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THE ROLE OF T LYMPHOCYTES IN IMMUNITY TO *Plasmodium falciparum*

Enhancement of Neutrophil-Mediated Parasite Killing by Lymphotoxin and IFN-γ: Comparisons with Tumor Necrosis Factor Effects[1]

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Human neutrophils were treated with IFN-γ or lymphotoxin and their interaction with the asexual blood forms of *Plasmodium falciparum* was studied by a radiometric assay and microscopy. The results showed that human neutrophils inhibited the growth of *P. falciparum* and killed the parasite via a phagocytic mechanism. The cytokines significantly augmented the neutrophil-mediated killing of the parasite. When compared with the effects of TNF-α similar results were seen although IFN-γ and TNF-α were effective at 10-fold less the concentration of lymphotoxin. The maximum neutrophil-mediated parasite killing was seen in the presence of immune serum and the cytokines. These findings suggest that T cells regulate the antimalarial activity of neutrophils.

Acquired immunity to malaria probably requires the development of cell-mediated responses as well as protective antibody. Although there is ample evidence for the participation of T lymphocytes in protective immunity to malaria, the actual mechanisms by which the lymphocytes are able to control the growth of the parasite and resolve the infection remain to be defined (1). One attractive possibility is that the cytokines produced by T cells activate effector cells such as macrophages and neutrophils for killing of the parasite. Antibody is also important for opsonization of the parasite (2-4).

Neutrophils are abundant in the circulation and phagocytosis of the malaria parasite by peripheral blood neutrophils has been reported (5, 6). Neutrophils have also been found in the brain lesions where sequestration occurs in *Plasmodium falciparum* malaria (7). In vitro studies have demonstrated that human neutrophils interact with opsonized *P. falciparum* infected RBC as well as merozoites, and kill all stages of the parasite via a phagocytotic mechanism (3, 8-11). Cytokines released by activated T cells could modulate the antiplasmodium activity of neutrophils. It has been shown that the cytokines, LT,[3] and IFN-γ, released by T lymphocytes enhance the neutrophil response in these cells to a range of soluble and particulate stimuli (12-17). In this study we have investigated the effects of LT and IFN-γ on the killing of asexual intraerythrocytic forms of *P. falciparum* by human neutrophils.

MATERIALS AND METHODS

Reagents. Human rLT (sp. act. 1.2×10^8 U/mg, >99% purity), rIFN-γ (sp. act. 2×10^7 U/mg, >99% purity), and rTNF-α (sp. act. 6×10^7 U/mg, >99% purity) were produced by Genentech Inc. (South San Francisco, CA) and kindly provided by Dr. G. R. Adolf from Boehringer, Ingelheim, Vienna, Austria. These products contained <0.01% ng/ml endotoxin as determined by *Limulus* amoebocyte lysate assay. Two other preparations of IFN-γ were also used. Purified human IFN-γ produced from buffy coats by induction with A23187 and mezerein, 1×10^6 U/mg, was obtained from Sigma (St. Louis, MO) and purified human IFN-γ produced by T lymphocytes stimulated with PHA and purified to remove contaminating proteins such as IL-2 and B cell growth factor (5×10^5 U/ml) was obtained from Cellular Products (New York, NY).

Murine mAB (IgG) to human LT, TNF-α, and IFN-γ were provided by Dr G. R. Adolf (Boehringer Ingelheim, Vienna, Austria). Goat anti-mouse IgG was obtained from Cooper Biomedical (Malvern, PA).

RPMI 1640 medium (Flow Laboratories, N.S.W., Australia), HBSS, and Medium 199 (GIBCO Laboratories, Grand Island, NY), Giemsa (Gurr, BDH, Poole, England), [^{3}H]hypoxanthine (Amersham Corp, Arlington Heights, Ill.), D-glucose (BDH Chemicals, Victoria, Australia), *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes buffer) (Sigma, St. Louis, MO) were obtained. All reagents were prepared in pyrogen-free distilled water and stored according to the manufacturers' instructions.

