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Systemic Endothelial Activation Occurs in Both Mild and Severe Malaria

Correlating Dermal Microvascular Endothelial Cell Phenotype and Soluble Cell Adhesion Molecules with Disease Severity

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Fatal *Plasmodium falciparum* malaria is accompanied by systemic endothelial activation. To study endothelial activation directly during malaria and sepsis *in vivo*, the expression of cell adhesion molecules on dermal microvascular endothelium was examined in skin biopsies and correlated with plasma levels of soluble (circulating) ICAM-1, E-selectin, and VCAM-1 and the cytokine tumor necrosis factor (TNF)-α. Skin biopsies were obtained from 61 cases of severe malaria, 42 cases of uncomplicated malaria, 10 cases of severe systemic sepsis, and 17 uninfected controls. Systemic endothelial activation, represented by the up-regulation of inducible cell adhesion molecules (CAMs) on endothelium and increased levels of soluble CAMs (sCAMs), were seen in both severe and uncomplicated malaria and sepsis when compared with uninfected controls. Plasma levels of sICAM-1, sVCAM-1, and sE-selectin correlated positively with the severity of malaria whereas TNF-α was raised nonspecifically in malaria and sepsis. Immunohistochemical evidence of endothelial activation in skin biopsies did not correlate with sCAM levels or disease severity. This indicates a background of systemic endothelial activation, which occurs in both mild and severe malaria and sepsis. The levels of sCAMs in malaria are thus not an accurate reflection of endothelial cell expression of CAMs in a particular vascular bed, and other factors must influence their levels during disease. *(Am J Pathol 1998, 152:1477–1487)*

Infection with the malaria parasite *Plasmodium falciparum* is characterized by sequestration of parasitized red blood cells (PRBCs) in the microvasculature of vital organs.[1] The adhesion of infected erythrocytes to the endothelium of these vessels is mediated by specific receptor-ligand interactions and is linked to the pathogenesis of malaria; sequestration of PRBCs in the brain is significantly associated with development of cerebral malaria, one of the most severe complications of the disease.[2,3] Several host molecules that allow adhesion of infected erythrocytes have been identified *in vitro*[4,5] and their distribution examined in postmortem tissues in fatal malaria.[6,7] The expression of the putative sequestration receptors intercellular adhesion molecule 1 (ICAM-1), E-selectin, and vascular cell adhesion molecule 1 (VCAM-1) is increased in several different tissues in fatal malaria, and in the brain, the distribution of sequestered PRBCs co-localizes with these receptors.[8] The up-regulation of markers for endothelial cell (EC) activation in the brain, muscle, lung, and kidney reflects a pattern of widespread endothelial activation in fatal malaria. This may result from the systemic release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-1, and interferon-γ, which are known to activate endothelial cells *in vitro*[9] and cause systemic endothelial activation in animal models of sepsis.[10,11] Levels of TNF-α are raised in African children with falciparum malaria and correlate with the severity of disease.[12,13]

Cell adhesion molecules (CAMs) expressed on endothelial cells have physiological roles in directing leukocyte adhesion and trafficking to sites of inflammation.[14–16] These molecules are also released as soluble forms into serum, either because of shedding from the endothelial cell surface or differential mRNA splicing to form a truncated, soluble form with no cytoplasmic anchor sequence.[17] The levels of soluble (circulating) ad-

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hesion molecules have now been measured in normal patients and shown to be raised in a wide variety of diseases,[18,19] due to release from a spectrum of cell types, including lymphocytes and endothelial cells.

Soluble (s)ICAM-1, VCAM-1, and E-selectin concentrations are raised in the sera of falciparum malaria patients from Africa and South America. sICAM-1 and sE-selectin were significantly raised in acute nonsevere malaria compared with controls.[20,21] In another study of Gambian and Danish patients with either *P. falciparum* or *P. vivax* malaria, levels of sE-selectin, sICAM-1, and sVCAM-1 were raised and correlated positively with the severity of the malaria.[22] Soluble CAMs were also raised in patients with vivax malaria and bacterial meningitis. However, not all studies have confirmed the relationship between severity of malaria and levels of ICAM-1.[23] In a study of Gambian children, sICAM-1 levels were higher in the 246 malaria-infected patients compared with 156 non-malaria-infected controls, but there were no differences between the levels in children with severe, cerebral disease compared with uncomplicated malaria, or between fatal cases and survivors of cerebral malaria.[24]

We set out to test whether endothelial cell activation occurs in nonfatal malaria and whether it correlates with disease severity. We chose two complementary approaches: direct examination of dermal endothelial cell phenotype *in vivo* by immunohistochemistry and measurement of the levels of soluble CAMs (sCAMs) in these same patients.

Deep organ sampling in living patients for such a study is unethical, and so minimally invasive techniques were necessary to obtain tissue with vessels for study. Skin and muscle biopsies have been used clinically to diagnose and study several diseases during life, including skin disorders and myopathies. Immunohistochemistry has been used on skin biopsy specimens from graft-*versus*-host disease and delayed hypersensitivity reactions to examine immune cell localization and endothelial phenotype on dermal microvessels. [25–27] TNF-α administered exogenously has been shown to modify the phenotype of the dermal vasculature,[28] and we have assumed that all endothelium is equally accessible to bursts of TNF-α and other cytokines released at schizogony. We carried out a prospective study of adult Vietnamese patients with severe and nonsevere malaria and sepsis and of uninfected controls. We obtained skin biopsies and blood from these patients to examine the phenotype of dermal endothelium and to correlate this with the plasma levels of sCAMs as surrogates for endothelial activation in deeper vascular beds.

