BLOOD-BRAIN BARRIER FUNCTION IN CEREBRAL MALARIA IN MALAWIAN CHILDREN

HEIDI BROWN, STEPHEN ROGERSON, TERRIE TAYLOR, MADALITSO TEMBO, JAMES MWENECHANYA, MALCOLM MOLYNEUX, AND GARETH TURNER

Malaria Research Group, Nuffield Department of Clinical Laboratory Sciences, The John Radcliffe Hospital, Oxford, United Kingdom; Malaria Project and Wellcome Trust Research Laboratories, Queen Elizabeth Central Hospital and College of Medicine, Blantyre, Malawi; Department of Internal Medicine, Michigan State University, East Lansing, Michigan; School of Tropical Medicine, University of Liverpool, Liverpool, United Kingdom

Abstract. Cerebral malaria (CM) is a serious complication of Plasmodium falciparum infection. Binding of parasitized erythrocytes to cerebral endothelium plays a key role in disease pathogenesis. Central nervous system signs and symptoms (coma, seizures, raised intracranial pressure) predominate in African children, whereas in adults, multiorgan system failure is more common. In this study we investigated whether changes in blood-brain barrier (BBB) structure and function are compatible with the signs and symptoms observed in Malawian children with CM. Immunohistochemistry on autopsy brain tissues from eight cases of CM showed activation of endothelial cells and macrophages, and disruption of endothelial intercellular junctions in vessels containing sequestered parasitized erythrocytes, but no gross leakage of plasma proteins. Examination of the partition of albumin between circulating plasma and the cerebrospinal fluid from 72 cases of CM showed subtle but measurable changes compatible with impaired BBB function in malaria. These findings suggest that BBB breakdown occurs in areas of parasite sequestration in CM in African children.

INTRODUCTION

A subset of children with cerebral malaria (CM) exhibit brain swelling and increased intracranial pressure, both of which are associated with a poor prognosis.1 Although Macgrath proposed the permeability hypothesis of CM over fifty years ago,2 it remains unclear whether brain swelling in patients with CM is due to changes in blood-brain barrier (BBB) function.

In murine models of CM, there is no doubt that BBB disruption occurs. Protein leakage is detectable using Evans blue dye or radioactive tracers,3 and occurs very early in the course of infection.4 This is due to major ultrastructural disruption of endothelial cells, as shown by electron microscopy.5 These changes in BBB permeability may result in myelin disruption6 and convulsions.7 Murine CM differs from the human disease in a number of important ways8 The observed vascular effects may result from the presence of large numbers of leukocytes within cerebral vessels, which are not seen in human CM.

The evidence for the contribution of BBB changes to the pathogenesis of human CM is less clear. Although petechial and ring hemorrhages in the brain in CM are suggestive of disturbed BBB function,9 studies in Thai adults suggest that otherwise the barrier is grossly intact.9,10 There is no appreciable leak of radiolabeled tracers into the cerebrospinal fluid (CSF).10 lumbar puncture CSF pressures are normal in most patients, and computed tomography scans of adults who survive CM show no cerebral edema.9 The role of cerebral edema in the pathogenesis of CM in African children is uncertain, with some studies showing a low incidence of widespread interstitial cerebral edema, as judged by brain weights and by macroscopic and histological criteria.11 A computed tomography study of Kenyan children comatose with CM showed that a subset exhibit brain swelling and raised intracranial pressure, in the absence of vasogenic edema.1

In the majority of CM cases there are some signs of microvascular changes in the brain. Endothelial cells show upregulation of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin.12,13 Electron microscopic studies of CM have shown a variety of morphological changes suggestive of endothelial activation.14 A recent immunohistochemical study of Vietnamese adult fatal malaria cases showed that there are specific changes in the distribution of intercellular junction proteins in vessels containing sequestered parasitized red blood cells (PRBC).15 This is associated with leakage of fibrinogen into the local brain parenchyma, and the activation of perivascular macrophages.

