Niger J Paed 2015; 42 (2): 142 –146

ORIGINAL

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Serum Levels of Pro-inflammatory Cytokines in relationship to outcomes in Children with P. falciparum malaria, in Nnewi-South east Nigeria

DOI:http://dx.doi.org/10.4314/njp.v42i2.14

Accepted: 12th February 2015

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Abstract: *Background and Objective:* In *P. falciparum* malaria (PFM) infestation there are marked changes in cytokine production as the body mounts an immune response to it. Hence we set out to study these changes.
Methods: A total of 158 cases of PFM among children attending the paediatric unit of our hospital and 56 healthy controls were studied. Children with febrile illness were screened for malaria using 10% Giemsa stained blood smear. Patients with positive smears were recruited; co-infected patients – those infected by another organism in addition to plasmodium specie.- were excluded. Whole blood was collected, some into plain tubes for serum cytokine testing and some into EDTA bottles for complete blood count and parasite density (PD) determination. Controls with asymptomatic parasitaemia were excluded.
Results: Using the World Health Organization criteria for defining severe malaria; we identified 15 cases of severe and 143 cases of uncomplicated PFM. Significantly elevated levels of interleukin-1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) were seen in the uncomplicated and severe forms of PFM. It was observed that the elevated cytokine values correlated with PD (in uncomplicated PFM but not in the severe forms). The difference between PD/absolute monocyte count (AMC) ratio was not significant (p=0.13); while PD/platelet count (PC) and PC/AMC ratios were significant (p=0.01, and 0.03 respectively) when compared between uncomplicated and severe disease.
Conclusion: Our data seems to suggest that subjects with an adequate immune response to the parasite density, in terms of pro-inflammatory cytokine levels, presented with uncomplicated disease; while those who have an inadequate response presented with severe disease. The ratios of (PD/PC) and (PC/AMC), in the positive and negative directions respectively, may be predictors of increased disease severity. These observations may have implications for predicting disease outcome and PFM therapy.

Key Words: plasmodium falciparum malaria, pro-inflammatory cytokines, Parasite density/Platelet count ratio, Platelet count/Absolute monocyte

Introduction

TNF-α has been implicated in the pathogenesis of malaria fever and appears to play a central role; complemented by the effects of other cytokines such as IL-1 and IL-6[1,2]. Following infection by *P.falciparum,* the pro -inflammatory cytokines such as TNF-α and interferon (IFN)-γ are produced during the early stages of the host immune response[3]. These mediators elicit anti-parasitic activities resulting in inhibition of parasiteamia and stimulation of phagocytosis to enhance parasite clearance[4]. At the later stages of infection, anti-inflammatory cytokines such as IL-10 are produced and down regulates the potentially pathogenic inflammatory responses that are important for controlling parasiteamia.[5] The presence of increased type-1 cytokines, including IFN-γ, IL1, IL-2, IL-12, and TNF-α has been confirmed in infected individuals[3]. Parasite factors such as the density and their ability to infect a high percentage of erythrocyte can correlate positively to the synthesis of inflammatory cytokines, and consequently contribute to the severity of the disease[6]. Other lines of evidence, however seem to suggest that parasite density do not correlate with clinical symptoms and by extension severity[7,8]. Thus this present study involving aged matched Nigerian children with severe and uncomplicated malaria, sought to examine cytokine production and to determine whether differences in serum cytokine levels correlated with varied disease severity; and if these varied manifestation can be predicted by simple laboratory parameters such as parasite density, platelet count and absolute monocyte count

Subjects, materials and methods

Study site and subject enrollment

Serum and sequestrated whole blood were obtained from 210 Nigerian children (3 months to 144 months) attending children outpatient clinic (CHOP), children emergency room (CHER) and hospitalized patients of our hospital in Nnewi, (population,4,177,828 - National Bureau of Statistics,[9]) Anambra State, South- East Nigeria, between March and December, 2011. Nnewi has an intense seasonal transmission -March to December of *Plasmodium falciparum* malaria (PFM). Cases were classified based on criteria described by World Health Organization[10] thus: 143 subjects had uncomplicated malaria, 15 had complicated malaria and 52 subjects without malaria served as the control group.

At enrollment, relevant clinical features including vital signs were assessed and documented by the attending paediatrican. Clinical information obtained was entered into standardized forms. For the patients who were febrile axillary temperature above 37.4^{0}C) a drop of blood through finger prick was obtained to make peripheral blood smears for microscopy – both thick and thin- for diagnosis and speciation respectively. The smear was stained with 10% Giemsia stain for 10 minutes and examined under the microscope immediately (using x100 objective). The number of parasites per 200 white blood cells and the number of parasites per microliter of blood cells determined (parasite density), the presence of malaria pigment (haemozoins) were checked for. A blood slide was considered negative after scanning through 100 fields and no malaria parasite was encountered. Differential WBC and morphological examination of cells were done. The result was communicated immediately to the attending doctor for management of the patient. The patients that have positive smears were recruited as subjects.

