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Chloroquine-induced inhibition of the production of TNF, but not of IL-6, is affected by disruption of iron metabolism

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SUMMARY

There is now considerable evidence that cerebral malaria may be related to the over-production of tumour necrosis factor (TNF). Nevertheless, our knowledge is very poor concerning the biological events which lead up to this TNF over-production. Furthermore, interleukin-6 (IL-6) is produced in large amounts during malaria infection and seems to have inhibitory action on TNF production. Anti-malarial drugs were investigated for their ability to interfere with TNF and IL-6 secretion by human non-immune macrophages stimulated by lipopolysaccharides (LPS) or *Plasmodium falciparum* culture supernatant. Macrophages were pretreated with chloroquine, quinine, proguanil, mefloquine or halofantrine before stimulation. TNF and IL-6 production were suppressed in a dose-dependent manner when macrophages were treated with chloroquine, but not with other anti-malarial drugs. Considering that chloroquine probably acts via lysosomotropic mechanisms, and that iron metabolism may interfere with the non-specific immune response, we focused our attention on these biochemical events in order to investigate the mechanisms by which chloroquine inhibits cytokine production. Our results demonstrated that chloroquine-induced inhibition of TNF and IL-6 production is not mediated through a lysosomotropic mechanism, and that chloroquine probably acts on TNF secretion by disrupting iron homeostasis. Inhibition of IL-6 production seems not to be mediated through these pathways. These observations suggest that chloroquine may help to prevent cerebral malaria whatever the drug sensitivity of the parasite strain, and may provide new tools for an anti-disease therapy regardless of the emergence of parasite multi-drug resistance.

INTRODUCTION

Cerebral malaria is probably related to an over-stimulation of the immune system and the cytokine network.[1] It has been previously demonstrated that tumour necrosis factor (TNF) plasma levels were significantly higher in patients who died from cerebral malaria than those who recovered.[2] We and others have demonstrated that TNF secretion by human macrophages could be induced by soluble and heat stable antigens from *Plasmodium falciparum* culture supernatant, providing evidence for a direct relationship between parasite and TNF.[3,4] Although interleukin-6 (IL-6) does not seem to play a major role in the pathogenesis of cerebral malaria, its plasma level follows the rise and the decrease of parasitaemia in patients with acute falciparum malaria.[5] Inasmuch as IL-6 significantly reduces TNF production, the secretion pattern of these cytokines seems to be closely related.[6]

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The dramatic extension of *P. falciparum* chloroquine resistance and the difficulties encountered in obtaining an anti-malaria vaccine have lead us to investigate the efficiency of anti-malaria drugs to modulate TNF and IL-6 secretion by human non-immune macrophages *in vitro*. The aim of this approach was to provide evidence that some already available drugs could enhance patient survival by inhibition of cytokine secretion, whatever the efficiency of these drugs at causing parasite death. Considering the large amounts of chloroquine used in malaria endemic areas, and the fact that this drug penetrates easily into macrophages, we have first investigated chloroquine diphosphate salt in our model. This was followed by an investigation of quinine, proguanil, mefloquine and halofantrine as they are the most widely used anti-malarial drugs.

MATERIALS AND METHODS

Chemical reagents

Chloroquine (diphosphate salt; Sigma, St Louis, MO), halofantrine chlorhydrate (kindly provided by Smith Kline & French,

Paris, France), mefloquine (kindly provided by La Roche, Basel, Switzerland), quinine–resorcine bichlorhydrate (Lab. Labaz, Paris, France), proguanil hydrochloride (ICI Pharma, Clamart, France) were used at various amounts related to concentrations observed *in vivo*. Lipopolysacharide (LPS), pentoxifylline and ammonium chloride (NH_4Cl) were purchased from Sigma.

Preparation of mononuclear cells

Blood from normal healthy volunteers who had never been exposed to malaria infection was collected into citric acid–dextrose (pH 7·2). Circulating mononuclear cells were isolated using a previously described method:[7] mononuclear cells were separated by centrifugation on Ficoll, washed three times, total cell counts made in a haemocytometer, and cells differentiated by staining. Cells were then plated in 24-well tissue culture dishes (Becton Dickinson, Oxnard, CA) in RPMI-1640 supplemented with 10% human serum and incubated at 37° in a CO_2 incubator. Non-adherent cells were removed by repeated washings with RPMI. Adherent cells were kept in culture medium for 5 days. Viability was determined by exclusion of trypan blue dye before and after incubation with maximal drug concentrations in all experiments. Each experiment was conducted with macrophages from a single individual. All the comparisons between supernatants or antigens have been made with macrophages from the same donor. Each test was conducted in triplicate, and negative (RPMI alone) or positive (LPS) controls were systematically included. Each experiment has been repeated with macrophages from different donors. Individual differences in the response of the macrophages were observed, but the pattern of cytokine secretion remained unchanged.

