

CHARACTERIZATION OF MAURER'S CLEFTS IN *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES

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Abstract. In 1902 Georg Maurer was the first to publish a detailed description of Giemsa-stained structures in the cytosol of *Plasmodium falciparum*-infected erythrocytes, today known as Maurer's clefts. Later when clefts were seen by electron microscopy, the description was modified to also include these, which has caused disagreement over the composition of Maurer's clefts. For that reason, Maurer's clefts were characterized during intraerythrocytic development of *P. falciparum* by simultaneously staining cytosolic structures with antibodies using indirect immunofluorescence assays and with Giemsa. At least three groups of antigens, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1)/RIFIN/SURFIN, *P. falciparum* histidine-rich protein 2 (PfHRP2), and exported proteins 1 and 2 (Exp1 and Exp2), were detected in distinct Giemsa-stained structures in the cytosol of infected erythrocytes, but PfHRP2 and Exp1/Exp2 were not found in clefts by transmission electron microscopy. Therefore, Maurer's clefts as defined by staining with Giemsa comprise a number of cytoplasmic structures and antigens not included in structures called clefts and seen by electron microscopy.

INTRODUCTION

In 1902 Georg Maurer reported from Medan-Deli in Sumatra on light microscopy studies of blood smears obtained from patients with *Plasmodium falciparum* malaria and stained with a Romanowsky dye (prototype of Giemsa).¹ He discovered the presence of multiple-stained structures in the infected erythrocyte (IE) cytosol, and provided a detailed description in words as well as with drawings.^{1,2} As a result, these cytosolic structures have been called Maurer's clefts (MCs).^{2,3}

As *P. falciparum* develops within the erythrocyte, extensive modifications of the host cell cytosol occur. New membranous structures, such as protein trafficking vesicles, are synthesized and exported outside of the parasitophorous vacuole (PV).^{4–6} The cytosolic structures have been suggested to function in both protein transport and sorting and have been suggested to be MCs.^{3,7–9} However, the latter definition is restricted because it only includes structures seen by electron microscopy (EM) and immunofluorescence assay (IFA).^{7,10–12} This more recent definition has caused some confusion because the MCs, as described by Maurer using light microscopy and Giemsa stain, is used in the diagnosis of malaria and serves as the gold standard to discriminate *P. falciparum* from other *Plasmodium* species.

Plasmodium falciparum antigen Pf332 and *P. falciparum* skeleton-binding protein 1 (PfSBP1) have been found in EM clefts and in IFA cytosolic structures. This suggests that MCs are involved in the transport of antigens to the surface of the erythrocyte, or to distal compartments closely associated with the plasma membrane. Transport vesicles carrying clonally variant surface antigens (i.e., *P. falciparum* erythrocyte membrane protein 1 [PfEMP1]/RIFIN/SURFIN) to their final destination at the IE surface through the cytosol in small single vesicles (SSVs), large multimeric vesicles (LMVs), and large spindle-like vesicles (LSLVs) have also been identified.^{6,13,14} These and other proteins (Sar1p, Sec31, Sec23, PfEMP3) co-

localize with Pf332 and PfSBP1 in the IE cytosol, whereas other groups of antigens such as exported protein 1 (Exp1), exported protein 2 (Exp2), and histidine-rich protein 2 (HRP2) are translocated to other subcellular compartments assumed not to be part of the MC system as seen by EM.^{6,7,12,15–17}

We have characterized MCs using the original staining protocol of Maurer and demonstrate that the same structures he saw can be detected in IFA. We show that some MCs are involved in cytosolic trafficking of the antigenically variant surface antigens (RIFIN, PfEMP1), and antigens Pf332, SURFIN, and Sar1p as previously suggested. Furthermore, we report that distinctly shaped MCs carry PfHRP2, Exp1, and Exp2. Our study presents data showing that neither Pf332 nor PfSBP1 alone are unique markers for all the different types of MCs following the original definition of an MC. The MCs as shown by staining with Giemsa comprise cytoplasmic structures and antigens not included in structures called clefts as seen by EM.

