KILLING OF BLOOD STAGE *PLASMODIUM VINCKEI PETTERI* BY SPLEEN MACROPHAGES THROUGH L-ARGININE DEPENDENT MECHANISM

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Abstract. A series of experiments was carried out to investigate the involvement of the L-arginine-dependent effector mechanism (LADEM) in the killing of the blood stages of the rodent malaria parasite, *Plasmodium vinckei petteri*, by activated spleen macrophages *in vitro*. *P.v.petteri*-infected red blood cells were co-incubated with spleen macrophages from normal mice which had previously received 10^8 *Mycobacterium bovis* (BCG) 5 days earlier, in the presence of 0.1 μg/ml LPS with and without 0.1 mM L-NMMA, an L-arginine analogue which inhibits LADEM, for 16 hours. The viability of the parasites was assessed according to their infectivity following inoculation into experimental mice. Incubation of parasites with spleen macrophages in the presence of LPS without L-NMMA reduced the parasite viability to about 3%. When L-NMMA was included in the culture, inhibition of parasite killing was observed, resulting in an increase of parasite viability to about 21%. These data provide evidence to suggest that spleen macrophages play an important role as effector cells in the immune mechanisms against *P.v.petteri* infection, and that the parasite killing of these cells, at least in part, was mediated by LADEM.

INTRODUCTION

There is considerable evidence to suggest that mononuclear phagocytes play a major role as effector cells in cell-mediated immune responses against blood stage malaria infections. This assumption is based on a number of observations: Firstly, resistance to malaria can be augmented by pre-treatment with micro-organisms, microbial extracts or heterologous parasites, to induce non-specific immune responses mediated by mononuclear phagocytes (Clark *et al*, 1976, 1977; Cox, 1982). Secondly, mononuclear phagocytes are activated during the course of malarial infection both in human and experimental animals, for example, monocytes recovered from patients with *P. falciparum* have significant increases in Fc receptor expression (Ward *et al*, 1984). Similarly, splenic macrophages recovered from mice during the course of infection with a variety of murine malaria species have been shown to be activated as indicated by enhanced phagocytosis of infected and non-infected erythrocytes (Shear *et al*, 1979) and production of oxygen metabolites (Wozencraft *et al*, 1985; Dockrell *et al*, 1986; Stevenson *et al*, 1992). Thirdly, *in vitro* studies have demonstrated that co-culturing of human (Jones *et al*, 1989) or murine (Taverne *et al*, 1982; Ockenhouse and Shear, 1984) malaria parasites with mononuclear phagocytes, or their supernatants (Wozencraft *et al*, 1984), bring about inhibition in parasite growth.

The precise mechanisms by which mononuclear phagocytes act as effector cells during malarial infection are still unclear. Mononuclear phagocytes are known to be cytotoxic to tumor cells, bacteria, fungi and parasites, and are able to secrete more than 100 molecules which are important in the modulation of immune responses (Nathan, 1987). Experimental evidence also indicates that mononuclear phagocytes can kill malaria parasites through the release of reactive oxygen intermediates (Clark and Hunt, 1983; Cox and Millot, 1984) although mice exhibiting a defect in reactive oxygen intermediate release can also overcome *P. chabaudii* infection (Cavacini *et al*, 1989). In addition, tumor necrosis factor alpha (TNF-α), which is produced by macrophages, has an indirect killing effect on malaria parasites (Jensen *et al*, 1987; Taverne *et al*, 1987; Rockett *et al*, 1988). Our unpublished observations indicate that the ability of spleen macrophages from *P.v.petteri*-immunized mice to synthesise nitrogen oxides increases during the later stages of infection. In this study, we present evidence that spleen macrophages from

normal mice which have received BCG 5 days earlier can kill blood stage *P.v. petteri in vitro*, and that this killing is, at least in part, mediated by the L-arginine-dependent effector mechanism.

MATERIALS AND METHODS

Parasites

Plasmodium vinckei petteri (2CR) was obtained from Professor D Walliker, University of Edinburgh, maintained in TO Swiss mice and frozen in liquid nitrogen until being used.

