**TITLE**

**The interleukin-1 balance is associated with clinical severity, blood-brain barrier permeability, neuroimaging changes and outcome in encephalitis**

**RUNNING TITLE**

**Interleukin-1 and severity in encephalitis**

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**ABSTRACT**

**WORD COUNT 250**

***Background.***

Encephalitis is parenchymal brain inflammation, commonly due to herpes simplex virus (HSV). Key host inflammatory mediators and relationship with blood-brain barrier (BBB) permeability, neuroimaging changes and outcome are poorly understood.

***Methods.***

We measured 38 mediators in serum (n=78) and cerebrospinal fluid (n=37) of encephalitis patients, including 17 HSV. Outcome measures were Glasgow coma and outcome scores, CSF:serum albumin ratio reflecting (BBB) permeability, and temporal lobe volume on magnetic resonance imaging in HSV cases.

***Results.***

Serum IL-1RA was elevated in those with a good outcome (*P* = 0.004), and in HSV the CSF IL-1:IL-1RA ratio was associated with worse outcome (*P* = 0.009); a concentration greater than -0.55pg/mL had high specificity and sensitivity forpoor outcome (100% and 83%, *P* = 0.015). Temporal lobe volume had a negative correlation with serum IL-1RA (p=0.012) and positive correlation with serum IL-1 and CSF IL-1 (p=0.0003 and p=0.007). A normal coma score was associated with elevated IL-10 in serum of HSV patients (p=0.007) and CSF of all (p=0.016); IL-10 correlated inversely with BBB permeability (*P* = 0.005).

***Conclusions.***

A pro-inflammatory cytokine response is associated with greater clinical severity, BBB permeability, and neuroimaging damage in encephalitis. IL-1 antagonists should be investigated as adjunctive treatment in encephalitis.

**Key words.**

Encephalitis, cytokine, chemokine, herpes simplex, blood-brain barrier

**WORD COUNT 3213**

**BACKGROUND**

Encephalitis is a pathological inflammation of the brain parenchyma. The most common cause is herpes simplex virus (HSV), which predominantly affects the temporal lobes, and has a 10-30% mortality despite effective antiviral treatment, with neurological morbidity in at least 60% of survivors [1, 2]. Aciclovir effectively reduces viral load but does not inhibit immune-mediated pathogenesis [3]. There is mounting evidence that the host inflammatory response, particularly cytokines and associated mediators, may play a key role in pathogenesis [4, 5]. These mediators act both directly on neuroglial cells and also orchestrate permeability of the blood brain barrier (BBB) and the influx of leucocytes, which is a key process in inflammation [6, 7].

Interleukin (IL) -1 is the prototypical pro-inflammatory cytokine and is elevated in murine and cell culture models of viral encephalitis resulting in fever, increased BBB permeability, and the production of additional pro-inflammatory mediators [3, 8, 9]. Many of these actions are directly opposed by the IL-1 receptor antagonist (IL-1Ra) and IL-10 [10-12]. However, these models only provide a limited representation of host responses and the importance of investigation of clinical samples has been highlighted [13-16].

The key mediators underlying this inflammatory response remain unclear. Previous clinical studies have assessed a few mediators in a limited number of cases [4, 5, 14]. Moreover, the downstream effects on BBB permeability and parenchymal damage on neuroimaging have not been assessed [4, 5, 14]. In addition, HSV only accounts for 20% of cases and it is not known if these mediators are associated with disease severity or BBB permeability in encephalitis more broadly [17]. These mediators act in concert and the importance of assessing their relative abundance has been demonstrated [18, 19]. Therefore, the pro- to anti-inflammatory cytokine ratio has been reported, for example IL-6:IL-4 and IL-6:IL-10 ratios have strong correlations with outcome in Japanese encephalitis and cerebral malaria, respectively [14, 20].