Materials and Methods

Sample and Data Collection

The experimental protocol was approved by the Scientific and Ethics Committee of The Center for Tropical Diseases, Ho Chi Minh City, Viet Nam. Consent was obtained from all patients (or their immediate relatives when the patient was comatose) before sample collection. Clinical details were taken for all cases at the time of sample collection, and all subsequent analyses were carried out blind to these details. Skin biopsies were obtained from 42 cases of uncomplicated malaria and 61 cases of severe malaria, and plasma samples were taken from 21 and 42 patients of these groups, respectively. For comparison, we examined 10 cases of systemic sepsis from a variety of causes. Malaria was excluded in each by repeated peripheral blood smears, and systemic sepsis was confirmed on blood culture. We also used skin samples from 13 Vietnamese and 4 English controls. These were collected at elective surgical operations from the periphery of the incision, in cases with no evidence of infection or neoplasm.

Skin biopsies from malaria- and non-malaria-infected patients were collected from the volar surface of the forearm or leg after local infiltration of 1% lignocaine anesthetic using a disposable sterile 3-mm skin biopsy punch (Steifel Labs, UK). Wound hemostasis was achieved with a 3/0 nylon suture, removed after 7 days. The wound was checked for infection at this stage, and no complications were observed.

Immunohistochemistry

Skin biopsies were snap frozen in liquid nitrogen and stored at −70°C until use. Frozen sections were cut using a cryostat, and immunohistochemistry was performed using a standard alkaline phosphatase anti-alkaline phosphatase (APAAP) method.[29] The panel of monoclonal antibodies used is detailed in Table 1 and included the following classes: 1) constitutive endothelial markers (eg, CD31 and CD34), 2) inducible endothelial markers (eg, E-selectin and VAP-1), 3) vascular endothelial adressins (eg, HECA-452), 4) host receptors for *P. falciparum*-infected erythrocytes (eg, ICAM-1 and CD36), 5) leukocyte subset markers (eg, CD4 and CD68), 6) cytokines (eg, TNF-α), and 7) *P. falciparum* parasite marker (MSP-1).

Slides were blinded, and the degree and distribution of staining was assessed independently by two observers (G.D.H. Turner and S.B. Fox). Results were compared, and when there was a disagreement, the section was reviewed to assign a final score.

Quantitation of Circulating sCAMs and TNF-α levels in Plasma

Peripheral venous blood samples were collected into lithium/heparin tubes and spun down immediately at 5000 rpm for 10 minutes at 4°C. The plasma was aliquotted and frozen to −70°C until use. Levels of the soluble adhesion molecules ICAM-1, VCAM-1, and E-selectin were measured using a commercially available ELISA (R&D Systems, Oxon, UK) in accordance with the manufacturer's instructions. The quantitative standards were diluted in fresh plasma at 1/20. Initially, all of the patients' plasma samples for sICAM-1 were diluted at 1/20 in the test buffer provided. Subsequent dilutions were made at 1/50 for sICAM-1 and sE-selectin and 1/100 for sVCAM-1. All samples were tested in duplicate. The color was de-

Table 1. Monoclonal Antibody Panel Used for Immunohistochemistry

Antigen	Clone	Form	Source
CD31 (PECAM)	JC70A	Neat hybridoma Supernatant (SN)	Dako, Glostrup, Denmark
CD34	TÜK3	SN	Dako
EN4 antigen	EN4	SN 1/5	Prof. C. Spry
P-selectin (CD62P)	SPU18.1	Mouse ascites diluted 1/500 in TBS (Asc 1/500)	Dr. H. Nieuwenhuis
vWf (FVIIIRA)	F8/86	SN	Dako
ICAM-1 (CD54)	15.2	SN	Dr. N. Hogg
CD36	ESIVC7	Asc 1/250	Dr. E. van der Schoot
E-selectin (CD62E)	1.2B6	Asc 1/200	Dr. D. Haskard
VCAM-1 (CD106)	1.4C3	SN	Dr. D. Haskard
HLA class I	W6/32	SN	Dako
HLA class II	NFK-1	Asc 1/400	Dr. S. Fuggle
Thrombospondin 1	189/53.2	Asc 1/50	Dr. J. Dawes
MSP-1 (*P. falciparum*)	M195/19.8	Asc 1/250	Dr. C. Newbold
Macrophage (CD68)	KP-1	SN	Dako
Neutrophil (elastase)	NP57	SN	Dako
B cell (CD22)	Tδ15	SN	Dako
T cell (CD3)	UCHT1	SN	Dako
TNF-α	52B83	Asc 1/100	Dr. W. Buurman
TNF-α receptor (p55 and p75)	5H5 and 4C8	Asc 1/100 cocktail	Dr. W. Buurman
Interferon-γ	MD-1	Asc 1/200	Dr. P. Van der Meide
MECA-79 antigen	MECA-79	SN	Dr. E. Butcher
HECA-452 antigen	HECA-452	SN	Dr. E. Butcher
VAP-1	VAP-1	Asc 1/150	Dr. M. Salmi
Negative control	Isotypes	Asc 1/200	Dr. C. Newbold

veloped using a tetramethylbenzidine substrate and read on a Titertek plate reader at absorbance 450 nm corrected at 620 nm.

