Bee Venom Phospholipase A$_2$ Induces Stage-specific Growth Arrest of the Intraerythrocytic Plasmodium falciparum via Modifications of Human Serum Components*

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Phospholipases A$_2$ (sPLA$_2$s) are enzymes that catalyze the hydrolysis of the sn-2 acyl bond of glycerophospholipids to produce free fatty acids and lysophospholipids. They are important for signal transduction processes, general lipid metabolism, and membrane remodeling (for review, see Refs. 1 and 2). From this superfamily of secreted and cytosolic enzymes, classified in 1997 into nine groups (3), the groups I, II, and III are all secreted, low molecular mass (13–18 kDa), Ca$^{2+}$-dependent PLA$_2$s (4). The pancreatic group IB is expressed at high level in the pancreas but has also been detected in various other tissues (5, 6), and apart from its role in the digestion of dietary lipids, it has been implicated in biological activities such as cell proliferation (7), cell migration (8, 9) and eicosanoid release (10). Snake and insect venoms contain a wide variety of secreted PLA$_2$s (sPLA$_2$s), which can be potent toxins exerting deleterious effects such as myotoxicity, neurotoxicity, cardio-toxicity and inflammation (11, 12). Involvement of enzymatic activity in many of these effects is unclear because not all venomous PLA$_2$s display toxic effects, although they all have similar catalytic activities (13). In some cases, like the cytotoxicity of nigexine, a PLA$_2$ from cobra venom (14), or the activation of prostaglandin E$_2$ production in rat mesangial cells by the mammalian pancreatic PLA$_2$ (15), it was demonstrated that catalytic activity and toxicity or biological effects are unrelated. Furthermore, the identification of different membrane proteins that bind secreted PLA$_2$s strongly suggest that these enzymes could behave as ligands for receptors and might be responsible for other physiological functions than the catalytic one (16).

Malaria is a widespread parasitic disease. Its estimated incidence in the world is in the order of 300–500 million clinical cases each year, with mortality estimated to be more than one million (46). The causative agent of malaria is an intracellular protozoan from the genus Plasmodium. Of the four human Plasmodium species, Plasmodium falciparum is the one responsible for most of the severe physiopathology. Increasing resistance of the parasite to classical antimalarial drugs calls for new chemotherapeutic approaches based on a better understanding of Plasmodium biology and its interaction with the host. In humans, part of the P. falciparum life cycle takes place inside the erythrocyte, where it develops through the successive stages ring, trophozoite and schizont, within 48 h. During its intraerythrocytic maturation, the parasite undergoes a large re-organization of the host cell membrane by inserting newly synthesized proteins, forming “knob-like” electron-dense structures (17), and remodeling of the molecular species of phospholipid composition (18).

Secreted PLA$_2$s have been used as tools for studying the phospholipid asymmetry of the Plasmodium-infected erythrocyte membrane (19, 20). Apart from these structural studies, one study reported the toxicity of a chemically modified pig pancreatic PLA$_2$ toward Plasmodium knowlesi-parasitized erythrocytes (21); it was demonstrated that the fatty acylated PLA$_2$ acquired an enhanced penetrative power, leading to the selective elimination of the parasitized erythrocytes, which...
present an altered membrane lipid packing compared with healthy erythrocytes. To our knowledge, no study has investigated the perturbation of *Plasmodium* physiological processes by exogenous PLA₂ activity.

Owing to the many deleterious effects attributed to these enzymes and the variability of their mode of action, we were interested in testing the possibility that externally added PLA₂ might induce specific toxic effects on *P. falciparum*. In this study, we analyzed the toxicity of bee and snake venom enzymes (toxic PLA₂s) and hog and bovine pancreas enzymes (non-toxic PLA₂s) toward *P. falciparum* cultured in vitro. Toxic and non-toxic PLA₂s both killed the intraerythrocytic parasite, but with very different efficiencies, allowing a clear discrimination between the two categories of enzymes. Comparative analysis of the development of a synchronized culture of *Plasmodium* upon addition at different times of bee venom PLA₂, revealed that a low dose of the toxic enzyme is lethal to the young trophozoite stage only, whereas pancreatic PLA₂ kills the parasite at each developmental stage. We demonstrate that these specific effects can be reproduced by using human serum previously incubated with the corresponding PLA₂, and that purified lipoproteins lipolyzed by the bee venom PLA₂ are inhibitory to the parasite growth. Taken together, our results demonstrate that bee PLA₂ and hog PLA₂ modify serum lipoproteins in different ways, leading to the generation of specific lipid products toxic to the intraerythrocytic *Plasmodium* and presumably acting on different targets.

