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Original Papers

Visualization of Malaria Parasites in the Skin Using the Luciferase Transgenic Parasite, *Plasmodium berghei*

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Abstract: We produced a transgenic rodent malaria parasite (*Plasmodium berghei*) that contained the luciferase gene under a promoter region of elongation factor-1α. These transgenic (TG) parasites expressed luciferase in all stages of their life cycle, as previously reported. However, we were the first to succeed in observing sporozoites as a mass in mouse skin following their deposition by the probing of infective mosquitoes. Our transgenic parasites may have emitted stronger bioluminescence than previous TG parasites. The estimated number of injected sporozoites by mosquitoes was between 34 and 775 (median 80). Since luciferase activity diminished immediately after the death of the parasites, luciferase activity could be an indicator of the existence of live parasites. Our results indicated that sporozoites survived at the probed site for more than 42 hours. We also detected sporozoites in the liver within 15 min of the intravenous injection. Bioluminescence was not observed in the lung, kidney or spleen. We confirmed the observation that the liver was the first organ in which malaria parasites entered and increased in number.
Key words: imaging, luciferase, malaria, probing, skin stage, sporozoite

Introduction

Visualizing technology has improved our understanding of the biology of malaria parasites. Green fluorescent protein (GFP)-expressing malaria parasites have been produced and detected in the red blood cells, cultured liver cells, and midgut and salivary glands of the mosquito [1, 2]. Previous studies using GFP-expressing malaria parasites revealed how malaria parasites moved in the skin and invaded blood vessels during their migration to the liver [3, 4]. However, the fluorescence emitted by GFP was not strong enough to observe these parasites from outside of the animal.

To overcome this drawback, researchers have developed transgenic parasites that express luciferase; transgenic *P. berghei* expressing luciferase (TG-PbLuc) was initially developed [5, 6], followed by transgenic *P. yoelii*-expressing luciferase (TG-PyLuc) [7, 8]. Not only the blood stage, but also the liver stage in the development of malaria parasites could be observed using TG-PbLuc and TG-PyLuc, and the liver was confirmed as the first organ that malaria parasites reached, entered, and increased in number.

We succeeded in producing a similar TG-PbLuc and obtained results that were consistent with the findings of previous studies. However, we also found that our TG-PbLuc expressed stronger luciferase activity than that of the previous TG-PbLuc. When Anopheline mosquitoes infected with our TG-PbLuc sporozoites bit a mouse, luciferase activity could be detected in the skin of that mouse. When we injected 12,000 sporozoites intravenously into a mouse, luciferase activity in the liver could be detected from the outside within 15 minutes. We herein described how we followed TG parasites in the mouse body following the deposition of spozozoites.

Methods

Ethics statement

Animal experiments were performed in a humane manner after receiving approval from the Institutional Animal Experiment Committee of Jichi Medical University. Experiments were conducted in accordance with Institutional Regulations for Animal Experiments and the Funda-

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mental Guidelines for the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the Jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Mosquitoes and mice

The *Anopheles stephensi* Swiss 500 strain was reared in our laboratory [9]. BALB/c mice were purchased from SRL Japan (Shizuoka) and reared at 26°C with 60–70% relative humidity under a 13 h-light/11 h-dark cycle. The mice were anesthetized by a muscular injection of xylazine (5 mg/kg) (Bayer UK Limited, Bury St. Edmunds, UK) and pentobarbital (75 mg/kg) (Kyoritsu-seiyaku Co., Tokyo, Japan).

Transgenic malaria parasites

The *Plasmodium berghei* ANKA clone 2.34 [10], a rodent malaria parasite, was used as a template parasite. Regarding the firefly luciferase gene (Gene Bank ID AAV52871), we purchased the pGL4.11 luciferase reporter vector (Promega Co., Madison, WI, USA). The firefly luciferase gene was amplified by PCR using a pair of primers (Luc-BamF: ggggatccATGGAAGACGCCAAAAAC ATAAAG and Luc-BamR: ggggatccTTACACGGCGATC TTTCCGCCCTTC). The PCR product was digested by *BamHI* and ligated to the same restriction enzyme site in pL0011 (Malaria Research and Reference Reagent Resource Center, MR4 (http://www.malaria.mr4.org)). The plasmid was then digested by *ApaI* and integrated into the small subunit ribosomal DNA locus in *P. berghei* by single crossover recombination [11, 12]. The resultant transgenic parasite line, PbLuc expressed luciferase during all stages under the control of the elongation factor 1-α promoter [2].

