A Surface Phospholipase Is Involved in the Migration of Plasmodium Sporozoites through Cells*

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Plasmodium sporozoites, injected by mosquitoes into the skin of the host, traverse cells during their migration to hepatocytes where they continue their life cycle. The mechanisms used by the parasite to rupture the plasma membrane of the host cells are not known. Here we report the presence of a phospholipase on the surface of Plasmodium berghei sporozoites (P. berghei phospholipase; Pb PL) and demonstrate that it is involved in the establishment of a malaria infection in vivo. Pb PL is highly conserved among the Plasmodium species. The protein is about 750 amino acids, with a predicted signal sequence and a carboxyl terminus that is 32% identical to the vertebrate lecithin:cholesterol acyltransferase, a secreted phospholipase. Pb PL contains a motif characteristic of lipases and a catalytic triad of a serine, aspartate, and histidine that is found in several phospholipases. We have verified its lipase and membrane lytic activity in vitro, using recombinant baculovirus-expressed protein. To study its role in vivo, we have disrupted the P. berghei PL open reading frame and generated mutants in its active site. During an infection through mosquito bite, the infectivity of the knock-out parasites in the liver is decreased by ~90%. The prepatent period of the resulting blood infection is 1 day longer as compared with wild type. Further, the mutant sporozoites are impaired in their ability to cross epithelial cell layers. Thus, the Pb PL functions as a lipase to damage cell membranes and facilitates sporozoite passage through cells during their migration from the skin to the bloodstream.

Malaria is caused by protozoan parasites of the Plasmodium genus that are transmitted to humans through the bite of an infected Anopheles mosquito. As the mosquito probes for a blood vessel during its bite, it deposits parasite forms termed sporozoites underneath the skin (1). Sporozoites stay at the site of bite for at least 30 min (2), during which time they migrate through cells of the avascular tissue before they are able to invade the dermal blood vessels (3). After a short time in circulation and arrest in the liver sinoids, the sporozoites traverse Kupffer cells (4), the resident macrophages of the liver sinusoidal lining, and infect hepatocytes. This is an active process that probably involves the formation of a moving junction between the parasite and host cell membrane (5). This leads to the formation of a parasitophorous vacuole, in which the sporozoite develops into exoerythrocytic forms (EEFs). In contrast, sporozoite migration through tissue layers involves the breaching and subsequent repair of the cell plasma membrane (6). The mechanisms used to rupture the cell membrane are unknown.

In this paper, we characterize a protein, Plasmodium berghei phospholipase (Pb PL), that plays a crucial role in this process, during the migration of sporozoites from the site of mosquito bite in the skin into the bloodstream. Pb PL was found in a suppressive, subtractive hybridization scheme (7) that identified genes specifically up-regulated in salivary gland sporozoites that are highly infectious to the vertebrate host, compared with midgut sporozoites that are virtually noninfectious to the vertebrate host (8).

MATERIALS AND METHODS

Parasite Maintenance—Anopheles stephensi mosquitoes were fed on infected Swiss-Webster mice, and sporozoites were dissected from their salivary glands at days 18–21 postfeeding. Dissections of mosquito salivary glands were performed in RPMI medium containing 3% bovine serum albumin. The glands were mechanically disrupted, and the debris was pelleted after a spin at 500 rpm. Infectivity of salivary glands was similar for wild type, knock-out, and mutant parasites at ~4000 sporozoites/mosquito.

Construction of the Pb PL Gene Disruption and Modification Plasmid—For the gene replacement vector, two fragments of the Pb PL gene were ampliﬁed using PCR and cloned into either side of a dihydrofolate reductase/thymidylate synthase-green fluorescent protein cassette present in the vector pMD205-GFP, described previously as pPyrFlu (9). The 5' fragment was amplified using 5'-CTCGAGATAAGCTTAACCGCAAATAAACAC and 5'-CTCGAGACATTGAAAAATACCCTGGC. Both oligonucleotides contained sites for XhoI (in boldface type), allowing the product to be cloned into the XhoI site of the vector. The 3' fragment was amplified using 5'-GGATCCTTAATGACATTTTGCTG and 5'-ACTAGTGAATAGTGCGTCTG. These oligonucleotides contained sites for BamHI (in boldface type) and SpeI (in boldface type) respectively, allowing the product to be cloned into BamHI and SpeI sites of the vector. The insert was released using KpnI and SacII, for replacement of the genomic locus, thus generating a “knock-out” parasite.

