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Original Article

Malaria in cynomolgus monkeys used in toxicity studies in Japan

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Abstract: *Plasmodium spp.* protozoa cause malaria and are known to infect humans and a variety of animal species including macaque monkeys. Here we report both our experience with malaria recrudescence in cynomolgus monkeys (*Macaca fascicularis*) in a toxicity study and the results of a survey on *Plasmodium* infection in cynomolgus monkeys imported to Japan for laboratory use. A cynomolgus monkey from the toxicity study presented with severe anemia and *Plasmodium* protozoa in erythrocytes on a thin blood smear and was subsequently diagnosed with symptomatic malaria. In this animal, congestion and accumulation of hemozoin (malaria pigment) in macrophages were noted in the enlarged and darkly discolored spleen. As a follow-up for the experience, spleen sections from 800 cynomolgus monkeys in toxicity studies conducted between 2003 and 2013 were retrospectively examined for hemozoin deposition as a marker of *Plasmodium* infection. The origin of the animals included Cambodia, China, Indonesia, and Vietnam. Hemozoin deposition was confirmed in 44% of all examined monkeys. Monkeys from Indonesia showed the highest incidence of hemozoin deposition (approx. 80%). A high prevalence of *Plasmodium* infection in laboratory monkeys was also confirmed with polymerase chain reaction (PCR) by using *Plasmodium* genus-specific primers. Although Japan is not a country with endemic malaria, it is important to be aware of the prevalence and potential impact of background infection with *Plasmodium spp.* and recrudescence of symptomatic malaria in imported laboratory monkeys on pharmaceutical toxicity studies. (DOI: 10.1293/tox.2015-0051; J Toxicol Pathol 2016; 29: 31–38)

Key words: cynomolgus monkey, hemozoin, histopathology, *Macaca fascicularis*, malaria, *Plasmodium*

Introduction

Protozoa of the genus *Plasmodium* (*Plasmodium spp.*) are known to cause malaria in a variety of animals. Primates are known to be infected by more than 20 species of *Plasmodium spp.*[1, 2]. Macaques are one of the most commonly used nonhuman primates in preclinical safety studies, and among the species of malaria that are known to naturally infect macaques, *P. cynomolgi, P. semiovale* and *P. fieldi* infections are known to result in latent hepatocellular forms (hypnozoites) that can lead to "relapse". In addition, *P. inui* may persist in the erythrocytic form in cynomolgus monkeys for many years as a chronic and asymptomatic infection[1, 3]. Because cynomolgus macaques are known to be resistant to the plasmodial disease, acute clinical malaria is rare[4], and imported cynomolgus macaques are frequently infected subclinically with *Plasmodium*[1, 5, 6].

Since standard thin blood smears do not routinely identify latent infection due to the low level of parasitemia, it is possible that nonhuman primates with latent malaria infection are used in laboratories as "normal" animals[7]. In fact, in the United States, recrudescence of malaria has been identified in cynomolgus monkeys used in a 3-month toxicity study of an immune-modulating monoclonal antibody[7]. We also experienced recrudescence of malaria in a 3-month toxicity study of a drug, which is not an immune modulator, conducted in Japan, and we report the clinical and histopathologic characteristics in this paper.

There are limited reports about the prevalence of *Plasmodium spp.* infection in laboratory cynomolgus monkeys, possibly due to the fact that Japan is not a malaria endemic area and the fact that Japanese macaques are normally malaria-free[8, 9]. To investigate the status of *Plasmodium spp.* infection in cynomolgus monkeys in toxicity studies in Japan, we conducted a retrospective analysis by using splenic deposition of the malarial pigment hemozoin as an indicator of some historical level of *Plasmodium* parasitemia in individual animals. Malarial pigment or hemozoin is a crystalline insoluble substance produced and excreted by the parasites when they digest hemoglobin in red blood cells (RBCs) [10–12]. It is detected as small granules or clusters of granules in tissues or RBCs, which appear black-brown under normal light and birefringent under polarized light[3, 13]. It is known to continue to be present in tissues, predominantly in the spleen, even after the parasite has disappeared from the peripheral blood[14–16]. Thus it is considered an indicator of past malarial infection.

In this article, we report typical clinical and histopathologic findings of malaria recrudescence in cynomolgus monkeys and results of a retrospective survey of the *Plas-*

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modium spp. infection rate by using hemozoin as an indicator.