**EXPERIMENTAL PROCEDURES**

Materials—Phospholipases A₂ from *Aegyptiurus halys* venom and from hog pancreas were purchased from Fluka; the PLA₂ from *Crotalus adamanteus* and *Naja mossambica* venoms were from Sigma; the bee venom PLA₂ was purchased from Fluka and from Sigma. *p*-Bromophenacyl bromide (*p*-BPB) and L-α-lectin were purchased from Sigma. [³H]Hypoxanthine (10–30 Ci/mmol) was from ICN Pharmaceuticals, France. Affi-Gel 10 gel was purchased from Bio-Rad.

**Culture of *P. falciparum* and Synchronization Procedure—** *P. falciparum* Colombian strain FcB1 was used in all the experiments and was cultured desynchronized previously (23) in 5% CO₂ in an atmosphere with human red blood cells in complete medium. Complete medium consisted of RPMI 1640 (Life Technologies, Inc.) containing 25 mM Hepes, supplemented with 11 mM NaHCO₃, 100 mM penicillin, 100 μg/ml streptomycin, and 7% (v/v) compatible heat-inactivated human serum. The parasite cell culture was synchronized by Plasmagel (23) and sorbitol (24) treatments. Under our culture conditions, the life cycle of the parasite was 48 h (5). The density of the culture and appropriate pH.

**Determination of PLA₂ Activity—** Enzymatic assays on mixed micelles of phosphatidylcholine and Triton X-100 were performed according to Lobo de Araujo and Radvanyi (25). Briefly, the substrate solution was prepared by stirring 3.5 mM L-α-lecithin from egg yolk in 7 mM Triton X-100, 100 mM NaCl, 10 mM CaCl₂, and 55 μM phenol red; the pH of the solution was adjusted to 7.5 with 40 mM NaOH so that the absorbance reading at 558 nm was 1.8 to 2. The substrate solution (1 ml) was introduced into the titration cuvette, and the reaction was started by adding the enzyme (resuspended in H₂O at a known concentration) in a volume smaller than 50 μl. The difference in absorbance between the reference (substrate solution alone) and the sample cuvettes was monitored continuously at 558 nm using a Uvikon spectrophotometer (Kontron, Zurich, Switzerland). Under these conditions, a decrease in absorbance of 0.1 corresponds to the release of 0.01 μmol of fatty acid. 1 IU of PLA₂ hydrolyzes 1 μmol of L-α-phosphatidylcholine to L-α-lysophosphatidylcholine and a fatty acid per min at room temperature and appropriate pH.

**Effect of Toxic and Non-toxic PLA₂ on Parasite Viability—** An asynchrony culture of *P. falciparum* at 1–1.5% parasitemia and 4% hematocrit in complete medium was distributed (100 μl/well) in a 96-microwell plate. Increasing concentrations of PLA₂ resuspended in complete medium were added (100 μl/well), and the cells were allowed to grow in a candle jar system. After 24 h in culture, [³H]Hypoxanthine was added (0.5 μCi/well), and after an additional 24-h incubation period, cells were harvested on filters after a freeze-thawing cycle. Dried filters were submerged in a liquid scintillation mixture (OptiScint Hisafe) and counted in a 1450 Microbeta counter (Wallac). Growth inhibition was calculated from the parasite-associated radioactivity (incorporated into nucleic acids) compared with controls. The IC₅₀ was determined according to Desjardins et al. (26) as was the IC₁₀₀, minimum (IC₁₀₀,m = minimum enzyme concentration leading to 100% inhibition) of free PLA₂ in the supernatant using the IC₅₀ to grow in a candle jar system. After 24 h in culture, [³H]hypoxanthine incorporation test.
RESULTS

