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Original Research Article

Cytokine and Chemokine Responses Influencing the Outcome of Japanese Encephalitis in Paediatric Age Group

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ABSTRACT

The role of early innate and cellular immune response in determining the outcome of Japanese Encephalitis Virus infection is yet to be fully understood. An attempt was made to understand the immunological response to Japanese Encephalitis Virus infection in influencing the outcome variables.

Methods: Cytometric Bead Array (CBA) Human Flex set (BD Biosciences, San Jose, CA) were used for estimation of levels of four cytokines (IL-4, IL-6, IFN-γ and TNF-α) and two chemokines (IL-8 and RANTES) as per manufacturer's instructions.

Results: Of the biomolecules examined we found RANTES in 92%, INF-γ in 88%, IL-8 in 84% and IL-6 in 80% of CSF samples. Similarly 91 % of acute serum samples were confirmed for presence of IL-8 and IL-6. In acute serum samples the level of proinflammatory chemokine IL-8 was significantly higher in study subjects who developed disability in comparison to those who recovered completely. We also witnessed significantly higher level of TNF-α in CSF who recovered completely than the children who recovered with disability. A significantly higher level of IL-6 was found in 1-5 years age group compared to age group of 5-12 years.

Conclusions: The overall objective of this research work was to address the fundamental issues that underlie the immunological responses of JE in children. The immunological findings of our study corroborate that *cytokines and chemokines* play a decisive role in outcome of Japanese encephalitis infection. Present research findings have opened the possibility to work further on immunological response of this most cripple disease in children.

Key Words: Cytokine, Chemokine, Japanese encephalitis virus, Immune response, Interleukin

INTRODUCTION

Japanese Encephalitis is the most prevalent and significant mosquito born viral encephalitis of man. The immunological response to JE virus infection is a least explored area of research that has only been studied in detail recently. Both innate and adaptive immune responses are activated in response to JEV infection and role of immune response in determining the outcome of human JEV infection is still poorly understood. Only the humoral immune response during JE has been relatively well characterized. [1-3] The early innate immune response and the cellular immune response during JE have been poorly understood, 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. [4-6]

In this study an attempt was made to understand the immunological response of JE in children influencing the outcome variables.

MATERIALS AND METHODS

Study participants and sample collection:

Between January, 2011 to December, 2013 children with Acute Encephalitis Syndrome (AES) were investigated who were admitted to the pediatric ward of Assam Medical College & Hospital, Dibrugarh, Assam. For investigating AES cases, WHO case definition was adopted. All enrolled cases were worked up with the help of a predesigned and pretested proforma. After getting written informed consent 2mL of blood and CSF samples were collected in sterile empty vial. The samples were then transferred under cold chain to Regional Medical Research Centre Laboratory, ICMR, Dibrugarh and stored at -80°C for further analysis.

This study was approved by the Institutional Ethics Committee (Human) of Regional Medical Research Centre (ICMR), NE Region, Dibrugarh, Assam, India.

Serology: JE virus specific IgM antibodies were detected by IgM antibody capture-enzyme-linked immunosorbent assay kits obtained from the National Institute of Virology (NIV), Pune, India. Serological cross-reactions are common within the flaviviruses that is, Dengue, Japanese encephalitis and West Nile encephalitis and all are prevalent in this part of the country. [7] Therefore, the JEV-IgM positive samples were further tested for the presence of IgM antibody against other flaviviruses namely Dengue and West Nile by using Dengue IgM capture ELISA kit obtained from NIV, Pune, India and PanBio WNV IgM capture ELISA kit (Australia).

Laboratory criteria for confirmation of JE:

We adopted the WHO recommended method for laboratory confirmation of a JE virus infection. According to WHO, presence of JE virus-specific IgM antibody in a single sample of cerebrospinal fluid (CSF) or serum, as detected by an IgM-capture ELISA specifically for JE virus is a case of laboratory confirmed JE. [8]

For estimation of chemokine and cytokines in the current study we enrolled 33 JE positive children. It was possible to estimate chemokine/cytokine levels in 25 CSF and 33 acute serum samples of those 33 JE positive cases. CSF samples of 8 JE patients could not be collected due to medical contraindication of lumber puncture.

