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Sera from Patients with Falciparum Malaria Induce Substance P Gene Expression in Cultured Human Brain Microvascular Endothelial Cells

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Substance P is a pluripotent neuropeptide capable of inducing neurogenic inflammation, immunoregulation, and vasodilatation. In an effort to contribute to the understanding of the pathophysiology of cerebral malaria, we have evaluated the effects of sera obtained from patients suffering from severe or mild malaria and from a healthy donor with no previous history of exposure to malaria on the expression of the substance P gene by cultured human brain microvascular endothelial cells (HBMEC) and human umbilical-vein endothelial cells. PCR, Southern blotting, hybridization with an internal probe, and densitometry demonstrated that treatment of HBMEC with sera from patients with severe malaria caused remarkably increased expression of the substance P gene. In HBMEC, substance P was not significantly influenced by serum from a healthy donor. Substance P was expressed at almost undetectable levels in untreated HBMEC. Treatment of cultured human umbilical-vein endothelial cells with the same sera produced no signal. The influence of different sera on the expression of substance P by HBMEC suggests that substance P expression may be involved in events leading to the development of severe malaria.

The pathophysiology of cerebral malaria remains poorly understood. Functional alterations of the vascular endothelium due to interaction with parasitized erythrocytes or parasite products may play a significant role. Several hypotheses have been put forward to explain the pathophysiology of cerebral malaria (1, 28, 40–42). These include occlusion of cerebral capillaries and venules by infected erythrocytes and decreased microcirculatory flow due to hemoconcentration resulting from increased capillary permeability to fluids, intravascular coagulation, or the inability of nondeformable infected cells to pass through the microcirculation. The possible involvement of neuropeptides in the pathophysiology of severe malaria has so far been totally disregarded, despite accumulating evidence that neuropeptides such as substance P play a significant role in neurogenic inflammation, immunoregulation, and vasodilatation. Substance P is a bioactive neuropeptide and a member of a group of structurally related neuropeptides, the tachykinins. It plays an important role in the transmitter, inflammatory, and immunoregulatory functions of both the central and peripheral nervous systems (26, 31). It is produced in response to trauma or inflammatory stimuli and has an excitatory effect on both the central and peripheral neurons (21). Notably, substance P induces the synthesis of proinflammatory mediators such as interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-α), and IL-6 in various immunocompetent cells (16, 25, 32, 33). In endothelial cells, it stimulates the production of nitric oxide synthase, which in turn synthesizes nitric oxide, a potent vasodilator. The identification of substance P receptors on leukocytes (5) as well as on cells of the endothelium (14) and the demonstration that substance P can regulate leukocyte functions have lent further support to the concept that this neuropeptide plays an important role in inflammation. The proinflammatory cytokines (IL-1, IL-6, and TNF-α), which are induced by substance P, have been implicated in the pathophysiology of the hemodynamic disturbances seen in severe malaria. Recently, there has been a proposed role for nitric oxide in cerebral malaria (9). It has been postulated that severe malaria results from activation of a cytokine cascade as part of the host response.

In this study we have used cultured human brain microvascular endothelial cells (HBMEC) as a model system to investigate the in vitro effects of patient sera on the expression of substance P.

MATERIALS AND METHODS

Patients. All patients studied were nonimmune adult Europeans admitted to the Bernhard Nocht Institute for Tropical Medicine with imported cases of severe and mild falciparum malaria. Falciparum malaria was defined as severe if at least one of the following criteria were met (20): (i) impaired cerebral function, disorientation, drowsiness, and unconsciousness; (ii) pathologic global clotting tests, a prothrombin time activity of <50%, and a partial thromboplastin time of >45 s; (iii) impaired renal function and a serum creatinine level of >176 mM (2 mg/dl); (iv) respiratory insufficiency and an arterial oxygen tension of <70 mg Hg; and (v) hepatic damage and aspartate aminotransferase and/or alanine aminotransferase at or above 100 U/liter.