Parasite. Six isolates of *P. falciparum* were maintained under in vitro conditions in group O$^+$ human RBC in RPMI 1640 medium (supplemented with 10% heat-inactivated group AB$^+$ serum, 0.25% D-glucose, and 0.85% Tes buffer). These isolates were originally collected from malaria-infected individuals in Papua New Guinea and Soloman Islands. The cultures were gassed with 1% O_2, 5% CO_2 in N_2. Experiments were performed with synchronized and nonsynchronized cultures at 1×10^8 E/ml with 3 to 5% parasitaemia.

Sera. IS refers to serum obtained from individuals who were long term residents in malaria-endemic areas in Papua New Guinea or Soloman Islands. A total of 36 samples were used at different times. These samples contained high titers of anti-*P. falciparum* antibody (18) and <10 hemolytic units of C. NS were obtained from 15 group AB$^+$ Australians who had not been to malaria-endemic areas. A portion of this was heated at 56°C for 30 min to inactivate C activity and referred to as HNS. The mean immunoglobulin levels in IS and NS/HNS were 12.39 (IgG = 9.70) g/liter and 13.76 (IgG = 9.74) g/liter, respectively.

Preparation of neutrophils. Neutrophils were prepared from the peripheral blood of healthy volunteers by the rapid single-step technique (19) involving 30-min centrifugation of the blood on Hypaque-Ficoll medium (d = 1.114). These cells were >96% pure and >99%

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[3] Abbreviations used in this paper: LT, lymphotoxin; IS, immune serum; NS, normal serum; HNS, heat-inactivated NS; IRBC, infected RBC; MNL, mononuclear lymphocyte.

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viable as determined by the trypan blue exclusion method. However, it was not unusual to find our preparations to be of >99% purity. The main contaminant was the eosinophil. Contamination with mononuclear leukocytes was <1%. Neutrophils were suspended in Medium 199 at 2×10^7/ml.

Neutrophils (10^6 in 50 μl Medium 199) were treated with LT (1000 U) or IFN-γ (100 U) in 50 μl HBSS in microdilution plates (Linbro, Flow Laboratories, Inc., McLean, VA) at 37°C for 30 min. In some experiments neutrophils (10^6) were treated with a range of cytokine concentrations (1 U to 5×10^4 U).

Growth inhibition assay. This was assayed as described previously (3). Experiments were conducted in 96-well flat-bottomed microdilution plates comparing primarily nine different treatment groups. *P. falciparum* IRBC were incubated in the presence of IS (group 1), NS (group 2), HNS (group 3), neutrophils plus IS (group 4), neutrophils plus NS (group 5), neutrophils plus HNS (group 6), cytokine-treated neutrophils plus IS (group 7), cytokine treated neutrophils plus NS (group 8), and cytokine-treated neutrophils plus HNS (group 9). After the pretreatment of neutrophils with the relevant cytokines or HBSS, RBC (5×10^6 with 3 to 5% parasitemia in 50 μl of malaria medium) and 50 μl of serum or malaria medium were added to the wells and mixed. The plates were incubated for 2 h at 37°C in 5% CO_2 in air. The wells were pulsed with 1 μCi of [^{3}H] hypoxanthine and incubated for a further 18 h at 37°C. Individual well contents were then collected with the use of a semiautomated sample harvester (Titertek, Flow Laboratories, N.S.W., Australia), the [^{3}H]hypoxanthine incorporation measured by liquid scintillation and percent growth inhibition of the parasite calculated as described previously. Percent inhibition of growth of the parasite was calculated by the following formula: percent inhibition = (dpm of parasite with serum − dpm of parasite with serum interacted with neutrophils)/dpm of parasite with serum. Because neutrophils took up negligible amounts of the label during this period, it was possible to determine accurately the neutrophil-mediated inhibition of [^{3}H]hypoxanthine uptake by the parasites.