Parasites

Plasmodium falciparum was maintained in culture according to the method of Trager and Jensen.[8] Briefly, SGE1 (Gambia) strain was maintained in group A+ erythrocytes in medium RPMI-1640 (Gibco, Grand Island, NY) supplemented with 21 mM sodium bicarbonate, 25 mM HEPES buffer, 10 μg/ml gentamycin and 10% blood group A human serum. The medium was renewed daily. Parasitized red blood cells were maintained as shallow layers in 75-cm² tissue culture flasks at 37° in an atmosphere of 90% N_2, 5% CO_2 and 5% O_2. Synchronization with 5% sorbitol was carried out before experiments according to the method of Lambros and Vanderberg.[9]

Preparation of the culture supernatants

Culture supernatants were collected after 24 hr of culture (parasitaemia 4%). Soluble factors released by the parasites into the culture medium were isolated by ultracentrifugation, as previously described.[3] Culture supernatants were passed through a 0·2-μm Millipore filter and stored at −20°.

Stimulation of macrophages

After 5 days in culture medium, macrophages were treated at different times with various amounts of chloroquine, quinine, proguanil, mefloquine or halofantrine. The pH of the solutions was systematically controlled in culture medium. Cells were then stimulated overnight with 100 ng/ml LPS. For some experiments, mononuclear cells were stimulated with *P. falciparum* culture supernatant. Controls were provided by cells incubated overnight with medium alone or LPS alone. Supernatants were then collected and kept at −20° before TNF and IL-6 assays. The absence of contamination by endotoxin was assessed by adding control samples containing 1 μg/ml of polymyxine B for each experiment. We investigated macrophage viability during prolonged (48 hr) incubation with all the drugs. More than 90% of the macrophages remained viable. These controls provided evidence for the non-toxic effect of the anti-malarial drugs on human macrophages at the concentrations used.

TNF assay

TNF levels in culture supernatants were determined in duplicate by immunoradiometric assay (IRMA MEDGENIX, Fleurus, Belgium) following a modified protocol as described previously.[10]

IL-6 assay

IL-6 levels in culture supernatants were determined in duplicate using an immunoenzymatic assay (IL-6; Immunotech SA, Marseilles, France) according to the manufacturer's specifications.

Statistical analysis

Results are expressed as mean ± SE unless otherwise indicated. Results were compared using either Student's *t*-test for paired samples or the Wilcoxon signed rank sum test.

RESULTS

Chloroquine salt inhibits TNF secretion

Human macrophages were incubated with various amounts of chloroquine over a 2 hr time course. These cells were then stimulated with LPS or with *P. falciparum* culture supernatant. Data presented in Fig. 1 show that chloroquine salt inhibits TNF secretion by macrophages in a dose-dependent manner, as previously reported.[11] This effect was observed when stimula-

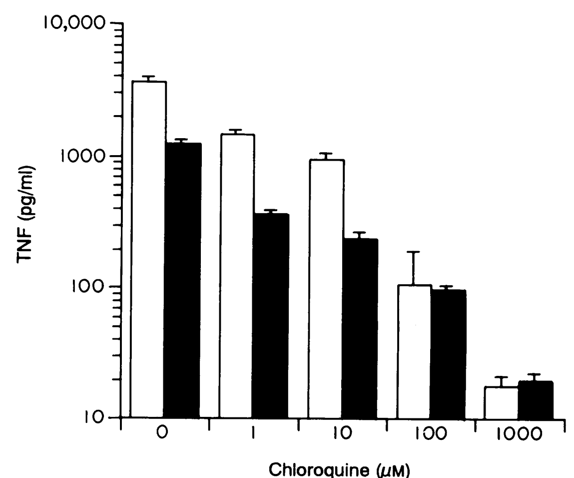

Figure 1. Chloroquine-induced TNF inhibition: macrophages were pretreated with various amounts of chloroquine for 2 hr. Then cells were stimulated overnight with LPS (0·1 μg/ml) (□) or *P. falciparum* supernatant (■). TNF levels were measured by immunoradiometric assay. Each test was performed in triplicate and results are expressed as means ± SE of the results obtained from five different experiments.