Study protocol. After obtaining informed consent from patients or their legal relatives (in cases of severely ill patients), blood samples were taken before the first dose of antimalarial therapy was administered. The study protocol did not interfere with the treatment of the patients, which was performed by independent physicians. Control blood samples were obtained from a healthy medication-free volunteer with no history of exposure to malaria. Venous blood was drawn in accordance with the hospital routine sampling procedure (17). Serum was separated within 15 min and immediately divided into aliquots, frozen, and stored at −70°C until use.

Isolation and culture of HBMEC. Primary cultures of HBMEC were derived from human brain specimens discarded from patients undergoing neurosurgical operations for various brain disorders. HBMEC were isolated following the procedure described by Risau (34). Briefly, meninges, white matter, and any recognizable vessels were removed and the brain tissues were thoroughly minced prior to enzymatic dispersion. The brain tissues were then digested with 0.3% collagenase (Biochrom) in isolation medium to dissociate microvessel fragments from surrounding tissue. The resulting cell suspension then underwent a density gradient centrifugation with 25% bovine serum albumin (Serva, Heidelberg, Germany) to separate myelin and cell debris from the microvessel fraction. These microvessels were again digested with 0.1% collagenase-dispase (Boehr-

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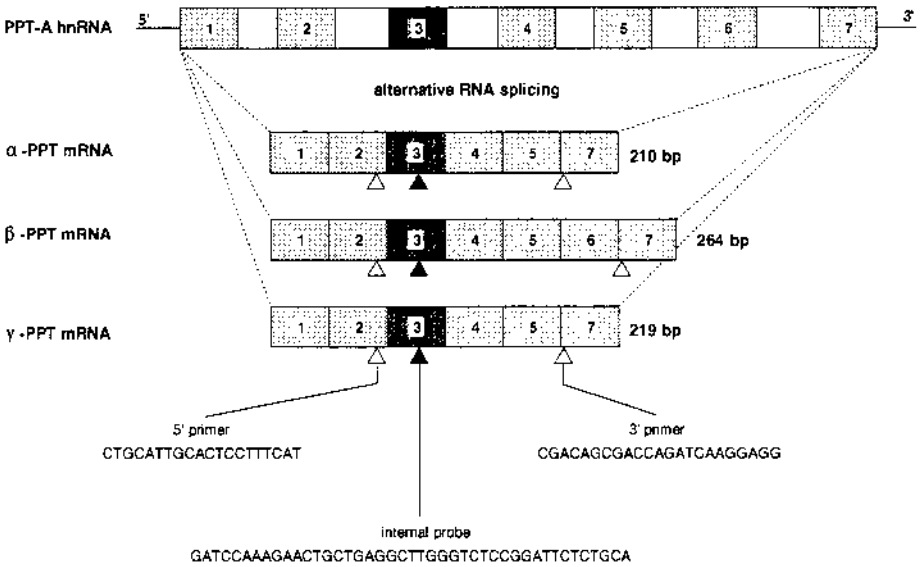


FIG. 1. Schematic alternative splicing of preprotachykinin (PPT)-A transcripts. Shaded boxes labelled with arabic numerals indicate exons. Unshaded and unlabelled boxes indicate introns. The approximate positions of the oligonucleotide primers are in exons 2 and 7, as indicated by open triangles. The internal probe is derived from exon 3, as indicated by the solid triangle. Introns and exons are not drawn to scale. hnRNA, heterogeneous nuclear RNA.

inger GmbH, Mannheim, Germany) to release single endothelial cells and small microvessel fragments, which were then cleansed of erythrocytes by Percoll gradient centrifugation (35% Percoll; Pharmacia). The cells were then resuspended in complete medium (high-glucose-content Dulbecco modified Eagle medium) supplemented with 1% antibiotic-antimycotic, 1% nonessential amino acids, 1% glutamine, 0.4% mercaptoethanol, and 1% sodium pyruvate (all from Gibco); 10% fetal calf serum (HyClone); 80 μg of endothelial cell growth factor (Bionetics, Rockville, Md.) per ml; and 50 μg of porcine heparin and were seeded onto collagen-precoated culture flasks (collagen type CLS II; Biochrom). Cells were grown in incubators at 37°C and 5% CO_2. The formation of confluent cell monolayers was monitored daily.