The effects of LT and IFN-γ on the neutrophils were also compared with the neutrophils pretreated with TNF-α (100 U), a cytokine produced by lymphocytes and monocytes, which has already been shown to increase neutrophil-mediated killing of *P. falciparum* (3).

In addition, the direct effects of LT, IFN-γ, and TNF-α on *P. falciparum*-infected E were compared, in the presence and absence of normal and immune serum, by using a range of concentrations (1 U to 5×10^4 U).

The effects of 1) neutrophils (1×10^6) with increasing numbers of infected E (1×10^5 to 1×10^7 RBC with 5% parasitemia) and 2) a constant number of E (5×10^6 RBC with 5% parasitemia) with increasing numbers of neutrophils (1×10^5 to 1×10^7) were also studied.

Light and electron microscopic examinations. Neutrophil-parasite interaction was studied by preparing cytocentrifuge smears of the microdilution plate contents after staining with Giemsa. Neutrophils and IRBC were also interacted at 5×10^6 RBC (5% parasitemia)/ 10^6 neutrophils in 10-ml sterile tubes while rotating on a nutator (Dickinson Company, Parsippany, NJ) for 30 min and centrifuged at $75 \times g$ (IEC HN-SAII bench centrifuge) for 10 min. The pellet was fixed in 1% gluteraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After postfixation in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, the pellet was dehydrated through a graded series of ethanol and embeded in Spurr's resin, polymerized at 70°C for 24 h, and sectioned using a Diatome diamond knife. Silver gold sections were mounted on copper grids, stained with uronyl acetate and lead citrate, and examined with the use of a Hitachi H-7000 transmission microscope.

Adsorption of cytokine preprarations. mAB for LT, IFN-γ, and TNF-α were applied to anti-mouse Ig-coated microtiter plates. Similarly, captured non-cytokine mAB-coated wells were used for mock adsorption of the preparation. In this procedure 10% sheep serum was used as a nonspecific blocking agent throughout (20). After mAB were bound, the wells were extensively washed. Then to the wells were added relevant cytokines (1500 U LT, 150 U TNF-α or IFN-γ) and the plates were incubated at 37°C for 30 min. The supernatants were collected and compared in the radiometric growth inhibition assay.

Heat inactivation of cytokines. The cytokine preparations were heated at 80°C for 30 min and compared with the nonheated cytokines. This was used as a method to distinguish between the effects of the cytokines from those of LPS contamination of the preparation based on the fact that LPS activity is resistant even at boiling temperatures.

Statistical analysis. The results were compared by the two-tailed Student's *t*-test.

RESULTS

The results of the radiometric assay showed that normal human neutrophils caused a small percent growth inhibition of *P. falciparum*, of which the highest inhibition was seen in the presence of immune serum (Table I). When a constant number of neutrophils (1×10^6) was reacted with increasing numbers of IRBC, in the presence of immune serum but no cytokines, there was an increased growth inhibition (27%) between 5×10^3 and 5×10^4 IRBC, which remained at this level with further increase in IRBC (up to 5×10^5). However, the parasite (2.5×10^5 IRBC) inhibition was markedly increased when the neutrophil number was increased, giving >80% inhibition with 1×10^7 neutrophils (Fig. 1). A similar trend was seen with normal serum but the amount of inhibition was much lower at all neutrophil concentrations.

The efficiency of parasite inhibition was also increased when the neutrophils (1×10^6) were interacted with IRBC in the presence of cytokines. The maximum percent growth inhibition of the parasite was observed when neutrophil-IRBC interaction occurred in the presence of cytokines as well as immune serum (Table I). Both IFN-γ and TNF-α were equally efficient in augmentation of neutrophils to inhibit *P. falciparum* growth. However a 10-fold higher concentration of LT was required to achieve a similar augmentation of parasite killing (Fig. 2, Table I).

