Proinflammatory Cytokines and Chemokines in Humans with Japanese Encephalitis

Peter M. Winter,1,2 Nguyen Minh Dung,3 Ha Thi Loan,1 Rachel Kneen,4 Bridget Wills,4 Le Thi Thu,1 Deborah House,4 Nicholas J. White,5 Jeremy J. Farrar,4 C. Anthony Hart,2 and Tom Solomon1,2

Departments of 1Neurological Science and 2Medical Microbiology and Genitourinary Medicine, University of Liverpool, United Kingdom; 3Hospital for Tropical Diseases and 4University of Oxford–Wellcome Trust Clinical Research Unit, Ho Chi Minh City, Vietnam; 5Wellcome Trust–Mahidol University–Oxford Tropical Medicine Research Programme, Mahidol University, Bangkok, Thailand

Background. Japanese encephalitis virus (JEV), the mosquito-borne flavivirus, annually causes an estimated 35,000–50,000 encephalitis cases and 10,000–15,000 deaths in Asia, and there is no antiviral treatment. The role played by the immune response in determining the outcome of human infection with JEV is poorly understood, although, in animal models of flavivirus encephalitis, unregulated proinflammatory cytokine responses can be detrimental.

Methods. We studied the innate, cellular, and humoral immune responses in 118 patients infected with JEV, of whom 13 (11%) died.

Results. Levels of interferon (IFN)–α, the proinflammatory cytokine interleukin (IL)–6, and the chemokine IL-8 were all higher in the cerebrospinal fluid (CSF) of the nonsurvivors than of the survivors (P < .04, P < .006, and P = .04, respectively), as were both the IL-6:IL-4 ratio in CSF (a marker of the balance of pro- and anti-inflammatory cytokines) and the level of the chemokine RANTES (regulated on activation, normally T cell expressed and secreted) in plasma (P < .03). In contrast, levels of immunoglobulin (Ig) M and IgG in CSF and of IgM in plasma were higher in the survivors (P < .035, P < .003, and P < .009, respectively). Levels of IFN-γ and nitric oxide did not vary with outcome.

Conclusions. During JEV infection, elevated levels of proinflammatory cytokines and chemokines are associated with a poor outcome, but whether they are simply a correlate of severe disease or contribute to pathogenesis remains to be determined.
have a secondary pattern of antibody response, with rapid pro-
duction of IgG to high titers. Thus, the IgM:IgG ratio can be
used to distinguish primary from secondary flavivirus infection
[10, 11]. Infection with JEV is followed by lifelong immunity
to the virus. Patients who fail to produce antibody are more
likely to have virus isolated from their CSF and are more likely
to die [9].

The early innate immune response and the cellular immune
response during JE have been less well characterized, although
interferon (IFN)–α, tumor necrosis factor (TNF)–α, and the
chemokine interleukin (IL)–8 have each been associated with
a bad outcome in small studies [12–14]. In animal models of
viral central nervous system (CNS) infection, Th2 responses,
characterized by the production of IL-4 (which supports the
differentiation of B cells for production of antibody), appear
to be protective [15, 16]. In contrast, Th1 responses, charac-
terized by the production of IFN-γ, favor the activation of
macrophages (which produce proinflammatory cytokines and
reactive oxygen and nitrogen intermediates) and cytotoxic T
cells, which may sometimes be detrimental [17, 18]. Recent
data from animal models of West Nile virus infection [19] and
of other flavivirus infection [20] suggest that there may be
immunopathology, which, if confirmed in humans, could point
the way toward future therapeutic targets. To examine whether
unregulated inflammatory responses play a role in the patho-
genesis of JE in humans, we prospectively studied a series of
Vietnamese patients. In addition to measuring the levels of the
cytokines IFN-α, IFN-γ, TNF-α, IL-4, and IL-6, we measured
the levels of the chemokines IL-8 and RANTES and the reactive
nitrogen intermediate nitric oxide (NO).

PATIENTS, MATERIALS, AND METHODS

Patients and setting. Between December 1994 and November
1998, children and adults with JE were investigated during
studies of patients with a suspected CNS infection who were
admitted to the pediatric and adult intensive care units of the
Hospital for Tropical Diseases (Ho Chi Minh City), an infec-
tious diseases referral hospital for southern Vietnam, where JEV
is endemic [21–24]. Study protocols were approved by the Sci-
cific and Ethical Committee of the Hospital for Tropical Dis-
eases, and consent was obtained from each patient’s accom-
panying relative. A CNS infection was suspected in patients
with fever or a history of fever and at least 1 of the following:
reduced level of consciousness (either a Glasgow coma score
≤14 or, for children <6 years old, a Blantyre coma score ≤4
[25]), severe headache, neck stiffness, tense fontanelle, focal
neurological signs, or seizures. Children between 6 months and
5 years old who had a single convulsion lasting <15 min and
who recovered consciousness within 60 min were considered
to have had a simple febrile convulsion and were not studied.

