

Immune Function of the Blood-Brain Barrier: Incomplete Presentation of Protein (Auto-)Antigens by Rat Brain Microvascular Endothelium In Vitro

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Abstract. The endothelial blood-brain barrier (BBB) has a critical role in controlling lymphocyte traffic into the central nervous system (CNS), both in physiological immunosurveillance, and in its pathological aberrations. The intercellular signals that possibly could induce lymphocytes to cross the BBB include immunogenic presentation of protein (auto-)antigens by BBB endothelia to circulating T lymphocytes.

This concept has raised much, though controversial, attention. We approached this problem by analyzing in vitro immunospecific interactions between clonal rat T lymphocyte lines with syngeneic, stringently purified endothelial monolayer cultures from adult brain microvessels. The rat brain endothelia (RBE) were established from rat brain capillaries using double collagenase digestion, density gradient fractionation and selective cytolysis of contaminating pericytes by anti-Thy 1.1 antibodies and complement. Incubation with interferon- γ in most of the brain-derived endothelial cells induced Ia-antigens in the cytoplasm and on the cell surface in some of the cells. Before the

treatment, the cells were completely Ia-negative. Pericytes were unresponsive to IFN- γ treatment. When confronted with syngeneic T cell lines specific for protein (auto-)antigens (e.g., ovalbumin and myelin basic protein, MBP), RBE were completely unable to induce antigen-specific proliferation of syngeneic T lymphocytes irrespective of pretreatment with IFN- γ and of cell density. RBE were inert towards the T cells, and did not suppress T cell activation induced by other "professional" antigen presenting cells (APC) such as thymus-derived dendritic cells or macrophages. IFN- γ -treated RBE were, however, susceptible to immunospecific T cell killing. They were lysed by MBP-specific T cells in the presence of the specific antigen or Con A. Antigen dependent lysis was restricted by the appropriate (MHC) class II product. We conclude that the interaction of brain endothelial cells with encephalitogenic T lymphocytes may involve recognition of antigen in the molecular context of relevant MHC products, but that this interaction per se is insufficient to initiate the full T cell activation program.

BRAIN microvascular endothelial cells have essential functions both in health and in disease. In the intact central nervous system (CNS),¹ endothelial cells form the blood-brain barrier (BBB), a tightly interconnected cellular monolayer, which contributes to the stability of the brain parenchymal microenvironment by strictly controlling the exchange of material between blood circulation and neuropil. Complex tight junctions, a low number of pinocytotic vesicles, a high electrical resistance and the presence of specific polarized transport systems distinguish brain endothelial cells from other endothelial cells in the body (Reese and Karnovsky, 1967; Coomber and Stewart, 1985, Crone

and Olesen, 1982). In addition to an intact basement membrane and pericytes that are found in most capillaries, the microvascular wall in the central nervous system contains tightly apposed astrocytic end feet (Brightman et al. 1982).

It has been tacitly assumed that the BBB endothelium is a complete barrier not only for macromolecules but also for all blood cells. This is certainly true for relatively inert cells like erythrocytes, thrombocytes, and resting lymphocytes. Recent work demonstrated, however, that activated T cells are able to cross the BBB quite readily (Wekerle et al., 1986b; Hickey and Kimura, 1988). It appears that these cells have a crucial role not only in immunosurveillance of the CNS but also as pathogenic mediators of autoimmune disease (Wekerle et al., 1986b), and, as in the case of retrovirus-associated neuropathies, as vehicles for viruses (Haase, 1986). In all these situations, contacts between circulating immune cells and BBB endothelia can be assumed to be the first step leading to CNS invasion.

1. *Abbreviations used in this paper:* APC, antigen presenting cell; BBB, blood-brain barrier; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FVIII:RAG, factor VIII-related antigen; GFAP, glial fibrillary acidic protein; IFN- γ , interferon- γ ; MBP, myelin basic protein; MHC, major histocompatibility complex; RBE, rat brain endothelium; TdR, thymidine.

To learn more about endothelial/lymphocyte interactions, we have studied the inducibility of Ia antigens and the antigen presenting capacity of BBB endothelia using purified rat CNS microvascular endothelia and antigen specific permanent syngeneic T lymphocyte lines. Our results show that BBB endothelia can be induced to some degree to express Ia-induction by interferon- γ (IFN- γ) treatment are competent report studying presentation of ovalbumin to specific T line cells (Pryce et al., 1989), BBB endothelia even after Ia-induction by interferon- γ (IFN- γ) treatment are completely unable to initiate an immunologically specific proliferation reaction of myelin basic protein (MBP)-specific T line cells. In contrast, the same Ia-induced BBB endothelia are readily and immunospecifically lysed by MBP-specific T cells in the presence of the relevant antigen.

Materials and Methods

Anti-rat Monoclonal Antibodies

The following mAbs were used: OX6, anti-rat major histocompatibility complex (MHC) class II (RT1.B); OX7, anti-Thy 1.1; OX8, anti-rat CD8; OX17, anti-rat MHC class II (RT1.D); OX18, anti-rat MHC class I. All were supplied by Serotec (Wiesbaden, FRG).

Isolation and Culture of Brain Endothelial Cells

Cerebral cortices from 420 Lewis rats (3–6 mo old) were used for the experiments described in this study. Microvascular endothelial cells were isolated using a modified combination of the methods of Williams et al. (1980) and Bowman et al. (1981), which is a further development of a method described recently (Hughes and Lantos, 1985; Male et al., 1987). Briefly, cortices from 10 brains were dissected free of meninges and white matter in buffer A (153 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 2.6 mM MgCl₂, 15 mM Hepes, pH 7.4, 10 mg/ml BSA). Using surgical blades, the tissue was minced until resuspendable by 10-ml pipettes. After centrifugation at 250 g for 5 min, the tissue pellet was resuspended in 0.3% (wt/vol) collagenase (CLS II; ~200 U/ml; Biochrom, Berlin, FRG), 10 mg/ml BSA in PBS (140 mM NaCl, 10 mM sodium phosphate, 0.2 mM CaCl₂, 0.2 mM MgCl₂, pH 7.1). The slurry was incubated for 90 min at 37°C and was resuspended twice during this incubation and once afterwards using 1-ml pipettes. After centrifugation at 250 g for 5 min, the pellet was resuspended in 40 ml of 25% (wt/vol) BSA in buffer A and centrifuged at 1,000 g for 20 min. While capillaries and blood cells formed a pellet, myelin floated as a thick band that was removed together with the supernatant, resuspended, and centrifuged again. The resultant pellet was processed separately, because it usually was contaminated to a varying degree with nonendothelial cells. The capillary pellet was resuspended in 5 ml 0.75% (wt/vol) collagenase, 10 mg/ml BSA in PBS and digested a second time for 90 min at 37°C to remove contaminating pericytes and astrocytes, both of which are embedded in the vascular basement membrane. Resuspending was repeated twice during this incubation. After centrifugation at 250 g for 5 min, capillary fragments were purified by centrifugation in Percoll density gradients exactly as described (Bowman et al., 1981). They were removed from the gradients and washed three times with DME (Gibco Laboratories, Heidelberg, FRG) containing 10% (vol/vol) FCS (Boehringer Mannheim GmbH, Mannheim, FRG). The fragments were then resuspended in 6 ml rat brain endothelium (RBE) medium, DME, enriched with 10% (vol/vol) calf serum 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% non-essential amino acids, sodium pyruvate (1 mM), L-glutamine (2 mM), 5 \times 10⁻⁵ 2-mercaptoethanol (all additives from Gibco Laboratories) and 1% (vol/vol) bovine retinal extract (D'Amore and Klagsbrun, 1984) as a crude source of endothelial cell growth factors. The material was then plated onto 35-mm tissue culture dishes (Falcon, Becton, Dickinson, München, FRG) that had been coated with collagen type I/III (Biochrom) or basement membrane matrix from bovine corneal endothelial cells as described by Gospodarowicz et al. (1986). The capillary fragments were allowed to attach for only 2 h in an incubator (37°C, 7% CO₂). The supernatant was removed and plated onto fresh coated 35-mm petri dishes.