The possibility that an endotoxin contamination is responsible for the stimulatory effects of neutrophils was tested. Heating of cytokines completely abolished any stimulatory activities of neutrophils (data not presented). Furthermore, mAB against LT, IFN-γ, and TNF-α neutralized the neutrophil stimulatory activity of the respective cytokines (data not presented). The mAB alone without the cytokine did not increase the percent growth inhibition of the parasite.

In this study, various isolates (Knob$^+$ and Knob$^-$) of *P. falciparum* from synchronous and asynchronous cultures were interacted with neutrophils. No significant differences were observed between the isolates in relation to their ability to adhere to neutrophils and consequent percent growth inhibition.

Light and electron microscopic studies (Fig. 3) repeatedly showed that the growth inhibition of parasite measured by the radiometric assay was associated with neutrophil-mediated phagocytosis and killing of the parasite. Neutrophils, individually or after formation of syncytia, adhered and phagocytosed merozoites, rings, and immature or mature schizonts. Although it was difficult to quantitate phagocytosis morphologically, it was evident that cytokine treatment resulted in an increased proportion of neutrophils phagocytosing *P. falciparum*. For example in the presence of IFN-γ, 42.5 ± 8.2% of neutrophils contained at least one engulfed parasite compared with only 13.3 ± 5.8% for untreated cells. This resulted in an increase in the average number of parasite phagocytosed.

The cytokines did not cause any degeneration (morphologically) or growth inhibition (radiometric assay) when directly reacted with IRBC, in the presence of normal and immune serum when used at concentrations of 1 U to 5×10^4 U.

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TABLE I

Inhibition of [H^3]hypoxanthine uptake by P. falciparum after interaction with neutrophils that were pre-incubated with LT, IFN-γ, and TNF-α or diluent in the presence of IS, NS, and HNS

	Neutrophils											
	Cytokine-activated									Normal		
	IS with:			NS with:			HNS with:			IS with: diluent (36)	NS with: diluent (36)	HNS with: diluent (36)
	LT (22)[a]	IFN-γ (8)	TNF-α (36)	LT (15)	IFN-γ (8)	TNF-α (35)	LT (15)	IFN-γ (8)	TNF-α (36)			
Percent growth inhibition of parasites (mean ± SEM)	41.7 ±0.7	41.1 ±2.3	43.3 ±1.2	17.5 ±1.3	22.7 ±2.1	18.6 ±2.6	11.2 ±3.2	14.2 ±6.3	13.1 ±4.0	26.7 ±2.7	15.5 ±1.8	7.9 ±1.6
	A	B	C	D	E	F	G	H	I	J	K	L

Statistics (Student's t-test two-tailed)

Not significant: A vs B; C vs D; E vs F

Significantly different: (A, B, C) vs (G, H, I) ($p < 0.001$); (E, F) vs K ($p < 0.05$); (A, B, C) vs (G, H, I) ($p < 0.001$); (D, E, F) vs I ($p < 0.001$); (A, B, C) vs J ($p < 0.005$); (G, H, I) vs L ($p < 0.05$); (A, B, C) vs K ($p < 0.001$); K vs L ($p < 0.05$); (A, B, C) vs L ($p < 0.001$); J vs L ($p < 0.001$); (E, F) vs I ($p < 0.05$); H vs I ($p < 0.05$)

[a] No. of experiments.

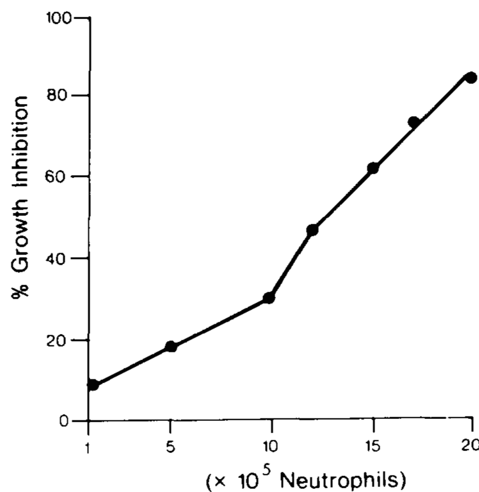

Figure 1. Effects of neutrophil number on the inhibition of [^{3}H]hypoxanthine uptake by *P. falciparum* in the presence of IS.