Materials and Methods

Case report

Thirty-two cynomolgus monkeys (3 to 6 years old, 16 males and 16 females, 4 animals/group/sex, imported from China) received oral administration of compound A (at 3 dose levels) or vehicle and were necropsied after a 13-week dosing period. Parameters evaluated included clinical signs, hematology (including thin blood smear examination) and macroscopic and microscopic pathology. Thin blood smears were stained with Wright stain, and RBCs were examined. Organs and tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and routinely processed for microscopic examination of hematoxylin and eosin (H&E)-stained sections. The levels of parasitemia were determined semiquantitatively by examination of blood smears.

In order to identify hemozoin, H&E-stained spleen sections were examined under normal and polarized light, and Berlin blue staining was also conducted. Hemozoin is usually ingested by tissue macrophages and present in the spleen, especially in the red pulp, for a long period. It is observed as intracellular black-brown small crystalline granules under normal light and birefringent granules under polarized light[16] in the H&E-stained sections and negative for Berlin blue staining. Hemozoin is morphologically different from hemosiderin or lipofuscin. Hemosiderin is an intracellular pigment characterized by light brown amorphous to small globules in macrophages, and lipofuscin is observed as an accumulation of brown fine granules in H&E-stained sections. Hemozoin is also different from melanin pigments, which are normally observed as yellowish to dark brown fine granules to globules in H&E-stained sections and are not observed in RBCs. Hemosiderin, lipofuscin and melanin pigments do not show birefringent under polarized light. The levels of hemozoin deposition in the spleen were categorized into 4 grades as follows: −, not observed, 1+, slight (a few foci of hemozoin deposition); 2+, moderate (multiple foci of hemozoin deposition with/without minimal lymphoid hyperplasia); and 3+, marked (diffuse deposition of hemozoin with lymphoid hyperplasia and congestion). Representative serial sections of the spleen were depigmented by pretreatment with 3% aqueous ammonia in 70% alcohol[17, 18] for 3 hours or 5% HCL in 95% alcohol overnight to discriminate hemozoin from other pigments, especially from formalin pigments. Discrimination of hemozoin from formalin pigment was accomplished by observation of the intracellular localization of the hemozoin pigment and by bleaching the pigment with ammonia–ethanol and alcohol hydrochloride acid, as described above. Formalin pigment is also bleached by ammonia-ethanol[17–19], but it is not reported to be easily bleached by 5% alcohol hydrochloride acid. In addition, the tissues examined in this study had been fixed in neutral buffered formalin and processed within a few days according to the recommended procedure to reduce formalin pigments[20].

Plasmodium spp. infection survey

H&E-stained spleen sections of 800 cynomolgus monkeys (406 males and 394 females, including 32 monkeys reported in the present case report section, 2–6 years old) used as control or treated animals in toxicity studies of various test articles conducted between 2003 and 2013 were examined under normal and polarized lights. The origins of the monkeys were China (407 monkeys), Cambodia (46 monkeys), Indonesia (135 monkeys), and Vietnam (212 monkeys). The findings were categorized into 4 grades depending on the degree of hemozoin deposition as above (from − to 3+).

PCR detection of a genus-specific small subunit ribosomal RNA gene of Plasmodium spp.

Thin blood smears stained with Wright Giemsa and spleen sections stained with H&E as well as whole blood samples stored at −80°C were collected from 11 male cynomolgus monkeys (derived from Cambodia) in a toxicity study. For polymerase chain reaction (PCR) detection of genus-specific small subunit ribosomal RNA (ssrRNA) gene of *Plasmodium spp.*, DNA was extracted from 200 microliters of whole blood using a DNeasy® Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) and stored at −20°C until use. The positive control DNA sample was extracted from the frozen liver of the animal with clinical malaria described in the case report section. DNA templates of whole blood samples collected from mice experimentally infected with *P. yoelii* and noninfected mice were also used as positive and negative controls, respectively. A nested PCR reaction was conducted by using genus-specific primers reported by Snigh *et al.*[21] (rPLU1, 5′-TCAAAGATTAAGCCATGCAAGTGA-3′, and rPLU5, 5′-CCTGTTGTTGCCTTAAACTTCC-3′, for forward and reverse primers in the 1st PCR and rPLU3, 5′-TTTTCTATAAGGATAACTACGGAAAAGCTGT-3′, and rPLU4, 5′-TACCCGTCATAGCCATGTTAGGCCAATACC-3′, for the forward and reverse primers in the 2nd PCR). For the 1st PCR reaction, 8 µL of template was added to 17 µL of reaction master mix containing 12.5 µL of THUNDERBIRD® SYBR® qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) and 6 pmol of each primer. Amplification was performed by using a Takara PCR Thermal Cycler (TP600, Takara Bio Inc., Otsu, Japan) with the following thermal profile: 94°C for 1-min; 40 cycles of 15 sec at 94°C, 15 sec at 60°C and 30 sec at 72°C; 7 min at 72°C. Two microliters of the 1st amplification product was applied to the 2nd PCR reaction as the DNA template, and amplification was performed with the following thermal profile for PCR: 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C. The PCR products were confirmed with electrophoresis using agarose gel.

