Clin. exp. Immunol. (1992) **88,** 473–477

Effect of lymphocytic infiltration on the blood–retinal barrier in experimental autoimmune uveoretinitis

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(Accepted for publication 29 January 1992)

SUMMARY

Using an experimental model of autoimmune uveoretinitis, we have examined the relationship of T cell infiltration in the retina to blood–retinal barrier (BRB) breakdown. Sensitive quantitative *in vivo* techniques were used to examine BRB permeability to sucrose, a low mol. wt non-transported solute. Electron microscopy was also used to localize extravasated horseradish peroxidase, a macromolecular visual tracer, from the retinal vasculature and to identify the route by which any leakage was occurring. No increase in BRB permeability was found prior to lymphocytic infiltration. By day 10 of the disease inflammatory cells could be seen within the structurally intact retina, which was shortly followed by an increase in the permeability of the BRB to sucrose. Only later in the disease process, when damage to the photoreceptor layer became apparent, did extravasation of the macromolecule HRP occur. At no stage of the disease process was there any detectable damage to inter-endothelial tight junctions. The size-dependancy of tracer extravasation in the initial stages of the disease is indicative of a paracellular route being responsible for the increase in BRB permeability. In later stages of the disease some evidence of horseradish peroxidase filled 'vesicle-like' profiles was observed. We suggest that the devastating complication of BRB breakdown in ocular inflammation is a direct consequence of lymphocytic infiltration.

Keywords lymphocytes blood–retinal barrier blood–brain barrier permeability experimental autoimmune uveoretinitis

INTRODUCTION

Ocular inflammation in humans is thought to be a T cell-mediated disorder [1] and has devastating effects on vision. This is partly as a result of breakdown of the normally tight blood–retinal barrier (BRB), which is often not amenable to currently available treatment. The experimental model of posterior uveitis in the Lewis rat, experimental autoimmune uveoretinitis (EAU), is also a T cell-mediated eye disease, in which severe ocular inflammation occurs as a result of systemic immunization with soluble retinal antigen in Freund's complete adjuvant (FCA) [2].

EAU shares many similar features to the experimental model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), which is similarly induced by systemic administration of myelin basic protein (MBP) in FCA. Both EAU and EAE can also be induced by the systemic administration of *in vitro*-activated T cells specific for an antigen in the target organ [3]. However, it is not fully understood how these activated T cells cross the tight barriers and how this affects barrier integrity.

In EAE, it has been suggested that activated T cells damage the integrity of the vascular endothelium as the primary insult resulting in widespread haemorrhage within the CNS and in clinically detectable disease [4]. This is supported by the observation that in irradiated leucopenic animals, haemorrhage as well as clinical disease were prevented by treatment with an anti-CD4 MoAb [4]. Several other lines of evidence also indicate that EAE may correlate with blood–brain barrier (BBB) disruption [5,6], including suppression of the increased permeability of the BBB seen in EAE by the α_1-adrenoreceptor antagonist prazosin. The effect of prazosin was attributed to inhibition of local vasoconstrictive events early in the establishment of delayed-type hypersensitivity reactions [7]. Prazosin administration resulted in reduction in retinal vascular leakage in EAU but had no effect on the severity of the ocular inflammation [8]. Whether barrier disruption in inflammatory disorders of the eye and brain is a result of cellular infiltration [9] or a result of the release of inflammatory mediators [10] requires further clarification.

Recent studies measuring BBB permeability in EAE suggested that increased permeability of capillaries and venules occurred several days before cellular infiltration [11]. Using radioactive mannitol, Daniel *et al.* [12] demonstrated an increase in BBB permeability in EAE prior to lymphocytic infiltration around blood vessels, and postulated that BBB disruption is involved in the development of the clinical lesions [12]. This would suggest that the breakdown of BBB integrity is

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not directly due to the activated encephalitogenic T cells, as has been suggested by Sedgewick & Mason [4]. Furthermore, the route of extravasation of visual tracers, as with most cases of disruption of the barriers, is not clear; both paracellular and vesicular routes [13–16] have been proposed.

In this study, sensitive quantitative *in vivo* techniques were employed to measure BRB permeability in EAU to sucrose, a low mol. wt solute, and to correlate any changes with lymphocytic infiltration into the retina. In addition, we used a macromolecular visual marker to identify the site of any increased permeability in the retinal vasculature and the route by which extravasation was occurring.

