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Plasmodium yoelii: Influence of immune modulators on the development of the liver stage

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ABSTRACT

Plasmodium sporozoites suppress the respiratory burst and antigen presentation of Kupffer cells, which are regarded as the portal of invasion into hepatocytes. It is not known whether immune modulation of Kupffer cells can affect the liver stage. In the present study, we found that sporozoites inoculated into Wistar rats could be detected in the liver, spleen, and lung; however, most sporozoites were arrested in the liver. Sporozoites were captured by Kupffer cells lined with endothelial cells in the liver sinusoid before hepatocyte invasion. Pretreatment with TLR3 agonist poly(I:C) and TLR2 agonist BCG primarily activated Kupffer cells, inhibiting the sporozoite development into the exoerythrocytic form, whereas Kupffer cell antagonists dexamethasone and cyclophosphamide promoted development of the liver stage. Our data suggests that sporozoite development into its exoerythrocytic form may be associated with Kupffer cell functional status. Immune modulation of Kupffer cells could be a promising strategy to prevent malaria parasite infection.

1. Introduction

Malaria remains one of the most devastating diseases in the world, with 40% of the population at risk. Approximately 300–500 million new cases occur every year, resulting in 1–3 million deaths annually. After inoculation by the anopheles mosquito, *Plasmodium* sporozoites rapidly invade hepatocytes and transform into liver schizonts containing large numbers of merozoites; after release from hepatocytes, they infect erythrocytes, causing the symptoms of malaria. In contrast, the liver stage is clinically silent and regarded as a promising stage to prevent malaria.

Most sporozoites inoculated by an anopheles mosquito bite enter a blood vessel and move to the liver. Some sporozoites drain into a lymph vessel, but the trip is stopped in the proximal lymph node (Amino et al., 2006). Sporozoites were previously thought to pass through the fenestration of endothelial cells (Shin et al., 1982); however, growing evidence suggests that Kupffer cells interspersed among endothelial cells are the portal for sporozoites to invade hepatocytes (Barnwell, 2001). Intravital microscopy has demonstrated that sporozoites can actively invade Kupffer cells (Frevert et al., 2005). By confocal and electron microscopy, sporozoites found enclosed in a Kupffer cell vacuole did not co-localize with lysosomal markers, thus they avoid phagosomal acidification and are allowed safe passage through Kupffer cells (Pradel and Frevert, 2001). Kupffer cell deficiency in op/op mice decreases sporozoite infection by reducing the number of portals to the liver parenchyma (Baer et al., 2007). Target disruption of *SPECT*, a novel microneme protein, reduced the infectivity of sporozoites to the liver by inhibiting cell passage (Ishino et al., 2004). After safely passing through the Kupffer cells, sporozoites traverse several hepatocytes and develop into the exoerythrocyte stage in the liver (Mota et al., 2001).

The function of Kupffer cells is suppressed during sporozoite passage through the liver sinusoid. Secretion of interleukin (IL)-12 and up-regulation of costimulatory molecules, such as CD80 and CD86, were inhibited in Kupffer cells when infected with wild-type sporozoites; however, this did not occur after infection with irradiation-attenuated sporozoites (Steers et al., 2005). Very recently, the sporozoite circum-sporozoite protein (CSP) was shown to suppress the respiratory burst of Kupffer cell, and sporozoites were even able to induce Kupffer cell apoptosis and anti-inflammatory cytokine release (Klotz and Frevert, 2008; Usynin et al., 2007).

As the resident macrophages of the liver, Kupffer cells are poised to initiate the innate immune response against a variety of infectious agents, including bacteria (Vazquez-Torres et al., 2004) and viruses (Wu et al., 2007). However, it is unknown whether modulation of Kupffer cell function can influence the establishment of the *Plasmodium* liver stage. In the present study, we pretreated Wistar rats with immune modulators prior to inoculation with sporozoites to investigate their effect on the development of exoerythrocytic stage.

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2. Material and methods

2.1. Plasmodium and reagents

In our laboratory, *Plasmodium yoelii* BY265 are routinely passed through *Anopheles stephensi* (Hor strain). Sporozoites were purified by the density gradient centrifugation method (Pacheco et al., 1979) from *A. stephensi* at 14–17 days after infection with *P. yoelii* BY265. Polyinosinic:polycytidylic acid or polyinosinic–polycytidylic acid sodium salt (poly[I:C]), Bacillus Calmette-Guerin (BCG) lysate, dexamethasone, and cyclophosphamide were all purchased from Sigma–Aldrich.

2.2. Sporozoites labeling with ^{125}I

Purified sporozoites were labeled with ^{125}I (China Institute of Atomic Energy) according to the lactoperoxidase method (Karonen et al., 1975). Wistar rats ($n = 3$ per group) were injected with 5×10^5 viable ^{125}I-labeled sporozoites via tail vein. At each time point (2, 4, 6, 8, 10, and 18 h), the liver, lung, and spleen of three age and sex-match rats were immediately isolated after anesthetized; sporozoites were detected as counts per minute (cpm) using an FJ-2008 radiation detector. In addition, Wistar rats ($n = 3$ per group) were anesthetized and infused with ^{125}I-labeled sporozoites through the liver portal vein or right ventricle at 3 ml/min and 7 ml/min. The eluted sporozoites were collected through the precaval vein; the arrest rate of sporozoites by liver, lung, and spleen was calculated as:

$$\frac{\text{Input of sporozoites (cpm)} - \text{output of sporozoites (cpm)}}{\text{input of sporozoites (cpm)}} \times 100\%.$$

