Digestive vacuole of *Plasmodium falciparum* released during erythrocyte rupture dually activates complement and coagulation

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Short title: DVs activate complement and coagulation in malaria

Supplemental online material: 3 video clips
Abstract

Severe *P. falciparum* malaria evolves through the interplay between capillary sequestration of parasitized erythrocytes, deregulated inflammatory responses and hemostasis dysfunction. Upon rupture, each parasitized erythrocyte releases not only infective merozoites, but also the digestive vacuole (DV), a membrane-bounded organelle containing the malaria pigment hemozoin. We report that the intact organelle, but not isolated hemozoin, dually activates the alternative complement and the intrinsic clotting pathway. Pro-coagulant activity is destroyed by phospholipase C treatment, indicating a critical role of phospholipid head groups exposed at the DV surface. Intravenous injection of DVs caused alternative pathway complement consumption and provoked apathy and reduced nociceptive responses in rats. Ultrasonication destroyed complement-activating and pro-coagulant properties in vitro, and rendered the DVs biologically inactive in vivo. Low molecular weight dextran sulfate blocked activation of both complement and coagulation, and protected animals from the harmful effects of DV-infusion. We surmise that in chronic malaria, complement activation by and opsonization of the DV may subserve a useful function in directing hemozoin to phagocytic cells for safe disposal. However, when the waste disposal system of the host is overburdened, it is speculated that the DV may transform into a trigger of pathology and then represents a potential therapeutic target in severe malaria.
Introduction

Severe malaria evolves through the interplay between capillaric sequestration of parasitized erythrocytes, a deregulated inflammatory response and hemostasis dysfunction\textsuperscript{1-5}. The responsible molecular mechanisms remain the subject of debate, and identification of the triggering events to this day still constitutes a major open challenge in science. Seminal findings on complement activation in malaria were published in the 1970's. Complement turnover was shown to be triggered in human patients suffering from severe malaria\textsuperscript{6}, and experiments in monkeys demonstrated that complement consumption coincided with schizont rupture\textsuperscript{7}. Clinical data have now accumulated to show that substantial complement activation occurs in human patients\textsuperscript{8} and enhanced early complement activation in experimental human malaria has directly been demonstrated\textsuperscript{9}. Experiments in a murine model of cerebral malaria also suggest a pathogenic role of complement activation. C5 deficiency protects mice against cerebral malaria\textsuperscript{10}, in which dysregulation of the terminal complement sequence is apparently centrally involved\textsuperscript{11}.

Hemozoin, which is formed in the digestive vacuole of developing intra-erythrocytic parasites, has emerged as a possible trigger of inflammation. This assumption is primarily based on the fact that hematin, considered to represent the synthetic analog of hemozoin\textsuperscript{12}, provokes inflammatory responses in macrophages\textsuperscript{13,14} and activates the alternate complement pathway\textsuperscript{15}. Also suggestive is the finding that malarial pigment colocalizes with fibrin in tissues\textsuperscript{16}. However, when the erythrocytic schizonts rupture, the malarial pigment is still surrounded by the membrane of the digestive vacuole and it is the organelle rather than free hemozoin that naturally gains contact with the host environment\textsuperscript{17,18}. 

We have recently reported that when parasitized erythrocytes rupture in blood, the DVs are rapidly phagocytosed by polymorphonuclear granulocytes (PMN)\(^9\). Phagocytosis only occurred in active serum and complement factor C3 was found attached to the surface of the organelle. It followed that the DV might directly activate complement and experiments were performed to explore this possibility. These led to novel findings that are reported herein. It is shown that the DV is endowed with the capacity to dually activate the alternative complement and the intrinsic clotting pathway. Possibly, efficient opsonization of the DV is initially beneficial because it enables the host to rapidly dispose of the waste product. However, transition to a pathologic state may occur as the disposal system is overrun. Unreigned activation of complement and coagulation may then contribute to events underlying the pathogenesis of severe malaria.

**Methods**

Banked human blood, group A Rh\(^+\) RBC, and group A human serum were continuously supplied by the Transfusion Center of the University Medical Center Mainz. Aliquots of human serum were stored at -20°C until use. Serum was heat-inactivated for 30 min, 56°C, for use in routine parasite culture. Active serum was used where indicated.

**Antibodies and Reagents**

Rabbit anti-human C3c antibody was from DakoCytomation GmbH (Hamburg, Germany), monoclonal antibody clone 979/143 against C5b-9 neoantigen (purified IgG, 10 mg/ml) from this laboratory\(^20\). Monoclonal antibody 1E1 specific for MSP1\(_{19}\) was a kind gift from A. Holder, MRC, London\(^21,22\). Alexa fluor 594 conjugated goat anti-mouse IgG, donkey anti-rabbit IgG, Alexa fluor 488 conjugated goat anti-mouse IgG and BCECF-AM were obtained...
from Invitrogen, Karlsruhe, Germany. Monoclonal antibody against RBC band 3 protein, protease inhibitor E64 (L-trans-epoxy-succinyl-leucylamido-(4-guanidino)butane), Hoechst 33342 and Low molecular weight-dextran sulfate (LMW-DXS; MW5000) were purchased from Sigma (Taufkirchen, Germany).

