Development of Severe Pathology in Immunized Pregnant Mice Challenged with Lethal Malaria Parasites

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Pregnant women are highly susceptible to malaria infection because of their low immunity and are at increased risk of maternal illness or death, in addition to spontaneous abortion, stillbirth, premature delivery, and low birth weight. However, the detailed pathogenesis of maternal malaria remains unclear. In this study, we evaluated a mouse model that shows similar severe pathological features of pregnant women during Plasmodium falciparum infection and investigated the pathogenesis of maternal malaria. Pregnant mice immunized by infection with an attenuated parasite, Plasmodium berghei XAT, were more susceptible to virulent P. berghei NK65 challenge-infection than were nonpregnant mice and showed high levels of parasitemia and a poor pregnancy outcome associated with placental pathology, such as accumulation of parasitized red blood cells, in the late phase of pregnancy. Notably, the pregnant immune mice challenged/infected with P. berghei NK65 developed liver injury associated with microvesicular fatty infiltration in late pregnancy. The pathological features were similar to acute fatty liver of pregnancy. Higher levels of gamma interferon and nitric oxide (NO) were found in plasma from pregnant immune mice infected with P. berghei NK65 than in plasma from nonpregnant mice. These findings suggest that development of liver injury and placental pathology in pregnant immune mice challenged/infected with P. berghei NK65 is accompanied by enhanced production of pro-inflammatory cytokines.

Malaria is the most devastating parasitic disease of humans in tropical and subtropical regions, resulting in an estimated 0.6 to 1 million deaths per year (1). The populations at greatest risk of developing severe pathology are children under the age of 5 years and pregnant women in areas where Plasmodium falciparum is endemic (2–4). Every year, approximately 50 million women living in areas where malaria is endemic become pregnant. An estimated 10,000 of these women and 200,000 of their fetuses or infants die annually as a result of malaria during pregnancy (1, 2). Malaria during pregnancy is a major public health problem in areas of endemicity, especially in Africa.

People living in regions where malaria is endemic acquire protective immunity against malaria parasites and often show asymptomatic infection. However, women are highly susceptible to malaria infection because of their low immunity during pregnancy and are at increased risk of maternal illness or death (5, 6). Malaria during pregnancy, in addition to maternal illness or death, is implicated in the occurrence of spontaneous abortion, stillbirth, premature delivery, and low birth weight (6). Recently, it has been demonstrated that pregnant mice infected with lethal Plasmodium berghei parasites show a feature similar to placental pathology and subsequently poor pregnancy outcome (7, 8).

Cerebral malaria (CM) or respiratory distress syndrome has been reported to be an infrequent but relevant cause of maternal death in women living in an area of sub-Saharan Africa where malaria is endemic (9). Previous studies using a mouse model have demonstrated that the development of experimental CM (10–14) or respiratory distress syndrome (15–17) is associated with the host immune response. However, the details of the pathogenesis of immunopathology, such as CM and respiratory distress syndrome, in pregnant women living in regions where malaria is endemic remain unclear. A mouse model of the immunopathology of pregnant women living in regions where malaria is endemic has not yet been established.

Plasmodium berghei NK65 causes a lethal infection in mice. C57BL/6 mice infected with P. berghei NK65 show increased parasitemia in the early phase of infection and suffer from liver injury, and all mice subsequently die within 2 weeks postinfection. The development of liver injury involves MyD88, interleukin-12 (IL-12), gamma interferon (IFN-γ), and CD8+ T cells (18–20). In contrast, P. berghei XAT is a low-virulence derivative from P. berghei NK65 (21). Mice infected with P. berghei XAT show low levels of parasitemia but become cured spontaneously within 5 weeks postinfection. Mice cured of P. berghei XAT infection have acquired protective immunity that completely suppresses the severe pathology caused by P. berghei NK65 (22, 23).

To investigate whether pregnant women who have acquired protective immunity against malaria parasites develop immunopathology during P. falciparum infection, it is necessary to establish a new mouse model. Because mice cured of P. berghei XAT infection have acquired protective immunity against malaria parasites, female mice were immunized by infection with P. berghei XAT in this study. Next, the immune female mice were mated with male mice and challenged/infected with lethal P. berghei NK65 parasites. In this study, we investigated whether pregnant mice that acquired protective immunity against malaria parasites developed liver injury during lethal P. berghei NK65 infection.