For the gene modification vector, full-length Pb PL was amplified from P. berghei wild type genomic DNA using the following primers that contain sites (in boldface type) for NotI and SpeI respectively: 5'-ATAAGATGCGGGCGCTTAAAATATGTTGAAAAATTCATG and 5'-AACTAGTATGGCAAGCATTTCTCTTAAATATACTATTATAACTGTTGAGATTTTTGGCTTATT. The product was cloned into a described previously targeting vector pMD 205 (10) using NotI and SpeI. The active site amino acids (Ser495, Asp700, and His728) were mutated to alanine using the QuikChange site-directed mutagenesis kit (Stratagene), following the manufacturer’s protocol. Each mutation was

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1 The abbreviations used are: EEF, exoerythrocytic form; Pb PL, Plasmodium berghei phospholipase; ORF, open reading frame; PLA2, phospholipase A2; PC, phosphatidylcholine; MDCK, Madin-Darby canine kidney; WT, wild type; LCAT, lecithin:cholesterol acyltransferase; KO, knock-out.
marked by a unique restriction enzyme site. We also introduced a stop codon at amino acid 5 of the PL gene. The resulting plasmid was used for parasite transfection after linearization with 

**Reverse Transcription—**RNA was extracted from 1 million sporozoites using the micro-FAST-TRACK kit (Invitrogen). Equal amounts were reverse transcribed either in the presence or absence of reverse transcriptase using the Advantage reverse transcriptase-PCR kit (Stratagene). The cDNA obtained was used as template in PCRs to amplify circumsporozoite (CS) and Pb PL using gene-specific oligonucleotides. For the CS gene, the following primers were used: CSFor, 5'-CTTATATCCAAAGCCGCGC; CSRrev, 3'-ACATTTACCTTTTATAC. For the PL gene, the following primers were used: PB62, 5'-CTGCAGATTAGAAAAATACCGTTCC; PB75, 5'-AATCTAGTATCGAAGCTTCTTGT.

**Parasite Transfection and Selection—**P. berghei transfection and selection of clones was performed as described previously (11). In order to differentiate between the Pb PL modified parasite clones that had undergone insertion and carried the mutation and those in which the mutation had been corrected, we enabled the mapping encompassing the Pb PL open reading frame (ORF) from mutant parasites and subjected the PCR product to restriction digestion with the introduced enzymes (data not shown). The presence of the amino acid substitutions was confirmed by sequencing of the modified Pb PL fragment.

**Antibody Preparation and Indirect Immunofluorescence Assay—**Antibodies were raised in mice against a gluthathione S-transferase-Pb PL fusion protein. DNA encoding amino acids 540–610 of Pb PL were amplified using the following oligonucleotides: PB110, 5'-TCCCCCGGGTAAATTATTACC; PB111, 5'-CGCGCTGGATGCTTGGAGCAAGTTC. The product was cloned upstream of gluthathione S-transferase vector (Amersham Pharmacia Biotech, UK). The recombinant protein was purified on an SDS-polyacrylamide gel and used for immunization of Balb/c mice. Salivary gland sporozoites were air-dried and fixed in 4% formaldehyde for 10 min at room temperature. Sporozoites were stained with anti-Pb PL antisera at a dilution of 1:200 at 37 °C for 1 h, followed by incubation at 37 °C for 1 h with Alexa Fluor® 488-conjugated anti-mouse secondary antibody. Cryoimmunoelectron Microscopy—Salivary glands from P. berghei-infected A. stephensi mosquitoes were fixed for 2 days at 4 °C in 0.2% paraformaldehyde in 0.1 M HEPES, pH 7.4, infiltrated, frozen, and sectioned as described previously (12). The sections were labeled first with mouse anti-Pb PL antibodies (1:50 in PBS, 1% fish skin gelatin) (Department of Cell Biology, Medical School, Utrecht University, Utrecht, The Netherlands), before examination with a Philips CM 120 electron microscope (Eindhoven, The Netherlands) under 80 kV.