2.3. Cryo-scanning electron microscopy

After administration of 5×10^6 sporozoites through the portal vein, Wistar rat ($n = 3$ per group) livers were removed at 10, 20, and 30 min and immediately infused with 15 ml Minimum Essential Medium to remove red blood cells. The livers were then fixed in 3% glutaraldehyde, cut into small pieces, and re-fixed in 2% glutaraldehyde. After overnight fixation at 4 °C, liver were soaked in 12.5%, 25%, and 50% ice-cold dimethyl sulfoxide (DMSO) for 1 h in sequence, and dehydrated in a series of increasingly concentrated ethyl alcohol dilutions. Finally, cryofractured livers were visualized under JSM-T-300 scanning electron microscope to observe the sporozoite journey through the liver.

2.4. Pretreatment of immunomodulators

Wistar rats ($n = 5$ per group) were injected with PBS as control or poly(I:C) (5, 10, and 20 mg/kg), BCG (2×10^7, 4×10^7, and 6×10^7 U), dexamethasone (1.5, 2, and 5 mg/kg), and cyclophosphamide (200 mg/kg). Twenty-four hours later, purified sporozoites (5.39×10^5 for BCG- and poly(I:C)-treated rats; and 2.25×10^5, 4.0×10^5, 6.2×10^5, or 1.2×10^6 for dexamethasone- and cyclophosphamide-treated rats) were injected into the tail vein of each rat. The effect of immunomodulators was then assessed by of exoerythrocytic form (EEF) quantitation.

2.5. Quantitation of Plasmodium exoerythrocytic stage

Livers were isolated from Wistar rats 48 h after sporozoite inoculation, and then fixed in Carnoy's fixative, embedded in paraffin wax, cut into 5-μm thick sections, and stained with Giemsa. The *Plasmodium* exoerythrocytic form was counted in a continuous section (at least 800 mm^2) under light microscopy, and the percentage of EEF development was calculated according to the following formula:

$$\text{Percentage of EEF development} = \frac{\text{volume of fresh liver} \times (1 - \text{shrink rate}) \times \text{number of EEF}}{\text{detected area} \times 0.0005\ \text{cm} \times \text{number of inoculated sporozoites}} \times 100\%.$$

The degree of shrinkage during fixation and embedding was calculated as: $\frac{\text{volume of dehydrated liver}}{\text{volume of fresh liver}}$, the mean shrink rate in present experiment was 31.5%.

2.6. Statistical analysis

All data were analyzed by paired-sample-*t*-test with SPSS12.0 software. Data are expressed as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Distribution of sporozoites in liver, lung, and spleen

Although it is well known that sporozoites specifically target the liver, little is known about the distribution of sporozoites in other organs, such as the spleen and lung. We injected **^{125}I**-labeled sporozoites and determined their distribution in the liver, lung, and spleen from 2 to 18 h after injection. At 2 h after inoculation, most sporozoites were deposited ($P = 0.0002$) in the liver (1.68 ± 0.15%) compared with lung and spleen (0.55 ± 0.34% and 0.23 ± 0.08%, respectively) (Fig. 1). At 18 h after inoculation, almost no signal was detected in either lung or spleen, but obvious signal were still present in the liver (0.78 ± 0.48%) (Fig. 1).

To observe by which sporozoites were retained in the liver, Wistar rats were anesthetized, and both liver and lung were immediately infused with sporozoites at different flow rates through the liver portal vein or right ventricle, respectively. When the infusion flow rate was increased from 3 ml/min to 7 ml/min, sporozoites were not significantly reduced in the liver (81.61 ± 6.33% vs 81.34 ± 11.03%, $P = 0.94$); however, approximately half of the sporozoites were lost in the lung (67.39 ± 2.60% vs 33.74 ± 9.26%, $P = 0.035$) (Table 1), indicating that sporozoites arrested in liver were more resistant to infusion than those in the lung.

3.2. Arrest of sporozoites by Kupffer cells in liver

Cryo-scanning electron microscope was used to visualize the passage of sporozoites in the liver after inoculation of sporozoites

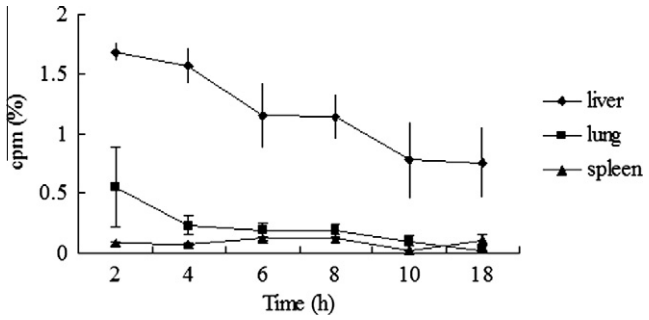

Fig. 1. Differential distribution of sporozoites in liver, lung, and spleen. After inoculation of 5×10^5 viable ^{125}I-labeled sporozoites through the tail vein of Wistar rats, sporozoites were detected in liver (♦), lung (■), and spleen (▲) at 2, 4, 6, 8, 10, and 18 h. Three rats were used for each time point; three individual experiments were performed. Data are expressed as mean ± SEM. $P = 0.002$.