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Selective elimination of malaria infected erythrocytes by a modified phospholipase A_2 in vitro

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Pig pancreatic phospholipase A_2 does not act on normal erythrocytes, but the membrane penetrating capacity is enhanced by the covalent attachment of one fatty acyl chain to Lys-116 of the enzyme. Taking advantage of the impaired packing of phospholipids in the membrane of *Plasmodium* infected erythrocytes it was demonstrated that a lauric acid derivative of phospholipase A_2 is capable of exclusively attacking the infected erythrocytes in vitro, leaving the uninfected cells undisturbed. The chemically modified phospholipase A_2 appeared to cause death of the parasite in cell cultures of infected erythrocytes.

Erythrocytes play an essential role in a particular part of the complex life cycle of *Plasmodium falciparum*, when they serve as host cells in which the parasite feeds, grows and asexually multiplies. This is the pathological part of the cycle and is generally seen to offer potentially interesting possibilities of destroying the parasite, thereby breaking the fatal cycle [1]. Many studies therefore aim to unravel the parasite induced changes in the structural and functional characteristics of the membrane of the infected erythrocyte. Changes in phospholipid asymmetry in the plasma membrane of both uninfected [2,3] and infected cells [4–6] have been reported previously, but these changes could not be confirmed when studied under more gentle conditions [7,8]. Nevertheless, studies on the host cell membrane, involving spin-labeled phospholipid analogues [9,10], fluorescent lipids [11,12], immunological techniques [13] or a variety of biochemical techniques [7], all indicate major disturbances in lipid bilayer organization as expressed by enhanced membrane fluidity, enhanced transbilayer mobility and a decrease in lateral surface pressure.

It has been recognised that water soluble phospholipases are only able to attack their substrates, organized in a lipid mono- or bilayer, when the enzymes are able to penetrate into that layer [14,15]. Studies involving monomolecular films of phospholipids, learned that each individual phospholipase has a characteristic penetration capacity that can be expressed by the maximal lateral surface pressure of such a film above which the enzyme is unable to degrade its substrate. Using highly purified phospholipases of which the penetration capacity had been calibrated by the above mentioned method, it was estimated that under physiologic conditions of tonicity, temperature and pH, the surface pressure in the outer membrane leaflet of the human erythrocyte is approx. 33 dyn/cm [14]. Consequently, pig pancreatic phospholipase A_2 for which the monolayer technique indicated the limiting pressure to vary from 12 to 16.5 dyn/cm depending on the phosphatidylcholine substrate used [14,16], appeared to be unable to attack the intact red cell. It has recently been shown that the weak penetrating properties of this pancreatic phospholipase A_2 can be considerably improved by the covalent attachment of a long acyl chain to Lys-116, the enhancement in capacity to penetrate into monolayers being proportional to the length of this chain [16]. The palmitoyl derivative, palmitoyl-lysine^{116}-ε-amidinated pancreatic phospholipase A_2(Pal-116-AMPA), easily degrades its substrates in the outer monolayer of the intact human erythrocyte at a relatively high rate. The lauric

Abbreviations: Lau-116-AMPA, lauroyl-lysine^{116}-ε-amidinated porcine pancreatic phospholipase A_2; AMPA, fully-ε-amidinated porcine pancreatic phospholipase A_2; AMPREC, fully-ε-amidinated porcine pancreatic *pro*phospholipase A_2; PC, phosphatidylcholine.

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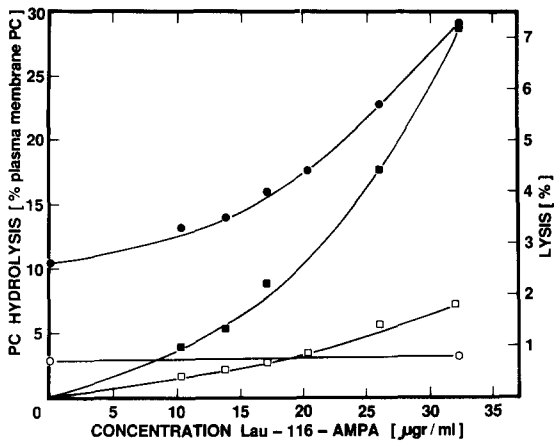

Fig. 1. Lau-116-AMPA induced haemolysis (○, ●) of, and phosphatidylcholine hydrolysis (□, ■) in, uninfected (○, □) and infected (●, ■) erythrocytes from monkeys, parasitized with *Plasmodium knowlesi*. *P. knowlesi* infected cells (95–100% parasitaemia) were obtained as previously described [17]. Samples of 70 µl of uninfected and 100% schizont infected packed cells were incubated at 5% haematocryt in 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM Tris-HCl (pH 7.4) for 1 h at 37°C under gentle agitation. Lau-116-AMPA was present at the concentrations indicated. Following incubation, cells were pelleted (5 min at 380 g) and the supernatant was used to determine the extent of haemolysis from its optical density at 418 nm and by comparing this value with that of an appropriately diluted sample of a 100% lysate prepared from the corresponding, either uninfected or infected, batch of cells. Phospholipase action was arrested by adding 0.5 ml 100 mM EDTA to the packed cells. Lipids were extracted by subsequent application of the procedures according to Rose and Oklander [28] and to Folch et al. [29] and separated by two-dimensional TLC on precoated silica gel plates (Merck, Darmstadt, F.R.G.; No. 5721) using the developing solvents according to Broekhuyse [30]. The acid developing mixture that was used for the second run, was freshly prepared every day to ensure a good separation between phosphatidylserine and lysophosphatidylethanolamine. The phosphorus content was determined according to the principle of Rouser et al., [31]. The percentage hydrolysis of each glycerophospholipid, as achieved by treatment of the intact cells with Lau-116-AMPA, was calculated as previously described [7]. The figure shows the results of a typical experiment.