We found here that pregnant mice immunized with nonlethal P. berghei XAT were more susceptible to lethal P. berghei NK65...
Materials and Methods

Animals and parasites. Female and male C57BL/6 (B6) mice at 5 to 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan). The experiments were approved by the Experimental Animal Ethics Committee of Kyorin University School of Medicine, Tokyo, Japan, and all experimental animals were kept at the animal facility in a specific-pathogen-free unit with sterile bedding, food, and water.

Malaria parasites were stored as frozen stocks in liquid nitrogen. *P. berghei* NK65 is a high-virulence strain and was originally obtained from M. Youdi (New York University Medical Center, New York, NY, USA). *P. berghei* XAT is a low-virulence derivative of *P. berghei* NK65 (21). Using standard methods of reverse genetics in *P. berghei* (24, 25), mCherry, a red fluorescent protein, and a green fluorescent protein (GFP) were integrated into the *c-ssu-rRNA* locus on chromosome 5 of *P. berghei* NK65 and *P. berghei* XAT, respectively. Expression of mCherry or GFP was controlled by the HSP70 promoter.

Infections and mating. Parasitized red blood cells (pRBCs) of mCherry-expressing *P. berghei* NK65 or GFP-expressing *P. berghei* XAT were generated in donor mice inoculated intraperitoneally with each frozen stock of parasites. The donor mice were monitored for parasitemia daily and bled for experimental infection in ascending periods of parasitemia. Experimental mice were infected intravenously with *1 × 10^8* pRBCs of a given parasite strain. Female B6 mice at 5 to 6 weeks of age were infected with nonlethal GFP-expressing *P. berghei* XAT. One female mouse on day 30 after infection with GFP-expressing *P. berghei* XAT was mated for 1 day with a male mouse aged >8 weeks and examined for the presence of a vaginal plug on the next morning. The mice immunized by GFP-expressing *P. berghei* XAT (IM) with or without a vaginal plug were challenged with mCherry-expressing *P. berghei* NK65 (IM + NK).

Parasitemia. Blood was observed by microscopic examination of methanol-fixed tail blood smears stained with 3% Giemsa stain diluted 1:20 and examined for parasitemia daily and bled for experimental infection in ascending periods of parasitemia. Experimental mice were infected intravenously with *1 × 10^8* pRBCs of a given parasite strain. Female B6 mice at 5 to 6 weeks of age were infected with nonlethal GFP-expressing *P. berghei* XAT. One female mouse on day 30 after infection with GFP-expressing *P. berghei* XAT was mated for 1 day with a male mouse aged >8 weeks and examined for the presence of a vaginal plug on the next morning. The mice immunized by GFP-expressing *P. berghei* XAT (IM) with or without a vaginal plug were challenged with mCherry-expressing *P. berghei* NK65 (IM + NK).

Parasitemia. Blood was observed by microscopic examination of methanol-fixed tail blood smears stained with 3% Giemsa stain diluted with phosphate buffer, pH 7.2, for 45 min. The number of pRBCs in 250 RBCs was enumerated when parasitemia exceeded 10%, whereas *1 × 10^8* RBCs were examined when mice showed lower parasitemia. The percentage of parasitemia was calculated as follows: {number of pRBCs/total number of RBCs} * 100. To determine the proportion of GFP-expressing *P. berghei* XAT parasites in peripheral blood, blood was diluted (1: 5,000) with fluorescence-activated cell sorting (FACS) buffer and evaluated by flow cytometry. Data were analyzed with a FACS Calibur cytometer (BD Biosciences, San Jose, CA, USA) using FlowJo for Windows, version 7.1.3.

Measurement of hematocrit and fetus weight. For hematocrit measurement, blood was obtained from pregnant uninfected, pregnant IM, and pregnant IM + NK mice on day 16 postmating. Blood from uninfected nonpregnant, nonpregnant IM, and nonpregnant IM + NK mice was obtained at the same time as from pregnant mice. Tail blood (50 μL) was collected into a heparinized capillary tube and centrifuged at 12,000 × g for 5 min with a microhematocrit centrifuge (HC-12A; Tomy, Tokyo, Japan). Hematocrit was expressed as the percentage of blood cells in the total volume of blood. Fetus weight was measured using a balance (TE1502S; Sartorius, Göttingen, Germany).