Estimation of Cytokine, Chemokine levels in JE patients:

Cytometric Bead Array (CBA) Human Flex set (BD Biosciences, San Jose, CA) were used for estimation of levels of four cytokines (IL-4, IL-6, IFN-γ and TNF-α) and two chemokines (IL-8 and RANTES) as per manufacturer's instructions. Briefly, 50 µl of a mixture of bead population with distinct fluorescence intensities, coated with specific antibodies for capturing different cytokines was incubated with 50 µl of serum / CSF samples for 1.5 hr at room temperature in the dark. The complexes were washed with the wash buffer provided in the kit and the cytokine-captured beads were mixed with 50µl of phycoerythrin-conjugated detection antibodies to form sandwich complexes. After incubation for 1.5 hr at room temperature in the dark, followed by a washing step, the samples were subjected to flow cytometry (BD FACS Aria II, BD biosciences, San Jose, CA) and the data was acquired for the samples. In each run of the assay cytokine/chemokine standards provided by the manufacturer were included to construct standard curves using the FCAP Array v3 software. A single set of diluted standards was used to generate a standard curve for each analyte and to extrapolate the level of cytokines/chemokines

Statistical analysis:

A χ^2 or Fisher's exact test was used to analyze categorical variables. *P* values of less than 0.05 were considered as statistically significant. All statistical

analyses were performed with the use of Statistical Package for Social Sciences (SPSS) software, IBM SPSS - version 20.

RESULTS

Study Participants:

It was possible to estimate chemokine/cytokine levels in 33 JE positive cases. Of these 32 were survivors and 1 was non survivor. Among the survivors 23 were recovered completely and 9 were recovered with disability at discharge. Among the survivors, 59.4% were male and the age groups mainly affected were 5 to 12 years (Table 1).

Table 1: Demographic profile of patients with confirmed JE (n = 33)

Category	Survivors (n= 32)
Age in years	
<1	1(4.3%)
1 to 5	12(37.5%)
5 to 12	19 (59.4%)
Sex	
Male	19 (59.4%)
Female	13 (40.6%)

Table 2: Clinical features of study participants with confirmed JE (n = 33)

Category, Characteristics	Survivors (n= 32)		Nonsurvivors (n=1)	P
	Recovered completely n=23 (71.9%)	Recovered with disability n=9 (28.1%)		
Clinical Features				
Fever	23(100%)	9 (100%)	1(100%)	
Altered Sensorium	20(87%)	9 (100%)	1(100%)	.5409
Headache	10(43.5%)	3 (33.3%)		.7036
Irritable	23(100%)	9 (100%)		
Vomiting	4(17.4%)	2 (22.2%)		1
Abnormal behaviour	23(100%)	9 (100%)		
Diarrhoea	1(4.3%)	2 (22.2%)		.1839
Seizure	22(95.7%)	8 (88.9%)	1(100%)	.4899
Glasgow Coma Scale (GCS) ≤ 8	17(73.9%)	6 (66.7%)	1(100%)	.6853
Signs of meningeal irritation	14(60.9%)	3 (33.3%)	1(100%)	.2433

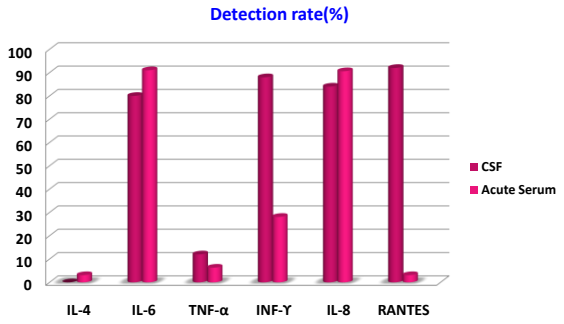
Clinical Profile:

All the participants were presenting with vivid signs and symptoms of AES. The most common presenting symptoms were moderate to high grade fever, irritable and abnormal behavior (100%). Other common clinical features among the completely recovered participants were Altered Sensorium in 87%, Seizure in 95.7%, Signs of meningeal irritation in 60.9%, Glasgow Comma Scale (GCS) ≤ 8 were found in 73.9% and Headache in 43.5%. While recovered with disability group were presenting with Altered Sensorium in 100%, Seizure in 88.9%, Signs of meningeal irritation in 33.3%, Glasgow Comma Scale (GCS) ≤ 8 in 66.7% and Headache in 33.3% (Table 2).

Detection of Cytokines and Chemokines in CSF and Serum:

Out of the six biomolecules tested the detection rate was higher for RANTES (92%), IFN-γ (88%), IL-8 (84%) and IL-6 (80%) in CSF samples. The percentage of patients with undetectable levels of cytokines in CSF was 100% for IL-4 and TNF-α 88% (Figure 1).