MATERIALS AND METHODS

Parasites and cultures. The laboratory-adapted parasites used in this study were of the *P. falciparum* rosette-forming clone FCR3S1.2.¹⁸ They were kept in continuous culture according to standard procedures with erythrocytes (O⁺) at a 5% hematocrit supplemented with 10% B⁺ Rh⁺ serum in buffered culture medium.^{19,20} Cultures were synchronized in 5% sorbitol for 10 minutes as previously described.^{20,21} Fresh clinical *P. falciparum* isolates were obtained from venous blood samples of malaria patients attending the Mulago Hospital, Kampala, Uganda.⁶ Informed consent was obtained from the patients and/or their guardians.

Conventional Romanowsky/Giemsa staining. Parasite cultures were harvested at the stage of interest and centrifuged for 30 seconds at 500 × g. Approximately 2 μL of the pelleted cells were used to prepare thin smears on glass slides. Smears were left to air dry at room temperature for up to 24 hours, and fixed for 5 seconds in 100% methanol. To visualize cytosolic structures in the IE cytoplasm, the thin smears were incubated in 5% Giemsa (Merck, Rahway, NJ) diluted in

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phosphate buffer (Merck; pH 7.2–7.4) for 30–90 minutes according to the original procedure.¹

Giemsa is a modified version of the Romanowsky dye, which was used by Maurer in 1902, with azure added to the methylene blue, and methanol and glycerol added for increased solubility and stability.^{22–24} The original Romanowsky dye (developed in 1891) was based only on methylene blue and eosin, and was thus rather unstable and insoluble. The addition of glycerol and methanol to the stain preserve the staining pattern the optimum pH (7.2–7.4) is used. The basic azure and methylene blue stains acidic structures blue and eosin stains basic structures, i.e., most proteins, red.

Sera and specific antibodies. The anti-RIFIN antiserum (#565) was produced in rabbits immunized with synthetic 20-amino acid peptides conjugated to the carrier protein keyhole limpet hemocyanin.²⁵ Antibodies to PfEMP1 were obtained by immunizing rats with glutathione-S-transferase fusion proteins comprising fragments from the highly conserved C-terminal acidic-terminal sequence of the PfEMP1 encoded by the *var* gene FCR3S1.2var1.²⁶ Human monoclonal antibody (MAb) 33G2 recognizes the sequential epitope VTEEI present in the *P. falciparum* antigens Pf332 and Pf155/ring-infected erythrocyte surface antigen.^{27,28} The lack of developmental overlap in the expression of these two proteins in the parasite enables the use of the 33G2 for the specific detection of Pf332.¹² Monoclonal antibodies mAb5.1, mAb7.7, and mAb2G12 were used for the specific detection of Exp1, Exp2, and PfHRP2, respectively.^{29–32}

Transmission electron microscopy (TEM) and immunoelectron microscopy (IEM). Parasites grown to trophozoite stages (20–28 hours) were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 2 hours. The fixation buffer was replaced by phosphate buffer and the cells were centrifuged and further processed for immunoelectron microscopy as described elsewhere.¹⁴

Indirect immunofluorescence assay for the detection of intracellular proteins. Synchronous parasite cultures were harvested every fourth hour during the 48-hour life cycle, washed three times with phosphate-buffered saline (PBS), and diluted to a density of 10^6 cells/mL. Monolayers were prepared on 15-well multitest slides (ICN Biomedical Inc., Costa Mesa, CA) as previously described.⁶ Monolayers were incubated 30 minutes with primary antibodies diluted in PBS, washed three times in PBS, and incubated 30 minutes with secondary antibodies conjugated with Alexa-488 or FITC fluorophores. All incubations were at room temperature in a humid chamber. Cover slips were mounted with anti-fading solution (20% DABCO; Sigma, St. Louis, MO [in glycerol]) and the slides were analyzed with a 100× oil-immersion lens and a 10× ocular in a Nikon (Tokyo, Japan) Optiphot 2 ultraviolet microscope equipped with suitable barrier filters.

Labeling of lipids and proteins and staining with Giemsa. To study the location of lipids relative to that of proteins and Giemsa-stained structures (MCs), Bodipy-ceramide-TR lipids were incorporated as previously described, and monolayers were prepared in the dark.⁶ After air drying, the monolayers were fixed for one hour with 4% PFA in PBS or with 4% PFA with 0.1% glutaraldehyde (GDA) in PBS, and washed three times in PBS before the protein of interest was labeled with Cy3- or Alexa-488 fluorophores. The slides were analyzed with a 100× oil-immersion lens and

a 10× ocular in an Optiphot 2 ultraviolet microscope equipped with suitable barrier filters. No anti-fading reagent was used. The IFA images were captured from defined areas of the slides and exact coordinates were noted, followed by the removal of the cover slip and drying of the cell monolayers. Giemsa staining was subsequently performed as described above and complementary images of identical areas as for the IFA were captured.

