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Soluble products of inflammatory reactions are not induced in children with asymptomatic *Plasmodium falciparum* infections

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SUMMARY

A proportion of children with *Plasmodium falciparum* infection have a high parasitaemia without accompanying fever, indicative of different clinical thresholds of parasitaemia. Higher levels of IL-10, IL-1Ra and sIL-4R but not sIL-2R were found in children with *P. falciparum* malaria, compared with levels in children with asymptomatic *P. falciparum* infections and in healthy children. Concentrations of IL-10 and IL-1Ra were correlated with levels of parasitaemia, but the association of cytokine levels with disease was independent of the association with parasitaemia. Children may tolerate a high parasitaemia by neutralizing the parasite-derived toxins. When studying potential anti-toxic molecules we found that children with symptomatic infections had lower concentrations of a phospholipid-binding molecule, β_2-glycoprotein I (β_2-GPI), compared with children with asymptomatic infections or healthy children. In conclusion, cytokines were found in much higher concentrations in children with symptomatic *P. falciparum* malaria than in children with asymptomatic infections, whilst the former had lower concentrations of β_2-GPI.

Keywords *Plasmodium falciparum* malaria asymptomatic infections cytokines β_2-glycoprotein I

INTRODUCTION

Asymptomatic *Plasmodium falciparum* infections are common in African children [1,2]. The risk of developing clinical symptoms normally increases with increasing levels of parasitaemia, but a number of African children carry a high level of parasitaemia but remain without symptoms. It is presently unknown whether these children, if not treated, would develop symptoms within the coming hours or days after examination, or whether they would assume a low level of asymptomatic parasitaemia lasting for weeks or months, possibly a kind of anti-disease immunity [3]. If these children have some degree of anti-disease immunity this might arise from the development of neutralizing antibodies to the malaria 'toxins', believed to be released at schizogony and which stimulate cytokine production by host mononuclear cells [4,5]. Alternatively, children who remain non-febrile despite a high level of parasitaemia may have developed resistance to the cytokine-inducing action of the malaria toxins or to the effects of the cytokines.

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To investigate these possibilities we have measured β_2-glycoprotein I (β_2-GPI), antiphospholipid antibody and cytokine levels in Gambian children with *P. falciparum* malaria, asymptomatic *P. falciparum* infections or without demonstrable *P. falciparum* infections.

MATERIALS AND METHODS

Donors and blood sampling

The study was carried out between October 1993 and May 1994 in a rural area near to the town of Farafenni, The Gambia. Parents or guardians gave informed consent for the participation of their children in the study, which was approved by The Gambia Government/Medical Research Council Ethical Committee.

Three donor groups were defined by their clinical status at the time of blood collection, which took place during the rainy season: (i) children with symptomatic *P. falciparum* infections. These children had an axillary temperature >37·5°C, *P. falciparum* parasitaemia and no other obvious causes for their fever. Some of these children had an additional blood sample collected during the dry season in May 1994; none of the children had fever at this time; (ii) children with asymptomatic *P. falciparum*

Table 1. Donor characteristics of (i) children with symptomatic *Plasmodium falciparum* infections, (ii) children with asymptomatic *P. falciparum* infections, and (iii) healthy children

Group	(i) Children with symptomatic infections		(ii) Children with asymptomatic infections		(iii) Healthy children	
No.		33		41		29
Age (years)	Mean	3·4	Mean	3·3	Mean	3·2
	s.d.	2·0	s.d.	1·3	s.d.	1·0
Parasitaemia (no./μl)	Median	35·000	Median	7·000	–	
	25%	15·000	25%	1·500		
	75%	90·000	75%	18·125		
Axillary temperature (°C)	Mean	39·3	Mean	36·6	Mean	36·7
	s.d.	0·9	s.d.	0·3	s.d.	0·4
Haematocrit	Mean	29·0	Mean	27·6	Mean	32·1
	s.d.	5·5	s.d.	4·6	s.d.	3·7

infections. These children had *P. falciparum* parasitaemia, an axillary temperature of <37·5°C, and were well. Some of these children also had an additional blood sample collected during the dry season in May 1994; (iii) healthy children without fever and without demonstrable parasites in their peripheral blood using blood film diagnosis.