Optimal purity of RBE cell cultures was obtained by selectively lysing by antibody/complement treatment the main contaminating cells (astrocytes

and pericytes) that both express on their surface the Thy 1.1 antigen. Thy 1.1 antiserum was obtained by weekly injecting i.p. C3H/f mice (Thy 1.2) with 3 \times 10⁷ AKR/J thymus cells (Thy 1.1). After 10 wk of immune serum from 30 mice was pooled, heat inactivated, and tested for specific cytotoxicity against AKR/J thymus cells (positive controls) and RBE monolayers (negative controls), this antiserum was then used for the killing as follows. RBE cultures were washed twice with DME, then incubated with Thy 1.1 antiserum (1:50) for 40 min at 37°C. After washing twice with DME, rabbit complement (Behringwerke, Marburg, FRG.) was added (1:10). The killing was done at 37°C, in a 10% CO₂ atmosphere. After ~14 d, the treated cells had reached confluency. They were passaged into collagen-coated 96- or 48-well plates (Costas, Tecnomara, Fernwald, FRG; or Nunc, Wiesbaden, FRG) or coated 35-mm dishes at a split ratio of 1:3 using 0.05% trypsin, 0.02% EDTA (Gibco Laboratories).

Isolation and Culture of Other Rat Cells

Pure cultures of pericytes were obtained by prolonged culture of the regular endothelial cell preparation obtained as described above without the Thy 1.1/complement lysis step. Pericyte survival and proliferation was favored by selective culture conditions (Orlidge and D'Amore, 1986) using uncoated dishes, long attachment time, and simple DME supplemented with 10% (vol/vol) FCS. They were identified by their morphology, lack of factor VIII-related antigen (FVIII:RAG) and glial fibrillary acidic protein (GFAP) staining and of expression of Thy 1.1 and smooth muscle actin.

Epididymal fat pad endothelial cells were isolated from adult Lewis rats according to the method of Madri and Williams (1983). Primary cultures of these cells and cloned epididymal fat pad endothelial cells from Wistar rats (a generous gift of Dr. John Castellot, Harvard Medical School) were used for control experiments. Rat skin fibroblasts grew out from explants of adult Lewis rat skin. Macrophages were obtained by peritoneal lavage of adult Lewis rats using a solution of 0.34 M sucrose in PBS (6 ml/rat).

T Lymphocyte Lines

The T lymphocyte lines L.OA, L.RBP, and Z82 have been described before (Sun and Wekerle, 1986; Wekerle et al., 1986a; Ben-Nun et al., 1981). They have been isolated from Lewis rat immunized against ovalbumin (L.OA), rat MBP (L.RBP) or guinea pig MBP (Z82), respectively. The lines were isolated and propagated by periodically alternating antigen-dependant activation episodes with T cell growth factor driven propagation phases. All lines express the markers of the CD4⁺ T cell subsets, and recognize their antigen in the molecular context of MHC class II, Ia determinants. L.RBP and Z82 lyse APC upon antigen presentation, whereas line L.OA is not lytic (Sun and Wekerle, 1986).

T Cell Proliferation Assay

Antigen presentation was assessed by confronting T lines in vitro with monolayer forming endothelial cells or fibroblasts, or, as a positive control, with thymic antigen presenting cells (APC) in flat bottom 96-well plates. Endothelial cells or fibroblasts were seeded into the culture wells in the presence or absence of IFN- γ (50 U/ml RBE medium), 48 h before the antigen presentation test. Then, the medium was replaced by the complete lymphocyte culture medium, composed of DME, enriched with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids 1% (vol/vol), 5 \times 10⁻⁵ M mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin (all from Gibco Laboratories, Heidelberg, FRG), supplemented with 1% normal fresh rat serum and appropriate antigen. Unless otherwise indicated, the cultures contained 2 \times 10⁴ T line cells, and in the positive control cultures 2 \times 10⁶ Lewis thymus cells irradiated with 3,000 R, as "professional" APC. After 48 h, the cultures were labeled with ³H-TdR (TdR, thymidine) (0.2 μ Ci/well; Amersham, Braunschweig, FRG). After overnight incubation the cultures were harvested with a Titertec Multiple Harvester (Flow Laboratories, Irvine, Scotland) and radioactivity was determined with a β scintillation counter (Kontron, Basel, Switzerland).

T Cell-mediated Cytotoxicity Assay

To quantify the lysis of RBE by MBP-specific T line cells, we used a conventional ⁵¹Cr-release assay. RBE were seeded in 0.5 ml RBE-medium into precoated 48-well plates (Costar) and grown to confluency. Ia-expression was either induced by adding IFN- γ (50 U/ml, 48 h) or IFN- γ containing Con A culture supernatant (20%, 72 h; Fierz et al., 1985), the induced cul-