MATERIALS AND METHODS

Animals

Female Lewis rats (St Thomas' Hospital, London) weighing 150–200 g were immunized with purified bovine retinal S-antigen [17]. The antigen was emulsified (1 : 1) in FCA (GIBCO, Paisley UK), enriched with 2·5 mg/ml of *Mycobacterium tuberculosis* (strain H37Ra). A total volume of 100 μl/rat containing 50 μg of S-antigen was injected into the foot-pad of each rat and 5×10^9 *Bordetella pertussis* organisms, necessary for reproducible induction of disease, were given in 300 μl PBS intraperitoneally. Control animals were given FCA alone in the foot-pad with or without killed pertussis organisms intraperitoneally.

Permeability studies with sucrose

Animals in all groups were killed at one of three time-points after immunization: days 7–8; 9–10; and 11–12. At least six animals were used in each group at each time-point and permeability studies carried out as described previously [18]. The rats were anaesthetized with inactin (100 mg/kg intraperitoneally (Byf, Hamburg, Germany) and polyethylene cannulae filled with heparinized isotonic saline were inserted into the femoral artery and vein. Blood pressure measurements were made by connecting the arterial cannula to a strain gauge transducer (Lectromed UK) and arterial pCO_2, pO_2 and pH were measured with a pH blood gas analyser.

At time 0, 40 μCi of ^{14}C-sucrose (Amersham International; specific activity 460–660 mCi/mmol) in 300 μl saline were injected via the i.v. cannula and sequential blood samples were taken via the arterial cannula. After 15 min, 25 μCi ^{125}I-albumin (Amersham International; specific activity 2·5 μCi/mg) were given as a bolus intravenously to quantify intra-vascular volume [19]. The rat was killed by decapitation 5 min later, after taking a final blood sample. The eyes were enucleated and the retina removed as described [18].

Permeability surface area products (PA) were calculated for the BRB in each animal as previously described [19] and the mean PA $\pm$ s.e.m. for animals in each group calculated.

Permeability studies with horseradish peroxidase (HRP)

Rats in each group and at days 11, 12, 13, 14, 17, 18, 21 and 26 post-immunization ($n = 30$) were anaesthetized with pentobarbitone (50–60 mg/kg intraperitoneally). The anti-histamine diphenhydramine was injected intraperitoneally (0·5 mg/kg) and, after 10 min, 50 mg of HRP (Sigma type II) in 200 μl of saline were injected intravenously. After 5 min the thorax was opened and a cannula was inserted via the left ventricle into the proximal ascending aorta. The vasculature was perfused with one half-strength Karnovsky's fixative at a rate of 25 ml/min. The descending aorta was tied off and after 3 min the flow rate of fixative was reduced to 12 ml/min. After 15 min of fixation the eyes were removed and placed in fixative at 4°C overnight. The retinae were then prepared for electron microscopy as follows: an incision was made through the sclera behind the ciliary body and extended 360°. The cornea and lens were removed and the posterior eye cups thoroughly washed in cacodylate buffer and embedded in 3% agar just prior to setting. One hundred-micrometre sagittal sections of the posterior eye cup were cut at the level of the optic nerve on a vibroslice (Campden Instruments, UK). These sections were then incubated with diaminobenzidine (DAB; 0·25 μg/ml) for 10 min to produce the electron-dense HRP reaction product. The sections were postfixed in 1% osmium textroxide for 1 h and processed conventionally for transmission electron microscopy. Finally, they were flat-embedded in resin between two aluminium foil-coated glass slides. After the resin had set, the slides were separated and small blocks of retina were cut from the flat 100 μm sections and glued to larger trimmed resin blocks, for placing in the microtome.

Thick sections were cut and stained with toluidine blue and viewed under the light microscope. Thin sections were cut from selected areas, placed on copper grids without heavy metal staining and observed on an Hitachi H600 transmission electron microscope.