Endothelial activation and focal BBB disruption may affect neurological function in the immediate perivascular region. Therefore, in this study we have used immunohistochemistry to examine the cerebral vasculature in fatal CM in Malawian children. We looked for upregulation of ICAM-1 as a marker of endothelial cell activation, and examined the distribution of three cell junction proteins (ZO-1, occludin, and vinculin) to determine junctional integrity. We used three plasma proteins as markers of leakage into the brain parenchyma: fibrinogen, C5b-9, and IgG. Two antibodies were used which recognize the antigens macrophage scavenger receptor and sialoadhesin, which are induced on macrophages in response to activation and exposure to plasma proteins, respectively. We also examined the levels of plasma proteins in the CSF as an indicator of BBB breakdown and intracerebral IgG production.

MATERIALS AND METHODS

Case selection and sample collection. Plasma and CSF samples were taken from pediatric patients admitted to the Malaria Project in the Department of Paediatrics, Queen Elizabeth Central Hospital, Blantyre, Malawi. Ethical approval for this study was given by The College of Medicine Research Committee, College of Medicine, University of Malawi. Informed consent was obtained from parents and guardians for the inclusion of all patients in this study. Control samples were collected from the Departments of Bac-
teriology (CSF) and Biochemistry (plasma) of the Oxford Radcliffe Hospital, Oxford, United Kingdom. These were the residua of diagnostic medical samples taken from pediatric patients, as approved by the Central Oxford Research Ethics Committee.

Plasma and CSF samples were collected from patients shortly after admission. Clinical data for these patients are given in Table 1. Plasma/CSF samples were considered as paired if they were collected on the same day. Lithium heparin was used as the anticoagulant for plasma collection. Samples were frozen as soon as possible after collection and were stored at −70°C. Control CSF samples were used if they were clear and colorless, contained negligible numbers of erythrocytes and leukocytes, and were free from microorganisms. Control plasma samples were included if biochemical results were within the normal range.

Tissues for immunohistochemistry were collected at autopsy, as soon as possible after death (median post-mortem interval = 6.2 hr; range = 3.1–14.5 hr). Clinical data are shown in Table 2. Samples of cerebral cortex and brainstem (medulla) were taken from eight cases of CM. A control group consisted of cerebral cortex from patients who had died from causes other than infectious or neurological disease (one Malawian child with severe non-malarial anemia, and seven British adults dying in the United Kingdom of non-cerebral disease). Tissue samples for immunohistochemistry were frozen in embedding compound (Bright Ltd., Huntingdon, United Kingdom) and stored at −70°C.

**Immunohistochemistry.** Indirect immunohistochemistry was performed on acetone-fixed 10 μm cryostat sections as described previously using antibodies shown in Table 3. A biotinylated secondary antibody was used and binding was visualised using DAKO ABC detection system (DAKO Ltd., High Wycombe, UK) with 3,3'-diaminobenzidine as the chromagen. Sections were counterstained using hematoxylin.

**Radial immunodiffusion assays.** A 2% agarose solution (Promega, Southampton, UK) was prepared in phosphate-buffered saline (PBS) with 0.02% sodium azide containing rabbit antiserum (DAKO, Ltd.) at 1:1,000 for anti-albumin, and 1:500 for anti-IgG. Four ml of antibody-agarose mixture was pipetted onto plain microscope slides, 75 × 25 mm, and allowed to solidify. Three wells, 1.8 mm in diameter, were punched into the agarose on each slide. Serial dilutions of human albumin or whole IgG standards (Sigma, Poole, United Kingdom) from 1–125 μg/ml were prepared in PBS/azide. Plasma samples were diluted at 1:500 for assessment of albumin and 1:250 for IgG. Cerebrospinal fluid was used at 1:10 for albumin and undiluted for IgG. Five μl of either standards or samples were placed in agarose wells and incubated for 48 hr at room temperature in a humid chamber. Slides were washed in five changes of PBS/azide over 48 hr, then completely air-dried. They were stained in 0.025% Coomassie brilliant blue R in 50% methanol:45% water: 5% acetic acid until rings were visible, then destained in 87% water: 8% acetic acid: 5% methanol until the background was clear. The assay generates a halo of immunoprecipitate whose diameter is proportional to the concentration of antigen. A standard curve was produced by plotting halo diameter against log₁₀ antigen concentration of the standards. Sample concentrations were determined by reference to this curve. Assays were performed twice for each sample.