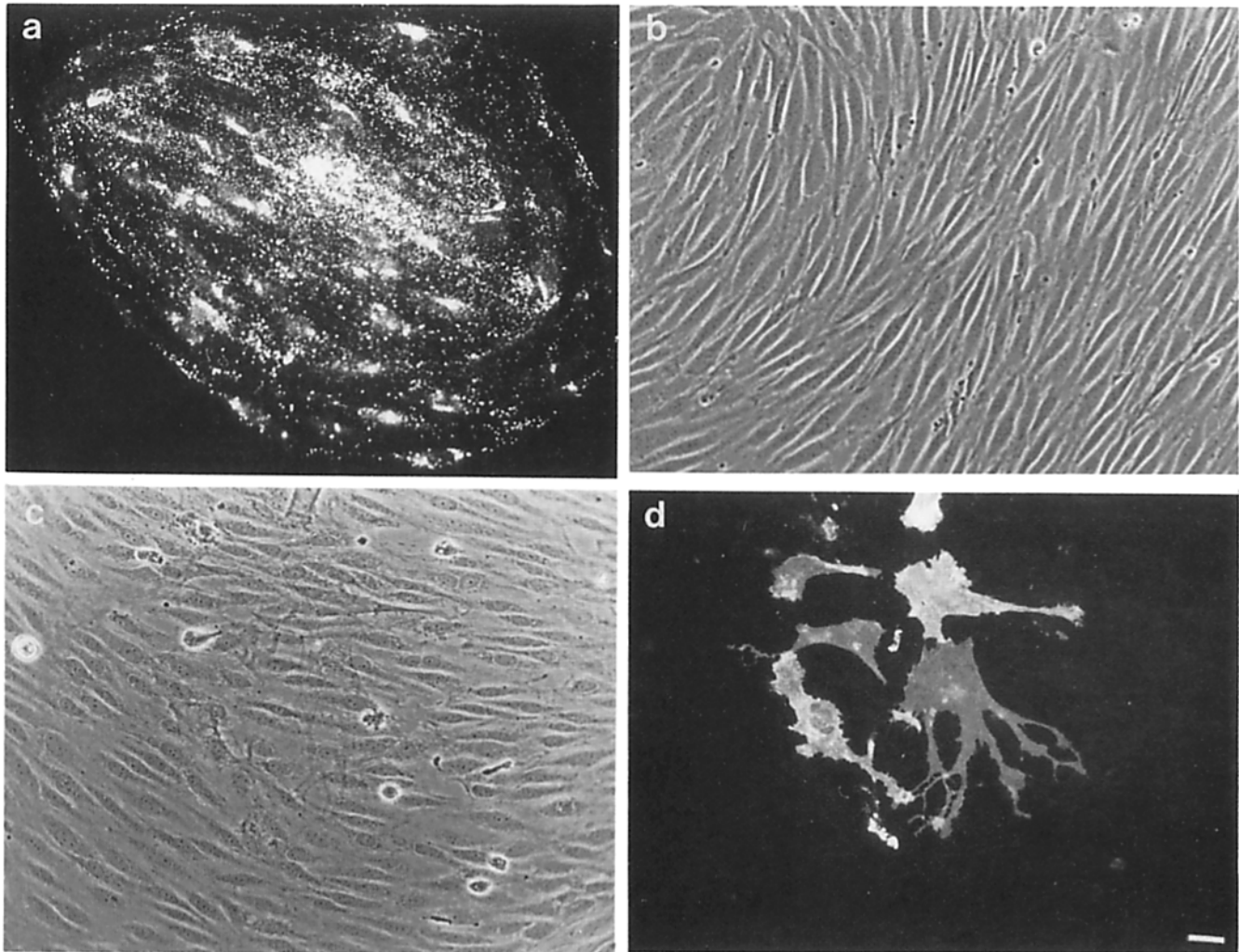


Figure 1. Characterization of RBE cell cultures. A colony of FVIII:RAG positive endothelial cells in primary culture (a) that grow and form a typical contact-inhibited monolayer (b). Thy 1.1 antigen was expressed by pericytes growing underneath a monolayer of Thy 1.1 negative endothelial cells; phase-contrast (c) and indirect immunofluorescence (d) of the same field. Bar, 50 μ m.

tures were labeled with 10 μ Ci $\text{Na}^{51}\text{CrO}_4$ (Amersham, Braunschweig, FRG) for 20 h. After two rinsings and an incubation interval of 2 h, followed by a third rinsing, T cells were added. 18 h later the supernatants were removed and the sediments were lysed with 20% SDS. Radioactivity of supernatants and lysates was determined in a γ counter (Kontron Analytical, Everett, MA). Specific ^{51}Cr -release was calculated as follows:

$$\frac{\text{cpm}_{\text{test}} - \text{cpm}_{\text{spont}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{spont}}} \times 100$$

$\text{cpm}_{\text{spont}}$ = spontaneous ^{51}Cr -release of target cells in absence of effector T cells

cpm_{max} = maximal ^{51}Cr -release of target cells in absence of effector T cells after lysis with 20% SDS

cpm_{test} = ^{51}Cr -release in test cultures with effector T cells.

Astrocyte Clone F10

This astrocytic clone originated from a brain monolayer culture that had been established and maintained for 8 mo as described (Manthorpe et al., 1979). After multiple transfers, within 8 mo of permanent culture, the monolayers had a homogeneous morphological appearance resembling monolayers of "protoplasmic" astrocytes. The cells weakly stained with anti-GFAP antibodies. Monolayers were dissociated and the cells were seeded into flat-bottomed 96-well plates (Falcon Labware) at a density of 0.3 cells/well. We used DME/10% (vol/vol) FCS. At a cloning efficiency