All experiments were performed according to the Eisai Animal Care and Use Committee's approved protocols.

Table 1. Changes in Hematologic Parameters after 13 Weeks of Dosing

Sex	Parameter/ groups	RBCs (106/μL)	Hemoglobin (g/dL)	Hematocrit (%)	Reticulocytes (109/μL)	WBCs (103/μL)	Lymphocytes (10^3/μL)	Incidence of parasitemia		
								−	+	++
Males	Control	5.645 ± 0.319	14.20 ± 0.56	43.58 ± 1.72	60.75 ± 17.61	8.268 ± 1.630	4.870 ± 0.942	4	0	0
	Low	5.908 ± 0.140	13.68 ± 0.75	44.15 ± 2.48	66.38 ± 27.43	9.530 ± 2.884	4.760 ± 0.568	3	1	0
	Middle	5.357 ± 0.168	12.27 ± 0.35	41.07 ± 1.10	145.07 ± 77.14	25.667 ± 20.237	17.527 ± 16.087	2	2	0
	High	3.753 ± 1.911	9.10 ± 4.47	31.38 ± 14.92	181.70 ± 104.36	9.208 ± 3.034	4.655 ± 1.265	2	0	2
Animal with Symptom		0.89	2.4	9	116.9	2.76	8.04	NA	NA	1
Females	Control	5.248 ± 0.428	13.10 ± 0.95	41.53 ± 3.84	63.70 ± 36.19	12.683 ± 9.370	9.185 ± 7.803	4	0	0
	Low	5.208 ± 0.079	12.13 ± 0.67	40.10 ± 1.64	51.40 ± 7.07	10.095 ± 3.304	5.283 ± 1.618	4	0	0
	Middle	4.858 ± 0.288	11.55 ± 0.65	38.65 ± 2.00	71.65 ± 19.97	13.498 ± 8.965	8.518 ± 8.215	4	0	0
	High	4.370 ± 0.447	10.23 ± 0.94	36.20 ± 3.84	170.95 ± 43.76	26.175 ± 20.864	20.033 ± 19.656	3	1	0

The number of animals examined was 4/sex/group. Values are shown as the mean ± SD for each group and actual values for the animal with symptomatic malaria. Severity of parasitemia: −, no parasite observed in red blood cells (RBCs); +, a few RBCs infected with parasite; ++, many RBCs infected with parasite. NA = not applicable WBCs: white blood cells.

Results

Case report

In a 13-week toxicity study, one animal in the high-dose group showed marked decreases in body weight (−15% compared with the pre-dosing value) and decreased spontaneous activity in the last few days of dosing. Hematological examination revealed marked decreases in erythrocyte count (pre-dosing period, 6.05×10^6/μL; week 13, 0.89×10^6/μL) and hematocrit (pre-dosing period, 51.5%; week 13, 9.0%), indicating severe anemia (Table 1). Blood smear examination demonstrated the presence of hemoparasites morphologically consistent with *Plasmodium spp.* (Table 1 and Fig. 1). The spleen and liver were massively enlarged and darkly discolored at necropsy. Deposition of black-brown pigments was observed microscopically mainly in the macrophages in the spleen and Kupffer cells in the liver along with Berlin blue-positive hemosiderin (Fig. 2a–c) and was accompanied by lymphoid depletion in the spleen (Fig. 2a). The pigment, which consisted of clusters of black-brown granules, was birefringent under polarized light (Fig. 3a and b), negative for iron staining (Berlin blue, Fig. 3c) and depigmented by alkaline or acid bleaching (Fig. 3d–f). These findings were consistent with the characteristics of hemozoin (malaria pigment)[17, 18].

All other animals were clinically normal until scheduled necropsy. However, there were slight decreases in erythrocyte count and hematocrit with increased reticulocytes and *Plasmodium* parasitemia at the middle and high doses (Table 1). Dose-dependent increases in the incidence and amount of hemozoin pigment in the spleen, liver, and bone marrow with lymphoid hyperplasia and congestion in the spleen (Fig. 4b–d), consistent with typical findings in *Plasmodium* infection, were also noted in the middle- and high-dose groups.