RESULTS

Lymphocytic infiltration

No lymphocytic infiltration was seen before day 10, and all eyes at days 7, 8 and 9 were histologically normal. A few inflammatory cells were seen in some of the eyes at day 10 which were mononuclear and lymphocytic in appearance. More consistent infiltration of inflammatory cells, especially around the larger vessels, was seen from day 11 onwards with retinal damage occurring from day 13 onwards (Fig. 1). First indications of

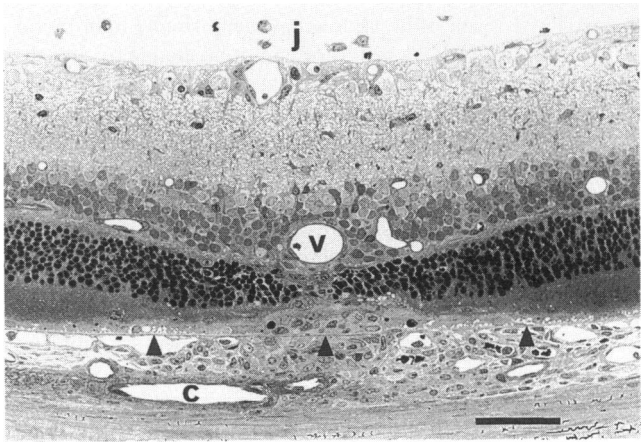

Fig. 1. Toluidine blue section of the retina from an animal in the second week of disease. Note the numerous inflammatory cells thoughout the retina and extensive damage of the tissue around the retinal vessel (v). Many inflammatory cells surround this vessel and large numbers appear to be migrating across from the choroidal vasculature (c) adjacent to it. Bruchs membrane appears intact (arrowheads). Inflammatory cells are also present in the vitreous (j). Bar = 50 μm.

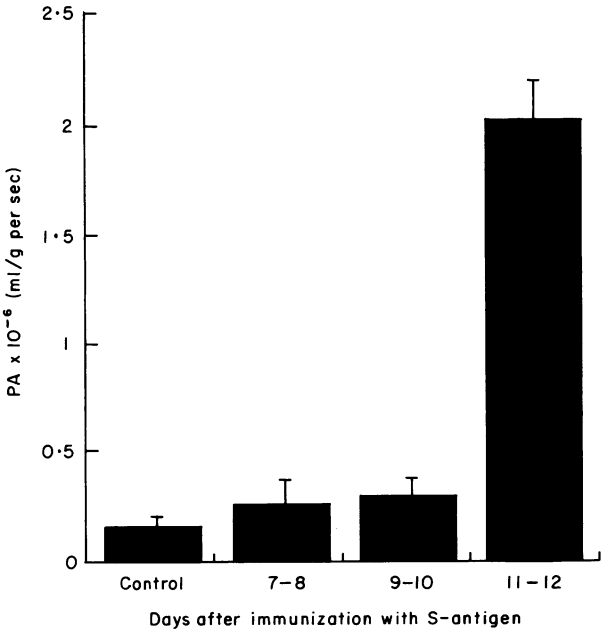

Fig. 2. Significant increase ($P < 0.001$) in permeability to sucrose occurring at the blood–retinal barrier from day 10–12 post-immunization with S-antigen.

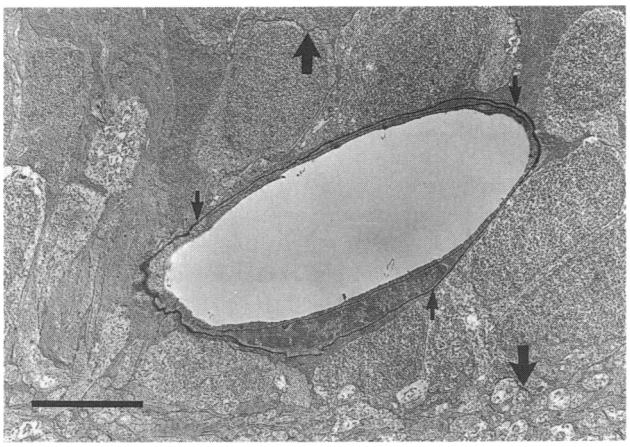

Fig. 3. Transmission electron micrograph of retinal microvessel from the retina of an animal in the second week of disease. Extravasated horseradish peroxidase can be seen flooding the basement membrane (small arrows) and extending into the parenchymal extracellular space (large arrows). Bar = 5 μm.

structural damage occurred in the photoreceptor layer particularly at regions in close proximity to a vessel (Fig. 1). All animals immunized with retinal S-antigen developed obvious ocular inflammation with initial disruption of the photoreceptor cell layer from day 13 onwards, leading to total destruction of the retina. The control groups remained histologically normal.