Mice

Female outbred, 6-8 week old LACA mice (purchased from Tuck and Son, Buttlebridge, Essex) and Balb/C mice (purchased from Harlan Olac, Bicester, Oxon) were used. They were free from hemoprotozoa, and kept in groups of six, fed and watered *ad libitum*.

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma Chemical Co. N^G-monomethyl-L-arginine (L-NMMA, Sigma Chemical Co) was kindly provided by Professor FY Liew, University of Glasgow.

Spleen macrophage preparation

Spleen macrophages extracted from mice injected with 10^8 BCG 5 days earlier were used. Spleen leukocytes were suspended in complete medium at 5×10^7 cells/ml, plated on Sigmacoat-treated dishes and incubated at 37°C and 5% CO_2 for 2 hours. The nonadherent cells were washed off and the remaining adherent cells were incubated for a further 4 hours. After washing, the adherent cells were incubated in PBS at 4°C for 30 minutes, and detached by gentle flushing with ice-cold PBS. The detached cells were then washed with warmed RPMI, resuspended in complete medium, and plated in the wells of 24 well culture plates at 1×10^7 cells/well. The plates were incubated at 37°C and 5% CO_2 in a humidified incubator for 24 hours.

The effects of spleen cells on parasite viability

Red blood cells from infected mice with parasitemias of about 3-5% were washed in RPMI and resuspended in complete medium at 1×10^6 infected cells/ml. The parasitemia was then adjusted to 1% using normal mouse red blood cells. 0.75 ml of this parasite suspension was plated in triplicate wells of 24 well culture plates containing:

a) spleen macrohages + 0.1 μg/ml LPS + 0.1 mM L-NMMA,
b) spleen macrophages + 0.1 mM L-NMMA,
c) spleen macrophages + 0.1 μg/ml LPS,
d) spleen macrophages,
e) medium + 0.1 μg/ml LPS,
f) medium + 0.1 mM L-NMMA,
g) medium only,
a') supernatant of a,
c') supernatant of c.

Plates were incubated at 37°C and 5% CO_2 in a humidified incubator for 16 hours and the parasite viability was examined.

Evaluation of parasite viability

The viability of the parasites was evaluated according to the infectivity of the infected erytrocytes following inoculation into experimental animals. The contents of each well were mixed gently, and 0.2 ml injected intravenously into LACA mice. The parasitemias were monitored by examination of Giemsa-stained tail blood smears prepared daily. The precise number of viable parasites that had been injected was calculated by reference to a standard curve of the correlation between the number of parasites injected and the time taken to reach 0.5% parasitemia.

RESULTS

Incubation with various numbers of BCG-activated spleen macrophages

In a series of preliminary experiments, 2.5×10^5 parasites/well were incubated for 16 hours with various numbers of LPS-stimulated spleen macrophages from 1×10^4 - 10^7 cells/well. It was apparent that the presence of an effective number of spleen macrohages was required for killing to occur. $1 \times$

10^5 cells or fewer per well were insufficient to produce killing in this assay, whereas incubation with 1×10^6 and 1×10^7 spleen macrophages/well reduced the parasite viability to about 70% and 3% respectively (Fig 1). 1×10^7 spleen macrophage were then used in the subsequent experiments.

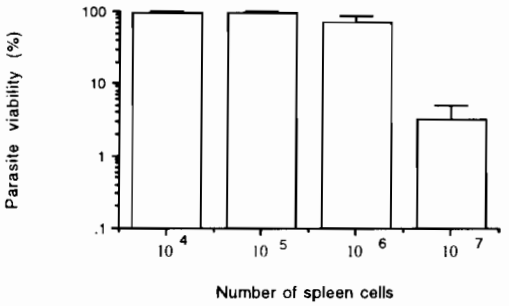

Fig 1–Effect of incubation with various numbers of spleen macrophages on the survival of *P.v.petteri* survival. 2.5×10^5 *P.v.petteri*-infected red blood cells were incubated with various numbers of spleen macrophages (1×10^4 - 10^7) in complete medium in the presence of 0.1 μg/ml LPS, at 37°C and 5% CO_2 for 16 hours. At the end of the incubation, the viability of the parasites was assessed by reference to a standard infectivity curve. Each value represents the mean and SD of the percentages of parasite viability from triplicate wells.