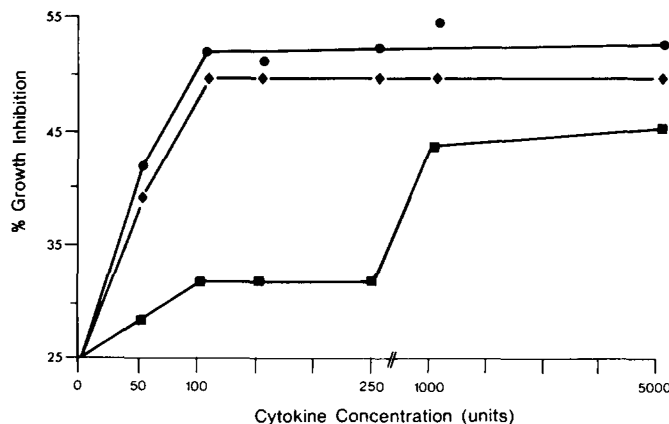

Figure 2. Effects of different concentrations (1 to 5 × 10^3 U/10^6 neutrophils) of LT (■——■), IFN-γ (♦——♦), and TNF-α (●——●) on the anti-*P. falciparum* activity of neutrophils in the presence of IS, expressed as the mean percent inhibition of [^{3}H]hypoxanthine uptake by the parasite. All the cytokines were compared within the same experiment by using neutrophils from one individual. Growth inhibition by neutrophils in the absence of cytokines was 25%.

DISCUSSION

The results of radiometric assays established that human neutrophils inhibited the growth of *P. falciparum* asexual blood forms. The microscopic studies confirmed that the parasites were phagocytosed and consequently killed by the neutrophils. Although this occurred even in the absence of the C and antimalarial antibodies, opsonization by immune serum significantly increased the percent phagocytosis, a finding consistent with previous observations (2–4). It was possible to further enhance the killing of *P. falciparum* by increasing the number of neutrophils. However, neutropenia has been reported in malaria infections due to redistribution of neutrophils away from peripheral circulation (21), thus this may not be relevant to the in vivo inhibition of parasitaemia. Therefore it is important to note that the efficiency of parasiticidal activity of a constant number of neutrophils was significantly enhanced by LT, IFN-γ and TNF-α which was associated with an increased proportion of neutrophil phagocytosing the parasite. The cytokine-augmented killing of the parasite was optimal in the presence of immune serum. It is likely that the antimalarial antibodies in immune serum are responsible for opsonizing the parasite, because the serum contained high titers of antimalarial antibodies and previously we have demonstrated that the Ig fraction of this serum promotes the neutrophil-mediated parasite damage (3).

As shown in Table I, there was no significant difference between the optimum percent phagocytosis caused by either LT, IFN-γ or TNF-α. However, IFN-γ and TNF-α were effective at 10-fold less the concentration of LT. The neutrophil stimulatory characters of these cytokines was not due to endotoxin contamination as cytokine-specific mAB neutralized the activities and heat inactivation (80°C, 30 min) completely abolished the neutrophil-augmentation property of the cytokines. IFN-γ and LT are predominantly produced by T lymphocytes (22–24), whereas TNF-α is produced predominantly by macrophages but also to some extent by T cells, large granular lymphocytes, monocytes, and some cells of nonhemopoetic origin (25–27).

