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High levels of circulating IL-10 in human malaria

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SUMMARY

IL-10 is a monocyte/lymphocyte derived cytokine which has been shown to inhibit certain cellular immune responses such as delayed hypersensitivity. In particular, the production of tumour necrosis factor (TNF), IL-1 and IL-6, which are involved in malaria pathology, are strongly inhibited by IL-10. Accordingly, we examined whether IL-10 could be involved in a human acute parasitic infection such as *Plasmodium falciparum* malaria. Human IL-10 levels in plasma were determined by two-site ELISA method, taking care to avoid non-specific reactions due to autoantibodies. Fourteen cerebral, 11 severe, and 20 mild malaria cases had mean IL-10 levels of 2812, 2882 and 913 pg/ml, respectively, while 98% of healthy individuals had undetectable (less than 100 pg/ml) circulating IL-10. Thirteen of the 25 cerebral/severe cases had >2000 pg/ml. In 11 hospitalized patients, circulating IL-10 levels were found to return to virtually normal levels 7 days after antimalarial chemotherapy when biological and clinical malaria features had disappeared (mean levels fell from 3880 to 333 pg/ml). Further studies are required to determine whether these elevated levels of IL-10 play a beneficial role by reducing the parasite-induced inflammatory response, or a detrimental one by decreasing the cellular immune responses.

Keywords IL-10 ELISA *Plasmodium falciparum* cerebral malaria tumour necrosis factor-alpha

INTRODUCTION

Malaria remains a major public health problem because of its important morbidity and mortality. Cerebral malaria represents the most dramatic and life-threatening complication of the disease [1]. Recent studies have shown the pivotal role of tumour necrosis factor-alpha (TNF-α) in the pathogeny of the disease [2]. In particular, the plasma levels of this cytokine have been correlated with the severity of the disease [3]. Other monocyte/macrophage-derived cytokines such as IL-1 and IL-6 have also been detected in the plasma of malaria patients [4,5].

Human IL-10 was recently found to be produced by monocytes [6,7] as well as T and B lymphocytes [8,9]. IL-10 was found to act rather as an 'antiinflammatory' agent able to block the production of IL-6, TNF-α, IL-1 and other cytokines by monocytes/macrophages [6]. This property, together with an inhibitory effect on the expression of MHC class II antigens on monocytes/macrophages results in a strong inhibition of the antigen-presenting capacity of these cells, and a consequent inhibition of T lymphocyte proliferation [10]. In addition, IL-10 was recently shown to induce the proliferation of B lymphocytes and most notably their differentiation into plasma cells secreting immunoglobulins at high rate [11,12]. Taken together, IL-10 inhibits DTH reactions and stimulates humoral responses.

Recent studies carried out in mice both *in vivo* and *in vitro* have suggested a regulatory role for IL-10 in the mediation of susceptibility to acute parasitic infections. In particular, IL-10 inhibits the microbicidal activity of interferon-gamma (IFN-γ)-treated macrophages against intracellular parasites such as *Toxoplasma gondii* [13], *Trypanosoma cruzi* [14] and *Leishmania major* [15], and the extracellular killing of *Schistosoma mansoni* schistosomulas [13–16]. This effect is associated with inhibition of the production of the toxic nitrogen oxide metabolites [17]. Although *P. falciparum* is an intraerythrocytic parasite, it may be cleared from the blood by phagocytosis and killed by oxygen intermediates released by macrophages. These results prompted us to examine whether IL-10 could also be involved in a human acute parasitic infection such as severe malaria. The present study demonstrates the presence of high levels of circulating IL-10 in cerebral, severe, and mild malaria.

PATIENTS AND METHODS

Population

The study was conducted at two different sites. In Antananarivo (Madagascar), a malaria-endemic area, the clinical and biologi-

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cal status of patients was recorded only at admission to hospital. In French hospitals (Hôpital Bichat, Paris, and Hôpital de la Croix Rousse, Lyon) patients could be followed for 7 days. All patients included in the study had *P. falciparum* infection, and parasitaemia was counted on Giemsa-stained thick smears. On day 0 (D0) patients underwent a physical examination and were classified, according to a modification of the WHO criteria [18], into three groups: cerebral malaria: patients with unrousable coma; severe malaria: patients with hyperparasitaemia (above 5%), anaemia, neurological signs or hypoglycaemia; mild malaria: patients with positive parasitaemia but without the clinical features of severe malaria.