Figure 1: Detection of Cytokines and Chemokines in CSF & Serum samples



In acute serum our study recorded higher detection rate (91%) each for IL-6 and IL-8. In contrast, percentages of patients with undetected levels of cytokines and chemokines in acute serum were 93.8% for TNF-α and 71.8% for IFN-γ while 96.8% each for IL-4 and RANTES. There was no significant difference of all the tested biomolecules in CSF and acute serum of patients who were recovered completely

with that of those patients who were recovered with disability (P value >0.05).

Comparisons of cytokine and chemokine levels among JE survivors according to outcome

Cytokine/ Chemokine levels in CSF:

CSF samples of 25 JEV infected patients were tested for Cytokine and Chemokine levels. Of which 17 were recovered completely and 9 were recovered with disability.

The patients who were recovered completely had the greater level (mean ± SD pg/ml) of chemokine IL-8 (596±500) and cytokine IL-6 (408±496.6) compared to patients who were recovered with disability IL-8 (519 ± 645.6) and IL-6 (279.6 ± 430.6).

The levels of other cytokine and chemokines were RANTES (145 ± 254.9), INF-γ (111.4 ± 111.5) in completely recovered group while in recovered with disability group levels were RANTES (183 ± 330.6) and INF-γ (111.5 ± 51.5).

However, all these differences in both the group were statistically insignificant (p>.05).

Conversely, CSF showed significantly higher level of TNF-α in patients recovered completely as compared to those recovered with disability (p= .041). We did not find IL-4 in any of the CSF of survivors (Figure 2).

Figure 2: Comparison of Cytokines and Chemokines in JE survivors according to out come (pg/ml CSF)

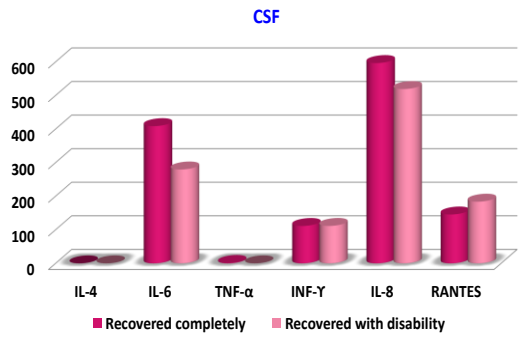
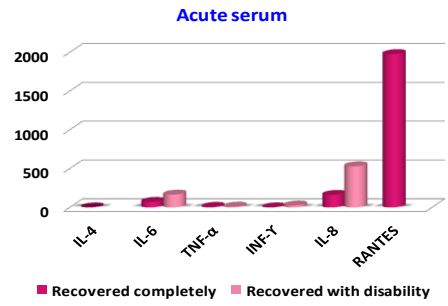


Figure 3: Comparison of Cytokines and Chemokines in JE survivors according to out come (pg/ml Serum)



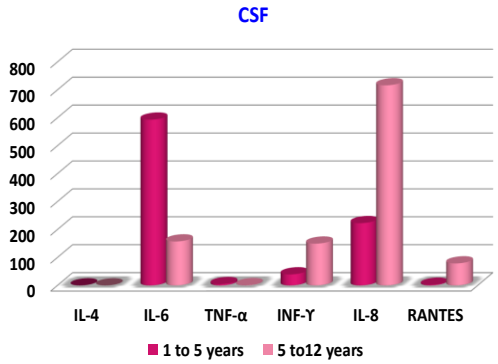
Cytokine / Chemokine levels in acute serum:

We measured chemokines and cytokine levels in 33 acute serums, of which 23 were recovered completely and 9 were recovered with disability.

In acute serum samples, we found significantly lower level of proinflammatory chemokine IL-8 in patients who recovered completely in comparison to recovered with disability group (Figure 3). 161±179.6 vs. 530.5±519.9 (p=.008).

None of the IL-4, IL-6, IFN-γ and RANTES were found significantly different between two patients group in acute serum, (p >.005).

