Trop. Med., 41 (4), 197-214, December, 1999

Both Glycolipid and Protein Components are Required for *Plasmodium falciparum* induced TNF-α and IL-1β Production in Human Monocytic Cells

Hla Myat MON, Haruki UEMURA, Shusuke NAKAZAWA and Hiroji KANBARA

Department of Protozoology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Abstract: Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are the endogenous pyrogens which mediate fever in malaria. The excessive production of TNF-α is associated with pathology of human malaria. The nature and properties of malaria antigens, which stimulate monocyte to secrete these cytokines were studied *in vitro* using human monocytic cell line THP-1. THP-1 cells produced the cytokines in response to *Plasmodium falciparum* malaria antigens similar to the response of peripheral blood monocytes. Malaria parasite components of infected erythrocytes and their culture supernatant were separately analyzed. Soluble and insoluble components of the infected erythrocytes and their culture supernatant stimulated cytokine production by THP-1 cells. Acid, base and pronase treatments of malaria culture supernatant greatly reduced the cytokine inducing activity, suggesting that both glycolipid and protein components are essential for cell stimulation. Considering the ultrafiltration results together, we assume that a complex of glycolipid and protein stimulates host cells to induce cytokine secretion. Application of Triton X-114 solubilization and phase separation procedures to the infected erythrocytes revealed that the membrane-free hemozoin pellet did not have any stimulation activity, whereas the hydrophobic components seemed to contribute to TNF-α and IL-1β production.

Key words: Plasmodium falciparum, glycolipid, TNF-α, IL-1β, THP-1

INTRODUCTION

Malaria remains one of the most important diseases of the world giving rise to an intense negative impact on socioeconomic development. One of the important factors for the clinical symptoms in acute *Plasmodium* malaria is an increased level of tumor necrosis factor-α (TNF-α) (Clark et al., 1989; Kwiatkowski et al., 1990; Scuderi et al., 1986). In both human and murine malaria cases, the development of severe complications such as cerebral malaria is usually associated with excessive production of TNF-α (Grau et al., 1987; Kern et al., 1989; Kwiatkowski et al., 1990). Furthermore, a direct correlation was observed be-

Received for publication, February 8, 2000
Contribution No. 3846 from the Institute of Tropical Medicine, Nagasaki University

tween the circulating TNF levels, the parasite density, and the severity of the disease, particularly in non-immune individuals (Kern et al., 1989). IL-1β together with TNF-α are known as endogenous pyrogens (Dinarello et al., 1988) responsible for characteristic periodic febrile episodes of malaria.

Several *in vivo* and *in vitro* studies have been carried out to observe the nature and properties of the parasite antigens and their effects on host responses in malaria infection (Bate et al., 1989; Jakobsen et al., 1991; Kristensen and Jakobsen, 1996; Taverne et al., 1990). It has been shown that malaria exoantigens liberated at the time of schizont rupture stimulate the release of TNF from the host mononuclear cells (Kwiatkowski et al., 1989). Among the malaria exoantigens or toxins, glycosylphosphatidylinositol (GPI) moiety has been identified as a dominant soluble toxin of *P. falciparum* (Schofield and Hackett, 1993; Tachado et al., 1997). The other virulence factor, which induces TNF-α and IL-1β, is malaria pigment or hemozoin (Pichyangkul et al., 1994). The cytokine inducing activity of malaria pigment has been detected irrespective of phagocytosis. We previously reported the TNF-α release in response to both soluble and insoluble components of *P. falciparum* infected erythrocytes (Nagao et al., 1996). In the present study we partially characterized the soluble and insoluble fractions of *P. falciparum* antigens to evaluate their nature and to identify the dominant components that contribute to exert cytokine production from the human monocytic cells.

Production of proinflammatory cytokines in malaria infection has been studied *in vitro* using fresh human peripheral blood monocytes (Allan et al., 1993; Picot et al., 1990; Taverne et al., 1990). In this report we present the usefulness of a human monocytic cell line THP-1 as an alternative to fresh human monocytes to study TNF-α and IL-1β production induced by malaria antigen.

Materials and Methods

Reagents

All-trans-Retinoic Acid, 1α, 25-dihydroxyvitamin D3 (Calcitriol), both from Wako Pure Chemical Industries, Ltd., Japan, and Phorbol 12-Myristate 13-Acetate (PMA) obtained from Calbiochem, USA, were used for induction of cell differentiation. Bacterial lipopolysaccharide (LPS, *Escherichia coli* 055:B5) was from DIFCO Laboratories, USA. Pronase from *Streptomyces griseus* was purchased from Boehringer Mannheim GmbH. Sodium deoxycholate, Polyoxyethylene (20) sorbitan monolaurate (Tween 20) were obtained from Wako Pure Chemicals, Ltd. Japan. Triton X-100 (Kishida Chemicals Co., Ltd. Japan), and Nonidet P-40 (Iwai Kagaru Co., Ltd. Japan) were also used. Polymyxin B and Ocytlphenoxypoly-ethoxy ethanol (Triton X-114) were purchased from Sigma, USA. Recombinant interferon-γ (IFN-γ) was obtained from Genzyme, USA.

Preparation of P. falciparum antigens

The *P. falciparum* FCR3 strain was maintained in long-term culture according to the method previously described (Trager and Jensen, 1976). Briefly, parasites were cultured in

group O erythrocytes adjusted to 5% hematocrit using RPMI 1640 medium (Gibco, U.S.A.) supplemented with 10% blood group O human serum and 25 μg/ml gentamicin in 25 cm^2 tissue culture flasks (Cellstar, Greiner Labortechnik, Germany) and kept at 37℃ in a moist atmosphere of 5% O_2, and 5% CO_2. Medium was renewed daily. Synchronization was done with 5% sorbitol according to Lambros and Vanderberg, 1979.

Following synchronization for 36-48 hr, culture containing mainly schizont-infected erythrocytes with a parasitemia of 4-5% was centrifuged at 5,000 x g for 5 min. The culture supernatant was passed through 0.20-μm filter units (Minisart, Sartorius, Germany) to remove any contaminating particles.

The remaining packed red cells were mixed with four volumes of sterile water and lysed by freeze-thawing three times. The soluble fraction of infected erythrocyte lysate was obtained by centrifugation at 10,000 x g for 10 min. The insoluble fraction containing malaria pigment was washed with PBS three times and resuspended to the same volume of soluble lysate fraction with RPMI 1640 medium.

We generally obtained 30 ml of culture supernatant and 7.5 ml each of soluble and insoluble lysate fractions from 6 parasite cultures.

Ultrafiltration of culture supernatant

One portion of 0.20-μm membrane filtered culture suppernatant was subjected to ultrafiltration through Centricon YM-10 and YM-100 Centrifugal filter devices (Amicon Bioseparations) with 10,000 and 100,000 molecular weight (MW) cut-offs respectively.