RESULTS

Analysis of MCs using Giemsa and the protocol of Maurer.

Giemsa staining was performed on thin smears of erythrocytes infected with the *P. falciparum* FCR3S1.2 clone. Synchronous parasite cultures were harvested every fourth hour during development from newly invaded rings (four hours) to bursting schizonts (48 hours). A detailed time-course analysis of the cytosol of the IE showed that the first MCs, seen as a few small weakly stained dots (0.1–0.2 μm), appeared at approximately eight hours post-invasion (Figure 1A). More dense structures of a variable size and shape became visible as the parasites developed into 16–20 hour trophozoite stages, which concurs with the original description of MCs in the stages referred to as large rings.¹ These structures further increased in number as well as in size, and accumulated at the outer periphery of the IE as the parasites developed into late trophozoite and early schizont stages (> 32 hours post-invasion) (Figure 1A). A mixture of both small and large MCs were observed in most of the trophozoite IE, although in some cells the structures were of similar size and

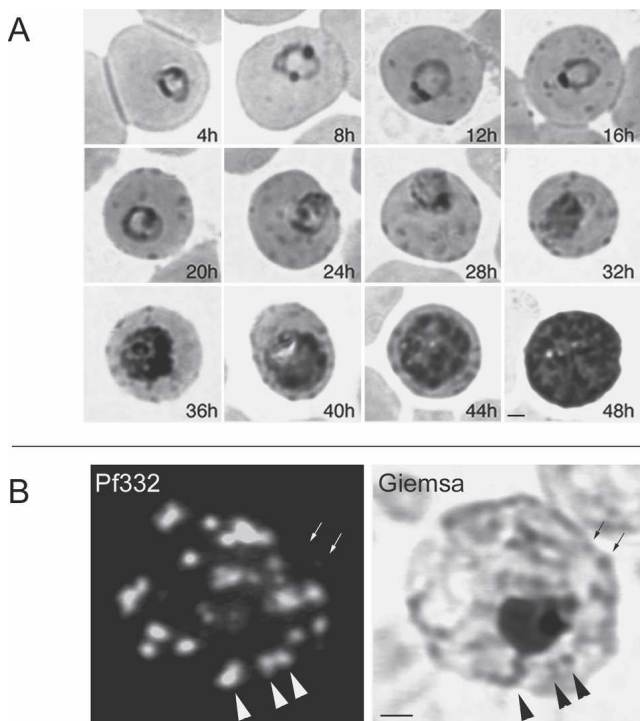


FIGURE 1. Maurer's clefts as originally described in Giemsa-stained infected erythrocytes and as seen with the *Plasmodium falciparum* marker Pf332. **A**, Time-course study of Giemsa-stained parasite culture smears. **B**, Co-localization of antigen Pf332 and Giemsa staining. Some co-localizing structures are pointed out (arrowheads) as are some non-overlapping structures (arrows). Scale bars = 1 μm.

in some IEs no MCs were seen. Our observations were confirmed in IEs from fresh patient isolates.

Identification of MCs using antigen Pf332 as a marker. The postulated MC marker protein Pf332 was reported to be transported to the IE cytosol at approximately 18 hours post-invasion. In contrast, we observed small cytosolic structures stained with Giemsa (i.e., MCs) as early as eight hours post-invasion.^{6,12} To further investigate this finding, we developed a new double-staining technique for sequential IFA labeling of proteins followed by staining with Giemsa (see Materials and Methods). This confirmed that the Giemsa-stained MCs could be detected in the cytosol much earlier than Pf332,⁶ but as the parasites developed into mature trophozoite stages an overlap was seen between the Pf332 staining and Giemsa staining (Figure 1B, arrowheads). In some cases, a number of MC structures were observed to be lacking Pf332 signal (Figure 1B, arrows).