Sample collection

Venous blood was drawn into an EDTA tube for full blood count, and examination for malaria parasites. Plasma was immediately collected and frozen at −70°C.

The study was conducted on the surplus of blood withdrawn to perform diagnostic procedures prescribed by a physician.

Cytokine assay

Plasma IL-10 levels were determined by a two-site sandwich ELISA format, kindly provided by John Abrams (DNAX, Palo Alto, CA) [19]. One assay quantified both human IL-10 (hIL-10) and BCRF1 (viral IL-10), while the other one was specific of BCRF1. Both assays relied on the same catcher antibody MoAb 9D7, and the specificity was determined by the tracer biotinylated antibody MoAb 12G8 for the hIL-10 and BCRF1 ELISA and MoAb 6B11 for the BCRF1 ELISA. Rat anti-IL-10 MoAb 9D7 was coated at 5 μg/ml for 2 h at 37°C, in 100 μl, in 96-well flat-bottomed microtitre plates (Nunc Immunoplate, Roskilde, Denmark). After 3 h saturation with bovine serum albumin (BSA), plasma diluted 1:2 in PBS 0·05% Tween 20 was incubated 2 h at room temperature. The standards consisted of Chinese hamster ovary (CHO)-derived purified hIL-10 for the IL-10 ELISA and Cos7-derived BCRF1 for the BCRF1 ELISA. The tracer biotinylated MoAbs were then incubated for 2 h at room temperature: MoAb 12G8 was added at 0·05 μg/ml and MoAb 6B11 was added at 0·1 μg/ml. The binding of the biotinylated antibodies was revealed with the alkaline phosphatase-conjugated streptavidin (Tago, Burlingame, CA)/phosphatase substrate (Sigma, St Louis, MO) system as previously described [19]. The lower detection levels were 100 and 50 pg/ml for the hIL-10 and the BCRF1, respectively. As a control to eliminate the non-specific signal given by rheumatoid factor, a non-relevant biotinylated anti-IFN-γ MoAb (MoAb B27 at 0·05 μg/ml) was used as a second MoAb, for each sample. All samples giving positive reactions under these conditions were eliminated from the study. Normal levels of IL-10 in plasma were determined (i) in 63 European blood donors, and (ii) in 43 Africans living in a rural malarial-endemic area in Burkina Faso, who had a negative thick smear and showed no clinical signs of malaria for at least 2 weeks before blood sampling.

Plasma TNF-α levels were assayed by radioimmunoassay (Medgenix, Fleurus, Belgium).

Statistical analysis

The Mann–Whitney test was used to compare IL-10 values among different groups, with use of Wilcoxon paired test to compare results in acute disease and convalescence. Spearman's rank test was used to assess the relationship between IL-10 and TNF-α. Results are expressed as mean ± s.e.m.

RESULTS

Patients

Among 47 Madagascan patients, 12 had cerebral malaria, 11 had severe, and 24 mild malaria, but because of non-specific scores in IL-10 assay, four, four and eight patients respectively were removed from the study. Of the patients admitted to French hospitals, six suffered from cerebral malaria (one died after 3 days), four had severe malaria, while the remaining four presented with a mild malaria (see Table 1). This unusually high proportion of non-specific scores is most probably due to the hyperstimulation of antibody production, in particular rheumatoid factor, which is described in malaria [20]. All patients were given intravenous quinine (8 mg/kg) every 8 h. Those with cerebral malaria were given a loading dose of 10 mg/kg quinine over 4 h. After 2 days, or when permitted by the clinical status of patient, quinine was given orally for a total duration of 7 days.

Levels of IL-10 in control groups

Of 63 European blood donors, three were excluded for positive reaction with BCRF1. In the remaining 60, the level of IL-10 in the plasma was below the detection limit of 100 pg/ml. Of 43 African controls, eight subjects positive for BCRF1 and rheumatoid factor, and four positive only for BCRF1, were excluded. Among the remaining 31, 27 had less than 100 pg/ml IL-10, while four showed levels of IL-10 between 100 and 189 pg/ml (mean 140 ± 0·03 pg/ml). For statistical analysis the value of 100 pg/ml was assigned to patients displaying undetectable IL-10 levels. The mean value for the 91 healthy individuals was therefore 112 ± 4·18 pg/ml.