**P. falciparum culture and isolation of DVs and merozoites.** *P. falciparum* clone 3D7 was maintained as a synchronous continuous culture and digestive vacuoles were isolated and banked in 50% glycerol at -20°C as described\(^\text{19}\). DVs were enumerated using a hematocytometer (CELL-DYN Ruby, Abbott, Wiesbaden, Germany; Software version 2.0ML). Hemozoin was isolated as described\(^\text{19}\), and heme was quantified by measuring absorbance at 405 nm as described\(^\text{15}\).

To isolate merozoites, cultures were tightly synchronized twice with a 4 h lap using 5% sorbitol. Naturally liberated merozoites and DVs were isolated from the supernatants of enriched cultures as above. DVs were first selectively sedimented by centrifugation at 2000 rpm for 5 min and the merozoites were then obtained by subsequent centrifugation at 5000 rpm for 5 min in a table top centrifuge. Merozoites were washed three times with RPMI and stored frozen at -20°C.

**Isolation of parasitophorous vacuole membrane-enclosed merozoite structures (PEMS).** Enriched, late stage pRBC cultures were suspended to 0.2% hematocrit in culture medium containing 10% inactivated serum in the presence of 15 µM E64 for approximately 8 h as described\(^\text{23}\). The culture was centrifuged at 250xg for 5 min to remove intact cells. The supernatant was then centrifuged at 1600 x g for 5 min to remove intact late schizonts. The
supernatant was then centrifuged at 2500xg for 10 min to pellet the PEMS. The pellet was washed twice with RPMI and twice with PBS and used for complement consumption tests.

**Complement consumption experiments.** A) Consumption during pRBC rupture: Enriched cultures of late stage pRBC were suspended in Veronal-buffered saline (VBS with 0.25 mM Ca\(^{2+}\) and 0.8 mM Mg\(^{2+}\), Virion/Sirion, Würzburg, Germany) containing 20% active normal human serum (NHS) at 50% hematocrit. Cells were incubated at 37°C and at hourly intervals, small aliquots were collected and analyzed for hemolysis and complement consumption, which was detected by hemolytic assays using sensitized sheep erythrocytes and expressed as % relative consumption as previously detailed\(^24\). Background absorption derived from lysed parasitized cells were determined in parallel controls using heat-inactivated serum, and subtracted from absorption values of the consumption assay. Two-dimensional immunoelectrophoretic analysis of C3 conversion was performed as described\(^24\). In two experiments, 10 mM EGTA/2 mM Mg\(^{2+}\) were added to prevent classical pathway activation, and complement turnover was assessed by C3 immunoelectrophoresis. Classical pathway CH\(_{50}\) of the serum employed was in the normal range of 70-80 HU/ml. B) Consumption with isolated DVs: DVs were incubated with 10% or 20% NHS in the presence or absence of 10 mM EGTA/2 mM Mg\(^{2+}\). C3 conversion was analysed in 20% NHS after 20 min, and consumption of hemolytic activity with either sensitized sheep erythrocytes or rabbit erythrocytes in 10% serum after 60 min at 37°C.

**Staining procedures.** Hoechst 33342 was used to stain DNA. BCECF-AM (10 µM) was added to pRBC cultures or to isolated DVs for 30 min. Cells and DVs were then washed three times and fluorescence microscopy immediately was performed on air-dried, unfixed smears.
Staining of merozoites and DVs following schizont rupture. Tightly synchronized cultures containing 8-10% late stage pRBCs were enriched and diluted to 0.2% hematocrit in active serum without addition of fresh RBC to prevent reinvasion of merozoites. Cultures were closely monitored for hemolysis and, immediately after the majority of schizonts ruptured, were centrifuged at 800 rpm for 5 min. The supernatants were centrifuged at 5000 rpm for 10 min in a Sorvall RC2B centrifuge to sediment the merozoites and DVs. Pellets were resuspended in RPMI and centrifuged twice at 2000 rpm for 1 min in a table top centrifuge to remove the RBC. Merozoites and DVs were then sedimented at 5000 rpm for 5 min. Pellets were resuspended in RPMI and thin smears were prepared on glass slides, air-dried and fixed with methanol for 10 seconds or in 2% paraformaldehyde for 10 min at room temperature and stained with anti-C3c or anti-C5b-9 as described below.