Controls were recruited from healthy afebrile clients attending the infant welfare clinic (Immunization Clinic) of the hospital and from apparently healthy clients who came in for routine checkup and medical examination. The control subjects were of the same age brackets as the patients (3 months to 144 months). Smears were also made as in the study group and examined for possible presence of asymptomatic parasiteamia; those with negative smears for *P. falciparum* were enrolled as controls.

Study protocols were reviewed and approved by Nnamdi Azikiwe University Teaching Hospital Ethics Committee. Subjects and controls were recruited following due ethical consent from their parents/guardians.

Further laboratory procedures

For all recruited subjects who have met the clinical and parasitological criteria, and also for the control subjects, 5ml of venous blood sample was withdrawn; 2ml into tubes containing EDTA and 3ml into sterile plain tubes. The sequestrated blood sample was used for full blood count determination which was carried out using Sysmex KX-21N automated haematology analyzer machine manufactured by Sysmex Corporation 1-5 Wakinohama-Kigandori, Chuo-ku, Kobe 651-0073, Japan. The 3ml blood sample collected into the plain tubes was left to stand in a vertical position for 1hour at 25^{0}C and allowed to clot. This was then centrifuged at 1000 x g for 10 minutes. The separated serum was dispensed in four aliquots and stored frozen at –70^{0}C until cytokine testing was performed in less than 3months of collection.

Measurement of circulating cytokines

The levels of TNF-α, IL-I, and IL-6 in the serum were determined using Enzyme linked inmmunosorbent assay, (ELISA) Kit procured from Abcam, Cambridge, United Kingdom, according to manufacturer's recommendations. The choice of this method was informed by its high sensitivity and the fact that micro (100µl) quantities of samples are required. The test kits are sensitive enough to detect cytokines levels less than 0.8pg/ml and cross reactivity has not been observed for any other proteins tested. All quality controls measures as prescribed by the manufacturer were observed.

Statistical analysis

Statistical analysis was performed with SPSS version 10; (SPS Inc., Chicago IL). Descriptive statistics were expressed as means and standard deviations while the student's t- test and Chi square analysis were used for testing differences in cytokines levels between clinical groups. Comparison of intra group differences was done using a post-hoc Bonferoni multiple comparative analysis while associations were tested using the Pearson's linear regression for bivariate correlation. The level of statistical difference was set at $p < 0.05$.

Results

A total number of five hundred and fifty-three (553) febrile children presenting at peadiatrics unit of our hospital were screened for possible presence of malaria parasiteamia according to the study protocol between the months of March and December 2011. One hundred and fifty-eight (158) patients tested positive for *P. falciparum* by microscopic examination of Giemsa stained blood slide, giving a prevalence of 35% (158/553). Five (5) patients had mixed infection of *P. falciparum* and *P. malariae* species (5/553) placing a prevalence of 3% for *P. malariae* species. No other species were seen. Using the criteria for severe malaria,[8] (anemia with Hb level 5g/dL, acute respiratory distress, renal failure, prostration, shock, abnormal bleeding and/or disseminated intravascular coagulation, (parasitaemia >2,000/µl of blood, repeated generalized seizures. 15 cases were identified as severe malaria – 5 with anemia (as described above), 2 with acute respiratory distress, 3 with generalized seizures and 5 with high parasitaemia (as described above). The mean duration of symptom before presentation was 3 days in this category and 2.2 days in the uncomplicated group. The mean ages for the various

groups – control, uncomplicated and complicated malaria – were 29.5 ± 39.28, 33.9 ± 34.37 and 40.9 ± 25.77; the difference between them were not statistically significant (p= 0.51). Table 1 shows the hematological parameters of the subjects studied according to their categories. A post-hoc multiple comparative analyses revealed that the control group had significantly (P<0.01 or) lower WBC but higher haemoglobin concentration (Hb) and haematocrit compared to the uncomplicated and complicated malaria groups respectively; but did not indicate any significant differences when percentages of neutrophil values were compared between groups.

Table 2 shows a statistically significant relationship in the means of serum pro-inflammatory cytokines in control, uncomplicated and complicated malaria groups of subjects.

Table 1: Haematological parameters of children with complicated and uncomplicated malaria compared with healthy control group.