Isolation and culture of HUVEC. Primary cultures of human umbilical-vein endothelial cells (HUVEC) were established from five pooled freshly delivered umbilical cords obtained from natural and cesarian births. The endothelial cells were isolated by cannulation and subsequent enzymatic digestion of the inner lining of the vessel with 0.2% collagenase (Cooper Biomedical) for 15 min at 37°C in 5% CO_2. Primary cultures were propagated in the presence of medium 199 and RPMI 1640 (1:1) supplemented with 20% fetal bovine serum (HyClone Laboratories), 80 μg of endothelial cell growth factor per ml, 50 μg of porcine heparin per ml, 2 mM glutamine, 10,000 U of penicillin per ml, 10,000 U of streptomycin per ml, and 25 U of fungizone per ml. HUVEC were serially passaged twice per week by harvesting with trypsin-EDTA and seeding at a split ratio of 1:2 in 1.5% gelatin-precoated 75-cm^3 culture flasks (Falcon).

Identification of endothelial cells. The endothelial identities of both HBMEC and HUVEC were confirmed by morphologic criteria (typical cobblestone morphology), positive immunofluorescent staining with a specific anti-human von Willebrand factor antibody (19), and selective uptake of Di-I-Ac-LDL (acetylated low-density lipoprotein labelled with the fluorescent dye 1,1-dioctodecyl-1-3,3,3′,3′-tetramethylinocarbo-cyanine perchlorate; BTI, Stoughton, Mass.) as described by Voyta et al. (39). Labelling was performed according to the manufacturer's specifications. Uptake of Di-I-Ac-LDL was visualized with standard rhodamine excitation emission filters on a Dialux 20 ethidium bromide fluorescence microscope (Leitz, Wetzlar, Germany).

Treatment of cells (stimuli). The effects of different sera on substance P gene expression were studied by adding 1 ml of patient sera to 9 ml of culture medium and incubating HBMEC and HUVEC for 6 h. Cells challenged with sera from healthy volunteers and those not challenged with sera served as negative controls.

RNA isolation. At the end of the culture period, the test media were removed, the monolayers were washed twice with ice-cold diethyl pyrocarbonate-treated phosphate-buffered saline (pH 7.4), and the cells were dissociated by mechanical means in the same buffer. Total cellular RNA was isolated from cultured HBMEC and HUVEC according to the procedure of Chomczynski and Sacchi (6). Briefly, cells were lysed in 4 M guanidine isothiocyanate–25 mM sodium citrate–0.5% N lauroyl-sarcosine–0.1 M β-mercaptoethanol. This was followed by acid phenol chloroform extraction, precipitation with isopropanol, reextraction with chloroform, and ethanol precipitation. Total cellular RNA was quantitated spectrophotometrically at 260 nm. Polyadenylated RNA was prepared with a commercial kit (Poly A Tract mRNA isolation system; Promega, Heidelberg, Germany).

Reverse transcription. One microgram of poly(A)$^+$ RNA from each of the treated and untreated cells was used as template for the single-stranded cDNA in each reaction. A total of 0.5 μg of oligodeoxyribosylthymine$_{12\text{-}18}$ (Promega) was used as primer for the reverse transcription reactions. Poly(A)$^+$ RNA and oligodeoxyribosylthymine in a final volume of 10 μl were denatured for 5 min at 65°C and quickly cooled on ice. The above-described reaction mixtures were used for single-stranded cDNA synthesis in a final volume of 20 μl from a master mixture containing 1× reverse transcription buffer (50 mM Tris [pH 8.15 at 41°C], 6 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 1 mM deoxynucleoside triphosphate [dNTP; Boehringer], 14 U of RNasin [Promega], 200 U of avian myeloblastosis virus reverse transcriptase [Promega]). Synthesis was carried out for 1 h at 42°C. The reaction mixtures were heat inactivated at 70°C for 10 min.