The predominant link with the immune memory of the host is considered to occur through IFN-γ which is released by T lymphocytes, usually after a second exposure to Ag of microbial origin (28). In *Plasmodium chabaudi*-infected mice, early response to the parasite is dominated by inflammatory type $CD4^+$ cells which produce IFN-γ (29), and this cytokine has been detected in the serum of mice within hours of infection with *Plasmodium berghei*

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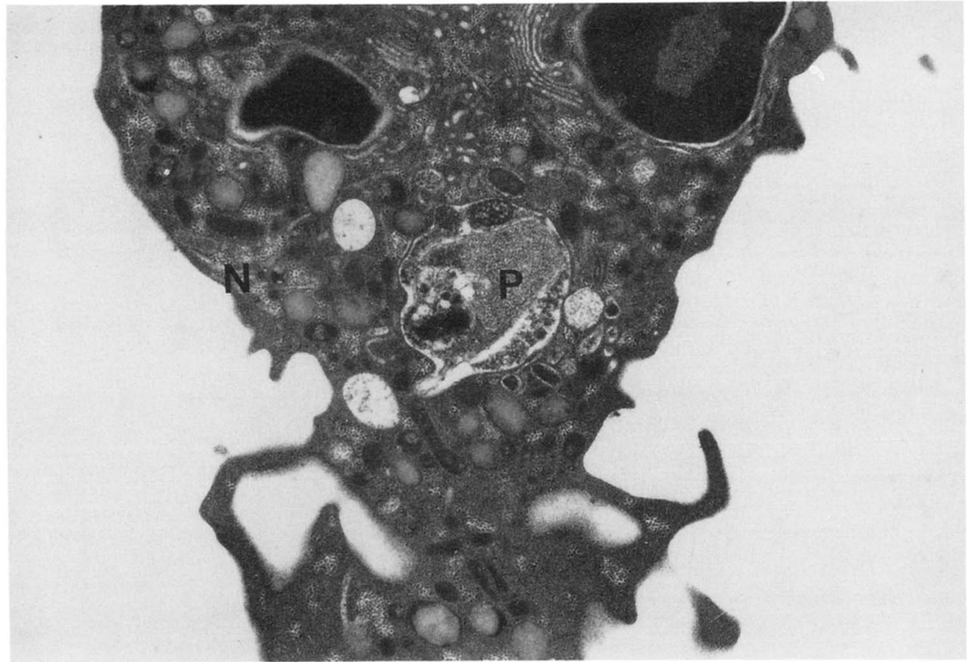

Figure 3. Electron micrograph of a lymphotoxin-treated neutrophil (*N*) with phagocytosed *P. falciparum* (*P*). Assay conducted in the presence of immune serum.

(30). IFN-γ has also been detected in the serum of patients shortly after an acute attack of *P. falciparum* (31, 32). The significance of the production of IFN-γ has been related to the host protection as well as the pathogenesis of cerebral malaria. When human IFN-γ was administered to chimpanzees infected with *Plasmodium vivax* (33) and rhesus monkeys infected with *Plasmodium cynomolgi* (34) diminished parasitemias have been observed. When *P. chabaudi adami*- (35) and *Plasmodium yoelii*- (36) infected mice were treated with repeated injections of IFN-γ, the parasitemia was reduced and degenerative forms appeared. These observations have been described as the effects of IFN-γ against the exoerythrocytic forms of *Plasmodium* (37). However, the results of the present study indicate that IFN-γ had no direct parasiticidal activity in vitro, but it increased the neutrophil-mediated killing of intraerythrocytic forms of *P. falciparum*. IFN-γ also increased the human monocyte-mediated killing of intraerythrocytic *P. falciparum* (38), and augmented neutrophil-mediated killing of bacteria (39), fungi (40, 41), and *Entamoeba histolytica* (12). It has been suggested that IFN-γ acts on human neutrophils through a complex, long lasting mechanisms and the cytokine treatment prolongs neutrophil survival at 37°C in a fully functional state (16). IFN-γ increase the protein synthesis in human neutrophils (26, 42), and augments the respiratory burst (15–17, 43, 44) and lysosomal enzyme release (15, 44). These effects may be due to the increased neutrophil-mediated phagocytosis of *P. falciparum* observed in our study. The molecular basis of the effects of IFN-γ on neutrophil respiratory burst has not been fully described. Modification of either surface recepter expression (44), alterations of transducing systems involved in stimulus response coupling or the kinetic properties of the NADPH-oxidase system may all be involved (45).

