



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: www.elsevier.com/locate/apjtm

Document heading doi:

Cytokine levels in patients with chikungunya virus infection

Chintana Chirathaworn^{1*}, Yong Poovorawan², Somrat Lertmaharit³, Norra Wuttirattanakowit⁴¹Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand²Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand³Department of Preventive and Social Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand⁴Narathiwatratchanakharin Hospital, Narathiwat, Thailand

ARTICLE INFO

Article history:

Received 10 October 2012

Received in revised form 15 November 2012

Accepted 15 December 2012

Available online 20 August 2013

Keywords:

Chikungunya virus

Cytokine

Interleukin-6

Granulocyte-macrophage colony-stimulating factor

Monocyte chemotactic protein 1

ABSTRACT

Objective: To investigate cytokine profile in patients with chikungunya virus (CHIKV) infection.**Methods:** Twenty eight pairs of serum samples collected from CHIKV infected patients during the outbreak of chikungunya fever in South Thailand in 2008 were obtained. A multiple cytokine assay for detection of 17 cytokines was performed. **Results:** In the acute stage of CHIKV infection, the patients had significantly higher levels of interleukin-6, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, monocyte chemotactic protein 1 and tumor necrosis factor alpha than the control ($P<0.001$, $P=0.023$, $P=0.015$, $P<0.001$ and $P=0.024$, respectively). When the disease developed to the recovery stage, the patients had significantly lower levels of interleukin-6, granulocyte-macrophage colony-stimulating factor, monocyte chemotactic protein 1 and macrophage inflammatory protein beta than in the acute stage ($P<0.001$).**Conclusions:** This study provides additional information that these cytokines could play roles in pathogenesis of CHIKV infection and could be used as disease biomarkers or drug targets.

1. Introduction

Chikungunya fever (CHIKF) is an acute illness caused by chikungunya virus (CHIKV). CHIKV is a linear single-stranded positive sense RNA virus belonging to the genus Alphavirus of the family Togaviridae. The virus is transmitted by *Aedes aegypti* and *Aedes albopictus*. The disease was first discovered in Tanzania in 1952 and the first case reported in Asia was in Bangkok of Thailand in 1958. CHIKF has disappeared for around 30 years. Recently, outbreak of this infectious disease has emerged in various countries in South-East Asia, the Pacific region and Europe^[1–4]. The recent outbreak in Thailand was in South Thailand in 2008^[5]. Clinical manifestations of patients with CHIKV infection include fever, skin rash and severe

arthralgia. CHIKV infection is usually not fatal but severe polyarthralgia may persist for several weeks or months. Clinical complications like neurological syndrome were reported. Currently, there is no specific anti-viral drug available for treatment of this infectious disease^[6,7].

The mechanism underlying disease pathologies induced by CHIKV infection is still unknown. Since the discovery of CHIKV, most reports on CHIKV infection were about the epidemiology and virus genotyping. There were only a few reports on immune responses and pathogenesis induced by this virus. It has been suggested that persistent arthralgia could be the result of host inflammatory response. Increased interleukin (IL)-1 alpha and IL-6 have been shown to correlate with disease severity^[8]. We have previously shown that IL-18 was increased in CHIKV infected patients^[9]. IL-18 is an interferon (IFN) gamma inducing cytokine. IL-18 could be induced in order to enhance Th1 response in patients. Moreover, we have shown that the level of IL-18 binding protein (IL-18BP), a natural regulator of IL-18, was also increased in CHIKF patients. IFN gamma is produced in response to IL-18, whereas IL-18BP is induced by IFN gamma. IFN gamma provides the negative feedback for IL-18 suppression by inducing IL-18BP production. Imbalance of IL-18 and IL-18BP production could be the underlying

*Corresponding author: Chintana Chirathaworn, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

Tel: 66-2-256-4132

E-mail: chintana.ch@chula.ac.th

Foundation project: This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1155A), Thailand Research Fund (DPG5480002), the Commission on Higher Education, Ministry of Education, and the Center of Excellence in Clinical Virology, Chulalongkorn University, Centenary Academic Development Project.

cause of inflammatory diseases.

In this study, we are interested in further investigating the levels of other cytokines in patients with CHIKV infection. The multiplex cytokine assay for determination of 17 cytokines simultaneously was performed.