**Statistical analyses.** The albumin index was calculated as: [albumin]$_{CSF}$/[albumin]$_{plasma}$. The formula used to generate the IgG index was: (IgG)$_{CSF}$ × [albumin]$_{plasma}$/([albumin]$_{CSF}$ × [IgG]$_{plasma}$). Both of these formulas produce ratios from which the geometric means and 95% confidence intervals

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (months)</th>
<th>Sex</th>
<th>Clinical diagnosis</th>
<th>Time from admission to death (hr)</th>
<th>Post-mortem interval (hr)</th>
</tr>
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<tbody>
<tr>
<td>Non-infected case</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP19</td>
<td>28</td>
<td>M</td>
<td>anemia</td>
<td>4</td>
<td>12.1</td>
</tr>
<tr>
<td>Malaria cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP9</td>
<td>16</td>
<td>M</td>
<td>CM</td>
<td>23.3</td>
<td>4.5</td>
</tr>
<tr>
<td>MP11</td>
<td>29</td>
<td>F</td>
<td>CM, anemia, pneumonia, sepsis</td>
<td>27</td>
<td>13.0</td>
</tr>
<tr>
<td>MP15</td>
<td>8</td>
<td>F</td>
<td>CM, anemia</td>
<td>10</td>
<td>6.2</td>
</tr>
<tr>
<td>MP16</td>
<td>51</td>
<td>F</td>
<td>CM</td>
<td>4.5</td>
<td>3.1</td>
</tr>
<tr>
<td>MP23</td>
<td>30</td>
<td>F</td>
<td>CM</td>
<td>7.3</td>
<td>14.5</td>
</tr>
<tr>
<td>MP25</td>
<td>44</td>
<td>F</td>
<td>CM</td>
<td>11.3</td>
<td>5.9</td>
</tr>
<tr>
<td>MP26</td>
<td>30</td>
<td>M</td>
<td>CM</td>
<td>48</td>
<td>6.6</td>
</tr>
<tr>
<td>MP27</td>
<td>20</td>
<td>M</td>
<td>CM, anemia</td>
<td>2.5</td>
<td>4</td>
</tr>
</tbody>
</table>

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**Table 1**

Clinical data for patients from whom cerebrospinal fluid and plasma samples were taken

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Male</th>
<th>Median age range yrs</th>
<th>Matched pairs CSF/plasma</th>
<th>Extra CSF samples</th>
<th>Extra plasma samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>12</td>
<td>50%</td>
<td>8 (1–199)</td>
<td>11</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>All CM</td>
<td>72</td>
<td>50%</td>
<td>39 (6–138)</td>
<td>47</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>CM nonsurvivors</td>
<td>25</td>
<td>60%</td>
<td>40 (14–82)</td>
<td>11</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>CM survivors</td>
<td>47</td>
<td>44.7%</td>
<td>38 (6–138)</td>
<td>36</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

CSF = cerebrospinal fluid; CM = cerebral malaria.
were calculated for each patient group. Statistical analysis consisted of one-factor analysis of variance followed by post-hoc testing with Fishers PLSD for multiple comparisons between groups. All calculations were made using the Statview 4.1 program (Abacus Concepts Inc., CA).

**RESULTS**

Expression of cell adhesion molecules on cerebral endothelium. Platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) was constitutively expressed on cerebral endothelium in control and malaria cases, and levels were unaffected by the presence of sequestered PRBC (data not shown). By contrast, ICAM-1 expression was weak on endothelium in United Kingdom and Malawian control brains (Figure 1A). However, in cases of malaria there was generalized upregulation of ICAM-1 both on vessels containing PRBC and those without (Figure 1B). This result was seen in grey and white matter and was consistent between brain regions.

Cell junction alterations. Staining for the cell junction proteins, ZO-1, occludin, and vinculin was constitutively high in control brains (Figures 1C, E and G). In cases of CM, however, there was focal loss of staining topographically associated with the presence of sequestered parasites in vessels in both the cortex and brainstem (Figures 1D, F and H).