A total of 103 children were enrolled in the study (Table 1). Thirty-three children (18 females and 15 males) had symptomatic malaria, 41 children (16 females and 25 males) had asymptomatic *P. falciparum* infections, and 29 were healthy children (13 females and 16 males).

Children with malaria or with asymptomatic *P. falciparum* infections were treated with chloroquine.

Thick blood smears were stained with Fields stain and thin blood smears were stained with Giemsa. Parasite density was calculated per 100 high-power fields as described previously [6]. Serum samples were frozen and kept frozen at −20°C for 1–2 months in The Gambia. The samples were then transported on dry ice to Denmark and stored at −70°C until they were analysed.

Determination of cytokines and soluble cytokine receptors

ELISA kits were used as specified by the manufacturers to measure IL-10 (Endogen, Cambridge, MA) and sIL-2R (T Cell Sciences, Cambridge, MA). Assay sensitivities were 15 pg/ml IL-10 and 50 U/ml sIL-2R.

IL-1Ra and sIL-4R levels were measured by ELISA as previously described [7,8]. Assay sensitivities were 30 pg/ml.

Determination of β_2-GPI

The concentrations of β_2-GPI were determined by rocket immunoelectrophoresis as described previously [9].

Determination of anti-phospholipid antibodies

Levels of anti-phosphatidylinositol (PI) IgG and IgM antibodies were measured by an indirect ELISA as described previously [7,10]. Two different procedures were used—procedures A and B. Fetal calf serum (FCS) was used in procedure A. It contains β_2-GPI, and antibodies may react with a phospholipid–β_2-GPI complex. Procedure B employs casein rather than FCS. Antibodies are more likely to react with free phospholipids in this system than in procedure A.

Laboratory standards including sera with high antibody titres against PI were used, and control wells without serum (background value) were included. To account for day-to-day variations, results were expressed in ELISA units (EU) calculated as $100 \times ((OD_{sample} - OD_{background})/(OD_{positive\ control} - OD_{background}))$.

Statistical analysis

The Mann–Whitney rank sum test was used for intergroup comparisons of concentrations of cytokine, cytokine receptor and β_2-GPI because of the skewed data distributions (Kolmogorov–Smironov test). Five markers were investigated and P values <0·01 were therefore considered significant.

Student's t-test was used for intergroup comparisons of anti-phospholipid antibody measurements. Four different assays were analysed and P values <0·01 were therefore considered significant.

The levels of IL-10, IL-1Ra and β_2-GPI in children with symptomatic or asymptomatic infections were compared after correction for levels of parasitaemia using analysis of covariance following logarithmic transformation of all variables.

The Spearman rank order correlation coefficient (r) was used for evaluation of parameter associations. P values <0·005 were considered significant. All calculations were performed using SigmaStat (Jandel Scientific, San Rafael, CA) or SAS (SAS Institute, Gary, NC) software.

RESULTS

Characteristics of the donor groups

In the three study groups the mean age was similar. Parasite density was significantly higher in children with symptoms than in those with asymptomatic infections ($P < 0{\cdot}001$). Levels of parasitaemia were correlated with axillary temperature ($r = 0{\cdot}505$, $P < 0{\cdot}001$).

Cytokine levels in children with symptomatic or asymptomatic P. falciparum *infections*

The serum concentrations of IL-10 (Fig. 1), IL-1Ra and sIL-4R, but not of sIL-2R, were significantly higher in children with malaria than in children with asymptomatic infections or in healthy children (IL-10 and IL-1Ra, $P < 0{\cdot}001$; sIL-4R, $P < 0{\cdot}01$) (Table 2).