2. Materials and methods

2.1. Serum samples

Serum samples were obtained from patients who visited Narathiwatratthanakharin provincial hospital, Narathiwat, Thailand. The patients presented acute febrile illness and blood samples were sent to the Center of Excellence in Clinical Virology, Chulalongkorn Hospital for diagnostic purposes. The remaining sera were preserved at -70°C until further use. Baseline clinical data including the history of present illness, physical examination and complete blood count were collected. Sera from patients with suspected CHIKV infection were confirmed by ELISA for anti-CHIKV IgM and semi-nested RT-PCR for CHIKV RNA as previously reported^[10].

Twenty eight pairs of patient sera positive in both IgM ELISA and semi-nested RT-PCR were included in this study. The acute sera were collected 2–6 d after the onset of fever. The convalescent sera were obtained 5–13 d after the acute sera were obtained. Sera from 20 donors at the National Blood Center, Thai Red Cross Society, Thailand, were included as controls.

2.2. Multiplex cytokine assay

The levels of 17 cytokines, namely, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12, IL-13, IL-17, G-CSF (Granulocyte colony-stimulating factor), GM-CSF (Granulocyte-macrophage colony-stimulating factor), IFN gamma, MCP-1 (Monocyte chemotactic protein 1) (CCL2), MIP-1 beta (Macrophage inflammatory protein 1 beta) (CCL4) and TNF (Tumor necrosis factor) alpha, were determined using Bio-Plex Multiplex cytokine assay kit (BioRad, Hercules, USA) according to the manufacturer's instruction. Briefly, beads coating with antibodies specific to all cytokines were added into a pre-wet filter plate followed by 2-time washing. Standard solutions or samples were added into each well and incubated for 30 min at room temperature. The plate was then washed 3 times before biotin-labeled detecting antibodies were added. After 30 min incubation and another 3-time washing, streptavidin labeled with phycoerythrin was added and the plate was further incubated for 10 min. The plate was then washed and the beads were re-suspended in assay buffer. Results were analyzed by the Bio-Plex System (BioRad, Hercules, USA).

2.3. Statistical analysis

The difference between levels of cytokines in the patients and control groups was analyzed by Mann-Whitney U test.

For the comparison between the levels of cytokines in acute and convalescent groups, Wilcoxon signed-rank test was used.

3. Results

The levels of all 17 cytokines determined in this study are shown in Table 1. The levels of IL-6, G-CSF, GM-CSF, MCP1 and TNF alpha were significantly higher in the acute sera than in the control sera ($P<0.001$, $P=0.023$, $P=0.015$, $P<0.001$ and $P=0.024$, respectively). The levels of IL-6, GM-CSF, MCP-1 and MIP-1 beta were significantly higher in the acute sera than in the convalescent sera ($P<0.001$), suggesting that the levels of these four cytokines declined during the convalescent stage. Comparison of IL-6, GM-CSF and CCL2 levels in the control, acute and convalescent sera are also shown in Figure 1.

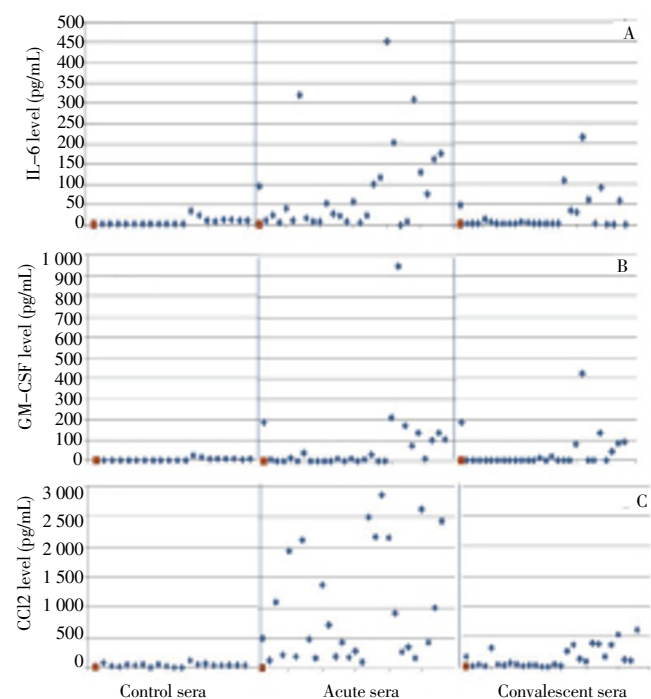


Figure 1. Levels of interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte chemotactic protein 1 (CCL2).