Preparation of infective mosquitoes

A mouse was injected intraperitoneally with 2×10^6 of TG parasite-infected red blood cells. Three days later, the parasitemia of the mouse reached 1–3%. Female *An. stephensi* mosquitoes, which had emerged 5–7 days earlier, were allowed to feed on the anesthetized mouse for 30 min at 20°C. Unfed mosquitoes were removed, and the blood-fed mosquitoes were reared at 20°C.

Deposition of sporozoites into a mouse by infective mosquitoes

Three infective mosquitoes were placed in a 15 ml-plastic tube (Corning Incorporated, NY, USA), and the head of the tube was covered with gauze. Mice were anesthetized, and the hair on the abdomen was shaved. To encourage mosquitoes to feed at this spot, we applied rubber tape with a hole 3 mm in diameter on the abdomen of the mouse. Infective mosquitoes were allowed to feed through the gauze and the hole in the rubber tape. Only one mosquito typically occupied the place to feed during the experimental period. Blood feeding was not permitted because we raised the plastic tube every 12 seconds. Mosquitoes deposited saliva and sporozoites in the skin of the mouse, but could not feed on blood during the 12-second period. This was repeated 10 times, and as a result, sporozoites were deposited in a limited area in the abdominal skin of the mouse.

Detection of malaria parasites in mice using the *in vivo* imaging system (IVIS)

IVIS (Xenogen Co., Alameda, CA, USA) was used as described previously [5, 13]. After probing by infective mosquitoes or artificial injections, anesthetized mice were peritoneally injected with 2 mg of D-luciferin firefly (Biosynth Biochemica & Synthetica, Staad, Switzerland) and were placed in the IVIS camera box for five minutes to count the bioluminescence of luciferin. Emission was accumulated and intensity was expressed as color. If transgenic malaria parasites were deposited in the skin, luciferin bioluminescence was detected at the skin site as an emitting spot. We could not observe each parasite in the skin because of the diffusion of photons in the tissue. We estimated the number of parasites using the sum of the counts from bioluminescence around each site.

Collection of sporozoites

Mosquitoes were dissected 14 to 16 days after the infective blood meal from an infected mouse, and the salivary glands were removed. RPMI 1640 medium was used as a dissecting solution. Ten pairs of salivary glands were collected in a 1.5 ml-Eppen-tube and crushed with a pestle. The parasite burden was estimated by counting a part of the sample using a hemocytometer. Fifty to 200,000 sporozoites were typically collected from ten pairs of salivary glands.

Estimation of the number of sporozoites at probing sites

Different numbers of sporozoites (0, 100, 1,000, and 10,000) were prepared in 20 μl of RPMI 1640 medium. Sporozoites were injected into the skin of the abdominal area of anesthetized and shaved mice. Bioluminescence was measured at each site of artificial injection. Three equations were then prepared from the bioluminescence results. Sixteen mice were probed by infective mosquitoes through a hole 3 mm in diameter. The bioluminescence of the spots was measured and the number of sporozoites in the skin was estimated using these equations.

Heat treatment

A Kyu-kit was purchased from Sennen-Kyu Co., Ltd. (Tokyo, Japan), and heat treatment was performed as described previously [10]. Infective mosquitoes were allowed to probe through the 3-mm hole as described above (12 seconds × 10 times). We confirmed that sporozoites had been deposited in the mouse skin by IVIS. Kyu was then placed on the deposited site. Probing by infective mosquitoes took three minutes. We then injected luciferin into the mouse and placed it in the IVIS box in order to confirm the deposition of sporozoites. This procedure required nine minutes. After confirming that sporozoites stayed at the skin spot, the Kyu treatment was initiated. Increasing the appropriate temperature to weaken sporozoites took three minutes. Thus, 15 minutes were needed to deposit sporozoites and heat them in the skin. Ten mice were used in this experiment. As a control, Kyu was placed on a separate location in 6 mice.