Plasma protein distribution. We detected no staining for fibrinogen, IgG, and C5b-9 around vessels in CM cases compared to control brains (data not shown). Both C5b-9 and IgG were virtually absent from the brains of non-malaria cases (Figures 2A and B insets). In cases of malaria, there were high levels of intravascular staining for C5b-9 and IgG in association with sequestered PRBC (Figures 2A and B, main figures).

Macrophage markers. CD68 was constitutively expressed by perivascular macrophages and microglia in control cases, whereas macrophage scavenger receptor and sialoadhesin were expressed only at very low levels (data not shown). CD68 staining of CM brains showed macrophages within white matter hemorrhages (Figures 2C and D) and large numbers of intravascular monocytes commonly associated with sequestered PRBC (Figure 2C). The expression of scavenger receptor and sialoadhesin was very weak in these lesions (Figures 2E and F), although both were expressed on intravascular monocytes and perivascular macrophages in CM (Figures 2G and H). Expression of scavenger receptor and sialoadhesin on perivascular macrophages was induced in CM cases associated both with vessels containing sequestered PRBC and those without.

Albumin and IgG indices. The albumin index results are shown in Figure 3A. The values are grouped according to whether the CM patients survived or died. Both CM groups showed significantly higher albumin indices than the control group (survivors: \( P = 0.0002 \); fatalities: \( P = 0.0001 \)). There was no significant difference between the two disease groups. IgG index results are shown in Figure 3B and Table 4. Neither CM survivors nor fatalities showed IgG indices significantly elevated above control values. In fact, the survivor group had a significantly lower IgG index than controls (\( P = 0.02 \)).

**DISCUSSION**

In this study we used immunohistochemistry to examine changes in the cerebral microvasculature in cases of CM in Malawian children. Firstly, we investigated adhesion molecules as evidence of endothelial cell activation. Constitutive expression of PECAM-1 validated the comparison of endothelial cell antigen expression between cases by demonstrating the lack of post-mortem degradation. As seen in Vietnamese adults, \(^{12,13,15}\) there was generalized upregulation of ICAM-1 on cerebral vessels in CM, indicating endothelial cell activation unrelated to the site of sequestration or pathology. Junctional protein staining, however, demonstrated specific changes in the vicinity of PRBC sequestration. Tight junctions, which include the proteins ZO-1 and occludin, are particularly important for maintaining the integrity of the BBB.\(^ {17}\) Adherens junctions, which contain the protein vinculin, are also involved in cell attachment. Our results show focally reduced staining of all three cell junction proteins in CM in vessels containing many sequestered PRBC. Loss of
Figure 1. Light micrographs (×200) showing immunohistochemistry on cryostat sections of human brain from a normal control (A, C, E, and G) and cases of cerebral malaria (B, D, F, and H). Sections were stained with antibodies recognizing InterCellular Cell Adhesion Molecule-1 (A and B), ZO-1 (C and D), occludin (E and F) and vinculin (G and H). In Figures C, E, and G, arrows highlight examples of junctional proteins at the borders of normal endothelial cells. Arrows in figures B, D, F, and H indicate sequestered parasitized red blood cells within vessels.