Correlation between RIFINs/PfEMP1 and IEM clefts and Giemsa-stained MCs. The suggestion that MCs are involved in trafficking and sorting of antigenically variable proteins destined for the IE surface (PfEMP1/RIFINs) motivated further studies of these proteins in relation to the Giemsa-stained structures. To investigate the relationship between the original MCs (as described by Maurer in 1902) and the clefts seen by EM (currently also acknowledged by some as MCs), we performed IEM, IFA, and Giemsa studies of parasites at early- and mid-trophozoite stages. The IEM studies were performed on trophozoite IEs fixed in 4% PFA without GDA, a protocol that preserves epitope recognition of the antigens, although some morphology is lost. Membranous structures in the cytosol and at the erythrocyte membrane were found, and the bulk of RIFIN/PfEMP1 was seen in IEM clefts and at the surface membrane (Figure 2A, arrowheads), but also in smaller structures of dense material (Figure 2A, arrow).

In experiments using dual staining with IFA and Giemsa, we found that in ring-stage parasites (< 16 hours), the small cytosolic dots that stained with RIFIN- or PfEMP1-specific antibodies (SSV, 0.1–0.2 μm) were also stained with Giemsa (MCs) (Figure 2B, arrowheads). As the parasites developed further (> 16 hours post-invasion), co-localization of LMVs and LSLVs with intensely stained MCs of variable size and shape was almost complete (Figure 2C, arrowheads). However, consistent with our observation with Pf332, we found that a number of Giemsa-stained structures in most IEs at all stages lacked RIFIN or PfEMP1 (Figure 2B and C, arrows). This demonstrates the existence of compartments consisting of other proteins/molecules able to bind Giemsa.

Co-localization of PfHRP2 with Giemsa-stained structures other than PfEMP1/RIFINs. Apart from the surface bound vesicular transport in SSV/LMV/LSLVs, other protein-containing compartments have been identified in the IE cytosol, including non-vesicular PfHRP2 aggregates.³³ As previously described, we detected PfHRP2 trafficking in the IE cytosol as diffusely stained material that occasionally was in larger aggregates (approximately 2–3 μm).³³ These aggregates, which were present only in IEs seen later than 28 hours post-invasion, co-localized with Giemsa-stained structures (Figure 3A, arrows). We also observed co-localization of PfHRP2 and the LMVs carrying PfEMP1/RIFIN/Pf332 in some of the IEs, but not in others (Figure 3C). Furthermore, labeling of PfHRP2 and RIFINs prior to staining with Giemsa