Levels of free circulating plasma TNF-α were measured using a previously characterized sandwich ELISA technique.[13] Sterile 96-well plates were coated with mouse monoclonal antibody against human TNF-α and incubated overnight at 4°C. They were then washed four times with PBS/0.2% Tween 20 (wash buffer), and 50 μl of neat plasma from each sample was aliquotted in duplicate, along with precharacterized quantification standards diluted in fresh human plasma. The plates were incubated for 2 hours at 37°C with shaking and then washed four times, and 50 μl of the second-layer antibody (a polyclonal rabbit anti-TNF-α serum diluted 1/100 in PBS/Tween plus 2% normal goat serum) was added. This was incubated for 1 hour at 37°C with shaking and washed three times. The third-layer antibody was a goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, Poole, UK) diluted 1/2000 in PBS/Tween plus 2% human serum. This was plated at 50 μl per well and incubated for an additional 45 minutes at 37°C. The wells were washed six times and developed with the substrate pNNP (Sigma) at a concentration of 5 mg/ml in diethanolamine buffer. A 100-μl aliquot of substrate was added per well, and the plate was incubated at 37°C for 15 minutes. The resulting color was measured on the pre-equilibrated Titertek plate reader at absorbance 405 nm. Values were converted into TNF concentrations using a standard curve drawn from the precharacterized quantitative standards.

Statistical Analysis

All statistical calculations were carried out using StatView 4.0 (Berkeley, CA) and Stata 4.0 (College Station, TX) software packages. The precharacterized quantitative standards from each ELISA were used to construct interpolated curves of optical density against concentration of sCAMs. The spectrophotometric reading for each sample was then converted to a concentration of sCAMs in nanograms per milliliter. Each ELISA was supplied with a separate test sample for an internal positive control, and this fell within the predicted range in all cases. The means of the duplicate readings were calculated and the samples unblinded. The concentrations of sICAM-1, sE-selectin, sVCAM-1, and TNF-α were then plotted as scattergrams for each patient within the four disease groups, and summary statistics were calculated for the groups (16 sets).

The distribution of concentrations of each molecule for each disease group (12 sets) was tested for normality using the Shapiro-Wilks *W* test. The distributions did not follow a normal distribution in the disease groups. To compare the levels of sCAMs and TNF-α between disease groups, the following statistical techniques were used: 1) nonparametric analysis of variance (Kruskall-Wallis test) and 2) nonparametric analysis of trend within the groups of controls, mild malaria, and severe malaria.[30] Statistical tests allowed for multiple comparisons using a Bonferroni correction.

To see whether the presence of endothelial activation within the severe malaria group predisposed to particular clinical complications, the presence or absence of clinical complications was compared with the presence or absence of dermal endothelial activation, using Fisher's exact test. Finally, to compare the two different methods used to judge endothelial activation (the presence of endothelial activation as judged from immunostaining and the levels of sCAMs/TNF-α), Fisher's exact test was used to judge how predictive an increased sCAM level

Table 2. Summary of Clinical Details of Patient Groups

Group	Diagnosis	Median age in years (range)	Median duration of illness in days (range)
Controls, n = 13	ENT cases and minor surgery. Cases with infection or inflammation were excluded	31.3 (9–54)	N/A
Sepsis, n = 10	Pyelonephritis (2), meningococcemia (2), leptospirosis (2), pneumonia (3), typhoid fever (1). Bacteremia on blood culture with clinical shock or Bone's criteria for sepsis syndrome. Proven negative for *P. falciparum* on at least two blood smears	30.4 (11–78)	5.7 (1–14)
Mild malaria, n = 42	Proven *P. falciparum* positive on blood smear. All were in-patients on the acute malaria ward with no complications of severe malaria	26.5 (15–46)	6 (1–13)
Severe malaria, n = 61	Proven *P. falciparum* positive on blood smear. All were in-patients on severe malaria ward with one or more complications of severe malaria as defined by WHO criteria	30 (15–72)	4.5 (1–14)

was of increased CAM expression in the skin biopsy, and *vice versa*.

Results

Clinical Details

The patients with severe malaria were defined by the presence of one or more diagnostic criteria from the World Health Organization guidelines.[31] Patients with nonsevere (mild) malaria had positive blood smears and were considered to require admission to hospital but had no signs of severity. The details of the sepsis patients are shown in Table 2. The baseline demographic characteristics did not differ significantly between groups.

Immunohistochemistry of Control Skin Biopsies

Dermal microvessels in control cases from both Vietnam and United Kingdom showed expression of the constitutive endothelial markers CD31, von Willebrand factor (vWf), and EN4 (Table 3). They were uniformly positive for class I HLA and CD36, and all cases showed constitutive expression of ICAM-1. There was no staining for the inducible endothelial adhesion molecules E-selectin or VAP-1, and only scanty weak staining for VCAM-1 in 2/17 control cases. There was variable but appreciable staining for P-selectin and CD34 in most control cases, but little thrombospondin staining was seen on endothelium. Class II HLA staining was seen in most control cases on both vessels and dermis. Cellular immune markers showed the expected distribution of macrophages throughout the dermis and epidermis, along with minimal $CD4^+$ cells in the dermis, but no neutrophil or B cell staining. Control staining for MSP-1, a *P. falciparum* marker, was uniformly negative. Thus, dermal microvessels from control cases in this study had a phenotype of $CD36^+$, ICAM-1^+, VCAM-1^-, E-selectin$^-$, TSP^-. Staining for TNF-α, interferon-γ, HECA-452, MECA-79, and VAP-1 was not seen in control cases.