Figure 2. Light micrographs of sections of human cerebral cortex from normal controls (insets to Figures A and B) and cases of cerebral malaria (A+B main Figures, and C–H). Micrographs show the results of immunohistochemistry for IgG (A), C5b-9 (B), CD68 (C and D), scavenger receptor (E and G), and sialoadhesin (F and H). Magnification = ×100 (A+B); ×200 (C–F); ×400 (G+H).
cell junction proteins, particularly ZO-1 and occludin, is evidence of activation of endothelial cells and disruption of BBB integrity. However, the distribution of fibrinogen, IgG, and the C5b-9 complement membrane attack complex does not suggest widespread leakage of plasma proteins into the brain parenchyma in these cases. This contrasts with the situation in Vietnamese adults where the uptake of fibrinogen by perivascular astrocytes indicates significant BBB disruption prior to death. IgG and C5b-9 were not seen in the perivascular region in Malawian cases, but were detected at high levels on PRBC and monocytes within cerebral vessels. Despite the lack of plasma proteins detectable around vessels with disturbed junctional complexes, immunohistochemistry for scavenger receptor and sialoadhesin indicate that BBB breakdown has occurred in these cases. Both antigens are restricted to sub-populations of macrophages. Sialoadhesin is not present in the normal central nervous system but is induced by a factor present in plasma. Thus, upregulation of sialoadhesin demonstrates that plasma proteins have crossed the cerebral endothelium. Macrophage scavenger receptor is involved in the uptake by macrophages of a wide range of molecules. It too is absent from the normal central nervous system and induction is evidence of macrophage activation. There was widespread induction of both antigens on perivascular macrophages in the Malawian cases, even in vessels containing no sequestered parasites. This strongly suggests that these cells have been activated by contact with leaked plasma proteins. In areas of hemorrhage, however, where CD68 staining demonstrates the presence of activated microglia and recruited monocytes, the expression of these two antigens was relatively low. This is surprising since a cerebral hemorrhage is a lesion where plasma proteins are certain to be present in the central nervous system parenchyma. Inhibitory factors, such as transforming growth factor-β, are present within these hemorrhages, and may suppress induction of these antigens. Alternatively, the macrophages may be damaged or dying, due to ischemia within the hemorrhage, for example.

The results of radial immunodiffusion assays of paired CSF/plasma samples from cases of CM showed a small but significant increase in the albumin index in all CM patients, regardless of disease outcome. There are several possible explanations for this. One is that there may be a difference between the normal albumin index of the disease population (Malawian children) and that of the United Kingdom controls, as a result of differences in nutritional status, for example. However, there is no evidence currently available to support this, and the disruption of endothelial cell junctions and activation of perivascular macrophages suggest that the difference is a real effect of the disease. Albumin may leak across the endothelium in areas of parasite sequestration, where junctional integrity appears to be lost, to produce this effect on the albumin index. The lack of immunohistochemical evidence for plasma proteins in the perivascular region suggest that IgG synthesis is not occurring within the brain parenchyma. This supports previous findings showing that few B-lymphocytes are seen in human post-mortem brain tissue from cases of CM (Louvrier K and others, unpublished data). In addition, B lymphocyte-deficient mice develop murine CM. This evidence suggests that intrathecal antibody production is not a significant factor in the pathophysiology of this disease.

In summary, these results show that in cases of CM in Malawian children, there is generalized activation of cerebral endothelial cells and focal disruption of intercellular junctions. There is a small but significant increase in the albumin index in the CM groups. This suggests that limited BBB breakdown has occurred, but this is unlikely to account for the degree of brain swelling seen in CM. Instead, the effects of limited BBB disruption may be subtle and localized. These cases show generalized and focal changes in
endothelial cell phenotype, and perivascular macrophage activation. This directs our interest to the vasculature, the interface between the intracellular parasitic and the central nervous system, and suggests that localized vascular effects may be an important pathological mechanism in CM.

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Authors’ addresses: Heidi Brown and Gareth Turner, Malaria Research Group, Nuffield Department of Clinical Laboratory Sciences, Level 4 Academic Block, The John Radcliffe Hospital, Headington, OX3 9DU, UK, Tel: +44 1865 222039, Fax: +44 1865 222912. Email. gareth.turner@cellsci.ox.ac.uk. Stephen Rogerson, Madalitso Molyneux, Liverpool School of Tropical Medicine, Pembe Place, Liverpool, L3 5QA, UK, Tel: +44 151 708 9393. Fax: +44 151 708 9007. Terrie Taylor, Department of Internal Medicine, Michigan State University, B315-C W. Fee Hall, E. Lansing, MI 48824; Tel: 517 353 8975. Fax: 517 432 1062. Email: taylorl@com.msu.edu.

Reprint requests: Dr. Gareth Turner, Nuffield Department of Clinical Laboratory Sciences, Level 4 Academic Block, The John Radcliffe Hospital, Headington, OX3 9DU, UK

REFERENCES