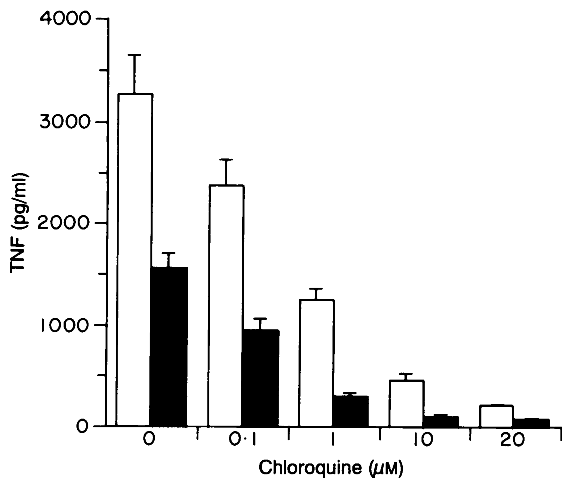

Figure 2. Chloroquine-induced TNF inhibition: macrophages were pretreated with lower amounts of chloroquine (0·1–20 μM) for 24 hr. Then cells were stimulated overnight with LPS (0·1μg/ml) (□) or *P. falciparum* supernatant (■). TNF levels were measured by immunoradiometric assay. Each test was performed in triplicate and results are expressed as means ± SE of the results obtained from five different experiments.

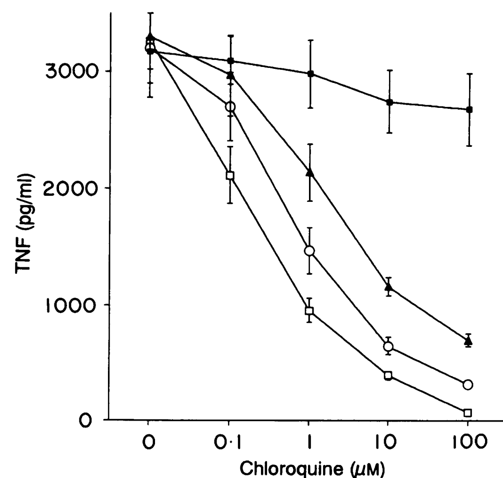

Figure 3. Time-course of chloroquine-induced TNF inhibition: macrophages were incubated with various amounts of chloroquine either 2 hr before (□), at the same time (○), 2 hr after (▲) or 4 hr after (■) stimulation by LPS (0·1 μg/ml). Cell supernatants were harvested after 12 hr incubation and TNF levels were determined by immunoradiometric assay. Each test was performed in triplicate and results are expressed as means ± SE of the results obtained from five different experiments.

tion was induced either by LPS or parasite culture supernatant. The inhibition of TNF secretion was not due to a cytotoxic effect on the monocyte monolayers, as more than 90% of the cells were viable throughout the course of the experiments.

To determine the effect of lower concentrations of chloroquine, we incubated monocytes for 24 hr with or without chloroquine at concentrations ranging from 0·1 to 20 μM, before stimulation with LPS or parasite supernatants (Fig. 2). We observed that TNF secretion was suppressed even at the lowest concentration tested (0·1 μM). This indicated that chloroquine exerts an inhibitory effect at clinically achievable concentrations, between 0·2 and 0·4 μM. The inhibitory effect was optimum when chloroquine was added to macrophages prior to stimulation (Fig. 3). Two hours preincubation with chloroquine (0·1 μM) before stimulation was enough to obtain a significant decrease in TNF secretion. Inhibition was still evident when chloroquine and LPS were both added to macrophages at the same time. Partial inhibition was observed when chloroquine was added 2 hr after stimulation. After 4 hr, no inhibitory effects of the drug could be detected.

When macrophages were treated with chloroquine for 2 hr, the medium was replaced once with RPMI without chloroquine, and the macrophages kept in culture without any drug for 24, 48 and 72 hr before stimulation by LPS, we observed that chloroquine remained able to inhibit TNF secretion, even after 72 hr (Fig. 4).