Luciferase activity of PbLuc after the death of parasites

We adopted a sonication method to follow luciferase activity after the death of PbLuc parasites. Four Eppendorf tubes containing 4,000 PbLuc sporozoites in 0.8 ml of RPMI 1640 medium were prepared. One tube was sonicated for 1 second. A subsequent sonication of 1 second was applied 5 seconds later. This was repeated for a total of 5 times. To avoid increases in temperature, these procedures were conducted in an ice-water container. The next tube was treated 3 times, and the next was treated 1 time. The last tube was not sonicated. The tube content of 0.2 ml (1,000 sporozoites) was placed in three wells of a 96-well plate (Corning Incorporated, NY, USA). Bioluminescence was measured after the addition of 75 μg luciferin to each well.

Removing the skin probed by infective mosquitoes

One mouse was probed by an infective mosquito and the deposition of sporozoites was confirmed by IVIS. The skin at the proved site was then removed. As a control, an intact patch was removed from the abdominal skin. Bioluminescence was detected in both the removed skin and the mouse.

Injection of sporozoites into the tail vein of mice

Different numbers of sporozoites were prepared in 50 μl of RPMI 1640 medium. Sporozoites were injected into the tail veins of anesthetized mice. A total of 2 mg of luciferin was then injected and bioluminescence was observed by IVIS. Mice were subsequently killed and the liver, lung, kidney and spleen were removed. Each organ was cut into pieces and placed in each well of a 12-well plate (Corning Incorporated) with 0.8 ml of RPMI 1640 medium. Bioluminescence was recorded after the addition of 300 μg luciferin to each well.

Statistical analysis

Data were analyzed using the Student's *t*-test. Differences in rates between the two groups were evaluated by the chi-square test or Fisher's exact test. A p value of less than 0.05 was considered to be significant.

Results

Monitoring one mouse after probing by an infective mosquito

A mouse was monitored with the IVIS camera 0 h, 12 h, 24 h, 42 h, 72 h and 96 h after probing by an infective mosquito (Fig. 1). The probed site expressed bioluminescence after at least 42 h. This result suggested that some parasites remained alive for 42 hours after probing by the mosquito. The liver site became bright after 24 h and a strong emission was observed after 42 h. The whole body of the mouse expressed bioluminescence after 72 h, suggesting that parasites started circulating at the erythrocyte stage. A marked increase in luciferase activity was observed after 96 h; however, parasitemia of the peripheral blood was only 0.001% at this time point.

Estimation of the number of sporozoites at probing sites

We prepared different numbers of sporozoites and injected the mouse skin at the abdominal site. We found that the bioluminescence of each spot was dependent on the number of sporozoites injected. Even 100 sporozoites could be detected in the mouse skin (Fig. 2a). We measured the bioluminescence of these spots, then prepared three equations from the bioluminescence results (Fig. 2b).

We measured the bioluminescence of each spot probed by mosquitoes (16 mice) and estimated the number of sporozoites using the equations described in the Methods section. According to our estimation, the bite of one infective mosquito under the experimental conditions led to the deposition of between 34 and 776 (median 80) spotozoites in the skin (Fig. 2c).

Effects of heat treatment on malaria parasites

The mice that underwent heat treatment at the deposition site did not develop malaria parasites (Fig. 3a). However, the mice that underwent heat treatment at a site other than the deposition site developed malaria parasites in the body (Fig. 3b). Nine out of the 10 mice that underwent