Staining of isolated DVs. DVs were incubated with 10% active human serum for 1 h at 37°C, or with 10% inactive serum as controls. DVs were washed with PBS and thin smears were made on glass slides. After fixation, the slides were washed with PBS and then blocked in 3% BSA for 1 h at room temperature. The slides were washed and incubated with rabbit polyclonal C3c antibody (1:250 dilution), monoclonal anti-human C5b-9 (1:250 dilution), monoclonal anti-human band 3 (1:1000 dilution) or monoclonal anti-MSP1 (1:100 dilution) in blocking buffer for 1 h at room temperature. Non-immune rabbit serum and isotype-matched mouse IgG were employed as respective controls. Following incubation, the slides were washed and then incubated with Alexa fluor 594 conjugated goat anti-mouse IgG, donkey anti-rabbit IgG or Alexa flour 488 conjugated goat anti-mouse IgG (Invitrogen, Karlsruhe, Germany; all at 1:500 dilution)) for 1 h at room temperature. Nuclei were stained with Hoechst 33342 for 5 min at room temperature. Samples were viewed in a Zeiss fluorescence microscope (Axiovert 200M, Carl Zeiss GmbH, Jena, Germany, Software
Western blotting. RBC ghosts were prepared by lysis of RBC with ice-cold 5 mM sodium phosphate buffer pH 8.0. RBC ghosts, isolated merozoites and DVs were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and probed with mouse anti-MSP1 (1:200) and mouse anti-band 3 antibodies (1:5000). Horseradish peroxidase-conjugated goat anti-rabbit (New England Biolabs, Frankfurt, Germany), goat anti-mouse or goat anti-human antibodies (both Santa Cruz biotechnology, Heidelberg, Germany) were used at a dilution of 1:10 000 for detection, and bands were visualized by enhanced chemiluminescence (Roche, Manheim, Germany).

Flow cytometry. DVs were incubated in 10% NHS for 1 h at 37°C and washed three times with PBS. Following incubation in 3% BSA/PBS for 30 min, they were stained for C5b-9 as described above and analyzed in a FACS Scan™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Controls consisted of identical DV preparations treated with heat-inactivated serum.

Determination of clotting times in recalcified plasma. Citrated blood was centrifuged and the cell-free plasma was diluted with 1 vol of saline. DVs were incubated with plasma for 1 min at 37°C, and reactions were initiated with the addition of 10 mM CaCl$_2$$. Clotting times were measured in a Coagulometer KC10A micro (Amelung GmbH, Lemgo, Germany).

Prothrombinase assay. Assessment of thrombin formation was performed in a two-step amidolytic substrate assay$^{25}$. The DVs were preincubated in a solution containing 150 mM
NaCl, 50 mM Tris (pH 7.4) and 0.1% BSA with 1 nM factor Va, 6.2 pM factor Xa and 2 mM CaCl$_2$ at 25°C for 5 min. The reaction was initiated by addition of 1 µM prothrombin and incubated for 5 min at 25°C. The reaction was stopped by addition of EDTA to 10 mM final concentration. The DVs were removed by centrifugation and thrombin formation was assessed at 405 nm, immediately after the addition of chromogenic substrate S-2238 (0.1 mM, Chromogenix Instrumentation Laboratory, Milano, Italy) in a kinetic microplate spectrophotometer (Labsystems Multiskan RC, Haverhill, MA, U.S.A. with Genesis software).

**Inhibition assays with low molecular weight-dextran sulfate (LMW-DXS).** Low molecular weight-dextran sulfate (MW5000) was employed at the given concentrations in complement consumption and coagulation assays. For in vivo experiments 6 mg LMW-DXS (in PBS) were injected i.p. 45 min prior to injection of 5 x 10$^9$ DVs.

**Animal experiments.** Male Sprague Dawley outbred rats [Crl:CD(SP)] were purchased from Charles River Laboratories (Sulzfeld, Germany) and introduced into experiments with a body weight of 60-70 g. Intravenous injections (300-350 µl) were performed after catheterization (24 G) of the lateral tail vein. All experimental protocols were approved by responsible authority (Landesuntersuchungsamt Koblenz, approval No 23 177-07/G 10-1-034) and conducted according to EU guidelines for the care and use of laboratory animals. Three video clips showing the behavioral reactions of the animals can be found in the supplemental online material.

**The hot plate test.** The hot plate test was adopted from the method of Woolfe and McDonald$^{26}$. In brief, a rat is introduced into an open-ended plexiglass cylinder (18 cm
diameter) to confine the animal to the heated surface of the hot plate apparatus. The plate is adjusted to 56°C. When the pain threshold is reached, the rat starts to react by licking the fore or hind paws. Normal reaction times are in the range of 10 sec. Reaction times are recorded with a maximum cut-off of 30 sec to avoid tissue damage.

**Statistical analysis**

The assumptions for normality and equal variance were verified with SigmaStat 3.1 software (Erkrath, SYSSTAT, Germany). Dependent on the experiment either Repeated Measure ANOVA or One-Way ANOVA was followed by the Bonferroni test. All results represent mean ± SD of at least three independent experiments, if not indicated otherwise. P values of < 0.05 were classified as statistically significant.

**Results**

**Complement activation occurs on the surface of DVs but not on merozoites**

In vitro culture of *P. falciparum* was undertaken conventionally and late stage parasitized RBC (pRBC) were enriched (Figure 1A, Giemsa stain). BCECF-AM, a membrane-diffusible acetylated carboxyfluoresceine derivative, becomes entrapped intracellularly after its cleavage by cytoplasmic esterases that are absent in RBC but present in nucleated cells and DVs. Entrapment occurs only when an intact biological membrane is present to limit its egress from the respective compartment. Gratifyingly, we found that merozoites and DVs could both be stained and were then clearly distinguishable from each other (Figure 1B). As to be expected, hemozoin characterized the DV while Hoechst 33342 stained merozoites but not the DVs (Figure 1C). DVs and merozoites could thus be easily distinguished. More importantly,
intactness of the DV membrane, simply probed with the fluorescent dye, would subsequently emerge as the decisive element underlying the singular biological properties of the DV reported herein.