Histological examination and measurement of parameters of liver injury. Livers and blood were obtained from pregnant uninfected, pregnant IM, and pregnant IM + NK mice on days 12 and 16 postmating. Blood from uninfected nonpregnant, nonpregnant IM, and nonpregnant IM + NK mice was obtained at the same time as that from pregnant mice. Placentas and fetuses were obtained from pregnant uninfected, pregnant IM, and pregnant IM + NK mice on day 18 postmating. Mice were killed, and the livers, placentas, and fetuses were removed. These tissues or fetuses were fixed in 10% buffered formalin and embedded in paraffin or were fixed in 4% paraformaldehyde and frozen at −80°C. Six-micrometer-thick sections were stained with hematoxylin and eosin (H&E) or with Sudan IV. The thick sections stained with Sudan IV were photographed at ×400 magnification using an All-in-One fluorescence microscope (BZ9000; Keyence Japan, Osaka, Japan). The fat droplets in the photographs were counted using BZ-II Analyzer software (Keyence Japan). The blood was centrifuged at 500 × g for 10 min. The resulting supernatants were stored at −20°C and used as plasma. The levels of aspartic aminotransferase (AST), alanine aminotransferase (ALT), and glucose in plasma were determined at Nagahama Life Science Laboratory (Shiga, Japan).

ELISA of cytokines and specific antibodies and NO assay. An enzyme-linked immunosorbent assay (ELISA) for the detection of IFN-γ or IL-10 in plasma was carried out as described previously (23). A rat anti-mouse IFN-γ monoclonal antibody (MAb) (clone R4-6A2; ebiosis) and a rat anti-mouse IL-10 antibody (clone JES5-16E3; ebiosis) were used as the capture antibodies. A biotinylated, rat anti-mouse IFN-γ MAb (clone XMG1.2; ebiosis) and rat anti-mouse IL-10 MAb (clone JES5-2A5; ebiosis) were used as the detecting antibodies. The concentrations of cytokines in plasma were calculated from standard curves prepared using known quantities of murine recombinant IFN-γ (Genzyme, Boston, MA, USA) and IL-10 (Pierce, Rockford, IL, USA). The nitric oxide (NO) concentration in plasma was measured using the Quantichrom nitric oxide assay kit (BioAssay Systems). The malarial antigens were prepared from the erythrocytic stages as described previously (26, 27). Malaria-specific antibodies in the plasma of mice were assayed using soluble antigens from *P. berghei* XAT as the capture antigens. Peroxidase-coupled anti-mouse IgG (Zymed, South San Francisco, CA, USA) was used to detect specifically bound IgG. The reaction mixtures were visualized by peroxidase-conjugated streptavidin (Zymed) and the substrate, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Wako, Osaka, Japan).

Statistical analysis. Student’s *t* test and one-way analysis of variance (ANOVA) were performed using Statcel (OMS, Saitama, Japan). *P* < 0.05 or *P* < 0.005 was set as indicating a statistically significant difference.

Results

Suppression of protective immunity against malaria parasites during pregnancy. We investigated the effect of immunization with GFP-expressing *P. berghei* XAT on pregnancy outcome. After confirmation that *P. berghei* XAT parasites were not present in the peripheral blood of female mice on day 30 postinfection, mice were mated with male mice for 1 day and pregnancy outcome was determined. Although mice immunized with GFP-expressing *P. berghei* XAT showed increased parasitemia in the late phase of pregnancy (Fig. 1A), pregnancy outcome was similar to that in pregnant uninfected mice.