PCR. Ten microliters of the reverse transcription mixtures was amplified by PCR. Reactions were performed with recombinant *Taq* polymerase (5 U per tube; Promega) in a PCR buffer containing two primers (1.0 μM each), 10 mM Tris-HCl, 50 mM KCl, 30 mM $MgCl_2$, and four dNTPs (200 μM each). The temperature profile was 1 min at 94°C (denaturation), 2 min at 60°C (annealing), and 1 min at 72°C (extension).

PCR exonic primers for substance P were constructed from the cDNA sequence (15). The primers were derived from exons 2 and 7 as shown in Fig. 1, and the internal probe was derived from exon 3, also as shown in Fig. 1.

Oligonucleotides were synthesized on a Biosearch-Milligen cyclone DNA synthesizer (Eschborn, Germany) and corresponded to nucleotides 186 to 208 (5′ primer) and nucleotides 431 to 449 (3′ primer) of the published human β-preprotachykinin-A cDNA sequence (15). The β-actin gene was used as a housekeeping gene to ensure the quality of the mRNA and reagents used in the reactions and as a standard for comparisons. Amplification of the β-actin gene was performed under the same conditions with the following primers (30): β-actin sense, TCACCAACTGGGACGACATG; β-actin antisense, AGGCTG TGCTATCCCTGTAC; and β-actin probe, CAGCCATGTACGTTGCTATC.

We utilized reverse transcriptase PCR to detect substance P gene expression because the expression of substance P gene was below the threshold of detection by any other accessible method. Identical amounts of starting material were subjected to 30 PCR cycles.

Analysis of PCR products. After completion of the PCR, the amplified DNA fragments were chloroform extracted, ethanol precipitated, and resuspended in water. Ten-microliter aliquots of PCR products were fractionated on 1.5% agarose (GIBCO BRL Life Technologies) gels. The fractionated DNA was transferred to Hybond N membranes (Amersham) essentially as described by Sambrook et al. (35). Briefly, the gels were soaked in 0.5 M NaOH–1.5 M NaCl to denature the DNA, neutralized in 1 M Tris-HCl (pH 8.0)–1.5 M NaCl, and transferred in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by capillarity. After transfer, DNA was covalently linked to the nylon membrane by exposure to UV light. To confirm sequence specificity of human substance P amplification, blots were probed with [γ-^{32}P]dATP T-4 kinase-labelled (Promega) internal hybridization oligonucleotide probe corresponding to part of

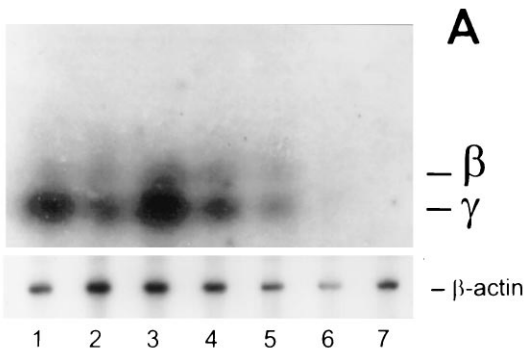
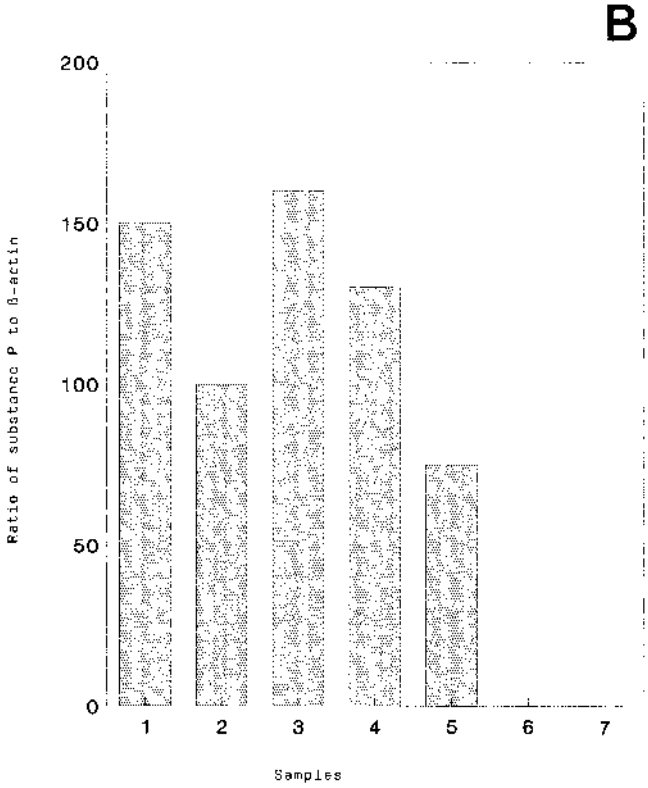