For study patients, a detailed history was taken, and a clinical
examination, including a full neurological examination, was
performed by a member of the study team. On admission to
the hospital, a lumbar puncture (LP) was performed in the left
lateral position, and, if the patient was calm, opening pressures
were measured with a spinal fluid manometer. LP was delayed
for convulsing patients or for those with clinical signs of raised
intracranial pressure. CSF samples were collected for cell counts
and for differential, protein, glucose, lactate, Gram stain, bacterial
culture, viral culture, and cytokine assays, as described below.

Diagnosis of JE. Levels of IgM to JEV were evaluated in
acute and convalescent serum and CSF samples by use of a
rapid IgM dot-enzyme immunoassay that distinguishes between
antibodies to JEV and dengue virus [24]. Levels of IgM and
IgG were subsequently measured by use of a double sandwich
capture ELISA [10, 26, 27]. For serum and CSF samples, ≥40
U of IgM to JEV (with IgM to JE virus greater than IgM to
dengue virus) was considered to be evidence of an acute JE infection;
for paired samples, an increase from <15 U to >30 U was
considered to be evidence of an acute JE infection [10, 26,
27]. These assays, which have high sensitivity and specificity
after the first few days of illness, have become the accepted
standard for diagnosis of JE [11, 28]. An IgM:IgG ratio ≥1.8:1
was considered to be evidence of a primary flavivirus infection,
whereas a ratio <1.8:1 was considered to be evidence of a
secondary flavivirus infection (i.e., the patient had been pre-
viously infected with a different flavivirus) [10]. These criteria
were derived for distinguishing between primary and secondary
dengue virus infection and have not been formally assessed for
JEV infection [10, 21]. Therefore, we also applied more-recently
devised criteria for distinguishing between primary and sec-
ondary infection, in which a secondary infection is diagnosed
if the level of IgG to dengue virus in serum is ≥35 U, and a
primary infection is diagnosed if the level of IgG to dengue
virus is ≤35 U, in ≥2 samples, with the last sample collected
during the first week of illness [7]. Although these newer criteria
have been validated against hemorrhage-
nation inhibition assays, they have the disadvantage of being
unable to diagnose a primary infection in patients who die
during the first week of illness. For isolation of JEV, autopsy
material and acute serum and CSF samples were inoculated into
Aedes albopictus C6/36 cells, rhesus monkey kidney (LLC-
MK2) cells, and Vero cells [6, 23].

Determination of cytokine, chemokine, and NO levels. Se-

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rum samples (2 mL) were collected in EDTA tubes and were centrifuged, and the plasma was separated. CSF and plasma samples were stored at −70°C until they were transported to the United Kingdom on dry ice for analysis. When possible, follow-up samples were also collected. Samples from the patients who presented within 2 weeks of the onset of illness were considered to be acute samples. CSF and plasma samples were analyzed for levels of IL-4, IL-6, IL-8, TNF-α, and IFN-γ by use of the Duo ELISA kit (R&D Systems); for levels of RANTES by use of a Quantikine kit (R&D Systems); and for levels of IFN-α by use of a human ELISA kit (Endogen), according to the manufacturers’ instructions. Each plate included a standard curve of recombinant human cytokine and negative controls. After ultrafiltration with Ultrafree-MC centrifugal filter units with PTGC polysulfone membranes (Millipore) to ensure protein removal, levels of NO in CSF and plasma were quantified by the measurement of total NO, using a Greiss-based reaction and a Total NO kit (R&D Systems). Samples were measured in duplicate, and the mean value was used for analysis. The thresholds of detection were as follows: for IL-4 and IL-8, 10 pg/mL; for TNF-α, 4.4 pg/mL; for IFN-γ and IFN-α, 3.0 pg/mL; for IL-6, 0.7 pg/mL; and for NO, 1.35 μmol/L.