**Expression of Pb PL in the Baculovirus System—**PyPL was amplified using the following oligonucleotides, which contain sites in (boldface type) for BamHI and XbaI, respectively, and introduce a His6 tag at the carboxyl terminus of the recombinant protein: PB112, 5'-GGAGATTATACGC; PB113, 5'-TCTTAGATCCATTAATTATAC and 5'-GGAGATTATACGC. The PCR product was cloned into pAcGP67A baculovirus transfer vector (BD Pharmigen) using BamHI and XhoI. SF9 insect cells were co-infected with the PyPL transfer vector and linearized baculovirus genomic DNA, following the manufacturer’s protocol (BD Pharmigen). After initial passages in SF21 cells, further infection was carried out in High Five cells. The supernatant containing recombinant protein was harvested 129 h postinfection. The supernatant was centrifuged to remove cell debris and used for further analysis. Expression of PyPL-His (6×) in the supernatant was confirmed through Western blotting using an anti-His monoclonal antibody (data not shown).

**Enzymatic Assay for Phospholipase A2 (PLA2) and Hemolysis Activity—**The assay for phospholipase activity is based on the method described for human lecithin:cholesterol acyltransferase (LCAT) (13). In mammals, LCAT is a serum enzyme that acts as a cholesterol esterifying enzyme. Phosphatidylcholine was used as substrate in the lipase reaction. The resulting species, lyso-PC, free fatty acid, and unphosphorylated PC, were separated by thin layer chromatography and measured by liquid scintillation counting. For the assay of PLA2, 50 μg of enzyme were incubated with 10 μg of phosphatidylcholine in 1 ml of reaction mixture containing 50 μM calcium. The reaction was started by the addition of 0.1 M MgCl2. At the time of measurement, 20 μl of reaction mixture was spotted on a silica gel plate and developed in a solvent system consisting of chloroform/methanol/acetic acid/water (60:38:3:1). The reaction was performed at 37 °C for 3 h. The product, lyso-PC, was identified by comparison with authentic standards.

**RESULTS**

**Identification of the P. berghei PL Gene—**Pb PL (previously termed UIS10) was identified in a subtractive hybridization screen for genes expressed preferentially in P. berghei salivary gland sporozoites (7). Such genes are expected to be important for invasion of the mammalian host, since their expression is up-regulated in the highly infectious salivary gland sporozoites but not in the noninfectious midgut sporozoites.

BLAST homology searches with Pb PL of PlasmodiumDB and the Malaria Genome Project (available on the World Wide Web at www.sanger.ac.uk/Projects/P_berghei) revealed homologs in Plasmodium yoelii, a primate parasite Plasmodium knowlesi, and the human parasite Plasmodium falciparum (Fig. 1A). A search of the GenBank™ nonredundant data base revealed that the Plasmodium PL is most similar to mammalian LCAT, a member of the PLA₂ family of serine lipases.