Figure 4: Cytokines and Chemokines levels in JE patients according to age group (pg/ml CSF)

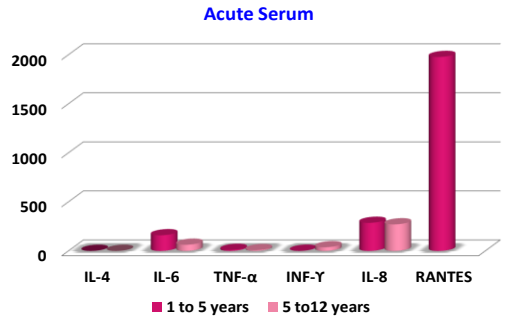


Cytokine /chemokine levels in relation to age group:

In CSF: The levels of all the three cytokines IL-8, INF - γ, TNF-α were found to be higher in 1 to 5 years age group in CSF than 5 to 12 years age group (table-4) except RANTES which was higher in age group 5 to 12 years. However, none of them

were found statistically significant. In contrast a significantly higher level of IL-6 (mean±SD, 593.5±564) was found in 1to 5 years age group as compared to age group 5 to 12 years. We could not confirm presence of IL-4 in any of the CSF samples in any age group (Figure 4).

Figure 5: Chemokine and Cytokines levels in JE patients according to age group (pg/ml serum)

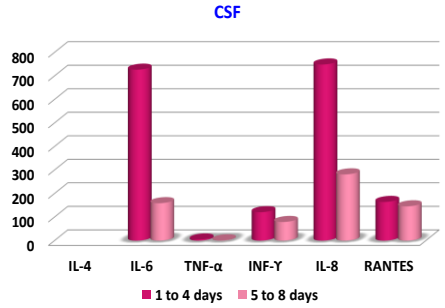


In acute serum: Among the all detected cytokines and chemokines none were found significantly higher according to age group in acute (Figure 5) serum.

Cytokine /chemokine levels according to duration of illness:

The mean levels of all the six cytokines / chemokines were analyzed according to date of collection of CSF from the day of onset of illness (Figure 6). Of the six cytokines studied mean levels of IL-6, TNF-α, INF- γ, IL-8, and RANTES found to be elevated in 1 to 4 days of illness than 5 to 8 days of illness. The mean level of IL-6, TNF-α, INF- γ, IL-8, and RANTES was

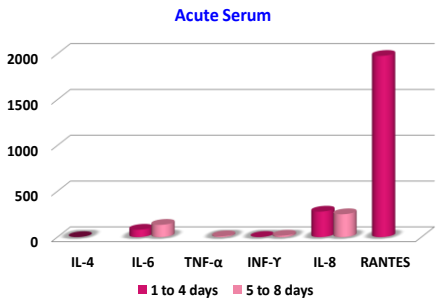
Figure 6: Chemokine and Cytokines levels in JE patients according to duration of illness (pg/ml CSF)



726.7 ± 940.3, 4.7 ± 4.5, 120.9 ± 101.8, 748.8 ± 563.6 and 164.5±257.4 pg/ml respectively in 1to 4 days from onset of illness while the mean level in 5 to8 days of illness was 159.1 ± 203.0, 1.08 ± , 79.3 ± 82.2, 79.3 ± 82.2 and 145.8 ± 338.3 pg/ml respectively. However, our study revealed that these elevation were statistically insignificant (p>.05). We could not confirm level of IL-4 in any of the CSF samples.

Furthermore, while analyzing the mean levels of chemokines and cytokines in acute serum samples none were found significantly elevated or lowered in respect to day of collection from disease onset (Figure 7).

Figure 7: Cytokines and Chemokines levels in JE patients according to duration of illness (pg/ml serum)



DISCUSSION

The present study was conducted to see the immunological response influencing the outcome variables of the JE cases in paediatric age group. We studied the markers of innate, humoral and cellular immune response in regard to JEV infection. It was possible to estimate chemokines/cytokine levels in 25 CSF and 33 acute serum samples of JE positive cases. All JE positive cases were confirmed on detection of JE specific IgM antibody in CSF and/or serum by MAC ELISA. Bio-molecules examined were IL-4, IL-6, TNF-α, INF-γ, IL-8 and RANTES. Of these IL-8 and RANTES were chemokines.

Of the six bio-molecules tested higher detection rate of IL-8 and IL-6 was found in CSF and acute serum. IL-6 and IL-8 are believed to contribute to the antiviral response indirectly by modulating different

aspects of immune response. [9] Similarly, this may be explained that higher detectable rate of IL-6, IL-8 in our study may have role in recovering the patients as none of our study participants died except one.

Earlier studies confirmed that the levels of TNF-α, IL- 8 and INF- γ have been associated with poor outcome. [4-6] We found IL-8 in 84% and IFN- γ in 88% of CSF and the level was IL-8(596±500.0 pg/ml) among completely recovered patients vs 519.645±645.6 pg/ml in recovered with disability patients. Similarly IFN- γ levels were 111.4±111.5 and 111.5±51.5pg/ml in recovered completely and recovered with disability respectively.