Permeability of the BRB to sucrose

Permeability at the BRB to sucrose was assessed up to day 12 post-immunization because after that time the retina became so

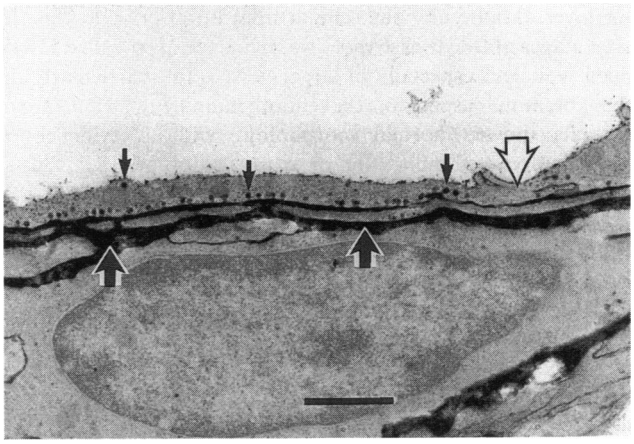

Fig. 4. Transmission electron micrograph of retinal vessel from the retina of an animal in the second week of disease. Extravasated horseradish peroxidase (HRP) filling the basement membrane (large solid arrows), and part of the way along a complete tight junction (large open arrow). HRP-filled vesicular-like profiles or pits (small arrows) can be seen in the endothelium, particularly at the abluminal aspect. Bar = 1 μm.

inflamed and thickened that it became impossible to ensure adequate separation from the choroid. Animals receiving FCA with or without pertussis did not show any increase in PA to sucrose over that described for the normal BRB [18]. No increase in permeability at the BRB was seen in the 7–8 and 9–10 day groups, but by day 11–12 a large increase occurred ($P < 0.001$) (Fig. 2).

Retinal vascular volumes, calculated from the ^{125}I-albumin results, demonstrated no increase over control values when the PA was found to be increased. This also suggests that extravasation of albumin in the inflamed eye up to day 12 was minimal.

Permeability studies of the BRB to HRP

Areas in the retina containing obvious blood vessels were identified on the thick sections stained with toluidine blue and more than 300 thin sections cut in these areas. All blood vessels identified in the thin sections by electron microscopy were looked at in detail to examine the integrity of the tight junctions and for extravasation of tracer into the parenchyma.

The tight junctions in virtually all sections examined in all eyes showed few obvious abnormalities, being apparently morphologically intact. Only very rarely could junctions be identified in which HRP could be traced along its entire length. In addition, no extravasation of HRP outside the blood vessel wall was seen, except when the retina was grossly disrupted later on in the inflammatory process (eyes from day 13 and later). At these more advanced stages of the disease inflammatory cells could be seen adhering to vascular endothelium and migrating into the parenchyma. In these regions HRP reaction product could be seen flooding the basement membrane of many of the vessels and extending into the parenchymal extracellular space (Fig. 3). Most of the vessel endothelium remained relatively normal in appearance apart from occasional thickened or tall endothelial cells at points of severe inflammatory cell migration in larger vessels. In addition, there was an apparent increase in the number of luminal microvilli. HRP extravasation only appeared when there was evidence of damage to the photorecep-

tor layer, despite obvious cellular infiltration prior to this. In later stages of the disease there was evidence of so-called HRP-filled 'vesicles', especially in larger vessels and particularly on the abluminal aspect of the endothelium (Fig. 4). Control animals showed normal morphology without evidence of inflammatory cell infiltration or extravasation of HRP.

DISCUSSION

We have demonstrated that breakdown of the integrity of the BRB only occurs in association with lymphocytic infiltration in the retina and does not occur prior to this event. In addition, the initial increase in permeability was only demonstrable with the small tracer sucrose as no increase in permeability could be detected with ^{125}I-albumin or HRP. That this is a true increase in BRB permeability and not just due to vasodilatation is shown by the normal retinal vascular volumes found at the time of the increased PA values. Only when gross destruction of the retina became apparent did extravasation of the larger molecular weight tracers occur. That BRB breakdown occurred concomitantly with lymphocyte infiltration supports earlier work on the BBB in EAE which indicated that these two events were linked [4,14]. However, other studies with EAE have reported that BBB dysfunction occurs 1–2 days prior to cellular infiltration [11,12]. These contradictions may be the result of a genuine difference between the BRB and the BBB in EAU and EAE, respectively. Equally likely is the possibility that these differences are due to a lack of spatial and temporal resolution in detecting the small, initial barrier disturbances and infiltrating cells.