Pb PL encodes a protein of 755 amino acids with a predicted signal sequence. The carboxyl terminus of Pb PL (amino acids 331–755) is 32% identical and 55% similar to human LCAT (Fig. 1A). The region of identity includes the GXSGX motif that is found in all serine lipases and the catalytic triad of a serine, histidine, and aspartate that is found in phospholipases such as LCAT (19). In mammals, LCAT is a serum enzyme that...
FIG. 1 Alignment of amino acid sequences of PLs from *P. berghei* (Pb PL) (Pb_303f12qlc in www.plasmoDB.org), *P. yoelii* (Py PL) (MALPY01249 in www.plasmoDB.org), *P. falciparum* (Pf PL) (GenBank™ accession number CAG25105), *P. knowlesi* (Pk PL) (pkn504e06qlc in www.sanger.ac.uk), and human LCAT (GenBank™ accession number P04180). Identical residues are boxed and shown in dark shading, whereas similar residues are shown in light shading. The carboxyl terminus (amino acids 331–755) of *P. berghei* PL is 32% identical and 55% similar to human LCAT. The conservation includes the GXSXG motif (line above) that is found in all lipases. In addition, Pb PL also contains the Asp (D) and His (H) amino acids (asterisks) that together with the Ser (S) form the active site in LCAT. In contrast to conservation of the carboxyl termini, the amino termini (amino acids 31–330) of PL molecules are much more divergent. Amino acids 1–30 are predicted to be signal peptide.
catalyzes a two-step reaction in which it first acts as a lipase to hydrolyze a fatty acid from the sn-2-position of PC and then transfers it to a cholesterol moiety to form a cholesterol ester. In contrast to the carboxyl terminus, the amino terminus of Pb PL (amino acids 1–330) is not homologous to any other protein in the data base. In fact, even in various Plasmodium species, there is much less conservation among the amino termini compared with the carboxyl termini (Fig. 1).

**Pb PL Is Expressed on the Surface of Sporozoites**—Expression and subcellular localization of Pb PL was investigated using antisera directed against the carboxyl terminus of Pb PL. Indirect immunofluorescence microscopy shows that Pb PL is present in sporozoites. The corresponding phase-contrast (middle panel) and overlay images (bottom panel) are shown. Sporozoites were stained with primary antibodies against Pb PL followed by staining with Alexa Fluor 488-conjugated secondary antibody. Cryoimmunoelectron microscopy localizes Pb PL to the surface of sporozoites in wild type sporozoites (top panel). This staining is absent in Pb PL knock-out sporozoites (bottom panel).

**Heterologously Expressed Pb PL Has Phospholipase and Membrane Lytic Activity in Vitro**—The conservation of the GXSXG motif and the SDH catalytic triad and similarity with LCAT suggested that Plasmodium PL may have a phospholipase A₄-like activity. In order to test this hypothesis, we expressed Pb PL in a baculovirus expression system. Supernatant from tissue culture cells infected with the Pb PL baculovirus was tested for activity in an assay using labeled PC as substrate. As a positive control, we used purified, recombinant human LCAT (a kind gift from Dr. John S. Parks), which hydrolyzed ~43.5% of free fatty acid in 1 h, producing a specific activity of 2.25 nmol of free fatty acid hydrolyzed/h/mg (data not shown), which is in close agreement with values reported previously (20). Heterologously expressed Pb PL hydrolyzes about 5% of fatty acid in 3 h, demonstrating phospholipase activity of about 3.6 nmol of free fatty acid hydrolyzed/h/ml (Fig. 3A) in a time-dependent manner (Fig. 3B). Pb PL activity appears to be calcium-independent, since it is unaffected by the presence of the Ca²⁺ chelator, EGTA, in the medium (data not shown). As a negative control, we used supernatant from cells infected with baculovirus expressing the kinase domain of the insulin receptor (14), which shows minimal lipase activity.

The product of PC lysis, lyso-PC, possesses membrane lytic activity (21). Therefore, we used a hemolytic assay to test whether Pb PL can disrupt cell membranes. As shown in Fig. 4, heterologously produced Pb PL leads to hemolysis (Fig. 4A) in a concentration-dependent manner (Fig. 4B) and time-dependent manner (Fig. 4C), whereas the control supernatant shows little...
hemolysis, indicating that Pb PL can act as a phospholipase and cause lysis of cell membranes.