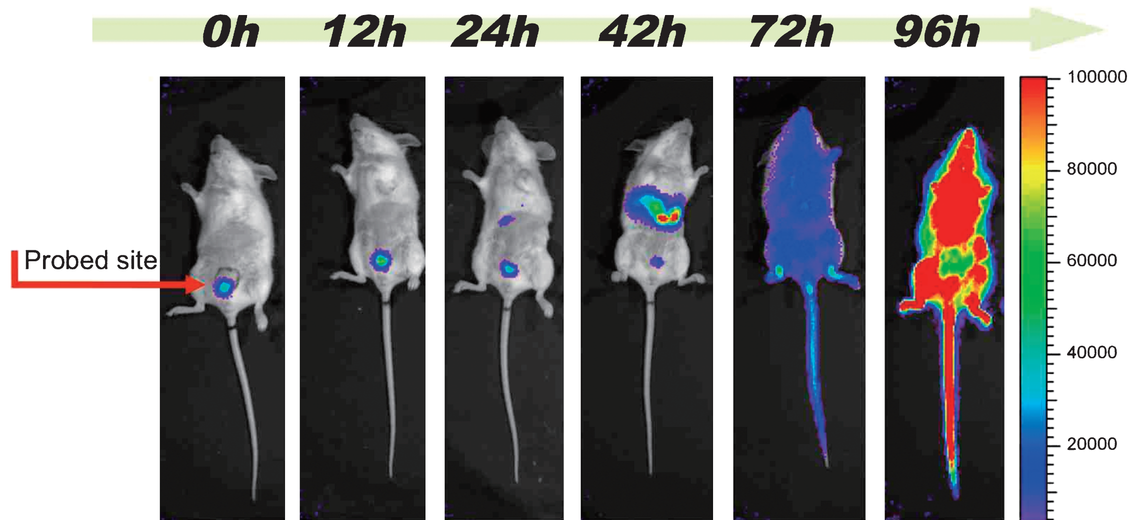

Fig. 1. Observation of one mouse for 4 days. One BALB/c mouse was probed by an infective mosquito with PbLuc sporozoites. Bioluminescence at the probing site continued for 42 hours. Strong emission appeared in the liver site after 42 hours. Bioluminescence spread to the whole body after 72 h. The intensity of the emission markedly increased after 96 h; however, parasitemia in tail vein blood was 0.001%.

heat treatment did not develop malaria. On the other hand, five out of the six mice that underwent the heat treatment at a separate site developed malaria parasites. These results indicated that the heat treatment weakened sporozoites in the skin (Fig. 4).

Luciferase activity of PbLuc after the death of parasites

Luciferase activity was markedly reduced when sporozoites were sonicated five times (Fig. 5). Even after one second of sonication, luciferase activity was clearly reduced. A correlation was observed between the death of parasites and luciferase activity. Thus, these results demonstrated that luciferase activity could be used as an indicator for the survival of parasites.

Removing the skin at the site of sporozoite deposition

When skin at the deposition site was removed after mosquito probing, sporozoites were detected in the removed skin, and some sporozoites were also observed in the peritoneal membrane (Fig. 6). This result confirmed that probing by infective mosquitoes deposited sporozoites not only in the skin but also under the skin. Jin et al. [4] reported similar findings using GFP parasites.

Intravenous injection of sporozoites

Sporozoites were injected intravenously, followed by luciferin 1 minute later. Mice were placed in the IVIS camera box, and bioluminescence was recorded for 5 minutes. The emission of sporozoites was observed in the liver as a mass when 12,000 or 48,000 sporozoites were injected but not when only 1,200 were injected. The intensity of emissions peaked 7 to 12 minutes after the sporozoite injection (Fig. 7a). Four organs (the liver, lung, kidney and spleen) were then removed and bioluminescence was recorded in each organ. Of these, only the liver emitted bioluminescence (Fig. 7b). These results demonstrated that sporozites attached to or were trapped in the liver within 15 minutes of entering the blood stream.

