Faecal peritonitis causes oedema and neuronal injury in pig cerebral cortex

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ABSTRACT

Encephalopathy is a common complication of sepsis. However, little is known about the morphological changes that occur in the brain during sepsis. Faecal peritonitis was induced in pigs that were killed 8 h later and frontal cortex samples were taken immediately after death. The tissue was investigated using light and electron microscopy and compared with frontal cortex samples taken from sham-operated controls. Septic pigs had 49.5% more perimicrovessel oedema than sham pigs. However, the tight junctions between cerebral microvessel endothelial cells appeared morphologically intact in both septic and sham pigs. Sepsis also resulted in neuronal injury, disruption of astrocytic end-feet and swollen, rounded erythrocytes. These morphological changes may be sufficient to underlie the clinical features seen in septic encephalopathy.

INTRODUCTION

Septic shock is the commonest cause of death in the Intensive Care Unit [1]. Progressive sepsis causes multi-organ failure and the mortality of patients with three or more failing organs approaches 100% [2]. The adverse effects of sepsis on the liver, lung, kidney and heart have been studied extensively [3–9]. There is panendothelial injury from persistent, uncontrolled inflammation which causes the deposition of fibrin and the formation of thrombi in microvessels [10]. As a result, these organs become underperfused [8] and accumulate activated leucocytes [3], especially degranulating neutrophils [5] which release lysosomal enzymes and superoxide radicals.

Although 50% of patients with progressive systemic sepsis develop encephalopathy, which is a marker for poor prognosis, little is known about the effects of sepsis on the brain [11]. Septic encephalopathy (SE) is a diffuse brain dysfunction. Its clinical features vary from mild confusion to coma and its pathogenesis is unclear. SE is associated with breakdown of the blood–brain barrier, since patients have high protein levels in the cerebrospinal fluid [12], and in septic rodents 125I-albumin [8], 14C-amino acids [13] and colloidal iron oxide [14] pass from the circulation into the brain parenchyma. However, the nature of the blood–brain barrier impairment in SE is unknown. Increased pinocytosis by cerebral microvessel endothelia and swelling of the astrocytes have been reported following electron microscopy of brains from rabbits with endotoxaemia [14], but these early experiments lacked haemodynamically matched controls. Therefore, in an attempt to investigate the effect of SE on the blood–brain barrier, the morphology of frontal cortex

Key words: astrocytes, cerebral microvessels, faecal peritonitis, neuronal injury, oedema, septic encephalopathy, ultrastructure.

Abbreviation: SE, septic encephalopathy.

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in an established porcine model of sepsis [5–7,9] was investigated by light and electron microscopy.

**METHODS**

**Surgical procedures**

Anaesthesia was induced in 10 adolescent middle white pigs (25–30 kg) by intramuscular injection of azaperone (2.0 mg/kg, Janssen-Cilag Ltd, High Wycombe, Bucks, U.K.) and intraperitoneal injection of metomidate (10.0 mg/kg, Janssen-Cilag Ltd). A tracheotomy was performed, each pig was intubated and anaesthesia was maintained throughout surgery with a mixture of 50% O$_2$, 48% NO$_2$ and 2% halothane (Zeneca Pharmaceuticals, Wilmslow, Cheshire, U.K.). Catheters were inserted into the internal jugular vein and descending aorta and a Swan Ganz catheter (Baxter Edwards Critical Care, Irvine, CA, U.S.A.) advanced into the pulmonary artery. Faecal peritonitis was induced in five pigs as follows: after laparotomy, an opening was made in the caecum and 35 ml of its contents aspirated and diluted to 50 ml with normal saline. The caecotomy was then closed and the faecal solution spread around the peritoneum. Five sham pigs underwent laparotomy without caecotomy and the induction of faecal peritonitis. After closure of the laparotomy in the pigs with faecal peritonitis and the sham controls, the lungs were ventilated (Harvard Ventilator, Harvard Ltd, Edenbridge, Kent, U.K.) with room air. The tidal volume (12–15 ml/kg) was adjusted to keep the arterial partial pressure of CO$_2$ at 35–45 mmHg (4.7–6.0 kPa). Anaesthesia was monitored continuously and, at signs of lightening, was maintained throughout surgery with a mixture of 50% O$_2$, 48% NO$_2$ and 2% halothane (Zeneca Pharmaceuticals Ltd, Wilmington, Delaware, U.S.A.) to compensate for peritonitis-induced fluid loss from the blood. This study was authorized by the U.K. Secretary of State under the provisions of the Animals (Scientific Procedures) Act 1986.