Enzymatic and chemical treatments of culture supernatant

Malaria culture supernatant and YM-100 retentate were incubated with pronase (200μg/ml) for 16 hr at 37℃ followed by inactivation of the enzyme at 95℃ for 10 min. The control samples, without pronase addition but 95℃ for 10 min treatment were included. NaOH deacylation was carried out in the final 0.2 N condition at 56℃ for 2 hr. The reaction was terminated by neutralization with 5 N HCl. Next portion of the samples were incubated in 0.2 N HCl at 56℃ for 2 hr followed by neutralization with 5 N NaOH. Portions of treated samples were reconstituted with serum supplemented RPMI 1640 medium and tested for cytokine inducing activity.

Acetone precipitation of the proteins

The samples were mixed with 8 volumes of cold acetone and kept at -20℃ overnight. After centrifugation at 3,000 x g for 15 min at 4℃, the pellet was reconstituted to the original volume with serum-free RPMI 1640. Protein recovery was confirmed by quantitation using Bradford method and SDS gel electrophoresis.

Detergent treatments of soluble and insoluble fractions of infected erythrocytes

The insoluble fraction obtained from sequential centrifugation of *P. falciparum* culture was aliquoted and treated twice with non-ionic detergents Tritox X-100, Tween 20, Nonidet

P-40 and ionic detergent Sodium deoxycholate at 1% concentration in separate tubes. Detergent treatment was followed by washing two times with phosphate-buffered saline (PBS). Every step of treatment and washing was accompanied by sonication. The final pellets were reconstituted with RPMI 1640 medium.

Triton X-114 (TX-114) temperature-induced phase separation was carried out following a modified protocol as previously described (Smythe et al., 1990) for infected erythrocytes lysed in water. To the sample was added 0.5% TX-114 in Tris-buffered Saline (TBS) and incubated at 4℃ for 90 min. The supernatant was collected after an initial centrifugation at 10,000 x g for 30 min at 4℃ and layered on 6% sucrose containing 0.06% TX-114 followed by incubation at 37℃ for 5 min. The aqueous and detergent phases were collected after centrifugation at 900 x g for 5 min at 37℃ and precipitated by acetone as previously described. The remaining precipitate after the first TX-114 treatment was washed with 0.5% TX-114 in TBS for three times and resuspended in the same buffer. After sonication, the sample was overlayered on a 50% sucrose in order to remove any membrane fragments and centrifuged at 100,000 x g for 45 min at 4℃. The final pellet containing malaria pigment was reconstituted to the original volume with RPMI 1640 medium after washing with the same medium. The TX-114 phase separation procedure is summarized in Figure 8A.

Isolation of human fresh peripheral blood monocytes

Fresh human peripheral blood monocytes were isolated from buffy coats of blood gorup O (kindly provided by Japanese Red Cross Society) following a modified protocol previously described (Moriuchi et al., 1996). Briefly, the buffy coat was mixed with an equal volume of PBS, and layered on Ficoll-Paque Research Grade (Pharmacia Biotech, USA) in centrifuge tubes and centrifuged at 900 x g for 30 min at room temperature. Mononuclear cells were recovered from the intermediate layers between the plasma and the Ficoll-Paque, pooled in one centrifuge tube, washed with PBS three times, suspended in RPMI 1640 without serum supplement and seeded on plastic tissue culture plates (Falcon, Beckton Dickinson, USA). Following 1-2 hr incubation at 37℃ in humidified 5% CO_2 and 5% O_2, nonadherent cells were removed by repeated washing with PBS. Adherent cells were collected and reconstituted with RPMI 1640 supplemented with 10% heat-inactivated group O human serum followed by incubation in 48-well tissue culture plates (Costar, USA) at the concentration of 2 x 10^5 cells per well for 2-3 hr. More than 95% of adherent cells were monocytes as confirmed by Giemsa-stained smears and were used as fresh monocytes for cytokine analysis. Some fresh monocytes were cultured in the same condition for three days before the experiments with or without supplementation with 1,000 units/μl of recombinant IFN-γ, a macrophage activator. Viability of the three days old cells was more than 95%, observed by Trypan blue exclusion.

Cell culture

The human monocytic and myeloid leukemia cell lines THP-1, U937, HL60, K-562 and KY821A3 obtained from Health Science Research Resources Bank (Japan) were maintained at 37℃, 5% CO_2 and 95% relative humidity in RPMI 1640 medium supplemented with 10%

heat-inactivated fetal bovine serum and 25 μg/ml gentamicin. Viability of the cells was observed by Trypan blue exclusion.

Monocyte stimulation with malaria antigen preparations

Malaria culture supernatant was diluted five times in serum-free RPMI 1640 medium before incubation with fresh monocytes. Dilution of malaria antigen preparation was determined by preliminary dose-response experiments. Stimulated culture supernatants were collected at the indicated time points, centrifuged at 2,000 x g for 5 min to remove the sediments and stored at -20℃ before the assays for TNF-α, IL-1β, and IL-6.

Stimulation was also carried out with the monocytic cell lines HL60, U937, THP-1, K-562 and KY821A3 (5 x 10^4 cells per well). For differentiation, cells were preincubated with PMA or *all-trans*-Retinoic Acid and vitamin D3 combination in RPMI 1640 medium for 24 hr. Inducers were used at 10^{-7} M. The RPMI 1640 medium with the inducers was removed and replaced with the medium containing the test samples. The supernatants were collected after 48 hr and assayed for TNF-α and IL-1β. Polymyxin B (100 U/ml) was used to prove that the cytokine production was not due to endotoxin contamination.

Cytokine assays

The production of TNF-α, IL-1β, and IL-6 was measured using enzyme-linked immunosorbent assay (ELISA) kits (Cytoscreen, Biosource International, USA) following the manufacturer's specifications. The detection limits were 15.6 pg/ml for TNF-α, IL-6 and 3.9 pg/ml for IL-1β.

RESULTS

TNF production from human monocytic cell lines stimulated by malaria antigens

Several previous works were reported that *P. falciparum* infected red blood cells and their culture supernatant induced human monocytes to release TNF. We demonstrated that schizont stage culture supernatant of *P. falciparum* stimulates the increasing secretion of TNF-α up to 24 hr as well as IL-1β and IL-6 from fresh human peripheral blood monocytes *in vitro* (Figure 1). However, TNF-α release from the three days old monocytes was dramatically decreased to less than 1/10 th of fresh monocytes, although IFN-γ activation could augment the cell responses to some extent (Figure 2). To use monocytes for this experiment, we had to obtain fresh human blood constantly and it was difficult to completely remove the non-adherent lymphocytes from adherent monocytes even with repeated washes, providing about 95% purity of the cells.