FIG. 2. (A) Southern blot analysis of PCR products of mRNA of HBMEC incubated with different sera. The filters were hybridized and labelled with substance P and β-actin probes. All cells were incubated for 6 h. Lanes 1 and 3, incubation with sera from patients with severe malaria; lanes 2, 4, and 5, incubation with sera from patients with mild malaria; lane 6, incubation with serum from a healthy donor; lane 7, incubation of untreated cells. Products were of the expected masses for the substance P primers used. The β-actin gene was used as the housekeeping gene for comparison. (B) Ratio of the substance P gene to the β-actin gene. Densitometric values were determined from the blots in panel A and are presented as the ratios of substance P to β-actin signals expressed as percentages. The numbers at the bottom correspond to the lane numbers in panel A.

substance P-encoding exon 3 of human cDNA (nucleotides 236 to 280) (15) (Fig. 1). Hybridization conditions were standard; hybridization was performed at 60°C overnight and was followed by stringent washing. Autoradiograms were exposed for 6 to 12 h to XAR-5 film (Kodak).



RESULTS

Remarkably increased expression of the substance P gene was observed in cells treated with severe malaria patient sera (Fig. 2A, lanes 1 and 3). The levels of substance P gene expression in HBMEC challenged with mild malaria sera (Fig. 2A, lanes 2, 4, and 5) were evidently less than those in HBMEC treated with sera from patients with severe malaria. HBMEC challenged with serum from a healthy donor with no previous history of exposure to malaria (Fig. 2A, lane 6) showed no or a negligibly low level of expression. No substance P gene expression was observed in unchallenged cells (Fig. 2A, lane 7). We studied the effects on substance P gene expression of sera from an additional seven patients with severe malaria. As shown in Fig. 3, there was enhanced expression of the substance P gene in all samples.

β-Actin gene expression was readily detected in all samples after overnight exposure as shown in Fig. 2A and Fig. 3. The β-actin signals did not vary significantly among the samples, indicating reasonable consistency in mRNA extraction, cDNA synthesis, and amplification among the different samples.

In order to confirm variations in substance P gene expression among the samples corresponding to the lanes shown in Fig. 2A, densitometric values were determined from the blots and expressed as ratios of substance P to β-actin signals. The results were then expressed as percentages (Fig. 2B).

DISCUSSION

The results presented in this paper demonstrate that sera from patients with falciparum malaria induce the expression of substance P gene by HBMEC in vitro. The expression of substance P gene was not significantly influenced by serum from a healthy donor. Our results suggest that severe falciparum malaria patient sera contain malarial exoantigens or neurohumoral factors which provoke the enhanced expression of the substance P gene by HBMEC. On the other hand, sera from mild falciparum malaria patients may not have contained the hypothesized mediators, or the concentrations of such mediators may have been so small that they did not elicit the expression of the substance P gene in detectable amounts.