Naturally liberated DVs were isolated from supernatants of hemolysed pRBC. As expected from the literature, they presented as platelet-sized particles that could be enumerated in a hematocytometer (Figure 1D). Staining with BCECF-AM could still be undertaken following their isolation and storage at -20°C in glycerol (Figure 1E), attesting to the remarkable stability of their membranes. Staining for DNA was negative (Figure 1F). In order to exclude potential contaminations of the DV preparation either with erythrocyte or merozoite fragments, the presence of the RBC anion transporter (band 3) and the merozoite surface protein 1 (MSP1) was analysed. The band 3 protein was not detectable by immunofluorescence on the DVs surface (Figure 1G). Instead, staining could only be observed on erythrocytes when these were artificially added as controls (Figure 1H). This finding was confirmed by immunoblot analysis demonstrating the lack of band 3 on DVs as well as on purified merozoites (Figure 1I, 1J). Presence of MSP1 was examined by Western blots of purified merozoites and DVs. The latter carry the C-terminal 19 kDa fragment of MSP1 but not the full length protein, which was readily detectable on merozoites but virtually absent in the DV preparations (Figure 1K).

To determine whether complement activation would occur upon schizont rupture, late stage pRBC were allowed to lyse in the presence of active, non-immune serum, and complement activity, C3 turnover and pRBC hemolysis were recorded (Figure 2A). No changes occurred prior to schizont rupture, but onset of hemolysis was accompanied by C3 turnover and a fall
in complement activity. These findings accorded with early observations of Glew et al. that complement consumption coincided with schizont rupture in infected monkeys\(^7\).

In order to identify the activation pathway, schizont rupture was analysed in the presence of 10 mM EGTA/2 mM Mg\(^{2+}\), which prevents classical pathway activation. As shown in Figure 2A complement activation indeed occurred and could thus be attributed to the alternative pathway (Figure 2A, lowest panels). At the same time, these experiments showed that Ca\(^{2+}\) was dispensable at the final stage of parasite development and release from the RBC. Because the alternative complement sequence is not triggered by soluble molecules, merozoites and DVs were examined for the presence of assembled C5b-9, the prime marker of membrane-associated complement activation\(^{20,29}\). Positive stainings were observed on the DVs but never on merozoites (Figure 2B). The DV membrane was thus identified as the critical site of complement activation. To exclude any additional requirement of soluble activators, experiments were conducted with isolated, washed DVs. Figure 3A shows a dose response experiment with matching data for complement consumption and C3 turnover in human serum. Decreases in total complement activity with concomitant C3 turnover were already provoked by low numbers (\(\sim 10^7/\text{ml}\)) of DVs. Activation occurred in the presence or absence of EGTA/Mg\(^{2+}\). The depicted panels are from an experiment with EGTA/Mg. When the fluid phase SC5b-9 was measured in the supernatants, a bell shaped dose response curve was observed that indicated a shift to the membrane bound state as the number of DVs increased. A representative example is shown in Figure 3B. All DVs stained positively for C3 (Figure 3C) and C5b-9 (Figure 3D). The latter finding was also confirmed by FACS analysis which impressively demonstrated that all DVs stained positive for the terminal complement complex (Figure 3D, lower panel).
Contact of the intact DV membrane with plasma is required for complement activation

To discern whether direct contact of the DV with plasma was required for complement activation, intact parasitophorous vacuoles and purified hemozoin were compared for their complement activating capacity with DVs prepared from naturally ruptured pRBC or from trophozoites. Parasitophorous vacuoles were prepared by permitting late stage parasitized RBC to lyse in the presence of the protease inhibitor E64\textsuperscript{23}. When the released parasitophorous vacuoles membrane-enclosed merozoite structures (PEMS, Figure 4A) were tested, they were found to be devoid of complement activating properties (Figure 4D). Control experiments showed that E64 itself did not suppress complement activation by isolated DVs. To test whether activation required the presence of an intact DV membrane, DVs were isolated from hemolysis supernatants or from trophozoite-stage infected RBC\textsuperscript{27,30}. The latter were prepared by saponin-lysis of late-stage infected RBC and, as known from the literature\textsuperscript{27,30}, such preparations contained some contaminating RBC-membrane debris (Figure 4B). Both preparations had similar complement-activating properties which were lost upon sonication, and this finding correlated with detectability of C3 and C5b-9 exclusively on the intact DV surface but never on any surrounding membrane debris (data not shown). Centrifugation of the sonicates through Percoll led to retrieval of purified dispersed hemozoin crystals (Figure 4C), which were also devoid of complement activating properties when tested at concentrations of 10 – 1000 µg heme/ml (Figure 4D). Thus, activation of complement required direct contact of intact DV membranes with serum and could not be mimicked by isolated hemozoin, which was also found to be devoid of procoagulant activity (data not shown). The fact that unfractionated, sonicated lysates had no activity reiterated that the activating effects could not have derived from protein or DNA contaminants in the DV preparations.
**DVs activate the intrinsic clotting pathway**

A number of mechanisms involving the extrinsic clotting pathway are mainly being discussed as the cause of hemostasis dysfunction in malaria patients. However, intrinsic pathway activation also appears to be involved and late stage pRBC reportedly support the assembly of multimeric coagulation complexes. We here demonstrate that the DV similarly has direct procoagulant activity.