Level of IL-10 in malaria patients

Figure 1 shows the plasma IL-10 levels of patients on admission in the three malaria groups. The IL-10 levels in the 14 patients suffering from cerebral malaria were extremely high, with a mean value of 2812 ± 626 pg/ml and a wide range: 8038–100 pg/ml. These levels were significantly higher than those of controls ($P = 0{\cdot}0004$). The mean plasma levels of IL-10 in 11 patients

Table 1. General characteristics of patients: age (median, range) and parasitaemia (median, range).

	Cerebral	Severe	Mild
Madagascar			
n	8	7	16
Age (years)	23 (8–36)	20 (5–42)	13 (2–41)
Parasitaemia (%)	2·15 (0·4–13·7)	7·80 (0·3–15·5)	1·55 (0·01–4·1)
French hospital			
n	6	4	4
Age (years)	45 (25–60)	36 (28–47)	39 (36–45)
Parasitaemia (%)	7·2 (0·4–13·7)	13·8 (2–28·2)	3·7 (2·9–4·2)

Only patients who showed specific reactivity for human IL-10 are represented.

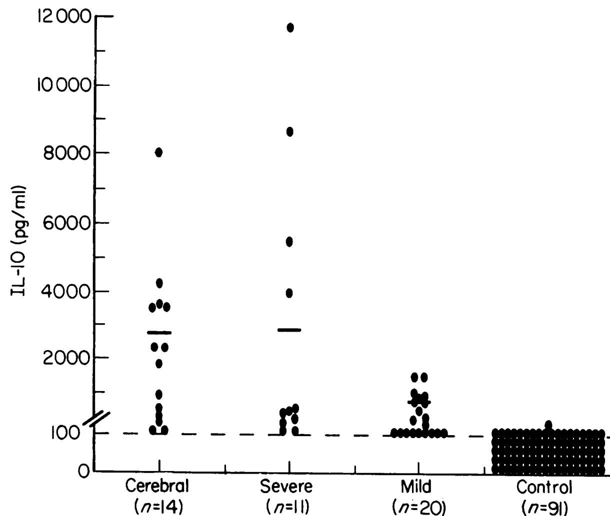

Fig. 1. Distribution of admission IL-10 levels in three groups of patients. Horizontal bars show mean values.

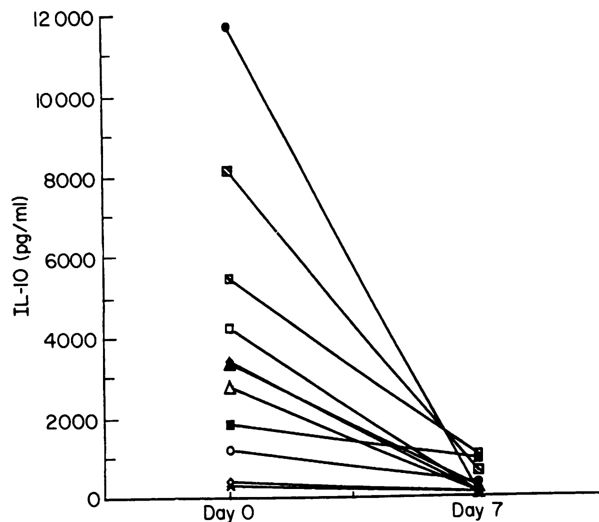

Fig. 2. Comparison of IL-10 values at D0 and D7 in patients followed in French hospitals.

suffering from severe malaria were not significantly different from those observed in the cerebral malaria group (2882 $\pm$ 1229 pg/ml, range 11705–100 pg/ml). The levels of IL-10 observed in patients with cerebral/severe malaria were very high, and 13 of them displayed more than 2000 pg/ml IL-10, although three patients with cerebral malaria and two patients with severe malaria had low levels of IL-10 (between 100 and 200 pg/ml). Twenty patients with mild malaria had much lower plasma IL-10 values than those with cerebral/severe malaria. Nonetheless, these patients displayed levels of IL-10 which were significantly raised above the normal controls (mean 913 $\pm$ 281 pg/ml, range 4600–100 pg/ml; $P = 0.0001$). Among the 14 patients from the French hospitals, 11 were monitored 7 days after treatment for any change in plasma IL-10 levels (one died after 3 days, two had non-specific score in the IL-10 assay on day 7). As shown in Fig. 2, their levels of circulating IL-10 were

