might be induced by the leakage of soluble products such as inflammatory cytokines generated by the cellular reactions established in the adjacent VR spaces is not supported by the findings. Early and occasionally extensive demyelination was found around vessels with little or no inflammatory cuffing (Fig. 1a). More significant in this regard were the subependymal and subpial foci, where oedema, microglial and astrocyte activation, immune complex concentration and early myelin phagocytosis were present in the absence of ependymal or pial concentrations of inflammatory cells (Fig. 2e).

These studies show that full-blown cellular and humoral immune reactions occur in the perivascular VR spaces of vessels traversing intact myelinated parenchyma in early acute MS. The VR space has been considered as a well defined immunological site distinct from the brain, where macrophages bearing MHC class II antigens may trap antigens carried to this location via the circulation of CSF and extracellular fluids [11]. The close association of these cells and their products thus provides the conditions occurring in lymph nodes for the development of a full immunolog-

Fig. 2. (a–g) 'Pre-demyelinating' lesions; Virchow-Robin spaces. T cells and astrocytes. The sub-ependymal lesion. (a and inset) Cryostat section. Cerebral vein with cuffed, dilated VR space in normal appearing white matter. Double label for CD4+ (brown) and CD8+ (purple) T cells. CD4+ cells are confined within the VR space and small numbers of CD8+ cells appear to be crossing the glia limitans. Biotinylated anti-CD4 (MT 310, mouse IgG) with streptavidin-HRP-AEC, plus anti-CD8 (RFT8, mouse IgM), with anti-mouse IgM, phosphatase-NBT. *Inset*: T cell specificity/sensitivity control. Ficoll purified peripheral blood leukocytes, labelled in parallel with MS tissues. CD4+/CD8+ ratio = 2.15 +/− 0.11, SEM. (b and b′) Spinal cord vein with cellular VR cuffing (b); double labelling for CD4+ (brown) and CD8+ (purple) T cells. The partially demyelinated perivascular zone is defined in the cryostat section using polarised light (b′), and is seen to be almost free of T cells. (c) Cryostat section labelled for complement C3d-FITC. Venules with dilated VR spaces show typical positive reactions on the glia limitans. Outlying activated astrocytes show strong reactions for C3 in the cell cytoplasm. (d and d′) Paraffin section: perivascular parenchyma. Giant astrocytes positive for C3d-FITC (d), and IgG-HRP-AEC (d′). (e) Cryostat section, showing the central canal of the cervical cord, and oedema in the sub-ependymal grey matter. Complement C3d-HRP. Minor reactions are typically found on the ependymal surface (arrows), and more strongly within surrounding parenchymal microglia. (f) Cerebral vein with Virchow-Robin cuffing. Large bodied activated macrophages and the glia limitans are strongly positive for complement. C3d-HRP-DAB. (g) Small cerebral arteriole. The glia limitans and VR cells show strong reactivity for complement. C3d-HRP-DAB.

ical response. This suggests that the putative complement fixing-inciting antigen, that is concentrated in the VR space for processing, may in excess, spill across the glia limitans into the adjacent parenchyma, where it induces demyelination. The ependyma and pia which are freely permeable to large molecules [12] would permit direct periventricular and subarachnoid penetration of antigen from the CSF.

The activation of resident microglia by immune complexes could be expected to powerfully induce myelinolytic enzymes such as metalloproteinases. This is an attractive hypothesis for a mechanism for demyelination and has been favoured by Brosnan et al. [13], because, in experimental allergic encephalomyelitis, myelin loss is believed to be a "bystander" effect exerted by proteinases produced by macrophages stimulated by T cells. Raine [14] has emphasised the fundamental difficulty of the "bystander" hypothesis: none of the many and varied chronic and acute inflammatory reactions of the CNS that have been studied, have induced plaques. Barnett and Prineas [9] have recently argued along similar lines for a more specific and novel pathogenic attack on myelin and on the oligodendrocyte. If an extrinsic antigen with myelinolytic properties, such as a bacterial lipase were involved, this could provide the missing element of specificity for the myelin sheath and with it, potentially, the oligodendrocyte.

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