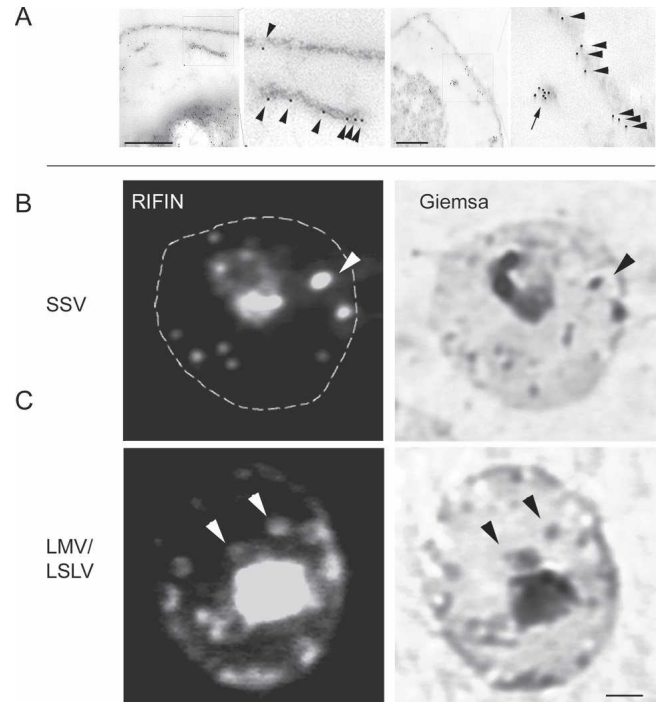


FIGURE 2. *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) and RIFINs in immunoelectron microscopy (IEM) clefts and in Giemsa-stained Maurer's clefts. **A**, cytosolic structures found by transmission electron microscopy contain RIFIN antigens. In paraformaldehyde-fixed parasites, gold-labeled RIFINs are detected in IEM clefts and other cytoplasmic vesicles (**arrows**) as well as at the surface membrane of the infected erythrocyte (IE) (**arrowheads**). **B** and **C**, IEs probed with antibodies specific to RIFIN polypeptides or PfEMP1 incubated with Alexa-488-conjugated secondary antibodies, and sequentially stained with Giemsa. Similar patterns were seen (here RIFINs are shown). **B**, Typical small dots (small single vesicles [SSVs]) are indicated in ring-stage parasites approximately 12 hours post-invasion, and **C**, larger structures (large multimeric vesicles [LMVs]) as well as irregular-shaped surface-associated structures (large spindle-like vesicles [LSLVs]) are shown in trophozoite stage parasites at 20–28 hours post-invasion. Selected regions of co-localization are indicated (**arrowheads**) as well as non-overlapping structures (**arrows**). Scale bars = 1 μm .

showed that all structures stained for either of these proteins, alone or simultaneously, were stained with Giemsa (Figure 3C).

Co-localization of membranous structures with parasitophorous vacuole membrane (PVM) buds and SSV/LMV/LSLVs and with Giemsa. The term MCs was earlier used to describe different membranous structures, such as acridine orange-stained vesicles or the PVM buds with Exp1 and Exp2.^{30,34} To investigate whether these structures were also stained with Giemsa, we performed triple staining of lipids, proteins, and Giemsa-stained structures in the *P. falciparum* FCR3S1.2 clone harvested at trophozoite stages (18–28 hours) as described in the Materials and Methods. Exp1 and Exp2 were associated with lipids, as shown by the incorporation of TR-labeled Bodipy-ceramide, and co-localized with Giemsa-stained material in the IE cytosol (Figure 3D).

DISCUSSION

Giemsa-stainable structures are present in the cytoplasm of erythrocytes infected by *P. vivax* and *P. ovale* (Schuffner's