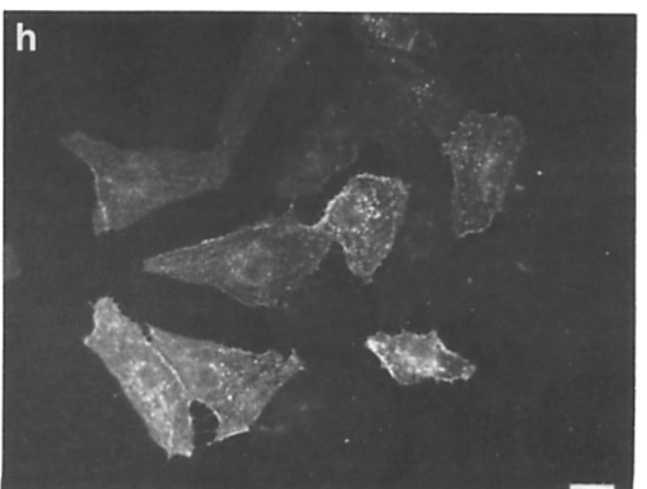
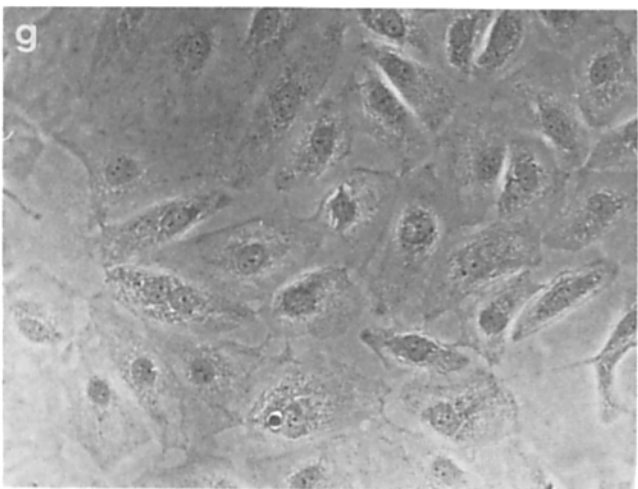
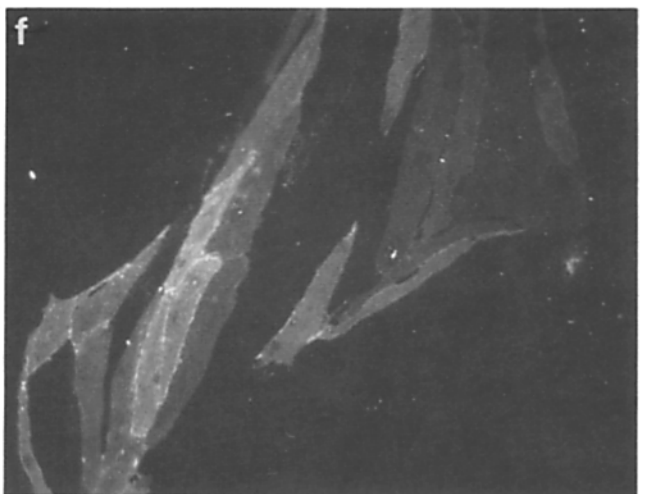
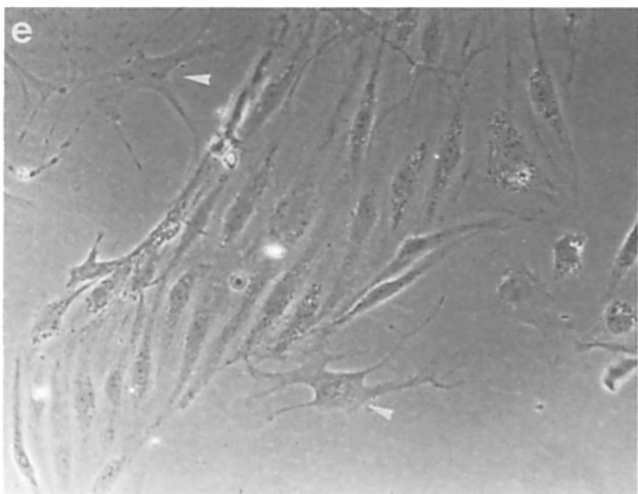
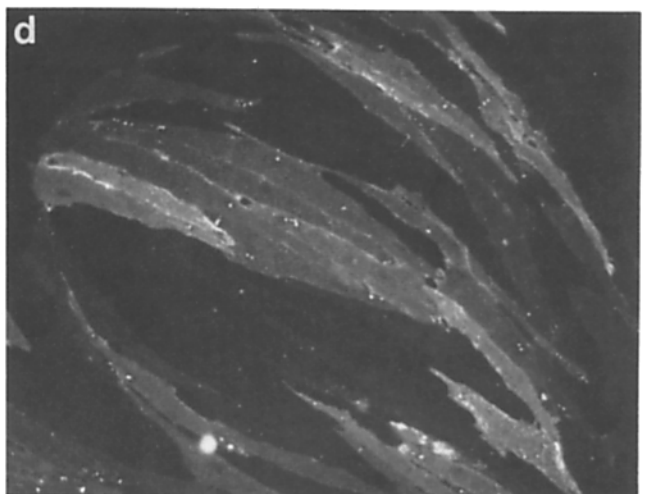
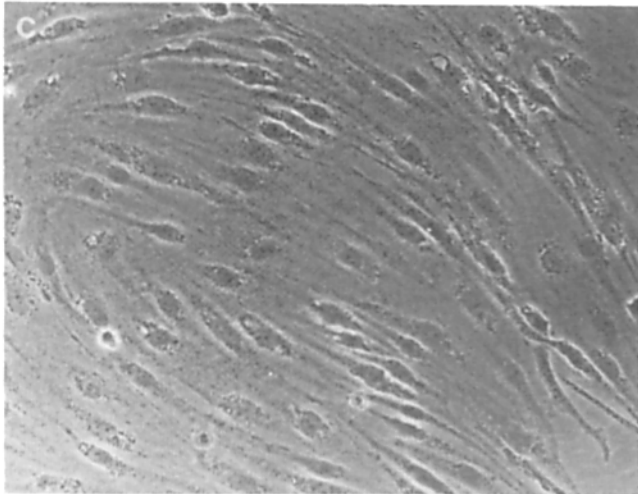
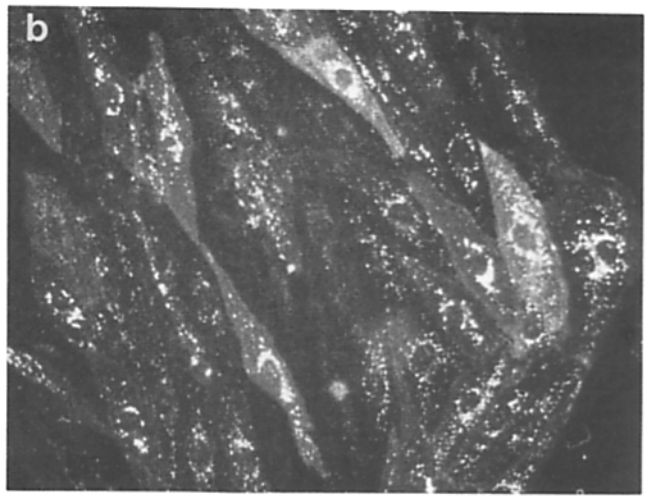
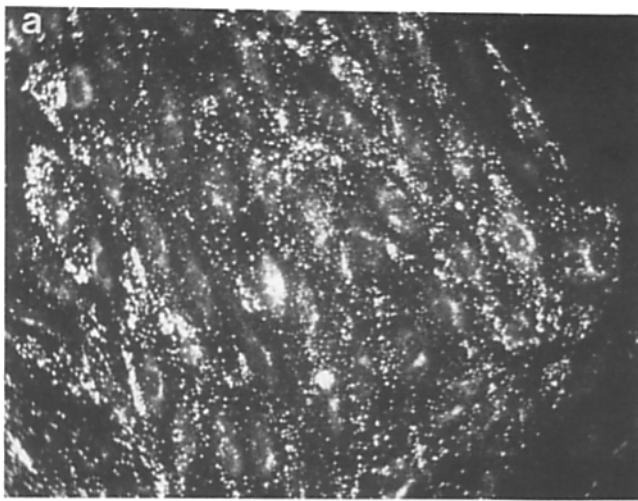
approaching 100%, homogeneous GFAP⁺ cell colonies could be selected and propagated. One of these cell colonies is clone F10, which had been propagated in vitro for another 12 mo before use in these experiments.

Immunocytochemistry

Cells were washed three times with PBS and fixed for 10 min using 4% (wt/vol) paraformaldehyde at room temperature or methanol at -20°C (for permeabilized cells). All further steps were carried out at room temperature. Cells were washed again and incubated with primary antibodies for 1 h, washed, incubated with secondary antibodies for 1 h, washed, and embedded in 50% (vol/vol) glycerol. In some experiments live cells were incubated with primary antibodies before fixation. Double staining was done as described previously (Risau and Lemmon, 1988). Antibodies and dilutions were rabbit anti-FVIII:RAG (Behringwerke), 1:100; rabbit anti-GFAP (a generous gift of Dr. Lawrence Eng, Stanford University), 1:200;

Table I. Characterization of Cultured Brain-derived Cells

Cells	Markers		
	FVIII:RAG	GFAP	Thy 1.1 (OX7)
Endothelium	+	-	-
Pericytes	-	-	+
Astrocytes	-	+	\pm



mouse monoclonal anti-smooth muscle actin (Renner GmbH, Dannstadt, FRG), 1:100; mouse monoclonal anti-rat Thy 1.1 (MRC OX7; Serotec), 1:100; mouse monoclonal anti-rat Ia (MRC OX6, Serotec, Camon GmbH, Wiesbaden, FRG), 1:100.