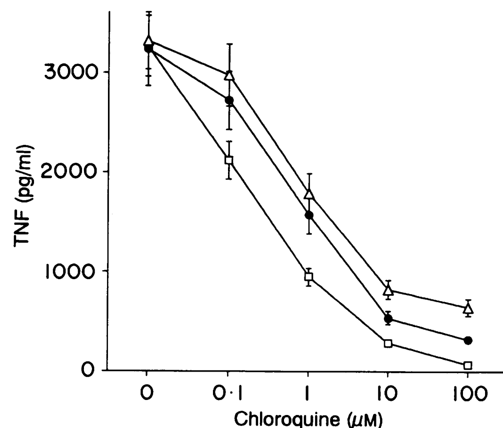

Figure 4. Effect of resting time after 2 hr chloroquine incubation on TNF secretion. Macrophages were incubated with various amounts of chloroquine, then media were renewed once with RPMI without chloroquine and cells kept in culture for 24 hr (□), 48 hr (●) or 72 hr (△). Cells were then stimulated with LPS (0·1 μg/ml), incubated for 12 hr before TNF levels were determined. Each test was performed in triplicate and results are expressed as means ± SE of the results obtained from five different experiments.

The weak base properties of chloroquine

Chloroquine salt and NH_4Cl act as weak bases and accumulate in acidic intracellular compartments, including lysosomes and endosomes. They increase vesicle pH rapidly (within 3–5 min) and interfere with the functions of the acid vesicle system.[12] To determine if weak base effects are involved in TNF down-regulation by chloroquine, we compared the effect of NH_4Cl on TNF secretion by LPS-stimulated macrophages. NH_4Cl failed to inhibit LPS-induced TNF secretion (Fig. 5). This raised the possibility that chloroquine inhibits TNF secretion through a non-lysosomotropic effect.

Effect of other drugs on TNF secretion

Pentoxifylline is a methylxanthine derivative which inhibits the production of TNF by peripheral monocytes/macrophages[13] and which prevents the development of cerebral malaria in

Table 1. TNF and IL-6 inhibition by anti-malarial drugs

Drugs*	Concentrations	Plasma levels†	% inhibition‡	
			TNF§	IL-6¶
Quinine bichlorhydrate (μg/ml)	0·5	10 μg/ml	0	0
	1		8±5	0
	10		24±11	3±2
	100		45±12	8±3
Mefloquine (μg/ml)	1	1 μg/ml	0	7±3
	10		12±3	8±2
	100		15±2	44±9
Halofantrine chlorhydrate (μg/ml)	0·3	0·3 μg/ml	0	0
	3		5±3	4±2
	30		13±2	0
Proguanil hydrochloride (μg/ml)	1	170 ng/ml	5±2	0
	10		7±3	0
	100		15±5	0
Pentoxifylline (mg/ml)	0·01	NT**	13±4	NT
	0·1		42±11	NT
	1		87±9	NT
	10		99±12	NT
Ammonium chloride (M)	10^{-7}	NT	0	NT
	10^{-6}		0	NT
	10^{-5}		4±3	NT
	10^{-4}		1±2	NT

* All the drugs were diluted in sterile RPMI medium and passed through a 0·2 μm Millipore filter. Stock solutions were kept at −20° before use.

† Plasma levels are expressed as mean of concentrations observed during treatment with conventional doses. No data are available for pentoxifylline and ammonium chloride.

‡ TNF and IL-6 are expressed as per cent of inhibition compared to maximum cytokine levels obtained when macrophages were not incubated with drugs. Results are expressed as means ± SE of the results obtained from three different experiments. Cells were incubated with drugs for 2 hr (§) or 4 hr (¶) before LPS.

** NT: Not tested.

susceptible mice infected with *P. berghei* Anka strain without affecting parasitaemia.[14] In our hands, LPS-induced TNF secretion was suppressed when macrophages were preincubated with various amounts of pentoxifylline, as previously described,[13] providing evidence for the reliability of the model used.

