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Differential changes in junctional complex proteins suggest the ependymal lining as the main source of leukocyte infiltration into ventricles in murine neurocysticercosis

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Abstract

The blood brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCB) limit the influx of immune mediators and bloodstream compounds into the central nervous system (CNS). Upon injury or infection, the integrity of these barriers is compromised and leukocyte infiltration occurs. The BCB is located in the choroid plexuses (CPs) found within ventricles of the brain, and it is considered one of the main routes of cellular infiltration into the CNS into healthy individuals. Our group recently showed that in a murine model of neurocysticercosis (NCC), there is a moderate increase in infiltration of leukocytes into ventricles, but the BCB is hardly compromised. To elucidate the role played by CPs and surrounding ependyma in leukocyte infiltration at ventricular sites, we analyzed changes in the expression of junctional complex proteins in animals intracranially infected with *Mesocystoides corti*. The results indicate that infection does not change the expression pattern of junctional complex proteins in CPs, but structural alterations and disappearance of these proteins were evident in ependyma adjacent to the internal leptomeninges. The kinetics and magnitude of these changes directly correlated with the extent of leukocyte infiltration through ependyma and with the expression and activity of MMPs. The results of this study indicate that the anatomical elements of the BCB are minimally disrupted during the course of murine NCC. Thus, most of the leukocytes infiltrating ventricles appear to extravasate through pial vessels located in the internal leptomeninges juxtaposed to the ependyma layer and then traverse the ependyma cells. In addition, MMP activity seems to be involved in this process. These results provide evidence for a previously undescribed entry route for leukocytes into the CNS.

Keywords

neurocysticercosis; blood-cerebrospinal fluid barrier; ependyma; choroid plexus; tight junctions; adherens junctions

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1. INTRODUCTION

An intact blood brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCB) are essential for maintenance of a healthy CNS, and disruption of these barriers is usually associated with enhancement of the neuroinflammatory process (Engelhardt et al., 2001; Hawkins and Davis, 2005; Petty and Lo, 2002; Strazielle et al., 2000). Our previous findings have shown that in mouse brain infected with the larval form of the helminth *Mesocostoides corti*, the kinetics of disruption of the BBB and BCB differ according to the vascular bed and anatomical site analyzed (Alvarez and Teale, 2006). Further analysis showed that disruption of the BBB in pial and parenchymal vessels, which are two known routes of leukocyte extravasation in the CNS, is directly associated with dislocation and/or disappearance of junctional complex proteins and that the kinetics of these changes correlate with expression of active MMP-9 by infiltrating leukocytes (Alvarez and Teale unpublished observations).

A third route of leukocyte infiltration into the CNS occurs in the choroid plexuses (CPs) (Ransohoff et al., 2003). The CPs are located within the brain ventricles and are comprised of a highly specialized ciliated epithelium enclosing a stroma with fenestrated blood vessels lacking BBB properties (Speake et al., 2001). The main function of the CPs is to secrete cerebrospinal fluid (CSF) which is important to control intracranial volume adjustment, to buffer extracellular fluid ions and other solutes and in general to support brain homeostasis (Strazielle et al., 2000). The cellular boundary created by the CPs that separate the blood and CSF is what is described as the BCB (Speake et al., 2001). The anatomical basis of the BCB is partly due to the apical tight junctions (TJs) located in between choroid epithelial cells (Cserr and Bundgaard, 1984). The TJ proteins in these cells include zonula occludens 1 (ZO1), occludin, claudin-1, -2, -11, and low levels of claudin-5 (Lippoldt et al., 2000a; Lippoldt et al., 2000b; Wolburg et al., 2001). Adherens junctions (AJs) proteins are another group of junctional complex proteins able to mediate cell adhesion and partially regulate paracellular permeability in endothelium and epithelium (Bazzoni and Dejana, 2004; Brown and Davis, 2002). The presence of AJ proteins have been examined in the CPs, and cadherin, α -catenin and β -catenin are detectable in choroid epithelial cells (Lippoldt et al., 2000a).

Another important epithelium for controlling CNS homeostasis is the ependyma which regulate the transport and metabolism of molecules from the blood to the brain as well as in the opposite direction (Del Bigio, 1995). Ependyma also have transport systems and distinct enzymes able to remove endogenous and exogenous toxins from the nervous system (Abramovitz et al., 1988; Cammer et al., 1989; Carder et al., 1990). In addition, ependyma express ZO1, occludin, cadherin, α -catenin and β -catenin which are some of junctional complex proteins known to provide the anatomical basis of the CNS barriers (Lippoldt et al., 2000a; Petrov et al., 1994). These chemical and mechanical elements provide ependyma the properties to act as a filtration barrier that protects the brain from potentially harmful substances present in the CSF (Kuchler et al., 1994). Although ependyma seem to work as another barrier in the CNS, its role during inflammation and leukocyte extravasation remains to be elucidated.