L-arginine dependent killing by spleen macrophages

To determine whether an L-arginine-dependent mechanism was involved in the killing, *P.v.petteri*-infected red blood cells were incubated with spleen macrophages in the presence of LPS with and without N^G-monomethyl-L-arginine (L-NMMA), an L-arginine analogue which blocks the biological synthesis of nitrogen oxides from L-arginine. The parasite viability was assessed according to the infectivity following inoculation into experimental mice.

As shown in Fig 2, the parasitemia curves of both mice which had received injection of parasites previously incubated with spleen macrophages in the presence of LPS with (A), and those without (C) L-NMMA, are shifted to the right, to different degrees, compared with control mice which had received parasites incubated in the medium only

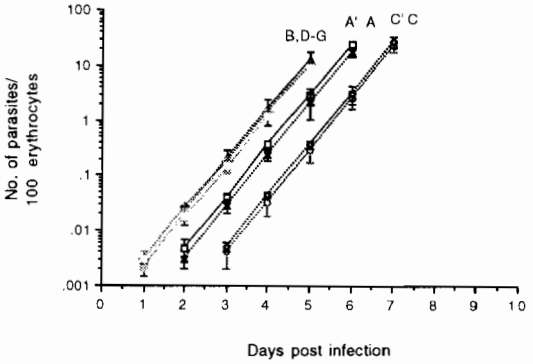

Fig 2–The infectivity of *P.v.petteri* infected red blood cells after co-incubation with LPS stimulated spleen macrophages. Infected blood with 1% parasitemia were suspended in complete medium at 1×10^8 RBC/ml. This suspension was plated at 0.25 ml/well, in 24 well culture plates containing:

A. 0.25 ml of 10^7 spleen macrophages, 0.1 μg/ml LPS, 0.1 mM L-NMMA
B. 0.25 ml of 10^7 spleen macrophages, 0.1 mM L-NMMA
C. 0.25 ml of 10^7 spleen macrophages, 0.1μg/ml LPS
D. 0.25 ml of 10^7 spleen macrophages
E. 0.25 ml of 0.1 μg/ml LPS
F. 0.25 ml of 0.1 mM L-NMMA
G. 0.25 ml of medium only
A'. 0.25 ml of supernatant of A.
C'. 0.25 ml of supernatant of C.

The plates were incubated at 37°C and 5% CO_2 in a humidified incubator for 16 hours 0.2 ml of the contents of each well was injected intravenously into 6-8 week old female LACA mice, and the parasitemias were monitored daily. Each point represents the mean and SD of the number of parasites/100 cells from 3 mice.

(G). When the precise number of viable parasites was calculated (Table 1), the average parasite viability of A (with L-NMMA) was 21.5% and C (without L-NMMA) was 3.1%. In control wells, in which parasites were incubated with spleen macrophages in the absence of LPS (D), no reduction of parasite viability occurred.

To determine whether the presence of cells was necessary for the killing, parasites were incubated in cell-free supernatants of spleen macrophages cultured in the presence of LPS with and without L-NMMA. The average viability of these parasites was insignificantly higher (A' and C'), compared to those incubated in wells in the presence of cells.

Table 1

The calculated parasite viability after coincubation with spleen macrophages in the presence of LPS with or without L-NMMA. 10^5 *P.v.petteri* infected red blood cells which had been coincubated with stimulated spleen macrophages for 16 hours, were inoculated intravenously into LACA mice, and the parasitemias monitored daily. The actual numbers of viable parasites injected was determined using a standard reference curve.

Well code*	Day of 0.5% parasitemia	No. of viable parasites (%) ± SD	Viability (%)
A	4.3463	21,480 ± 789	21.5
B	3.4927	104,667 ± 10,221	104
C	5.2425	3,103 ± 118	3.1
D	3.5843	91,009 ± 9,976	91
E	3.5533	96,941 ± 8,622	96
F	3.4822	102,028 ± 8,214	102
G	3.5693	93,816 ± 9,666	94
A'	4.1747	27,307 ± 782	27.3
C'	5.1373	3,843 ± 164	3.8

* see Fig 2.