**Tissue removal and processing**

Eight hours after the induction of anaesthesia, the pigs were killed by injection of a lethal dose of phenobarbitone (0.6 ml/kg, Animal Care Ltd, York, U.K.). A 3-cm hole was bored through the frontal bone, the meninges were incised and reflected and a fixative solution (3% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4 at room temperature) was poured on to the surface of the brain. A block of frontal cortex (approximately 1 cm$^3$) extending from the pial surface to the underlying white matter was removed, cut into 1-mm thick strips perpendicular to the brain surface and immersed in fixative for 1 h. The tissue was then rinsed in cacodylate buffer and post-fixed in cacodylate buffered 2% osmium tetroxide for 2 h. It was then washed in cacodylate buffer, immersed in 30% ethanol containing 2% uranyl acetate for 30 min, dehydrated through graded alcohols and processed into Spur's resin (Agar Scientific Ltd, Stansted, Essex, U.K.). The tissue blocks were then coded so that subsequent analysis could be performed ‘blind’.

**Light microscopy and image analysis**

Semi-thin sections (2 μm) of the embedded tissue were cut, picked up on glass slides, stained with a mixture of 0.5% Toluidine Blue, 0.5% Methylene Blue and 1% boric acid for 1 min, washed, dried, placed on cover slips and then viewed in a light microscope. Twenty images were captured from the tissue of each pig and imported into an image analysis system (Macintosh Performa 630 running NIH image software (public domain). Each image contained a microvessel (cerebral vessel with a diameter of ≤ 10 μm) in cross-section. The following measurements were made in triplicate from each image: A, area of oedema surrounding the microvessel; B, total microvessel area enclosed by the basement membrane; and C, area of microvessel lumen. Mean values were obtained and then endothelial cell area (B − C) and the percentage microvessel area occupied by oedema (A/B × 100%) were calculated for each image. The experimental codes were broken and mean values calculated for each pig.

**Electron microscopy**

Thin sections (80–100 nm) were cut from the resin-embedded tissue blocks perpendicular to the surface of the cortex, picked up on copper grids and stained with uranyl acetate [15] for 20 min followed by Sato’s lead stain [16] for 20 min. The sections were examined in a Zeiss 900 electron microscope and photomicrographs were taken of tissue from each pig.

**Statistical analysis**

Values are expressed as means ± S.E.M. The data from septic and sham pigs were compared using Student’s t-test.

**RESULTS**

**Light microscopy**

Oedema was present around cortical microvessels but not around larger vessels. There was significantly ($t = 3.34$, $P = 0.01$) more perimicrovessel oedema in the frontal cortices of pigs with faecal peritonitis than in sham controls (Figure 1). Faecal peritonitis had no effect
Cerebral oedema and neuronal injury in sepsis

Figure 1 Effect of sepsis on the mean cross-sectional areas of microvessel lumina (Lu), microvessel endothelial cells (En) and perimicrovessel oedema (Oe) in the frontal cerebral cortex of pigs 8 h after the induction of faecal peritonitis, compared with sham-operated controls. *P < 0.05 for both experimental groups. n = 5 for both experimental groups.