Disruption and Modification of the Pb PL Locus—To study the function of Pb PL in vivo, we created two cloned lines of transgenic parasites. The first was a null mutant (KO) in which the Pb PL ORF was disrupted through the insertion of a dihydrofolate reductase/thymidylate synthase gene cassette (Fig. 5A). In the null mutant, gene disruption was verified using Southern hybridization of BamHI and SphI-digested genomic DNA, using the Pb PL ORF as probe. As expected, the Pb PL probe gave rise to a 2.5-kb band in the WT population but a 0.5-kb band in the KO line (Fig. 5B). To confirm the loss of Pb PL expression, we reverse transcribed first strand cDNA from Pb PL KO sporozoites and used it as template for amplification of Pb PL message. The lack of a product indicates the absence of Pb PL RNA (Fig. 5C). As a control for the integrity of the cDNA template, we used it to amplify a product from another sporozoite gene, the CS gene. The loss of protein expression was confirmed by a loss of staining in indirect immunofluorescence and cryoimmuno-EM in KO sporozoites as compared with wild type ones (Fig. 2A).

In the second mutant line (Mut), the putative catalytic site was mutated (S495A, D700A, and H728A) through the insertion of a mutant Pb PL gene into the wild type genomic locus (Fig. 6A). The generation of the mutant line was verified through Southern hybridization analysis of NsiI and XbaI-digested genomic DNA. The probe (Pb PL ORF) produced a single 2.9-kb band in WT and two bands (6.9 and 2.5 kb) in the Mut line (Fig. 6B). The Mut line was confirmed to have Pb PL message through gene-specific PCR amplification using reverse transcribed cDNA from Pb PL Mut sporozoites as template (Fig. 6C). Mut sporozoites displayed normal expression of Pb PL (Fig. 6D).

Pb PL KO and Mut Sporozoites Have Reduced Infectivity—Asexual stages of Pb PL KO and Mut parasites are viable, morphologically indistinguishable, and developmentally similar in to wild type parasites. Sporozoites from KO and Mut parasites formed in oocysts and invaded mosquito salivary glands in numbers similar to WT. They also displayed normal gliding motility. To investigate the effects of the KO on the development of EEFs, we infected a human hepatocyte cell line, HepG2, with equal numbers of KO and WT sporozoites. KO parasites developed into EEFs that were similar in number and gross morphology to WT EEFs (data not shown). Therefore, we conclude that KO sporozoites undergo normal development in the hepatocytes.

To test the infectivity of KO parasites to the mammalian host, we infected groups of rats with sporozoites via either intravenous injection or bites of mosquitoes that were equally infected with either wild type or KO parasites. When infected by bite, the KO parasites were detected in blood 1 day later...
Fig. 5. A, replacement of the Pb PL locus by a double crossover event. Schematic representations of the Pb PL genomic locus, integration plasmid, and the resulting disrupted locus are shown. DHFR, dihydrofolate reductase; GFP, green fluorescent protein. B, genomic Southern blot hybridization of WT and the knock-out (Pb PL KO) parasites is shown. The probe used for hybridization is represented by a red line in A. The full-length PL ORF was used as a probe. Integration of the targeting plasmid causes reduction in size of a 2.5-kb fragment in WT parasites to a 0.5-kb fragment in the KO parasites. Results from three KO clones are shown. C, there is complete loss of PL message in the KO parasites. The PCR product amplified from Pb PL is represented by the green line in A. RNA from PL KO sporozoites was reverse transcribed into cDNA, either in the presence (+) or absence (−) of reverse transcriptase (RT). It was then used as template to amplify PL and CS messages. For both sets of amplification reactions, water was used as negative (−ve) control, and wild type P. berghei genomic DNA (gDNA) was used as positive control for the PCR. D, indirect immunofluorescence (IFA) confirms the loss of Pb PL protein expression in mutant sporozoites. The corresponding phase-contrast (middle panel) and overlay images (bottom panel) are shown.

than WT parasites (Fig. 7A). Similar results were obtained in two independent experiments. In contrast to a bite infection, there was no difference in the prepatent period of KO and WT parasites when the rats were infected by intravenous injection. Identical results were obtained for the Mut line, indicating that the phenotypic effect of the KO line is a result of the loss of enzymatic activity of Pb PL.