Levels of parasitaemia were correlated with levels of IL-10 ($r = 0{\cdot}648$, $P < 0{\cdot}005$) and of IL-1Ra ($r = 0{\cdot}500$, $P < 0{\cdot}005$).

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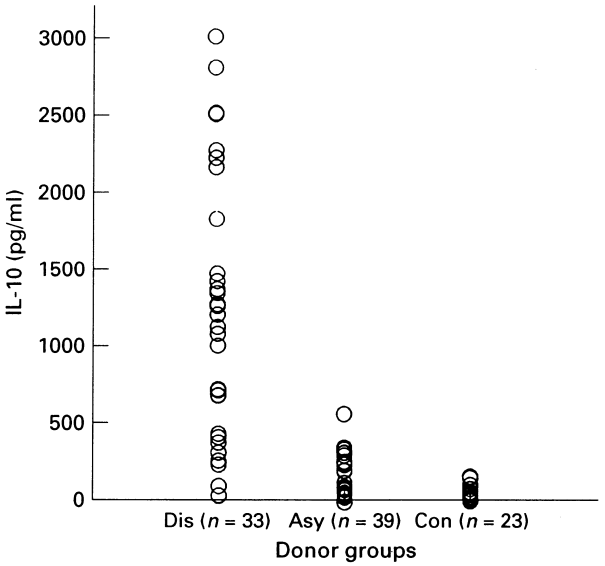

Fig. 1. IL-10 concentrations in three groups of Gambian children

IL-1Ra with disease was maintained after correction for levels of parasitaemia (analysis of covariance, $P < 0{\cdot}01$).

When comparing children with *P. falciparum* infections, 25 of 26 with IL-10 concentrations exceeding 500 pg/ml had symptoms giving a specificity of >96%. Twenty-five of 33 children with symptoms had IL-10 concentrations exceeding 500 pg/ml, giving a sensitivity of 75%.

Axillary temperature was correlated with levels of IL-10 ($r = 0{\cdot}709$, $P < 0{\cdot}005$) and of IL-1Ra ($r = 0{\cdot}651$, $P < 0{\cdot}005$).

Concentrations of IL-10 were correlated with levels of IL-1Ra ($r = 0{\cdot}703$, $P < 0{\cdot}005$).

Levels of β_2-GPI (Table 2)

Serum concentrations of β_2-GPI were significantly lower in children with malaria than in children with asymptomatic infections or in healthy children ($P < 0{\cdot}001$ for each comparison).

Concentrations of β_2-GP1 were inversely correlated with levels of parasitaemia ($r = -0{\cdot}360$, $P < 0{\cdot}005$).

The negative association between concentrations of β_2-GPI and disease manifestations was maintained after correction for levels of parasitaemia (analysis of covariance, $P < 0{\cdot}01$).

Concentrations of β_2-GP1 were inversely correlated with temperature ($r = -0{\cdot}467$, $P < 0{\cdot}005$), IL-10 levels ($r = -0{\cdot}470$, $P < 0{\cdot}005$) and sIL-2R levels ($r = -0{\cdot}401$, $P = 0{\cdot}001$).

Levels of anti-phospholipid antibodies

No significant difference in levels of IgM reactivities was found in children with asymptomatic infections, in children with clinical malaria and healthy children when measured according to procedure A (data not shown). When measured according to procedure B, both children with malaria and those with asymptomatic infections ($P = 0{\cdot}001$, respectively, $P = 0{\cdot}002$) had higher IgM reactivities to PI than healthy children (data not shown).

