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Oral administration of vitamin D and importance in prevention of cerebral malaria

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ABSTRACT

Cerebral malaria (CM) is a serious and fatal malaria-associated syndrome caused by the development of an overwhelming proinflammatory response. Vitamin D (Vit.D; cholecalciferol) has regulatory functions associated with both innate and adaptive immune responses. Prevention is better than cure, in this experiment, we evaluated prophylactic oral Vit.D as a means of preventing CM presentation before infection of C57BL/6 mice with *Plasmodium berghei* ANKA (PbA) by modulating the host proinflammatory response. Mice that were supplemented with oral Vit.D has reduce death rate and ameliorated the integrity of the blood brain barrier. Prophylactic oral vitamin D relieved the symptoms of brain malaria and avoided death, gained valuable time for the diagnosis and treatment post infection. The robust Th1 response was attenuated in the Vit.D + PbA group. Furthermore, T-cell trafficking to the brain was diminished before PbA infection using Vit.D. The results suggest that Vit.D supplementation mediates the development of an anti-inflammatory environment that improves CM severity. In summary, the use of Vit.D as a nutritional supplement in malaria-endemic regions may help reduce the severity and mortality of CM.

1. Introduction

The World Health Organization (WHO) estimates that 216 million cases of malaria occurred globally in 2016, resulting in 445,000 deaths. An estimated 91% of all malaria deaths occurred in the WHO African Region, where children under 5 years old accounted for nearly 90% of these deaths [1]. It is well known that *Plasmodium falciparum* is the most prevalent malaria parasite in sub-Saharan Africa (SSA), which accounted for nearly 99% malaria infections [1]. As one of the most severe complications of the *Plasmodium falciparum* infection [2], cerebral malaria (CM) is a frequent cause of death and neurological disability among children in SSA [3]. More specifically, some studies indicate the median duration of disease onset is around 5 days [4,5]. However, most falciparum infection cases present clinical symptoms within one month or even a few months after exposure [5]. Treatment-seeking behaviour usually depends on the local economic status [6] and the health caregivers' educational level [7]. It causes most patients with *P. falciparum* being admitted after their clinical symptom appearances. Some patients do not even have a definite diagnosis until diagnosed with CM at the time of medico-legal autopsy [8]. Thus, prevention and control strategies aimed at preventing or ameliorating the severity of CM are vital to reduce childhood mortality and morbidity rates associated with *P. falciparum* infection. Furthermore, malaria and malnutrition are the top two causes of childhood morbidity and mortality in sub-Saharan Africa [9]. Some studies indicate that malnutrition has been suggested to influence susceptibility to and severity of malaria [10,11]. Studies of the relationship between malaria and nutrients have been focused on relatively common nutrients and micronutrients, such as vitamin A [12,13], iron [14–16], thiamine (vitamin B1) [17], vitamin D (VD; $1,25(OH)_2D_3$,$1,25D_3$) [18] and zinc [19], as well as protein energy malnutrition [20–22]. These studies demonstrate that nutrition may be an important factor in reducing the malaria morbidity and mortality.

Vit.D is an important micronutrient needed throughout life [23]. Vit.D insufficiency is a serious problem in southern Ethiopia and

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eastern African [24,25], where infectious diseases, such as malaria, HIV, and tuberculosis, are prevalent [26–33]. A recent study showed that Vit.D mediates the regulation of both innate and adaptive immune responses [34]. Specifically, Vit.D inhibits the membrane expression of MHC-II–complexed antigens and costimulatory molecules on antigen-presenting cells (APCs) [35,36], and inhibits the production of IL-12 and IL-23. In addition, Vit.D indirectly polarizes T-cells from Th1 and Th17 cells towards Th2 cells. These roles of Vit.D are of particular importance in the context of CM, which is associated with the development of a Th1 cell-mediated proinflammatory response. Regulatory T cells (Tregs) are critical in mediating immune responses elicited in response to infectious agents, such as viruses, bacteria, and protozoa [37–40]. Furthermore, Tregs help to regulate the polarized immune responses that occur as a result of CM. Therefore, induction of an appropriate and effective immune response to *Plasmodium* spp. after infection is needed for the subsequent control of this pathogen.

In recent study, intramuscular arteether-vitamin D combination may be the potential CM therapy [41]. Our team has also confirmed that oral administration of Vit.D after PbA infection can effectively inhibit the occurrence of experimental cerebral malaria (ECM) in mice [42]. However, the early intervention is critically important to ameliorate disease severity, since about 80% of the deaths take place within 24 h after acute CM patients are admitted [43]. Additionally, vector control, chemoprevention, and vaccination were recognised as the primary preventive measures to combat malarial infections [1]. We have a preliminary discovery of prophylactic oral vitamin D protecting the mice from early motality [42]. However, the preventive protecting mechanism of oral Vit.D is unclear. In this study, we focused on the preventive benefit of Vit.D on the ECM model. The results showed that Vit.D pre-treatment also significantly inhibited the pro-inflammatory immune responses associated with the development of ECM. This will provide a potential and beneficial strategy for the vitamin D prophylactic application and malaria preventive intervention.