Results

Characterization of Cultured Cells

Capillary fragments isolated by collagenase digestion of rat brain cerebral cortex (gray matter) and subsequent density gradient centrifugation adhered and spread rapidly on basement membrane matrix derived from bovine corneal endothelial cells. They also adhered to but spread less readily on collagen-coated plastic. Nevertheless, the number of contaminating nonendothelial cells could be reduced by using short plating times and collagen-coated dishes, and finally by serologically eliminating Thy 1.1 expressing contaminants. Colonies of endothelial cells, positive for FVIII:RAG (Fig. 1 *a*), started to proliferate after a delay of 5–8 d. They formed a typical confluent monolayer after ~14 d, dependent on the initial density (Fig. 1 *b*). Pericytes were the main contaminating cells (up to 5% of the cells) in cultures before Thy 1.1/complement treatment. They were identified by their typical morphology (well spread, flat, many processes, no contact inhibition); their slow growth; and absence of FVIII:RAG and GFAP but presence of smooth muscle actin (not shown). We found that those cells also expressed Thy 1.1 antigen, whereas endothelial cells were completely negative for Thy 1.1 (Fig. 1, *c* and *d*). Less than 5%, and often none, of the cells were GFAP positive, suggesting that astrocytes forming the vascular end feet were effectively removed by this isolation procedure. Some astrocytes, if present in cultures, also expressed Thy 1.1 antigen as determined by double staining for GFAP and Thy 1.1 (Table I). This was also observed in astrocyte cultures from neonatal rat brain (not shown). Both pericytes and astrocytes contaminants were removed by anti-Thy 1.1/complement lysis, resulting in cultures composed of almost 100% RBE.

Ia-Expression

Primary cultures and first passage endothelial cells, treated with IFN- γ (50 U/ml) for 3 d, expressed Ia-antigen. If cells were permeabilized and fixed by methanol treatment at -20°C, Ia-antigens were predominantly localized intracellularly in the perinuclear region of FVIII:RAG positive cells (Fig. 2, *a* and *b*). This confirms recently published results (Male et al., 1987). However, to present antigen in the appropriate context of major histocompatibility complex (MHC) products, Ia-antigens have to be present on the cell surface. We therefore tested whether different staining procedures would affect the detectability of the antigens. Indeed, if live cells were incubated with anti-Ia antibodies before fixation, Ia antigens were detected on the cell surface of endothelial

cells (Fig. 2, *c-f*). The staining pattern was, however, very heterogeneous. Only ~30–50% of the endothelial cells in a given culture expressed detectable Ia and the staining intensity was variable. Double staining for FVIII:RAG confirmed that the Ia-expressing cells were endothelial cells. Under the same conditions, epididymal fat pad endothelial cells were also inducible to express Ia antigens in heterogeneous densities, irrespective of whether primary cultures (Fig. 2, *g* and *h*) or passaged cells were used. Peritoneal macrophages, however, homogeneously expressed Ia after IFN- γ treatment. Pericytes did not respond to the same treatment by Ia expression (e.g., Fig. 2, *e* and *f*; other results not shown). When IFN- γ was omitted, there was no Ia expression in parallel cultures of endothelial cells, pericytes, and macrophages. Inhibition of prostaglandin synthesis by indomethacin had no effect.

Brain Microvascular Endothelia and Antigen Presentation

T Cell Proliferation. The principal ability of RBE to present protein antigens to specific syngeneic T line lymphocytes was examined by confronting a Lewis rat-derived T lymphocyte line specific for ovalbumin, L.OA, with Lewis brain endothelia in the presence or absence of ovalbumin, or the polyclonal T cell mitogen, Con A. These L.OA cells had been cultured in T cell growth factor-containing medium for 2 wk, to allow them to revert to a quiescent state of activation, and to deplete thymic APC that could have been carried over from the previous cycle of antigen/APC-dependent activation. Indeed, in the absence of any APC, the L.OA cells remained completely inactive irrespective of the presence of ovalbumin or Con A (Fig. 3, *a*). On the other hand, professional thymic APC presented ovalbumin and Con A in a highly activating manner to the responding L.OA T cells (Fig. 3 *f*). Lewis brain endothelial cells, which were derived from a practically pure secondary endothelial culture, were completely unable to induce T cell proliferation in the presence of antigen or mitogen, irrespective of Ia induction by pretreatment for 48 h with 50 U/ml IFN- γ (Fig. 3, *b* and *c*). This response pattern could be reproduced exactly with T cell lines specific for MBP and other antigens. RBE cells were thus as ineffective in antigen presentation, as were rat skin fibroblasts (Fig. 3, *d* and *e*).

To learn whether the endothelial inability to activate T cells was due to active suppression of T cell activation, passaged Lewis brain endothelia were cultured with T line L.RBP. This line, as described before (Sun and Wekerle, 1986), specifically recognizes rat MBP, and is capable of mediating experimental autoimmune encephalomyelitis (EAE) to syngeneic Lewis rats. In this case, however, the line cells were used only 4 d after antigen/APC activation. Fig. 4 shows that, at this stage, the T line cells had some spontaneous proliferation, in the absence of APC and antigen. Addi-

Figure 2. Ia expression by Lewis brain endothelial cells cultured in the presence of 50 U/ml IFN- γ . Double staining using FVIII:RAG antibodies (*a*) and anti-Ia antigens (OX 6; *b*) in methanol-fixed cells show the presence in the cytoplasm of Ia-antigens. Staining of live cells reveal heterogeneous surface staining (*c* and *e*, phase-contrast; *d* and *f*, indirect immunofluorescence of the same fields; arrowheads in *e* point to Ia-negative pericytes). Lewis epididymal fat pad endothelial cells also expressed Ia-antigens after gamma interferon induction (*g* and *h*). Bar, 50 μ m.