acid derivative, Lau-116-AMPA, on the other hand, which can penetrate a phosphatidylcholine monolayer packed at a pressure up to 20 dyn/cm, virtually fails to attack the (normal) human red cell [16].

In view of the above mentioned decreased packing of lipids in the membrane of *Plasmodium* infected red cells, we studied the possibility that Lau-116-AMPA might be able to attack infected cells leaving the uninfected ones undisturbed. Studies on erythrocytes obtained from *Plasmodium knowlesi*-infected rhesus monkeys and Cynomolgus monkeys showed that, in contrast to the uninfected cells, the 100% schizont infected cells were highly susceptible to Lau-116-AMPA (Fig. 1). As little as 45 µg Lau-116-AMPA per 70 µl packed cells induced the hydrolysis of approx. 30% of the phosphatidylcholine (Fig. 1), but none of the phosphatidylserine and -ethanolamine (not shown), present in the host cell plasma membrane. Although in the case of healthy erythrocytes, this extent of hydrolysis would not have given rise to any haemolysis [16], we observed 7% of lysis in the case of the parasitized cells (Fig. 1). It is essential to note here that the procedure we had to apply for the isolation of the parasitized cells [17], impairs their stability. This has the immediate consequence that those cells exhibit some haemolysis within a couple of hours when incubated at 37°C, even in the absence of a phospholipase. Hence, it was inappropriate to follow the Lau-116-AMPA induced hydrolysis of PC, and accompanying lysis of the parasitized cells, for more than one hour. Though still limited at this time point, the course of these events indicates that we are only observing the beginning of a major disruption of the host cell plasma membrane. When lyso-PCs and free fatty acids, the two split products of PC that can be generated in the outer membrane leaflet of intact erythrocytes by the action of phospholipase A_2, are subsequently removed, complete lysis of the cells is the inevitable result [18,19]. Also, it has been reported that lysophospholipids and fatty acids are rapidly transported to, and taken up by the parasite [20,21]. There are as yet no reasons to assume that the fate of these two hydrolysis products that are generated in the host cell plasma membrane by the action of the Lau-116-AMPA, would be any different and thus gives rise to the lysis of the infected cells as they are rapidly transferred to the parasite. In full agreement with the above, is the observation that the weakly penetrating derivative of pig pancreatic phospholipase A_2 in which all of the nine free ε-amino groups had been amidinated [22] (AMPA), was unable to attack the infected cells (data not shown).

As these studies clearly demonstrated that *Plasmodium*-infected erythrocytes are indeed specifically susceptible to Lau-116-AMPA, it became of interest to see what might happen to the parasite itself when this modified pancreatic phospholipase A_2 is present during its in vitro culturing. To that end, 7.6 µg of Lau-116-AMPA was added to 200 µl of a suspension of *P. falciparum* infected cells – haematocryt: 7, parasitaemia (percentage of infected cells): 2 – in RPMI 1640, supplemented with 10% human serum. It appeared that the growth of the parasite, - as determined by [^{3}H]hypoxanthine incorporation (1 h) into its nucleic acids [23], had been inhibited by some 50% when this suspension had been incubated for 4 h at 37°C. This concentration of Lau-116-AMPA (38 µg/ml), causing 50% inhibition of parasite growth under these conditions, is very similar to the concentration of this enzyme as used in the experiments depicted in Fig. 1. To gain more detailed information on the lethal effect that Lau-116-AMPA may exert on the parasite, *P. falciparum* was cultured for 24 h in a suspension of human erythrocytes containing various amounts of this modified phospholipase. Similar experiments were performed by using either

