Characterization of Membrane Permeability Alterations in *Plasmodium*-Infected Erythrocytes: Insight into Novel Mechanisms for Malaria Chemotherapy

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Introduction

Current malaria research is geared toward identifying novel targets for malaria chemotherapy because of the growing resistance of *Plasmodium falciparum* to existing drug options (Trager et. al., 1997). Potential targets not yet explored are the new permeability pathways induced by *Plasmodium* on host erythrocytes. These pathways confer increased permeability to inorganic ions including chloride, sodium, and calcium (Adovelande et al. 1993, Brand et al. 2003, Garcia et al. 1996, Lang et al. 2003), as well as organic solutes such as sorbitol (Tanneur et al. 2005), lactic acid, and hemoglobin-derived amino acids (Duranton et al. 2004). Evidence suggests that the primary function of these pathways is to allow for abundant access to nutrients and vitamins essential for parasite growth (Brand et al. 2003, Duranton et al. 2004), while facilitating elimination of metabolic waste products (Duranton et al. 2004). Due to the apparent dependence of *Plasmodium* survival on these transport pathways (Brand et al. 2003), targeting the pathways could be a potent method for inhibiting the blood stage life cycle of *Plasmodium* and, in turn, arresting disease progression.

However, the precise nature of the new permeability pathways induced by *Plasmodium* on host erythrocytes is ill-defined. That is, it is not clear whether the pathways are endogenous membrane proteins activated by *Plasmodium* or, alternatively, if they are xenoproteins encoded by *Plasmodium* and shuttled to the host cell membrane. Classification of these membrane channels is essential before pharmacological antagonists can be developed. Additionally, it is unclear how *Plasmodium* prevents premature cell death of erythrocytes, which is one of the expected consequences of parasite-induced new permeability pathways. Specifically, osmolyte influx via these pathways leads to a breakdown of plasma membrane asymmetry (Brand et al. 2003), which in normal erythrocytes triggers apoptosis (Lang et al. 2003). Obviously, it is crucial to understand the mechanisms by which *Plasmodium* avoids programmed cell death if we are to formulate ways to initiate parasite destruction. One possibility is that *Plasmodium* activates osmolyte pathways that allow for nutrient influx, while simultaneously inhibiting efflux pathways normally stimulated during apoptosis that allow for a decrease in erythrocyte volume. Research aimed at assessing this possibility is severely lacking.

Specific Aims

The purpose of this research effort is to find evidence to support the hypothesis that *Plasmodium* activates osmolyte influx pathways typically inactive in erythrocytes while simultaneously inhibiting efflux pathways normally stimulated during apoptosis. To this end, the individual goals of this study are 1) to discern the origin of the new permeability pathways induced by *Plasmodium* on human erythrocytes, and 2) to identify whether deficient apoptosis in *Plasmodium*-infected cells results from inhibition of endogenous efflux pathways. The origin of the new permeability pathways expressed in infected erythrocytes will be assessed through electrophysiological studies comparing plasma membrane permeability of infected erythrocytes with that of uninfected erythrocytes. To identify whether *Plasmodium* infection results in inhibition of endogenous osmolyte efflux pathways, cell volume studies will be performed using electronic sizing to characterize the responses of uninfected and infected erythrocytes to varying extracellular media.

Experimental Proposal

Discerning the Origin of Parasite-Induced Permeability Pathways: Electrophysiology Studies

The results of previous studies suggest that erythrocytes incur oxidative stress as a result of *Plasmodium* infection (Brand et al. 2003, Tanneur et al. 2005). One possibility not yet fully examined is that oxidative stress, in turn, activates endogenous channels in the host cell membrane, and that these channels are the new permeability pathways previously observed in infected cells. To test this hypothesis, the effects of oxidation on uninfected erythrocytes will be determined using electrophysiology. Presumably, if the new permeability pathways present in infected erythrocytes are indeed endogenous channels activated by oxidation, then treating uninfected erythrocytes with oxidizing agents should result in activation of permeability pathways *identical* to those observed in *Plasmodium*-infected cells, even in the absence of parasite infection. In contrast, if activation of new permeability pathways is *not* the result of oxidation, or if the ion channels activated by *Plasmodium* are xenoproteins, oxidation of uninfected cells should *not* result in activation of permeability pathways similar to those observed in infected cells.