The intrinsic clotting pathway is routinely tested by determination of activated partial prothrombin time, where an empiric mix of activators is added to citrated plasma and clotting times after recalcification are read. Long clotting times (>400-1000 sec) are observed if the activators are omitted. When DVs were used instead of the activators, dose-dependent shortening of clotting times was observed (Figure 5A). These results were corroborated in prothrombinase assays using isolated factors FVa, FII and FXa. In the conventional test, phospholipids are added to provide platforms for Ca$^{2+}$-dependent assembly of the prothrombinase complex FVa/FXa. The phospholipids were here omitted and replaced by DVs, which dose-dependently provoked thrombin formation (Figure 5B). Thus, the DV could directly assemble the key convertase of the clotting pathway. As with complement activation, this required only low numbers of DVs corresponding to less than 1% hematocrit. Prothrombinase assembly is generally mediated via Ca$^{2+}$-bridged interactions with phospholipid head groups. When DVs were treated with phospholipase C, procoagulant activity was indeed destroyed (Figure 5C), while complement-activating capacity remained intact (not shown).
Low molecular weight dextran sulfate (LMW-DXS) suppresses activation of complement and coagulation by the DV

Low molecular weight dextran sulfate blocks both the complement and coagulation cascade at micromolar concentrations that, in contrast to heparin, do not cause bleeding complications and are well tolerated in humans. If DV-induced activation of complement and clotting should indeed contribute to malaria pathogenesis, the existence of a well-established inhibitor could be of high interest. Therefore, we tested the effect of LMW-DXS on DV effects. LMW-DXS at concentrations of 10-100 µg/ml effectively inhibited DV-dependent activation of the intrinsic clotting pathway (Figure 6A) and abrogated assembly of prothrombinase complexes (Figure 6B). In accord with the literature, somewhat higher concentrations were required to block complement activation, but effects were also apparent at 100 µg/ml (Figure 6C).

DV s activate the alternative complement pathway and produce clinical symptoms in rats

Animal experiments were performed to obtain an indication whether the DVs also activated complement in vivo and might contribute to the development of clinical symptoms. These experiments were performed in rats because alternative complement pathway activity is extremely low in mice. Alternative pathway activity was determined using rabbit erythrocytes as targets. The first group of animals received 4-5 x 10^9 DVs injected into the tail vein. Nociceptive responses were determined with the hot plate test and behavioral reactions were filmed. Acute onset of clinical symptoms was observed in all cases, commencing 1-2 min after injection. Animals became lethargic, exhibited diminished reactions to tactile and acoustic stimuli, and nociceptive responses were retarded (Figure 7A and Supplement Video 1). Alternative complement pathway activity was reduced by > 85% in all animals (n = 5).
The second group of animals received the same batch and dose of DVs that, however, were sonicated prior to application. Alternative complement pathway activity remained unchanged in the serum of these animals, which strikingly also developed no clinical symptoms \((n = 3)\) (Figure 7A and Supplement Video 2). The third group of animals received 6 mg LMW-DXS intraperitoneally, which led to > 65% reduction in alternative pathway complement activity in plasma after 45-60 min. Remarkably, all animals \((n = 4)\) were fully protected from the detrimental effects of DV-infusion (Figure 7A and Supplement Video 3).

**Rapid cellular uptake of DVs by mononuclear cells in rats after intravenous injection**

To trace the fate of the DVs, rats were sacrificed after 4-6 h. DVs and PMN were rarely observed in blood smears (not shown). Paraffin-embedded sections of spleen and lung were investigated microscopically. Staining with hematoxylin and eosin revealed intracellular accumulation of DVs in the marginal zone of the spleen containing abundant macrophages (Figure 7B, left panel). This pattern became very impressive after polarization at lower magnification (Figure 7B, right panel). Likewise, intravascular accumulation of mononuclear cells with myriads of intracellular DVs could be observed in the lung (Figure 7C).

Collectively, these data demonstrated that free DVs are rapidly taken up by mononuclear cells.

**Discussion**

Although complement activation and coagulation defects during *P. falciparum* malaria have been recognized for decades, no single entity of parasite origin has yet been identified that might directly be involved in triggering these events. Rupture of each *P. falciparum* parasitized erythrocyte is accompanied by release of one DV into the circulation. Yet, while
the effects of DV uptake on macrophage function are under study, no significance has ever been attached to their presence in the circulation. This report is the first to reveal the DVs capacity to dually activate complement and coagulation. In severe malaria, parasitemia levels of several percent develop corresponding to $\geq 10^8$ cells/ml blood, and capillaric sequestration will further heighten the local load of DVs. Activation of both complement and coagulation became detectable at concentrations of $\sim 10^7$ DV/ml, and displayed simple dose-dependency with no prozone effects (Figure 3A, Figures 5A, B). Thus, increases in the load of DVs as occurring at sites of pRBC sequestration would be expected to simply augment activation of both enzyme cascades. Provocation of clinical symptoms will naturally depend on myriad local factors of the micro-environment, e.g. presence or absence of regulatory factors such as tissue factor and thrombomodulin$^{1-5}$. Transactivation events occurring between the coagulation system, complement, platelets and blood cells may then pave the way to final disaster.