A slight degree of hemozoin deposition was also observed in the spleen of some animals in the control and low-dose groups, indicating the animals in this cohort were subclinically infected by *Plasmodium spp.*

Plasmodium spp. infection survey

The incidences of hemozoin deposition in the spleen of 800 cynomolgus monkeys from Cambodia, China, Indonesia, and Vietnam are summarized in Table 2. Among the animals examined, the animal we reported in the case report section was the only one showing clinical anemia due to *Plasmodium* recrudescence. In animals with multifocal or diffuse hemozoin deposition (Grade 2+ [moderate] or 3+ [marked]), hemozoin pigments were easily observed, even by bright-field microscopy, as black-brown granules. Marked follicular hyperplasia or increased germinal centers indicating activation of lymphoid follicles with congestion in the red pulp were observed in some animals with moderate to marked hemozoin deposition but not in animals with fewer foci of hemozoin (Grade 1+). Hemozoin deposition was observed in 352 animals (44% of all animals examined). The hemozoin deposition rate was higher in monkeys from Indonesia (84%) than in monkeys from Cambodia (39%), China (29%) or Vietnam (47%). There were no apparent sex differences in the incidence of hemozoin deposition.

PCR detection of a genus-specific small subunit ribosomal RNA gene of Plasmodium spp.

In order to detect the presence of *Plasmodium spp.* protozoa in the peripheral blood, PCR of a genus-specific ssrRNA gene was conducted in 11 male monkeys together with examination of thin blood smears and hemozoin deposition in the spleen.

No *Plasmodium spp.* protozoa were identified in RBCs by examination of thin blood smears, but a slight (1+) degree of hemozoin deposition was noted in 2 animals (Table 3).

By PCR amplification of DNA template from whole blood samples, bands of the expected size (about 240 bp) were detected in all 11 animals (Fig. 5a).

No band was detected with the primers following amplification of DNA template of normal (noninfected) mice (Fig. 5b, mN lane, mouse negative control), but an expected band of about 240 bp was detected in mice experimentally infected with *P. yoelii* (Fig. 5b, mP lane, mouse positive control).

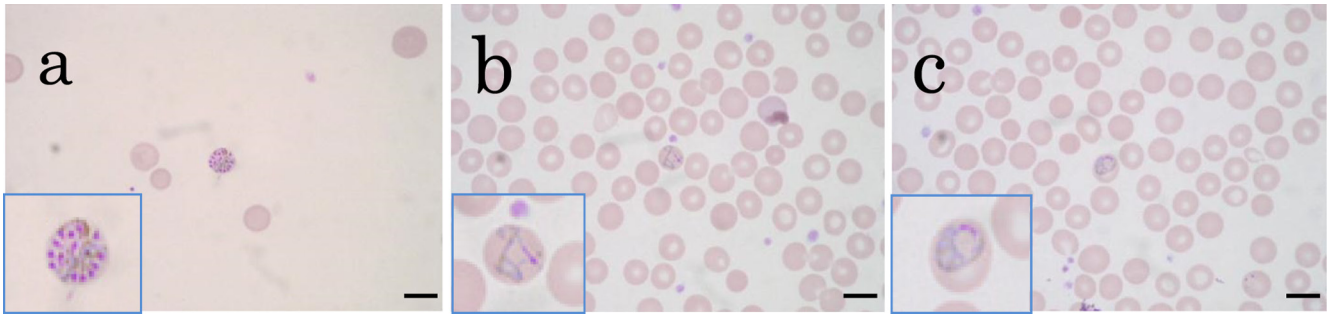

Fig. 1. Parasites in the red blood cells with characteristics of plasmodium spp. a, schizonts; b and c, trophozoites. Wright stain. Bars = 10 µm.

Discussion

Symptomatic malaria recrudescence was observed in one monkey used in a 13-week toxicity study. In the study, dose-dependent decreases in erythrocyte parameters were observed with increases in reticulocytes, incidence or level of parasitemia (Table 1) and hemozoin deposition (Table 2). The clinical and histological findings, including anemia, the presence of parasites in RBCs, enlargements and dark discoloration of spleen and liver, and hemozoin (malaria pigment) deposition, were compatible with *Plasmodium spp.* infection[7, 22, 23]. In the symptomatic animal, lymphoid depletion was observed in the spleen and was considered to be due to poor condition under severe anemia. In other animals with increased hemozoin accumulation, congestion and lymphoid hyperplasia were observed, which were considered compatible with the typical response to *Plasmodium* infection. Although the morphological appearance of schizonts and trophozoites resembled *Plasmodium inui*[1], the actual species of the protozoa was not identified because the pattern of fever and species-specific serological or genetic examinations were unavailable.