IFN-γ is believed to be responsible for the enhanced TNF-α levels in *P. falciparum*-infected individuals (46). It has been shown that IFN-γ increases the expression of receptors for TNF-α on various cells including monocytes (47, 48). When IFN-γ was injected into *Plasmodium vinckei*-infected mice, TNF-α production was greatly enhanced (49). The results of the present study have demonstrated that IFN-γ and TNF-α are equally capable of augmenting human neutrophils to kill *P. falciparum*. In the case of IFN-γ, because of the contamination of neutrophils by a small proportions (<1%) of MNL, it is possible that IFN-γ may act by inducing TNF-α production via these MNL. However, this is unlikely, because our studies (R. Staugas, A. Ferrante, and B. Rowan-Kelly, unpublished results) have shown that the number of contaminating MNL, in stimulation with IFN-γ, would be expected to produce <1 U of TNF-α. This quantity of TNF-α was shown in previous studies (3) not to be effective in significantly stimulating neutrophil antiplasmodial activity. When *P. chabaudi adami*-infected mice were treated with TNF-α with the use of an osmotic pump, the parasite degenerated and parasitemia was reduced (35). TNF-α-augmented antiparasitic activity of neutrophils (3, 12, 40) and macrophages (50, 51) has been reported and the possible mechanisms involved have been discussed (3, 52, 53). High levels of TNF-α increase the adherence of endothelial cells where sequestration of the IRBC occurs in the brain (46) and the role of IFN-γ and TNF-α in relation to pathogenesis of cerebral malaria has been addressed (46, 49).

No information is available whether LT is produced in malaria-infected individuals or if this cytokine plays a role in the context of malaria. However, in vitro experiments carried out in our laboratory showed that lymphocytes produced LT in the presence of *P. falciparum* schizont Ag (54) and the present study convincingly shows that when available at high concentrations LT could be as effective as TNF-α in mediating antiplasmodial activity. Although initially TNF-α and LT were described as having similar cytotoxic activities (55), more recent work has indicated that LT is less active on certain tumor lines (56, 57) and has shown at least 100-fold less activity than TNF-α in relation to endothelial cell adherence (58, 59) and release of various cytokines by fibroblasts (60),

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endothelial cells (59), and monocytes (61). The results of the present study showed that a 10-fold higher concentration of LT was required than TNF-α to obtain maximum neutrophil-mediated killing of *P. falciparum*. These results are comparable to the effects of LT and TNF-α in relation to oxygen radical formation by neutrophils (13). It has been shown that LT increases the ingestion of latex beads (17), respiratory burst, and release of lysosomal enzymes (15) by neutrophils, which may explain the enhanced LT-induced phagocytosis observed in the present study.

The precise role of IFN-γ, LT, or TNF-α in the host's defense mechanisms still remains to be defined. The results presented in this report as well as the others (3, 62, 63) indicate these cytokines have no direct *P. falciparum* activity. These cytokines, however, may have a role in the modulation of the immune response in vivo. Our results may explain the discrepancy between the absence of direct antiplasmodium activity of the cytokines in vitro but their ability to reduce parasitemia in vivo (35, 36, 63) and shed new light on the biologic significance of these mediators. The results presented in this report also indicate some aspects of involvement of T lymphocytes in protective immunity to malaria. Our data further support the concept that for effective immunity against *P. falciparum* there is a requirement for both products of the cellular and humoral immune responses.

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