Therefore, we undertook a study to assess the relationship between cytokines and associated mediators in cerebrospinal fluid and serum with clinical disease severity and BBB permeability. As HSV is the most common cause of encephalitis and, as there are stereotypic neuroimaging changes, we also assessed the relationship between these mediators and the volume of injury in this subgroup.

**METHODS**

**Study population and measurement of mediators**

Patients were recruited prospectively through the Health Protection Agency Aetiological Study of Encephalitis in England from 24 centres which each recruited over 2 years (2005-2008), and is described in detail previously [17]. Of 203 patients recruited, serum and/or CSF were used if >50L was available after diagnostic testing and an aliquot archiving. Samples were collected at recruitment, stored at -80oC and freeze-thaw cycles minimised. Thirty-eight mediators were assessed in duplicate using commercial cytometric bead array (Procarta ®, Affymetrix ®, Itay) and anlysed using BioPlex Manager 4.1 (Bio-Rad Laboratories ©, UK). The pro- to anti-inflammatory balance was assessed by the IL-1:IL-1RA and IL-1:IL-10 ratios [12, 14, 20].

The HPA study was approved by the North and East Devon Multicentre Research Ethics Committee (Reference: 05/Q2102/22). This sub-study was approved by the HPA Encephalitis Study Steering Group and also by the Pan-Manchester Research and Development Group for the University of Manchester.

**Clinical outcome measures and blood-brain barrier permeability**

The admission Glasgow coma scale (GCS) score was recorded and (15/15) defined as good and (<14/15) as poor [14]. The discharge Glasgow outcome scale (GOS) score was recorded, with good defined as 5/5 (minor-no disability) and poor as <4/5 (moderate disability-death) [5]. CSF white cell count of <5/L was defined as normal. As a marker of BBB permeability, the CSF:serum albumin ratio was determined on paired samples, using radial immunodiffusion (Binding Site, Birmingham, UK©) [27].

**Imaging outcome**

Magnetic resonance images (MRI) were analysed where available for patients with HSV encephalitis. Volumes were determined using stereology of temporal lobes on T1-weighted (0.72-0.94 mm resolution), indicating swelling, and T2-weighted images (0.45-0.54 mm resolution), indicating tissue damage; with a 5mm thickness using a grid-size of 10 voxels and established anatomical boundaries (EasyMeasure) [28, 29]. Preliminary analysis compared absolute volume on T1 with 18 healthy controls [30]. However, to account for individual differences, all analysis was undertaken with volumes as a percentage of total intracranial volume. Measurements were made in duplicate and by another blinded person [31].

**Statistical analysis**

Mann-Whitney U test, Kendall’s rank, linear regression and Pearson’s correlation coefficient were used (SPSS Inc© 2011 and GraphPad Inc© 2014), and p<0.05 defined as significant. Overall mediator data underwent a one-way hierarchical cluster analysis; data from each outcome group underwent nearest neighbour analysis, using Pearson’s correlation coefficient, to generate proximity matrices [19, 32]. A heat map was generated for those with a good and a poor outcome. A separate heat map was generated by subtracting the values of the proximity matrix for those with a good outcome from those with a poor outcome, and a heat map generated from this, as described previously [19,32].

To avoid undetectable levels or missing data bias, only mediators detected in the CSF or serum of >80% of the samples were analysed [26]. To minimise any potential for storage impact, concentrations of each mediator were median-centred for each patient [19, 26]. Therefore, the concentration of each mediator was expressed and analysed as a value relative to the median concentration of all of the mediators in that patient sample, as described previously [19,26].

