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Ring-stage malaria antigens induce high levels of tumor necrosis factor-independent interleukin 6 secretion in blood cells from patients with severe malaria

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Introduction

High concentrations in plasma of the cytokines tumor necrosis factor (TNF) and interleukin 6 (IL-6) in *Plasmodium falciparum* malaria have been widely documented (1-4). These cytokines seems to play a crucial and decisive role in malaria protection, and also in the pathogenesis and development of complications, such as the cerebral malaria syndrome (1, 4, 5). It has also been shown that different preparations of malaria antigens, as well as some isolated and purified antigenic components, induce the expression and secretion of both TNF and IL-6 in human mononuclear leucocytes or in murine peritoneal macrophages (6-8). Since IL-6 may be induced by TNF (9) and concomitant elevation of these two cytokines is simultaneously found in several conditions (2, 4, 10) it is assumed that the IL-6 production during malaria could be TNF-dependent. However, we have previously reported that the production of IL-6 and TNF induced by exogenous heat stable malaria antigens may be differently modulated by pentoxifylline (8). In addition, evidence has recently been provided that hemozoin (malaria pigment) may also differentially modulate the production of IL-6 and TNF in murine malaria (11). We have now extended our studies in human leucocytes by analysis of the IL-6 and TNF secretion in whole blood samples from 14 Gabonese children with severe malaria, stimulated for two hours with ring-stage antigens from *P. falciparum* parasites.

Patients and methods

Blood samples from 14 children with severe *P. falciparum* malaria (12) were obtained at the International Research Laboratory of the Albert-Schweitzer-Hospital in Lambaréné, Gabon. The clinical findings on admission from the 14 patients included in this study are summarized in Table 1. At least one of the following four criteria were present for the inclusion in this study: Hyperparasitemia (>250,000/µl), severe anemia (hemoglobin <5 g/dl or hematocrit <15%), hypoglycemia (glucose <40 mg/dl) or cerebral malaria (unrousable coma not attributable to another cause). For taking blood samples, ethical clearance was obtained from the International Foundation of the Albert-Schweitzer-Hospital in Lambaréné. Ring-stage *P. falciparum* antigens (RS-PfAg) were prepared from blood of different patients with very high parasitemia (>500,000 parasites/µl blood) following the procedure described elsewhere for *Plasmodium berghei* antigens (13). A control preparation without *P. falciparum* antigens was obtained by using exactly the same procedure with blood from aparasitemic individuals (control extract

Table 1:

Clinical findings and symptomatology of patients with severe *P. falciparum* malaria on admission.

Patient					Further
Number	Age *	Sex **	Parasitemia ***	Hemoglobin	Symptomatology ****
1	2y	m	320,000	11.1	DI, VO
2	6y	m	310,000	7.7	HU
3	5y	m	200	7.4	CM, CO, HG, VO
4	4y	m	270,000	7.9	HP
5	4y	f	500,000	10.9	DI
6	2y	m	10,500	4.0	HG, VO
7	3y	m	2,000	7.9	CM, CO
8	10y	f	300,000	9.4	VO, HP
9	3y	m	9,000	7.6	HG, HU
10	2y	m	800,000	10.4	CO, HP
11	2y	f	52,600	3.9	CM, HG, HP
12	7m	m	60,000	2.1	HG, DI
13	6m	m	540,000	7.1	HP, VO
14	5y	m	275,000	8.7	VO

* y = years · m = months
** m = male · f = female
*** Parasitemia is indicated as parasites/µl, and hemoglobin as g/dl
**** DI = diarrhea · VO = vomiting · HU = macroscopic hemoglobinuria
CM = cerebral malaria · CO = convulsions · HG = hypoglycemia
HP = hyperpyrexia

for stimulations with RS-PfAg). Whole blood stimulations were performed in blood samples from the 14 patients with severe malaria by using for each patient 4 different tubes, each containing 1 ml of blood obtained directly from venipuncture, and supplied with 10 µl of:

1. RS-PfAg preparation,
2. control extract,
3. phytohemagglutinin (PHA) at a concentration of 1 mg/ml PHA, and
4. without addition.

The blood was stimulated for 2 hours at 37.5°C and gently shaken every 30 minutes. After stimulation, the tubes were centrifuged and the supernatants were collected and frozen at -20°C until use in the IL-6 and TNF enzyme immunoassays (EIA) (Medgenix Diagnostics, Fleurus, Belgium). Statistical analysis was performed by using the Mann Whitney U-test.

Results

The 14 patients included in this study were children (age < 10 years) from the area of Lambaréné, Gabon, all of them presenting severe *P. falciparum* malaria. The symptomatology of these patients was relatively heterogeneous but representative for severe malaria in the studied area. The more common features were hyperparasitemia (8 cases) and hypoglycemia (5 cases). Three patients had severe anemia and three cerebral malaria (Table 1). In stimulations with *P. falciparum* ring-stage antigens, the observed IL-6 levels were twice as high as in stimulations with a control preparation from aparasitemic individuals ($p < 0.001$) (Figure 1A). Moreover, the IL-6 levels in the control stimulations were similar to the IL-6 plasma levels of the corresponding malaria patients, which indicates that the control preparations from aparasitemic individuals did not further induce IL-6. The TNF concentrations in the same supernatants were similar in both the samples stimulated with ring-stage antigens and in the controls ($p > 0.05$). They also were similar to the TNF plasma levels in the patients, indicating that the malaria antigens used did not induce any detectable TNF production after 2 hours stimulation (Figure 1A).