Cynomolgus macaques are known to be resistant to plasmodial disease, therefore, acute clinical malaria is rare[4], and imported cynomolgus macaques are frequently subclinically infected with *Plasmodium spp.*[1, 5, 6]. In the study described herein, in which clinical malaria was observed in a high-dose animal, hemozoin deposition was observed in the spleen of control and low-dose animals without systemic or hematologic changes. It is most likely that the cohort of animals used in the study was subclinically infected with *Plasmodium* at a high incidence and that symptomatic malaria in the high-dose animal was due to recrudescence of the subclinical infection. In fact, plasmodial recrudescence in cynomolgus monkeys has also been reported in another 13-week preclinical toxicity study with an immunomodulatory monoclonal antibody that inhibits trafficking of T-lymphocytes and macrophages[7].

In the present case, there was no hematological or histopathologic evidence of test article-related immunosuppression (Table 1).

Changes indicative of increased turnover of erythrocytes, including slight decreases in RBC parameters with an increase in reticulocyte count, were also noted in a longer toxicity study with this compound and *Plasmodium*-free monkeys in which *Plasmodium*-free was confirmed by PCR examination (data not shown). Reticulocytes and young RBCs are more susceptible to invasion by some *Plasmodium* species than metabolically older RBC populations[24, 25]. Increased production of RBCs as a compensatory response to loss can boosts parasite counts[26]. It is also reported that erythropoietin-induced reticulocytosis at an early stage of infection augments *Plasmodium* parasite multiplication and results in lethal infection in experimentally infected mice[27]. The test article-related increase in RBC turnover observed in this study may therefore be related to or permissive of the observed plasmodial recrudescence. Further investigation will be needed to elucidate the pathogenesis of the observed recrudescence.

Our retrospective survey of hemozoin deposition revealed a high incidence of hemozoin deposition in the spleen (44%) of cynomolgus monkeys imported for toxicology studies in Japan. Although it is difficult to know the true origin of monkeys because they may be traded across several countries, the incidence was highest in monkeys imported from Indonesia (84%), followed by those imported from Vietnam (47%), Cambodia (39%) and China (29%). The incidence of hemozoin deposition was comparable to or higher than the reported *Plasmodium* infection rate in cynomolgus monkeys diagnosed by blood smear examination, PCR analysis or serum antibodies[8, 9, 28–30]. Since splenic hemozoin deposition is known to remain even after the erythrocytic form of the malarial parasite becomes undetectable in blood[15, 16], hemozoin detection in the spleen is thought to be an indication of some historical level of *Plasmodium* parasitemia, but not of actual parasitemia in monkeys at the time of examination. An examination of Giemsa-stained thick blood smears are reported to be better than that of thin blood smears for the detection of low levels of parasitemia and remain a standard method for the diagnosis of malarial infection in nonhuman primates; however, it requires significant technical skill. Other new diagnostic methods have not been thoroughly evaluated in nonhuman primates[31]. To elucidate the actual prevalence of *Plasmodium* infection in cynomolgus monkeys, large-scale examination of peripheral blood with a combination of these methods will be needed.

