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Vitamin C Deficiency Fails to Protect Mice from Malaria

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Abstract: Nutritional deficiencies are frequent in malaria-endemic areas. It seems that micronutrient antioxidants play an important role in malaria parasite's proliferation. Thus, the effect of vitamin C deficiency on malaria infection was examined in mice. When vitamin C deficient mice, L-gulono-γ-lactone oxidase gene knockout mice which are unable to synthesize ascorbic acid, were infected with a lethal dose of *Plasmodium berghei* NK65-infected red blood cells, the knockout mice showed similar parasitemia kinetics and survival rates as wild-type mice. The results indicate that deficiency of vitamin C might not affect the development of the malaria parasite in mice.

Key words: free radical, malaria, vitamin C

The outcome of malaria infection is influenced by the host status, socio-cultural conditions, and the parasite condition [20]. Among them, it is believed that the host nutritional status plays an important role in the outcome of malaria infection [4, 18, 20]. In fact, 60% of malaria deaths in young children are attributable to undernutrition [3]. However, the relation between malaria symptoms and the nutrition condition still remains controversial. For example, vitamin E and selenium deficiencies might have protective effects [10, 21], while vitamin A, thiamine and pyridoxine deficiencies likely exacerbate the course of the infection [20]. However, the role of vitamin C deficiency on the outcome of malaria infection has not yet been fully elucidated. Ascorbic acid is a hydrosoluble non-enzymatic antioxidant, which is synthesized in the liver of most mammals from glucose. Guinea pigs and higher primates including humans lack the ability to synthesize vitamin C and must rely on efficient mechanisms for its absorption, transport, and maintenance within tissues. Newly absorbed ascorbate is distributed by blood into tissues [17]. Beside its antioxidant activity, vitamin C is involved in a number of cellular functions such as collagen biosynthesis and iron transport [9]. *In vitro* studies on the effect of ascorbate on the parasite stage of *Plasmodium* indicate that ascorbate enhances the development of young forms (ring stage), but it is detrimental to the advanced forms (schizont stage) [15, 16]. In addition, previous reports showed that mice deficient in ascorbate were more susceptible to bacterial or viral infection [6, 12] indicating the important role of vitamin C in the inflammatory response of the host.

In this report the role of vitamin C deficiency during malaria infection was examined using L-gulono-γ-lactone oxidase gene knockout mice (Gulo-knockout mice), which are unable to synthesize ascorbate [14].

Gulo-knockout mice [14], were purchased from Mutant Mouse Regional Resource Centers, Betheosda, MD, USA and established as a line with a C57BL/6J genetic background by backcrossing more than ten times. For

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breeding, Gulo-knockout mice were supplied with 300 mg/l of ascorbic acid (Sigma, St. Luis, MO, USA) in drinking water. Genotypes were determined by PCR analysis of DNA from tail tissue. DNA was extracted by using an automatic DNA isolation system machine (PI-5α, KURABO, Tokyo, Japan). PCR amplification was performed using Takara Ex taq (Takara, Shiga, Japan). The primers used were P2 (5'-CGCGCCTTAATTAAGGATCC-3'), P3 (5'-GTCGTGACAGAATGTCTTGC-3'), and P4 (5'-GCATCCCAGTGACTAAGGAT-3'). Reaction conditions consisted of a hot start of 95°C for 1 min, 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, followed by 72°C for 1 min. PCR products were visualized in 2% agarose gel, 330 bp for the mutant-allele and 230 for the wild-type allele, as described previously [14]. To determine vitamin C concentrations in mice, whole blood was collected by cardiac puncture at 4–12 weeks of age (n=4). Serum was obtained by centrifugation at 5,000 rpm for 5 min at 4°C, and the vitamin C concentration was assessed with a Ferric Reducing Ascorbate (FRASC) assay kit (Biovision, Mountain View, CA, USA). Hemoglobin concentration, packed cell volume and number of red blood cells were also recorded (n=6). Briefly, 5 μl of blood taken from the mouse tail vein were mixed with an isotonic buffer (Isotonac, MEK-510; NIHON KOHDEN, Tokyo, Japan) and analyzed with an automatic hematological analyzer (Celltac α, MEK-6358; NIHON KOHDEN). The mice used for the infectious experiments were supplied with tap water immediately after weaning. For the experimental infection, Gulo-knockout and C57BL/6J mice at 7 weeks of age (n=6), were intraperitoneally inoculated with 0.2×10^5 infected red blood cells (IRBCs) of *P. berghei* NK65. Parasitemia was monitored every other day by examination of Giemsa-stained blood smears collected from the tail vein. Survival rate was monitored every day after infection. For the reticulocyte count, 2 μl of whole blood taken from tail vein was mixed with 2 μl of brilliant crystal blue (Wako, Tokyo, Japan). Then, thin smears of sample were prepared, dried at room temperature and stained with Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany). The number of infected and uninfected reticulocytes as well as mature RBCs were counted under a light microscope. The animals used in