Immunohistochemistry on Malaria and Sepsis Skin Biopsies

Dermal microvessels in both malaria and sepsis cases showed similar expression of CD31, vWf, and EN4. CD34 staining was the same as in control cases. The staining of vWf was less widespread but stronger on ascending spiral vessels between the superficial and deep dermal plexus. Often the staining of vWf and P-selectin was not confined to the endothelial cell granules but released into the lumen and surface. Thrombospondin was either weakly expressed on endothelium or not seen, but was expressed strongly on myoepithelial cells around the sweat glands. In addition to these markers, all vessels in all cases were strongly positive for class I HLA, class II HLA, and ICAM-1. All vessels were $CD36^+$ as in control cases. There was evidence for endothelial activation in the increased expression of inducible endothelial markers in both severe and nonsevere malaria (Table 3 and Figure 1). VCAM-1 staining was seen in 73% and 69% of severe and nonsevere cases, respectively, and E-selectin in 39% and 43%. The induction of E-selectin and VCAM-1 was accompanied in some cases by the expression of the inducible endothelial cell adhesion molecule VAP-1[32] (in 45% and 62% of mild and severe cases, respectively). The distribution of these markers varied between cases but was more focal and weaker than ICAM-1 or CD31. ICAM-1 staining was very intense in some infected cases and showed increased staining on endothelium and epidermal cells compared with controls. There was no significant difference in the numbers of cases staining for VCAM-1 and E-selectin between the two malaria groups, indicating that endothelial activation in dermal microvessels is not specific to severe malaria. This was confirmed by the increased expression of these markers in non-malaria-infected sepsis cases. These showed evidence for endothelial activation with 80% positivity for E-selectin, 60% positivity for VCAM-1, and 40% for VAP-1, respectively. In addition to changes on the

Table 3. Summary of Immunohistochemistry Results

	Controls (n = 13)	Sepsis (n = 10)	Mild malaria (n = 42)	Severe malaria (n = 61)
Constitutive endothelial markers				
CD31	++100%	++100%	++100%	++100%
CD34	+82%	+80%	+/−90%	+/−95%
EN4 antigen	++100%	++100%	++100%	++100%
P-selectin	+100%	+100%	+84%	+92%
vWf	+100%	+100%	+100%	+100%
HLA class I	++100%	++100%	++100%	++100%
Inducible endothelial markers				
VAP-1	−0%	+/−40%	+/−45%	+/−61%
HLA class II	+100%	+100%	+97%	+95%
MECA-79	−	−	−	−
HECA-452 antigen	−	−	−	−
Putative sequestration receptors				
ICAM-1	+100%	+100%	+94%	+98%
CD36	+96%	+100%	+100%	+98%
E-selectin	−0%	+/−80%	+/−39%	+/−43%
VCAM-1	+/−12%	+/−60%	+/−69%	+/−73%
TSP-1	+/−6%	+/−10%	+/−16%	+/−8.3%
Malaria markers				
MSP-1 (*P. falciparum*)	−	−	+/−19%	+/−23%
Negative control (*P. chabaudi*)	−	−	−	−
Cellular markers				
Macrophage	++100%	++100%	++100%	++100%
Neutrophil	−0%	+/−20%	−5%	−4%
B cell	−	−	−	−
T cell	+100%	+100%	+100%	+100%
Cytokines				
TNF-α	−0%	+/−20%	−0%	+/−7.4%
TNF-α receptor	+/−24%	+/−70%	+/−76%	+/−55%
Interferon-γ	−0%	+/−10%	+/−20%	+/−24%

The number of cases showing staining for each marker in the different disease groups is shown, along with the average staining intensity for their expression. Thus, all cases showed strong endothelial staining for CD31, whereas 80% of cases of sepsis showed scattered weak staining for E-selectin.

endothelium, some up-regulation of HLA antigens and ICAM-1 on dendritic antigen-presenting cells in the epidermis was noted. This may represent an immune reaction, with presentation of soluble malaria antigens. However, little evidence for parasite sequestration was seen with MSP-1 staining positive in only 18% of mild and 23% of severe malaria cases. This may be due to the washing out of all erythrocytes for the section by infiltration of local anesthetic, or may indicate lower rates of sequestration in the skin despite the expression of adhesion receptors.

Reactivity of the antibodies against TNF-α was negative in controls and mild malaria, with weak staining in isolated cases of sepsis (2/10) and severe malaria (4/27) mainly in cells surrounding vessels rather then on endothelium. There was also isolated staining for interferon-γ in these groups (1/10 and 5/21, respectively, and also in 4/20 mild malaria cases). In contrast, there were appreciable levels of weak and scattered staining for TNF-α p55 and p75 receptors on endothelium in cases of malaria and sepsis. Cellular markers showed no influx of immune cells, such as B cells or neutrophils, in skin biopsies from malaria patients. There was constitutive staining for macrophages and T cells in all cases, but the degree and distribution of these were not increased when compared with controls.

Soluble Cell Adhesion Molecule Levels and Plasma TNF-α Concentrations

Levels of circulating sCAMs were in general increased in both sepsis and mild and severe malaria (Figure 2 and Table 4). The statistical comparisons of differences between the levels in the different disease groups are shown in Table 5.

sICAM-1

The concentration of sICAM-1 was elevated in all three disease groups. When compared with the control group, the rise in the sepsis and mild malaria group was not significant. sICAM-1 levels were significantly higher in severe malaria than mild malaria, sepsis, or controls. There was a significant trend across the control, mild malaria, and severe malaria groups for increasing disease severity to be associated with increasing sICAM-1 levels ($P < 0.01$). sICAM-1 was significantly greater in severe malaria patients than in sepsis, but there was no significant difference between levels in mild malaria and sepsis.

