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Measurement of some Th1, Th2 cytokines and white cell count in childhood haemoglobinopathies with uncomplicated malaria infection

Medição de alguns Th1, citocinas Th2 e contagem de células brancas em hemoglobinopatias de crianças com infecção da malária não-complicada

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Abstract
The relative balance between Th1 and Th2 cytokines appears crucial in the outcome of infections. We assayed some Th1 cytokines (interleukin-2 (IL-2) and gamma interferon (IFN-γ) and Th2 cytokines (IL-4 and IL-10) in homozygous haemoglobin (Hb) AA, heterozygous HbAS genotyped and sickle cell (HbSS) individuals with uncomplicated *P. falciparum* malaria to determine the group with the strongest cytokine response to *P. falciparum* malaria. Levels of Th1 and Th2 cytokines of 111 children with uncomplicated malaria and 89 healthy controls were determined by Enzyme Linked Immunosorbent Assay and haematological parameters were estimated using standard haematological techniques. Th1 and Th2 cytokine levels were significantly higher in HbAA, HbAS genotyped patients compared to their respective healthy controls (P<0.05). IFN ã, IL-2 and and IL-10 were significantly elevated in HbAA compared to HbAS and HbSS subjects (P<0.05). The mean haematological parameters (total white cell count, monocyte) of HbSS infected children were significantly higher compared to that of HbAA and HbAS subjects (P<0.05), however, their mean packed cell volume was significantly lower compared to others (P<0.05). Even though the role of cytokines in immune response is yet to be fully understood, these findings may suggest a stronger cytokine response in HbAA than HbAS and HbSS individuals when infected with malaria.
Keywords: Cytokines – Childhood haemoglobinopathies - Uncomplicated malaria.

Resumo
O relativo equilíbrio entre as citocinas Th1 e Th2 parece crucial para o resultado das infecções. Analisamos algumas citocinas Th1 (IL-2 (IL-2) e interferon-gama (IFN-γ) e citocinas Th2 (IL-4 e IL-10) da hemoglobina homozigoto (Hb) AA, HbAS heterozigotos genotipados e células falciformes (HbSS) em indivíduos) com malária não-complicada por P. falciparum, para determinar o grupo com a resposta mais forte de citocinas para malária por P. falciparum. Os níveis de citocinas Th1 e Th2 de 111 em crianças com malária não-complicada e 89 em controles saudáveis foram determinados por método imunoenzimático, e os parâmetros hematológicos foram estimados através de técnicas hematológicas. Os níveis de citocinas Th1 e Th2 foram significativamente maiores em pacientes HbAA e HbAS genotipados, em relação aos respectivos controles (P <0,05) . IFN-ã, IL-2 e IL-10 foram significativamente elevados em comparação com HbAA HbAS e HbSS indivíduos (P <0,05). A média dos parâmetros hematológicos (contagem total de leucócitos, monócitos) das crianças infectadas HbSS foi significativamente maior, se comparada à de HbAA e HbAS indivíduos (P <0,05), porém o volume corpuscular médio embaladas foi significativamente menor,se comparado aos outros (P <0,05). Mesmo que o papel das citocinas na resposta imune ainda esteja para ser totalmente compreendido, esses resultados podem sugerir uma resposta mais forte de citocinas em indivíduos que HbAA HbAS e HbSS, quando infectados com malária.
Palavras-chave: Citocinas – Hemoglobinopatias – Crianças – Malária não complicada.

INTRODUCTION

After the first description of sickle cell disease in a Grenadian dental student in Chicago in 1910, subsequent reports indicated that the disease was confined to people of African origin. Over the next 40 years, the clinical features were recorded in Americans, but it was not until the late 1940s that reports began to appear from Africa itself. These documented the high prevalence of the sickle cell trait throughout equatorial Africa and its geographical coincidence with the distribution of *Plasmodium falciparum* malaria. Studies from west, east, and central Africa showed that individuals with the sickle cell trait had a relative protection against malaria during a critical period of early childhood.[1]

A number of factors likely are involved and contribute in varying degrees to the defense against malaria in these individuals. Red cells from people with sickle trait do not sickle to any significant degree at normal venous oxygen tension. Very low oxygen tensions will cause the cells to sickle[2]. In malarious areas, the high frequency

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of haemoglobinopathies, such as sickle cell disease (SCD), support their protective role against *Plasmodium falciparum* malaria.[3,4,5]. However, in patients homozygous for sickle hemoglobin (SS), the persistence of unrecognizable *P falciparum* infection could trigger acute hemolytic events. [6]

Reports in the literature suggest a central role for inflammation in the disease process of sickle cell patients. Elevated basal leukocyte counts[7] are typical, including activated monocytes[8] and sickle cell children with the highest white blood cell counts are more likely to develop disease complications such as frequent pain and stroke.[9]