To determine whether LPS or L-NMMA alone had a killing effect, parasites were incubated in complete medium containing 0.1 μg/ml LPS or 0.1 mM L-NMMA. The viability of these parasites (E and F) was similar to that of those incubated in the medium only.

DISCUSSION

The results of these *in vitro* experiments demonstrate that incubation of *P.v.petteri*-infected red blood cells with 10^7 spleen macrophages in the presence of LPS results in a reduction of parasite viability to about 3%. When L-NMMA is included in the culture, the killing is inhibited, and the parasite viability is reduced to about 21%, indicating that parasite killing of spleen macrophages is mediated, at least in part, by an L-arginine-dependent and by implication of a nitric oxide dependent mechanism as L-NMMA is a specific inhibitor of nitric oxide production.

Previous studies have demonstrated that activated macrophages are able to synthesize nitrogen oxides (NO, NO_2^- and NO_3^-) by oxidizing the terminal gianidino nitrogen atoms of L-arginine (Hibbs *et al*, 1987, 1988). The importance of nitrogen oxides as effector molecules of parasite killing has been reviewed by James and Hibbs (1990) and Liew and Cox (1991). Since the NO molecule is very short-lived, its reactivity must necessarily be directed toward target cells in the immediate vicinity of the effector cells. In the present study, no significant difference was observed between the reduction of viability of parasites which had been coincubated with activated spleen macrophages and of those incubated with cell supernatant only (in the absence of cells). This suggests that the L-arginine-dependent parasite killing in this system is mediated by NO_2^- or NO_3^- rather than by NO, based on the fact that NO is unstable and oxidized quickly to NO_2^- and NO_3^- in an aerobic environment (Marletta *et al*, 1988; Stuehr *et al*, 1991; Granger *et al*, 1990). In addition, Rockett *et al* (1991) observed that nitric oxide at saturated concentrations did not inhibit the growth of *P. falciparum in vitro*, but its oxidation products (NO_2^- and NO_3^-) were toxic to the parasites in a millimolar concentration.

Malaria parasite killing via a nitric oxide-dependent mechanism has also been demonstrated *in vivo* by Taylor-Robinson *et al* (1993) using another rodent malaria parasite, *Plasmodium chabaudii*, in

mice. These authors raised several clones of TH1 and TH2 cells which they transfered to CD4-cell depleted mice, and found that TH1 cells restored immunological competence through the agency of nitric oxide.

The reduction of parasite viability observed following incubation with spleen macrophages in the presence of LPS in this assay possibly involved oxidative and other non-oxidative macrophage-killing mechanisms. Tumor necrosis factor (TNF), which is also produced by LPS stimulated macrophages, was probably also secreted in this system. This molecule, in turn, could induce the macrophages to release reactive oxygen species that destroy parasites through lipid peroxidation (Clark *et al*, 1986).

Previous observations indicate that the ability of spleen macrophages recovered from *P.v.petteri* immunized mice to synthesize nitrogen oxides are higher at the later stages of infection compared to the non-immunized group (Supargiyono, 1997). These observations, together with the results of the present study, suggest that the non-oxidative, L-arginine-dependent mechanisms probably act in concert with the oxidative, and probably with other killing mechanisms, bringing about successful reduction of parasitemia in immunized mice. This theory may also explain the ability of A/J mice which exhibit defects of the macrophage oxidative burst to successfully recover from *P. chabaudii adami* infection, as do in Balb/C mice (Cavacini *et al*, 1989).

L-arginine dependent destruction of malaria parasites has also been shown to be effective against the intrahepatic stage of *P. yoellii* (Nussler *et al*, 1991). These authors showed that TNF and/or IL-6 could induce inhibition of intrahepatic parasite development by hepatocytes through the L-arginine dependent mechanism. Mellouk *et al* (1991) working with *Plasmodium berghei* found that IFN-γ induced nitric oxide production inhibited the development of the parasite in primary culture of hepatocytes. Similar killing mechanism have also been suggested to play role in other parasitic infections, since some macrophage populations have been shown to kill *Leishmania major* (Green *et al*, 1990; Liew *et al*, 1990) and *Leishmania donovani* (Roah *et al*, 1991) amastigotes, schistosomula of *Schistosoma mansoni* (James and Glaven, 1989), and *Toxoplasma gondii* (Langermans *et al*, 1992), through a mechanism which is inhibited by L-NMMA, an L-arginine competitor. However, the way in which oxygenated-L-arginine-derived intermediates can affect intra- or extra-cellular targets remains an open question. It has been proposed that L-arginine-derived nitric oxide can react with the Fe-S groups, forming an iron-nitrosyl complex, causing the inactivation and degradation of the Fe-S prosthetic groups of aconitase, Complex I and Complex II of the mitochondrial electron transport chain (Lancaster and Hibbs, 1990; Pellet *et al*, 1990).