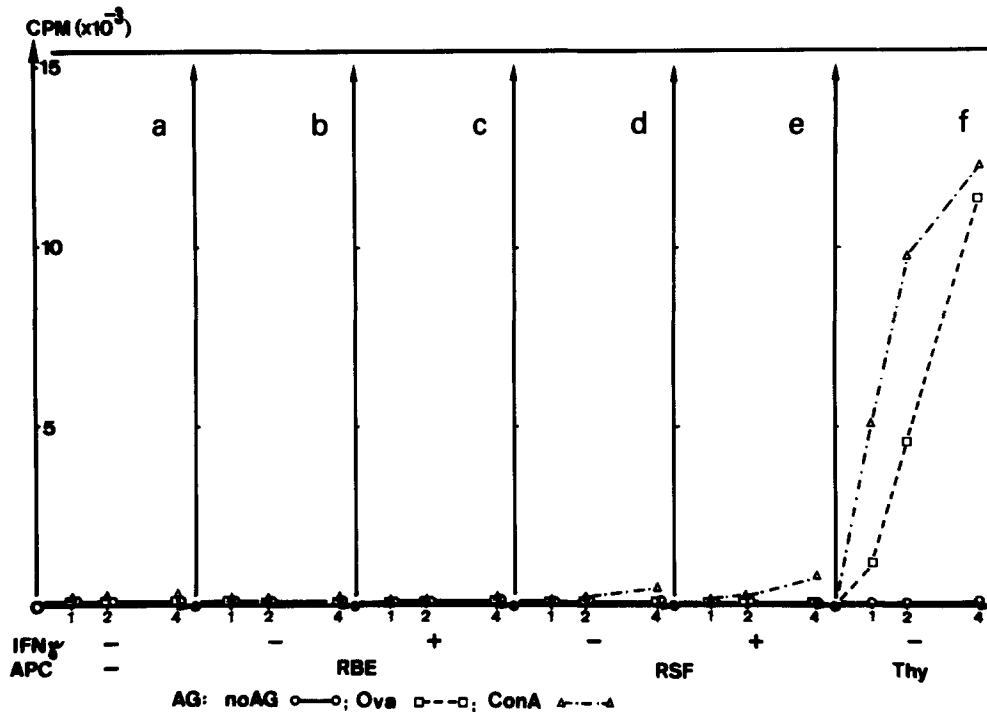


Figure 3. Presentation of ovalbumin (Ova) and Con A to Lewis-derived, ovalbumin-specific T line L.OA. Graded numbers of the metabolically resting L.OA lymphocytes ($1-4 \times 10^4$ cells/well/abscissa) were cultured in the presence or absence of RBE, rat skin fibroblasts (RSF), or irradiated thymic APC (Thy). RBE and RSF were precultured with or without IFN- γ (50 U/ml) for 48 h before addition of L.OA cells. ^3H -TdR incorporation is given in the ordinate.

tion of rat myelin basic protein (rat MBP) or Con A without exogenous APC resulted in increased proliferation, which indicates the persistence of intrinsic APC that had been carried over from the previous restimulation round. As expected, the monoclonal antibody OX6, directed against RT.1B product blocked antigen dependent Ia-restricted proliferation, but did not interfere with Ia-unrestricted activation by the mitogen Con A. Furthermore, addition of thymic APC strongly enhanced both Ia-restricted activation by rat MBP as well as mitogenic activation by Con A. It should be noted that a Lewis brain derived astrocyte clone, F10, which had been induced to express Ia by 48 h incubation with 50 U IFN- γ , also specifically presented rat MBP to the responder T line cells, and drove them into activation and proliferation. Much in contrast, however, purified RBE neither presented rat MBP, in a way to induce T cell proliferation, nor did they interfere with the spontaneous response pattern of the L.RBP cells. Antigen independent spontaneous proliferation was unaffected by the presence of endothelia, as was the MHC-restricted response to rat MBP. Preincubation of brain endothelia for 48 h with 50 U of IFN- γ did not alter this response pattern. Thus, the purified RBE neither actively induce antigen-specific T cell proliferation, nor do they suppress T cell proliferation in response to recognition of antigens presented by other APC.

Antigen-specific, MHC-restricted Cytolysis of Endothelia. Morphological screening of mixed RBE/T lymphocyte cultures suggested antigen-dependent interactions between both cell types. MBP-specific T line cells disrupted monolayers of IFN- γ pretreated RBE in the presence but not in the absence of their specific antigen MBP. We quantified the T cell dependent lysis of RBE using a conventional ^{51}Cr -release assay. T cell-dependent cytolysis of RBE reached a peak within 18 h and was dependent on the presence of MBP at

a minimal concentration of 20 $\mu\text{g}/\text{ml}$ (Fig. 5). There was no RBE lysis in the presence of control antigens, like ovalbumin. Furthermore, Ia expression on RBE cultures was a critical element. First, ^{51}Cr -release could only be observed with RBE pretreated with IFN- γ . Second, in pretreated cultures, the monoclonal anti-Ia antibody OX6 suppressed lysis almost completely, whereas the monoclonal antibody OX17 (which binds to an irrelevant MHC class II product) and anti-MHC class I antibody OX18 had no effect (Fig. 6). In contrast, the mitogen Con A that triggers polyclonal T cell activation of rat T lymphocyte line cells without MHC-restriction facilitated lysis to RBE cultures irrespective of pretreatment with IFN- γ . In variance to the encephalitogenic MHC-reactive T lines, the ovalbumin specific line L.OA caused only marginal isotope release.

Discussion

The endothelial BBB has a pivotal role as an interface connecting the nervous system with the immune system. This has been strikingly documented by experimental animal models of T lymphocyte mediated autoimmune disease in the central and the peripheral nervous system. Encephalitogenic T lymphocyte lines, monospecific for one critical epitope on MBP cause autoimmune disease exclusively restricted to the CNS, without involvement of adjacent peripheral nervous tissue (Wekerle, 1984; Tabira and Sakai, 1987). Conversely, T line cells recognizing a specific epitope on the P2 protein of peripheral nerve myelin, specifically attack peripheral nerves, but ignore adjacent CNS tissue (Izumo et al., 1985). In these models, the pathogenic T cells are injected i.v. and reach their relevant target organ via local endothelial vascular sheaths. Considering this and the fact that all pathogenic T lines described so far are mem-