The pathophysiology observed in severe malaria seems to be caused not directly by parasite product but by normal components of the immune response, mainly cytokines such as TNF-α, produced in excess (7, 11–13, 17, 20, 22, 24). These mediators are released from the host's monocytes and macrophages, probably in response to stimulation by substance P. Circulatory immunocompetent cells (lymphocytes, mast cells, and macrophages) may be under the direct influence of neuropeptides (32). Lotz and coworkers have demonstrated that substance P directly stimulates the release of cytokines IL-1, TNF-α, and IL-6 from lymphocytes and macrophages (25).

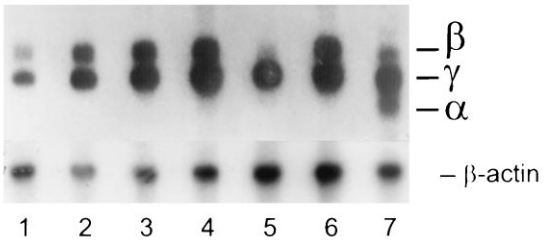


FIG. 3. Southern blot analysis of PCR products of mRNA of HBMEC incubated with severe malaria patient sera for 6 h. The filters were hybridized with substance P and β-actin probes. β-Actin was used as a housekeeping gene for comparison.

This implicates this neuropeptide in the inflammatory and immunoregulatory effects of IL-1, TNF-α, and IL-6. Convincing evidence from various studies indicates that the clinical manifestations of severe malaria are associated with the presence of high circulatory concentrations of TNF-α in serum (7, 11–13, 17, 20, 22, 24). Recent experimental studies have demonstrated that a phospholipid containing inositol, which is released from parasitized erythrocytes, induces the release of TNF-α from macrophages (3, 4, 23, 36, 38). This is compatible with current concepts of the pathophysiology of severe malaria. In endothelial cells, substance P and inositol monophosphate are known to stimulate the production of both TNF-α and nitric oxide (NO), which have both been implicated in the pathophysiology of cerebral malaria. Substance P and inositol may act independently or synergistically in the production of NO and TNF-α. It is currently assumed by some authors that NO plays a part in the development of cerebral malaria (2, 8, 9, 10). Some authors, on the other hand, argue that NO protects the organism against the development of cerebral malaria (18, 27, 29, 37). There may be an interplay of substance P, inositol, and proinflammatory cytokines in the pathogenesis of severe malaria. Thus, we hypothesize that the release of substance P and inositol may be the earliest pathophysiological events leading to the enhanced or exaggerated inflammatory response of cytokines, with subsequent triggering of the clinical symptoms seen in severe malaria. The failure of malaria patient sera to induce the expression of substance P gene expression by HUVEC suggests that substance P expression is limited to tissues of inflammatory or immunological significance. It is known that endothelial cells from different organs do not have the same physiological properties. Therefore, the stimulation of substance P production in HBMEC may reflect this difference. The vasodilatatory properties of substance P might be utilized by the host to correct the blood flow in the capillaries.