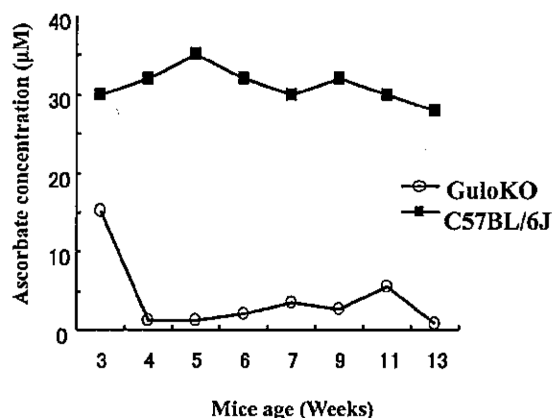

Fig. 1. Ascorbate concentration (μM) in serum of Gulo-knockout mice. Breeding pairs were supplied with 300 mg/l of ascorbic acid in drinking water. Gulo-knockout mice were maintained with tap water without ascorbate supplementation after weaning. n=4.

this study were housed in polycarbonate cages and maintained as specific pathogen-free animals in light/dark controlled environment (lights on from 5:00–19:00, lights off from 19:00–5:00) and air-conditioned rooms. The animal-room temperature was set at 24 ± 1°C with a humidity of 50 ± 10%. Mice had free access to standard laboratory chow (CE-2, CLEA Japan, Tokyo, Japan). The Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine reviewed the protocols and confirmed that the animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

Statistical analysis was performed using one-way variance analysis (S-plus6 software for Windows) (Insightful corporation, Seattle, WA, USA). Data are expressed as means of the standard error of the mean (SEM). *P*-values less than 0.05 were considered to be significant. For survival rates, data were analyzed by using the Kaplan Meier method.

After weaning at 21–25 days of age, when Gulo-knockout mice were supplied with tap water without vitamin C supplementation, ascorbate concentration in circulation of the knockout mice declined to residual levels at 4 weeks of age (Fig. 1). Hematological parameters of the Gulo-knockout mice remained stable within

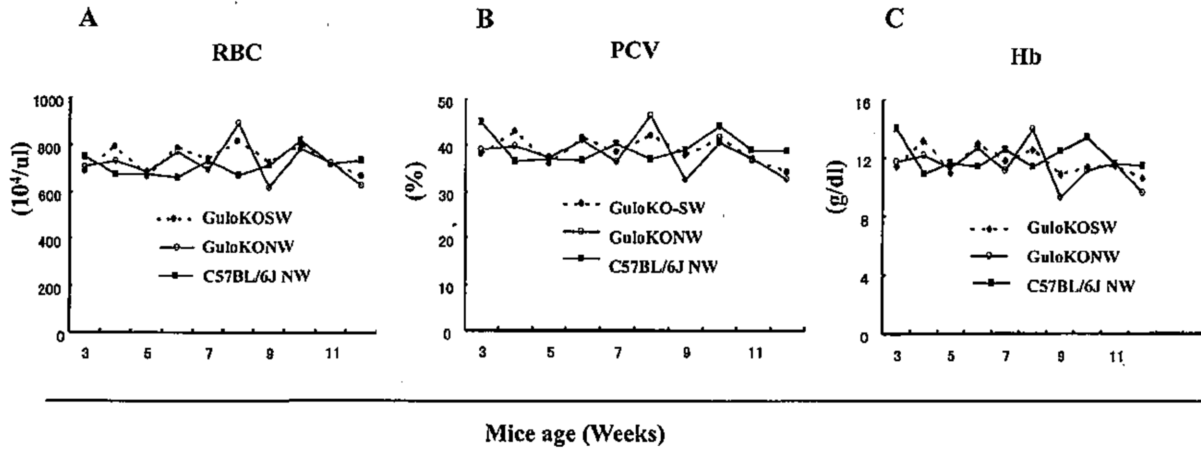

Fig. 2. Number of red blood cells (A), Packed cell volume (B), and hemoglobin concentration (C), of Gulo-knockout mice supplied with 300 mg/l of ascorbic acid (SW) or tap water (NW), and C57BL/6J mice supplied with tap water (NW). n=6.