Variables	Control Group (N = 52)	Uncomplicated Malaria Group (N = 143)	Complicated Malaria Group (N = 15)	F-Stat	P Value
Age (months)	29.5 ± 39.28	33.9 ± 34.37	40.9 ± 25.77	0.67	0.51
WBC (x109 /L)	4.8 ± 1.29	9.2 ± 5.02	13.2 ± 12.22	20.03	0.000*
RBC (x1012 /L)	4.8 ± 0.52	4.8 ± 4.20	3.0 ± 1.12	1.78	0.17
Haemoglobin(g/dl)	11.8 ± 1.23	10.4 ± 1.92	5.9 ± 3.63	51.71	0.000*
Haematocrit (l/l)	35.4 ± 3.65	32.6 ± 5.36	18.2 ± 9.58	59.52	0.000*
Platelet (x109 /L)	267.9±84.55	278.4 ± 142.9	125.7±54.01	9.92	0.000*
PCT (%)	21.0 ± 10.07	14.3 ± 14.54	3.6 ± 5.17	27.13	0.000*
MCV (fl)	74.1 ± 4.80	72.5 ± 10.03	58.2 ± 14.53	17.43	0.000*
MCH (pg)	24.6 ± 1.71	24.6 ± 16.34	19.8 ± 5.99	0.85	0.43
MCHC (g/l)	33.1 ± 1.53	32.2 ± 5.10	33.6 ± 2.75	1.38	0.25
RDW	16.6 ± 5.99	31.7 ± 23.67	19.8 ± 5.99	11.87	0.000*
MPV	8.5 ± 0.79	9.3 ± 1.23	8.3 ± 0.94	10.48	0.000*
PDW	11.3 ± 1.79	12.3 ± 3.75	11.2 ± 2.40	1.89	0.15
% Lymphocytes	54.9 ± 24.61	42.9 ± 16.51	48.1 ± 15.29	7.91	0.000*
% Monocytes	9.0 ± 3.10	11.2 ± 4.69	7.1 ± 5.06	8.57	0.000*
% Neutrophils	38.9 ± 10.72	45.7 ± 19.80	36.8 ± 22.69	3.59	0.029*
Parasite density (cells/ul)		4096.4±169.54	55008.2±941.12	-14.42	0.000*

Data is expressed as mean ± standard deviation. * Significant difference (P<0.05 or P<0.001). Abbreviations: WBC = White blood cell; RBC = Red blood cell; PCT = Plateletcrit; MCV = Mean cell volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; RDW = Red cell distribution width; MPV = Mean platelet volume; PDW = Platelet distribution width.

Table 2: Mean serum levels of the pro-inflammatory cytokines in control, uncomplicated malaria and complicated malaria groups of subjects.

Variables (Pg/Ml)	Control group (N = 52)	Uncomplicated malaria group (N = 143)	Complicated malaria group (N =15)	P Value
IL-1	45.6±37.04	177.9 ± 316.31	315.8± 233.71	0.001*
IL-6	48.0±35.27	492.3 ± 596.84	1275.3±605.37	0.000*
TNF-α	48.9±58.98	132.2 ± 229.42	369.0 ± 453.45	0.000*

Data is expressed as mean ± standard deviation. * Significant difference (P<0.001). Abbreviations: IL-1 = Interleukin - 1; IL-6 = Interleukin - 6; TNF-α = Tumor Necrosis Factor - Alpha.

Bonferroni comparison test showed statistically lower levels of IL-6 in controls compared to subjects with complicated and uncomplicated malaria, as shown in Table 3. The levels of TNF-α were statistically lower in controls compared to subjects with complicated malaria and similarly lower for subjects with uncomplicated malaria compared to those with complicated malaria, as shown in Table 3. The levels of IL-1 were statistically lower in controls compared to subjects with complicated malaria, but not in the other subjects as shown in Table 3. Pearson's linear regression test indicated significant positive correlations between parasite density level and all cytokines studied in the combined 'all' (test subjects) data and the uncomplicated malaria group as shown in Table 4. In the complicated malaria group, no significant correlations were observed between parasite density and all the cytokines (P>0.05). The difference between PD/absolute monocyte count (AMC) ratio, was not significant (p=0.13); while PD/platelet count (PC) and PC/AMC ratios were significant (p=0.01, and 0.03 respectively) when compared between uncomplicated and severe disease.