We tested several human monocytic cell lines for cytokine release induced by malaria antigens. THP-1, U937, HL60 and K-562, potentially shown to produce TNF-α or IL-1β, were incubated with malaria culture supernatant and assayed for TNF-α production in the medium. Lymphocyte-like cell line KY821A3 was included as a control. None of these cells secreted

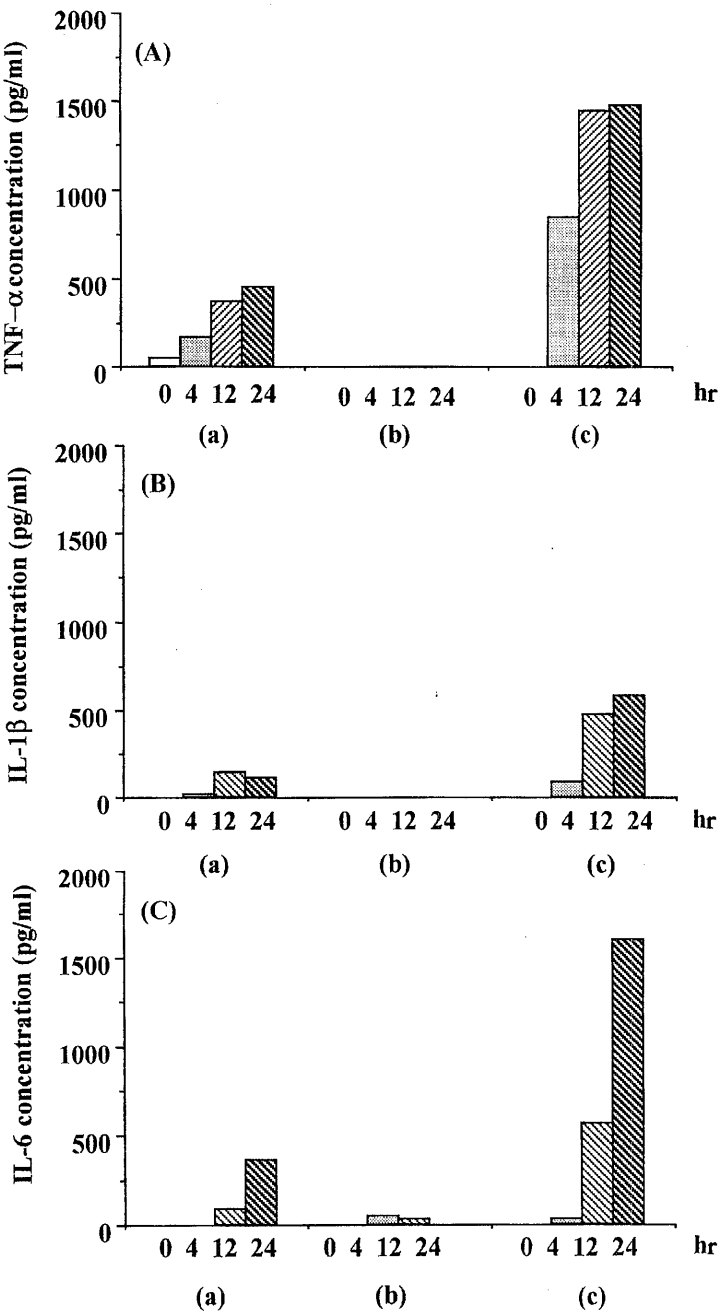

Fig 1. (A) TNF-α, (B) IL-1β, and (C) IL-6 production from fresh monocytes in response to stimulation by *P. falciparum* culture supernatant. Monocytes were incubated with (a) *P. falciparum* culture supernatant, (b) NIRBC, non-infected red blood cell culture supernatant, a negative control, and (c) LPS, bacterial lipopolysaccharide (1 μg/ml), as a positive control. Test samples were collected at 0, 4, 12 and 24 hr after incubation with the antigens.

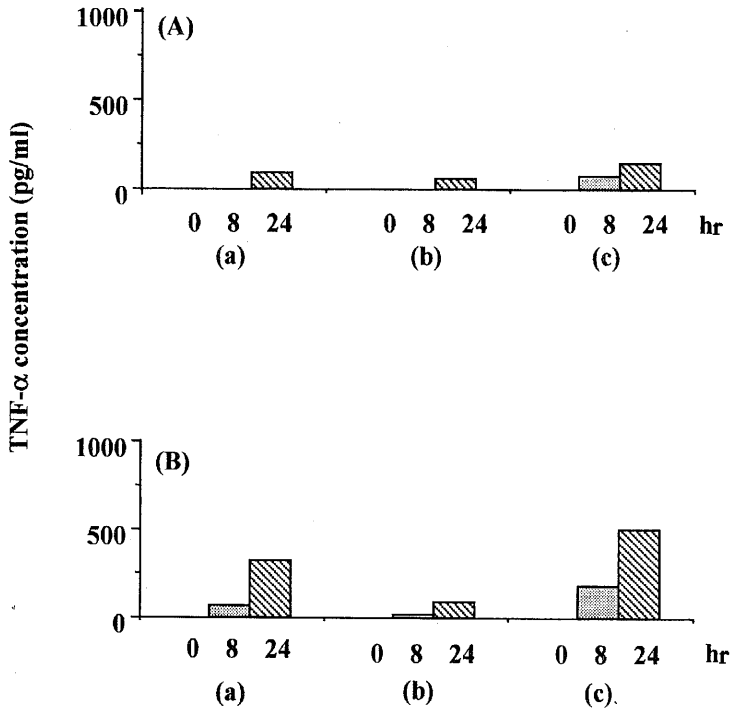

Fig 2. TNF-α production from three days old monocytes. (A) without IFN-γ incubation, and (B) with IFN-γ, induced by (a) *P. falciparum* malaria culture supernatant, (b) non-infected red blood cell culture supernatant, and (c) LPS (1 μg/ml). Test samples were collected at 0, 8 and 24 hr after incubation with the antigens.

TNF-α without the inducers (Figure 3). The cells were preincubated with either *all-trans*-Retinoic Acid plus vitamin D3 or PMA alone for 24 hr and then stimulated with malaria antigens for 48 hr. Incubation time for induction of cell differentiation and antigen stimulation were determined based on the results of preliminary experiments. THP-1 and U937 induced with PMA showed significant TNF-α production in response to malaria antigens, but HL60, K-562 and KY821A3 did not secrete the cytokine (Figure 3). Incubation of the cells only with inducers, either *all-trans*-Retinoic Acid plus vitamin D3 or PMA, did not induce any TNF activity. In some experiments, however, U937 released TNF-α in response to non-infected red cell preparations whereas THP-1 showed negligible activity (data not shown). Further experiments were carried out primarily using THP-1 cells differentiated with PMA. Similar to the fresh monocytes, IL-1β secretion was detected from induced THP-1 cells (Figure 4B).