Finally, it can be summarized that incubation of *P.v.petteri*-infected red blood cells with activated spleen macrophages in the presence of LPS for 16 hours reduced parasite viability to about 3%. Addition of L-NMMA in the culture system inhibited the killing, indicating the involvement of LADEM in these effector mechanisms. These data provide evidence to suggest that spleen macrophages play an important role as effector cells in the immune mechanisms against *P.v.petteri* infection, and that the parasite killing of these cells, at least in part, was mediated by an L-arginine-dependent effector mechanism.

REFERENCES

Cavacini LA, Guidotti M, Parke LA, Melankon-Kaplan J, Weidanz WP. Reassessment of the role of splenic leukocyte oxidative activity and macrophage activation in expression of immunity to malaria. *Infect Immun* 1989; 57 : 3677-82.

Clark IA, Allison AC, Cox FEG. Protection of mice against *Babesia* and *Plasmodium* with BCG. *Nature* 1976; 259 : 309-11.

Clark IA, Cox FEG, Allison AC. Protection of mice against Babesia spp and Plasmodium spp with killed *Corynebacterium parvum*. *Parasitology* 1977; 74 : 9-18.

Clark IA, Hunt NH. Evidence for reactive oxygen intermediates causing hemolysis and parasite death in malaria. *Infect Immun* 1983; 39 : 1-6.

Clark IA, Hunt NH, Cowden WB. Oxygen-derived free radicals in the pathogenesis of parasitic disease. In: *Adv Parasitol* 1986; 25 : 1-44.

Cox FEG. Non-specific immunity against parasites. *Clin Immunol Allerg* 1982; 2 : 705-20.

Cox FEG, Millott SM. The importance of parasite load in the killing of *Plasmodium vinckei* in mice treated with *Corynebacterium parvum* or alloxan monohydrate. *Parasitology* 1984; 89 : 417-24.

Dockrell HM, Alavi A, Playfair JHL. Changes in oxidative burst capacity during murine malaria and the effect of vaccination. *Clin Exp Immunol* 1986; 66 : 37-43.

Granger DL, Hibbs JB Jr, Perfect JR, Durack DT. Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J Clin Invest* 1990; 85 : 264-73.

Green SJ, Meltzer MS, Hibbs JB Jr, Nacy CA. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine dependent killing mechanism. *J Immunol* 1990; 144 : 278-83.

Hibbs JB Jr, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role of L-arginine deiminase and imino-nitrogen oxidation to nitrite. *Science* 1987; 235 : 473-6.

Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Communic* 1988; 157 : 87-94.

James SL, Glaven J. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J Immunol* 1989; 143 : 4208-12.

James SL, Hibbs JB Jr. The role of nitrogen oxides as effector molecules of parasite killing. *Parasitol Today* 1990; 6 : 303-5.

Jensen JB, Vande Waa JA, Karadsheh AJ. Tumor necrosis factor does not induce *Plasmodium falciparum* crisis forms. *Infect Immun* 1987; 55 : 1722-4.

Jones KR, Cottrell BJ, Targett GA, Playfair JHL. Killing of *Plasmodium falciparum* by human monocyte derived macrophages. *Parasite Immunol* 1989; 11 : 585-92.

Lancaster JR Jr, Hibbs JB Jr. EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc Nat Acad Sci USA* 1990; 87 : 1223-7.

Langermans JAM, van Der Hulst MEB, Nibbering PH, *et al*. IFN-γ-induced L-arginine dependent toxoplasmastatic activity in murine peritoneal macrophages is mediated by endogenous tumor necrosis factor-α. *J Immunol* 1992; 148 : 568-74.