To establish a mouse model of immunopathology, such as liver injury caused by maternal malaria, and placental pathology, mice immunized by infection with GFP-expressing *P. berghei* XAT (IM) with or without a vaginal plug were challenged/infected with lethal mCherry-expressing *P. berghei* NK65 parasites. As shown in Fig. 1B, nonpregnant IM mice infected with mCherry-expressing *P. berghei* NK65 (nonpregnant IM + NK) showed low levels of parasitemia; *P. berghei* NK65 parasites were completely eliminated within 30 days postinfection. In contrast, parasitemia of pregnant IM mice infected with mCherry-expressing *P. berghei* NK65 (pregnant IM + NK) increased significantly from day 14 postmating. Mice immunized with GFP-expressing *P. berghei* XAT showed recrudescence during late pregnancy (Fig. 1A). Therefore, it is possible that GFP-expressing *P. berghei* XAT would increase in pregnant IM + NK mice in late pregnancy. However, GFP-expressing *P. berghei* XAT was not increased during infection (Fig. 1C and Table 1). On day 16 postmating, the hematocrit of pregnant IM + NK mice was significantly lower than those in pregnant mice.
uninfected, pregnant IM, and nonpregnant IM+NK mice (Fig. 2A). In plasma from pregnant IM+NK mice on day 16 postinfection, the P. berghei-specific IgG level was lower than that in pregnant IM and nonpregnant IM+NK mice (Fig. 2B), suggesting that the protective immunity induced by primary infection with attenuated parasites was suppressed by pregnancy.

Development of placental pathology in late pregnancy. Fetus weight and the number of fetuses in the uterus of pregnant IM+NK mice were significantly reduced compared with those of pregnant uninfected and pregnant IM mice (Fig. 3A and B). Accumulation of pRBCs and a decreasing number of vascular branches were observed in the placentas of pregnant IM+NK mice on days 16 to 18 postmatting but not in pregnant uninfected and pregnant IM mice (Fig. 3C to H). In the placentas of pregnant IM+NK mice on day 16, the proportion of pRBCs tended to be higher than that in peripheral blood (Table 2). These results suggest that our mouse model reflected the severe placental pathology in pregnant women living in areas where malaria is endemic.

Development of maternal pathology in mice challenged with P. berghei NK65 in late pregnancy. To investigate whether pregnant IM+NK mice develop liver injury, we assessed plasma levels of AST, ALT, and glucose. Levels of AST and ALT in pregnant IM+NK mice on day 16 postmatting were higher than those in nonpregnant uninfected, pregnant uninfected, and nonpregnant IM+NK mice (Fig. 4A and B). In contrast, levels of glucose in pregnant IM+NK mice on day 16 postmatting were lower than those in nonpregnant uninfected, pregnant uninfected, and nonpregnant IM+NK mice (Fig. 4C). We performed a histopathological analysis of liver tissue (Fig. 5). Hepatic pathology was not observed in pregnant uninfected (Fig. 5A) or pregnant IM (Fig. 5B) mice in late pregnancy. Notably, extensive necrotic regions were observed in liver tissue from pregnant IM+NK mice on day 16 postmatting (Fig. 5C). The incidence of liver injury in pregnant IM+NK mice was ~75% on day 18 postmatting (Fig. 5D).

Accumulation of fat in hepatocytes in pregnant mice challenged with P. berghei NK65. By histopathological analysis at higher resolution with H&E staining, cytoplasmic microvesiculation was observed in liver tissue from pregnant IM+NK mice

| TABLE 1 Proportion of GFP-expressing P. berghei XAT in peripheral blood from IM+NK micea |
|-------------------------------|-------------------|-------------------|
| Day postinfection | Nonpregnant mice | Pregnant mice |
| | Total | P. berghei XAT | Total | P. berghei XAT |
| 12 | 0.35 ± 0.44 | 0.0030 ± 0.0001 | 0.09 ± 0.03 | 0.0230 ± 0.0002 |
| 15 | 0.12 ± 0.02 | 0.0002 ± 0.0000 | 0.54 ± 3.97 | 0.0122 ± 0.0001 |
| 19 | 0.01 ± 0.00 | 0.0000 ± 0.0000 | 53.3 ± 25.27 | 0.0000 ± 0.0000 |