Cytokine levels were determined as described in Materials and methods. Levels of IL-6 (A), GM-CSF (B) and CCL2 (C) in control, Chikungunya acute and convalescent sera are shown as indicated in the figure.

4. Discussion

It has been shown that the increased IL-1 beta and IL-6 and decreased RANTES (Regulated on activation in normal T cell expressed and secreted) levels correlated with disease severity^[8]. We have reported the increase of IL-18 and IL-18BP levels in patients with CHIKV infection which suggests the involvement of Th1 response in this infectious

Table 1

Levels of 17 cytokines in patients with chikungunya virus infection (pg/mL).

Cytokine	Acute sera		Convalescent sera		Control sera		Lowest standard concentration ^a
	Median	Range	Median	Range	Median	Range	
IL-1 beta	1.75	2.00–33.54	1.75	2.00–28.76	1.75	2.00–27.00	2.00
IL-2	0.91	1.00–67.39	0.91	1.00–161.42	0.91	1.00–31.00	1.00
IL-4	0.20	0.20–47.75	0.20	0.20–44.34	4.96	0.20–41.00	0.20
IL-5	2.22	2.00–91.37	2.22	2.00–174.70	2.22	2.00–25.50	2.00
IL-6	41.50 ^{***}	4.54–454.07	3.30 ^{†††}	4.54–214.89	1.95	2.00–34.5	2.00
IL-7	2.19	2.00–175.71	5.58	2.00–489.27	15.00	2.00–42.5	2.00
IL-8	6.20	2.00–1 838.34	9.02	2.00–1 093.60	31.50	2.00–253.50	2.00
IL-10	1.72	2.00–185.09	1.72	2.00–259.72	1.72	2.00–35.00	2.00
IL-12	1.86	2.00–229.33	1.86	2.00–1 602.25	1.86	2.00–29.5	2.00
IL-13	1.51	1.50–107.75	1.51	1.50–333.91	1.51	1.50–24.00	1.50
IL-17	4.47	4.00–43.7	4.47	4.00–56.03	4.47	4.00–40.00	4.00
G-CSF	5.27 [*]	5.00–1 555.13	5.27	5.00–1 266.04	5.27	5.00–38.00	5.00
GM-CSF	12.69 [*]	3.00–950.21	2.93 ^{†††}	3.00–427.07	2.93	3.00–25.00	3.00
IFN gamma	6.09	5.00–3 430.25	6.09	5.00–2 268.07	27.86	5.00–184.72	5.00
MCP-1	490.94 ^{***}	103.18–2 507.88	78.07 ^{†††}	4.44–616.10	44.82	2.00–121.00	2.00
MIP-1 beta	248.48	40.72–2 250.50	150.07 ^{†††}	15.75–514.04	289.66	39.40–104.50	1.00
TNF alpha	4.59 [*]	5.00–3 052.87	4.59	5.00–2 467.70	6.19	5.00–25.14	5.00

The symbols * and *** indicate the levels of cytokines were significantly higher in the patients than in the controls at the level of 0.05 and 0.001, respectively, and the symbol ††† indicates the levels of cytokines were significant lower in the convalescent sera than in the acute sera at the level of 0.001. ^aThe lowest concentration of standard used to prepare standard curve. When the actual cytokine level was lower than the lowest concentration of standard, the data range was expressed using the lowest concentration of standard as the minimum level.

disease[9]. Later, the measurement of cytokine levels in CHIKF patients by multiple cytokine detection assays has been reported. It has been shown that levels of CXCL9 (MIG, monokine induced by IFN gamma), CCL2, IL-6 and CXCL10 (IP10, IFN gamma-induced protein 10) were increased in acute phase patients and levels of CXCL9 and CXCL10 were possible markers for disease severity[11]. Another study demonstrated that IFN alpha and IL-6 were increased in acute phase, whereas IL-17 became detectable in chronic phase. Chaaitanya *et al.* determined levels of 30 cytokines in sera of acute, fully-recovered and chronic CHIKF arthritis patients. They found that the levels of IL-1, IL-6 and IL-10 were elevated in acute patients, whereas the levels of CCL2, IL-6, CXCL8 (IL-8), CCL3 (MIP-1 alpha), and CCL4 (MIP-1 beta) were high in chronic phase[12].