Leukocyte trafficking is a multistep process, in which various types of adhesion molecules, cytokines and chemoattractants are involved (van Buul and Hordijk, 2004). The last step of this process is known as diapedesis and involves leukocyte migration in between endothelial cells and through the basal lamina of blood vessels (Muller and Randolph, 1999). It has been postulated that activity of matrix metalloproteinases (MMPs) is essential to support the diapedesis process (Asahi et al., 2001; Gurney et al., 2006). Access of leukocytes into the CSF from the CPs is a more complex process because they first need to extravasate the fenestrated blood vessels, move through the CP stroma, and then migrate into the ventricular space by wedging through the junctional complexes of choroid epithelial cells (Ransohoff et al., 2003). This pathway has been described as one of the main routes of leukocyte migration into

the CNS during normal physiological conditions as well as injury (Carrithers et al., 2002; Provencio and Vora, 2005; Ransohoff et al., 2003). Multiple studies focusing on the migration of leukocytes through this route measure the phenotype and quantity of cells coming from the CPs into the CSF (Hintzen et al., 1995; Kivisakk et al., 2003; Svenningsson et al., 1995). However, a caveat to this interpretation, particularly in CNS disease states, is that leukocytes found in the CSF may derive from other sources since not all of the potential barriers to leukocyte infiltration have been sufficiently explored.

Recently, we showed that upon infection, CP disruption is minimal and does not correlate with the number of leukocytes found in ventricles. In the present study, we examined leukocyte infiltration in ventricles and correlate it with changes in the expression of junctional complexes in CPs vs ependyma. Changes in the expression of junctional complex proteins were determined at different post-infection times. The presence of parasitic antigens at ventricular sites was also tested using antibodies specific for *M. corti* molecules. Taken together, the results suggest that damaged subependymal vessels of the internal leptomeninges coupled with the juxtaposed disrupted ependyma explain the predominant source of leukocytes infiltrating ventricles rather than the CP.

2. MATERIAL AND METHODS

Animals

Female BALB/c mice 3–5 wk old were purchased from the National Cancer Institute Animal Program (Bethesda, MD). Animal experiments were conducted under the guidelines of the University of Texas System, The U.S. Department of Agriculture, and the National Institutes of Health.

Parasites and inoculations

M. corti metacystodes were maintained by serial intraperitoneal (i.p.) inoculation of 8 to 12 wk old female BALB/c mice. For intracranial inoculations, parasites were aseptically collected from the i.p. cavity of mice that had been infected for about 6 months. Harvested parasites were extensively washed in HBSS.

After that, the metacystodes (50 microorganisms) were suspended in 50 μ l of HBSS and injected intracranially into 3–5-wk-old female BALB/c mice using a 1-ml syringe and a 25-gauge needle (Cardona et al., 1999; Ding et al., 1997). The needle was inserted to a 2-mm depth at the junction of the superior sagittal and the transverse sutures. This allows insertion of the needle into a protective cuff avoiding penetration of the brain tissue. Control mice were injected with 50 μ l sterile HBSS using the same protocol. Before intracranial inoculation, mice were anesthetized intramuscularly with 20 μ l mouse mixture containing 100 mg/ml ketamine and 20 mg/ml rompum (Laboratory Animal Resource, University of Texas Health Science Center, San Antonio, TX). Animals were sacrificed at 1 d, 3 d, 1 wk, 3 wk and 5 wk after inoculation and analyzed for parasite burden and immune cell infiltration. Before sacrifice, animals were anesthetized with 25 μ l of mouse mixture and perfused through the left ventricle with 15 ml of cold PBS. Five or more animals were analyzed for each post-infection time with multiple sections analyzed per animal.

Tissue processing

Perfused brains were immediately removed, embedded in O.C.T. resin (Sakura, Torrance, CA) and snap frozen. Serial horizontal cryosections of 10 μ m in thickness were placed on silane prep slides (Sigma-Aldrich, St. Louis, MO). One in every five slides was fixed in formalin for 10 min at room temperature (rt) and stained with hematoxylin and eosin (H&E). The remainder of the slides was air dried overnight and fixed in fresh acetone for 20 seconds at rt. Acetone-

fixed sections were wrapped in aluminum foil and stored at -80°C or processed immediately for immunofluorescence.

Antibodies

Antibodies purchased from Zymed (San Francisco, CA) and generated in rabbits included: anti- β -catenin, anti- α -catenin, anti-pan-cadherin, anti-occludin, anti-claudin-1 (no cross reaction with claudin-3), anti-claudin-3 and anti-claudin-5. Goat-anti-JAM-A was obtained from R&D systems (Minneapolis, MN). Rat anti-Mac1 Alexa 647-conjugated was purchased from Serotec (Oxford, UK). The purified rabbit anti-fibronectin and rat anti-ZO1 antibodies were purchased from Chemicon International (Temecula, CA). Rabbit anti-MMP-8 was purchased from Triple Point Biologics (Forest Grove, OR). Finally, rabbit anti-laminin was purchased from SIGMA (Saint Louis, MO). Rabbit primary antibodies were detected with a Cy2-conjugated AffiniPure anti rabbit IgG, JAM-A with a Rhodamine red X (RRX)-conjugated AffiniPure anti goat IgG. ZO-1 was detected with a RRX and Cy2 conjugated AffiniPure anti-rat IgG purchased from Jackson ImmunoResearch (West Grove, PA). The lectin Isolectin-B4 (IB4) conjugated with Alexa 546 was purchased from Molecular Probes (Eugene, OR). Monoclonal antibody against *M. corti* was raised against supernatant collected after in vitro incubation of *M. corti* larvae for 3 days. The anti-MCS monoclonal antibody was labeled with Alexa 488 monoclonal antibody labeling kit from Molecular Probes (Eugene, OR) following manufacturer's protocol. All antibodies were titrated with brain sections from 2 wk infected animals to determine optimal concentrations prior to use. Spleen sections were used as positive controls in titration experiments.