**Statistical analysis.** For data that were not normally distributed, log transformations were applied when appropriate. Univariate analysis of baseline variables was performed by use of Student’s t test for normally distributed data and the Mann-Whitney U test for nonnormally distributed data. Spearman’s rank correlation was used to assess associations between continuous variables (Staview software; version 4.02; Abacus Concepts). Multiple logistic regression was used to assess the relationships between multiple variables and outcome (SPSS software; version 10). To examine changes in cytokine levels during the first few days of disease, median cytokine levels on days 2–3 of illness were compared with those on days 4–5 of illness.

**RESULTS**

**Patients.** One-hundred eighteen patients infected with JEV were studied, comprising 112 children (median [total range] age, 7 [1.6–14.9] years) and 6 adults (median [total range] age, 16 [15–75] years). At least 1 plasma sample was available for all of the patients. For 59 patients (54 of them children) it was possible to measure cytokine levels in CSF as well as in plasma, and for 25 patients it was possible to obtain a second plasma level ~48 h after the first. Thirteen (11%) patients died (median [total range] hours after admission, 74 [4–6536] h). Twenty-six (22%) had severe, 36 (31%) had moderate, and 22 (19%) had mild sequelae; 21 (18%) appeared to have made a full recovery when discharged from the hospital. A comparison of the clinical features of the nonsurvivors and survivors is shown in table 1. The nonsurvivors were significantly more likely to be comatose, to have rigidity spasms, to have abnormal oculocephalic reflexes, and to have elevated CSF opening pressures. The 59 patients for whom CSF samples were available were representative of all of the patients with regard to age (median [interquartile range {IQR}, total range] for the 59 patients, 7 [5–11, 1.6–75] years), length of illness (4 [3–6, 2–4] days), the number in a coma on admission (38 [64%]), and the number with fatal outcome (8 [14%]). JEV was isolated from the CSF of 1 patient with a relatively low IgM level and no detectable IgG; this patient died on the seventh day after admission to the hospital.

**Distribution of cytokine levels.** For most of the biomolecules, the distribution of levels in CSF and plasma was highly skewed to the left, because many samples had undetectable levels. The percentages of patients with undetectable levels in CSF were as follows: TNF-α, 23%; IFN-α, 63%; IFN-γ, 44%; IL-4, 27%; IL-6, 13%; and RANTES, 14%. The percentages of patients with undetectable levels in plasma were as follows: TNF-α, 66%; IFN-α, 67%; IFN-γ, 63%; IL-4, 37%; IL-6, 32%; and IL-8, 7%. Therefore, these data sets could not be transformed toward normality and were analyzed by use of nonparametric tests. However, levels of NO in CSF and plasma were detectable for all of the patients and were log transformable toward normality. In addition, levels of RANTES in plasma were normally distributed.

**Univariate analysis of cytokine levels and outcome.** Levels of IL-6, IL-8, and IFN-α were significantly higher in the CSF of the nonsurvivors than in that of the survivors, and TNF-α showed a trend toward higher levels in the nonsurvivors (table 2). In contrast, levels of IgM and IgG in CSF were higher in the survivors than in the nonsurvivors. Only in plasma were levels of RANTES significantly higher in the nonsurvivors than in the survivors, whereas levels of IgM in plasma were higher in the survivors than in the nonsurvivors. Levels of NO in both CSF and plasma were similar for the nonsurvivors and survivors. A level of IL-6 in CSF >250 pg/mL predicted a fatal outcome (sensitivity, 88% [95% confidence interval {CI}, 80%–96%]; specificity, 56% [95% CI, 43%–69%]) (P = .026). However, the best predictor of fatal outcome was a level of IgG in CSF <15 U (sensitivity, 92% [95% CI, 86%–98%]; specificity, 57% [95% CI, 46%–68%]) (P = .006).

**Primary versus secondary flavivirus infection.** Thirteen (14%) of the 91 patients with a primary flavivirus infection (as defined by the IgM:IgG ratio) died, compared with none of the 27 patients with a secondary infection (P = .038). For all of the patients, at least 1 plasma sample was available for analysis, and, for 59, a CSF sample was also available. A comparison of the cytokine levels in patients with a primary versus a secondary infection showed that, for most cytokines, the levels were similar. However, none of the 10 patients with a secondary flavivirus infection had detectable levels of IFN-α in CSF, com-
pared with 18 (46%) of the 39 patients with a primary infection \((P = .007)\). Subgroup analysis examining only the patients with a primary infection showed significantly higher levels of IL-6 in CSF \((P = .007)\) and of RANTES in plasma \((P = .04)\) (data not shown), compared with those in the patients with a secondary infection. Within this subgroup, median levels of IgM and IgG in CSF were lower in the nonsurvivors than in the survivors \((P = .004\) and \(P = .03\), respectively\). For the patients with a primary infection, a level of IL-6 in CSF \(>250\) pg/mL predicted a fatal outcome, with a sensitivity of 88% \((95\%\ CI, 79\%–97\%\) \) and a specificity of 53\% \((95\%\ CI, 39\%–67\%\) \) \((P = .055)\), and a level of IgG in CSF \(<15\) U also predicted a fatal outcome, with a sensitivity of 92\% \((95\%\ CI, 84\%–97\%\) \) and a specificity of 57\% \((95\%\ CI, 43\%–71\%\) \) \((P = .019)\).