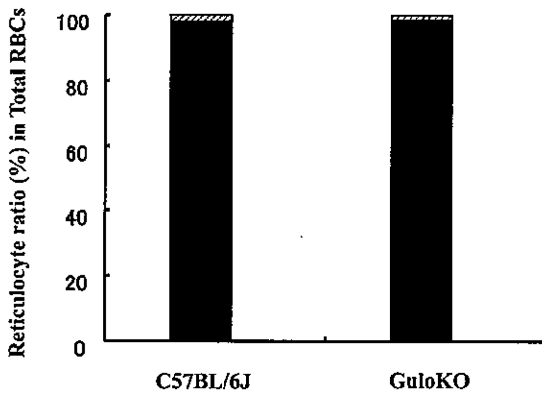

Fig. 3. Reticulocyte ratio in total RBCs in uninfected Gulo-knockout mice. n=6.

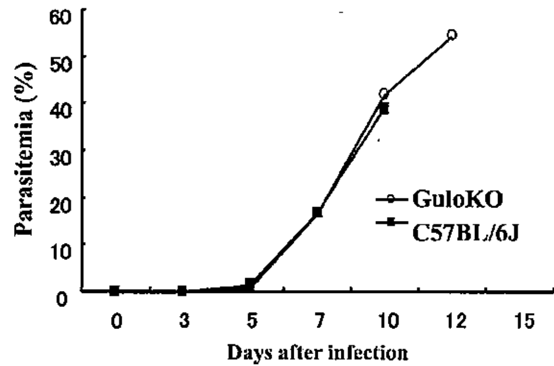

Fig. 4. Parasitemia kinetics of Gulo-knockout mice infected with 0.2×10^5 infected red blood cells (IRBCs) of *P. berghei* NK65. n=6.

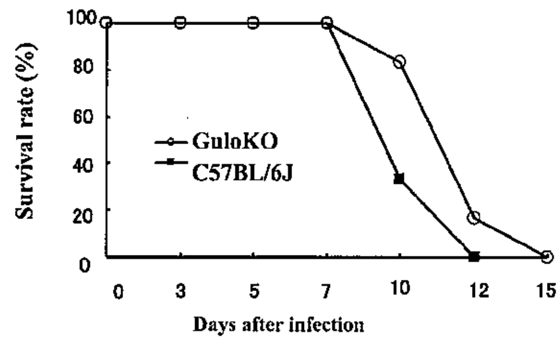

Fig. 5. Survival curve of Gulo-knockout mice infected with 0.2×10^5 IRBCs of *P. berghei* NK65. n=6.

normal ranges until 12 weeks old of age. Anemia was not observed in the Gulo-knockout mice (Fig. 2). Moreover, the ratio of reticulocytes in total RBCs, an indicator of the erythropoietic activity in the host, was not different between the genotypes (Fig. 3). Therefore, mice at 7 weeks of age were used for the infection experiments. As shown in Figs. 4 and 5, there were no significant differences in the parasitemia kinetics and survival rates between the Gulo-knockout and C57BL/6J mice after infection with *P. berghei* NK65. The first peak of parasitemia appeared on day 8 post infection, then the mice died until day 14 post infection in both groups. These results indicate that both groups had similar susceptibil-