Discussion

Rice et al. [14] reported that 500 cells expressing luciferase were necessary for luciferin emission to be detected by IVIS, and Jin et al. [4] demonstrated that the number of sporozoites injected into the ventral abdomen by an infective mosquito was approximatly 120. Therefore, we did not expect to be able to observe sporozoites in the skin immediately after their deposition by infective mosquitoes. Malaria parasites invade and then develop in liver cells. The number of parasites that developed in a liver cell after 42 hours was previously estimated to be between 1,500 and 2,500 [15]. Therefore, we observed mice using the IVIS 42 h after the mosquito bite and succeeded in observing a mass of parasites expressing luciferase in the liver. The only organ that expressed strong emission was the liver. We treated more than 20 mice using the same procedure, but no organs other than the liver were found to express bioluminescence after 42 h. These results were consistent with previous findings in which the liver was

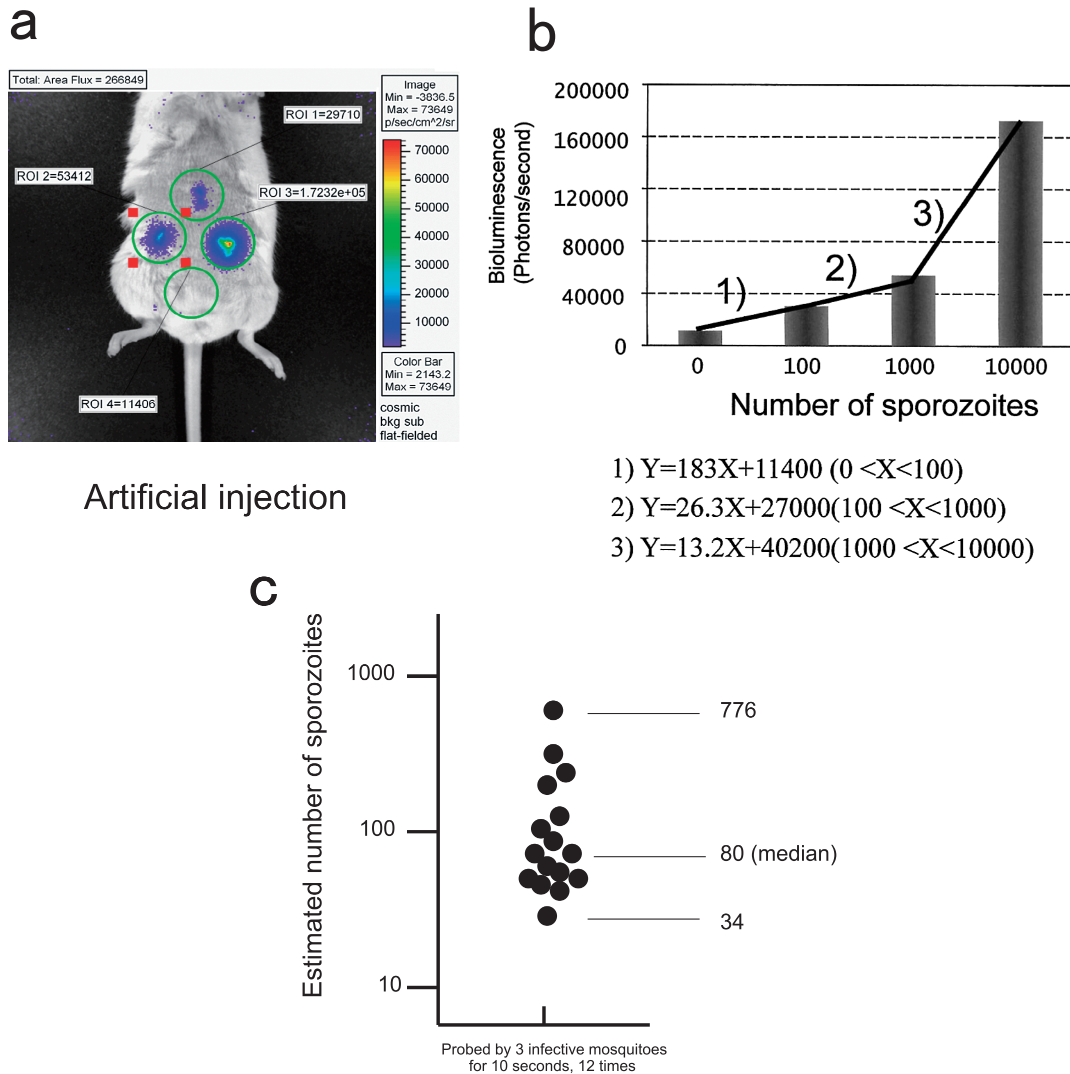

Fig. 2. Visualization of sporozoites injected artificially into the skin of a mouse. (a) Different numbers of sporozoites were artificially injected. Zero, 100, 1,000 and 10,000 sporozoites in 20 µl of RPMI 1640 medium were injected at the abdomen site. Bioluminescence was measured at each spot of the artificial injection. (b) Three equations were prepared from the bioluminescence results. (c) Sixteen mice were probed by infective mosquitoes through a 3-mm hole. Bioluminescence of the spots was measured and the number of sporozoites in the skin was estimated using the equations in Figure 2b.

shown to be the first organ in which malaria parasites developed [16].

We then directly injected sporozoites intravenously. Bioluminescence was observed in the liver immediately after the injection, but not in the spleen, lymph nodes or any other organ. This result suggested that the liver possessed specific ligands to which malaria sporozoites attach. We also confirmed that our transgenic parasites could be detected in the liver if more than 12,000 sporozoites were injected.

Luciferase emission could be detected in the skin 42 h and 72 h after probing. The sites corresponded to the site bitten by the mosquito 42 h or 72 h previously (Fig. 1, 3). We then attempted to expose mice that had just been bitten by an infective mosquito. We detected a strong emission at the site that had been bitten. This result suggested two possibilities: less than 100 cells in the skin could be detected by IVIS, or more than hundred sporozoites were injected by the mosquito.

We then prepared a series of solutions containing 0, 100, 1,000 and 10,000 sporozoites in 20 µl of medium and injected the skin of a mouse at four sites. Three spots containing sporozoites were detected with different emissions. A correlation was observed between the number of sporo-