All of the 27 patients classified as having a secondary flavivirus infection by the IgM:IgG ratio were also classified as having a secondary infection by the more-recent criteria \((\text{IgG} >35\) U\). Of the 91 patients classified as having a primary infection by the IgM:IgG ratio, 9 could not be classified by the more-recent criteria, and 6 were classified as having a secondary infection. Nonetheless, comparison of levels of cytokines in the patients classified as having a primary or a secondary infection using the more-recent criteria gave similar results. One \(7.7\%\) of the 13 patients with a secondary flavivirus infection had a detectable level of IFN-\(\alpha\) in CSF, compared with 11 \(39.3\%\) of the 28 patients with a primary infection \((P = .06)\).

### Relationship between biomolecules

Because failure to produce antibody is strongly associated with a fatal outcome during JE, we examined the relationship between cytokine and immunoglobulin levels. Levels of IL-8 and IL-6 in CSF were significantly inversely related to levels of IgM in CSF \((\rho = 0.424\) and \(\rho = 0.346\), respectively \); \(P = .005\) and \(P = .02\), respectively\), but none of the other biomolecules was correlated with levels of IgM or IgG in CSF. Repeating this analysis for only the patients with a primary infection \(\left(\text{as defined by the IgM:IgG ratio}\right)\) showed that levels of IL-8 and IL-6 in CSF both remained inversely correlated to levels of IgM in CSF \((\rho = 0.445\) and \(\rho = 0.495\), respectively \); \(P = .006\) and \(P = .003\), respectively\). For the patients with a secondary flavivirus infection, there was no correlation. Levels of IgM in plasma were significantly correlated with those of IL-8 \((\rho = 0.234\) and \(P = .02\)\), IFN-\(\alpha\) \((\rho = 0.245\) \(P = .018\)\), and TNF-\(\alpha\) \((\rho = 0.33\) \(P = .002\)\). For the patients with a primary infection but not for the patients with a secondary infection, levels of IgM in plasma remained significantly correlated with those of IFN-\(\alpha\) \((\rho = 0.255\) \(P = .031\)\) but not with those of IL-8 or TNF-\(\alpha\). As a measure of the balance between proinflammatory cytokines and humoral immunity and its relationship to survival, we determined, for