Immunofluorescence microscopy

Double and triple immunofluorescence staining was performed as previously described (Alvarez and Teale, 2006). Briefly, tissues were fixed in -20°C acetone for 10 min and then hydrated in PBS. Non-specific immunoglobulin binding was blocked by 30 min incubation at rt with serum from the same species that the fluorochrome conjugated antibodies were derived. Sections were incubated for 40 min with primary antibodies diluted in species specific serum. Sections were washed 7X for 3 min each after incubation with specified antibodies. Secondary antibodies were incubated for 30 min at rt when necessary. The second set of primary antibodies was then incubated with their respective secondary antibody. Negative controls using secondary antibodies alone were included in each experiment and found to be negative for staining. The third antibody included in some of sections was anti-Mac1 conjugated with Alexa 647. Sections were mounted using fluorsave reagent (Calbiochem, La Jolla, CA) containing $0.3\text{ }\mu\text{M}$ 4',6'-diamidino-2-phenylindole dilactate-DAPI (Molecular Probes, Eugene, OR). Fluorescence was visualized using a Leica DMR epifluorescent microscope (Leica Microsystems, Wetzlar Germany). Images were acquired using a cooled CCD SPOT RT camera (Diagnostic instruments Inc, Sterling Heights, MI), and they were processed and analyzed using Adobe Photoshop 7.0 (Adobe, Mountain View, CA).

In situ zymography

In situ zymography was performed to detect and localize gelatinolytic activity in brain sections. Sections that had been stored at -80°C were fixed and hydrated for immunofluorescent analyses. A solution containing DQ-gelatin or collagen (Molecular probes, Eugene, OR), which are MMP substrates heavily labeled with fluorochromes, were used to determine activity of MMPs in situ. These substrates are heavily quenched and upon digestion the increase in fluorescence is proportional to MMP activity. Sections were incubated 40 minutes at rt with $1\text{ }\mu\text{g/mL}$ of DQ-gelatin in reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl_2 , pH 7.6) or with 5 mg/mL of collagen-oregon 488 in PBS 1X, washed and subsequently followed by

immunofluorescent staining to correlate the levels of active and inactive MMPs in the samples. Images were acquired and processed as previously described.

3. RESULTS

Histological characterization of ventricles from *M. corti* infected mice

During the course of murine NCC, leukocytes migrate into the CNS through three vascular beds; pial, parenchymal and choroid vessels, but the number of cells infiltrating through the later appears relatively low (Alvarez and Teale, 2006). The limited extravasation from the CPs is in apparent contradiction to the number of leukocytes that are often present in ventricles. To analyze this further, entire brains from infected and mock infected mice were sectioned and whole brain sections analyzed by H&E staining and laminin immunostaining (Fig 1 and Fig 2). Ependyma juxtaposing parenchyma vs internal leptomeninges can be distinguished by the presence of laminin (basal lamina) in the later (Fig 1). Histological analysis showed a lack of inflammatory infiltrates in the subependyma of mock infected animals (Fig 2). In contrast, upon infection leukocytes can be detected in subependymal areas proximal to pial vessels. These leukocytes appeared to be migrating into ventricular spaces through breaks in the ependymal lining juxtaposing internal leptomeninges (Fig 1 and Fig 2). In addition, areas exhibiting such ruptures correlated with cell infiltration and a loss of laminin (Fig 2). The ependyma juxtaposing the parenchyma does not exhibit ruptures or leukocyte migration in mock or infected animals (Fig 2). Thus, subsequent analyses of infection induced changes in ependyma were focused on areas juxtaposing internal leptomeninges.

Detection of *M. corti* antigens in ventricular areas

We also studied the distribution of *M. corti* antigen in ventricular areas (Fig 3). Antigen was detected using two different types of antibodies. The first was a pool obtained from the sera of animals intracranially infected with the parasite for 3 weeks. The second was a monoclonal antibody directed against excretory/secretory molecules released by the parasite when cultured in vitro and termed MCS for *M. corti* supernatant. Antibodies from 3 wk infected animals labeled the parasites located in ventricular areas, and the material released by the parasite (Fig 3A). *M. corti* antigens were detected in close proximity to the CP (Fig 3B). Choroid vessels (red) were detected with Alexa 546 conjugated isolectin B4 (IB4). Parasite derived antigens were found within the ventricular cavity in general, at the surface and in between the ependymal cells surrounding the ventricle. Parasite antigen was also present in the subependyma and subarachnoid space and at the basal lamina of the ependyma (Fig 3A). MCS monoclonal antibody was utilized to confirm the pattern of staining obtained with polyclonal antibodies. MCS labeled the same areas as antibodies from 3 wk infected animals (Fig 3C and 3D). Thus, *M. corti* antigen is widely distributed in these areas.