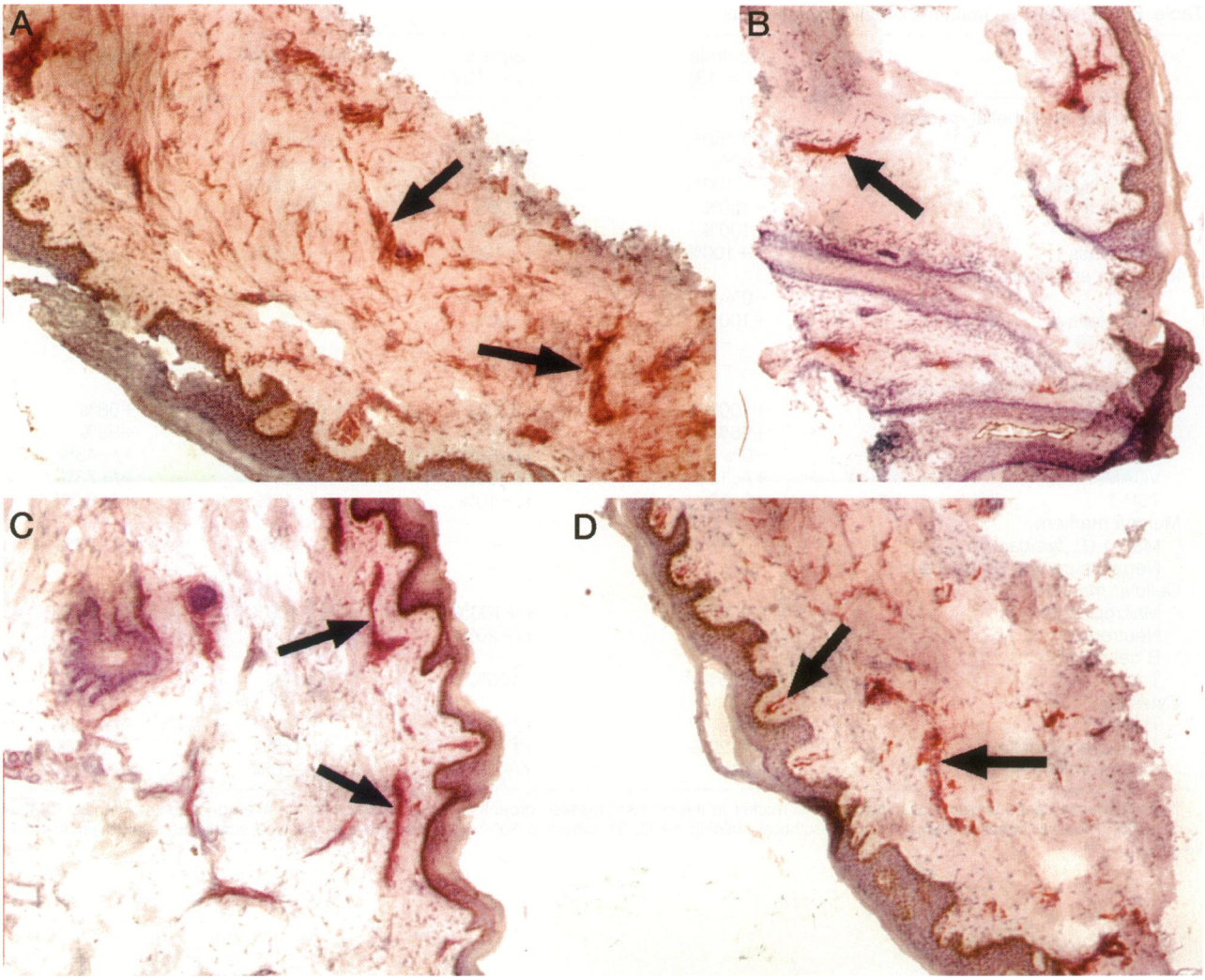

Figure 1. Immunohistochemical evidence for endothelial activation in skin biopsies. The pictures show immunohistochemistry using the APAAP technique staining. **A:** A control case showing constitutive expression of ICAM-1 on vessels and keratinocytes. **B:** A case of severe malaria showing induction of E-selectin staining. **C:** A case of mild malaria showing increased VCAM-1 expression. **D:** Scattered staining for VAP-1 in vessels from a case of sepsis

sVCAM-1

The results for sVCAM-1 were similar to those of sICAM-1. There was a significant rise in sVCAM-1 in both mild and severe malaria. In addition, the sVCAM-1 was significantly greater in mild malaria than in the sepsis group. sVCAM-1 showed the most significant tendency to increase with disease severity across the malaria groups ($P < 0.01$).

sE-Selectin

Levels of sE-selectin also showed a significant difference between severe malaria and controls. The highest individual levels were seen in patients from the sepsis group, especially one patient who had typhoid fever. The levels of sE-selectin were significantly higher in sepsis than in controls and also than in mild malaria. There was no difference in the levels between controls and mild malaria, but patients with severe malaria had significantly higher levels compared with controls, although not compared with sepsis.

TNF-α

Plasma concentrations of TNF-α were significantly increased in malaria patients compared with controls, but there was no association between TNF-α levels and disease severity in malaria. There was also no significant difference between the TNF-α levels in the sepsis and control groups, although this may partly have been due to the small sample size and correction for multiple comparisons (uncorrected $P < 0.04$). Thus, TNF-α was raised in disease, but in this study no differences were seen between groups.