<table>
<thead>
<tr>
<th>Category, characteristic</th>
<th>Nonsurvivors (n = 13)</th>
<th>Survivors (n = 105)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>6 (46)</td>
<td>60 (57)</td>
<td>.648</td>
</tr>
<tr>
<td>Median age (\text{IQR, total range},) years</td>
<td>7 (5.0–9.3, 2.0–14.0)</td>
<td>7 (5.0–10.0, 1.6–75.0)</td>
<td>.853</td>
</tr>
<tr>
<td>History</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median length of illness (\text{IQR, total range},) days</td>
<td>3 (2.8–4.3, 2.0–6.0)</td>
<td>4 (4.0–6.0, 2.0–14.0)</td>
<td>.015</td>
</tr>
<tr>
<td>Rigor</td>
<td>4 (31)</td>
<td>19 (18)</td>
<td>.277</td>
</tr>
<tr>
<td>Headache</td>
<td>11 (85)</td>
<td>64 (61)</td>
<td>.130</td>
</tr>
<tr>
<td>Rigidity spasm</td>
<td>7 (54)</td>
<td>15 (14)</td>
<td>.003</td>
</tr>
<tr>
<td>Convulsions</td>
<td>6 (46)</td>
<td>46 (44)</td>
<td>.999</td>
</tr>
<tr>
<td>&gt;1 convulsion</td>
<td>6 (46)</td>
<td>27 (26)</td>
<td>.186</td>
</tr>
<tr>
<td>Examination findings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median pulse (\text{IQR, total range},) beats per minute</td>
<td>120 (120–125, 100–130)</td>
<td>120 (100–120, 80–180)</td>
<td>.087</td>
</tr>
<tr>
<td>Median respiration rate (\text{IQR, total range},) breaths per minute</td>
<td>28 (28–35, 20–52)</td>
<td>28 (24–32, 20–52)</td>
<td>.184</td>
</tr>
<tr>
<td>Coma(\text{a})</td>
<td>12 (92)</td>
<td>59 (56)</td>
<td>.014</td>
</tr>
<tr>
<td>Able to localize pain</td>
<td>4 (31)</td>
<td>67 (64)</td>
<td>.022</td>
</tr>
<tr>
<td>Seizure on admission</td>
<td>3 (23)</td>
<td>11 (10)</td>
<td>.185</td>
</tr>
<tr>
<td>Rigidity spasms on examination</td>
<td>7 (54)</td>
<td>20 (19)</td>
<td>.012</td>
</tr>
<tr>
<td>Abnormal oculocephalic reflex</td>
<td>6 (46)</td>
<td>8 (8)</td>
<td>.001</td>
</tr>
<tr>
<td>Hemiparesis</td>
<td>2 (15)</td>
<td>11 (10)</td>
<td>.999</td>
</tr>
<tr>
<td>Any increased limb tone</td>
<td>9 (69)</td>
<td>43 (41)</td>
<td>.075</td>
</tr>
<tr>
<td>Any flaccid limbs</td>
<td>4 (31)</td>
<td>29 (28)</td>
<td>.755</td>
</tr>
<tr>
<td>CSF opening pressure &gt;20 cm of CSF(\text{b})</td>
<td>6 (33)</td>
<td>8 (9)</td>
<td>.007</td>
</tr>
<tr>
<td>Median CSF WCC (\text{IQR, total range},) cells/mL</td>
<td>105 (60–180, 14–200)</td>
<td>60 (20–120, 0–156)</td>
<td>.206</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of patients, unless otherwise noted. CSF, cerebrospinal fluid; IQR, interquartile range; WCC, white cell count.

\(\text{a}\) Either a Glasgow coma score <11, or, for children <6 years old, a Blantyre coma score <4.

\(\text{b}\) CSF opening pressure was measured in 13 nonsurvivors and 65 survivors.
Table 2. Cytokine, chemokine, nitric oxide (NO), and immunoglobulin levels on admission to the hospital, in nonsurvivors and survivors of Japanese encephalitis.