However, whether cytokines are involved as mediators of the inflammation of HbSS remains inconclusive, as there are only a few reports on the topic. Some investigators report elevated plasma levels of certain proinflammatory cytokines, for example, tumor necrosis factor-α(TNF-α)[10,11], supporting a role for cytokine driven inflammation. Others report normal levels of some of the same proinflammatory cytokines[12,13,14] and reduced levels of others such as interferon-γ(IFN-γ)[15]. However, some studies report high circulating levels of anti-inflammatory cytokines in the asymptomatic state[15]. Haemoglobin genotype is believed to contribute to malaria resistance alongside several other factors. Some cytokines have been reported to have antiplasmidial activities, in this study, we investigate some Th1 and Th2 cytokine response of HbAA, AS SS individuals which may affect malaria infection out come

MATERIALS AND METHODS

Study Design

A total of 111 venous blood samples from children with *Plasmodium falciparum* slide positive between the age of 1 and 5 years (37HbAA, 39 HbAS and 35 HbSS) who presented with symptoms of uncomplicated malaria (i.e. temperature below or equal to 39^0C, bitterness of mouth, joint pains, and lack of nervous system involvement) as well as 89 healthy controls (asymptomatic *Plasmodium sp* slide negative) (26 HbAA, 29 HbAS and 31 HbSS haemoglobin genotyped individuals) were collected between the months of March and June 2008 in Medical Centers in the Benin metropolis (Lahor Medical Centre, Suyi Hospital and Milestone Hospital) for this study. Benin City is a cosmopolitan town located in the southern region of Nigeria where malaria infection is endemic. Informed consent was obtained from the patients before blood samples were collected for analysis.

Malaria Parasites Screening

The 111 blood samples from symptomatic subjects and 89 from asymptomatic control subjects were used for the study. Thick blood films were made on a clean glass slide and stained with Giemsa stain for 15 minutes. The slides were air–dried and examined under X100 objective microscope lens for characteristic features of malaria parasites.[16]

Haemoglobin Genotyping

An aliquot of washed blood cells was transferred into a clean test tube and lyzed by adding few drops of water. Each sample was spotted on a cellulose acetate paper along side with control with an applicator stick. Then the cellulose acetate paper was then placed in an electrophoretic tank. Electrophoresis was run at 60 volt for 10 minutes. The haemoglobin types were determined by distance comparing with standard.[17]

Cytokine assay

Serum samples from *Plasmodium falciparum* slide positive and negative (control) were used for cytokines assay. IFN γ, IL-2, IL-4 and IL-10 cytokines levels were assayed using Enzyme Linked Immunosorbent Assays (ELISA or EIA)[18] reagents from MABTECH Inc. Company, Sweden.

Haematological Parameters

The haematological parameters of the subjects and controls (differential white blood cells, total white blood cells and packed cell volume) were assessed using the Swelab Automatic Counter (Boule Medical Stockholm, Sweden).

Statistical Analysis

Data are expressed as means and standard deviations. Statistical analysis was performed using Kruskal-Wallis-test (one-way ANOVA). *P* values are considered significant when *P* is <0.05.

RESULTS

Levels of Th1 cytokines, IL-2 and IFN-, and Th2 cytokines, IL-4 and IL-10, in serum were tested in 111 children with uncomplicated malaria and were compared with those detected in healthy children controls. IFN-γ, IL-2, IL-4 and IL-10 cytokine levels were significantly higher in HbAA, HbAS genotyped patients than values obtained in their respective healthy controls (P<0.05). IFN-γ, IL-2, and IL-10 levels were significantly elevated in HbAA than levels in HbAS and HbSS subjects (P<0.05) (TABLE 1).

The mean haematological parameters (total white cell count, monocyte) in HbSS infected children were significantly higher than values in HbAA and HbAS subjects (P<0.05), however, their mean packed cell volume was significantly lower than others (P<0.05) (TABLE 2).

DISCUSSION

P. falciparum malaria is responsible for over 1million deaths each year, mostly in children under the age of 5 living in sub-sahara Africa. The pathogenesis of severe

Table 1 - A comparison of the mean±SD Th1 and Th2 cytokine levels between children presenting with uncomplicated malaria symptoms and healthy control children (grouped by genotype).

		HbAA	HbAA CONTROL	HbAS	HbAS CONTROL	HbSS	HbSS CONTROL
Th1	IFN-γ (pg/ml)	65.21±17.79[a]	11.07±4.31	39.66±94.93[b]	15.27±7.38	26.38±64.45[b]	10.07±5.28
	IL2(pg/ml)	118.85±43.57[a]	9.61±7.11	69.06±46.40[b]	17.61±2.35	79.74±79.00[b]	11.49±7.08
Th2	IL4(pg/ml)	10.06±5.24[a]	4.30±2.68	19.59±3.22[b]	2.03±3.57	5.62±25.50[c]	5.48±1.42
	IL10(pg/ml)	18.34±5.07	3.73±4.72	19.23±4.74[b]	4.59±6.50	11.11±1.92[c]	5.61±3.57

Note: [a,b,c] show value with significant differences (P<0.05)

Table 2 - A comparison of the mean ±SD haematological parameters between subjects and controls.