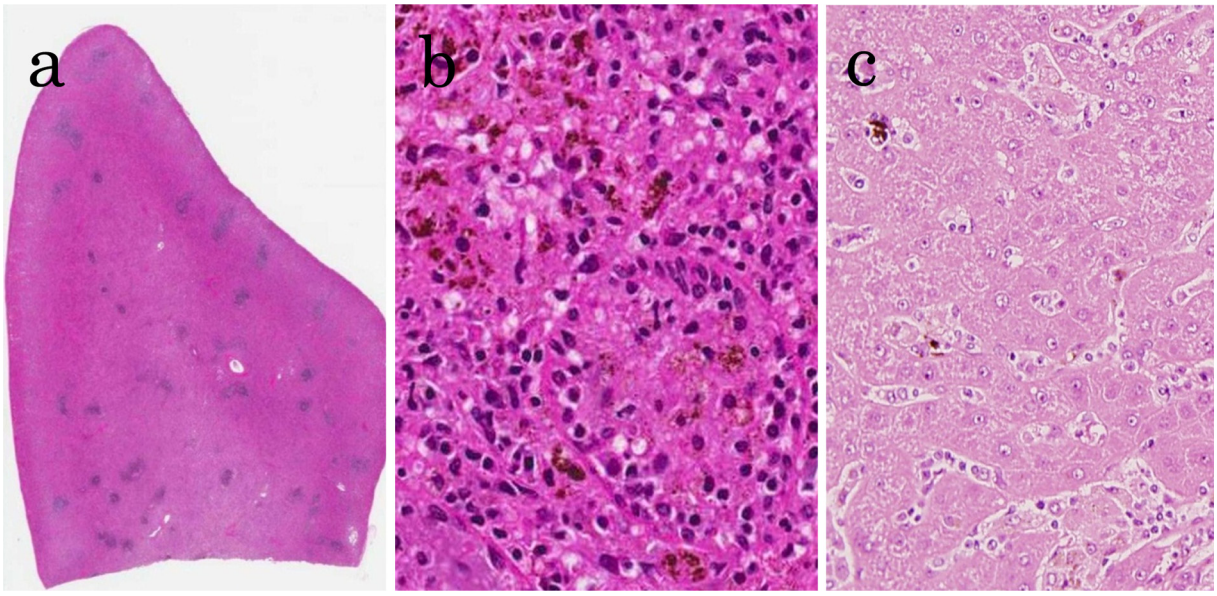

Fig. 2. Histopathology of the spleen (a and b) and liver (c) of the animal with symptomatic malaria. In the enlarged spleen, massive accumulation of macrophages in the red pulp and lymphoid depletion considered related to the poor physical condition in the animal were present (a). The macrophages in the red pulp contained blackish brown pigments (b). Blackish brown pigments were also observed in the hypertrophic Kupffer cells in the liver (c). H&E stain.