Junctional complex proteins in ependyma and choroid plexus of mock infected animals

To perform a systematic study in normal mice of the junctional complex proteins in CP and ependyma, we used antibodies specific for several junction proteins using in situ multicolor fluorescence microscopy. The TJ proteins used in this analysis included claudin-1, claudin-3, claudin-5, occludin, JAM-1 and ZO-1. All the TJ proteins evaluated in this study were present at ventricular sites of mock infected animals although the patterns of expression varied particularly between CPs and ependyma (Fig 4). ZO-1 was used as a reference for the location of junctional complex proteins in ventricular areas as it was detected in both CP and ependyma, although to a lesser extent in ependyma (Fig 4-Table 1). Claudin-1 was located mainly in the cytoplasm of choroid cells with lower expression apparent at cellular junctions themselves (Fig 4A). In contrast, claudin-1 was not detected in ependyma (Fig 4A-Table 1). Claudin-3 was also found in the cytoplasm of choroid cells, but its expression at the position of cellular attachment was higher than claudin-1, and it showed essentially perfect co-localization with ZO-1 in the

junctional complex (Fig 4B). Claudin-3 was not detected in ependyma (Fig 4B-Table 1). Occludin exhibits a similar pattern of staining in the CP when compared to claudin-1, but in contrast to claudin-1 and -3, it was also expressed in the cytoplasm and junctions between ependymal cells (Fig 4C-Table 1). Claudin-5 showed a unique pattern of staining in ventricles. The CPs expressed very low levels of claudin-5, almost undetectable in the junctional complexes (Fig 4D). Ependymal cells do not seem to express claudin-5, although it is expressed in pial vessels located under the ependyma (Fig 4D-Table 1). In addition, there appears to be a subpopulation of choroid vessels showing high expression of claudin-5 (Fig 4E), intermediate to low expression of β -catenin, α -catenin, ZO-1, cadherin, JAM-1 and very low expression of claudin-1. Finally, JAM-1 is found in the cytoplasm of the choroid cells, as well as in the junctional complexes of these cells (Fig 4F-Table 1).

The expression of AJ proteins in mock infected animals is also shown (Figs 4G,I-Table 1). Expression of cadherins was determined by using a pan polyclonal antibody. Cadherins were highly expressed at the junctions of ependymal cells (Fig 4G) whereas lower expression was found in CP and located more in the cytoplasm (Fig 4G). α and β -catenin showed a similar pattern of expression as pan-cadherin with lower expression in CP compared with ependyma (Fig 4F and 4I). Also, α and β -catenin localized more to interepithelial junctions in ependyma (Fig 4H), but were more localized to the cytoplasm in CP (Fig 4F and 4H). Clearly, the AJ proteins analyzed predominated more in ependyma of normal mice whereas the TJ proteins predominated in the CP (Table 1).

Upon infection the expression of junctional complex proteins changes in ependyma but not in choroid plexus

To determine changes in the expression of the junctional complex proteins at ventricular sites during the course of infection, mice were infected with *M. corti* and analyzed after 1 day, 3 days, 1 wk, 3 wks and 5 wks for the expression of junctional proteins by in situ immunofluorescent microscopy. In terms of the CPs, there were no apparent changes in the expression of TJ proteins analyzed at any of the post-infection times. Claudin-1 (Fig 5A), claudin-3 (Fig 5B), occludin (Fig 5C), claudin-5 (Fig 5D and 5E) and JAM-1 exhibited essentially identical staining patterns as compared to mock infected animals (Fig 4). In contrast, expression of TJ proteins found in the junctional complexes of ependyma adjacent to the internal leptomeninges differed from the mock infected animals. ZO-1 expression in the ependymal junctional complexes appeared dislocated (Fig 5C) or absent at distinct junctions (Fig 5H). Occludin expression also appeared discontinuous in the junctional complexes (Fig 5C). Thus, the TJ proteins expressed in the CPs appear unaltered by infection whereas substantial structural changes in TJ proteins are apparent in the ependyma.

Expression of AJ proteins in CP also showed no apparent differences between mock and infected animals (Fig 5F and 5G). However, expression and organizational changes of AJ proteins in ependyma juxtaposing internal leptomeninges were similar to results with TJ proteins. Changes were found in both cadherins and catenins, particularly in the ependyma lining the third and fourth ventricle with dislocated expression (Fig 5G) and absence of catenins (Fig 5F) and cadherins (Fig 5I) at distinct junctions. In addition, areas of the ependyma with no expression of junctional complex proteins showed evidence of leukocytes migrating from subependymal infiltrates to the ventricle (Fig 5I). Moreover, some of those inflammatory cells expressed AJ proteins (Fig 5G and 5I). These results suggest that upon infection with *M. corti* the junctional complexes of the BCB seem to be unaffected, whereas the junctional complex proteins of the ependyma were dislocated or undetectable compared with mock infected mice (Table I).