Nonparametric analysis of trend between the groups of controls, mild malaria, and severe malaria showed that for all three sCAMs there was a significant trend, with

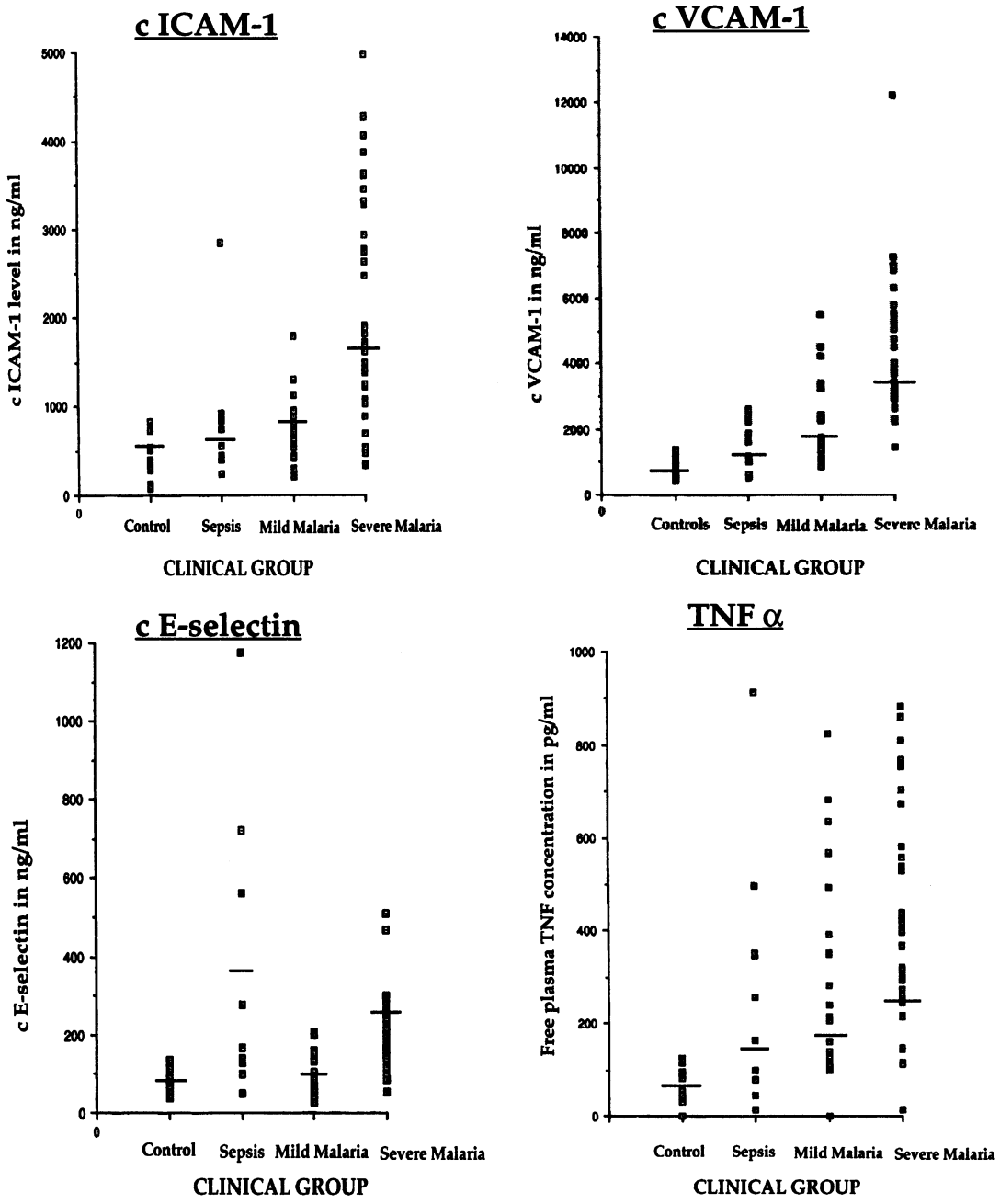

Figure 2. Levels of circulating cell adhesion molecules and TNF-α in the plasma of controls and different disease groups. The figure shows dot plots for each sCAM and TNF-α compared between the four study groups. The median value is shown for each distribution as a horizontal line.

sCAM levels increasing in relation to increased disease severity.

Comparison Between Immunohistochemistry and sCAM/TNF Levels as Measures of Endothelial Activation

The severe malaria group was divided into two groups according to the presence or absence of endothelial activation, as judged by staining for the inducible markers E-selectin, VCAM-1, or VAP-1. There were 21 cases positive for all markers examined and 38 cases that had one or more of these markers absent. The incidence of clinical complications was similar in the two groups (data not shown). This implies that the presence of endothelial activation is not significantly related to the incidence of particular syndromes of severe malaria, such as cerebral malaria or acute renal failure. There was no difference between mean disease duration in the two groups, suggesting that the difference in dermal CAM expression was not simply a result of patients being observed at different times in their illness.