We did not find any significant difference of IL-8 and IFN- γ in both the group but in variance to our observation a previous study had reported a difference between the levels of IL-8 in JE patients who died or had prolonged illness and in patients who recovered. [6] Similarly there was significantly elevated IL-8 in CSF of non-survivors compared to survivors. [10] Also in another study reported elevated level of IL-8 in CSF in viral encephalitis compared to controls. [11]

Further, in our study CSF showed significantly higher level of TNF-α in patients who were recovered completely than those who recovered with disability. This observation is in agreement with earlier studies [12] who observed elevated level of TNF-α in encephalitis patients. This may be explained that Th1 type of cytokines IL-8, IFN-γ tends to produce the proinflammatory responses responsible for killing intracellular parasites. Excessive proinflammatory responses can lead to uncontrolled tissue damage. [13] On the other hand excess Th2 type of anti-inflammatory cytokine response will counteract Th1 mediated microbicidal action. The optimum scenario would therefore seem to be that human immune system should produce a well balanced Th1 and Th2 response, suited to the immune challenge. In the present study significantly higher level of pro-inflammatory TNF-α may be optimum in favour of the JE patients who recovered completely without any residual sequlae.

Earlier studies confirm the presence of elevated level of IL-8, IL-6 in serum is associated with poor outcome. [4-6] In conformity to this the present study showed a significantly higher level of IL-8 in acute serum in patient who recovered with disability as compared to the recovered completely group. Similarly an elevated level of IL-6 was found in our study in acute serum samples of severely ill patients compared to completely recovered. Similar findings were also observed in earlier research. [10]

We found elevated level of RANTES in CSF samples in patient who developed disability at the time of discharge than completely recovered group. In our line other researcher also revealed an association of elevated level of plasma RANTES with a fatal outcome. [10] However, on the contrary in meningococcal disease RANTES was found to be protective. [14]

We compared the chemokines and cytokine levels in CSF of JE patients according to age group and found significantly higher level of cytokine IL-6 in 1-5 years age group than the age group of 5-12 years. This may be explained that in endemic region humoral immunity level is low in early childhood. As a compensatory mechanism IL-6 stimulates proliferation and differentiation of B cells and thereby play an important role in antibody mediated immune response which might contribute to the decrease in the viraemia during acute phase of illness. [15]

Of the six cytokines studied, the mean levels of IL-6, TNF-α, IFN- γ, IL-8 and RANTES in CSF showed a higher trend in 1-4 days as compared to 5-8 days of illness. Similar variations were also made by Winter *et al.*, in 2004 for IL-6, IL-8 and IFN- γ in CSF according to day of illness among survivors and non survivors of JE. However, no such trend was observed in serum samples. Cytokines produced by microglia cells and perivascular macrophages as a part of innate immune

response inherent to the CNS before the infiltration of inflammatory cells from periphery. [16] Therefore, this higher trend in CSF samples may be due to innate immune response and not due to adoptive immunity. [10]

Among all the cytokine and chemokines estimated in serum samples INF-γ and RANTES showed a higher trend in acute samples. This results in agreement with findings for CHIKV infection during which INF- γ and RANTES was higher in acute phase of illness. Further RANTES was the lone immune marker which was consistently lower up to 12 weeks of infection in comparison to first acute serum sample of Chikungunya patients. Similarly, for meningococcal diseases, during which RANTES appears to be protective. [17]

In contrast elevated levels of RANTES in plasma were associated with a fatal outcome of JE. [10] It would therefore be imperative to conduct an elaborative study to confirm the role of RANTES in flavivirus infection in relation to disease prognosis and outcome of JE.

CONCLUSION

The immunological findings of our study confirm that both innate and adaptive immune responses are activated in response to JEV infection. Our study further substantiates that *Cytokines and chemokines* play a decisive role in outcome of Japanese encephalitis in children which warrant an in depth investigation in this field.

Competing Interests: None declared.

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Authors' Contribution: GK conceived and designed the study protocol, involved with data and sample collection, data entry, performed the tests, undertook initial analysis, interpreted the data and drafted the manuscript. BRD was involved in sample collection, interpretation of data and developed a portion of the manuscript. PD coordinated the study, finally reviewed the manuscript and provided feedback in refining the content of the manuscript. All authors read and finally approved the final manuscript for submission.

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