Comparative Analysis of the Cytotoxicity of Different Secreted PLA2s toward P. falciparum—Two non-toxic, non-inflammatory PLA2s from hog and bovine pancreas (group IB) and four toxic PLA2s of venomous origin, the N. mossambica (group IA), A. halys and C. adamanteus (group IIA), and Apis mellifera (group III), were tested for their capacity to inhibit the intraerythrocytic development of P. falciparum. Asynchronous cultures of the FcB1 strain were grown at 1.5% parasitemia for one cell cycle (48 h) in the presence of various concentrations of PLA2. Parasite growth was checked via nucleic acid synthesis by [3H]hypoxanthine incorporation, and extent of hemolysis was measured by reading absorbance of the supernatants of the culture media at 420 nm. IC50 values obtained for each PLA2 as well as minimum PLA2 concentration leading to detectable hemolysis are given in Table I.

All PLA2s appeared toxic to P. falciparum, with IC50 values much lower than the minimum enzyme concentration required for 1% hemolysis. It should be noted, however, that the amount of released hemoglobin would hardly account for lysis being restricted to infected erythrocytes, owing to the low parasitemia in the test.

PLA2s from venoms appear much more toxic to P. falciparum than pancreatic PLA2s, with low IC50 values ranging from 1.1 pm to 0.2 nM. Values were translated in terms of enzymatic units after the amount of active enzyme present in each PLA2 commercial preparation had been checked by kinetic analysis of mixed micelles (Triton X-100/phosphatidylcholine) hydrolysis. The same differences in magnitude were found (see Table I); IC50 of toxic PLA2s ranged from 0.65 × 10−8 to 19.60 × 10−8 IU/ml, whereas IC50 of non-toxic PLA2s were 1.12 IU/ml (hog) and >0.15 IU/ml (bovine). Several conclusions may be drawn from these data. If added to cell culture, both sets of PLA2 (toxic and non-toxic) are able to stop parasite development; as previously shown (21), PLA2 concentrations effective in killing parasite are clearly much lower than concentrations required to lyse healthy red blood cells; pancreatic PLA2s are very inefficient in parasite killing compared with venom PLA2s. From these results, the bee venom (toxic) and hog pancreas (non-toxic) PLA2s were chosen for further investigations.

Inhibition of Parasite Development Is Dependent upon Enzymatic Activity of PLA2s—Because some situations have been reported where cytotoxicity or biological effects due to secreted PLA2s were independent of catalytic activity, we analyzed the importance of PLA2 enzymatic activity in Plasmodium growth arrest by chemical inhibition of the bee venom and hog pancreas enzymes by p-BPB (27). Reaction of PLA2s with p-BPB has been shown to be active site-directed, leaving the overall conformation of the protein mainly unchanged and free to interact with other molecules. Inhibition of the venom and pancreatic enzyme activities was measured in assays using mixed micelles as substrates. In our hands, PLA2 inhibition by p-BPB never reached 100%, and the best inhibition rates that could be obtained varied from 80 to 95%. Bee venom PLA2 and hog pancreas PLA2 used in the experiment presented in Fig. 1 were inhibited by 95 and 94%, respectively. Analysis of the growth inhibition of the FcB1 strain in the presence of p-BPB-treated enzymes (Fig. 1) shows that their lethal effect depends largely upon enzymatic activity in both cases, since the IC50 of bee PLA2 is increased by more than 2 log units upon enzyme inhibition and the IC50 of hog pancreas PLA2 by more than 1 log unit upon inhibition, even though both enzymes were not totally inactivated. The same results were obtained with different batches of phospholipases A2, making it most unlikely that a p-BPB-sensitive contaminant from the commercial preparation would be responsible for the observed effect. It must be noted that IC50 value of native bee PLA2 in Fig. 1 appeared higher (70 pm) than IC50 value in Table I (1.1 pm), indicating that some enzyme has been lost during the successive steps of the inhibition procedure.