There was no correlation between disease severity in malaria and dermal endothelial expression of ICAM-1

Table 4. Levels of Circulating CAMs and TNF-α in the Different Clinical Groups Compared with the Expression of These Markers in Skin Biopsies

	Immunohistochemistry				Median sCAM (ng/ml) or TNF-α (pg/ml) levels (95% CI)
	n	Marker	% cases with staining	*n*	
Control	13	ICAM-1	100%	12	386 (77.7–826)
		E-selectin	0%		88.5 (37.7–137)
		VCAM-1	12%		631 (428–1381)
		TNF-α	0%		67.3 (0.75–118)
Sepsis	10	ICAM-1	100%	10	800 (414–916)
		E-selectin	80%		222 (50.8–670)
		VCAM-1	60%		1413 (237–2608)
		TNF-α	20%		210 (15–450)
Mild malaria	43	ICAM-1	100%	21	625 (260–1293)
		E-selectin	39%		69.2 (56.7–10)
		VCAM-1	69%		1745 (890–4471)
		TNF-α	0%		227 (108–684)
Severe malaria	60	ICAM-1	100%	42	1741 (800–3982)
		E-selectin	43%		211 (113–297)
		VCAM-1	73%		3704 (2352–6766)
		TNF-α	7.4%		272 (130–760)

(which was constitutive), E-selectin, or VCAM-1. Both of these markers, and VAP-1, were increased in malaria and sepsis compared with controls, but there was no difference between their expression in patients with severe and uncomplicated malaria. Thus, there was a poor correlation between endothelial activation, as measured by immunohistochemistry, and disease severity in malaria. In contrast, the levels of sICAM-1, sVCAM-1, and sE-selectin were significantly higher in patients with severe malaria compared with mild malaria. Interestingly, the most specific marker for endothelial cells, sE-selectin, was not significantly raised in mild malaria compared with controls but was increased in severe malaria. Patients with sepsis also had significantly higher levels of sE-selectin than controls. The two cases of severe malaria who died did not have the highest sCAM or TNF-α levels.

To compare the two different measures of endothelial activation, the expression of ICAM-1, VCAM-1, E-selectin, and VAP-1 was compared within individual cases with the sCAM and TNF levels. To allow for the fact that one is a continuous and the other a discontinuous variable, the normal range of sCAMs was calculated from the control group as median ± 95% range. sCAM levels in individual disease cases were then assigned as normal or elevated when they fell outside this range. Comparisons of sCAM levels (normal or raised) *versus* immunohistochemical staining for CAMs (absent or present) were then carried out using Fisher's exact test (Table 6). Increases in sVCAM-1 and sE-selectin were associated with increased staining for these markers in the dermal microvasculature. It was not possible to analyze this association between ICAM-1 staining and other sCAMs, because dermal ICAM-1 expression was constitutive. There was no association between expression of any marker on immunohistochemical staining with the circulating levels of TNF-α.

Discussion

The precise mechanism of vital organ dysfunction in severe malaria is unknown,[33] but endothelial cell activation and the adherence of infected red cells to endothelium in the process of sequestration are considered to be important steps in pathogenesis. We have shown previously that there is systemic endothelial cell activation in fatal malaria, with up-regulation of key adhesion molecules involved in infected red cell adhesion, and have now extended this finding to nonfatal disease.

This study, performed in adult Vietnamese patients, confirms that systemic endothelial activation occurs during nonfatal malaria in life by direct examination of the endothelial cell phenotype *in situ*. Dermal microvascular cell phenotype has been used as a surrogate of endothelial cell activation in deep organs to examine the relationship of activation with the severity of disease, and this has been correlated with the levels of circulating CAMs. Endothelial activation occurs not only in severe malaria *in vivo* but also in nonsevere malaria and in sepsis. We found that raised levels of sCAMs correlated with the severity of malaria, in agreement with previous studies, but when we examined endothelial cell phenotype in skin there was no direct correlation between dermal endothelial cell activation and disease severity.

These observations can be interpreted in two ways. Systemic endothelial activation, as measured by examination of dermal microvascular endothelium, may not represent a pathophysiological mechanism that is specific to severe malaria. The significant correlation between the levels of endothelial-specific circulating CAMs (E-selectin and VCAM-1) and disease severity may result, not from increased production from activated endothelium, but from impaired clearance. Hepatic and renal impairment is common in adult severe malaria, although the clearance pathways of these molecules have yet to

Table 5. Statistical Comparisons of Levels of sCAMs and TNF-α between Different Clinical Groups

	Significance levels		
	Sepsis	Mild malaria	Severe malaria
sICAM-1*			
Control	NS	NS	$P < 0.005$
Sepsis		NS	$P < 0.005$
Mild malaria			$P < 0.01$
sVCAM-1*			
Control	NS	$P < 0.01$	$P < 0.005$
Sepsis		NS	$P < 0.002$
Mild malaria			$P < 0.01$
sE-selectin*			
Control	$P < 0.01$	NS	$P < 0.02$
Sepsis		$P < 0.01$	NS
Mild malaria			$P < 0.02$
TNF-α†			
Control	NS ($P < 0.04$)	$P < 0.02$	$P < 0.02$
Sepsis		NS	NS
Mild malaria			NS

The table shows the significance levels of comparisons of the levels of sCAMs and TNF between pairs of clinical groups. Each comparison was made using a two-group nonparametric test (Mann-Whitney *U* test). All *P* values were adjusted for multiple comparisons using the Bonferroni's correction. NS, not significant. A nonparametric test of trend was performed for each marker across the control, mild malaria, and severe malaria groups (the sepsis group was excluded from these trend analyses). In all cases where there was a significant trend (ie, ICAM-1, VCAM-1, and E-selectin), the levels of the markers increased with increasing disease severity.

*Nonparametric estimate of trend across malaria groups, $P < 0.01$.