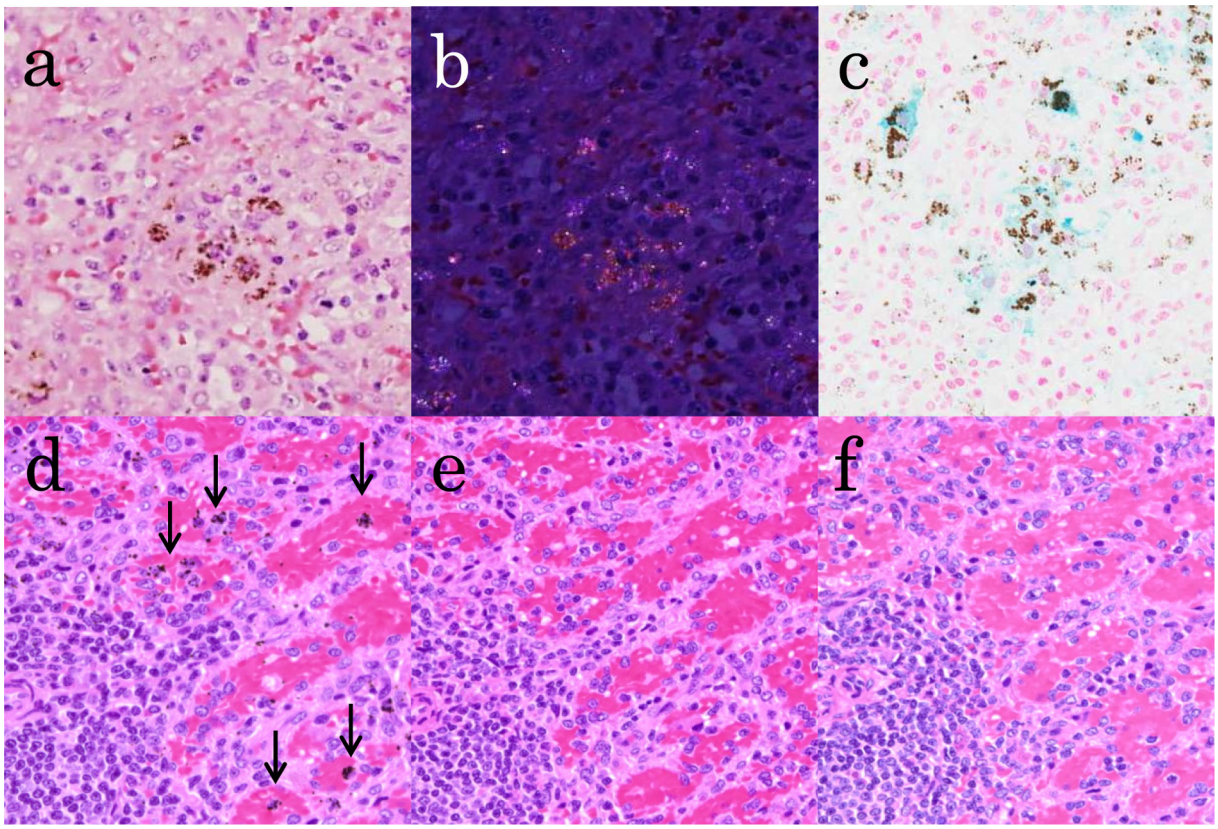

Fig. 3. Characteristics of hemozoin pigments in the spleen. The pigments were clusters of blackish brown granules under normal light (a). The same section and area as in a. The granules showed brilliant birefringent under polarized light (b). The hemozoin pigment was negative for iron staining and was observed along with Berlin blue–positive hemosiderin (c). It was depigmented by 3% aqueous ammonia in 70% alcohol (Kardasewitsch method) or 5% HCL in 95% alcohol (e and f). The same areas as in e and f without any digestion (d). Arrows = hemozoin. H&E stain (a, b, d–f) and Berlin blue stain (c).

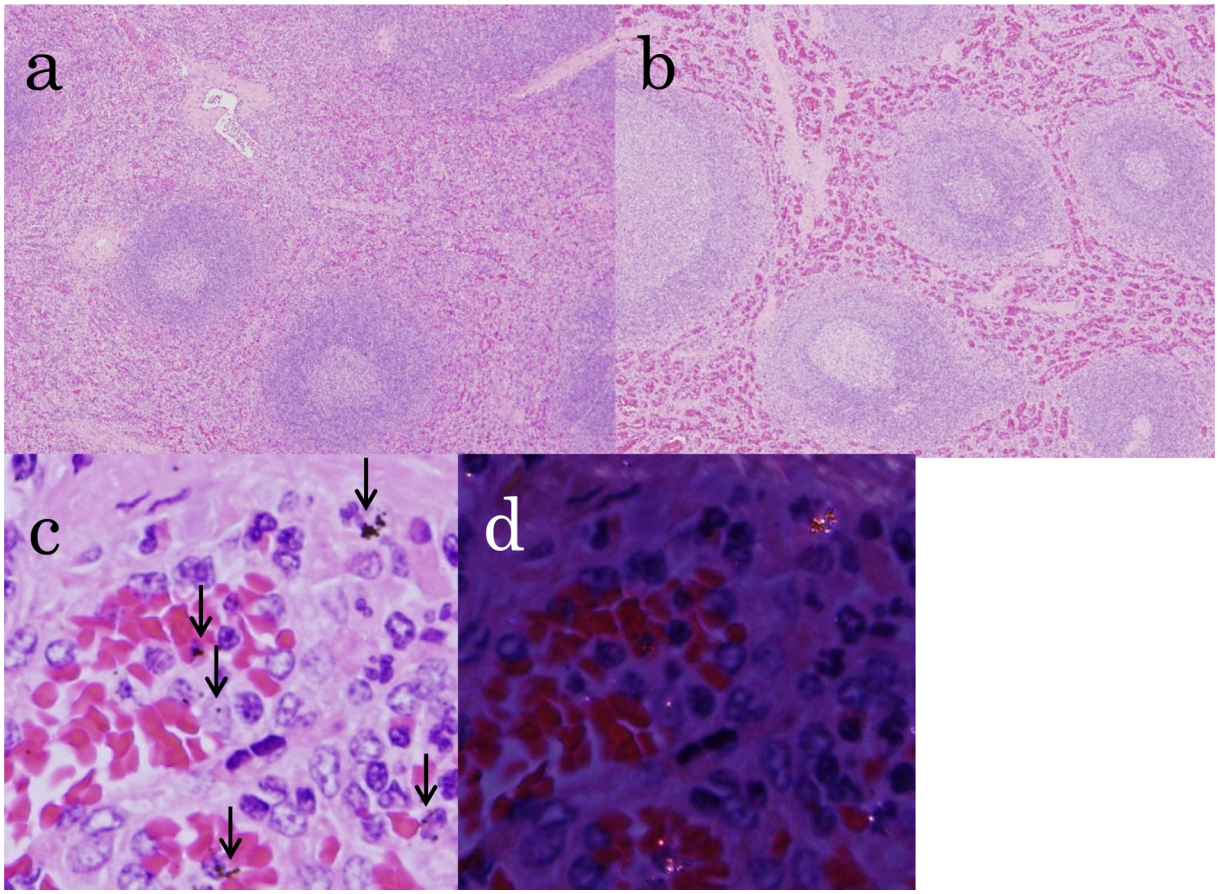

Fig. 4. Histopathology of the spleen of a control (a) and a middle-dose animal (b–d). In the middle-dose animal, lymphoid hyperplasia in the white pulp and congestion in the red pulp were observed (b) with hemozoin deposition in the red pulp under normal (c) and polarized (d) light. Arrows = hemozoin. H&E stain.