**RESULTS**

Of 203 patients meeting the clinical case definition of encephalitis, a total of 95 patients had sufficient serum and or CSF samples available for analysis. Serum samples were available for 78 patients; these comprised 38 with an infectious aetiology (17 HSV, 7 varicella zoster virus [VZV], 5 tuberculosis, 3 bacterial, 2 dual infection [1TB and HIV, 1 cryptococcus and VZV], 1 influenza A, 1 measles, 1 HIV, 1 toxoplasmosis); 20 immune-mediated cases (9 antibody-mediated, 8 acute disseminated encephalomyelitis [ADEM], 1 paraneoplastic, 1 vasculitis, 1 multiple sclerosis), and 20 of unknown aetiology. For 37 patients CSF was available, 20 with an infectious aetiology (12 HSV, 6 VZV, 1 JC virus, and 1 toxoplasmosis), 9 immune-mediated (5 ADEM, 3 antibody-mediated, 1 paraneoplastic), and 8 of unknown aetiology.

The following mediators were not identified in >80% of the cohort and were therefore removed from further analysis: TNFR1 and 2, eSelectin, CXCL9, IL-17a, and VEGF. In addition, in CSF the following mediators were not detected above the lower limits of quantification in >80% of the samples: G-CSF, GM-CSF, IFN2, IFN, leptin, IL-6. In serum the following mediators were also not detected above the limits of quantification in >80%: IFN2, IL-6.

**Mediators associated with clinical severity**

For all patients with encephalitis, comparing those with a normal or reduced GCS on admission, there was no significant difference in the IL-1 concentration in serum or CSF. However, the mediator most closely associated with GCS on admission was IL-10 (Table 1). For all patients, the concentration of IL-10 in the CSF was significantly higher in those with a normal GCS (p=0.016) and IL-10 also had a positive correlation with GCS (p=0.017) (Figure 1a). Assessing only the subset with HSV encephalitis, in the serum the mediator most closely associated with the GCS was also IL-10, which was higher in those with a normal GCS (p=0.007), and serum IL-10 also had a positive correlation with the GCS score (p=0.01) (Figure 1b).

For the cohort overall comparing those with a good and poor Glasgow outcome scale (GOS) score, there was no significant difference in IL-1 concentration in the CSF or serum. However, the serum mediator associated most significantly with outcome was IL-1RA (Table 2). Higher serum concentrations of IL-1RA were seen in those with a good as opposed to poor outcome (p=0.004). Also there was a significant positive correlation between IL-1RA and the GOS score (p=0.01) (Figure 1c). Assessing only the subset with HSV encephalitis, IL1RA was also the mediator most significantly associated with the GOS. The CSF IL-1:IL-1RA ratio was significantly higher in those with a worse outcome score (p=0.009). There was also a significant inverse correlation with GOS score (p=0.003)(Figure 1d). Applying a CSF IL-1:IL-1RA cut-off of greater than -0.55pg/mL had a high specificity and sensitivity for distinguishing patients with a good from a poor outcome (100% and 83% respectively, p=0.015).

**Figure 1. Relationship between Glasgow coma score on admission and IL-10 concentration in cerebrospinal fluid in patients with encephalitis (a) and serum IL-10 in those with HSV encephalitis. Relationship between Glasgow outcome score at discharge and serum IL-1RA in patients with encephalitis (c) and there cerebrospinal fluid IL-1:IL-1RA ration in those with HSV encephalitis (d)**

Hierarchical analysis of the data showed that the mediators clustered into three broad groups; cluster one contained IL-10 and IL-1RA, amongst other mediators, cluster two contained the chemokines CCL2, CCL3 and CXCL10, and cluster three contained IL-1 and IL-1 in addition to other mediators including the adhesion molecules VCAM and ICAM (Figure 2). There was a negative correlation between group one and group three mediators in those with a good outcome which was less apparent in those with a poor outcome, this was particularly highlighted in the extraction heat-map. This suggests that in those with a good outcome greater concentrations of the anti-inflammatory mediators IL-1RA and IL-10 were associated with lower concentrations of the pro-inflammatory mediators, IL-1 and IL-1, and that this relationship was less evident in those with a poor outcome.