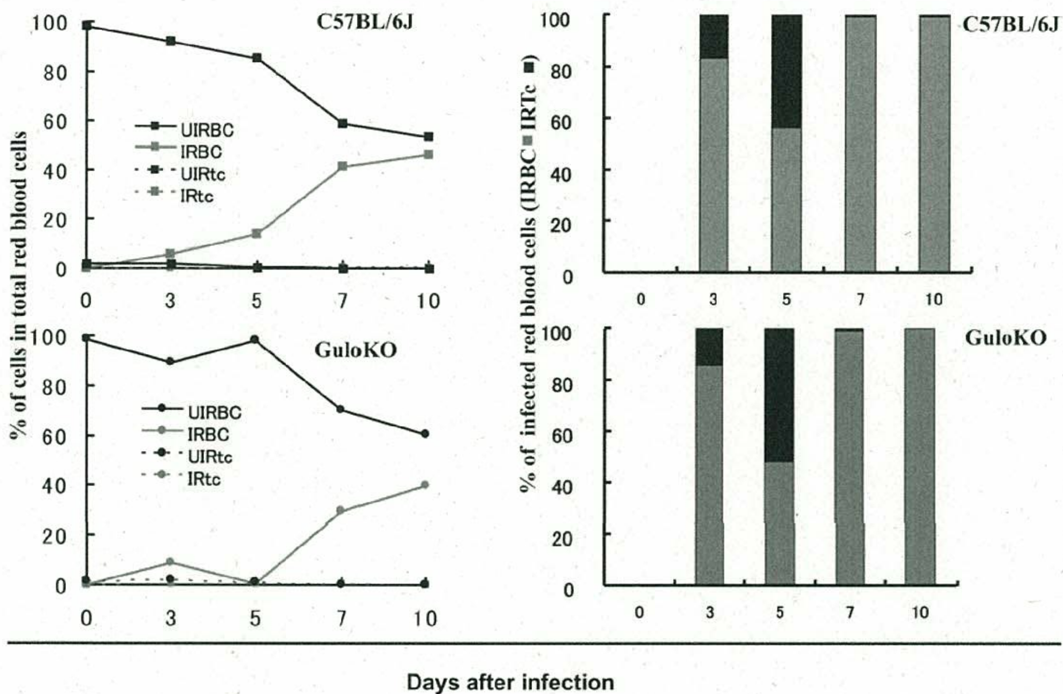

Fig. 6. Disposition of parasite invasion in RBCs of Gulo-knockout mice. Percentages of uninfected mature RBCs (UIRBC), IRBC, uninfected reticulocytes (UIRtc) and infected reticulocytes (IRtc) in total RBCs of C57BL/6J (upper left panel) and Gulo-knockout (lower left panel) mice, and percentages of infected mature RBCs (gray bar) and infected reticulocytes (black bar) in total infected RBCs of C57BL/6J (upper right panel) and Gulo-knockout (lower right panel) mice. n=6.

ity to *P. berghei* NK65 infection. In addition, the percentages of the infected reticulocytes as well as the infected mature RBCs in total infected RBCs were not different between the genotypes throughout the infection (Fig. 6). Parasite morphology and parasite life-cycle in 24 h was similar in both experimental groups (data not shown).

The role of vitamin C deficiency on the outcome of malaria infection has not been fully elucidated. To date actual knowledge is derived from *in vitro* models and field studies based on nutritional interventions in areas where malaria is endemic. *Plasmodium* susceptibility to ascorbate treatment *in vitro* has been demonstrated [15]. When a combined supplement of iron, thiamine, riboflavin, and vitamin C was given to children living in malaria-endemic areas, they developed parasitemias more severe than the infected children not given the supplemented diet [1]. Patients infected with *P. falciparum* showed higher concentrations of vitamin C and lower concentrations of vitamin E in serum than a healthy control group [7, 11]. On the other hand, it has been reported that vitamin C deficiency exacerbated malarial infection in monkeys [20]. In our present study, kinetics of vitamin C reduction in circulation were monitored because vitamin C can be rapidly recycled from its oxidized form, semidehydroascorbate, in the cytosol by *cytochrome b5* reductase and thioredoxin reductase or through spontaneous reaction with glutathione (GSH). This process may increase the antioxidant potential of the RBCs [13]. After withdrawal of vitamin C supplement, the concentration of ascorbate in circulation of the Gulo-knockout mice declined to residual levels within one week (Fig. 1). However, the residual levels of vitamin C might be the product of the recycling system mentioned above. Deficiencies of several antioxidants tend to protect against malaria infection due to higher *Plasmodium* sensitivity to the toxic effects of reactive oxygen species [5, 11, 13]. Therefore, it has been speculated that the absence of an important free radical scav-

enger such as vitamin C might trigger a redox imbalance in the RBC creating an environment that might be detrimental to the parasite's growth. However, parasite growth was not inhibited in the Gulo-knockout mice (Fig. 4), and parasitemia kinetics and survival rates in the Gulo-knockout mice were not significantly different from those of the wild-type animals ($P>0.05$). Moreover, the percentages of infected and uninfected reticulocytes were not different between the genotypes (Fig. 6). These results indicate that vitamin C deficiency did not induce nor accelerate anemia in the infected mice. *Plasmodium* appears to prefer to invade immature RBCs to avoid oxidative damage [5]; however, parasites infecting the Gulo-knockout and wild-type mice showed the same preference for reticulocyte and mature RBC invasion in the present study (Fig. 6). These results suggest that the contribution of ascorbic acid in the redox balance of the infected red blood cell is not essential for the development of the parasite *in vivo*. It has also been speculated that a parasite antioxidant system [8] would efficiently redress any redox imbalance provoked by vitamin C deficiency, and/or host compensate this deficiency by inducing several antioxidant systems [19]. Vitamin C as well as vitamin E possess strong free radical scavenger properties [2, 13]; however while vitamin E seems to be crucial for the parasite development and its deficiency is detrimental to parasite development [11, 21], vitamin C appears not to exert an effect on parasite development. Host and parasite ability to manage the redox imbalance may vary according to the antioxidant deficiency. In conclusion, although vitamin C is an important hydrosoluble antioxidant and its presence represents a major source of antioxidant activity in the host, its deficiency does not seem to affect the development of *P. berghei* NK65 in mice.

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