Our study originated from the observation that rupture of pRBC in active serum led to C3 conversion and to binding of C3b and C5b-9 to the DV, with conspicuous sparing of merozoites. The results were reproduced with isolated DVs, and attachment of C5b-9 indicated its presence in membrane-bound form. Direct contact between the DV membrane and serum was required for activation to take place, and PEMS, in which DVs remained encased within the PVM, were without effect. DVs isolated via saponin-lysis of late-stage infected RBC also activated complement. These preparations contained contaminating membrane material that, however, was not seen to stain positively for C3 or C5b-9. The findings do not entirely exclude that other membranes or organelles may also have complement activating properties. However, these yet remain to be identified. Quite remarkably, isolated hemozoin activated neither complement nor coagulation. This was
somewhat unexpected because hematin, which is considered to represent the synthetic analogue of hemozoin, activates the alternative complement pathway, albeit at very high concentrations\textsuperscript{15}. The possibility that the biological properties of hematin and hemozoin may not be identical merits close attention in the future.

Disruption of the DV membrane destroyed both complement-activating and procoagulant properties. Thus, our findings led to the question what particular membrane characteristics enabled the digestive vacuole but not the merozoite to activate both cascades. Information on the composition and organization of the DV membrane is currently not available. Such analyses are impeded by the fact that entities other than hemozoin are encased within the DV, including lipid bodies of poorly defined composition\textsuperscript{38}. However, we found that procoagulant activity was selectively destroyed by phospholipase C treatment and must thus be borne by phospholipid head groups. This finding is not surprising since multimolecular assembly of clotting enzymes is generally promoted by Ca\textsuperscript{2+}-bridged interactions with negatively charged phospholipid head groups. It is very likely that, in analogy with other activating surfaces, exposed phospholipid head groups similarly play essential roles. Further studies are needed to identify the responsible moieties, but the key recognition remains that an intact membrane is required for the DV to unfold both its complement-activating and procoagulant properties. The latter are intrinsically borne and not dependent on any interaction with platelets. Clotting was triggered in platelet-free plasma, and thrombin could be generated directly by incubating purified DVs with FXa and prothrombin.

Why complement activation in non-immune serum occurs exclusively on the DV remains to be clarified. GPI-anchored proteins including complement inhibitors\textsuperscript{39,40} have been shown to shuttle from the RBC to the parasitophorous vacuole membrane\textsuperscript{41-44}, and the possibility is
being examined whether they further be recruited to the merozoite surface to shield the
disease from complement attack. In any event, the DV might serve as a decoy for central host
defense elements. Indeed, we have found that complement activation marks the DV for
selective phagocytosis by neutrophil granulocytes, while merozoites are left free to reinvade
cells. This situation was found to persist in the presence of serum from malarial patients\textsuperscript{19}. Possibly, alternative pathway activation plays a first role in mediating selective opsonization
of DVs in non-immune serum. As specific antibodies appear, classical pathway activation
may be triggered on both the DVs and merozoites. The density of activated complement
components will consequently remain higher on the DV thereby sustaining its preferential
phagocytosis.

When DVs were injected into rat tail veins, complement consumption occurred within
minutes and the animals became lethargic and behavioral responses were impaired. The
clinical symptoms possibly derived from systemic activation of endothelial and phagocytic
cells with release of inflammatory molecules and mediators, which would be expected to
occur following triggering of the clotting\textsuperscript{5} and alternative complement pathway\textsuperscript{45}. The effects
of bolus DV-infusions were transient, reminiscent of LPS-injection, which also provokes
systemic inflammatory responses and complement activation\textsuperscript{46}. Termination of the reaction to
DV\textsubscript{s} might be explained by their rapid clearance by phagocytic cells. Indeed, PMN were
rarely seen in the bloodstream of the animals, and the massive uptake of DV\textsubscript{s} by tissue
phagocytes was impressive. At the onset of malaria infections, the low load of DV\textsubscript{s} is perhaps
first cleared by PMN. As the clearance capacity is overrun, tissue macrophages may come
into play. Malaria pigment is present in these cells in human patients\textsuperscript{16,47}, and our finding that
isolated DV\textsubscript{s} reside in tissue macrophages within hours after intravenous application also
accords with the concept. Dysfunction of these cells occurring after DV-uptake has been reported in several studies\textsuperscript{14,48-50} and likely also contributes to the pathogenesis of disease.

A major challenge facing the DV-complement activation theory is the fact that high parasite loads develop in many patients, particularly in Africa and the Pacific, without provoking severe symptoms. Just two speculations are advanced at this stage. First, the dynamics of DV release and removal may be important. Efficacy of phagocyte uptake of DVs is possibly subject to wide inter-individual variations. Second, genetic deficiencies in late complement components occur worldwide with varying and sometimes surprisingly high frequency\textsuperscript{51}. Heterozygotes are not prone to suffer from bacterial infections because serum complement activity is only lowered, but perhaps these individuals are protected against the effects of complement overactivation that lead to severe malaria.