Wauquier *et al.* investigated cytokine levels in patient sera collected on day 0, 1, 2, 3, 5 and 7 after disease onsets. They concluded that the levels of IP10, CCL2, CCL4, CXCL12 (stromal cell-derived factor-1 alpha), MIF (Macrophage migration inhibitory factor), IL-6, CXCL8 (IL-8), IL-16, IL-17, IL-13, G-CSF, GM-CSF, VEGF (Vascular endothelial growth factor), SCGF (Stem cell growth factor)-beta, PDGF (Platelet-derived growth factor) beta beta, IFN gamma, IFN alpha, IL-4, IL-7, and IL-12p40 were higher in patients than in controls[13]. However, the levels of some cytokines fluctuated from day 0 to day 7. For examples, IL-6 level was significantly higher in patients than in controls on day 1, 3, 4 and 7, whereas the level was not significantly higher on day 2, 5 and 6. Only the levels of IP10, CCL2, CXCL12, IL-1Ra, IL-2a, G-CSF, GM-CSF, VEGF, PDGF beta beta, IFN alpha 2, and IL-12 were significantly higher in patients than in

controls at every time point. In addition, the study in mouse model has shown the increases of CCL2, KC (Keratinocyte-derived cytokine), CCL4, RANTES, IL-6, IL-10, CSF (Colony-stimulating factor) 3, IL-2, IFN gamma, IL-17, CCL3, and IL-1 beta during acute myopathy induced by chikungunya virus[14].

In our study, the levels of CXCL8 and IFN gamma seemed to be higher in the CHIKF patients than in the controls. However, when the statistics were applied, only IL-6, G-CSF, GM-CSF, MCP-1 and TNF alpha were significantly higher in the patients than in the controls ($P < 0.05$ or 0.001). It has been shown that the levels of IL-6 and GM-CSF associated with persistent arthralgia[15,16]. We also found that the levels of these two cytokines were higher in the patients than in the controls. However, the levels of IL-6 and GM-CSF were significantly decreased in the convalescent sera. It was unfortunate that we could not perform the follow-up study to investigate whether these patients develop the chronic disease.

It has been shown that CCL2 inhibitor (Bindarit) treatment reduced inflammatory infiltrate in muscle and joints of mice infected with CHIKV. Moreover, mice treated with CCL2 inhibitor showed milder and shorter disease duration than control mice[17]. We have observed the increase of CCL2 in CHIKF patients. In addition, when CCL2 level in sera collected at various times was analyzed, we found that the level of CCL2 was significantly higher in patients than in controls since day 1 after fever onset (data not shown). Our data supported that CCL2 is a promising target for further study as a biomarker or drug target.

In the immune response to infection, Th1 cytokines

promote viral infected cell killing, whereas Th2 cytokines induce antibody response involving in viral neutralization. However, in our study, no significant difference of Th1 and Th2 cytokine levels was found between the patients and controls. It seems that the levels of IL-12 and IFN gamma, the cytokines responsible for Th1 response, were higher in the patient group but the difference was not significant.

IL-17 is a cytokine shown to be involved in pathogenesis of autoimmune arthritis[18]. In our study, the IL-17 level in 27 out of 28 patients was lower than detection limit. It has been shown that IL-17 was increased in symptomatic phase in a mouse model with severe myopathology[14]. However, in a human study, Chow *et al.* demonstrated that IL-17 became detectable during chronic phase. All samples included in our study were collected during day 0–16 after fever. It is a possible explanation why we could not detect the increased IL-17 in the CHIKF patients.

The contradictory results shown in cytokine studies may be due to the difference in number of samples investigated, specimen collection times and disease stages of patients included in each study. Among all the cytokine studies including ours, IL-6 and CCL2 were consistently found to be increased in patients with CHIKV infection. These cytokines are suggested to be biomarkers, cytokines involved in immunopathologies and drug targets. The future follow-up study that includes high number of samples collected on different times after infection and high number of patients in different disease stages will strengthen the roles of cytokines in immunopathologies induced by CHIKV infection.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We would like to express our gratitude to the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1155A); Thailand Research Fund (DPG5480002), the Commission on Higher Education, Ministry of Education; the Center of Excellence in Clinical Virology, Chulalongkorn University; Chulalongkorn University Centenary Academic Development Project; and King Chulalongkorn Memorial Hospital for their generous support.

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