**Figure 2. Heat-map representation of mediator interaction in the cerebrospinal fluid of patients with encephalitis with a good (a) and poor (b) outcome scores, and the subtraction heat-map (c).**

**Mediators associated with blood-brain barrier permeability**

Paired CSF and serum samples for determining the albumin ratio were available for 20 patients (8 infective, 6 immune-mediated, and 6 unknown aetiology). For all mediators in serum and for most mediators in CSF there was not a significant association with the CSF:serum albumin ratio (Table 3-supplementary online). However, the CSF mediator with the strongest negative correlation with the CSF:serum albumin ratio was IL10 (tau b [95% CI] -0.49 [-0.79- -0.20], p=0.0045) and that with the strongest positive correlation was VCAM (0.50 [0.20-0.81], p=0.0035) (Figure 3). There was no association between the CSF:serum albumin ratio and the GCS or GOS score. There was a trend towards a higher median [range] CSF albumin concentration in those with an elevated CSF white cell count (250 [67.5-905] vs 149 [50.65-348.5] mg/L, p=0.049).

**Figure 3. The relationship between the cerebrospinal fluid:serum albumin ratio and the concentration of vascular cell adhesion molecule and IL-10 in the cerebrospinal fluid of patients with encephalitis.**

**Mediators associated with temporal lobe volume in patients with HSV encephalitis**

An MRI was available for 8 patients with HSV encephalitis; 6 had an MRI scan on the first day of admission, one on day 13 and one on day 33. All images showed enlarged temporal lobes and tissue damage. Temporal lobe volume ranged between 9.5-13.9% of total intracranial volume, and global tissue damage ranged from 0.4-11.0%. This represents a 8.5% ± 0.9% increase in temporal lobe volume in comparison to normal values in healthy controls [32]. There was good intra-observer agreement (95% limit of agreement of 5%), but inter-observer agreement was less consistent (95% limit of agreement of 11%). Larger studies, which are suitably powered to address the question of the optimal number of investigators reviewing scans in encephalitis, are needed. There was no significant correlation between temporal lobe volume and the volume of damaged tissue. Serum mediator data were available for all cases and CSF data were available for 2 cases.

In serum there was a strong positive correlation between temporal lobe volume and IL-1 (p=0.0003) and a negative correlation with IL-1RA (p=0.012)(Table 4-supplementary online)(Figure 4). No other mediators in serum or CSF correlated with temporal lobe volume or damaged tissue volume. There was no correlation between either the temporal lobe volume or damaged tissue volume and the GCS or GOS scores.

**Figure 4. The relationship between temporal lobe damage, as a percentage of total brain volume, on magnetic resonance imaging and the concentration of IL-1 and IL-1RA in the serum of patients with HSV encephalitis**

**DISCUSSION**

This study has found that in a cohort of patients with encephalitis and a subset due to HSV, the balance between IL-1 and its antagonists, IL-1RA and IL-10, was associated with clinical severity, BBB permeability, and also the volume of temporal lobes in HSV. Specifically IL-1RA was associated with a better outcome in the cohort overall and those due to HSV, and also with reduced temporal lobe swelling in those with HSV. IL-10 was associated with a better coma score on admission in the cohort overall and also in those due to HSV. Elevated levels of IL-10 were also associated with a lesser degree of BBB permeability. These may be proxy markers of clinical severity and outcome, or may represent potential avenues for adjunctive therapy in HSV encephalitis, which may have implications of encephalitis more broadly.

Encephalitis is a devastating condition of brain parenchymal inflammation [33]. The majority of cases are due to infection, most commonly with HSV, although others are antibody-mediated or of unknown aetiology [2, 17]. The pathophysiology of the host inflammatory response is poorly understood and current treatments are limited to antiviral therapy for HSV and non-specific immune-suppression for antibody-mediated cases [2, 21].