To clarify the potential cytokine-inducing factors of infected erythrocytes and their culture supernatant, we examined the sequentially separated fractions of *P. falciparum* culture components. Both TNF-α and IL-1β secretion from THP-1 cells were detected after 48 hr incubation with malaria culture supernatant medium, and soluble and insoluble fractions of infected red blood cell lysates (Figure 4).

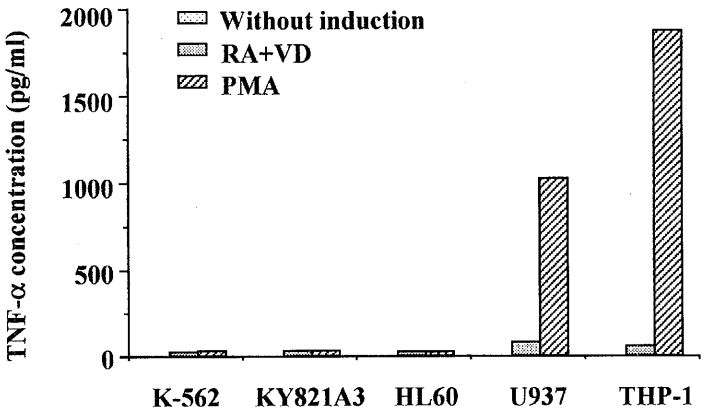

Fig 3. TNF-α production from human monocytic cell lines. Each cell line was incubated with malaria antigen (culture supernatant) with or without preincubation with inducers, *all-trans*-Retinoic acid plus vitamin D3 (RA+VD), or phorbol myristate acetate (PMA).

Importance of both glycolipid and protein components for monocyte stimulation

The malaria antigens secreted in the culture supernatant medium were characterized. *P. falciparum* culture supernatant was subjected to the YM-10 and YM-100 centrifugal devices. As shown in Figure 5, the filtrate fractions from both YM-10 and YM-100 could not induce any cytokine production from THP-1 cells, and the remaining retentate fractions kept the cytokine inducing activity. This result indicated that the soluble malaria antigens for cytokine production did not pass through the YM-100 membrane filter of 100,000 molecular weight cut-off.

P. falciparum culture supernatant and its YM-100 retentate fraction were treated with alkali, acid or pronase. Alkali treatment is often applied for deacylation of glycolipids and *P. falciparum* GPI-anchored structure is sensitive to this reaction. Incubation of malaria antigens in the acidic condition may also affect ester-linked acyl chains of GPI. Figure 6A shows the effect of those chemical and enzymatic treatments on malaria culture supernatant. The cytokine-inducing activity of the parasite antigens were decreased by alkaline and acid treatments. This implies the contribution of glycolipid component for cytokine production. In order to confirm that the chemically and physically treated samples did not induce any damages to host cells, we constantly observed the cell morphology. The cytokine inducing activity was partially decreased by heat treatment and pronase treatment followed by heat deprived its activity, suggesting that proteins are also essential for stimulation. We further analyzed which protein components, parasite-derived protein or serum protein, are sensitive to those treatments. The treated samples were reconstituted with serum supplemented RPMI 1640 before incubation with the monocytic cells. Partial recovery of cytokine activity was

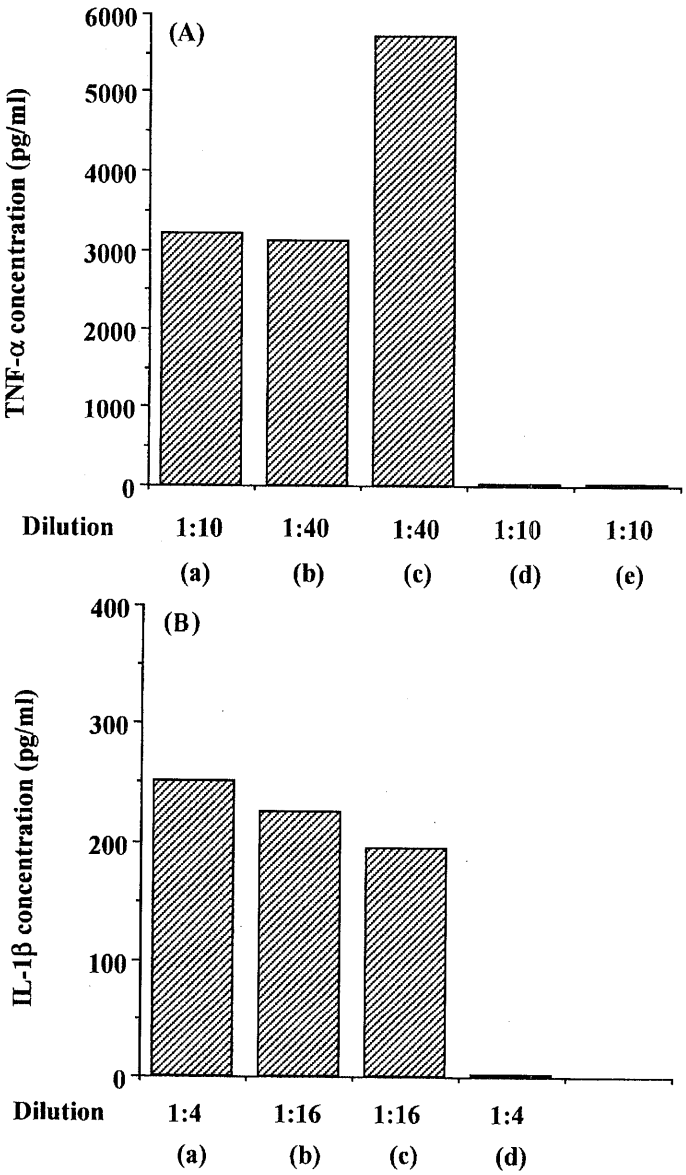

Fig 4. (A) TNF-α production from THP-1 cells in response to different preparations of malaria antigens. *P. falciparum* culture supernatant (5 ml) was obtained at 11% parasitemia. Infected erythrocyte lysate (1.25 ml) was collected by centrifugation. The remaining precipitate was reconstituted in 1.25 ml of RPMI 1640 medium. Ten times diluted (a) culture supernatant, and forty times diluted (b) soluble lysate fraction, and (c) insoluble precipitate were used for stimulation of the cells. (B) IL-1β production by four times diluted *P. falciparum* (a) culture supernatant, and sixteen times diluted (b) soluble lysate fraction, and (c) insoluble precipitate. Non-infected red blood cell (d) culture supernatant and (e) insoluble lysate were included as a control at ten times dilution for TNF-α and four times dilution for IL-1β assays.