It would appear from these findings that the route of tracer extravasation during the early stages of EAU is via the formation of paracellular channels. Size dependency has been used previously to distinguish between junctional and vesicular routes of extravasation following hyperosmolar opening of the BBB [20]. These small channels act as molecular sieves allowing the passage of sucrose into the extracellular space but not the high mol. wt tracers, HRP and albumin. These breaches in the BRB may be caused by the migration of lymphocytes through either the tight junctions or the endothelial cells themselves, as has been suggested in EAE [21]. Alternatively, the BRB may be disrupted indirectly by the release of vasoactive mediators that are known to alter vascular permeability [10,16].

In the later stages of EAU, extravasation of the high mol. wt molecules was detected, a finding which is consistent with BBB permeability in the later stages of EAE [21,22]. However, as with earlier studies on barrier permeability changes, there was no obvious ultrastructural alteration to the retinal vascular endothelial cell tight junctions with only occasional appearances of HRP-filled junctions or vesicular-like profiles in larger vessels (Fig. 4). The mechanism of barrier disruption at this stage of the disease is more likely to be the result of the release of inflammatory agents that are known to disrupt the BRB [10] and which have been reported to induce so-called 'vesicle' formation in the retinal vascular endothelium [13,15,23]. The paucity of tight junctions with HRP reaction product filling the complete length does not necessarily indicate that disrupted junctions are not involved in the breakdown of BRB integrity. The three-dimensional tortuosity of tight junctions is likely to limit the probability of obtaining, in one section, a complete junction from luminal to abluminal aspect. Moreover, the appearance of HRP-filled vesicular-like profiles may not be the result of induction of pinocytosis but due to sectioning through membrane invaginations or caveoli, tubular structures that are in continuity with the abluminal membrane [24]. However, this remains a contentious issue and requires further elucidation. There was little evidence of HRP leakage from the retinal pigment epithelium (RPE) into the photoreceptor layer even when photoreceptor damage was apparent. The ultrastructure and route of leakage of the RPE in BRB breakdown in EAU is currently under further investigation.

Exactly how inflammatory cells cross tight barriers is unknown. Penetration of the BBB by activated T cells is thought to be mediated by interactions between the lymphocyte and the endothelial cell via specific adhesion molecules [25,26] and has also been shown to be associated with enzymatic degradation of endothelial cell basement membranes [27,28]. Recent studies on the BBB in EAE suggest that inflammatory cells do not migrate via a paracellular route but penetrate through the endothelium close to tight junctions [21,29]. In a recent ultrastructural investigation of the BRB in EAU in the Lewis rat Dua *et al.* [30] also reported no apparent damage to the endothelial cell tight junctions but did describe tall 'high endothelial vein' (HEV)-like endothelium at regions of inflammatory cell migration. In the present study a small number of HEV-like endothelium were also seen in areas of severe infiltration although in many other areas of leucocyte infiltration the endothelium appeared relatively normal. These observations are also consistent with recent findings at the BBB in EAE [29] and in retinal endothelium in human posterior uveitis [31].

It is well known that T cells have to be activated to cross the BRB or the BBB [3,32]. Indeed, T lymphoblasts specific for ovalbumin enter the CNS as readily as those specific for myelin basic protein (MBP) [33]. This study demonstrates an increase in permeability asociated with the appearance of lymphocytes in the retina. It is therefore possible to hypothesize that the activated lymphoyctes are having an effect on the vascular endothelium either directly or via secretion of lymphokines. It has recently been suggested that in EAE barrier disruption is being mediated, at least in part, via cytokines acting directly upon BBB integrity [21,23]. The direct effect of cytokines upon the BBB or BRB is also beginning to be examined. Experimental administration of IL-2 has been reported to disrupt the BBB [34] and intra-vitreal injection of IL-1 and tumour necrosis factor (TNF) have been shown to lead to cellular infiltration and an increase in endothelial cell pinocytosis [23] in the retina. Preliminary studies on lymphokine secretion by activated T cell lines specific for retinal S-antigen *in vitro* [35] and *in vivo* [36] have shown that these cells can secrete IL-2, interferon-gamma and lymphotoxin. The effect of the latter two on BRB integrity is unknown.

Further studies are underway, examining the T cell/retinal endothelial interactions *in vivo* and the effects of various T cell lymphokines on BRB integrity. A greater understanding of the effect of the activated T cell on the BRB may allow future therapeutic intervention to prevent the devastating effects on vision which result from intraocular inflammation.

ACKNOWLEDGMENTS

We would like to thank Mr S. Davies and Mr R. Howes, Department of

Clinical Science, Institute of Ophthalmology, for the technical work they have contributed to this paper.

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