Despite antiviral therapy the mortality from HSV encephalitis is 10-30% and neurological sequelae are common, with <20% returning to work [1, 25]. There is mounting evidence from both animal models and clinical studies that the cytokine-mediated inflammatory response may play an important role in pathogenesis [3, 5, 14]. These mediators modulate the innate and adaptive inflammatory responses and facilitate BBB permeability, which is vital for leucocyte infiltration and oedema [15, 16]. However, the key mediators in HSV encephalitis, and more broadly, remain unclear. Both the numbers of patients and of mediators assessed has limited previous studies and markers of BBB permeability and parenchymal inflammation have not been assessed. The significance of many mediators, such as IL-1, is dependent on the relative concentration of natural antagonists and has also not been assessed [4, 5, 7, 12, 34]. An improved understanding of the pathophysiology of this inflammatory response could pave the way for utilisation of novel or existing adjunctive immunomodulatory therapies [22, 23].

Therefore, we analysed CSF and serum samples from patients prospectively recruited in a multi-centre study in England to determine whether mediator profiles correlated with clinical severity, and downstream markers of BBB permeability and also neuroimaging changes in the HSV subset. This study identified a potential role for the anti-inflammatory IL-1 antagonists, IL-1RA and IL-10. The serum concentration of IL-1RA was elevated in those with a better outcome score at discharge in the cohort overall and, of those with HSV, the ratio of IL-1:IL-1RA in the CSF was raised in those with a worse outcome. Moreover, in those with HSV encephalitis, the serum IL-1RA concentration was inversely associated with the volume of the temporal lobes on MRI, and serum IL-1 with a greater volume. CSF IL-10 was elevated in those with a higher admission GCS score and, in HSV, a higher concentration of serum IL-10 was found in those with a higher GCS. In addition, IL-10 was associated with lesser BBB permeability. That IL1RA correlated with temporal lobe volume but not damaged tissue indicates that there may be different mechanisms underlying these MRI changes. For example, as IL-10 was associated with both GCS and BBB permeability this may be through a common mechanism of raised intracranial pressure as both are reported to have a reciprocal relationship [14]; whereas the mechanisms underlying the action of IL-1 may be more broad.

The IL-1 family, particularly IL-1 are the prototypical pro-inflammatory cytokines. IL-1 and IL-1 act through the IL1-receptor 1, which is blocked by IL-1RA [10, 12]. In vitro studies of HSV infection have found marked up-regulation of IL-1 predominantly by human microglia [3, 12, 34, 35]. Also IL-1, IL-1 and IL-1RA have been identified in murine models of HSV encephalitis in association with cerebral oedema [36]. IL-1 has an important role in up-regulating many pro-inflammatory mediators including adhesion molecules and chemokines which further mediate BBB permeability [10, 12, 35]. Two previous studies have attempted to assess IL-1 in the CSF of patients with HSV encephalitis, and both did not identify concentrations above the limit of detection in the majority of the 20 and 9 adults in each study [5, 37]. Although, in both studies many patients received steroids and the timing of this is not always clear. As both steroid use and later sampling are both associated with lower levels of IL-1, this may account for these findings. Elevated concentrations of IL-10 were identified in the majority and this did not differ between those with moderate sequelae/death and those with mild/no disability, although it was not possible to assess this relative to the IL-1 concentration or BBB permeability [5, 37].