Whole blood stimulations were also performed using 10 µg/ml PHA as stimulant. Incubations without PHA served as controls for these stimulations and resulted in IL-6 and TNF levels identical to the plasma levels of malaria patients. The IL-6 levels obtained after PHA stimulation were similar or slightly higher than those found after stimulation with ring-stage antigens (Figure 1B). However, the TNF production was significantly increased to levels about ten times higher after stimulation with PHA than in incubations without PHA (plasma levels) ($p < 0.001$) (Figure 1B).

Discussion

Despite the heterogeneous symptomatology found in the 14 patients with severe malaria, the results obtained for further induction of TNF and IL-6 secretion in whole blood stimulations were found to be relatively homogeneous (SEM < 15% of the means). It indicates that the observed effects are common to the majority of patients with severe malaria, at least in the studied area. The induction of IL-6 production in blood leucocytes by *P. falciparum* ring-stage

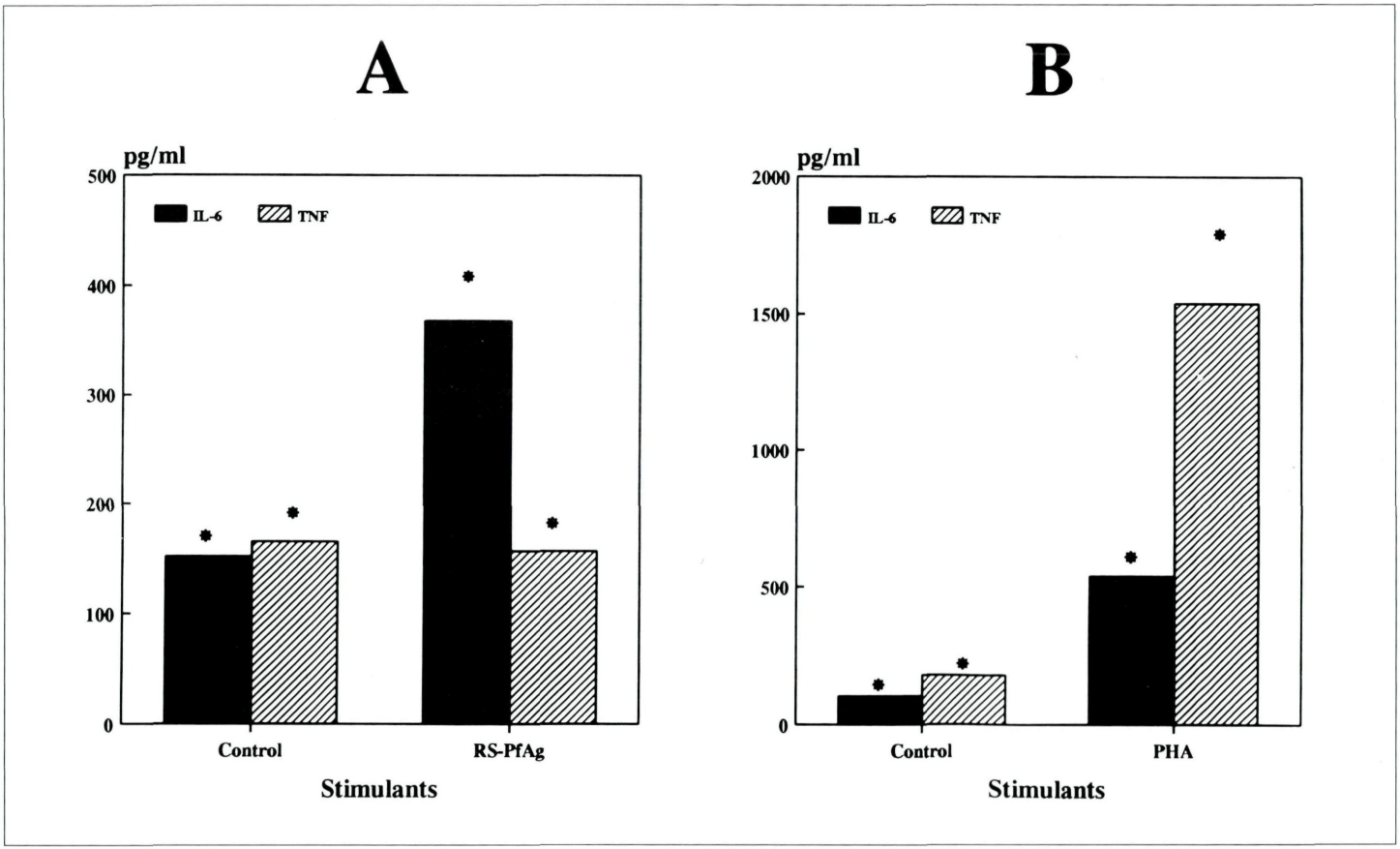

Figure 1:

IL-6 and TNF production in whole blood from 14 patients with severe *P. falciparum* malaria stimulated for 2 hours A) with ring-stage *P. falciparum* antigens (RS-PfAg) or B) with 10 µg/ml phytohemagglutinin (PHA)*, **.