To further quantify the difference in infectivity in the KO and WT parasites after a bite infection, we used real time PCR to measure parasite load in the liver by determining the levels of the parasite-specific 18 S rRNA (17). In mice infected with KO sporozoites, there was a decrease of ~90% in parasite load in the liver, compared with mice infected with WT sporozoites (Fig. 7B). The parasite loads in the liver were not significantly different when the animals were infected through an intravenous injection (Fig. 7C). This is in good agreement with the lack of difference in prepatent periods of blood stage infections.

Pb PL KO Sporozoites Have Reduced Ability to Traverse Cells—We hypothesized that the decrease in liver infectivity and increase in prepatent period of the KO parasites, infected through mosquito bite, was most likely a result of impairment in the ability of sporozoites to reach the liver. In order to test the migratory ability of KO sporozoites, we used an assay in which sporozoites have to traverse an epithelial cell barrier formed by a confluent monolayer of Mardin-Darby canine kidney (MDCK) cells. MDCK cells are plated in the top chamber of a Transwell filter system, whereas HepG2 cells are plated in the bottom chamber (6). Sporozoites are then added to the top chamber. Since confluent MDCK cells form very tight intercellular junctions, the number of EEFs formed in the HepG2 cell layer is proportional to the numbers of sporozoites that are able to traverse through the MDCK cell layer and is a reflection of the ability of sporozoites to cross cell barriers. As shown in Fig. 8A, KO sporozoites formed about 90% fewer EEFs compared with wild type sporozoites, suggesting that they are impaired in their ability to traverse cell layers.

DISCUSSION

The process of infection by Plasmodium sporozoites is poorly understood. Imaging of live sporozoites deposited after a mosquito bite (3) and other experimental approaches (1, 2) reveal that a majority of parasites are placed in the dermal tissue or in a pool of blood that forms while mosquitoes probe the skin vasculature. From there, the sporozoites have to make their way into blood vessels, a process that takes at least 30 min and requires the passage of parasites through skin cells.

In this paper, we present evidence that Pb PL plays an important role in this migration of sporozoites from the skin to the bloodstream. We hypothesize that Pb PL acts as a phospholipase on the surface of sporozoites that hydrolyzes PC (and possibly other phospholipids) present in host cell membranes and thus wounds the cell membrane and allows access into the cell to the migrating sporozoite. Pb PL action facilitates the passage of sporozoites through cells and enables Plasmodium infection in mammalian hosts.

This conclusion is based on genetic and biochemical lines of evidence. Disruption of the open reading frame or mutations in the catalytic site of the protein lead to a profound decrease in parasite infectivity when the parasites are delivered to the skin through mosquito bite. This is evidenced both by a sharp decrease in liver infection and an increase in prepatent period. The phenotype is most likely due to a defect in transmigration, which is observed, in vitro, by the decrease in the number of EEFs formed when Pb PL KO sporozoites, compared with wild type, have to cross epithelial cell layers. Inability to damage cell membranes and the resulting impairment in traversing cells is the most likely cause of the significant decrease in liver infection and the delay in the prepatent period of blood infection of Pb PL KO parasites.
Pb PL has lipase and membrane lytic activity. Given the sequence homology to LCAT in its carboxyl terminus, it is likely that Pb PL belongs to the PLA2 superfamily of phospholipases. Phosphatidylcholine is the favored substrate of phospholipases such as LCAT and is also the major phospholipid in mammalian cell membranes. Its hydrolysis by PLA2 results in