<table>
<thead>
<tr>
<th>Category, biomolecule</th>
<th>Nonsurvivors</th>
<th>Survivors</th>
<th>P\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median level in CSF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>25.3 (11.6–43.7, 0–60.0)</td>
<td>0 (0–13.0, 0–116.45)</td>
<td>.093</td>
</tr>
<tr>
<td>IL-4</td>
<td>36.5 (14.6–72.9, 0–175.0)</td>
<td>58.3 (0–87.5, 0–218.8)</td>
<td>.535</td>
</tr>
<tr>
<td>IL-6</td>
<td>1377.0 (418.0–1648.0, 193.0–2190.0)</td>
<td>174.1 (38.0–849.0, 0–2821.0)</td>
<td>.006</td>
</tr>
<tr>
<td>IL-8</td>
<td>1140.0 (420.0–1656.0, 190.0–3823.0)</td>
<td>286.0 (78.0–799.0, 0–4714.0)</td>
<td>.040</td>
</tr>
<tr>
<td>IFN-α</td>
<td>4.4 (0.4–13.9, 0–35.6)</td>
<td>0 (0–2.2, 0–53.3)</td>
<td>.044</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>60.0 (0–240.0, 0–255.0)</td>
<td>13.0 (0–180.0, 0–320.0)</td>
<td>.511</td>
</tr>
<tr>
<td>RANTES</td>
<td>32.2 (13.0–50.1, 3.1–95.4)</td>
<td>18.5 (6.0–47.6, 0–353.7)</td>
<td>.486</td>
</tr>
<tr>
<td>NO, mmol/L</td>
<td>97.6 (25.9–67.5, 18.0–503.5)</td>
<td>54.9 (30.7–69.0, 14.6–170.0)</td>
<td>.875</td>
</tr>
<tr>
<td>IgM, units</td>
<td>60.5 (33.5–60.5, 4.0–274.0)</td>
<td>162.0 (67.0–229.5, 0–412.0)</td>
<td>.035</td>
</tr>
<tr>
<td>IgG, units</td>
<td>5.5 (1.5–13.0, 0–17.0)</td>
<td>23.0 (7.5–60.0, 0–273.0)</td>
<td>.003</td>
</tr>
<tr>
<td>Median level in plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0 (0–17.5, 0–180.0)</td>
<td>0 (0–32.5, 0–3970.0)</td>
<td>.910</td>
</tr>
<tr>
<td>IL-4</td>
<td>23.9 (0.6–89.6, 0–478.4)</td>
<td>15.9 (0–90.9, 0–1530.4)</td>
<td>.691</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.5 (0–6.5, 0–997.7)</td>
<td>7.4 (0–26.4, 0–2466.2)</td>
<td>.160</td>
</tr>
<tr>
<td>IL-8</td>
<td>29.2 (21.3–92.4, 8.0–182.0)</td>
<td>54.0 (16.4–252.0, 0–5632.0)</td>
<td>.506</td>
</tr>
<tr>
<td>IFN-α</td>
<td>0 (0–0, 0–31.9)</td>
<td>0 (0–2.6, 0–31.7)</td>
<td>.201</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0 (0–5.0, 0–20.1)</td>
<td>0 (0–4.0, 0–2510.0)</td>
<td>.306</td>
</tr>
<tr>
<td>RANTES</td>
<td>11,312.0 (8823.0–15,704.0, 357.0–17,536.0)</td>
<td>8269.0 (6180.0–10,113.0, 0–29,224.0)</td>
<td>.031</td>
</tr>
<tr>
<td>NO, mmol/L</td>
<td>79.4 (41.0–61.5, 5.3–436.5)</td>
<td>119.6 (42.8–93.1, 18.8–631.6)</td>
<td>.121</td>
</tr>
<tr>
<td>IgM, units</td>
<td>31.5 (12.5–41.5, 2.0–187.0)</td>
<td>90.5 (36.0–148.5, 1.0–145.0)</td>
<td>.009</td>
</tr>
<tr>
<td>IgG, units</td>
<td>10.0 (4.0–13.0, 0–40.0)</td>
<td>11.0 (4.0–28.0, 0–188.0)</td>
<td>.463</td>
</tr>
</tbody>
</table>

NOTE. Data are median (interquartile range [IQR], total range) levels in picograms per milliliter, unless otherwise noted. For cerebrospinal fluid (CSF) levels, for nonsurvivors and for survivors; for plasma levels, for nonsurvivors and for survivors. TNF, tumor necrosis factor.

\* Mann-Whitney U test or t test on log-transformed data, as appropriate.

each patient, the ratios of the CSF levels of IL-6:IgM, IL-8: IgM, and RANTES:IgM and compared the ratios in the nonsurvivors and the survivors. The median CSF IL-6:IgM ratio was significantly higher in the nonsurvivors than in the survivors (median [IQR, total range], 54.9 [8.0–59.6, 1.6–389.8] vs. 2.3 [0.12–7.3, 0–244.7]) (P = .005). Higher ratios in the nonsurvivors were also found for CSF IL-8:IgM (P = .006) and CSF RANTES:IgM (P = .008). These findings remained significant when only the patients with a primary infection were examined. We looked at the ratio of the levels of IL-6 and IL-4 in CSF as a measure of the balance of pro- and anti-inflammatory cytokines. The median (IQR, total range) CSF IL-6:IL-4 ratio was 18.6 (8.8–39.7, 2.6–75.0) in the nonsurvivors and was 1.8 (0.2–8.6, 0–29.5) in the survivors (P = .005). For the patients with a primary infection, the median (IQR, total range) CSF IL-6:IL-4 ratio was 18.6 (8.8–39.7, 2.6–75.0) in the nonsurvivors and was 1.8 (0.5–7.9, 0–18.0) in the survivors (P = .004). There were positive correlations between most of the levels of pro- and anti-inflammatory cytokines in CSF or plasma (data not shown). We also looked for a correlation between the level of each biomolecule measured in CSF and plasma but found none, suggesting that levels in CSF represent local production within the CNS, rather than production in the periphery. To determine whether 1 cytokine level in CSF was associated with death independent of the other cytokine levels and of clinical features, variables associated with death in univariate analyses were included as independent variables in a stepwise logistic regression, with death as the dependent variable. The level of IL-6 in CSF remained an independent risk factor and was significantly associated with fatal outcome (odds ratio [95% CI], 1.002 [1.001–1.004]) (P = .008) in both forward-selection and backward-elimination methods.