The more effective killing of parasites by toxic PLA2s compared with non-toxic ones led us to question their respective mechanisms of lethal action. One possible explanation was the higher penetration power of toxic PLA2s into a lipid monolayer compared with non-toxic PLA2s. Indeed, the penetration power of PLA2 enzymes depends on lipid pressure of the layer (29), and disorganization of the plasma membrane lipid bilayer in parasitized cells is expressed in part by a decreased lateral surface pressure compared with intact erythrocytes. Moll et al. (21) report the preferential attack of Plasmodium-infected erythrocytes by a modified pig pancreas PLA2 with enhanced penetration power. However, if we hypothesize that killing of parasites strictly depends upon enzyme capacity to penetrate the erythrocyte membrane, we must notice that although all PLA2s tested kill parasites, the number of enzymatic units required to kill is very different between toxic and non-toxic PLA2s, suggesting that the substrate itself, i.e., the infected erythrocyte, might be “seen” differently by the two types of enzymes.

Synchronous Cultures of P. falciparum Are Not Equally Sensitive to Bee Venom and Hog Pancreas PLA2s—First of all, we analyzed the effects of bee venom and hog pancreas PLA2 on the intraerythrocytic cell cycle of P. falciparum. Parasites from the FcB1 strain were synchronized. Cultures of young rings at 1–1.5% parasitemia and 2% hematocrit were distributed into 96-well plates in RPMI containing 7% heat-inactivated human serum. The minimum concentration of PLA2 enzyme leading to 100% inhibition of the parasite growth (IC100m) was determined from curves of growth inhibition plotted as a function of PLA2 concentration (not shown). Bee PLA2 IC100m was set at 30 pm, and hog PLA2 IC100m was set at 500 nm. PLA2s at these concentrations were added to the cell cultures at different times of the 48 h cell cycle, and parasitemia from each well was determined on Giemsa-stained smears after 60 h. 100% re-invasion was determined from control cultures under the same conditions but in the absence of PLA2. Experiments were repeated twice with red blood cells and sera from different donors. Results are presented in Fig. 2. When added to culture medium early in the cell cycle (at the young ring stage), bee PLA2 prevented re-invasion, but when added beyond the 25–29-h period (i.e., the young trophozoite stage), bee PLA2 displayed only a slight inhibitory effect on parasite re-invasion. In contrast, almost no re-invasion was observed upon the addition of hog PLA2 to the culture, regardless of the time of addition, demonstrating that killing potencies of the two PLA2s at IC100m are different. Furthermore, we could notice from the observation of the Giemsa-stained smears that the healthy erythrocytes of the PLA2-treated cultures were shrunk, with an echinocytic shape, compared with the control erythrocytes.

The results obtained with bee venom PLA2 might be explained either by the necessity for a long period of time for the toxic activity to exert its effect (i.e., the longer the time of incubation with PLA2, the more deleterious the effect on cells) or by a specific targeting of the toxic effect to pre-schizogonic forms of the intraerythrocytic Plasmodium, whereas hog pancreas PLA2 would exert its toxic effect on any development stage.

PLA2-modified Culture Medium Is Toxic to Intraerythrocytic P. falciparum—Since in vitro cultivation of intraerythrocytic P. falciparum is performed in the presence of human serum, it was possible that phospholipids from serum lipoproteins might...
be substrates of PLA₂ as well as phospholipids from erythrocyte plasma membrane. To understand the biological mechanisms underlying the respective actions of bee and hog PLA₂s on intraerythrocytic Plasmodium, we checked first if hydrolysis of serum phospholipids might be the source of the observed effects. Bee venom and hog pancreas PLA₂s were coupled to Affi-Gel 10 beads and then tested for enzymatic activity on mixed Triton X-100/phosphatidylcholine micelles. 1% enzym-

![FIG. 1](image1)

Inhibition of the *P. falciparum* growth by native and p-BPP-treated bee and hog PLA₂s. To analyze the involvement of their enzymatic activity in the parasiticial effect of bee and hog PLA₂s, both enzymes were inhibited by treatment with *10⁻⁴ M* p-BPP. The efficiency of the inhibition was assessed by enzymatic assays on Triton X-100/phosphatidylcholine mixed micelles. An asynchronous culture from the FcB1 strain (parasitemia 1–1.5%, hematocrit 2%) was distributed in a 96-microwell plate and grown for 24 h in culture conditions in the presence of increasing concentrations of: 95% inhibited (filled circles) or non-inhibited (open circles) bee PLA₂ and 94% inhibited (filled squares) or non-inhibited (open squares) hog PLA₂. 0.5 μCl of [³H]Hypoxanthine was added per well for an additional 24-h period, then the cells were freeze-thawed, and incorporation of the radioactivity into nucleic acids was measured upon transfer of the macromolecules onto a membrane filter. Filters were counted in a Wallac counter. Parasite growth inhibition (%) was plotted against the concentration of PLA₂ in the culture medium. Values are the mean of three independent experiments.