Similarly, although procedure A identified no difference in IgG reactivities in children with malaria and those with asymptomatic infections, IgG reactivities in the *P. falciparum*-infected children were significantly higher than in healthy children ($P = 0{\cdot}001$) (data not shown). When IgG reactivities were measured according to procedure B, children with malaria had significantly higher IgG reactivities than both children with asymptomatic infections ($P = 0{\cdot}003$) and healthy children ($P = 0{\cdot}003$), while there was no significant difference between the two latter groups (Fig. 2). Reactivities of IgG to PI using procedure B were correlated with IL-10 levels ($r = 0{\cdot}373$, $P = 0{\cdot}003$) and inversely correlated with β_2-GPI ($r = -0{\cdot}355$, $P = 0{\cdot}005$).

Table 2. Cytokine, soluble cytokine receptor and β_2-glycoprotein I (β_2-GPI) concentrations in the three subsets of Gambian children

Group	(i) Children with symptomatic infections		(ii) Children with asymptomatic infections		(iii) Healthy children	
IL-10 (pg/ml)	Median	1265	Median	80	Median	43
	25%	627	25%	33	25%	<15
	75%	2233	75%	248	75%	60
	$n = 33$		$n = 39$		$n = 23$	
IL-1Ra (pg/ml)	Median	2750	Median	85	Median	525
	25%	875	25%	39	25%	425
	75%	9600	75%	524	75%	750
	$n = 31$		$n = 33$		$n = 18$	
sIL-2R (U/ml)	Median	6575	Median	4550	Median	5025
	25%	3325	25%	2600	25%	2300
	75%	8225	75%	5950	75%	6500
	$n = 32$		$n = 40$		$n = 18$	
sIL-4R (pg/ml)	Median	4267	Median	3357	Median	2795
	25%	3170	25%	2500	25%	1995
	75%	5913	75%	4076	75%	3905
	$n = 28$		$n = 38$		$n = 28$	
β_2-GPI (μg/ml)	Median	86	Median	112	Median	117
	25%	74	25%	103	25%	97
	75%	103	75%	129	75%	129
	$n = 27$		$n = 39$		$n = 29$	

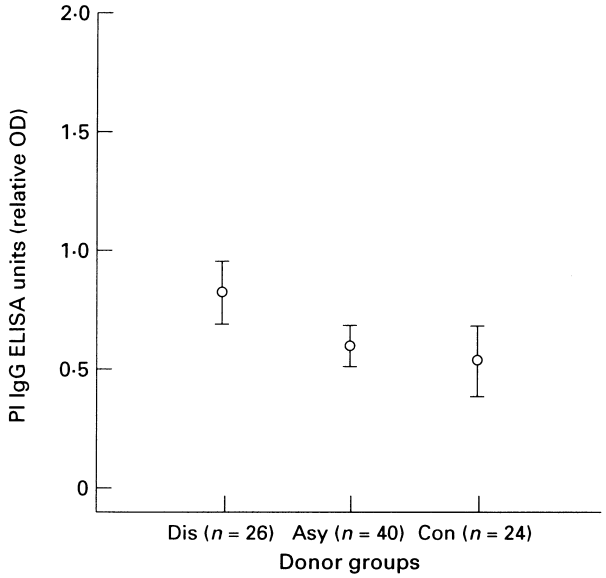

Fig. 2. Serum IgG anti-phospholipid antibody reactivities using procedure B with casein in three groups of Gambian children. The figure shows means and s.d. PI, Phosphatidylinositol.

Cytokine levels in children in the dry season

The serum concentrations of IL-10 and sIL-2R in children who had malaria in the rainy season were decreased in the following dry season ($P < 0{\cdot}001$) (Table 3). In this season the concentrations of IL-1Ra and sIL-4R in these children were similar to and the concentration of sIL-2R lower than the concentrations in healthy children during the rainy season. Serum concentrations of β_2-GPI were increased in the dry season compared with concentrations in the wet season in children who had malaria ($P = 0{\cdot}002$), while there was no seasonal variability in reactivities of IgM and IgG to PI.

DISCUSSION

The present study shows that children with symptomatic *P. falciparum* infection had much higher concentrations of the cytokines IL-10 and IL-1Ra than did children with asymptomatic infections, even when correcting for levels of parasitaemia.