To determine which of the above scenarios is correct, comparative studies between uninfected, oxidized erythrocytes and *Plasmodium*-infected erythrocytes will be performed by monitoring ion channel activity in the cell membranes of each cell type. This involves using a glass pipette tip attached to the cell membrane; a microelectrode present within the pipette detects current (ions) flowing through the membrane protein channels (Peterson et. al., 1986). By exposing cells to varying extracellular solutions, it is possible to discern the selectivity of the ion channels (i.e., what ions they allow to traverse the membrane) and, in turn, characterize permeability pathways active in cells.

These patch-clamp experiments will be performed on control cells (uninfected human erythrocytes), *Plasmodium*-infected cells, and oxidized,

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uninfected cells. Infected cells will be prepared by growing *Plasmodium falciparum* in complete liquid medium, and then the culture will be used to infect human erythrocytes (the technique used will be adapted from Trager et. al., 1997). Oxidized, uninfected cells will be prepared by treating human erythrocytes with the oxidizing agent *tert*-butylhydroperoxide (tBHP), as described by Brand et al. (2003). Patch clamp recordings of each cell type will then be obtained by bathing cells in a sodium chloride solution (control solution that mimics blood plasma), a medium in which chloride is replaced by the impermeable anion gluconate (to determine the degree to which each cell type is permeable to chloride), a medium in which sodium is replaced by the impermeable cation NMDG (to determine whether any component of the current carried across the membrane is carried by sodium), and a medium in which calcium is chelated by EGTA (to determine the degree in which each cell type allows for calcium influx).

It is expected that current recordings in all test solutions will be low for control cells, as uninfected erythrocytes have a low resting ion conductance (Huber et al. 2004). In contrast, it has previously been shown that *Plasmodium*-infected erythrocytes have a very high chloride conductance and modest cation conductance compared to uninfected cells (Brand et al. 2003). Therefore, high current recordings should be seen in the sodium chloride solution, whereas reduced currents are expected to be seen in the chloride-free, sodium-free, and low-calcium media. In all solutions, however, currents from infected cells should be greater than those observed in control cells. Lastly, it is expected that current recordings from oxidized, uninfected cells should match those of infected cells, if the hypothesis that oxidation results in activation of endogenous permeability pathways in the host cell membrane *is* indeed correct. If unexpected results are obtained and parallels are not observed, this would imply that the method used by *Plasmodium* to stimulate new ion conductance is something *other* than oxidation, or that the ion channels activated by *Plasmodium* are foreign, not endogenous. In order to verify such a result, however, alternate concentrations of the oxidizing agent tBHP will be tested and other oxidizing agents will be used to rule out the possibility that non-specific effects are occurring. That is, a small degree of oxidation might activate ion channel activity, whereas a pharmacological increase in oxidation might have an inhibitory effect.

It should be noted that there are limitations to what information can be drawn from the above experiment. One cannot conclude definitively that if oxidation of uninfected cells does not induce ion channel activity identical to infected cells that the mechanism responsible for activating new permeability pathways is not *Plasmodium*-induced oxidation. To explain, the oxidative events characteristic of *Plasmodium* infection might be different than those triggered by pharmacological agents i*n vitro*. Additionally, it is possible that although oxidation might have a role in activating new permeability pathways, other factors, such as parasite-derived enzymes, might also be involved. If this is the case, then the absence of these enzymes in uninfected cells would prevent expression of novel permeability pathways, even in the presence of oxidation.