2. Materials and methods

2.1. Mouse model of disease

Female C57BL/6 mice (6–8 weeks old) were purchased from the Beijing Animal Institute. PbA was kindly provided by Dr. Motomi Torii (Dept. of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan). Vit.D (cholecalciferol) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in soybean oil before use. Mice were randomly divided into three groups: mice that were uninfected by PbA, mice that were infected with PbA (PbA group), and then infected with PbA (Vit.D + PbA group). In parallel, the control group animals received the same volume of soybean oil. Infections were initiated by intraperitoneal injection of 1×10^6 PbA pRBCs. Mice in the Vit.D + PbA group were orally administered 50 μg/kg Vit.D before PbA infection once a day for 5 consecutive days. For the mortality and parasitemia experiments, 10 mice were used in each group. Parasitemia was monitored by counting the number of pRBCs per 1000 RBCs by light microscopy examination of Giemsa-stained thin blood smears [44]. Mortality was assessed daily. All experiments were performed in compliance with the local animal ethics committee.

2.2. Determination of cerebral pathology

When PbA-infected mice began presenting with neurological symptoms (usually at 5 days post infection (d.p.i.)), three mice from each group were sacrificed, and the integrity of the blood brain barrier (BBB) assessed. 200-μl 2% (wt/vol) Evans blue solution (Sigma, Lot# MKBH2094V) in phosphate-buffered saline (PBS) was injected intravenously into the mice. One hour later, mice were euthanized and the brains were isolated and incubated in 2 ml formamide for 48 h at 37 °C. The amount of Evans blue in 100 μl of brain tissue extracts was determined by measuring the absorbance at 630 nm [45].

Table 1
Primers sequences.

Target gene or mRNA	Primer 5′-3′	
	Forward	Reverse
β-Actin	GATTACTGCTCTGGCTCCTAGC	GACTCATCGTACTCCTGCTTGC
VCAM-1	CCTCACTTGCAGCACTACGG	CATGGTCAGAACGGACTTGG
ICAM-1	GGCAGCAAGTAGGCAAGGAC	CTGGCGGCTCAGTATCTCCT
CXCL9	CCGAGGCACGATCCACTACA	AGTCCGGATCTAGGCAGGTTTG
CXCL10	GCCGTCATTTTCTGCCTCAT	GCTTCCCTATGGCCCTCATT
CXCR3	AAGTGCCAAAGGCAGAGAAG	AAAGTCCGAGGCATCTAGCA
TNF-α	TATGGCTCAGGGTCCAACTC	CCCATTTGAGTCCTTGATGG
IFN-γ	TGATCCTTTGGACCCTCTGA	ACAGCCATGAGGAAGAGCTG

2.3. Total RNA extraction and real-time PCR

Total RNA was extracted from isolated brains and spleens by the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of total RNA were subjected to DNase I digestion. The total digested RNA was used for the reverse-transcription reaction with the appropriate oligo (dT) primers. One-fifth of the reverse-transcription reaction mixture was used for real-time PCR with primer sets specific for ICAM, VCAM, CXCR3, CXCL9, CXCL10, IFN-γ and TNF-α. PCR was performed with the SYBR Green PCR Master Mix for 40 cycles in an ABI PRISM 7700 apparatus (Applied Biosystems, Foster City, CA, USA). Primer sequences used here are described in Table 1. Threshold values were obtained by using the PE Biosystem software, and mRNA was quantified. The ratio of each target gene to the internal control (β-actin) was determined. Treated values were calculated on the basis of an untreated control (taken as 100%). The specificity of the PCR was confirmed by melt-curve analysis.

2.4. Measurement of cytokines by ELISA

For quantification of cytokines, splenocytes were harvested from mice and adjusted into 1×10^7/ml with RPMI-1640. Aliquots (5×10^6 cells/well) of the cell suspensions were seeded in 24-well flat-bottom tissue culture plates (Falcon) in triplicate in a humidified 5% CO_2 incubator. Levels of IFN-γ, TNF-α, and IL-10 in culture supernatants and plasma samples were measured by enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, MN, USA). Optical density (OD) values were measured at 450 nm using a microplate reader (Bio-Rad, USA). The concentration of cytokines in each sample was calculated via a standard curve generated using recombinant cytokines.

2.5. Flow cytometry analysis

To measure Th1-type cells ($CD4^+$ T-bet^+ IFN-γ^+), 10^7 fresh splenocytes cultured in RPMI-1640 were stimulated with 50 ng/ml PMA and 1 mM ionomycin (Sigma-Aldrich) in 12-well plates for 5 h at 37 °C and 5% CO_2 in the presence of 1 ml of brefeldin A (BD Biosciences) to inhibit cytokine secretion. Cells were harvested and their surfaces stained with FITC-conjugated anti-CD4 mAb (clone H1.2F3). Cells were fixed and permeabilized with an intracellular fixation kit (eBioscience), in accordance with the manufacturer's instructions. Then, cells were stained with anti-T-bet-PE (clone eBio4B10) and anti-IFN-γ-APC (clone XMG1.2). To identify Tregs, the PMA and ionomycin stimulated splenocytes were incubated with FITC-anti-CD4 and PE-anti-CD25 antibodies in 100 μl of PBS supplemented with 3% FCS. Cells were also stained for intracellular Foxp3 with APC-anti-Foxp3 antibody (clone FJK16s), as described above [46]. To determine the migration of $CD4^+$ and $CD8^+$ T cells to the brain, brain mononuclear cells were isolated from the brains on day 5 postinfection (p.i.) following a published procedure [47].