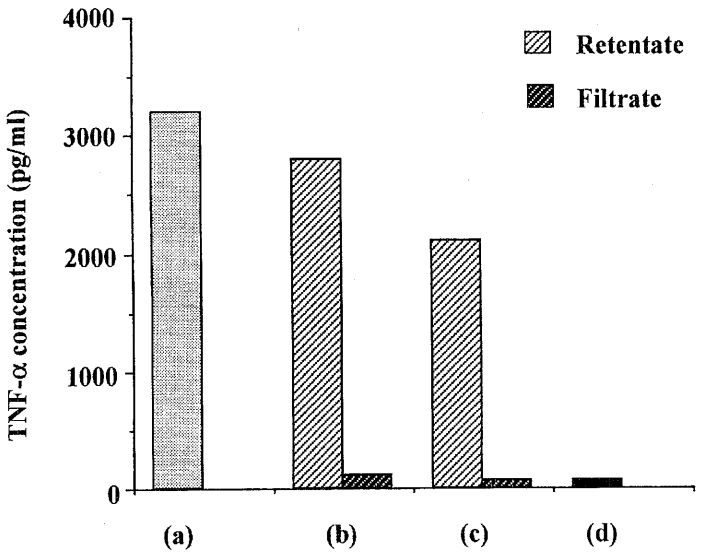

Fig 5. TNF-α production from THP-1 cells stimulated with (a) *P. falciparum* original culture supernatant, its ultrafiltrated sample using Amicon centrifugal devices, (b) YM-10 and (c) YM-100, (d) a control culture medium.

observed from heat-treated samples but not from pronase or alkali and acid treated samples (Figure 6B). This implies that parasite derived glycolipids and proteins, together with serum proteins are important for cell stimulation. Similar results were obtained using YM-100 retentate fraction.

Acetone precipitation of the proteins in the malaria culture supernatant greatly reduced the stimulatory activity as shown in Figure 8B. We did not detect the activity in acetone phase even after evaporation of the organic solvent.

Effect of detergent treatment on infected erythrocytes

TNF-α release in response to insoluble components of infected erythrocytes was previously reported (Pichyangkul et al., 1994; Nagao et al., 1996). In these papers it was shown that phagocytosis was not a major factor for cytokine release. However, it was still unclear which components of insoluble substrate stimulate monocytes for TNF-α production. We treated the insoluble component of infected erythrocytes with several detergents commonly used for solubilization of membrane proteins, and the remaining precipitates were assayed for cell stimulation after thorough washing with PBS. Repeated treatments of the precipitates in the presence of 1% non-ionic detergent, Triton X-100, Tween 20, Nonidet P-40 or 1% ionic detergent Sodium deoxycholate with combination of sonication could not completely remove the stimulation activity from the precipitates. Figure 7 represents these results showing the cytokine activities induced by Tween-20 and Sodium deoxycholate treated samples.

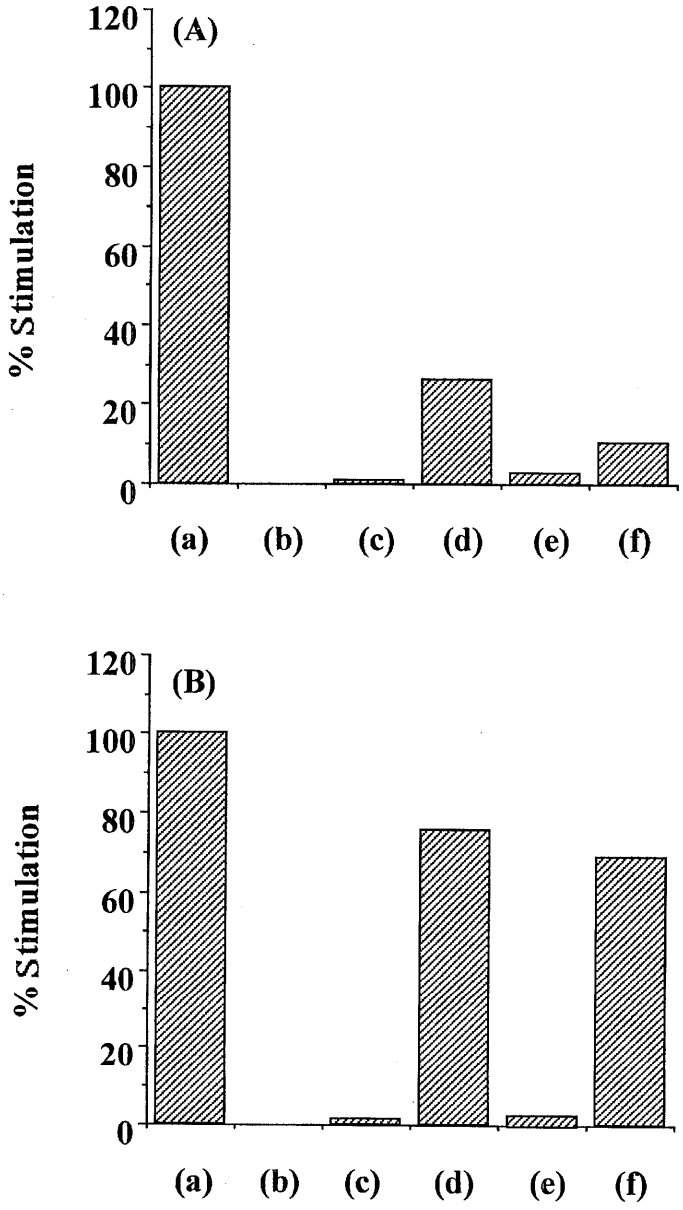

Fig 6. Effects of various treatments on *P. falciparum* culture supernatant (A) IL-1β production in response to *P. falciparum* culture supernatant incubated with the cells with serum-free RPMI 1640 medium. (a) original untreated sample, (b) 0.2 N NaOH at 56℃ for 2 hr followed by neutralization, (c) 0.2 N HCl at 56℃ for 2 hr followed by neutralization, (d) incubation at 56℃ for 2 hr, (e) pronase 200 μg/ml treatment followed by inactivation of the enzyme by incubation at 95℃ for 10 min, (f) incubation at 95℃ for 10 min. (B) IL-1β production stimulated by the same samples incubated with RPMI 1640 containing reconstituted human serum.

While analyzing the effect of detergent treatment, we realized that monocyte stimulation by malaria antigens was strongly inhibited by detergents. The precipitate fractions treated with any of the detergents could not stimulate the cytokine release if they were reconstituted with the medium without washing. This inhibitory effect could be removed by thorough washing with PBS (Figure 7).