a Total parasitemia was determined by microscopy as described in Materials and Methods. The proportion of P. berghei XAT-infected RBCs to total RBCs (P. berghei XAT parasitemia) was determined with a FACSCalibur cytometer as described in the legend to Fig. 1C. Note that a slight number of P. berghei XAT parasites existed on day 12 but that P. berghei XAT is not increased during P. berghei NK65 challenge infection. Results are expressed as means ± standard deviations of 5 to 8 mice.
anti-inflammatory cytokine IL-10 (Fig. 7). In plasma from pregnant IM+NK mice on day 16 postmatting, IFN-γ levels were significantly increased compared with those in pregnant uninfected, pregnant IM, and nonpregnant IM+NK mice (Fig. 7A). IFN-γ induces expression of inducible NO synthase mRNA and increases NO production by macrophages (28); therefore, we assayed plasma NO levels. Levels of NO in pregnant IM+NK mice on day 16 postmatting were higher than those in pregnant uninfected and nonpregnant IM+NK mice (Fig. 7B). In contrast, IL-10 levels in pregnant mice were higher than those in nonpregnant mice but were not significantly different among pregnant uninfected, pregnant IM, and pregnant IM+NK mice (Fig. 7C).

**DISCUSSION**

Malaria during pregnancy, in addition to maternal illness or death, has been implicated in the occurrence of spontaneous abortion, stillbirth, premature delivery, and low birth weight (6, 29). Using murine malaria parasites, valuable findings with regard to pathogenesis of placental pathology and the mechanism by which pRBCs bind to placenta tissue have been reported (7, 8). In contrast to placental pathology, the detailed mechanisms of the pathogenesis of maternal malaria remain unclear. In this study, we evaluated a mouse model of the immunopathology and placental pathology of pregnant women living in regions where malaria is endemic and investigated the pathogenesis of maternal malaria using the model.

Pregnant mice immunized with GFP-expressing *P. berghei*

FIG 4 Severe liver dysfunction in pregnant immune mice challenged with *P. berghei* NK65. Plasma AST (A), ALT (B), and glucose (C) levels on day 16 postinfection. Asterisks indicate a statistically significant difference (P < 0.05 compared with pregnant uninfected mice). Experiments were performed in triplicate, and representative data are shown. Results are expressed as means ± standard deviations of 3 to 5 mice.

TABLE 2 Proportion of pRBCs in peripheral blood and placenta in pregnant IM+NK mice on day 16 postmatting

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Peripheral blood</th>
<th>Placenta 1</th>
<th>Placenta 2</th>
<th>Placenta 3</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>43.83</td>
<td>47.44 ± 3.42</td>
<td>62.59 ± 5.95</td>
<td>41.93 ± 13.47</td>
</tr>
<tr>
<td>2</td>
<td>60.72</td>
<td>57.87 ± 8.90</td>
<td>69.19 ± 6.26</td>
<td>52.55 ± 5.78</td>
</tr>
<tr>
<td>3</td>
<td>26.66</td>
<td>70.67 ± 9.93</td>
<td>54.30 ± 7.38</td>
<td>50.49 ± 2.67</td>
</tr>
<tr>
<td>4</td>
<td>19.69</td>
<td>56.06 ± 13.09</td>
<td>63.62 ± 5.76</td>
<td>53.46 ± 10.01</td>
</tr>
<tr>
<td>5</td>
<td>26.24</td>
<td>57.76 ± 6.96</td>
<td>57.99 ± 4.90</td>
<td>57.09 ± 6.75</td>
</tr>
</tbody>
</table>

* Calculation of the proportion of pRBCs in peripheral blood is described in Materials and Methods. The proportion of pRBCs in placenta was calculated as (number of pRBCs/100 RBCs) × 100 and expressed as means ± standard deviations of five fields of H&E-stained placenta sections. A significant difference was shown between peripheral blood (n = 5) and placenta (n = 75) (P < 0.005).

Upregulation of proinflammatory cytokines and NO in pregnant mice challenged with *P. berghei* NK65. The development of liver injury caused by *P. berghei* NK65 infection involves the production of proinflammatory cytokines, such as IL-12 and IFN-γ, and cytotoxic CD8+ T cells (18–20). On the other hand, it is shown that IL-10 plays a suppressive role for the development of liver injury (23). Therefore, we assessed the levels of IFN-γ and the
XAT were more susceptible to mCherry-expressing *P. berghei* NK65 challenge/infection than otherwise identical conditioned nonpregnant mice. Their parasitemia increased in the late phase of pregnancy. Our findings suggest that protective immunity is suppressed during pregnancy and are consistent with previous studies showing that mice immunized with *P. berghei* followed by drug cure showed parasitemia and placental pathology during pregnancy (8).