Changes in junctional complexes of ventricular areas correlate with leukocyte infiltration

As the junctional complex proteins associated with the CP remain intact following infection; it was critical to assess the source of infection induced increases of mononuclear cells into the ventricles. To address this question the expression of junctional complex proteins was correlated with the infiltration of leukocytes in ependyma adjacent to internal leptomeninges, CPs and the subependymal vasculature. As macrophages represent one of the major leukocyte subsets infiltrating during infection, Mac1⁺ (CD11b⁺) cells were analyzed. In mock infected animals, junctional complex proteins are expressed in subependymal pial vessels. A representative image for ZO-1 and β -catenin is shown in Fig 6A (asterisk). As above, the higher expression of β -catenin relative to ZO-1 in the ependyma is evident. The structural organization of junctional proteins appears dislocated in subependymal pial vessels as early as 1 day of infection and relative to mock becomes increasingly disorganized with time post-infection (Fig 6B). In addition, the junctional complexes of the ependyma lining in proximity to pial vessels are also disrupted (Fig 6B). Analysis of the Mac1⁺ infiltrating leukocytes in these areas revealed Mac1⁺ cells moving from subependymal pial vessels towards the ependymal basal lamina after 1 week of infection (Fig 6C).

The active migration of leukocytes through the ependyma correlated with polarized activity of MMP-8 (Fig 6D), similar to our observations of leukocyte extravasation from pial and parenchymal vessels (Alvarez and Teale unpublished observations). As the infection progresses the number of Mac1⁺ cells increases, and they appear to be migrating into the ventricles through areas of the ependymal lining where the junctional complexes have been lost (Fig 6E), presumably due to the action of MMPs. The same type of analysis was done with the CPs. As shown in figures 2 and 3, the junctional complex proteins of the CP do not appear to be altered upon infection. To determine changes in the permeability of the BCB the extravasation of fibronectin was evaluated. Active extravasation of this serum protein was not found in the CPs of mock infected animals and detection of fibronectin was limited to some of the vessels in the CP stroma (Fig 6F). Upon infection, active extravasation of fibronectin was detected in subependymal pial vessels and juxtaposed ependyma (Fig 6G) correlating with the active migration of Mac1⁺ cells shown in Fig 6E. In contrast, fibronectin extravasation was very limited in the CPs. Consistent with this, a limited number of Mac1⁺ cells were seen moving from the choroidal stroma towards the ventricle early in the infection (Fig 6H) and examination of samples from chronically infected animals (3 to 5 wks) showed no apparent changes in the number of cells migrating from the CP into the ventricles. Similar results were found when T cells and dendritic cells were analyzed. These findings were further supported by the limited expression of MMP-8 in the few cells migrating through the CPs (Fig 6I).

4. DISCUSSION

Leukocyte infiltration into the normal and diseased CNS is known to happen through distinct anatomical sites and types of vasculature (Alvarez and Teale, 2006; Ransohoff et al., 2003). Infiltration of immune cells upon infection has been mainly studied in vessels of parenchyma and leptomeninges but less so in ventricles. Histological analyses of ventricular areas of infected animals showed a progressive increase in the number of mononuclear cells accumulating in these areas (Alvarez and Teale, 2006). The most obvious source of these cells would be the CPs, the site of the BCB, as this is the normal route for the few numbers of infiltrating leukocytes in the healthy brain. However, the low level of serum protein leakage and leukocyte extravasation detected in CPs of infected mice suggested an alternative site. The focus of this study was to determine the expression and organization of structural proteins that contribute to the barrier properties of ventricular sites in murine NCC in order to assess potential pathways followed by inflammatory cells entering the cerebrospinal fluid (CSF). This is

clinically important as approximately 30% of NCC patients have intraventricular metacestodes (Cuetter et al., 1997).

Our findings in mock infected animals confirmed previous data showing expression of the junctional proteins claudin-1, occludin, cadherin, α -catenin and β -catenin in both the junctional complexes and cytoplasm of the choroid cells, whereas ZO-1 is exclusively found at the interendothelial junctions between choroid cells (Lippoldt et al., 2000a; Wolburg et al., 2001). In addition, we report for the first time expression of TJ proteins claudin-3, -5 and JAM-1 in cytoplasm and junctions of choroid cells. By using various combinations of these antibodies, significant colocalization of these molecules as well an ordered structure was apparent, indicating that multiple junctional complex protein molecules contribute to the barrier function of the choroid epithelium. The vessels embedded in the CP differ from those in the brain as they are fenestrated and thought to lack junctional proteins and associated barrier properties (Brightman, 1968; Larter et al., 1999). However, our results using uninfected mice clearly show that at least some choroidal vessels express several junctional complex proteins including claudin-1, -5, ZO-1, JAM-1, β -catenin, α -catenin and cadherin perhaps suggesting that not all the capillaries in CPs lack barrier properties.