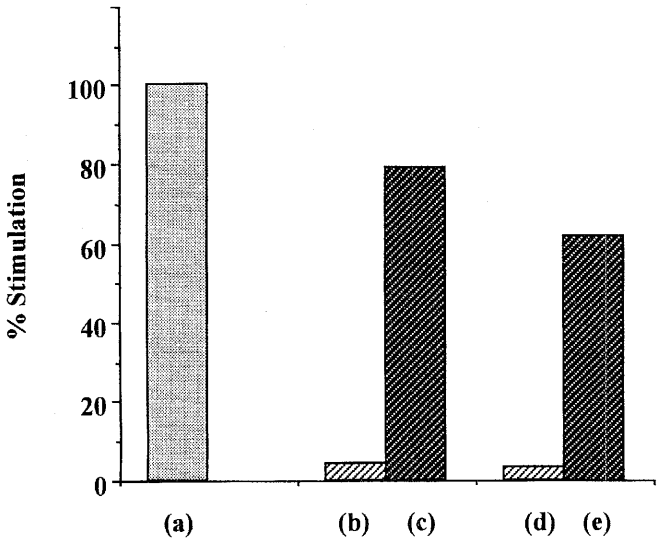

Fig 7. Effect of detergent treatments of insoluble fraction of *falciparum* infected erythrocytes on TNF-α production in THP-1 cells. Detergents were used at 1% concentration. (a) original untreated sample, (b) non-ionic detergent Tween-20 treatment without PBS washing, (c) followed by thorough PBS washing, (d) inonic detergent Sodium deoxycholate treatment without PBS washing, (d) followed by thorough PBS washing. All treated samples were reconstituted to the same volume with RPMI 1640. Their stimulation activity was compared with the insoluble fraction without detergent treatment.

Several experiments have been reported where glycosylphosphatidylinositols (GPIs) of *P. falciparum* are involved in TNF-α and IL-1β release from host monocytes and macrophages. We applied Triton X-114 temperature phase separation method to isolate GPI-anchored proteins and hemozoin (insoluble malaria pigment) from infected red blood cells (Figure 8A). The hydrophobic membrane proteins were precipitated from detergent phase by adding 8 vol. of cold acetone to prevent the inhibitory effect of the detergent. The detergent insoluble membrane-free hemozoin pellet was obtained by ultracentrifugation on sucrose cushion. These fractions were reconstituted with RPMI 1640 medium and used for THP-1 stimulation assay. Only slightly measurable level of activity was detected in the detergent

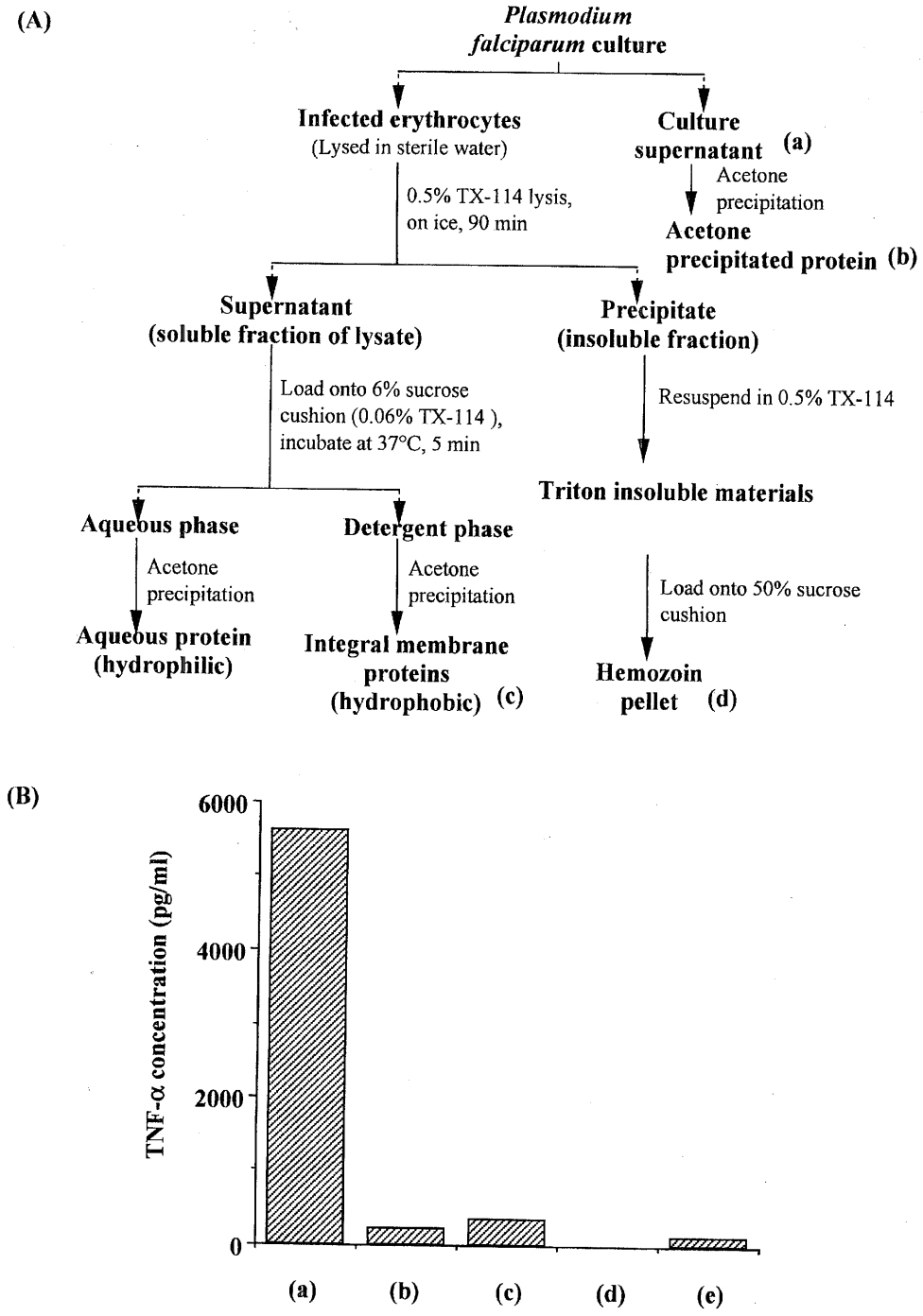

Fig 8. (A) Diagram of Triton X-114 temperature phase separation of *P. falciparum* culture components. (B) Effect of detergent treatment on malaria antigens in relation to TNF-α production from THP-1 cells. (a) Original malaria culture supernatant, (b) acetone treated culture supernatant, (c) TX-114 detergent phase of soluble lysate fraction, (d) insoluble membrane-free hemozoin pellet, (e) RPMI 1640.

phase proteins after acetone precipitation and no stimulation by insoluble hemozoin pellet (Figure 8B). We did not include the data of aqueous phase in Figure 8B, because after acetone precipitation it produced enormous amount of insoluble clumps, which interfered with the cytokine assay. However, the independent experiments with less amount of the aqueous phase precipitates indicated that these could not stimulate cytokine release from THP-1 cells. We did not detect the original stimulatory activity in any of these fractionated components from the infected red blood cells after acetone precipitation or hemozoin purification. Considering together the previous detergent treatment results that the stimulatory activity in the precipitate was not removed by the detergents, we presume that the substances important for cell stimulation are hydrophobic membrane components and solubilized in acetone layer.