Electron microscopy

Perimicrovessel oedema was evident in both experimental groups, but it was far more marked in septic pigs (Figures 2A and 2B) than in sham controls (Figure 2C). Microvascular endothelial cells appeared normal with morphologically intact mitochondria and intercellular tight junctions (Figure 2D) in all specimens. However, extravasated erythrocytes were occasionally observed in the cortical parenchyma of septic pigs. Within the lumina of cerebral microvessels from sham pigs, erythrocytes appeared normal (Figure 2C), but in septic pigs they were swollen and more spherical (Figure 2B). Astrocyte end-feet were occasionally slightly swollen in sham pigs, but in septic pigs they were frequently grossly swollen and their perimicrovessel membranes ruptured (Figure 2B). Scattered, apparently degenerating neurons were found in irregular clusters within the frontal cortex of septic pigs (Figure 2A). These neurons were dark, shrunken, contained pyknotic nuclei, multiple vacuoles and degenerating mitochondria (Figure 2E). The remaining neurons in the frontal cortex of pigs with faecal peritonitis appeared to have more ‘dilute’ cytoplasm than those in sham pigs. In sham pigs, the neuronal perikarya appeared normal, although some of their mitochondria were swollen and a few cytoplasmic vacuoles were occasionally present (Figure 2F). The morphology of synapses visible in both septic and sham pigs appeared normal.

DISCUSSION

Faecal peritonitis resulted in extensive perimicrovessel oedema in porcine frontal cortex. Although sham pigs exhibited some oedema, probably resulting from 8 h of anaesthesia and immobilization, there was 49.5% more oedema in septic pigs than in sham counterparts. The oedema in septic pigs was associated with gross swelling of perivascular astrocyte end-feet and their rupture and detachment from microvessel endothelial cell walls. Such swelling is known to result from disruption of the blood–brain barrier [17]. This perivascular brain oedema is likely to affect the passage of oxygen, nutrients and cellular waste between the blood and brain parenchyma and thus contribute to the symptoms observed in SE. In addition to their role in maintaining the blood–brain barrier, astrocytes transport energy substrates from the blood to neurons in proportion to the level of synaptic activity [18]. Therefore, the injury to astrocyte end-feet in SE may impair this metabolic coupling and adversely affect synaptic activity.

Dark, shrunken neurons were present in the frontal cortices of pigs after only 8 h of experimental peritonitis. This is unlikely to be an artefactual change, since such neurons were not evident in similarly processed tissue from sham-operated pigs. The cytoplasm of remaining neurons in the frontal cortices of septic pigs appeared ‘dilute’. These observations suggest that factors associated with the oedema or its causative agent are cytotoxic. It is not known whether the neuronal damage that occurs in the pig model also occurs in patients with SE or if long-term neurological/neuropsychiatric problems emerge in patients who recover from sepsis. These possibilities require investigation because the effects of SE in humans are currently considered to be reversible [19,20]. The dark neurons present after 8 h in the porcine model of sepsis probably represent early degenerative alterations that could be reversible [21–23]. However, since the duration of sepsis is considerably longer in critically ill patients than in the pig model, their neuronal damage may be even more severe.

The damage to the cerebral cortex resulting from 8 h of sepsis in the current study was in some ways less than that reported for hepatic sinusoids in the pig model 5 h after the induction of faecal peritonitis [6]. The hepatic sinusoids were found to be occluded by degranulating neutrophils, lymphocytes and activated Kupffer cells and there was endothelial hypertrophy and obliteration of the space of Disse [6]. Leucocyte exudation into the parenchyma of the frontal cortices of septic pigs was not observed in the current study, in agreement with the...
Figure 2  Electron micrographs showing the effect of sepsis on the frontal cerebral cortex 8 h after the induction of faecal peritonitis