The ventricles are encased in an epithelial lining or ependyma (Del Bigio, 1995). The ependyma of mock infected animals expressed the junctional proteins occludin, ZO-1, cadherin, α -catenin and β -catenin as previously reported (Lippoldt et al., 2000a). However, the expression of claudins-1, -3, and -5 were undetected, and the level of expression of ZO-1 was considerably less than that found in the CP epithelium. In general, the expression of TJ proteins was lower in ependyma as compared with CP epithelium. Conversely, the expression level of AJ proteins was higher. The ependyma has been considered a protective barrier between the CSF and the brain parenchyma (Del Bigio, 1995). However, the lower or undetectable expression of TJ proteins in the ependyma shown here suggests that this barrier is more permissive than the BCB of the CP.

Consistent with these predictions of the relative strength of the CP vs. the ependyma, upon infection there were no significant changes in the expression or structural morphology of the junctional complex proteins in CPs and the extravasation of serum proteins and immune cells was limited. Moreover, the expression of active MMPs is more limited in the CPs (Fig 6, Alvarez and Teale, unpublished observations). Previous studies are consistent with these findings. In rats with fluid shifts induced by head-down tilts there were no alterations in the TJs at the ultrastructural level or by immunofluorescence microscopy (Massequin et al., 2001). Also, in vitro cultures of choroidal cells treated with pro-inflammatory cytokines induced expression of active MMP-9 and MMP-2, but even under these conditions no alterations in the expression of TJ proteins were detected (Strazielle et al., 2003). In a study of experimental autoimmune encephalitis (EAE) the fenestrated capillaries of the CP remained intact, adhesion molecules ICAM-1 and VCAM-1 were not induced in these vessels and leukocyte migration across the endothelium could not be detected (Engelhardt et al., 2001). The apparent restrictive properties in the choroid vessels are complemented by the expression of junctional complex proteins reported in this study. Therefore, while the evidence indicates that extravasation of leukocytes through the CPs is a major route in the healthy brain; it is not in the diseased brain. Leukocytes that infiltrate the brain during normal homeostasis, particularly T cells, are thought to be involved in immunosurveillance and have a memory phenotype (Kivisakk et al., 2003). This process is likely to be regulated differently.

In contrast to the relative impermeability of the CPs, the bulk of cells infiltrating the ventricles appear to be migrating through disruptions in the ependyma and originate from subependymal vessels of the internal leptomeninges juxtaposing ependyma. Consistent with these results, analyses of the junctional protein complexes in ependyma of infected animals exhibited either

structural discontinuities or disappearance of the junctional complex proteins studied. Moreover, the changes in junctional complex proteins coincide with both the appearance of leukocytes extravasating the ependyma and MMP activity.

To determine if the differential change in the CP vs. ependymal barriers was due to the distribution of antigen, specific antibodies to parasite antigens were used. Parasitic antigens were found to be ubiquitous in the ventricular areas: on the apical side of the CPs, the ventricular space, surrounding ependymal cells and in the subependymal region. Therefore, it is unlikely that antigen distribution is involved in the observed permeability differences.

A recent study showed that T cells found in ventricles and lumbar CSF of healthy individuals have essentially the same phenotype and suggested that most of these cells come from the CPs although other ports of entry were not ruled out (Provencio and Vora, 2005). The same T cell phenotype was also found in the CSF of patients with inflammatory neurological disease (Giunti et al., 2003). However, it is known that CSF is actively secreted by the CP epithelium and circulates from the ventricles into the subarachnoid spaces before it is resorbed into the systemic circulation. Thus, CSF derived from a lumbar puncture would include leukocytes that had accumulated from pial vessels in the subarachnoid as well as those from the ventricular systems. Given the results of this study, many of the cells from the ventricles likely originate from a disrupted ependymal layer. Thus, the phenotype of cells in the CSF is a reflection of leukocytes coming from multiple sources.

To date three pathways have been identified for leukocyte migration into the CNS during disease. These include from the blood to CSF crossing the CPs, from the blood to subarachnoid spaces through pial vessels, and from the blood to the parenchyma through parenchymal vessels (reviewed in Ransohoff et al 2003). We propose a fourth route from these studies, i.e. from blood to the CSF through the disrupted ependymal layer by way of pial vessels in the internal leptomeninges.

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Figure 1. H&E and laminin staining of ventricular areas in *M. corti* infected brain

(A) H&E section of fourth ventricle (4V), ependyma juxtaposing parenchyma is depicted by the black dotted line and ependyma juxtaposing internal leptomeninges is indicated by the blue dotted lines. Arrowheads indicate major breaks in the ependyma where leukocytes move into the ventricle. Internal leptomeninges in proximity of the ventricle and surrounding areas are depicted by a continuous blue line and are characterized by extensive leukocyte infiltration (i). 3 wks infected animal 5X. (B) Immunostaining of laminin (red), glial fibrillar acidic protein-GFAP (green) and nuclei (blue) in 4V showing that ependyma juxtaposing parenchyma (white dotted lines) and internal leptomeninges can be distinguished by the presence of laminin in the later. 1 wk infected animal 10X. (C) Same image shown in B, but excluding nuclear staining