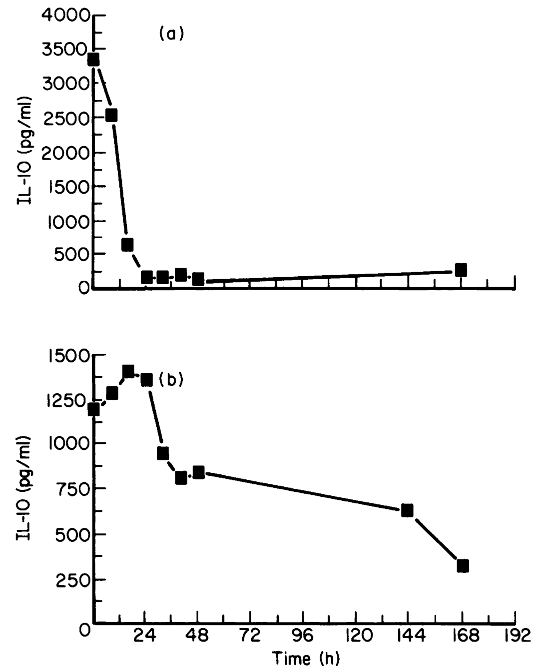

Fig. 3. Two types of IL-10 evolution in malaria patients followed in French hospitals. (a) Cerebral malaria case. (b) Severe malaria case. Time after admission and treatment.

greatly reduced from a mean level of 3880 pg/ml at day 0 to a mean level of 333 pg/ml ($P = 0.0007$). At day 7, all patients had a negative thick smear. Blood samples were drawn at various times during a 7-day period in seven patients. IL-10 levels decreased within 24 h in three patients (as shown in Fig. 3a for one cerebral malaria case), and more slowly in the four other patients (depicted in Fig. 3b, for one severe case). One patient who died after 3 days had circulating IL-10 levels between 2218 and 4404 pg/ml. During this period, the kinetics of the disappearance of IL-10 from the circulation did not correlate with either the severity of the disease or other parameters such as parasitaemia or fever (data not shown).

IL-10 and TNF

The TNF-α levels determined in all of these patients at the onset of the disease were 172·2 $\pm$ 186·4 ng/ml, range 6–910 ng/ml (normal value < 10 ng/ml). There was no correlation between the levels of IL-10 and TNF-α ($r = 0.37$, $P = 0.06$).

DISCUSSION

This study has measured the level of IL-10 in the plasma of patients suffering from acute and mild malaria. Most patients suffering from cerebral and severe malaria were found to display strikingly high levels of circulating IL-10, with mean values of 2812 and 2882 pg/ml, respectively. Indeed, 13/25 patients suffering from cerebral and severe malaria were found to display IL-10 levels higher than 2000 pg/ml, a level which we found earlier only in a single case of lymphoma (out of 150 lymphoma and 140 myeloma plasma tested) [21,22]. The detected IL-10 was of human rather than viral origin, as positive samples did not score in an ELISA assay specific for BCRF1. Patients with mild malaria also displayed elevated levels of circulating IL-10,

although much less than observed in patients with severe disease. A follow up of the patients with the most acute malaria showed that levels of circulating IL-10 decreased markedly within 1 week after treatment, with antimalarials and those who no longer showed clinical signs of malaria. Thus, there is a link between the presence of circulating IL-10 and the presence of clinical symptoms. However, there was no correlation between the levels of IL-10 and fever or parasitaemia. The decrease in IL-10 occurred between 24 h and a few days in a manner which was apparently not related to the severity of the disease. The very quick decrease of circulating IL-10 observed in three severely affected patients may explain why four patients suffering from cerebral/severe malaria had only low levels of circulating IL-10, as these patients may have been admitted in the hospital relatively late after the onset of the attack. It is difficult to determine precisely when during malaria circulating IL-10 becomes detectable, and cytokine secretion may be abrupt or gradual, sometimes preceding the onset of clinical manifestations [23]. The cell population producing IL-10 requires identification. These may be either monocytes, T/B lymphocytes, or another cell type not yet known to produce this cytokine. The presence of circulating IL-6 and TNF together with IL-10 in the early stage of malaria may suggest activation of monocytes, but this requires further investigation. Although IL-10 has been shown to inhibit the production of TNF-α by monocytes, there was no positive or negative correlation between the IL-10 and TNF levels in the plasma of malaria patients.

The role of the cytokine network in malaria remains to be elucidated. High concentrations of proinflammatory cytokines such as TNF, IL-1, IL-6 or nitric oxide products seem to be deleterious for the host, but at low doses these agents facilitate the clearance of the parasites. Whether high levels of IL-10 observed during malarial episodes are beneficial by reducing the inflammatory response, or detrimental by decreasing the cellular response, remains to be elucidated.

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