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REFERENCES

1. **Aikawa M.** 1988. Human cerebral malaria. Am. J. Trop. Med. Hyg. **39:**3–10.
2. **Asensio, V. C., H. Oshima, and P. B. Falanga.** 1993. Plasmodium berghei: is nitric oxide involved in the pathogenesis of mouse cerebral malaria? Exp. Parasitol. **77:**111–117.
3. **Bate, C. A. W., and D. P. Kwiatkowski.** 1994. Stimulators of tumor necrosis factor production released by damaged erythrocytes. Immunology **83:**256–261.
4. **Bate, C. A. W., J. Taverne, E. Roman, C. Moreno, and J. H. L. Playfair.** 1992. Tumor necrosis factor induction by malaria exoantigens depends upon phospholipid. Immunology **75:**129–135.
5. **Bolst, K. L.** 1988. Hormone and neuropeptide receptors on mononuclear leucocytes. Prog. Allergy **43:**68.
6. **Chomczynski, P., and N. Sacchi.** 1987. Single step of RNA isolation by acid guanidium thiocynate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159.
7. **Clark, I. A., G. Chaudhri, and W. B. Cowden.** 1989. Roles of tumor necrosis factor in illness and pathology of malaria. Trans. R. Soc. Trop. Med. Hyg. **83:**436–440.
8. **Clark, I. A., and K. A. Rockett.** 1994. The cytokine theory of cerebral malaria. Parasitol. Today **10:**410–412.
9. **Clark, I. A., K. A. Rockett, and W. B. Cowden.** 1991. Proposed link between cytokines, nitric oxide and human cerebral malaria. Parasitol. Today **7:**205–207.
10. **Grau, G. E., and S. de Kossodo.** 1994. Cerebral malaria: mediators, mechanical obstruction or more? Parasitol. Today **10:**408–409.
11. **Grau, G. E., P. H. Lambert, P. Vassalli, and P. F. Piguet.** 1989. Tumor necrosis factor (TNF) and pathology; its relationships with other cytokines. Schweiz. Med. Wochenschr. **119:**1756–1761.
12. **Grau, G. E., P. F. Piguet, P. Vassalli, and P. H. Lambert.** 1989. Tumor necrosis factor and other cytokines in complicated malaria: experimental and clinical data. Immunol. Rev. **112:**49–70.
13. **Grau, G. E., T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel, and P. H. Lambert.** 1989. Tumor necrosis factor and disease severity in children with falciparum malaria. N. Engl. J. Med. **320:**1586–1591.
14. **Greeno, E. W., P. Mantyh, G. M. Vercellotti, and C. F. Moldow.** 1993. Functional neurokinin 1 receptors for substance P are expressed by human vascular endothelium. J. Exp. Med. **177:**1269–1276.
15. **Harmar, A. J., A. Armstrong, J. C. Pascall, K. Chapman, R. Rosie, A. Curtis, J. Going, C. R. W. Edwards, and G. Fink.** 1986. cDNA sequence of human β-preprotachykinin, the common precursor to substance P and neurokinin A. FEBS Lett. **208:**67–72.
16. **Hartung, H. P., K. Wolters, and K. V. Toyka.** 1986. Substance P binding properties and studies on cellular responses in guinea pig macrophages. J. Immunol. **135:**3856.
17. **Hemmer, C. J., P. Kern, F. G. E. Holst, K. P. Radtke, R. Egbring, A. Bierhaus, P. P. Nawroth, and M. Dietrich.** 1991. Activation of the host response in human P. falciparum malaria: relation of parasitemia to TNF alpha, thrombin-antithrombin III and protein C levels. Am. J. Med. **91:**37–41.
18. **Jacobs, P., D. Radzioch, and M. M. Stevenson.** 1995. Nitric oxide expression in the spleen, but not in the liver, correlates with resistance to blood-stage malaria in mice. J. Immunol. **176:**5306–5313.
19. **Jaffe, E. A., L. W. Hoyer, and R. L. Nachman.** 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. J. Clin. Invest. **52:**2757–2764.
20. **Kern, P., C. J. Hemmer, J. Van Damme, H. J. Gruss, and M. Dietrich.** 1989. Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated plasmodium malaria. Am. J. Med. **87:**139–143.
21. **Krause, J. E., Y. Takeda, and A. D. Hershey.** 1992. Structure, functions, and mechanisms of substance P receptor action. J. Invest. Dermatol. **98(6):**2S–7S.