DISCUSSION

Several substances and antigens are identified to stimulate TNF production in malaria (Bate et al., 1989, Jakobsen et al., 1991; Kristensen and Jakobsen, 1996; Taverne et al., 1990). Among the soluble antigens are glycosylphosphatidylionsitol (GPI) moieties from the merozoite surface antigens MSP-1 and MSP-2 (Schofield and Hackett, 1993; Tachado et al., 1997), an antigen complex known as Ag7 (Jakobsen et al., 1991; Taverne at al., 1990), and ring-infected erythrocyte surface antigen (RESA) (Picot et al, 1993). Malaria pigment or hemozoin, an insoluble metabolic derivative of hemoglobin, is also reported to trigger TNF-α and IL-1β production (Pichyangkul et al., 1994). However, it has not been demonstrated which components are the dominant cytokine inducers of the parasites. Better understanding of malaria antigens or toxins is particularly important, as the inhibitory antibodies against malaria toxin might provide the basis for an anti-disease vaccine. Moreover, this will favor to find the way to develop a strategy acting directly against the cytokine-inducing antigens.

We first demonstrated that human monocytic cell line THP-1 is useful for the study of cytokine release by *P. falciparum* antigens. In the experiments using fresh monocytes, the levels of the cytokine production varied from one experiment to the other and this could be due to individual variation of the donors, although the kinetics of the cytokine production was consistent in all experiments. Moreover, the preparation of fresh monocytes for each experiment is a laborious work and the purified adherent cells always contain about 5% of non-adherent lymphocytes. THP-1 cells can be maintained in continuous culture and more easily set up for experiments by PMA induction. The induced THP-1 produces TNF-α and IL-1β in response to malaria antigens. We showed that soluble and insoluble fractions of infected erythrocytes and its culture supernatant stimulated comparably high levels of cytokine production from the THP-1 cells.

We then presented that both glycolipid and protein components were required for monocyte stimulation based on the analysis of culture supernatant. Physical heat treatment decreased the level of cytokine production. Depletion of either glycolipid or protein component by acid, alkaline or pronase treatment resulted in the loss of cytokine induction. Partial

recovery of stimulation activity was observed when the heat treated samples were used for monocyte stimulation in the presence of serum supplemented culture medium. However, recovery was not observed from the samples of acid, alkaline or pronase treatment. This suggests that infected erythrocyte derived glycolipids and protein components in associattion with serum proteins induce the host cells for cytokine production. The host cells may interact either with the complex of glycolipids and proteins, or with each component independently. In either case, native protein conformation was required for cell stimulation, as the inducing activity was partially reduced even by 2 hr incubation at 56℃. Some non-serum proteins seemed to be important as we did not detect the recovery of stimulation activity by serum supplementation to pronase treated samples, but these proteins seemed more resistant to heat.

On the other hand, highly purified GPI-anchors isolated from *P. falciparum* infected erythrocytes were shown to induce high levels of TNF and IL-1 from mouse macrophages (Schofield and Hackett, 1993; Tachado et al., 1997). The other reports showed that heat-stable antigens of malaria parasites induced the release of TNF from human monocytes and mouse macrophages (Bate et al., 1992; Picot et al., 1990). In these experiments, however, the malaria antigens were incubated with serum supplemented medium at the time of cell stimulation. These malaria antigens probably stimulated the host cells in association with serum proteins as has been mentioned above.

Our result of ultrafiltration experiment which showed the stimulatory activity was in the retentate supported the speculation that association complexes of glycolipids and proteins stimulate cytokine secretion from host cells. Molecular weight of protein free GPI moieties is less than 5 kDa and expected to pass through the YM-10 and YM-100 membranes. Some of the GPI components may exist in association with proteins and remained in the retentate fraction.

Deprivation of stimulatory activity by acetone precipitation of the proteins in malaria culture medium is another evidence of the importance of both acetone soluble glycolipid and insoluble protein components. Either of the single fraction did not stimulate cytokine production of THP-1 cells.

The likelihood of cooperation between the malaria parasite-derived glycolipids and protein components for cytokine production was also supported by our results of malaria pigment. Malaria pigment or crude hemozoin is composed of aggregated heme and a variety of attached components of parasite and host origin lipids and proteins (Arese and Schwarzer, 1997). We observed the cytokine stimulation by crude preparation of insoluble fraction of infected erythrocytes (malaria pigment). This activity was abolished by Triton X-114 and sucrose gradient separation (Figure 8), which removed the associated membrane fractions from the final hemozoin pellet. Therefore, the active component of the insoluble fraction was composed of heme and attached components. Similar result was reported that cytokine production was diminished by boiling and treatment with 2% SDS (Sodium dodecylsulfate) and completely abolished after treatment with non-specific protease XIV (Pichyangkul et al., 1994).

Our speculation that the malaria-derived glycolipids and serum proteins stimulated the host cells to induce cytokine release suggests a possible similarity to the mechanism of bacterial lipopolysaccharide (LPS) recognition in macrophages. LPS binds via lipid A to LPS-binding protein (LBP) that is normally present in blood and this LPS-LBP complex binds to CD14 present on macrophage surface (Ulevitch and Tobias, 1995). If indeed the malaria antigens stimulate host cells in similar manner as the LPS, this will raise several questions to be answered. What is the binding protein? What is the receptor on the cell surface? Even if it is the case, CD14 may not be the receptor. Malaria antigens may act via a different cell receptor from LPS. This is becuase TNF production by malaria antigens was not diminished by Polymyxin B, which neutralizes the activity of lipid A (data not shown). The insensitivity to Polymyxin B on malaria antigens for their cytokine inducing effect has been reported (Bate et al., 1989; Taverne et al., 1990). Furthermore, there is a report that macrophages from LPS-hyporesponsive C3H/HeJ mice could secrete TNF in response to malaria antigens comparable to that of normal CBA/J mice (Taverne et al., 1990).

Another possible mechanism is that hydrophobic malaria antigens are incorporated into serum lipoprotein complex, and this complex interacts with host cells then initiates the cascade of cell signaling pathway. Normally, the hydrophobic lipid components in blood are carried by low-density lipoprotein (LDL), a lipid monolayer particle with apo-B protein, which binds specifically to LDL receptor on the cell surface. If it is the case in malaria antigens, the particles incorporating hydrophobic *Plasmodium* antigens may interact with receptors in a specific or a non-specific manner. According to this hypothesis, inhibitory effect of detergents on the malaria antigen mediated cytokine production could be explained by detergent interference of lipid monolayer of lipoprotein particles.

In this paper, we presented the usefulness of human monocytic cell line THP-1 to study the cytokine inducing activity of malaria antigens. Partial characterization of malaria antigens suggested that parasite-derived glycolipids and protein components are important for cell stimulation activity. Further investigations to study the specific nature of cytokine inducing malaria antigens and the possible mechanism are of great value for anti-disease strategy.