If the major tenets of our hypothesis turn out to be correct, strategies to inhibit DV-dependent activation of complement and coagulation might have therapeutic potential. Activation of both cascades was found to be inhibited by LMW-DXS. In contrast to heparin, LMW-DXS does not cause bleeding complications and is well tolerated in humans. Indeed, the agent caused no side effects in rats but protected the animals from the harmful effects of very high doses of DV infusion. The agent was applied at the same concentrations as previously used in transplantation models\textsuperscript{36}. LMW-DXS has also been reported to suppress merozoite reinvasion\textsuperscript{52} and may therefore simultaneously fulfill dual beneficial functions.

It is intriguing that, having served its physiological purpose in the life cycle of the parasite, the DV should be endowed with the capacity to activate the two archaic enzyme cascade systems of the blood into which it is cast. Perhaps these events initially serve a protective
function by enabling the infected host to rapidly remove alien waste material. However, pathological consequences may evolve when the disposal machinery suffers overload, these events then contributing to the evolution of severe malaria. Future studies should disclose whether a novel determinant of parasite pathogenicity has been discovered that might also be targeted for therapy to the benefit of patients suffering from what still remains one of the most prevalent life-threatening diseases in the world.
Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 490 (S.B.), SFB 593 (K.L. and St. B), CRC877 (Ka.R.), the Cluster of Excellence "Inflammation at Interfaces" (Ka.R.) and the Thai Infectious Disease Network (P.D., S.C.B, S.B.). We are grateful to Anthony Holder for the kind gift of anti-MSP1 antibodies and to Johannes Müthing for valuable advice on the detection of sialylated glycans. The authors thank Walter Hitzler and Roland Conradi for continued supply of erythrocytes, banked human blood and human sera, Antje Canisius for excellent technical assistance, and Monika Wiedmann for outstanding secretarial work.


Competing Financial Interest

The authors declare that they have no competing financial interest.
References


Figure Legends

Figure 1. Differential staining of merozoites and digestive vacuoles (DV). (A) Giemsa stain of late-stage parasitized RBC (pRBC) undergoing schizont rupture. (B) Vital stain of the same culture with BCECF-AM showing intense staining of DVs (large arrow) alongside staining of merozoites (small arrow). (C) Same culture stained with Hoechst 33342 that detects the merozoites (small arrow) but spares the DVs (large arrow). (D) Giemsa stain of isolated DVs. Insert: recording of analysis in a hemocytometer with DVs presenting as particles with the size of platelets (PLT). (E) Staining of isolated DVs with BCECF. (F) No staining with Hoechst 33342. (G) Phase-contrast image of DVs to which RBC were added. (H) Same smear fluorescently stained for RBC band 3 protein. (I) Giemsa stain of purified merozoite preparation used in Western blots. (J) Western blots of RBC membranes, merozoites and DVs probed with antibodies against RBC band 3 protein and MSP1 (K). DV preparations contained very scant contaminations with MSP1. Arrows depict position of the respective molecular weight markers. Scale bars: 10 µm.

Figure 2. Complement activation occurs to completion on the surface of DVs, but not on merozoites. (A) Late stage pRBC were cultured and allowed to rupture in the presence of 10% active human serum. Complement activity and hemolysis were recorded over time and found to be inversely correlated. Two-dimensional immunoelectrophoresis confirmed that C3 turnover occurred at the onset of hemolysis concomitant to the fall in complement activity (6 h). Buffer controls (in the absence of pRBC) are shown in the second row of panels. The bottom two plates show C3-immunoelectrophoresis at 0 and 8 h from an experiment conducted with pRBC in the presence of 10 mM EGTA/2 mM Mg^{2+}. First dimension electrophoresis: left to right, second dimension immunoelectrophoresis: bottom to top. (B)
Synchronized late-stage pRBC were allowed to rupture in active human serum whereafter unlysed cells were pelleted and merozoites and DVs were harvested from the supernatants and stained for DNA or C5b-9. Upper left: phase contrast microscopy, upper right: Hoechst 33342 DNA stain, lower left: complement C5b-9 complex: lower right: merge. Note selective staining of DVs for C5b-9.

**Figure 3.** Isolated DVs activate the alternative complement pathway. (A) Addition of isolated DVs to serum in the presence of EGTA/Mg dose-dependently provoked complement consumption, assessed with rabbit erythrocytes, and C3 turnover. (B) Concentration of fluid phase SC5b-9 in serum spiked with increasing numbers of isolated DVs. (C) Staining of C3 on isolated DVs following incubation with serum. Upper row: Control, incubation in inactive serum. Lower row: Incubation in active serum. (D) Detection of C5b-9 on DVs following incubation with active human serum. Corresponding flow cytometric analysis shows staining of all DVs in the sample. Representative results of 4 independent experiments are shown.