Table 2. Summary Results of the Survey of Plasmodium Infection by Hemozoin Deposition

Origin of monkeys	No. of animals examined (male/female)	Grade and incidence (male/female)				No. of animals with hemozoin (1+ to 3+)	%
		–	1+	2+	3+		
Cambodia	46 (30/16)	28 (21/7)	16 (8/8)	2 (1/1)	0 (0/0)	18	39
China	407 (204/203)	287 (133/154)	70 (40/30)	37 (23/14)	13 (8/5)	120	29
Indonesia	135 (68/67)	21 (10/11)	38 (20/18)	58 (27/31)	18 (11/7)	114	84
Vietnam	212 (104/108)	112 (47/65)	75 (40/35)	23 (15/8)	2 (2/0)	100	47
Total	800 (406/394)	448 (211/237)	199 (108/91)	120 (66/54)	33 (21/12)	352	44

−, not observed; 1+, slight (a few foci of hemozoin deposition); 2+, moderate (multiple foci of hemozoin deposition with/without minimal lymphoid hyperplasia); 3+, marked (diffuse deposition of hemozoin with lymphoid hyperplasia and congestion).

Table 3. Severity of Hemozoin Deposition in the Spleen and Plasmodium Spp Detection in Peripheral Blood by Nested PCR in Male Cynomolgus Monkeys

Animal No.	1	2	3	4	5	6	7	8	9	10	11	CP
Parasitemia	–	–	–	–	–	–	–	–	–	–	–	++
Hemozoin level	–	–	–	1+	1+	–	–	–	–	–	–	3+
Plasmodium 18s rRNA	+	+	+	+	+	+	+	+	+	+	+	+

CP = cynomolgus monkey positive control, Severity of parasitemia: −, no parasite observed in red blood cells (RBCs); +, a few RBCs infected with parasite; ++, many RBCs infected with parasite. Severity of hemozoin level: −, not detected; 1+, slight; 2+, moderate; 3+, marked. PCR results: +, expected-sized product observed.

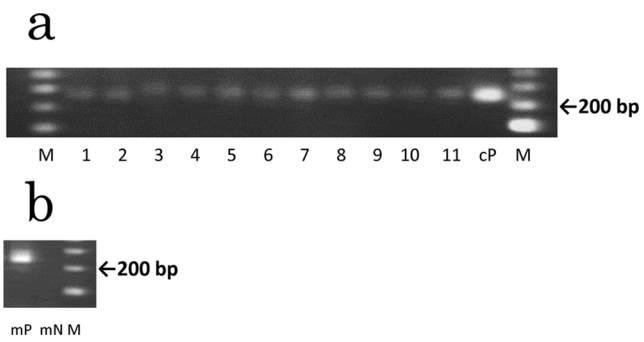

Fig. 5. Results of PCR amplification of genus-specific sequence of 18s rRNA of *Plasmodium* spp. The expected sizes of PCR products were detected with the primers designed by Snigh et al. (240 bp, a). The primers also detected experimentally infected *P. yoelii* in mice (b). M = molecular size marker (100 bp ladder), cP = cynomolgus monkey positive control, lane 1–11 = DNA samples from whole blood of the 11 monkeys, mP = mouse positive control, mN = mouse negative control.

In all 11 monkeys examined by PCR, *Plasmodium*-specific PCR products were detected, and 2 of them demonstrated the presence of splenic hemozoin. The reason why splenic hemozoin was observed in only 2 animals and not in the others is unclear, but a combination of degree and duration of parasitemia may be factors responsible for the level of hemozoin accumulation in the spleen. These results suggest that although no erythrocytic forms of the parasite were detected by thin smear examination, latent infection with low levels of *Plasmodium* parasitemia was present in this cohort of monkeys and persisted in some animals. To investigate the actual infection rate or prevalence of *Plasmodium spp.* in laboratory monkeys imported to Japan and the relationship of infection with splenic hemozoin, further investigation using more samples will be needed.

In conclusion, we reported our experience of recrudescence of malaria in one monkey used in a toxicity study. We also demonstrated a high incidence of splenic hemozoin deposition in laboratory monkeys imported to Japan, during the time period examined, as well as positive results from PCR amplification of a *Plasmodium* genus-specific ssrRNA gene in the peripheral blood of monkeys. All of these results together indicate that considerable numbers of cohorts of monkeys imported to Japan may be assumed to have some level of background infection with *Plasmodium spp.* It is important to be aware of these facts and to consider their potential impact on evaluation of the toxicologic properties of drugs.

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