Liew FY, Li Y, Millot S. Tumor necrosis factor-α synergizes with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. *J Immunol* 1990; 145 : 4306-10.

Liew FY, Cox FEG. Nonspecific defence mechanism: the role of nitric oxide. In: Ash C, Gallopher RB, eds. Immunoparasitology Today 1991; pp. A17-A21.

Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* 1988; 27 : 8706-11.

Mellouk S, Green SJ, Nacy CA, Hoffman S. IFN-γ inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. *J Immunol* 1991; 146 : 3971-6.

Nathan CF. Secretory products of macrophages. *J Clin Invest* 1987; 79 : 319-26.

Nussler A, Drapier JC, Renia L, *et al*. L-arginine-dependent destruction of intrahepatic malaria parasites in response to tumor necrosis factor and/or interleukin 6 stimulation. *Eur J Immunol* 1991; 21 : 227-30.

Ockenhouse CF, Shear HL. Oxidative killing of the intraerythrocytic malaria parasite *Plasmodium yoelii* by activated macrophages. *J Immunol* 1984; 132 : 424-31.

Pellet C, Henry J, Drapier JC. IFN-γ-activated macrophages: Detection by electron paramagnetic resonace of complex between L-arginine derived nitric oxide and non-heme-iron proteins. *Biochem Biophys Res Commun* 1990; 166 : 119-25.

Roach TIA, Kiderlen AF, Blackwell JM. Role of inorganic nitrogen oxides and tumor necrosis factor alpha in killing *Leishmania donovani* amastigotes in γ-interferon-lipopolysaccharide-activated macrophages from Lsh^s and Lsh^r congenic mouse strains. *Infect Immun* 1991; 59 : 3935-44.

Rockett KA, Targett GA, Playfair JHL. Killing of blood-stage *Plasmodium falciparum* by lipid peroxides from tumor necrosis serum. *Infect Immun* 1988; 56 : 3180-3.

Rockett KA, Awburn MM, Cowden WB, Clark IA. Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. *Infect Immun* 1991; 59 : 3280-3.

Shear HL, Nussenweig RS, Bianco C. Immune phagocytosis in murine malaria. *J Exp Med* 1979; 149 : 1288-98.

Stevenson MM, Huang DY, Podoba JE, Nowotarski ME. Macrophage activation during *Plasmodium chabaudii* AS infection in resistant C57BL/6 and susceptible A/J mice. *Infect Immun* 1992; 60 : 1193-201.

Stuehr DJ, Kwon NS, Nathan CF, Griffith GW. N^w-Hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J Biol Chem* 1991; 266 : 6259-63.

Supargiyono. Cell mediated immunity in malaria 6. Changes in the functional activities of resident bone marrow and spleen macrophages during *Plasmodium vinckei petteri* infection. *Indonesian Med J* 1997 (in press).

Taverne J, Dockrell HM, Playfair JHL. Killing of malarial parasite *Plasmodium yoelii in vitro* by cells of myeloid origin. *Parasite Immunol* 1982; 4 : 77-91.

Taverne J, Tavernier J, Fiers W, Playfair JHL. Recombinan tumor necrosis factor inhibit malaria parasite *in vivo* but not *in vitro*. *Clin Exp Immunol* 1987; 67 : 1-4.

Taylor-Robinson AW, Phillips RS, Severn A, Liew FY. TH1 and TH2 CD4+ T cells protect against *Plasmodium chabaudii* infection via nitric oxide and IgG1 respectively. 1997 (Submitted)

Ward KN, Warrell MJ, Rhodes J, Looareesuwan S, White NJ. Altered expression of human monocytes Fc receptors in *Plasmodium falciparum* malaria. *Infect Immun* 1984; 44 : 623-6.

Wozencraft AO, Croft SL, Sayer G. Oxygen radical release by adherent cell populations during the initial stages of a lethal rodent malarial infection. *Immunology* 1985; 56 : 523-31.

Wozencraft AO, Dockrell HM, Taverne J, Targett JA, Playfair JHL. Killing of human malaria parasites by macrophage secretory products. *Infect Immun* 1984; 43 : 664-9.