![FIG. 2](image2)

Comparative analysis of the rates of RBC re-invasion by *P. falciparum* grown in the presence of bee PLA₂ and hog PLA₂. Cells from the *P. falciparum* FcB1 strain were synchronized on a 4-h window and distributed at the young ring stage (3–7 h old) into a 96-well plate at 1–1.5% parasitemia and 2% hematocrit in complete medium. Bee PLA₂ (30 pM final) or hog PLA₂ (500 nM final) were added to wells at time 0 h (3–7-h-old parasites), 3 h (6–10-h-old parasites), 18 h (21–25-h-old parasites), 26 h (29–33-h-old parasites), and 42 h (45–49-h-old parasites). At *t* = 60 h, parasitemia from each well was determined on Giemsa-stained smears. 100% re-invasion was estimated from cells grown in the absence of PLA₂. Cells grown in the presence of bee PLA₂ are shown as black bars, and those grown in the presence of hog PLA₂ are shown as white bars.

<table>
<thead>
<tr>
<th>PLA₂ origin</th>
<th><em>A. mellifera</em> group III</th>
<th><em>N. mossambica</em> group IA</th>
<th><em>A. halys</em> group IIA</th>
<th><em>C. adamanteus</em> group IIA</th>
<th><em>Hog pancreas</em> group IB</th>
<th><em>Bovine pancreas</em> group IB</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IC₅₀</em> (μM)</td>
<td>&gt;1 × 10⁶</td>
<td>1.1</td>
<td>2.2</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>IC₅₀</em> (IU/ml)</td>
<td>&gt;1 × 10⁶</td>
<td>0.65</td>
<td>0.30</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Concentration of PLA₂ inducing 1% hemolysis</td>
<td>&gt;625 nm</td>
<td>&gt;285 nm</td>
<td>&gt;47 nm</td>
<td>≈7 μM</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

![Table 1](image3)

Comparative analysis between the *IC₅₀* values of toxic and non-toxic PLA₂s toward the *P. falciparum* in vitro growth. An asynchronous culture of the FcB1 strain of *P. falciparum* was grown in the presence of increasing concentrations of various snake (from *A. mellifera*, *N. mossambica*, *A. halys*, and *C. adamanteus*) and *Hog* pancreas PLA₂s, both enzymes were inhibited by treatment with *10⁻⁴ M* p-BPP. The efficiency of the inhibition was assessed by enzymatic assays on Triton X-100/phosphatidylcholine mixed micelles. An asynchronous culture from the FcB1 strain (parasitemia 1–1.5%, hematocrit 2%) was distributed in a 96-microwell plate and grown for 24 h in culture conditions in the presence of increasing concentrations of: 95% inhibited (filled circles) or non-inhibited (open circles) bee PLA₂ and 94% inhibited (filled squares) or non-inhibited (open squares) hog PLA₂. 0.5 μCl of [³H]Hypoxanthine was added per well for an additional 24-h period, then the cells were freeze-thawed, and incorporation of the radioactivity into nucleic acids was measured upon transfer of the macromolecules onto a membrane filter. Filters were counted in a Wallac counter. Parasite growth inhibition (%) was plotted against the concentration of PLA₂ in the culture medium. Values are the mean of three independent experiments.
Results confirmed that the modified serum was toxic to Plasmodium, experiments performed in the presence of increasing concentrations of the PLA2-treated medium and grown at 37 °C in culture conditions for 24 h. The initial concentration of [3H]hypoxanthine was added per well, and cells were grown for an additional 24-h period. Parasite growth was assessed by measuring incorporation of radioactivity and plotted against the concentration of the modified medium (here expressed in terms of dilution factor). IC50 values were calculated by extrapolation of the log2 dose-response curves.