Increased parasitemia in mice immunized with *P. berghei* followed by drug cure during pregnancy was observed (30) and was shown to correlate with levels of IgG specific for variant surface antigens (VSAs) on the surface of pRBCs (31). Decreased levels of *P. berghei*-specific IgG were found in pregnant IM/H11001 NK mice. These results may suggest that the decreased levels of IgG were due to immunosuppression by pregnancy. However, it is possible that they could also be caused by reduced free antibody due to the high levels of parasitemia in these mice. On the other hand, levels of parasitemia in multigravidas have been shown to be suppressed compared with those in primigravidas during pregnancy, suggesting that the protective immunity may be strengthened by multiple gestations/infections (8, 30, 31).

Poor pregnancy outcomes such as a decreased number of fetuses and reduced fetus weight were observed in pregnant IM+NK mice. In placentas from pregnant IM+NK mice, the typical features of placental pathology in pregnant women living in areas where malaria is endemic were observed. Using murine malaria parasites, accumulation of pRBCs and decreased numbers of vascular branches in placenta tissue have been reported in pregnant mice infected with lethal malaria parasites (32). These results suggest that a severe placental pathology also develops in immune mice challenged with lethal malaria parasites.

In pregnant IM+NK mice, the proportion of pRBCs in placenta was higher than that in peripheral blood. These findings suggest that lethal parasites might remain viable and/or replicate in the placenta during pregnancy, and pregnant IM+NK mice subsequently show high levels of parasitemia and placental pathology. *P. falciparum* adhesion to chondroitin sulfate A (CSA) and hyaluronic acid (HA) is associated with accumulation of pRBCs in placenta and is a key feature related to pathology (32, 33). Protection from placental malaria in women may be mediated by antibodies to the VAR2CSA protein belonging to the clon-
the anti-inflammatory cytokine IL-10 than did nonpregnant mice. A previous study showed that IL-10 is involved in suppression of immunopathology, such as liver injury (23, 39) and experimental cerebral malaria (40, 41). However, it was also shown that suppression of immunopathology is mediated by CD4+ natural regulatory T cells but not IL-10 (42). These findings suggest that IL-10 might not be essential for suppression of immunopathology during pregnancy.

Microvesicular fatty infiltration was detected in liver tissue from pregnant IM+NK mice on day 16 postmating. Acute fatty liver of pregnancy (AFLP) and hemolysis, elevated liver enzymes, and low platelet count (HELLP syndrome) are associated with significant maternal and fetal morbidity and mortality (43, 44). In previous studies, pregnant women infected with P. falciparum were shown to develop hepatitis (45) or HELLP-like syndrome (46–48). The patients proceeded to spontaneous abortion, stillbirth, and premature delivery. Further study using our mouse model of malaria during pregnancy could help to elucidate the mechanism by which liver dysfunction develops in pregnant women infected with Plasmodium spp.

In this study, we demonstrated that during pregnancy, mice immunized with attenuated parasites, P. berghei XAT, became susceptible to virulent P. berghei NK65 challenge/infection due to suppression of their immune responses, and pregnant IM+NK mice developed liver injury during the late phase of infection. Moreover, pregnant IM+NK mice also showed placental pathology comparable to that of mouse models reported in two independent studies (7, 8). These findings suggest that pregnant women who have acquired protective immunity against malaria parasites develop immunopathology during P. falciparum infection. On the other hand, the mechanism by which accumulation of parasites in the placenta leads to immunopathology and placental pathology remains unclear. To reduce the risk of maternal illness and death, and poor pregnancy outcome, further study should aim to elucidate the cause of maternal death and the mechanisms of pathogenesis in pregnant women living in areas where malaria is endemic.

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S.M., M.N., and F.K. designed the study; S.M. and M.N. performed the research; M.K. contributed reagents/materials/analysis tools; S.M., M.N., S.-I.I., M.K., and F.K. analyzed the data; and S.M., M.N., and F.K. wrote the paper.

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