**Relationship with clinical features.** Because raised intracranial pressure and cerebral edema are thought to be important in the pathophysiology of JE, we examined the relationship between proinflammatory cytokines and clinical features of brain stem dysfunction. The patients with a history of rigidity spasm (a clinical marker of brain stem dysfunction) had higher levels of NO in CSF than did those without a history of rigidity spasm (median [IQR, total range], 60.5 [36.9–73.3, 14.6–503.5] vs. 29.4 [23.1–39.3, 18.0–70.0]) (P = .006), but there was no other significant difference in levels of biomolecules in CSF according to clinical features suggestive of raised intracranial pressure. Six (46%) of the nonsurvivors and 25 (24%) of the survivors had pneumonia, but levels of cytokines in plasma and CSF were not different for the patients with or the patients without pneumonia. To examine the putative role played by cytokines and chemokines in recruiting inflammatory cells into
the CNS, the relationship between biomolecule levels and white cell counts in CSF was examined by use of Spearman’s rank correlation. This analysis showed an association between white cell count and levels of RANTES in CSF ($\rho = 0.544; P = .0002$) (figure 1), which remained when only the patients with primary infection were examined ($\rho = 0.476; P = .003$). Analysis when different white cell types within CSF was allowed for showed that the association between CSF lymphocyte count and levels of RANTES was stronger than that between CSF neutrophil count and levels of RANTES (data not shown). There was no significant association between levels of other biomolecules and CSF cell counts.

**Variation by day of illness.** In figure 2, the median levels of IL-6, IL-8, and IFN-α in CSF are compared for the non-survivors and survivors according to the day of illness. Comparison of the median levels on days 2–3 of illness with those on days 4–5 of illness showed that levels of IL-6 in CSF were initially similar in both patient groups but tended to increase during the first few days of illness in the nonsurvivors, compared with the levels in the survivors ($P = .042$). For levels of IFN-α in CSF, there was a trend toward higher initial levels in the nonsurvivors ($P = .089$), which subsequently decreased.

**DISCUSSION**

There is no established antiviral treatment for JEV, West Nile virus, or indeed any other member of the *Flavivirus* genus. However, in a range of animal models of viral CNS infection, including for West Nile virus [19] and other flaviviruses [20], there is evidence that some of the damage may be mediated by the immune response itself, which could have implications for possible future therapeutic targets. The present study, the largest investigation to date of cytokines and chemokines (both in terms of number of patients and number of parameters) for any acute viral encephalitis, has provided clinical data consistent with the hypothesis that an unregulated proinflammatory response could be detrimental in flavivirus encephalitis. In previous studies examining a single biomolecule during JE, levels of TNF-α, IL-8, and IFN-α have been associated with a poor outcome [12–14]. The present study has confirmed the importance of these molecules and has also shown the importance during JE of IL-6 (which increases blood-brain barrier permeability) and RANTES (which attracts lymphocytes). Although examining a wide range of markers of the innate, humoral, and cellular immune responses necessarily required multiple comparisons, it had the advantage of allowing us to examine the interactions between different components of the immune system. We decided not to formally adjust for multiple testing, because of the increased risk for type II errors associated with this type of adjustment (such that truly important differences may have been deemed nonsignificant) [29]. However, if, to allow for multiple comparisons, we take $P < .01$ rather than $P < .05$ to be significant, most of our observations on antibody production and the relationships with pro- and anti-inflammatory cytokines remain significant. It was intended that the molecules chosen would give us information on various aspects of the immune response, although, because of the multifunctionality of many biomolecules and the widespread interactions between them, our interpretation is, inevitably, overly simplistic. Other possible confounding variables include differences in the timing of when samples were obtained and differences in the availability and processing of the samples. Despite this, certain patterns are evident. First, the innate immune response—as indicated by the production of IFN—is important during JE. IFN-α was detected in the CSF of 37% of our patients, with a trend toward higher levels in the nonsurvivors, particularly during the first few days of illness (table 2 and figure 2). Few of the patients had elevated levels of IFN-α in plasma, perhaps suggesting that, by the time the patients had presented to the hospital, the levels had already decreased. IFN-α is a glycoprotein cytokine that has antiviral effects against JEV in vitro and in animal models [30] and that is thought to be protective; as such, it was considered to be a possible treatment in human disease. However, a recently completed double-blind placebo-controlled trial of recombinant IFN-α2a in Vietnamese children with JE showed that it did not improve outcome [22]. It is possible that this result occurred because the disease was already well established by the time the patients had presented to the hospital. The significance of the trend observed in our study toward higher levels of IFN-α in CSF during more-severe disease is not certain, but it may reflect higher viral load.