to better visualize ependyma juxtaposing parenchyma and internal leptomeninges. **(D)** H&E section of third ventricle (3V), ependyma juxtaposing parenchyma is depicted by the black dotted line and ependyma juxtaposing internal leptomeninges is depicted by the blue dotted lines. Internal leptomeninges in proximity of the ventricle and surrounding areas are depicted by a continuous blue line and are characterized by extensive leukocyte infiltration (i). A parasite (p) can be seen in the vicinity of 3V. Choroid plexus (cp). 3 wks infected animal 5X **(E)** Laminin (red), glial fibrillar acidic protein-GFAP (green) and nuclei (blue) staining in 3V showing that ependyma juxtaposing parenchyma (white dotted lines) and internal leptomeninges can be distinguished by the presence of laminin in the later (white arrowheads). The laminin antibody labels the cp stroma strongly. 1 wk infected animal 5X. **(F)** Same image shown in **E**, but excluding nuclear staining to better visualize ependyma juxtaposing parenchyma and internal leptomeninges.

Figure 2. H&E staining of mouse brain

The top left panel shows an axial section of mock infected brain indicating the locations of third (3V) and fourth ventricle (4V) 1X. Top right panels are magnified views of the 3V and 4V showing the location of choroid plexuses (cp) and ependyma (e) in mock infected animals; the black dotted line depicts ependyma juxtaposing parenchyma whereas the blue dotted line is depicting ependyma juxtaposing internal leptomeninges. Leukocyte infiltration is absent in ventricular areas of these animals (20X). Panel in the center shows the 4V of a representative 3 wk infected animal. Inflammatory infiltrates (i) are located in the subependymal region proximal to subependymal pial vessels (asterisks). Inflammatory leukocytes (I) appear to be traversing into the ventricular space through breaks in the e (arrowheads). The e is depicted by

a continuous dotted line (juxtaposing internal leptomeninges-blue and parenchyma-black) 20X. Bottom left panel shows the breaks (arrowheads) in the laminin positive layer below **e** juxtaposing internal leptomeninges of 3V in a 3 week infected animal. Bottom right panel is the same image on the left but includes nuclei staining (DAPI-blue); and the **e** is depicted by a dotted line with interruptions at breaking sites.

Figure 3. Detection of *M. corti* antigens in ventricles of infected animals

Antibodies from 3 wk infected animals (**A** and **B**) and MCS (**C** and **D**) were conjugated with Alexa 488 (green). All sections shown here come from 3 wks infected animals. Nuclear staining with DAPI is blue. (**A**) *M. corti* parasites (**p**) located in ventricle (**v**) and stained with serum from 3 wk infected animals. *M. corti* antigen is also detected in the surface (large arrowheads), inter-epithelium (small arrowheads) and basal lamina (arrows) of ependyma (**e**). 40X (**B**) *M. corti* antigens located in the choroid plexus (**cp**) surface. Choroid vessels were labeled with IB4 conjugated with Alexa 546 (red). 40X (**C**) Detection of *M. corti* antigen with MCS antibody in **v** and surface (large arrowheads), inter-epithelia (small arrowheads) and basal lamina (arrows) of **e**. 40X (**D**) MCS antibody labeling antigen in **cp** surface and in parasite **p** lodged in **v**. 40X

Figure 4. Expression of junctional complex proteins in ventricles of mock infected animals

The junctional protein ZO1 was highly expressed in the junctional complexes of choroid cells; but less so in **e**. ZO-1 was used as a frame of reference for location of other junctional complex proteins in ventricles. Nuclear staining with DAPI (blue) was done in all the sections, but excluded in some of the images for better visualization of the junctional complexes. (A) Claudin-1 was detected in the cytoplasm and to a lesser extent in the junctional complexes of **cp**, but not in **e**. 40X (B) Claudin-3 showed a similar pattern to claudin-1 except there was a higher expression at the cellular junctions colocalizing with ZO-1. Claudin-3 was not detected in **e**. 40X (C) Occludin had almost the same pattern of staining as claudin-1 in **cp**, but expression was also detected in **e**. 40X (D) Claudin-5 had a completely different pattern than the other TJ proteins. It was barely detectable in **cp** and not found in **e**. Expression was detected in the junctional complexes of subependymal pial vessels (arrowhead). 40X (E) Claudin-5 expression was also detected in a small group of choroid vessels (large arrowhead). Low levels of ZO-1 expression (small arrowheads) can be seen in **e**. 100X (F) JAM-A was found in the junctional complexes of **cp**, and it exhibited a punctate pattern of staining in the cytoplasm of choroid cells. No expression was detected in **e**. α -catenin was highly expressed at cellular attachment sites of **e** and moderate expression was seen in **cp**. 40X (G) Cadherin exhibited a similar distribution as α -catenin, highly expressed in **e** and moderately found in **cp**. 40X (H) High power view of ZO-1 expression in junctional complexes of **e** (arrowheads) with higher expression in **cp**. 100X (I) Same field as H, showing higher expression of β -catenin at the junctions of **e** compared with **cp** (arrowheads). Expression of β -catenin in **cp** is moderate. 100X.