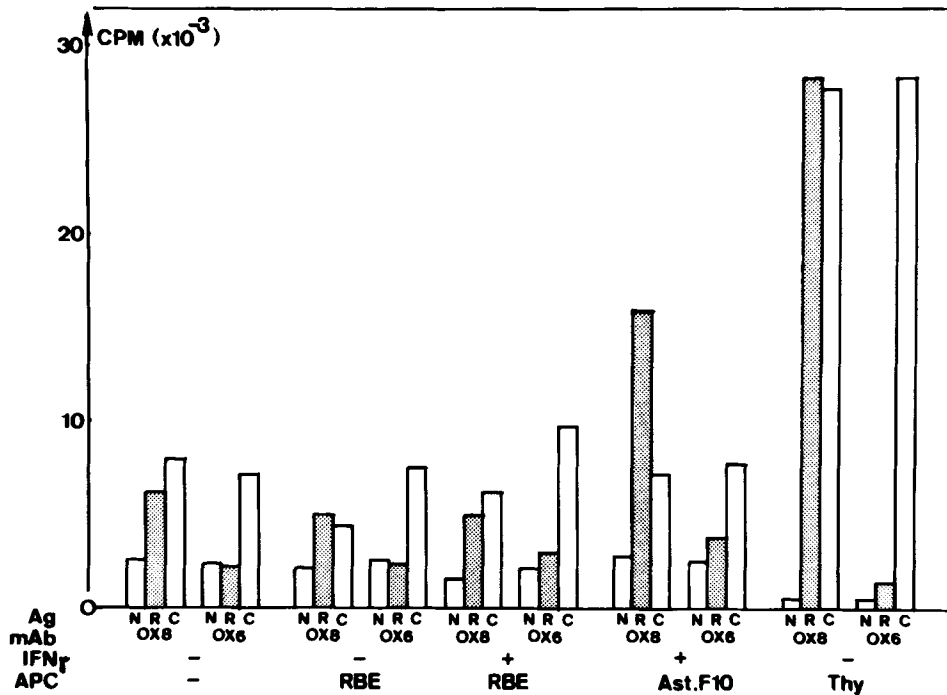


Figure 4. Presentation of rat MBP to Lewis-derived T line L.RBP (Wekerle et al., 1986) by Lewis RBE, a Lewis-derived astrocyte clone F10, and irradiated Lewis thymus APC. Monolayer APC were precultured for 48 h in the presence or absence of 50 U/ml IFN- γ . Presentation of antigen (N, no antigen; R, rat MBP; C, Con A) tested in the presence of monoclonal anti-Ia-antibody (OX6), or isotype matched anti-CD8 monoclonal antibody OX8 as control. The L.RBP T line cells were in a partly activated metabolic state 4 d after activation by antigen and APC. Part of the original APC were carried over as demonstrated by OX6 sensitive activation by rat MBP in absence of exogenously added APC (left side).

bers of the CD4 subset and hence recognize protein antigens in the molecular context of Ia determinants, inducibility of Ia on CNS endothelial cells attracts considerable interest. There is general agreement that in the normal CNS there are very few if any Ia positive cells (Hart and Fabre, 1981; Craggs and Webster, 1985), and interestingly, even after prolonged infusion of recombinant IFN- γ , extremely few CNS endothelia

become positive (Skoskiewicz et al., 1985; Momburg et al., 1986). There is, however, less agreement about the nature of Ia-positive cells in EAE. Most Ia-positive cells are macrophage-like cells (Wekerle, 1984; Sobel et al., 1984; Matsumoto and Fujiwara, 1986; Vass et al., 1986) often concentrated around the blood vessels and presumably derived from bone marrow precursors (Hickey and Kimura, 1988). Ia de-

Cytotoxic effect of Z82 line cells on RBEs

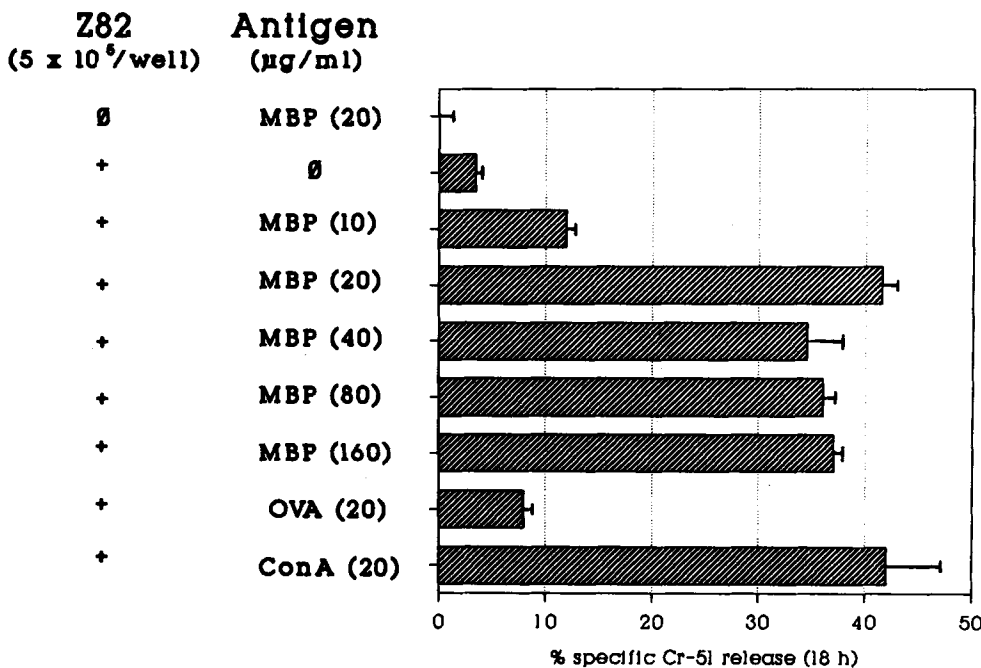


Figure 5. Cytotoxic effect of encephalitogenic, MBP-specific Lewis T cell line Z82 on Ia-induced MBP presenting syngeneic RBE, as dependent on antigen concentration. Ia induction by 72 h incubation with IFN- γ containing Con A culture supernatant (Fierz et al., 1985) before the ⁵¹Cr-release test. Isotope incorporation: 10 μ Ci/well Na⁵¹CrO₄, 20 h. Lysis was tested after 18 h of coincubation of the labeled target cells with Z82 cells and test antigen/mitogen. Isotope release calculated as indicated in Materials and Methods.

Cytotoxic effect of Z82 line cells on RBEs Inhibition by OX-6

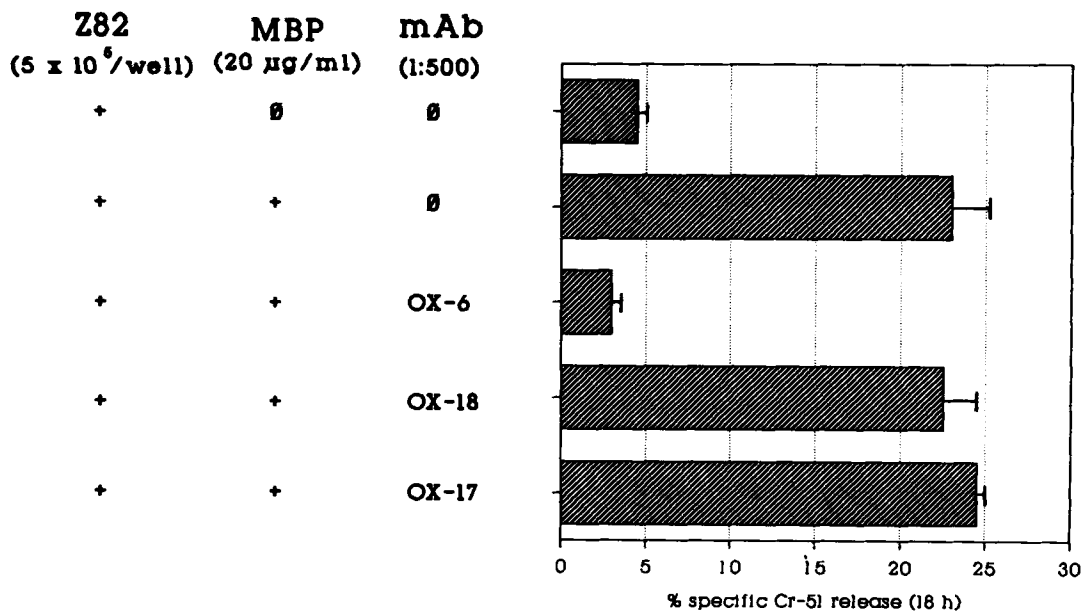


Figure 6. MHC restriction of brain endothelial lysis by encephalitogenic T lymphocytes. Assay as in Fig. 5. Monoclonal antibodies (details in Materials and Methods) were present throughout lysis period.

terminants on endothelial cells in EAE infiltrates have been observed by some (Sobel et al., 1984*a,b*; Sobel et al., 1987; Traugott et al., 1985), but not by others (Matsumoto and Fujiwara, 1986; Vass et al., 1986).