Results obtained with the other anti-malarial drugs used are described in Table 1. We observed that among drugs which are chemically close to chloroquine, quinine–resorcine bichlorhydrate (concentration range 0·5–100 μg/ml; plasma level achievable *in vivo* 10 μg/ml) does not exhibit TNF inhibition except for very high concentrations (100 μg/ml); mefloquine (concentration range 1–100 μg/ml; plasma level 1 μg/ml), halofantrine chlorhydrate (concentration range 0·3–30 μg/ml; plasma level 0·3 μg/ml) and proguanil hydrochloride (concentration range 1–100 μg/ml; plasma level 170 ng/ml) had no inhibitory effect. There was no enhancement of the effect of chloroquine when it was included with proguanil hydrochloride (data not shown).

IL-6 inhibition by anti-malarial drugs

Similar experiments were performed to observe the effect of anti-malarial drugs on IL-6 production (Table 1). IL-6 secretion, like TNF, could be induced by LPS or parasite stimulation.[15] Preliminary experiments provided evidence that IL-6 levels reached a maximum after 24 hr incubation with stimulants (data not shown). Cells were incubated with the various drugs for 4 hr before stimulation by LPS. Cell supernatants were harvested 24 hr after LPS stimulation and kept at −20° before IL-6 measurement. Chloroquine inhibited IL-6 secretion at the same doses as those which inhibited TNF secretion. Mefloquine reproduced an effect at the highest concentration, whereas none of the others drugs modulated IL-6 production.

Chloroquine-induced TNF inhibition is regulated by iron metabolism

To evaluate interactions between iron and chloroquine, macrophages were pretreated with desferrioxamine (50 μM), an iron-specific chelating agent. Desferrioxamine was first added and the cultures were left to stand for 30 min before the addition of chloroquine (20 μM). After incubating for 2 hr, macrophages were washed with RPMI-1640 and stimulated with LPS. We next investigated if LPS-induced cytokine secretion and chloroquine-induced cytokine inhibition were directly affected by iron

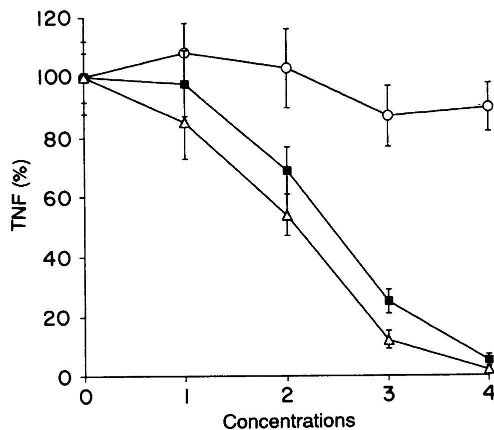

Figure 5. Dose–response curve depicting progressive inhibition of TNF secretion by macrophages pretreated with four concentrations of chloroquine (■) (0·1, 1, 10, 100 μM) and pentoxifylline (△) (0·01, 0·1, 1, 10 mg/ml), but no effect of ammonium chloride (○) for any concentration (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M). Cells were incubated with drugs for 4 hr before exposure to LPS (12 hr). TNF is expressed as per cent of the maximum level obtained when macrophages were stimulated with LPS in the absence of chloroquine, ammonium chloride or pentoxifylline. Results are expressed as means ± SE of the results obtained from three different experiments.

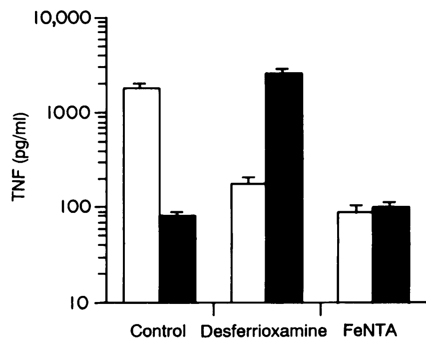

Figure 6. Chloroquine-induced TNF inhibition by macrophages pretreated with desferrioxamine or FeNTA: macrophages were incubated with desferrioxamine (50 μM) or with FeNTA (50 μM) for 2 hr (□). Then chloroquine (20 μM) was added to each well cell, plates were kept at 37° for 2 hr more before cells were stimulated overnight with LPS (0·1 μg/ml) (■). TNF levels were measured by immunoradiometric assay. Each test was performed in triplicate and results are expressed as means ± SE.

overloading. Macrophages were incubated with iron in the form of ferric nitrilotriacetate (FeNTA), which remains soluble at neutral or alkaline pH, and which is not affected by increases in intracellular pH induced by chloroquine.[16]

First, we observed that desferrioxamine by itself inhibited LPS-induced TNF secretion ($P < 0·001$) (Fig. 6). Secondly when macrophages were preincubated with desferrioxamine, chloroquine failed to suppress LPS-induced TNF secretion but actually enhanced it. Surprisingly, we observed that LPS-induced TNF secretion was directly inhibited by FeNTA (50 μM) ($P < 0·005$), but that chloroquine did not potentiate this TNF inhibition (Fig. 6). Neither desferrioxamine nor FeNTA were able by themselves to induce TNF or IL-6 production in non-stimulated macrophages.