Developmental Stages of P. falciparum Are Not Equally Sensitive to Hog and Bee PLA2-Induced Cytotoxicity—We checked whether the particular profile of the inhibitory effect observed on Plasmodium growth in vitro was toxic per se; Plasmodium growth was inhibited to the same extent depending on the concentration of modified serum in the culture medium and despite the presence of 7% intact serum (not shown).

Fig. 3. Inhibition of the P. falciparum growth by bee PLA2- treated serum. Complete culture medium containing 14% human serum was incubated at 37 °C with 130 µM Affi-Gel beads-immobilized bee PLA2, for 1 h (open circles), 3 h (filled circles), 6 h (open squares), and 17 h (filled squares). Asynchronous cells of the P. falciparum FC1 strain were distributed at a 1–1.5% parasitemia and a 2% hemocytometer into a 96-well plate in the presence of increasing concentrations of the PLA2-treated medium and grown at 37 °C in culture conditions for 24 h. The initial concentration of [3H]hypoxanthine was added per well, and cells were grown for an additional 24-h period. Parasite growth was assessed by measuring incorporation of radioactivity and plotted against the concentration of the modified medium (here expressed in terms of dilution factor). IC50 values were calculated by extrapolation of the log2 dose-response curves.

Fig. 3. Inhibition of the P. falciparum growth by bee PLA2-treated serum. Complete culture medium containing 14% human serum was incubated at 37 °C with 130 µM Affi-Gel beads-immobilized bee PLA2, for 1 h (open circles), 3 h (filled circles), 6 h (open squares), and 17 h (filled squares). Asynchronous cells of the P. falciparum FC1 strain were distributed at a 1–1.5% parasitemia and a 2% hemocytometer into a 96-well plate in the presence of increasing concentrations of the PLA2-treated medium and grown at 37 °C in culture conditions for 24 h. The initial concentration of [3H]hypoxanthine was added per well, and cells were grown for an additional 24-h period. Parasite growth was assessed by measuring incorporation of radioactivity and plotted against the concentration of the modified medium (here expressed in terms of dilution factor). IC50 values were calculated by extrapolation of the log2 dose-response curves.

To ascertain if the mode of action of hog PLA2 toward erythrocytes also involved modification of the human serum, 40 nM of enzyme was added to complete culture medium and left at 37 °C for 30 h to allow serum modification. From the previous determination of hog PLA2 IC50 (given in Table I), the 40 nM concentration was chosen from the inhibition curve as the maximum concentration that does not inhibit the growth of asynchronous cells within a 48-h incubation. We reasoned that repetition of the IC50 experiment in the presence of complete medium pre-treated with a harmless amount of enzyme which led to growth arrest would imply that the serum had been modified by the catalytic action of the phospholipase and had become toxic to Plasmodium.

As can be seen in Fig. 4, the presence of hog PLA2-treated culture medium during the same periods of time as bee PLA2-treated medium on the same cultures led in every case to negligible re-invasion rates. Giemsa-stained smears at different times of the experiment revealed numerous lysed infected red blood cells, which were not observed upon the addition of bee PLA2-treated medium, suggesting that toxicity of the hog PLA2-modified medium would lead to selective lysis of the infected cells.

From here it came out clearly that results obtained with culture mediums pre-modified by the PLA2s are similar to results obtained with the enzymes themselves. Indeed, we show here that distinct effects on the parasite development already observed with bee and hog PLA2 can be reproduced with complete medium pre-incubated with respective PLA2s.

In our experimental conditions, cytotoxicity of both PLA2s toward the infected erythrocytes occurs via modification of serum phospholipids. The modification in size and shape of the healthy erythrocytes (shrinkage and echinocytosis) that had been observed upon incubation of culture with PLA2s was also observed in the presence of the modified serum. This might be attributed to the incorporation of phospholipid by-products into the erythrocyte plasma membrane, since it has been reported that externally added lysophosphatidylcholine induces echinocytosis of erythrocytes (30).

