SHORT REPORT: LETHAL MALARIA IN CYTOSOLIC PHOSPHOLIPASE A2- AND PHOSPHOLIPASE A2IA-DEFICIENT MICE

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Abstract. Lipid mediators play important roles in the pathogenesis of malaria. Phospholipase A2s are enzymes involved in the production of lipid mediators. Plasmodium berghei ANKA causes cerebral malaria in CL57B/6 mice, and we recently produced cPLA2-deficient mice with this background. With the expectation of reduced pathogenicity, we performed experimental infection in these mice. Unexpectedly, the infected mice developed cerebral malaria and died at the same time as the control mice. These observations suggest that secretory PLA2s rather than cPLA2 may be involved in the aggravation, although possible compensation by the induction of other enzymes has not been excluded. The present findings are expected to help clarify the involvement of various phospholipase A2s in malaria.

Several lines of evidence indicate that lipid mediators known as eicosanoids are involved in the pathogenesis of malaria. Clark and Hunt showed that peritoneal macrophages from mice infected with murine malaria produced increased amounts of arachidonate metabolites.1 A report of successful treatment of human cerebral malaria with prostaglandin I2 has been published.2,3 Intervention in murine malaria with aspirin, a prostaglandin synthesis inhibitor, suggested that prostaglandins are protective against cerebral malaria, while leukotrienes aggravate this disease.4 In human malaria caused by Plasmodium falciparum, the levels of prostaglandin E2 in plasma and cyclooxygenase-2 in blood mononuclear cells are inversely related to disease severity.5 Focal accumulation of cyclooxygenase-1 (COX-1)- and COX-2-expressing cells was reported in human cerebral malaria.6 Cyclooxygenases are the enzymes that convert arachidonic acid to prostaglandins. However, the exact roles of lipid mediators in malaria remain to be elucidated.

Lipid mediators, including prostaglandins and leukotrienes, are all produced from arachidonic acid, which is released from phospholipids in membranes by phospholipase A2s. Phospholipase A2s are enzymes that digest the sn-2 ester bond of phospholipids. Increasing numbers of these enzymes, many of which are involved in inflammation, have been identified. Among them, cytosolic phospholipase A2 (cPLA2) is a key enzyme that is specific for arachidonic acid.7 When activated by micromolar calcium and the phosphorylation of serine by mitogen-activated protein kinase (MAPK) and mitogen-activated protein kinase kinase (MAPKK), the enzyme is translocated to perinuclear membranes via binding to vimentin. This process is presumed to be important in the production of lipid mediators in inflammation. We have produced cPLA2-deficient mice that displayed reduced bronchial asthma and injury due to cerebral infarction.8-10 Recently, the cPLA2-deficient mice were backcrossed to CL57/B6 mice for 10 generations. It is well established that infection of CL57/B6 with the P. berghei ANKA strain causes severe malaria symptoms in the mice one week after infection, when parasitemia is approximately 20%, resulting in the death of the mice. In contrast, BALB/c or C3H mice survive this period without severe symptoms and die later due to severe anemia and prostration.11

In an attempt to elucidate the mechanisms of aggravation of malaria, we induced experimental infection in cPLA2-deficient mice. We expected that cPLA2-deficient mice would not acquire severe malaria because of the abolition of the production of lipid mediators in inflammation pathways in these mice. However, contrary to our expectation, the infected cPLA2-deficient mice showed severe symptoms and died at just the same time as the control mice.

Eight cpla−/− female mice (6–8 weeks old) and 10 age-matched cpla/+ mice were used for the experiments. Throughout the experiments, the mice were treated in a humane way. Animals were anesthetized with ether before they were humanely killed. The experiments were performed according to the guidelines of the Animal Center of Nihon University.

The P. berghei ANKA strain was kept frozen in liquid nitrogen and propagated in mice once by intraperitoneal injection. Mice were infected by intraperitoneal injection of 1×10⁶ infected erythrocytes into the experimental mice.

Mice were carefully observed twice a day and parasitemia was estimated by Giemsa-stained slides of thin blood smears made from a drop of tail blood every other day. For pathologic studies, organs were obtained from two cpla−/− mice and three cpla+/+ mice killed on day 6 after infection or from mice under observation immediately after death. Survival of six deficient mice and seven control mice was observed.

Parasitemia showed similar exponential increases in both the deficient and control mice (Figure 1). Body weight remained unchanged during the period of the experiment, which indicated that the mice did not die of prostration (Figure 2). Symptoms in the mice began with coat ruffling and general immobility, followed by partial paralysis, fitting, hyperventilation, coma, and death, the severity of which was essentially the same in both groups.