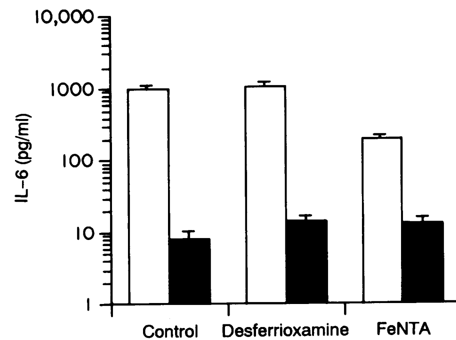

Figure 7. Chloroquine-induced IL-6 inhibition by macrophages pretreated with desferrioxamine or FeNTA: macrophages were incubated with desferrioxamine (50 μM) or with FeNTA (50 μM) for 2 hr (□). Then chloroquine (20 μM) was added to each well cell, plates were kept at 37° for 2 hr more before cells were stimulated overnight with LPS (0·1 μg/ml) (■). IL-6 levels were measured by immunoenzymatic assay. Each test was performed in triplicate and results are expressed as means ± SE.

LPS-induced IL-6 secretion was directly inhibited by FeNTA (50 μM) ($P < 0·005$), but was not inhibited by desferrioxamine (Fig. 7). On the other hand, LPS-induced IL-6 secretion was still inhibited by chloroquine when macrophages were pretreated with desferrioxamine or FeNTA.

DISCUSSION

In the past 10 years evidence has emerged that stresses the importance of macrophages and their cytokine products in the pathophysiology of human cerebral malaria.[1,2] Therefore, it can be argued that mechanisms for inhibiting their production are important to study. A number of agents have been shown to inhibit cytokine production, including IL-4,[17] pentoxifylline[13] or glucocorticoids.[18] We investigated the effect of chloroquine and most of the anti-malarial drugs easily available, on TNF and IL-6 production.

LPS- and parasite culture supernatant-dependent TNF and IL-6 production were reduced by more than 70% by chloroquine at concentrations achievable in humans. Time-course experiments demonstrated that the inhibition of TNF and IL-6 were maximal when chloroquine was added only 2 hr prior to stimulation. Moreover, this effect could be observed to a lesser extent when chloroquine was added up to 2 hr after LPS or parasite culture supernatants.

To analyse the time-course of this effect further, macrophages were incubated for 2 hr with chloroquine before washing and then were stimulated with LPS. We observed that TNF and IL-6 inhibition seemed to be partly irreversible, as the cells exhibited diminished responsiveness to LPS 72 hr after chloroquine has been removed.

Chloroquine has been previously shown to inhibit IL-1 secretion, presentation of antigens by accessory cells, to depress mitogen responsiveness and natural killer cell cytotoxicity and to display anti-inflammatory properties.[19] Recently, the action of prophylactic doses of chloroquine (600 mg base/week for 6 weeks) has been shown to inhibit the phagocytosis of IgG-

coated sheep red blood cells.[20] It seems conceivable that chloroquine used at pharmacological concentrations could inhibit cytokine over-production *in vivo* and that the observation made here is likely to be clinically relevant. However, the molecular basis for the activity of the drug is still unknown.