**Figure 4.** Preparations of parasitophorous vacuoles membrane-enclosed merozoite structures (PEMS), intact DVs and free hemozoin. (A) Merozoites and DVs encased in the parasitophorous vacuole from pRBC following rupture in the presence of the protease inhibitor E64, which inhibits lysis of the parasitophorous vacuole membrane but not lysis of the erythrocyte membrane. (B) DVs isolated from trophozoites. (C) Hemozoin crystals isolated from sonicated DVs by centrifugation in Percoll. Scale bar: 5 μm. (D) Complement consumption tests were performed with materials (A) to (C) above using 10% NHS. DVs were used as positive control and similar concentrations of heme were employed throughout. Neither PEMS nor isolated hemozoin had complement activating capacity. One representative assay out of three independent experiments is shown with SD from triplicate determinations.
Figure 5. DVs directly activate the intrinsic clotting pathway. (A) Clotting time after recalcification of 50% plasma was significantly accelerated in the presence of DVs. Clotting times of the respective buffer controls were taken as the 100% reference in each experiment. Data are expressed as mean ± SEM of five independent experiments. (B) Isolated DVs dose-dependently enhanced thrombin generation in the prothrombinase assay (n=4 ± SEM). Controls without DVs did not induce any thrombin generation and are not shown. (C) Procoagulant activity of DVs is sensitive to phospholipase C treatment. Clotting times of 50% citrated plasma were determined after recalcification in the presence of Veronal buffered saline (VBS), VBS plus PLC, DVs, or DVs after PLC treatment. Clotting time was significantly accelerated in the presence of DVs (*p<0.05 versus control). This effect was abolished after PLC treatment (op<0.05). Data are expressed as mean ± SD of three independent experiments.

Figure 6. Low molecular weight dextran sulfate (LMW-DXS) abrogates procoagulant and complement triggering action of DVs. (A) Clotting times were determined after recalcification of 50% citrated plasma in the absence (control) or presence of DVs and LMW-DXS at depicted concentrations. DVs provoked a significant reduction in clotting time (*p<0.05 versus control) which was significantly reversed by 10 µg/ml of LMW-DXS (p<0.05). 100 µg/ml LMW-DXS completely prevented clot formation despite the presence of DVs. Data are expressed as mean ± SD of three independent experiments. (B) LMW-DXS abrogates prothrombinase assembly on DVs. Prothrombinase assays were performed in the presence of 5 x 10^7 DVs/ml and in the absence or presence of LMW-DXS at the given concentrations, and thrombin generation was determined after 2 and 5 min (n=3 ± SD). 100 µg/ml DXS abolished prothrombinase assembly (*p<0.05 versus control). (C) Inhibition of DV-dependent C3 turnover by 100 – 1000 µg/ml LMW-DXS. Control (upper left panel): 20%
NHS incubated for 30 min, 37°C. 10⁸ DVs/ml were added to 20% NHS in the absence or presence of LMW-DXS at the depicted concentrations. Inhibition of C3 turnover (arrows) was observed at 100 µg/ml LMW-DXS. Results shown are representative for three independent experiments.

**Figure 7. Effect of intravenous injection of isolated DVs in rats.** (A) Reduction of nociceptive responses. The hot plate test was used in which rats were placed in jars warmed to 56°C. Latency periods until licking of the paws were measured. Results are depicted as the quotient of reaction times measured after DV infusion to reaction times determined before infusion in each animal. One group of animals received a bolus injection of 5 x 10⁹ DVs (n = 5). The second group received an injection of 5 x 10⁹ sonicated DVs (n = 3). The third group received 6 mg LMW-DXS i.p. 45 min prior to injection of 5 x 10⁹ DVs (n = 4). All experiments were performed with one and the same banked DV pool. DVs provoked a significant increase in latency time (*p<0.05 versus control) which was significantly decreased in the presence of LMW-DXS ("p<0.05). Bars represent mean values with SD. (B-C) Rapid cellular uptake of DVs by mononuclear cells. Paraffin-embedded sections of (B) spleen and (C) lung were stained with hematoxylin & eosin. (B) Intracellular accumulation of DVs in the marginal zone (asterisk) containing abundant macrophages (left panel; magnification x252). This pattern is illustrated in the right panel after polarization at lower magnification (x63). (C) Intravascular accumulation of mononuclear cells (left panel, arrowhead, magnification x252) containing intracellular DVs (right panel, arrowhead, magnification x630).
Figure 1
Figure 2

A

B
Figure 3

A

B

C

D

% relative consumption

DV x 10^-6/ml

0

100

0

8

16

32

63

125

250

500

SC5b-9 (ng/ml)

0

1

2

3

4

5

6

DV x 10^-6/ml

phase contrast

C3

merge

phase contrast

C3

merge

counts

log fluorescence intensity

10^0

10^1

10^2

10^3

10^4

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Figure 4

A. PEMS

B. DVs from trophozoites

C. hemozoin

D. % relative consumption

- DV
- PEMS
- DVs from trophozoites
- hemozoin
Figure 5

A

B

C

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Figure 7

A

![Chart showing latency times after and before infusion with different treatments: DVs, DVs sonicated, and LMW-DXS.](chart)

B

- **spleen**
  - H&E
  - pol
  - * indicates significant differences

C

- **lung**
  - H&E
  - H&E
  - Arrowheads indicate specific structures or areas of interest.
Digestive vacuole of Plasmodium falciparum released during erythrocyte rupture dually activates complement and coagulation

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