Bee PLA2-Treated Lipoproteins Are Toxic to P. falciparum—Serum phospholipids are found in four classes of lipoproteins: chylomicrons, VLDL, LDL, and HDL. To analyze the respective roles of the lipoprotein classes as substrates of the bee venom PLA2 and their involvement in the cytotoxic effect toward Plasmodium, we prepared enriched fractions of HDL, LDL, VLDL/chylomicrons and proteins from human heat-inactivated serum. Each fraction was then submitted to hydrolysis by Affi-Gel-coupled bee PLA2 for 17 h at 37 °C. Such lipolyzed lipoproteins and PLA2-treated protein fraction were tested for cytotoxicity toward the FC1 strain of P. falciparum in the presence of 7% normal serum via the [3H]hypoxanthine incorporation test. Results of the experiments (Fig. 5) show that all the lipoprotein fractions became toxic to Plasmodium upon hydrolysis by bee venom PLA2, with IC50 values at 100 ± 3.4 µg/ml for treated HDL, 50 ± 6.1 µg/ml for treated LDL, and 5 ± 0.5 µg/ml for treated VLDL/chylomicrons. The protein fraction...
Stage-specific Toxicity of Bee Venom PLAr toward P. falciparum

FIG. 4. Analysis of the respective susceptibilities of P. falciparum developmental stages to hog and bee PLAr-modified serum. Synchronized cultures of the P. falciparum FcB1 strain (synchronization windows: 0–3 or 0–5 h) were incubated at different times of the intraerythrocytic development and for various periods of time (ranging from 6 to 28 h, horizontal arrow) in bee PLAr- or hog PLAr-treated serum at the final 1/4 dilution in complete culture medium. Re-invasion rates at the end of the cell cycle were determined from Giemsa-stained smears of cells incubated with bee PLAr-treated serum (B) and hog PLAr-treated serum (H). 100% re-invasion was measured from control cultures in the absence of PLAr-modified serum. ND, not determined.

Discussion

To our knowledge, secreted phospholipases A2 have largely been used to study the phospholipid asymmetry of Plasmodium-infected erythrocytes, but only one report has dealt with the toxic effects of secreted PLAr on intraerythrocytic Plasmodium (21), which describes the selective elimination of Plasmodium knowlesi-infected simian red blood cells by a chemically modified (fatty acylated) pig pancreatic PLAr. In this report, the possible role of serum phospholipids as substrates of PLAr in culture conditions was not explored, and we present here a different approach to investigate PLAr cytotoxicity toward Plasmodium. First, we have systematically analyzed the cytotoxic effect of four venomous and two pancreatic native secreted PLAr. The results enable a clear distinction between the two categories of enzymes. Much less enzymatic units from the four venom PLAr can induce P. falciparum growth arrest compared with pancreas PLAr (approximately 10^{-5}–10^{-3} IU/ml versus >0.1–1 IU/ml, respectively). The higher capacity of venom PLAr to penetrate the membrane of parasitized erythrocytes might have explained these results, because maximal surface pressures at which bee PLAr, C. adamanteus PLAr, and pig PLAr can hydrolyze monomolecular films of phospholipids are, respectively, 35.3, 23.0, and 16.5 dynes/cm (29), and the membrane lateral pressure of P. knowlesi-parasitized erythrocytes is 20 dynes/cm (21). Extension to P. falciparum-infected cells would lead to discrimination between venom and pancreas PLAr, only the former being able to hydrolyze membranes at 20 dynes/cm.

In contradiction with this interpretation, we show here that PLAr are indirectly cytotoxic toward P. falciparum, with PLAr-modified lipoproteins being major actors in parasite killing. Serum lipoproteins are targets of the PLAr, although concomitant enzymatic hydrolysis of phospholipids from the membrane of infected erythrocytes cannot be excluded. Such modifications of the lipoproteins are toxic to Plasmodium. The three fractions, HDL, LDL, and VLDL/chylomicrons, are able to inhibit parasite growth upon bee PLAr hydrolysis. However, when one considers the respective molecular masses of the particles (HDL, 400 kDa; LDL, 3000 kDa; VLDL, 7500 kDa; chylomicron, 5 x 10^6 kDa) and their respective content in apoproteins (HDL, 50%; LDL, 25%; VLDL, 10%; chylomicron, 1%) versus their content in phospholipids (HDL, 22%; LDL, 22%; VLDL, 18%; chylomicron, 7%), it appears that the VLDL/chylomicron fraction is the more toxic one, with the lowest amount of phospholipids. Additional investigations are now needed to decipher the respective effects of the three lipoprotein fractions on the parasite cycle.