Fig. 6. A, modification of the Pb PL locus by a single crossover event. Schematic representations of the Pb PL genomic locus, integration plasmid, and the resulting modified locus are shown. The modified locus contains two copies of the PL locus. The first copy, under the control of the endogenous regulatory 5' untranslated region of Pb PL, expresses full-length protein whose active site has been mutated. The second copy is not expressed, since it is missing the 5'-untranslated region and contains a stop codon at the 5'-end. DHFR, dihydrofolate reductase. B, in a genomic Southern hybridization using Pb PL ORF as probe, the modified locus gives rise to two hybridizing fragments, 6.9 and 2.5 kb, whereas the WT locus gives rise to a single one of 2.9 kb. C, the expression of the modified PL gene in the mutant clone was verified through reverse transcription-PCR. RNA from PL Mut and WT sporozoites was reverse transcribed into cDNA, either in the presence (+) or absence (−) of reverse transcriptase (RT). It was then used as template to amplify PL and CS messages. For both sets of amplification reactions, water was used as negative (−ve) control, and wild type P. berghei genomic DNA (gDNA) was used as positive control for the PCR. D, indirect immunofluorescence (IFA, left panel) confirms the presence of Pb PL protein in mutant sporozoites. The corresponding phase-contrast (middle panel) and overlay images (right panel) are shown.
the formation of lysophosphatidylcholine, which causes membrane lysis (21) or changes membrane fluidity. This conclusion is supported by the observation that Pb PL makes membranes more fragile, as demonstrated by its hemolytic activity. The presence of Pb PL homologs in various Plasmodium species suggests that its function is conserved.

Pb PL contains a signal sequence at its amino terminus. Although the mechanisms that anchor Pb PL to the sporozoite surface are unknown, Pb PL, similar to other lipases, contains hydrophobic regions, which could interact with lipid bilayers. The amino-terminal portion of Pb PL does not resemble any other protein in the data base. In fact, the amino termini of phospholipase molecules from various Plasmodium species show much less homology to one another compared with the highly conserved carboxyl termini. It is possible that they serve as prosequences that are proteolytically cleaved during protein processing.

Membrane lytic activity has been observed in the pathogenesis of many microbial infections. In bacteria such as Pseudomonas aeruginosa, secreted lipolytic lipases are important virulence factors. Strains expressing the enzymes have greater cytotoxic effects and disseminate faster in host tissue as compared with strains without such lipases (22). One such lipase, PLA2, encoded by ExoU, has membrane lytic and cytotoxic effects (23, 24). In Helicobacter pylori, a mutant disrupted in the gene encoding PLA2 has decreased hemolytic activity and is impaired in the colonization of the gastric mucosa (25). In protozoa, such as Entamoeba histolytica, Cryptosporidium parvum, and T. gondii, cytotoxic and hemolytic effects have been associated with phospholipase activity. In E. histolytica, loss of virulence is associated with a decrease in PLA2 activity (26). In C. parvum (27) and T. gondii (28–30), a phospholipase might be involved in host cell invasion.

Once in the bloodstream, the sporozoites have to traverse...
through Kupffer cells present in the liver sinusoidal layer in order to infect hepatocytes (4, 31). However, the Pb PL knock-out and mutant parasites are not defective when delivered by intravenous injection, indicating that Pb PL activity is not required to penetrate Kupffer cells. There are two possible explanations for this paradox. One is that Kupffer cell invasion occurs via the formation of a parasitophorous vacuole. Using confocal and electron microscopy, previous studies have found sporozoites encapsulated in a vacuolar membrane, within the Kupffer cell cytoplasm (4). This vacuole is nonacidic in nature and allows sporozoite to survive intact for several hours. Thus, entry of sporozoites into Kupffer cells does not require membrane disruption, and Pb PL would not be expected to play a major role in liver infection. The second possibility is that cell traversal in the liver utilizes molecules and mechanisms other than Pb PL. A recent report implicates a novel protein, SPECT (sporozoite microneme protein essential for cell traversal) in the liver utilizes molecules and mechanisms other than Pb PL (31). SPECT is present in sporozoite micronemes, and spect-disrupted sporozoites are impaired in ability to passage through cells. However, the mechanism of SPECT action is unknown. It is likely that sporozoite entry and exit from cells may well result from the combinatorial action of several molecules acting in concert or succession. Different parasite molecules may be required for the invasion of various target host cells.

Further studies are required to unravel the process of cell wounding by sporozoites. In addition, another intriguing question is the nature of the signals and molecular mechanisms that lead the parasite to switch from the “cell wounding” strategy used to migrate through host tissues to the “vacuole formation” strategy that leads to the formation of the intracellular parasitophorous vacuole, where sporozoites finally transform into EEFS within hepatocytes.

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