It might be argued that the inhibition of TNF and IL-6 production by chloroquine could be induced by a mechanism related to its weak base properties. Cationic amphiphilic compounds like chloroquine can accumulate in acidic intracellular compartments including lysosomes and endosomes. The resultant elevated pH can cause major alterations in intracellular trafficking, affecting receptor-mediated endocytosis, intralysosomal digestion, exocytosis and the biosynthesis of secretory proteins.[21] The intralysosomal concentration of chloroquine has been shown to reach 1000 times that of its extracellular concentration but it has been recently demonstrated that reacidification of lysosomes occurs only 3 hr after chloroquine treatment and that chronic chloroquine treatment is not associated with alkalinization of hepatocyte lysosomal pH.[22] These data provided evidence against a major role for weak base properties in chloroquine-dependent TNF inhibition. In our experiments macrophages were washed and incubated 12 hr or more after chloroquine treatment, providing enough time to lysosome reacidification. Moreover, quinoline-containing drugs (e.g. chloroquine, quinine, mefloquine) accumulate in intracellular acid vesicles because of their weak base properties, and many members of this family (including quinine and mefloquine) increase vesicle pH at their biologically active concentrations,[23] but did not inhibit TNF and IL-6 secretion as demonstrated here. NH_4Cl, a weak base amine which accumulates in acidic compartments to the same extent as chloroquine, did not prevent TNF or IL-6 production. Taken together, these findings support the hypothesis that chloroquine-induced cytokine inhibition is not mediated through a lysosomotropic mechanism.

Nevertheless, considering that the intracellular transport and mobilization of iron depend upon an acidic environment,[24] and that lactoferin, an iron-binding protein, inhibits TNF, IL-1β and IL-2 production by macrophages,[25] we assumed that chloroquine could inhibit cytokine secretion through interference with iron metabolism. Iron deficiency or iron overload are associated with malfunction of the immune response, so it may be that iron has a regulatory role on this response.[26-28] When macrophages where preincubated with desferrioxamine, an iron chelator, we observed first that LPS-induced TNF secretion was subsequently decreased, secondly that chloroquine-induced TNF inhibition was completely reversed and thirdly that desferrioxamine seemed not to have any effect on LPS-induced IL-6 secretion. Our results lead us to suggest that chloroquine may inhibit TNF secretion by disrupting the normal iron metabolism, as LPS-induced TNF secretion by macrophages pretreated with desferioxamine is dramatically enhanced by chloroquine. Chelating iron suppressed the inhibitory effect of chloroquine on TNF secretion. Moreover, when macrophages where pretreated with FeNTA, which hold iron in a soluble form at neutral or alkaline pH, LPS-induced TNF secretion was reduced to the same extent with or without chloroquine.

We may now be in a position to consider that TNF secretion is directly dependent on intracellular iron metabolism, and that chloroquine acts on TNF secretion by disrupting iron homeostasis outside lysosomal compartments, as enhancement of intralysosomal pH seems not to be related to the chloroquin[e] effect.

It is not surprising that IL-6 seems not to be regulated b[y] iron metabolism, as this cytokine plays a different role in th[e] induction of the immune response and may itself have [a] regulatory effect on TNF secretion.[29] However, despite almos[t] half a century of use and extensive studies, we still do no[t] understand how chloroquine works at the molecular level on th[e] malaria parasite.[30] Thus, one might speculate that the chloroquine effect on cytokine secretion is not only related to iro[n] metabolism, and that other mechanisms could be involved.

Chloroquine has been shown to inhibit cholera toxin induced secretory effect on rat intestinal vascular endothelium.[3] Nevertheless chloroquine tested in cholera patients did no[t] diminish the severity or duration of the disease,[31] because it ha[s] to be administered before the beginning of the fluid loss. It is no[t] known whether chloroquine may be used prophylactically t[o] prevent cholera symptoms, but this kind of prophylaxis directed against pathophysiological mechanisms instead o[f] against pathogens themselves, seems to be similar to that whic[h] we are trying to develop to prevent TNF over-productio[n] during cerebral malaria.

Our *in vitro* observations suggest that chloroquine may b[e] able to prevent cerebral malaria in humans. If TNF is the ke[y] mediator ultimately responsible for mortality, then it is logica[l] to hypothesize that moderate chloroquine plasma levels shoul[d] contain serum TNF levels below the threshold at which cerebra[l] malaria and death occur. Although such extrapolation from *i[n] vitro* experiments should be made with caution, this approac[h] could provide new tools for an anti-disease therapy regardless o[f] the emergence of *P. falciparum* multi-drug-resistant strains. W[e] may then be in a position to consider whether chloroquine ca[n] be used as a prophylactic agent against the clinical severity o[f] malaria and whether it can transform an attack of malaria int[o] an unpleasant experience, but not a life-threatening one. A larg[e] study has begun in an endemic area to provide clinical evidenc[e] for the efficiency of moderate levels of chloroquine.

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