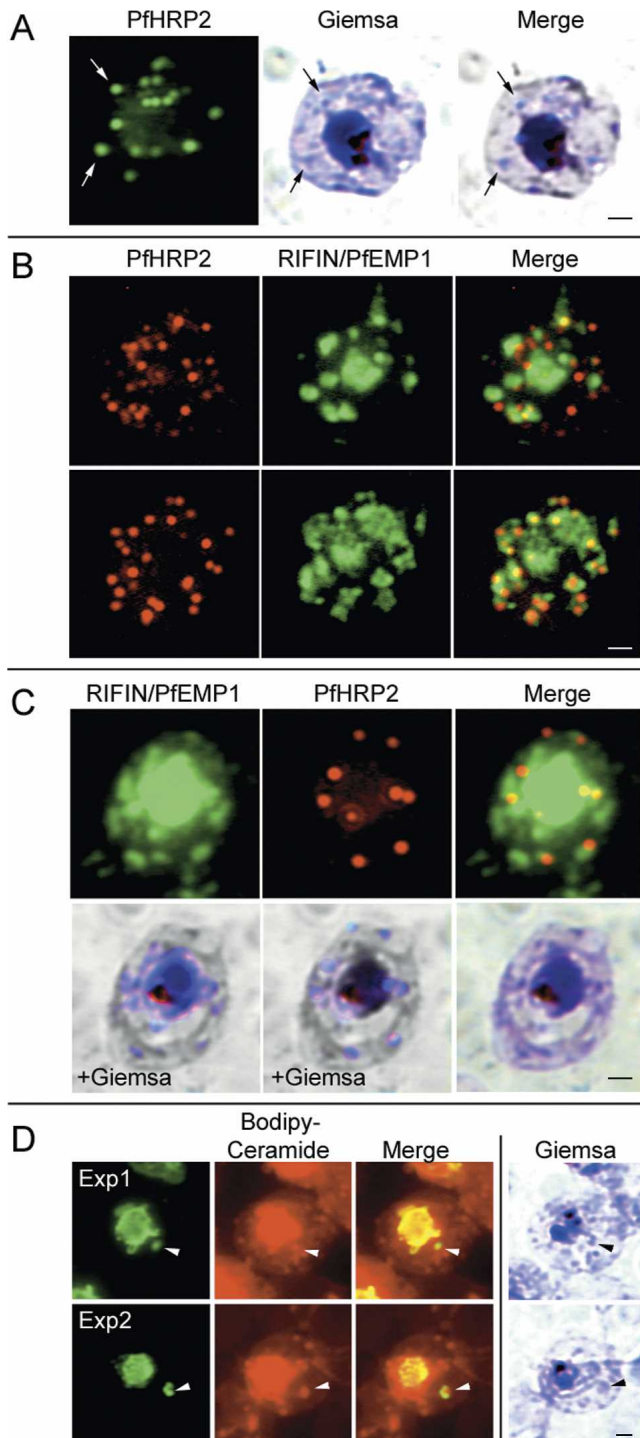


FIGURE 3. Maurer's clefts consist of proteins as well as lipids. **A–C**, Cytosolic *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) is coincident with structures stained with Giemsa (Maurer's clefts) and occasionally with *P. falciparum* erythrocyte membrane protein 1 (PfEMP1)/RIFINs. Simultaneous protein and Giemsa staining of FCR3S1.2 parasites at trophozoite stages was performed as described in the Materials and Methods. **A**, Alexa-488 labeled HRP2 (green) and Giemsa-stained Maurer's clefts. Co-localization is shown in merged images (blue, arrows). **B**, Dual staining of HRP2 and PfEMP1/RIFINs show different patterns that occasionally overlap. **C**, All HRP2 and PfEMP1/RIFIN structures co-localize with Giemsa staining. **D**, Parasites of the FCR3S1.2 clone were harvested at trophozoite stages (20–28 hours post-invasion) and protein, lipid, and Giemsa labeled as described in the Materials and Methods. Immunofluorescence analysis with Alexa-488-labeled ex-

ports, James's dots) but MCs are specific for *P. falciparum*-infected erythrocytes. MCs were first described in 1902 as a few small dots present in erythrocytes infected with ring-stage parasites, and 10–12 larger spots or blotches with a blurred outline, often hollow and looped, in later developmental stages.^{1,2}

It seems reasonable to assume that in early stage IEs (< 8 hours post-invasion), when the concentration of parasite proteins in the IE cytosol is low, staining with Giemsa will be absent or weak. When the parasite reaches the peak of intracytoplasmic protein transport and expression of parasite-derived molecules on the IE surface (approximately 24–30 hours post-invasion), the cytosolic protein content will also be at its maximum and staining will be more intense. Thus, the number of MCs found in the IE cytosol will generally reflect the level of protein content during *P. falciparum* maturation.

In this study, we have analyzed the characteristics of the various MC structures by Giemsa staining, as originally described and according to the conditions used in 1902.^{1,2} In detailed time-course analyses, we detected a few small cytosolic dots in the young ring-stage IE and larger irregularly shaped dots in IEs with more mature parasites. Since antigen Pf332, which is currently used as an MC marker, is not detectable in the IE cytosol using IFA until > 18 hours post-invasion, we developed a new staining technique to investigate the relationship between Giemsa- and IFA-stained structures.^{8,35} We were able to visualize co-localization of Pf332/RIFIN/PfEMP1 in Giemsa-stained MCs in trophozoite stage parasites. RIFIN/PfEMP1 were also co-localized with Giemsa-stained structures in younger stage parasites (rings < 16 hours), as were PVM buds (Exp1/Exp2) and protein aggregates (PfHRP2). None of these structures were previously included in the concept of MCs, using Pf332 or PfSBP1 as reference markers, although earlier IEM studies with Exp1/Exp2 suggested this finding.^{30,36} These data suggest that protein antigens and lipids participate in the composition of the MC, as determined by staining with Giemsa.

Early EM studies of the ultrastructure of the *P. falciparum* IE cytosol identified several membranous structures originating from the intracellular developing parasite.^{10,11,37} Among these were EM clefts seen in IEs with trophozoite stages, which was suggested to be identical to the structures Georg Maurer described, and referred to today as MCs.^{3,9,10} In the past decade, interest has focused on surface-exposed antigens, the mechanisms behind their routes through the IE cytosol, and the involvement of these EM clefts. The megadalton antigen Pf332 was found in IEM clefts, as well as at the surface of trophozoite stage IEs. Thus, these structures were presumed to have a role in parasite protein transport to the surface.^{12,35} More recent studies of PfSBP1, a protein associating with the erythrocytic ligand LANCL1 as well as with PfEMP1, showed the presence of a non-surface exposed protein in IEM clefts and LMVs.^{7,38} Today PfSBP1 is generally

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ported protein 1 (Exp1) or exported protein 2 (Exp2) was performed on infected erythrocytes with incorporated Bodipy-Ceramide-TR, which labels membranes (red), prior to further drying and staining with Giemsa. Merged images show co-localization of proteins and lipids (yellow), as well as with Giemsa-stained structures (blue). Scale bars = 1 μ m.