Direct studies of Ia induction in, and antigen presentation by BBB endothelial cells are complicated by several factors. It should be noted that CNS blood vessel walls are composed of more than one cell type. Endothelia, pericytes, smooth muscle cells, but also bone marrow-derived elements (Hickey and Kimura, 1988), and astrocytes are intimately associated with the BBB (White et al., 1981). At least some of these cells can be induced in vitro to express MHC class II determinants and thus are potential APC (Pober et al., 1983; Hart et al., 1987; Fontana et al., 1984; Fierz et al., 1985; Wong et al., 1985; Hirsch et al., 1983). Contamination of endothelial cultures with any of these cells must be expected in the absence of stringent selection of culture conditions and of the careful characterization of the cells arising in the cultures.

We have directly investigated Ia inducibility of CNS derived endothelia and their capacity to immunogenically present protein (auto-)antigens to T lymphocytes using highly purified CNS endothelia on the one hand, and antigen monospecific permanent T lymphocyte lines on the other.

Rat brain-derived microvascular endothelia were completely unable to activate monospecific syngeneic T line cells by immunogenic presentation of foreign or autoantigens. This incapacity was not due to suppression of T cell activity by downregulatory mediators (for example, prostaglandins), but rather seemed to reflect immunological inertness of the endothelia. Moreover, the incapacity of RBE of inducing antigen specific T cell proliferation was not because of their inability to synthesize and express Ia antigens, which are known to restrict antigen presentation to T cells. Treatment

of endothelia for 48 h in vitro with optimal doses of IFN- γ led to marked, though not abundant, and variable expression of Ia determinants on the surface membrane of endothelia.

Our results that are in line with a previous report by Pryce et al. (1989) do not necessarily conflict with other work demonstrating full scale antigen presentation by BBB cells (McCarron et al., 1985; McCarron et al., 1987). This work relied on protease dissociated microvascular isolates on the one hand, and APC-depleted primed spleen cells on the other. Although Ia may indeed have been induced on endothelia in these cultures, minor numbers of professional or inducible APC-associated with the BBB cells have not been excluded.

Inducibility of Ia on the plasma membrane is necessary, but certainly not sufficient for an individual cell to present antigen to T lymphocytes. It should be noted that to be recognizable by T cells, a protein antigen has to be engulfed, cleaved, and reexpressed on the surface in association with membrane MHC antigens (Unanue and Allen, 1987). Hence, the capacity of endocytosis and intracellular protein sorting is a second critical function of any APC. Ia positive, fixed APC are able to present only small peptides, but no intact protein antigens (Shimonkevitz et al., 1983) and the same has been reported for L cells transfected with MHC class II genes (Shastri et al., 1985). Moreover, thyrocytes were shown to be Ia inducible, but yet failed to effectively present antigen (Ebner et al., 1987; Lorenz and Allen, 1988; Lorenz and Allen, 1989). Finally, we recently showed that within the panel of immunoglobulin producing B cell hybridomas, some were able to present antigen to T cells, whereas others, with comparable Ia expression, were not (Zhang et al., 1988).

Our finding that brain-derived endothelial cultures are susceptible to antigen specific cytolysis by MBP-reactive

cytotoxic T line cells but fail to activate these lymphocytes to proliferation, strongly argues against a total defect of endothelial cells to process and present protein antigen to immune cells. It should be noted that the molecular events triggered in an antigen recognizing T lymphocyte are complex. They include changes in the lymphocytic plasma membrane immediately upon antigen recognition, but also activation of "late activation genes" after a period of up to 48 h. (Crabtree, 1989). Cytolytic effects, both perforin dependent and lymphotoxin-mediated, are early effects, preceding the signals required for blastogenesis and proliferation. These are not provided by the antigen presenting endothelia. The nature of the deficient late signals is unknown, and their definition will require detailed sequential analyses of T cell activation in the present lymphocyte endothelium system.

Our studies were focused on using optimally purified endothelial and lymphoid cells to study lymphoendothelial interactions. We paid less attention to the marked developmental plasticity of CNS endothelia. It should be noted that BBB endothelia in situ are highly specialized showing unusual enzyme patterns, membrane receptors, complex intercellular junctions, and lack of pinocytotic vesicles (Coomber and Stewart, 1985; Crone and Olesen, 1982; Nagy et al., 1984; Jefferies et al., 1984; Risau et al., 1986a,b). This specialization seems to be the result of microenvironmental induction (Stewart and Wiley, 1981; Risau et al., 1986b; Janzer and Raff, 1987) that is lost after a few passages in vitro (our unpublished results).

At present, we cannot exclude that the same conditions that induce endothelial specializations like formation of tight junctions (Tao-Cheng et al., 1987) or enzymes (Beck et al., 1986) also enhance the immunological potential of RBE. Yet, some experimental evidence argues against antigen presentation by specialized BBB endothelial cells in situ. In chimeric rats that were "constructed" by transplantation of (semi-)allogeneic bone marrow to irradiated hosts, EAE could be transferred with MBP specific T cell lines, which were MHC compatible with the bone marrow graft, whereas compatibility with the recipient was less crucial. This implied that bone marrow-derived perivascular monocytes/macrophages, rather than endothelial cells had a role in local presentation of myelin autoantigen (Hickey and Kimura, 1988; Hinrichs et al., 1987; Ting et al., 1983). Furthermore, work from our laboratory indicated that radiolabeled T cells enter the BBB irrespective of their antigen specificity. During the first day after i.v. injection, autoimmune, MBP-specific T cells were not found at higher frequencies in the CNS than ovalbumin specific T cells that certainly did not have an opportunity to recognize "their" antigen on the BBB endothelium of their normal recipient rat (Wekerle et al., 1986b; Meyermann et al., 1987).

Taken together, our present data do not support the concept that antigen presentation by BBB endothelial cells is the critical first signal telling circulating T cells to cross the BBB. We rather propose that other, contact dependent intercellular interactions, possibly involving more than one set of cell interaction molecules, are responsible for the induction of T cell entry into the CNS. Antigen presentation by BBB endothelium may have, however, a role in late stages of progressing inflammatory CNS disease. In EAE for example, at a time when locally infiltrated MBP specific T lymphocytes have already initiated a strong response against my-

elin presenting astrocytes or microglial cells (Wekerle et al., 1986b), local BBB endothelium may be induced to express Ia antigens by the high local concentrations of lymphokines released from activated lymphocytes. The endothelia may then present myelin degradation products, which may be available in unusually high quantities as a consequence of the myelin specific inflammatory reaction. As a consequence, the activated local BBB endothelial cells would present MBP to the cytolytic T cells and be destroyed. The rapid destruction of the BBB, which is typical for early phases of clinical EAE, would be the result of this interaction.

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