In our study it is not surprising that the IL-1 antagonists were found at higher concentrations and with more consistent associations than IL-1, as it is well recognised that up-regulation of IL-1 in the CNS is early and transient, and also that the action is predominantly autocrine and paracrine, with a lesser spill-over into the peripheral circulation [12]. As the early up-regulation of IL-1 production may be pivotal, future studies require assessment of critical timeframes within which any therapy may be efficacious, as has been found in murine models of glucocorticoid therapy [25, 38]. A degree of IL-1 production may be protective in HSV encephalitis in the absence of aciclovir treatment, as IL-1 knock-out mice succumb to fatal infection [38]. However, IL-1 has not been demonstrated to suppress infection of human astrocytes with HSV and associated viruses [3]. Exogenous IL-1RA (Anakinra©) is currently contraindicated in active infection but the risk of promoting viral replication for herpesviridae may be low [39]. Interestingly, HSV has been used as a gene-therapy vector to induce IL-1RA production ameliorating experimental autoimmune encephalomyelitis [40]. In addition a phase 2 trial of IL-1RA demonstrated improved outcomes in patients with inflammation due to cortical infarcts [22]. Moreover, IL-1RA expression and corresponding IL1 inhibition due to glatiramer acetate has been found to reduce CNS inflammation in multiple sclerosis [41]. Inhibition of IL-1 production has also been demonstrated with corticosteroids [35]. Indeed, steroids were routinely used to treat HSV encephalitis before aciclovir was established and in one study of 45 aciclovir-treated patients, not receiving corticosteroids was associated with a poor outcome [42, 43]. Moreover, administration of adjunctive steroids in a murine HSV model reduced neuroimaging changes without increasing viral load [44].

This study also identified a potentially important role for IL-10. One previous study identified higher CSF concentrations of IL-10 in patients with HSV encephalitis than in non-infectious controls, although this did not assess disease severity [4]. This potent anti-inflammatory cytokine produced by glial cells and lymphocytes reduces pro-inflammatory cytokines production, particularly IL-1, and promotes survival signalling, including in *in vitro* studies of HSV infected human microglia [13, 45]. Also IL-10 was elevated in a murine encephalitis model due to Japanese encephalitis virus, and to correlate inversely with IL-1 and histopathological changes [8, 9]. Moreover, exogenous IL-10 also decreases cyclooxygenase-2 production, which is important for BBB permeability, and also to reduce neuronal death in murine models of viral encephalitis [45, 46]. In a BBB model, exogenous IL-1 has been associated with increased expression of adhesion molecules and permeability, as determined by the endothelial electrical resistance [47]. This supports our finding that the mediator with the strongest negative correlation with BBB permeability was IL-10 and that with the strongest positive correlation was VCAM. However, IL-10 did not correlate with outcome suggesting that BBB permeability is only part of the determinant of neurological injury, which is also due to cytotoxic oedema. The combined volume of vasogenic and cytotoxic oedema seen on MRI was most closely associated with IL-1 and IL-1RA.

High levels of IL-10 potentially increase susceptibility to intracellular pathogens [48]. However, IL-10 treatment may reduce pro-inflammatory cytokines and infiltrate in murine HSV keratitis without impairing viral clearance [49]. Interestingly the protection from otherwise fatal murine HSV encephalitis demonstrated with intravenous immunoglobulin is not achieved in IL-10 knock-out mice [50]. Our study did not identify any association with IL-6 or the IL-6:IL-4 ratio, and IL-6 was not identified above the limits of detection in >20% of the cohort. Although identified in previous studies of severe infection, these have been with viraemia, with Japanese encephalitis virus, or a parasitaemia, with malaria [14, 20]. This is pathophysiologicaly different from HSV encephalitis in which brain parenchymal dysfunction follows neurotropic migration [2].

This study adds to our understanding of the complex interplay of cytokines and associated mediators in encephalitis in relation to disease severity, and also identified associations between these same mediators and BBB permeability and degree of parenchymal swelling on neuroimaging. These may represent modifiable mediators with the potential to improve outcomes in HSV encephalitis and encephalitis more broadly and warrant further investigation in animal models, which may open the door to clinical studies.

**Acknowledgements**

BDM is an NIHR Academic Clinical Lecturer. This work was supported by the laboratory team at the Vaccine Evaluation Unit, Public Health England, Manchester, UK. The authors are thankful for the expert advice on this work and manuscript from Professor Dame Nancy Rothwell.

**Funding**

BDM received support as part of an NIHR Doctoral Research Fellowship. TS received support from the NIHR Health Protection Research Unit in Emerging and Zoonotic Infections at Liverpool. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England.

**Conflict of interest statement**

The authors do not report any conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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