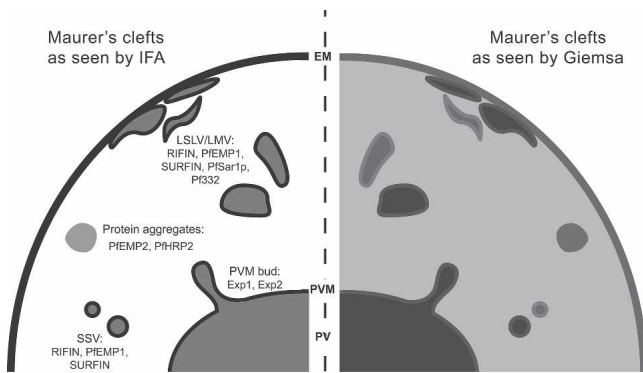


FIGURE 4. Illustration of transport routes (immunofluorescence assay [IFA] and Giemsa-stained structures, i.e., Maurer's clefts), in the cytosol of an infected erythrocyte. Specific detection of proteins in the infected erythrocyte using IFA techniques visualizes a variety of different cytosolic structures. These structures are also stainable with the Giemsa. Small vesicles or soluble proteins freely diffusing in the cytosol will in part generate the weak Giemsa staining of the same structure, whereas accumulated proteins are seen as dots or blotches, i.e., Maurer's clefts. EM = erythrocyte membrane; LSLV = large spindle-like vesicles; LMV = large multimeric vesicles; PfEMP2 = *Plasmodium falciparum* erythrocyte membrane protein 2; PfHRP2 = *P. falciparum* histidine-rich protein 2; PVM = parasitophorous vacuole membrane; Exp1 = exported protein 1; Exp2 = exported protein 2; SSV = small single vesicles; PV = parasitophorous vacuole.

accepted as an MC marker, and since it is detected in the IE cytosol as early as 4 hours post-invasion it might be responsible for the early Giemsa-stainable structures.⁷ However, with the exception of one study, a number of studies report that in structures where PfEMP1, RIFIN, MAHRP, PfEMP3, PfSar1p, PfSBP1, and occasionally knob-associated histidine-rich protein co-localize, there was no staining with the PVM bud markers (Exp1/Exp2). This suggests that with PfSBP1 as a marker, most but not the entire range of MCs as detected with Giemsa, are labeled.^{6,8,39}

Interestingly, PfSBP1 has also been detected in *P. chabaudi* and *P. berghei* IEs, which suggests that MC-like structures can also develop in *Plasmodium* species other than *P. falciparum*. The presence of MCs has also been implicated in gametocyte IEs and in sporozoites because of the finding of PfSBP1 in these stages.^{40,41} However, the naming MC of these structures in non-*P. falciparum* species and in parasite stages Maurer never studied may be misleading. Furthermore, it has recently been reported that the MC structures in which PfSBP1 is located, remain after the gene encoding this protein is inactivated.⁴²

Some cytosolic parasite proteins (i.e., PfEMP1) have been described to localize in MCs as well as in membrane whorls called the tubovesicular network (TVN), which suggests a relationship between these structures.^{43,44} However, proteins in TVN have been reported in serial section EM studies, whereas the MC association was detected by co-localization with PfSBP1 in IFA studies.^{3,43} The relationship between TVN and Giemsa-stained structures has not yet been clarified.

In conclusion, we have shown that Pf332 and PfSBP1 are not markers for the entire array of MCs because there are other intra-cytoplasmic structures that stain with Giemsa but lack both of the polypeptides. In agreement with a theory that MCs are lipid-rich compartments, we have also shown that lipid markers stain some MCs but not all, and this is probably

due to the fact that some proteins can be transported independent of lipids (PfHRP2).⁹ Until it can be proven that all structures stained by Giemsa correspond to a single molecular marker, staining with Giemsa will continue to be the optimal marker for MCs. Therefore, we suggest that structures that comprise the MCs should be given names (e.g., EM clefts, LMV) that will easily distinguish them from the original description of MCs stained with Giemsa. Figure 4 shows a schematic illustration of all the Giemsa-stainable structures characterized in this study.

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