Table 3: Bonferonni Post-Hoc multiple comparison test

Variables	Control verses Uncomplicated P - value	Control verses complicated P-value	Uncomplicated verses Complicated P-value
IL1	0.008	0.002	0.183
IL6	0.000	0.000	0.000
TNF-α	0.071*	0.000	0.000

Abbreviations: IL-1 = Interleukin - 1; IL-6 = Interleukin - 6; TNF-α = Tumor Necrosis Factor - Alpha.* Non significant difference (P> 0.05)

Table 4: Bivariate correlation between parasite density level and the pro-inflammatory cytokines in study subjects

Variables	All test subjects		Uncomplicated malaria Group		Complicated malaria Group	
	Coeffi-cient	P	Coeffi-cient	P	Coeffi-cient	P
Parasite Density vs IL-1	0.161	0.043	0.169	0.043	0.077	0.784
Parasite Density vs IL-6	0.433	0.000	0.308	0.000	0.441	0.100
Parasite Density vs. TNF-α	0.170	0.033	0.268	0.001	-0.345	0.207

Discussion

P. falciparium malaria is characterized by marked changes in cytokine production arising from immune responses to infection[1,2]. Malaria outcomes vary from mild to severe disease[11]. This study documented elevated serum levels of pro-inflammatory cytokines in malaria infection. This study also found distinct differences in cytokine production correlating with disease severity. Our results seem to suggest that individuals who had an adequate immune response to the parasite density they were infested with, in terms of proportionate cytokine response, presented with uncomplicated disease; while those who has an inadequate response presented with severe disease. Thus the clinical category of severity of malaria may be an indication of the degree of cytokine response to the infection. This observation shows that indeed the pathology observed in malaria is not directly as a result of the activities of the invading organism but the response of the individual to it[12]. Excess production of TNF-α may be responsible for the clinical and pathology (such as fever) seen in malaria. Previous studies had shown IL-6 as an important pro-inflammatory cytokine that is unregulated by TNF-α and acts in concert with other inflammatory mediators to control parasitaemia[13].

This study found that both IL-6 and IL-1 appear to correlate with disease severity since elevated levels were noted in the severe malaria patients compared to the matched uncomplicated cases, as well as healthy controls. This observation agrees with previous studies[11,14].

We also observed that elevation in TNF-α level is associated with high density of *P. falciparium* infection, suggesting a correlation with level of parasiteamia. This finding could be responsible for the statistical difference observed in the mean TNF-α between the severe and control groups. Chotivanich and co-workers had in their previous study demonstrated that parasite factors such as the parasite density and their ability to infect high percentage of erythrocytes correlate positively with the rate of synthesis of inflammatory cytokines and disease severity[6] and consequently result in fatal outcome[11,12,14]. Other lines of evidence have demonstrated that increasing cytokine concentrations were coincident with rise in asexual parasiteamia, suggesting also that there is a causal relationship between onset of blood stage infection, initiation of the immune response and subsequent parasite growth[15]. However, Walther and colleagues[15], also noted that expression of these pro-inflammatory cytokines that brings about parasite control on the parasite proliferation comes at the cost of developing clinical symptoms, suggesting that the initial innate response may have far reaching consequences on disease out come. This highlights the question of balance proposed by a number of scholars; Kresmer *et al.*[16], Perkins *et al.*[17], Tiago *et. al*[18]. Although this study did not determine the levels of type 2 cytokines, it is evident that the pathological alterations and outcome of the infection depend on the reciprocal regulation of type 1 and type 2. Type 1 cytokines predominantly mediate cellular immune response as a result play important roles in delayed-type hypersensitivity and in infections by intracellular pathogens (such as malaria). These include interleukin-1 (IL-I), gamma interferon, interleukin-12 (IL-I2), and tumor necrosis factor-β (TNF-β)[19]. Type 2 cytokines are important in humoral immune response and infections that involve the development of hypergammaglobulinaemia, increased immunoglobulin E and eosinophilia, they include interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-I0), interleukin-13 (IL-I3)[19]. Both of these cytokine types are produced by a number of cell types including CD4+ (T helper-1 and 2), CD8+ , Natural killer (NK), T and B cells[20]. Malaria infection has been reported to tilt the balance towards the production of more type 1 cytokines[20]. More so, marked imbalance between these two kinds of cytokines has been variously associated with differences in severity of the infection[14,15]. Therefore in severe, acute infections such as malaria, the ability to mount an effective innate response may have implication on survival as our data set demonstrated that the cytokine response of those with severe outcome compared to their parasite density seem to have been blunted. The significant difference between PD/PC and PC/AMC ratios in uncomplicated and severe disease seems to suggest that these ratios used in combination with other clinical methods, may be useful in predicting disease outcome. More work, however needs to be done, using larger data sets, to establish this.

Conclusion

Subjects with an adequate immune response to the parasite density, in terms of proportionate increase in pro-inflammatory cytokine levels, presented with uncomplicated disease; while those who have an inadequate response - in terms of disproportionate increase in pro-inflammatory cytokine levels, presented with severe disease. This observation makes immunotherapy an option for the treatment of malaria. More so, disease severity, may be predicted by ratios such as PD/PC and PC/AMC in combination with other clinical methods. More work, which should include anti-inflammatory cytokines, need to be done to corroborate this finding.

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