Survival after challenge infection is shown in Figure 3. Kaplan-Meier analysis of the survival time using the Gehan
generalized Wilcoxon method showed that death occurred at a similar time ($P = 0.86$), with no significant difference between the deficient and the control mice. The weights of various organs (brain, lungs, liver, spleen, and kidneys) showed no significant difference between the two groups, which showed that the pathophysiology, at least in context of malarial infection, is not so different irrespective of the presence or absence of cPLA2.

Extensive pathologic studies of tissues showed remarkable reactive responses in both the deficient and control mice just after death, while essentially the same but milder responses were found in both types of mice killed on day 6 (Figure 4). In brain tissue, several features including microhemorrhage and vascular distention with parasitized erythrocytes and macrophages were seen to the same extent both in wild-type and vascular distention with parasitized erythrocytes and macrophages were seen to the same extent both in wild-type and control mice (Figure 4A–E). In addition to staining with hematoxylin and eosin, immunohistochemical staining using B cell-, T cell-, and macrophage-specific antibodies (Figure 4C) was done. Such staining showed no significant difference in the cellular infiltration pattern between the two groups. The main inflammatory cells in brain tissue were macrophages in the vascular lumen or in the brain parenchyma around microhemorrhage foci. There was a slight increase in T and B lymphocytes in the cerebral vasculature. Immunohistochemical analysis for cPLA2 showed ubiquitous expression of cPLA2 in neuronal cells (Figure 4F–H), which is consistent with the results of a previous report\(^{13}\) in wild-type mice with and without malarial infection. Staining intensity was essentially the same in both groups. There was no expression of cPLA2 in cells in the vascular lumen in infected wild-type mice, which indicates that cPLA2 may not play critical roles in cerebral malaria pathophysiology. In the ovaries, results were consistent with those of a previous report.\(^{13}\) In the spleen, we observed distortion of follicular architecture accompanied by numerous immunoblasts and germinal center formation, which represent intense immunologic reactions (Figure 4L and K). In liver and lungs, there were increases of Kupffer cells and macrophages with malarial pigment (Figure 4M–P). In the lungs, almost no alveolar exudate or stromal response that might have had an effect on mouse survival was found. Overall, there was no significant change that indicated the cause of death, except for several findings compatible with cerebral malaria in both wild-type and cpla−/− mice.

Prostaglandins and leukotrienes are major lipid mediators that function in inflammation. They are produced from arachidonic acid by cyclooxygenases and 5-lipoxygenase, respectively. Arachidonic acid, a key metabolite, is released from phospholipids by phospholipase A\(_2\)-s, which digest the ester bond of the sn-2 fatty acids of phospholipids. A growing number of phospholipase A\(_2\)-s have been identified. They are divided into secretory, cytosolic, and Ca-independent PLA\(_2\)-s.\(^7\) Phospholipase A\(_2\) was first isolated from pancreatic juice as an enzyme that digests phospholipids in food (group IB PLA\(_2\)). The 14-kD phospholipase A\(_2\)-IIA was then purified from exudates of animals with experimental inflammation. Subsequent searches of nucleic acid sequence databases resulted in identification of 10 secretory PLA\(_2\)-s. With regard to intracellular PLA\(_2\)-s, cPLA\(_2\) alpha was cloned as a high molecular weight PLA\(_2\)-.\(^7,14\)

Cytosolic phospholipase A\(_2\) alpha is an 85-kD molecule located ubiquitously in the cytoplasm of all cell types examined except for mature lymphocytes.\(^7\) cPLA\(_2\) beta and cPLA\(_2\) gamma have also been cloned. cPLA\(_2\) alpha and cPLA\(_2\) beta have an N-terminal C2 domain, which is critical for a Ca\(_2+\)-dependent association with phospholipid membranes. cPLA\(_2\) gamma contains an isoprenylation site for membrane binding. Activated cPLA\(_2\) specifically releases arachidonic acid, which is metabolized to prostaglandins and leukotrienes. Because this is the first enzyme in the cascade leading to the production of various lipid mediators, its deficiency is thought to abolish the subsequent production of prostaglandins and leukotrienes. Actually, cPLA\(_2\)-deficient mice show marked reductions in the production of prostaglandin E\(_2\), leukotriene B\(_4\), and leukotriene C\(_4\) by activated peritoneal macrophages.\(^6,9\)