(A) Perimicrovessel oedema (po) and dark neurons (dn) in a pig with faecal peritonitis. × 1260. (B) A cerebral microvessel in a pig with sepsis, showing perimicrovessel oedema (po) comprising swollen and disrupted astrocytic end-feet. The microvessel endothelial cell (end) surrounded by a basement membrane (bm) appears normal, but within the vessel lumen, the erythrocytes [red blood cells (rbc)] can be seen to be swollen. × 3200. (C) A cerebral microvessel in a sham-operated pig. The endothelial cell (end) basement membrane (bm) is lined by non-oedematous astrocyte foot processes (afp). × 3200. (D) Intact trilaminar junctions (arrows) between cerebral microvessel endothelial cell (end) processes, ending in a juxta-luminal zonula occludens (zo) in a septic pig. The microvessel lumen contains an erythrocyte [red blood cell (rbc)] and perimicrovessel oedema (po) is present outside the endothelial cell basement membrane (bm). × 38000. (E) A dark neuron in a septic pig containing a pyknotic nucleus (pn), numerous vacuoles and degenerating mitochondria. × 3000. (F) A neuron in a sham-operated pig with a normal nucleus (n), slightly swollen mitochondria and a few vacuoles. × 3000.
Previous observation that intracerebral injection of various chemotactic cytokines fails to cause leucocyte infiltration of murine brain parenchyma [24]. During sepsis, cytokines such as tumour necrosis factor \( \alpha \) and interferon \( \beta \) are released from macrophages and lymphocytes and play a major role in the initiation, propagation, regulation and suppression of immune and inflammatory responses [25,26]. Normal central nervous parenchyma is an immunologically privileged site and is uniquely resistant to leucocyte accumulation [27]. Moreover, although septic shock is associated with a reduction in the perfusion of many organs [4], the brain may be protected from hypoperfusion by the selective redistribution of blood flow from other parts of the body [28]. However, in the current study, the erythrocytes of septic pigs were often enlarged and rounded. This finding is in agreement with those of previous studies [29,30] which showed that, in sepsis, activated leucocytes generate oxygen free radicals that damage the erythrocyte plasma membranes. Damaged erythrocytes may be unable to squeeze through cerebral microvessels and thus impair cerebral perfusion in sepsis.

Since extensive liver damage occurs in the pig model of sepsis [6], the cerebral changes observed in the current study may be secondary to hepatic failure, a well-recognized cause of encephalopathy [31]. Perivascular cerebral oedema and damage to astrocyte end-feet have been reported both in patients who died from fulminant hepatic failure [32] and in rabbits with galactosamine-induced hepatic failure [33]. Both hepatic failure [34] and sepsis [25,26] are characterized by high levels of circulating cytokines and tumor necrosis factor \( \alpha \). Human and primate brain endothelium suggest that cytokines increase blood–brain barrier permeability. In our experiments, the intercellular tight junctions appeared intact. In an early study using rabbits, Clawson et al. [14] found that endotoxaemia caused brain oedema without apparently damaging the tight junctions. Addition of tumour necrosis factor \( \alpha \) to bovine cerebral endothelial cultures also increased permeability without affecting the morphology of tight junctions [35].

Faecal peritonitis is common, and many patients with this problem require ventilation and the administration of intravenous colloids to maintain constant pulmonary artery occlusion pressures. Pigs were used in the current study since they are of comparable size to humans. Their size allows monitoring of haemodynamic variables [37] in a similar way to that of patients in an Intensive Care Unit. Haemodynamic parameters are difficult to measure in rodents, which are also relatively resistant to sepsis from their own faeces [37]. The similarities between our experimental model and the clinical situation suggest that the perivascular cerebral oedema present in septic pigs may also occur in septic patients in an Intensive Care Unit. The systemic effects of sepsis may arise not only from faecal peritonitis but also from other infections [38]. Since encephalopathy may develop in septic patients with Gram-negative bacteraemia, Gram-positive bacteraemia, fungaemia and no causative organism identified [11], our findings may be relevant to all septic patients, not only those with faecal peritonitis.

Although faecal peritonitis results in perimicrovessel oedema, disruption of astrocyte processes, neuronal degeneration and erythrocyte damage in the frontal cortices of pigs, the mechanisms by which they occur remain to be elucidated. Endothelial cell tight junctions appear morphologically intact in sepsis, but their functional status is not clear. Since the molecular structure of the tight junction is known [39], immunohistochemistry using antibodies against occludins may show whether these glue-like tight junction proteins are disrupted in sepsis, allowing microvessel leakage and the development of oedema. If the junctions remain functionally ‘tight’ in sepsis, then some other route such as pinocytosis must be involved in the formation of perimicrovessel oedema. It is not known whether the factors causing the oedema directly are responsible for the other pathological changes present in the septic pig frontal cortex or whether they develop in response to the oedema. Whatever the cause, they are likely to contribute to the symptomatology of SE, and it is important to determine whether the different inotropic agents commonly used to treat patients with septic shock modulate the effects of sepsis on the brain.

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