22. **Kwiatkowski, D.** 1990. Tumor necrosis factor, fever and fatality in falciparum malaria. Immunol. Lett. **25:**213–216.
23. **Kwiatkowski, D., J. G. Cannon, K. R. Manogue, A. Cerami, A. Dinarello, and B. M. Greenwood.** 1989. Tumor necrosis factor production in *Falciparum malaria* and its association with schizont rupture. Clin. Exp. Immunol. **77:** 361–366.
24. **Kwiatkowski, D., A. V. Hill, T. Sambou, P. Twumasi, J. Castracane, K. R. Manouge, A. Cerami, D. R. Brewster, and B. M. Greenwood.** 1990. TNF concentration in fatal complicated malaria, non-fatal cerebral malaria and uncomplicated Plasmodium malaria. Lancet **336:**1201–1204.
25. **Lotz, M., J. H. Vaughan, and D. A. Carson.** 1988. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. Science **241:** 1218–1220.
26. **McGillis, J. P., M. L. Organist, and D. G. Payan.** 1987. Substance P and immunoregulation. Fed. Proc. **46:**196–199.
27. **Mellouk, S., S. L. Hoffman, Z.-Z. Liu, P. de la Vega, T. R. Billiar, and A. K. Nussler.** 1994. Nitric oxide-mediated antiplasmodial activity in human and murine hepatocytes induced by gamma interferon and the parasite itself: enhancement by exogenous tetrahydrobiopterin. Infect. Immun. **62:**4043–4046.
28. **Miller, L. H., M. F. Good, and G. Milon.** 1994. Malaria pathogenesis. Science **264:**1878–1883.
29. **Naotunne, T. S., N. D. Karunaweera, K. N. Mendis, and R. Carter.** 1993. Cytokine-mediated inactivation of malarial gametocytes is dependent on the presence of white blood cells and involves reactive nitrogen intermediates. Immunology **78:**555–562.
30. **Ng, S.-Y., P. Gunning, R. Eddy, P. Ponte, J. Leavitt, T. Shows, and L. Kedes.** 1985. Evolution of the functional human β-actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol. Cell. Biol. **5:**2720–2732.
31. **Payan, D. G.** 1989. Neuropeptides and inflammation: the role of substance P. Annu. Rev. Med. **40:**341–352.
32. **Payan, D. G., D. R. Brewster, and E. J. Gotzel.** 1986. Specific stimulation of T lymphocytes by substance P. J. Immunol. **131:**1613–1615.
33. **Payan, D. J., D. R. Brewster, A. Miissirian-Bastian, and E. J. Goetzl.** 1984. Substance P recognition by a subset of human T lymphocytes. J. Clin. Invest. **74:**1532–1539.
34. **Risau, W.** 1990. Isolation and culture of brain endothelial cells. J. Cell Biol. **110:**1757–1766.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Schofield, L., and F. Hackett.** 1993. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J. Exp. Med. **177:** 145–153.
37. **Seguin, M. C., F. W. Klotz, I. Schneider, J. P. Weir, M. Goodbary, M. Slayter, J. J. Raney, J. U. Aniagolu, and S. J. Green.** 1994. Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated Plasmodium berghei infected mosquitoes: involvement of interferon γ and $CD8^+$ T cells. J. Exp. Med. **180:**353–358.
38. **Taverne, T., C. A. W. Bate, D. A. Sarkar, A. Meager, G. A. W. Rook, and**

J. H. L. Playfair. 1990. Human and murine macrophages produce TNF in response to soluble antigens of *Plasmodium falciparum*. Parasite Immunol. **12:**33–43.
39. **Voyta, J. C., D. P. Via, C. E. Butterfield, and B. R. Zetter.** 1984. Identification of endothelial cells based on their incorporation of acetylated low-density lipoprotein. J. Cell Biol. **99:**2034–2040.
40. **Warrell, D. A.** 1987. Pathophysiology of severe Falciparum malaria in man. Parasitology **94:**S53–S76.
41. **Warrell, D. A., M. E. Molyneux, and P. F. Beales.** 1990. Severe and complicated malaria. Trans. R. Soc. Trop. Med. Hyg. **84**(suppl. 2)**:**1–52.
42. **White, N. J., and M. Ho.** 1992. The pathophysiology of malaria. Adv. Parasitol. **31:**84–149.

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