Although the unexpected results obtained in the present study are difficult to explain, they provide the basis for some speculations about the responsible mechanism. One possibility is that the enzyme deficiency was compensated for by the induction of cPLA\(_2\)-beta, cPLA\(_2\)-gamma, or hitherto unidentified enzyme(s).\(^{15,16}\) However, if one considers the difference
between the localization of cPLA2 beta and cPLA2 gamma in normal tissues and that of cPLA2 alpha, this seems unlikely. Conversely, it is well known that secretory PLA2s IIA and V (sPLA2) are involved in inflammation and protection against bacterial infection. In both adult and juvenile malaria patients, an increased level of phospholipase A2 has been reported. The increased enzyme level was shown immunologically to be group II secretory phospholipase A2. Whether this enzyme is protective against malaria or simply associated with the disease is unknown. The mice used in our experiments are also deficient in phospholipase IIA due to a frame shift mutation in the corresponding gene. Infection with P. berghei ANKA causes accelerated death in CL57B/6 and 129SvJ mice, which are phospholipase IIA deficient. However, C3H and BALB/c mice, which have normal phospholipase IIA, survive the early phase of this infection and die later due to severe anemia and prostration. One reported exception to this pattern are B10.RIII mice, which have a defective phospholipase IIA gene but which rarely acquire cerebral malaria. Since the deficient mice used in the present study were originally produced in strain 129/SvJ, it is from a mouse without infection with Plasmodium berghei ANKA parasites by intraperitoneal injection. Dashed line = cytosolic phospholipase A2-deficient mice; Solid line = control mice.

FIGURE 3. Survival after challenge infection with 10^6 Plasmodium berghei ANKA parasites by intraperitoneal injection. Dashed line = cytosolic phospholipase A2-deficient mice; Solid line = control mice.

FIGURE 4. Histopathologic evaluations of wild and cytosolic phospholipase A (cpla-/-)–deficient mouse tissues obtained immediately after death (only F is from a mouse without infection with Plasmodium berghei ANKA). A, Wild-type mouse brain showing vascular distention and mononuclear cells and erythrocytes with malarial pigments in the lumen (hematoxylin and eosin stained). B, Brain as in A in a cpla-/- mouse. C, Immunohistochemical analysis of a cpla-/- mouse brain using an MAC-3 monoclonal antibody showing macrophages in distended vascular lumens. D, Brain tissue of wild-type mouse showing microhemorrhage characteristic of cerebral malaria. E, Brain as in D in a cpla-/- mouse. F and G, Immunohistochemical analysis using an anti-cPLA2 polyclonal antibody showing cPLA2 expression in neuronal cells in wild-type mice without (F) and with (G) malarial infection. There is no expression in inflammatory cells in vascular lumen in wild-type mouse with infection. H, Neuronal cells as in G in a cpla-/- mouse. There is almost no non-specific staining with the anti-cPLA2 antibody. I and J, Immunohistochemical analysis of ovaries in wild-type (I) and cpla-/- (J) mice. Results are consistent with those in a previous report. K, Spleen in a wild-type mouse showing hyperplastic reactions with numerous immunoblasts, which represent an intense immunologic reaction. L, Spleen as in K in a cpla-/- mouse. M, Lungs of a wild-type mouse showing an increase in macrophages with malaria pigments, but with almost no alveolar exudate or stromal reactions. N, Lungs as in L in a cpla-/- mouse. O, Liver showing numerous Kupffer cells with malarial pigments in a wild-type mouse. P, Liver as in O in a cpla-/- mouse. (Magnification × 200 in A, B, D, E, F, G, H, K, L, M, N, O, and P; × 400 in C; and × 100 in J and L)
FIGURE 4. Continued.
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tor-α or interleukin-1β. The possible involvement of sPLA₂-IB receptor in malaria is also of great interest.

Recently, the use of genome-wide polymorphism markers resulted in the mapping of the genetic loci in mice that are responsible for the control of parasitemia and cerebral malaria. This novel approach is expected to provide a powerful tool to decipher the genetic basis of malaria resistance. However, gene knockout mice are important for pinpointing the critical gene(s) involved in resistance to and pathogenesis of malaria.

In conclusion, experimental infection of cPLA₂- and PLÅ₂-IIA-deficient mice in a C57B/6 background resulted in the rapid death of these mice, as it did in control mice. It is expected that further studies of phospholipase A₈ will reveal more details of the pathogenesis of lethal malaria and the involvement of lipid mediators.

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