*) As controls served: A) a control preparation from aparasitemic individuals (in RS-PfAg stimulations), or B) an incubation without PHA (in stimulations with PHA).

**) The IL-6 and TNF production were measured in supernatants by using an enzyme immunoassay (EIA) and expressed in pg/ml. The results are presented as means of the 14 examined patients (bars), and standard errors of the means (SEM) (stars).

antigens surprisingly occurred in the absence of a simultaneous upregulation of TNF. This observation accorded with previous reports about the different modulation of TNF and IL-6 during malaria, either by pentoxifylline in vitro (8) or by hemozoin in murine malaria (11). In this study, our results show that the production of IL-6 by leucocytes from patients with severe malaria may be directly induced by malaria antigens and not via the secretion of TNF. However, the influence of other related immunomodulators like soluble TNF receptors and interleukin 1 remains to be elucidated. The control stimulations performed with PHA show that the blood leucocytes from patients with severe malaria are still capable of producing high amounts of TNF, and that in severe malaria, the lack of further response to the used ring-stage antigen preparations is not due to maximal stimulation resulting in high concentrations of circulating TNF and subsequent exhaustion. It has previously been described that ring-stage *P. falciparum* antigens like the group of ring-infected erythrocyte surface antigens, and especially the Pf155/RESA protein, can induce TNF production in human macrophages after prolonged stimulation (12-60 hours) (14). This may also happen in whole blood stimulations of malaria patients, but the incubation time of 2 hours is probably not long enough for this observation. In addition, the stimulatory effect of Pf155/RESA was found to be concentration dependent and the presence of other antigenic components in the preparation we used may compensate the effects of a single type of antigen. Moreover, it is also probable that a prolongation in the stimulation time over 2 hours, as performed in the mentioned report (14), would show high levels of both TNF and IL-6 in our whole blood stimulations, but this would not exclude that both cytokines are independently induced by malaria antigens. In conclusion, the presented findings provide further evidence for an independence of IL-6 and TNF secretion

during malaria, and indicate different and independent modes of induction of TNF and IL-6 in severe *P. falciparum* malaria.

Summary When whole blood samples from 14 Gabonese children with severe *Plasmodium falciparum* malaria were stimulated for 2 hours in the presence of ring-stage *P. falciparum* antigens, high levels of interleukin 6 (IL-6) secretion were detected in the supernatants, while no detectable increase could be observed in the same supernatants for tumor necrosis factor (TNF) production. On the contrary, control stimulations of the same blood samples with phytohemagglutinin, a potent stimulator known to induce high levels of inflammatory cytokines, led to three fold higher TNF than IL-6 concentrations in the supernatants. These IL-6 levels were similar to the IL-6 levels induced with ring-stage malaria antigens. These results indicate that strong IL-6 secretion may be directly induced by ring-stage malaria antigens, and that the IL-6 secretion seems to be TNF-independent during severe *P. falciparum* malaria.

Key words Cytokines, malaria antigens, *Plasmodium falciparum*, blood leucocytes, enzyme immunoassays.

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Zusammenfassung

Ring-Stadium Malaria-Antigene induzieren hohe Spiegel von Tumornekrosefaktor-unabhängiger Interleukin 6-Sekretion in Blutzellen von Patienten mit schwerer Malaria

Vollblutproben von 14 Gaboner Kindern mit schwerer *Plasmodium falciparum*-Malaria wurden mit Ringstadium-Malaria-Antigenen stimuliert. Dabei wurden hohe Spiegel von Interleukin 6 (IL-6)-Sekretion in den Überständen nachgewiesen, während keine festellbare Zunahme der Tumornekrosefaktor (TNF)-Produktion in denselben Überständen beobachtet werden konnte. Im Gegenteil dazu führten Kontrollstimulationen von denselben Vollblutproben mit Phytohämagglutinin (PHA), bekannt als starker Stimulator von inflammatorischen Zytokinen, zu dreifach höheren TNF- als IL-6-Konzentrationen in den Überständen. Die beobachteten IL-6-Spiegel in den Stimulationen mit PHA und mit Malaria-Antigenen waren ähnlich. Diese Ergebnisse zeigen, daß eine starke IL-6-Sekretion direkt durch Ringstadien-Malaria-Antigene induziert werden kann und daß die IL-6-Sekretion während schwerer Malaria weithin TNF-unabhängig zu sein scheint.

Schlüsselwörter Zytokine, Malaria-Antigene, *Plasmodium falciparum*, Blutleukozyten, Enzymimmuntests.

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