Acknowledgments

We thank Japanese Red Cross Society for the provision of buffy coats to isolate fresh monocytes and Prof. G.L. Enriquez for the comments of the manuscript. This work was supported by a Grant-in-Aid for Scientific Research (C) 10670232 from the Ministry of Education, Science, Sports, and Culture of Japan. Hla Myat Mon is in receipt of PhD scholarship from Japanese Government, Ministry of Education, Science, Sports, and Culture (Monbusho).

References

1) Allan, R.J., Rowe, A. & Kwiatkowski, D. (1993): *Plasmodium falciparum* varies in its ability to induce tumor necrosis factor. Infect. Immun., 61, 4772-4776

2) Arese, P. & Schwarzer, E. (1997): Malarial pigment (haemozoin): a very active 'inert' substance. Ann. Trop. Med. Parasit., 91, 501-516

3) Bate, C.A.W., Taverne, J. & Playfair, J.H.L. (1989): Soluble malarial antigens are toxic and induce the production of tumour necrosis factor *in vivo*. Immunology, 66, 600-605

4) Bate, C.A.W., Taverne, J., Roman, E., Moreno, C., & Playfair, J.H. (1992): Tumour necrosis factor induction by malaria exoantigens depends upon phospholipid. Immunology, 75, 129-135

5) Clark, I.A., Chaudhri, G. & Cowden, W.B. (1989): Roles of tumour necrosis factor in the illness and pathology of malaria. Trans. R. Soc. Trop. Med. Hyg., 83, 436-440

6) Dinarello, C.A., Cannon, J.G. & Wolff, S.M. (1988): New concepts on the pathogenesis of fever. Rev. Infect. Dis., 10, 168-189

7) Grau, G.E., Fajardo, L.F., Piguet, P.F., Allet, B., Lambert, P.H. & Vassalli, P. (1987): Tumor necrosis factor (Cachectin) as an essential mediator in murine cerebral malaria. Science, 237, 1210-1212

8) Jakobsen, P.H., Hviid, L., Theander, T.G., Riley, E.M., Grellier, P., Brun, L.S., Dalsgaard, K., Schrevel, J. & Jepsen, S. (1991): Isolation and characterization of a soluble antigen complex of *Plasmodium falciparum* with pyrogenic properties. APMIS., 99, 21-29

9) Kern, P., Hemmer, C.J., Van Damme, J., Gruss, H.J. & Dietrich, M. (1989): Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria. Am. J. Med., 87, 139-143

10) Kristensen, G. & Jakobsen, P.H. (1996): *Plasmodium falciparum*: Characterization of toxin-associated proteins and identification of a hemoglobin containing parasite cytokine stimulator. Exp. Parasitol., 82, 147-154

11) Kwiatkowski, D., Cannon, J.G., Manogue, K.R., Cerami, A., Dinarello, C.A. & Greenwood, B.M. (1989): Tumour necrosis factor production in *Falciparum* malaria and its association with schizont rupture. Clin. Exp. Immunol., 77, 361-366

12) Kwiatkowski, D., Hill, A.V.S., Sambou, I., Twumasi, P., Castracane, J., Manogue, K.R., Cerami, A., Brewster, D.R. & Greenwood, B.M. (1990): TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet, 336, 1201-1204

13) Lambros, C., & Vanderberg, J.P. (1979): Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol., 65, 418-420

14) Moriuchi, H., Moriuchi, M., Combadiere, C. & Murphy, P.M. (1996): $CD8^+$T-cell-derived soluble factor(s), but not β-chemokines RANTES, MIP-1α, and MIP-1β, suppress HIV-1 replication in monocyte/macrophages. Proc. Natl. Acad. Sci. USA, 93, 15341-15345

15) Nagao, T., Uemura, H., Yanagi, T., Oishi, K., Nagatake, T., & Kanbara, H. (1996): Loss of tumor necrosis factor production by human monocytes in falciparum malaria after their maturation in vitro. Am. J. Trop. Med. Hyg., 55, 562-566

16) Pichyangkul, S., Saengkrai, P. & Webster, H.K. (1994): *Plasmodium falciparum* pigment induces monocytes to release high levels of tumor necrosis factor-α and interleukin-1β. Am. J. Trop. Med. Hyg., 51, 430-435

17) Picot, S., Peyron, F., Deloron, P., Boudin, C., Chumpitazi, B., Barbe, G., Vuillez, J.P., Donadille, A. & Ambroise-Thomas, P. (1993): Ring-infected erythrocyte surface antigen (Pf/155RESA) induces tumour necrosis factor-alpha production. Clin. Exp. Immunol., 93, 184-188

18) Picot, S., Peyron, F., Vuillez, J.P., Barbe, G., Marsh, K. & Ambroise-Thomas, P. (1990): Tumor necrosis factor production by human macrophages stimulated in vitro by *Plasmodium falciparum*.

Infect. Immun., 58, 214-216

19) Tachado, S.D., Gerold, P., Schwarz, R., Novakovic, S., McConvilles, M. & Schofield, L. (1997): Signal transduction in macrophages by glycosylphosphatidylinositol of *Plasmodium*, *Trypanosoma*, and *Leishmania*: Activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. Proc. Natl. Acad. Sci. USA, 94, 4022-4027

20) Taverne, J., Bate, C.A.W., Kwiatkowski, D., Jakobsen, P.H. & Playfair, J.H.L. (1990). Two soluble antigens of *Plasmodium falciparum* induce tumor necorsis factor release from macrophages. Infect. Immun., 58, 2923-2928

21) Taverne, J., Bate, C.A.W., Sarkar, D.A., Meager, A., Rook, G.A.W. & Playfair, J.H.L. (1990): Human and murine macrophages produce TNF in response to soluble antigens of *Plasmodium falciparum*. Parasite Immunol., 12, 33-43

22) Trager, W. & Jensen, J.B. (1976): Human malaria parasites in continuous culture. Science, 193, 673-675

23) Schofield, L. & Hackett, F. (1993): Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J. Exp. Med., 177, 145-153

24) Scuderi, P., Sterling, K.E., Lam, K.S., Finley, P.R., Ryan, K.J., Ray, C.G., Petersen, E., Slymen, D.J. & Salmon, S.E. (1986): Raised serum levels of tumour necrosis factor in parasitic infections. Lancet, 2, 1364-1365

25) Smythe, J.A., Murray, P.J. & Anders, R.F. (1990): Improved temperature-dependent phase separation using Triton X-114: Isolation of integral membrane proteins of pathogenic parasites. J. Methods Cell Mol. Biol., 2, 133-137

26) Ulevitch, R.J. & Tobias, P.S. (1995): Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Ann. Rev. Immunol., 13, 437-457