If the immune response is unable to control JEV replication in the periphery, a brief viremia is thought to occur, followed by viral spread across the blood-brain barrier into the CNS [31]. A series of studies has shown that failure to produce antibody is associated with an increased risk of death [7, 9, 32];
in one study, such patients were more likely to have virus isolated from their CSF [9]. In contrast, patients previously infected with a different flavivirus (in our setting, most often dengue virus, presumably) rapidly produce antibodies to epitopes that are common to dengue virus and JEV and are less likely to die [7, 8]. This anamnestic response to common flavivirus antigens is thought to explain the reduced rates of clinical attack and case fatality for JEV in dengue-immune subjects [9, 33–35]. However, prior flavivirus exposure is unlikely to be the only explanation for the differing antibody responses in the nonsurvivors and survivors in the present study, because, even among the patients with a primary infection (i.e., excluding the patients with prior flavivirus exposure), antibody responses (as determined by levels of IgM and IgG in CSF and of IgM in plasma) varied with outcome.

The immune response to viral infection of the CNS differs from that in the periphery, because of the potentially damaging consequences of cellular cytotoxicity, altered vascular permeability, and the influx of inflammatory cells to the brain [18, 36]. In the present study, levels of proinflammatory cytokines and chemokines were elevated in the CSF, plasma, or both of many of the patients, and higher levels of some of them (IL-6, IL-8, and TNF-α in CSF and RANTES in plasma) were found in the nonsurvivors, compared with those in the survivors. Because the nonsurvivors tended to present earlier, some of the differences between the 2 groups may be a reflection of dif-

Figure 2. Variation in cytokine and chemokine levels in cerebrospinal fluid (CSF), according to the day of illness. On the left, variations by day are shown; on the right, days 2–3 of illness are compared with days 4–5 of illness. IFN, interferon; IL, interleukin.
ferences between when the samples were obtained. However, that levels of IL-6 and IL-8 in CSF tend to increase over time argues against the theory that the earlier presentation of the nonsurvivors explains these higher levels. Moreover, a comparison between the time course in the nonsurvivors and that in the survivors showed that IL-6 levels were initially similar but that production then increased significantly for the nonsurvivors (figure 2). Several studies have shown that cytokines are produced by cells intrinsic to the CNS (especially microglial cells and perivascular macrophages) as part of the innate immune response to viral infection before the infiltration of inflammatory cells from the periphery [36]. The levels measured in the present study may, therefore, reflect this innate response, not an adaptive immune response. However, histological studies of patients who died of JE and of West Nile encephalitis have confirmed that perivascular cuffing often occurs with an acute inflammatory infiltrate of CD4+ and CD8+ lymphocytes [37–39], suggesting that, later in the disease process, adaptive immune responses are likely important. In the present study, we found, interestingly, that levels of RANTES, a chemokine that attracts lymphocytes and can cause nonspecific activation, in CSF were correlated with lymphocyte counts in CSF, whereas elevated levels of RANTES in plasma were associated with a fatal outcome. This result contrasts with findings for meningococcal disease, during which RANTES appears to be protective [40]. In the present study, the patients with clinical signs of brain swelling had significantly higher levels of NO, a reactive nitrogen intermediate produced by activated macrophages as part of the respiratory burst. However, a randomized placebo-controlled trial of dexamethasone for the control of cerebral swelling in 55 Thai children with JE failed to show any benefit [41].

The associations between immune markers and fatal outcome observed in the present study do not necessarily imply causality. In a previous study of JE, fatality was related to isolation of the virus from CSF [9], suggesting that the vigorous immune response we observed may simply be a response to high virus replication. In experimentally infected mice, cytokine, as part of the innate immune response, is produced in the CNS in proportion to the titer of the infecting virus [17]. Clearly, this important question needs to be addressed for JE. Interestingly, in some patients who died of JE, immunohistochemical studies reveal viral antigen in neurons but no associated inflammation [38], suggesting that, in these patients, virus-induced neuronal dysfunction due to apoptosis or another mechanism may be important. In summary, the present study has shown that there are correlations between levels of proinflammatory cytokines and chemokines and fatal outcome during JE, but whether these are simply a correlate of severe disease or actually contribute to pathogenesis remains to be determined. A better understanding of the role played by viral and immunological factors during JE may direct us toward new treatments.

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References