	HbAA	HbAA CONTROL	HbAS	HbAS CONTROL	HbSS	HbSS CONTROL
TWBC(cells/mm^3)	8159.24±877.60	5570.89±9670	9714.56±867.09	7313.87±545.4	21226.54±345.56	9750.0±997.6
NEUT (%)	36.44±6.57	47.77±18.52	48.79±16.74	42.68±8.49	31.00±25.08	47.40±7.69
LYMPH(%)	54.55±7.68	49.54±61.10	42.71±16.92	52.68±9.48	61.37±43.44	45.84±6.00
MONO(%)	8.16±2.03	3.67±2.68	8.57±1.60	3.45±2.59	8.70±3.76	4.40±1.12
EOS(%)	0.42±1.04	1.52±1.68	1.73±1.17	1.35±1.40	1.43±1.43	1.10±0.11
PCV(%)	34.42±3.01	43.48±5.65	34.64±18.06	37.69±7.62	18.30±13.00	30.81±0.68

Note : TWBC = Total White Blood Cells; NEUT = Neutrophils; LYMPH = Lymphocytes; MONO = Monocytes; EOS = Eosinophils; PCV= Packed Cell volume.

malaria is not fully understood[19]. As highlighted by Long and others[20], cytokines play an extremely significant role in both the prevention and exacerbation of severe diseases. There is ample evidence that higher levels of inflammatory cytokines are associated with a higher likelihood of fatal cerebral malaria.

Our study revealed that IFN-γ, IL-2, IL-4 and IL-10 cytokine levels were significantly higher in HbAA, HbAS and HbSS genotyped patients than their respective healthy controls (P<0.05). Further more, Th1 and Th2 cytokines considered in this study were significantly elevated in HbAA than in HbAS and HbSS subjects (P<0.05). Higher levels of the cell mediators in the HbAA individuals depict their immunocompetence. However, clinical malaria infection is common in these individuals than in the heterozygote HbAS whereas in patients homozygous for sickle hemoglobin (SS), *P. falciparum* infection could trigger acute hemolytic[6] events. The elevated cytokine levels in HbAA subjects could be as a result of higher parasitaemia that is usually obtained in HbAA individuals.[16] Nevertheless, the outcome of malaria infection may not rest soly on cytokine levels, several other factors have been identified and are found to proffer resistance to malaria. Sickle cell trait (genotype HbAS) confers a high degree of resistance to severe and complicated malaria [21]. To some extent it almost certainly relates to the peculiar physical or biochemical properties of HbAS red blood cells: invasion, growth, and development of *Plasmodium falciparum* parasites are all reduced in such cells under physiological conditions in vitro and parasite-infected HbAS red blood cells also tend to sickle a process that may result in their premature destruction by the spleen[22]. In as much as these cytokines play an important role in the prevention of malaria, their pathologic role is being serious investigated by researchers. Higher levels of some pro-inflammatory cytokines in Hb AA, HbAS and HbSS infected children against their respective control confirm the ability of malaria antigen to ignite an immune response, however, the outcome of this response is determined by the activities of the anti-inflammatory Th2 cytokines. Several reports have it that an unbalance activities of pro and anti-inflammatory cytokines accounts immensely in the severity of the infection. Both type 1 and type 2 cytokines are both required for adequate protection, likely encompassing different mechanisms finely tuned in time and intensity. Type 1 cytokines are important in controlling early parasitemia, although they need to be counterbalanced later in the infection by a type 2 response which leads to antibody production. Pathogenesis of malaria is a complex process in which a common outcome might be reached by different routes. For example, various proinflammatory cytokines that clearly play a role in CM may be redundant, making it difficult to unequivocally assign to them a pathogenic role in all clinical situations. although animal models, most notably knockout mice, have been paramount to our understanding of the role of cytokines in malaria by providing much valuable information, it is still controversial whether they can reproduce all of the features of human malaria.[23]

The haematological indices of our subjects revealed that total white cells and monocyte count of HbSS was significantly elevated. Proliferation of these cells is coordinated by cytokines, strikingly; individuals in this group had lower levels of Th1 and Th2 cytokines[8]. These findings are in line with that of West and others [7] who reported that elevated basal leukocyte counts are typical, including activated monocytes [8] and HbSS sickle cell children with the highest white blood cell counts

are more likely to develop disease complications such as frequent pain and stroke.[9]

CONCLUSION

In conclusion, the role of cytokines in malaria infection is cannot be overlooked. Although our findings suggest that HbAA individuals may present with a stronger pro and anti-iflammatory cytokine response in malaria infection as against the HbAS and HbSS individuals, they are however more predisposed to severe malaria.

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