Determining if Plasmodium Inhibits Apoptotic Volume Decrease: Cell Volume Studies

It is well known that apoptosis, or programmed cell death, occurs by activation of osmolyte efflux pathways that result in volume decrease (Lang et al. 2003, Okada et. al., 2001). This, in turn, reduces cells to a size that is easily engulfable by phagocytic cells. One cellular signal known to trigger apoptosis is a breakdown of membrane asymmetry, which occurs following translocation of phosphatidyl serine from the inner leaflet of the plasma membrane, where it is predominantly if not exclusively localized under normal conditions, to the outer leaflet of the membrane (Lang et al. 2003). This translocation event has been shown to occur in *Plasmodium*-infected erythrocytes. However, unlike in uninfected cells, it does not trigger apoptosis (Brand et al. 2003). A possible explanation for this observation is that *Plasmodium* inhibits membrane channels crucial for osmolyte efflux and subsequent apoptosis.

To test this possibility, control cells and *Plasmodium*-infected cells (prepared as described above) will be exposed to an extracellular solution that should stimulate volume regulatory efflux pathways similar to those activated during programmed cell death. Specifically, each cell type will be exposed to an isosmotic (control) solution and a hypotonic solution, and the effects of this exposure on cell volume will be monitored using a Coulter Counter. This machine electronically sizes and counts cells based on the change in resistance that occurs as cells pass through an aperture opening. Cell volume is directly proportional to this change in resistance (www.beckman.com). By monitoring the reduction in cell size following exposure to varying experimental media, it will be possible to discern whether osmolyte efflux pathways involved in volume decrease are active in *Plasmodium*-infected cells.

It is expected that exposure to a dilute medium will result in cell swelling of both control and *Plasmodium*-infected cells due to the unavoidable influx of water. Control cells, which are able to counteract cell swelling by activating efflux pathways (Okada et. al., 2001), should gradually recover from swelling and approach steady-state cell size. However, if the efflux pathways necessary for this recovery are inhibited by *Plasmodium* as hypothesized above, then infected cells should lack the compensatory mechanism that offsets cell swelling and should remain swollen. It is also possible that *Plasmodium* cells lyse in hypotonic solution due to dramatic cell swelling. Again, this would indicate that the efflux pathways required for volume regulation are inactive. Alternatively, if *Plasmodium* infection does *not* correspond with inhibited efflux pathways, then the response of infected cells to hypotonic challenge should match that of uninfected cells (i.e., cell volume recovery should proceed). If this turns out to be the case, then one could conclude that *Plasmodium* does *not* bypass apoptosis by inactivating efflux pathways. Therefore, other potential mechanisms by which *Plasmodium*-infected cells avoid premature destruction will be examined. For example, phosphatidyl serine translocation might actually *assist* in *Plasmodium* infection if it allows *Plasmodium*-infected erythrocytes to cytoadhere more effectively to endothelial cells and thus evade the host immune system, in particularly the spleen. This could be assessed using cytoadhesion, flow-based assays such

as those described by Cooke et. al. (1995) and Cooke et al. (1995).

Although the cell volume study described above will provide insight into how *Plasmodium* prevents the erythrocyte death that typically accompanies phosphatidyl serine translocation, it is limited in some respects. Specifically, apoptotic events can be simulated by activating volume regulatory mechanisms because the efflux pathways involved in apoptosis are believed to be identical to those involved in regulatory volume decrease. However, the exact mechanics of apoptotic volume decrease are not completely understood and may vary slightly from other cell volume regulatory processes. Additionally, it is possible that *Plasmodium* inactivates efflux pathways normally active in erythrocytes, while activating *other* efflux pathways (such as those that would allow for waste elimination). Clearly, if these *Plasmodium*-induced efflux pathways could somehow be recruited during volume recovery, this would confound the results of cell volume studies. Therefore, although the findings of this experiment can be applied to apoptotic events, such applications must be done with some degree of skepticism.

Conclusion

Studies aimed at characterizing the alterations in erythrocyte membrane properties induced by *Plasmodium* infection and the mechanisms by which *Plasmodium* compensates for the adverse consequences of these alterations are beneficial to the field of malaria research. They will assist in elucidating the underlying processes involved in growth and survival of *Plasmodium* during blood stage infection, which are a crucial aspect of malaria pathophysiology. Further, more complete knowledge of how *Plasmodium* evades the host immune response and apoptosis will facilitate our ability to recognize, track, and prevent malaria. And, most importantly, this information could be applied when developing novel mechanisms for malaria treatment that bypass current limitations in the field.

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