†Nonparametric estimate of trend across malaria groups, NS.

be characterized. This view is supported by studies examining the correlation between *in vivo* immunohistology and soluble CAM markers of endothelial activation during cardiac transplant rejection.[34,35] These found that, whereas the tissue expression of ICAM-1 on endomyocardial biopsies increased significantly in histologically severe types of rejection, there was no differential expression of E-selectin or VCAM-1. There was also no correlation between sICAM-1 and rejection. Thus, the measurement of circulating adhesion molecules cannot be taken in these two studies as an accurate measure of tissue-specific levels of expression of the same molecule. A recent study of the kinetics of sICAM-1 production in relation to endothelial expression in different organs in the mouse has confirmed that sICAM levels cannot be directly linked to endothelial ICAM-1 expression.[36]

The alternative interpretation of the results is that changes in skin endothelium do not reflect pathological changes occurring in those vascular beds most important in the disease process. There are certainly phenotypic differences between the resting and activated endothelial cell phenotype in dermal vessels compared with other tissues,[37,38] particularly compared with the brain microvasculature, which expresses very low levels of CD36, scattered low levels of ICAM-1, and no P-selectin.[8] In addition, we cannot comment on temporal variations in the level of expression of endothelial receptors. To try and address this issue, levels of sCAMs within the cerebrospinal fluid are being measured in an attempt to look more closely at the vasculature of the brain.

The physiological role of sCAMs in malaria is unclear. Because several of the CAMs measured also act as potential endothelial adhesion receptors for parasitized erythrocytes, sCAMs may theoretically also bind to infected erythrocytes. Whether this can inhibit adhesion to endothelial cells is unknown, but attempts to block cytoadherence with sICAM-1 *in vitro* have proved unsuccessful.[39]

Table 6. Comparisons of sCAM Levels and Immunohistochemistry as Predictors of Endothelial Activation

Comparison	Group A	Group B	Group C	Group D	Odds ratio	Relative risk	*P* value
VCAM-1 and sICAM-1	15	10	22	32	2.18 (0.75–6.44)	1.47 (0.94–2.23)	NS
VCAM-1 and sVCAM-1	14	12	9	45	5.83 (1.89–19.3)	3.23 (1.60–6.47)	<0.01
VCAM-1 and sE-selectin	17	7	20	35	4.25 (1.25–13.67)	1.95 (1.26–3.01)	<0.01
VCAM-1 and TNF-α	12	14	15	39	2.23 (0.76–6.62)	1.66 (0.91–3.02)	NS
E-selectin and sICAM-1	26	14	10	30	5.57 (1.92–16.6)	2.6 (1.45–4.66)	<0.02
E-selectin and sVCAM-1	16	23	4	35	6.09 (1.62–24.4)	4.02 (1.47–10.89)	<0.005
E-selectin and sE-selectin	27	12	10	30	6.75 (1.56–4.93)	2.77 (1.56–4.93)	<0.005
E-selectin and TNF-α	17	20	10	31	2.63 (0.91–7.75)	1.88 (0.99–3.58)	NS
VAP-1 and sICAM-1	25	12	10	14	2.92 (0.89–9.77)	1.62 (0.96–2.74)	NS
VAP-1 and sVCAM-1	15	22	5	17	2.32 (0.62–9.12)	1.78 (0.75–4.23)	NS
VAP-1 and sE-selectin	22	14	12	12	1.57 (0.49–5.1)	1.22 (0.76–1.87)	NS
VAP-1 and TNF-α	20	17	6	19	3.73 (1.07–13.45)	1.65 (1.06–2.50)	NS

The table shows an analysis of sCAM levels and immunohistochemistry for predictive value over all clinical groups. This analysis used 2 × 2 contingency tables to compare the proportion of cases with raised sCAM/TNF-α compared to increased inducible endothelial markers. For each case, the level of sCAMs or TNF-α was assigned as raised or within normal range when compared with the median ± 95% of the control population, as shown in Table 4. Combining this with the presence or absence of immunohistochemical staining for the marker, the table for each comparison took the following form:

	sCAM level normal	sCAM level raised
Marker absent	Group A	Group B
Marker present	Group C	Group D

Using Fisher's exact test, the *P* value, odds ratio, and relative risk were calculated for each comparison and are shown in the table with ranges for relative risk and odds ratio. All *P* values were adjusted for multiple comparisons using the Bonferroni correction. NS, not significant. Thus, for example, there was no relationship between staining for VAP-1 and the sVCAM-1 levels, but sE-selectin was significantly greater in the cases with E-selectin staining.

An important adjunct to this study is the finding that all patients from this study group in Viet Nam had immunologically detectable sICAM-1 in their serum. A polymorphism in the expressed amino-terminal sequence (domain 1) of the ICAM-1 molecule has recently been identified in African patients, which predisposes them to cerebral malaria.[40] This sequence change abolishes the epitope recognized by the BBA4 antibody, one of the pair used in the ELISA kit used in this study to detect sICAM-1. Thus, as all Vietnamese patients tested had detectable sICAM-1, we conclude that the polymorphism is certainly substantially less frequent in the Vietnamese than the Kenyan population, where the polymorphic gene frequency is more than 30%.

In conclusion, widespread endothelial activation is a feature of malaria in life as it is in fatal malaria[8] and sepsis.[41,42] Surrogate measurements of endothelial cell phenotype such as sCAM levels are correlated with the severity of disease but need to be interpreted with care as they may not reflect changes in individual target organs. Examination of endothelial cell phenotype and function in individual tissues will be necessary to determine the relationship of endothelial activation and dysfunction to the different clinical manifestations of severe malaria. The correlation of sCAM levels with disease severity is likely to reflect a nonspecific generalized endothelial activation in response to infection, rather than a specific pathophysiological mechanism in severe malaria.

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