Secretion of IL-10 and IL-1Ra are induced by inflammatory mediators, including tumour necrosis factor-alpha (TNF-α). Although these cytokines down-regulate inflammatory reactions, they are valuable markers of inflammation as they are secreted in high concentrations. Several cell types produce IL-10 and IL-1Ra, but monocytes are likely to be one of the main sources. Increased concentrations of IL-10 have previously been detected in children with malaria [11], in particular in children with severe malaria, and we have found that the concentrations of IL-1Ra were associated with disease severity in Gambian children with malaria [7].

The concentrations of TNF-α or IL-6 in the study samples were not determined, since we measured only low or undetectable concentrations of these cytokines in sera from children with mild or asymptomatic *P. falciparum* infections on a previous occasion [7].

In accordance with earlier suggestions [1], our data indicate that children have different clinical thresholds for *P. falciparum* infections. It is not known why some children tolerate high levels of parasitaemia without developing fever. Children with asymptomatic infections may have peripheral resistance to cytokine activities, but our data suggest this is not the case, as almost all children with malaria had high levels of IL-10 while children with asymptomatic infections had low levels of IL-10, slightly higher than the levels detected in healthy children.

Parasites may have different capacities to induce inflammatory mediators such as TNF-α [12]. Children may have different capacities to mount a cytokine response [13], or they may have factors blocking the malaria toxin-mediated stimulation of inflammatory reactions. If antitoxic malaria immunity exists it is likely to be composed of several different factors [14]. The parasite toxin has not yet been characterized in detail, but appears to contain PI [15]. The serum protein β_2-GPI binds to negatively charged phospholipids and may therefore bind to the malaria parasite toxin. Although binding of β_2-GPI to this toxin has not been reported, we have previously shown that concentrations of β_2-GPI are significantly decreased in children dying of cerebral malaria [7]. In this study, we found that children with asymptomatic *P. falciparum* infections had higher concentrations of β_2-GPI compared with children with malaria. Further studies characterizing the potential of β_2-GPI to block the activity of the malaria parasite toxin seem justified.

IgM antibodies against PI may block parasite-induced TNF secretion [16,17] and may therefore protect against clinical disease [3,18], but IgM reactivities also correlate with the severity of disease [10]. The potential role of these IgM antibodies in protecting against disease was not demonstrated in this study.

When performing ELISA with casein (procedure B), higher IgG reactivities against PI were found in children with disease than in children with asymptomatic infections. Apparently, IgG antibodies are not associated with protection against disease, but their potential role in modulating disease severity needs further investigation.

We have previously reported that the concentration of sIL-2R was associated with both disease severity and levels of parasitaemia [8], but concentrations of sIL-2R were not higher in children with symptomatic infections than in children with asymptomatic infections. We suggest that early inflammatory reactions induced by *P. falciparum* infections mainly involve monocytes/macrophages

Table 3. Seasonal variations in cytokine, soluble cytokine receptor and β_2-glycoprotein I (β_2-GPI) concentrations in children with clinical malaria

Groups	Dry season		Wet season	
IL-10 (pg/ml)	Median	20	Median	1475
	25%	<15	25%	728
	75%	41	75%	2633
	$n = 13$		$n = 13$	
sIL-2R (U/ml)	Median	2875	Median	7275
	25%	1650	25%	5050
	75%	4325	75%	8725
	$n = 12$		$n = 12$	
β_2-GPI (μg/ml)	Median	108	Median	79
	25%	97	25%	69
	75%	122	75%	94
	$n = 13$		$n = 13$	

and possibly endothelial cells, while T cell activation may occur later in the infection and may be associated with the intensity of inflammatory reactions and disease severity.

In conclusion, cytokines induced by inflammatory reactions were found in much higher concentrations in children with malaria than in children with asymptomatic *P. falciparum* infections, while children with malaria had lower concentrations of β_2-GPI.

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