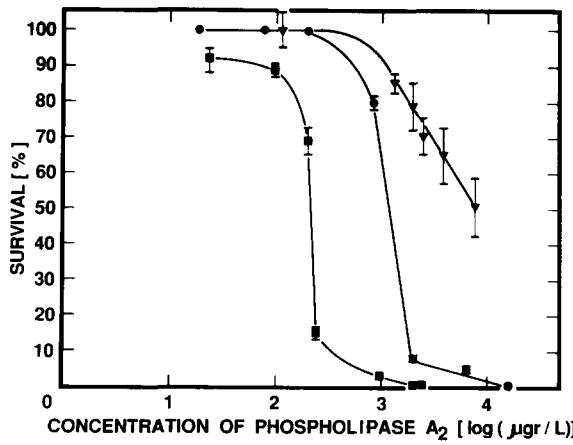

Fig. 2. Effect of phospholipases A_2 on the in vitro growth of *Plasmodium falciparum*. *P. falciparum* was cultured in mirowell plates according to Jensen and Trager [32], using a 1.5–2.0% suspension of human erythrocytes in 200 μl RPMI 1640 medium, containing 10% human AB^+ serum. Experiments were started by the addition of various amounts of phospholipases A_2 as indicated; parasitaemia (percentage of infected cells) started at approximately 1%. *Naja naja* phospholipase A_2 (■) was from Sigma Chemical Company (St. Louis, MO, U.S.A.); AMPA (▼) and Lau-116-AMPA (●) were prepared as described by Van der Wiele et al. [24]. After 24 h of incubation of the cells in the presence of phospholipase A_2, parasite growth was assayed according to Desjardin et al., [23] by adding 0.5 μCi [^{3}H] hypoxanthine (Amersham; specific radioactivity 14.3 Ci/mmol) and continuation of the incubation for another 24–26 hours. Cells were subsequently lysed and the parasite nucleic acids recovered on a filter by using a cell harvester (Dynatech). Radioactivity of the tritium-labeled nucleic acids was determined in a Beckman 3801 liquid scintillation spectrometer. Results shown are the means ± S.E. of triplicate assays, performed on samples that had been derived from the same cell suspension. IC_{50} values obtained from experiments involving different red cell suspensions may vary by a factor 3, probably due to different stages of parasite development or growth.

Naja naja phospholipase A_2 or AMPA. The corresponding dose-response curves of the effects that these phospholipases A_2 have on the survival of the parasite are shown in Fig. 2. Microscopic examination of smears from suspensions treated with a dose of Lau-116-AMPA which causes complete inhibition of parasite growth (Fig. 2), in fact showed the complete absence of parasites. In agreement with its higher penetration power, when compared to Lau-116-AMPA, *N. naja* phospholipase A_2 [16] is much more effective in killing the parasites than the lauroyl derivative of the pancreatic enzyme is. It should be recalled, however, that in contrast to Lau-116-AMPA, the snake venom phospholipase A_2 also attacks the uninfected cells. Some inhibition of growth could also be achieved with AMPA, but a complete elimination of the parasites could not be reached at the highest concentration (7.3 μg/ml) tested (Fig. 2). On the other hand, the growth of the parasite appeared to develop normally when cultured in the presence of 1 μg of lauric acid per ml, which (in molar terms) is about 50-times as much as the highest concentration of Lau-116-AMPA used (compare Fig. 2). Similarly, *P. falciparum* growth was not affected by Lau-116-AMPREC [24], when this enzymatically inactive precursor of Lau-116-AMPA was present at concentrations identical to those used for Lau-116-AMPA. These observations indicate that the lethal effect that Lau-116-AMPA exerts on the parasite is indeed related to its enzymatic activity. It is also worth noting that incubation of healthy human erythrocytes for 24 h in the *Plasmodium* medium and in the presence of an amount of Lau-116-AMPA that would have caused a complete elimination of the parasite, degraded as little as 7% of the PC in those cells. This agrees well with the observations from experiments in which either the total number of cells or the relative number of parasitized cells was varied, showing that under the conditions in Fig. 2 the amount of Lau-116-AMPA that is needed to cause a 50% reduction in parasite survival was rather strictly correlated to the number of infected cells only (results not shown). Furthermore, it appeared that the lethal effect that Lau-116-AMPA exerts on the parasite, was not at all affected when the amount of AB^+ serum present in the *Plasmodium falciparum* culture was varied from 5 to 15%, which most likely precludes that serum (lipoproteins) play(s) an intermediary role in this process.

Taken together, our studies demonstrate that Lau-116-AMPA exclusively affects the infected cell and, equally important, that its action causes the death of the parasite in vitro. Although it may be tempting to speculate that this modified pancreatic phospholipase A_2 may open new ways of strategy in the fight against malaria, it should be realised that its potential application still has to await the answer on a number of questions and the solution of some pertinent problems. For instance, it still has to be ascertained that Lau-116-AMPA is unable to affect other blood cells such as leucocytes, similarly as it is unable of attacking healthy erythrocytes. Preliminary experiments already indicated that exposure of intact platelets (10 mg wet weight, isolated from sheep blood according to Sakariassen [25]) to Lau-116-AMPA (4 I.U., total volume 1.0 ml) did cause neither any appreciable extent of phospholipid degradation (less than 2% of the PC, if any), nor lysis of the cells when determined by the release of lactate dehydrogenase [26]. One of the major problems that has to be solved yet, however, is to prevent a direct attack of the enzyme on the serum lipoproteins [27]. Indeed, incubation of defibrinated whole blood from a healthy individual together with an appropriate amount of Lau-116-AMPA, showed considerable degradation of the serum glycerophospholipids, but did cause neither lysis of the red cells, nor hydrolysis of any of their phospholipids. Nevertheless, the essentially absolute selectivity by which Lau-116-AMPA attacks only those erythrocytes that have been infected by the *Plasmodium* para-

site, warrants further research to overcome those problems.

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