Most intriguing here is the difference observed between the effects on parasite development due to serum modified by bee PLAr or hog PLAr. Intraerythrocytic Plasmodium is sensitive to the toxic effect of bee PLAr-hydrolyzed serum only during a short period of its development, situated between 19 and 26 h of the cycle, i.e. at the young trophozoite stage. All stages, however, are sensitive to hog PLAr-treated serum. Our observations suggest that, in the latter case, serum by-product(s) might act through preferential lysis of the infected erythrocytes compared with the healthy ones, without discrimination between the developmental stages of the intraerythrocytic parasite. Here, modification of the biological properties of the red cell membrane upon parasite invasion might explain increased sensitivity to destabilizing by-product(s). Such a hypothesis of a possible cytolytic effect by the products of PLAr-mediated phospholipid lysis is reinforced by a study demonstrating that two group II phospholipases A2, the PLAr from the venom of Crotales durrissus terrificus and the human secreted PLAr, are indirectly cytolytic in the presence of exogenous phospholipids (31).

Hydrolysis of phospholipids from lipoproteins by PLAr leads to three by-products: free fatty acids and lysophospholipids, generated by cleavage of the ester linkage at the sn-2 position of the phospholipids, plus the modified lipoprotein. The net electric charge of the lipoprotein is altered due to an increase in negative surface charges. It is known that oxidized lipoproteins can be highly toxic toward cells (32, 33). What is not well known is whether the toxicity of oxidized lipoproteins relies on a single compound or is produced by the combined action of several compounds generated from the phospholipids during the oxidation process. Although secreted PLAr are generally considered to display almost no preference for different types of naturally occurring phospholipid head groups or structure of the fatty acyl chains attached to the glycerol backbone (34), it has been reported that there is a marked specificity of the human secretory type II PLAr for phosphatidic acid (35) and preferential release of arachidonic acid by the type I and type II
PLA₂ when it is linked to phosphatidylethanolamine, among other phospholipid subclasses (36).

To explain the specific effect of the bee PLA₂-modified culture medium compared with the hog PLA₂-treated medium on Plasmodium development, we must infer that the two PLA₂s generate a different compound(s) from the serum lipoproteins. Lysophospholipids and free fatty acids produced by PLA₂ enzymatic cleavage of phospholipids are important regulatory molecules and have been extensively studied as precursors of eicosanoids and platelet-activating factor (for a review, see Refs. 37 and 38). Besides, different biological effects have been attributed to fatty acids, like the cytotoxic/cytostatic effects on a number of tumor cells (39, 40) or the modulation of some membrane transporter activity, like that of the erythrocyte Ca²⁺-ATPase (41). Lysophospholipids as well have been reported to display somewhat similar effects, like the inhibition of the human erythrocyte Na⁺,K⁺-pump activity by lysophosphatidylcholine (42, 43). The trophozoite stage, the target of the bee PLA₂ activity product, is metabolically very active and, thus, might present greater sensitivity to any of these various lipid actions.

Although nothing is known about possible toxic effects of lysophospholipid molecules toward Plasmodium, antimalarial properties of fatty acids have been previously reported: n-3 and n-6 polyunsaturated fatty acids kill P. vinckei petteri and P. falciparum in vitro and Plasmodium berghei in vivo (44), and C18 fatty acids are toxic to P. falciparum in vitro and to Plasmodium vinckeii petteri and Plasmodium yoelii nigeriensis in vivo (45). In the former case, parasiticidal effects could be attributed to the susceptibility of the polyunsaturated fatty acids to oxidation, whereas Krugliak et al. (45) report no effect of C18 fatty acids on lipid peroxidation, ATP levels or transport through the parasite-induced permeability pathways could be found.

In this respect, fine analysis of the respective lipid composition of bee and hog PLA₂-lypopolysed lipoproteins is being attempted in order to obtain direct information on the nature of the generated molecules. Identification of the one or few molecule(s) selectively released from lipoprotein phospholipids by bee PLA₂, should offer a double interest: qualitative information about the venom toxin ability to modify human lipoproteins and a means of deciphering biological mechanisms responsible for the toxic effects on Plasmodium.

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