Abbreviations: **e**: ependyma, **cp**: choroid plexus, **LV**: lateral ventricle, **3V**: third ventricle.

Figure 5. Expression of junctional complex proteins in ventricles of *M. corti* infected animals
 Expression of TJ and AJ proteins was evaluated at different post-infection times. ZO1 expression seems unaltered in the junctional complexes of **cp**, but not in **e**. Nuclear staining with DAPI (blue) was done in all the sections, but excluded in some of the images for better visualization of the junctional complexes. (A) Claudin-1 expression did not change in the **cp** of infected animals. 40X (B) The pattern of expression seen for claudin-3 during infection was very similar than in mock infection. 40X (C) Occludin expression showed no differences between *M. corti* infected and mock infected animals in **cp**. Dislocated expression of occludin and ZO-1 was found in the junctional complexes of **e**. 40X (D) Claudin-5 expression was very low in intact junctional complexes of choroid cells. Unidentified structure in the choroid cells nuclei was labeled with claudin-5 antibody. 100X (E) A small number of choroid vessels (arrowhead) maintained claudin-5 expression during infection, as observed in mock. 100X (F) JAM-1 expression in **cp** showed no differences between parasite infected and mock infected animals. Expression of α -catenin showed dislocation of the junctional complexes in **e**; changes in expression were not detected in **cp**. 40X (G) Junctional complex proteins ZO-1 and α -catenin have dislocated expression in **e**. In inflammatory infiltrates (**i**) located under the **e**, the expression of α -catenin was also apparent. 40X (H) Holes in the ependymal lining (arrowheads) are detected when ZO-1 expression was studied. No changes in the junctional complexes of **cp** were found. 100 (I) Same field as H, but showing pan-cadherin staining. Cadherin positive leukocytes in **i** appear to migrate into the ventricles through holes in the ependymal lining (arrowheads). The pattern of cadherin expression in **cp** is similar between parasite infected and mock infected animals. 100X
 Abbreviations: **e**: ependyma, **cp**: choroid plexus, **LV**: lateral ventricle, **3V**: third ventricle, **4V**: fourth ventricle, **i**: infiltrate.

Figure 6. Leukocyte infiltration through ependyma and choroid plexus

Nuclei were stained with DAPI (blue) (A) ZO-1 and β -catenin expression in the junctional complexes of subependymal pial vessels (asterisk) and **e** lining in **4V**. Mock infected animal. 100X (B) Similar area to A, showing dislocated expression of ZO-1 and α -catenin in subependymal vessels (asterisks) and **e** after 1 day of infection. 100X (C) Junctional complex proteins, ZO-1 and β -catenin were expressed in subependymal vessels (asterisks) and **e** of **4V**. Mac1⁺ cells (white) are seen in ependymal and subependymal areas. 1 wk infected animal. 40X (D) In situ zymography seen by gelatin (**gel**) digestion (green) showed active polarized expression of MMP-8 in leukocytes (arrowhead) traversing the ependymal lining after 1 wk of infection. Inactive MMP-8, showing no degradation of **gel** is detected in some ependymal cells (red). 40X (E) Massive infiltration of Mac1⁺ cells into **4V** through holes (arrowheads) in the cadherin positive **e**. 3 wk infected animal. 20X (F) In mock infected animals, fibronectin (**Fibro**) is confined to the fenestrated vessels (asterisks) located in the **cp**. Serum and leukocyte extravasation is not detected in the lumen of **LV**. 40X (G) **Fibro** extravasation is found in subependymal vessels (asterisk) and moving into **3V** through disrupted **e** (arrows). Low extravasation was detected in **cp** (arrowhead). 3 wk infected animal. 40X (H) Mac1⁺ cells (arrowheads) appear to be moving from **cp** into ventricles after 3 days of infection. 100X (I) In situ zymography showing active MMP-8 expression in **e**, seen as **gel** digestion (green). Low levels of MMP-8 are detected in the **cp**. 1 wk infected animal. 40X

Abbreviations: **e**: ependyma, **cp**: choroid plexus, **3V**: third ventricle, **LV**: lateral ventricle, **4V**: fourth ventricle, **i**: infiltrate.

Relative levels or changes in junctional complex proteins expressed in ventricular areas of mock and *M. cori* infected animals.

Junctional proteins ^a	Mock		Infected	
	ependyma	CP	ependyma	CP
TJs	claudin1	+++	-	+++
	claudin3	-	-	+++
	claudin5	-	-	+/-
	occludin	+++	altered ^c	+++
	JAM-A	-	-	+++
	ZO1	+	altered ^c	+++
AJs	pan-cadherin	+++	altered ^c	+
	α-catenin	+++	altered ^c	+
	β-catenin	+++	altered ^c	+

CP: choroid plexus

^aJunctional proteins were determined by using the antibodies described in Materials and Methods.

^bThe relative expression of junctional proteins was arbitrarily assigned and represent: (-) absent, (+/-) scarce to (+++) abundant.

^cThe modifications in these junctional proteins relate more to positional changes of the structural proteins relative to each other rather than changes in levels of expression. However, in a number of interepithelial junctions, these proteins were absent.