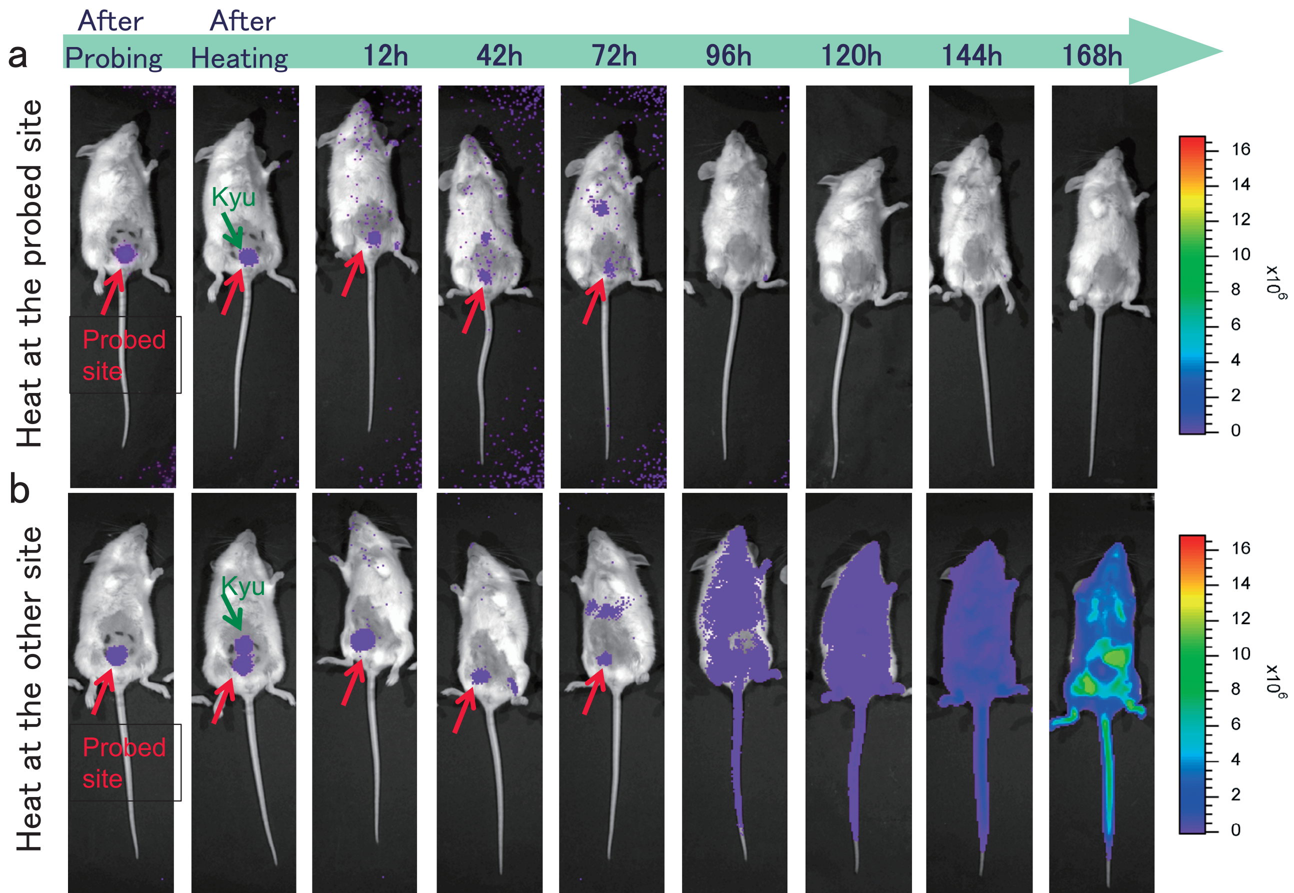

Fig. 3. Heat treatment effects on malaria parasites. (a) One mouse was probed by an infective mosquito. After confirming the deposition of sporozoites, Kyu was placed on the biting site. Weak emission appeared in the liver after 72 h, but could not be detected after 96 h. (b) One mouse was probed by an infective mosquito. After confirming the deposition of sporozoites, Kyu was placed on a different site. A new spot appeared near the original spot because of the heating effects of Kyu. Malaria parasites increased in number in the liver after 72 h and spread to the whole body after 96 h.

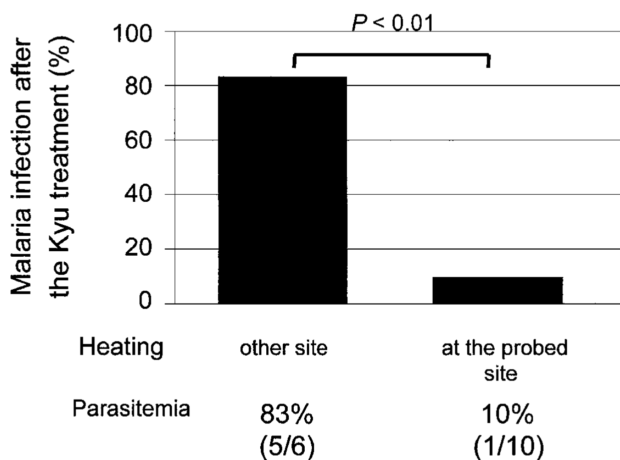

Fig. 4. Heat treatment effects on the probing site. Without the heat treatment at the probing site, the number of malaria parasites increased in 5 out of 6 mice, whereas 9 out of ten mice were protected by the heat treatment at the probing site. This difference was confirmed by Fisher's exact test.

zoites and the density of bioluminescence. Thus, we confirmed that less than 100 sporozoites could be detected in the skin with IVIS. We also established that approximately 30–800 sporozoites were injected into the skin by an infective mosquito.

Based on these results, we considered our transgenic parasites to express higher luciferase activity than other transgenic parasites. We currently cannot explain why our PbLuc expressed higher luciferase activity. We used the same promoter region of elongation factor-1α as that used in a previous study [2]. In any case, we obtained a good tool for research at the malaria skin stage.

We followed luciferase emission at the skin site following probing. Sporozoites were detected at 0 h, 24 h and 42 h with slight decreases in emission. This result indicated that a few sporozoites succeeded in entering the blood stream and were transported to the liver, while most sporozoites injected remained at the skin site. Yamaguchi et al. [17] demonstrated that most sporozoites had moved from

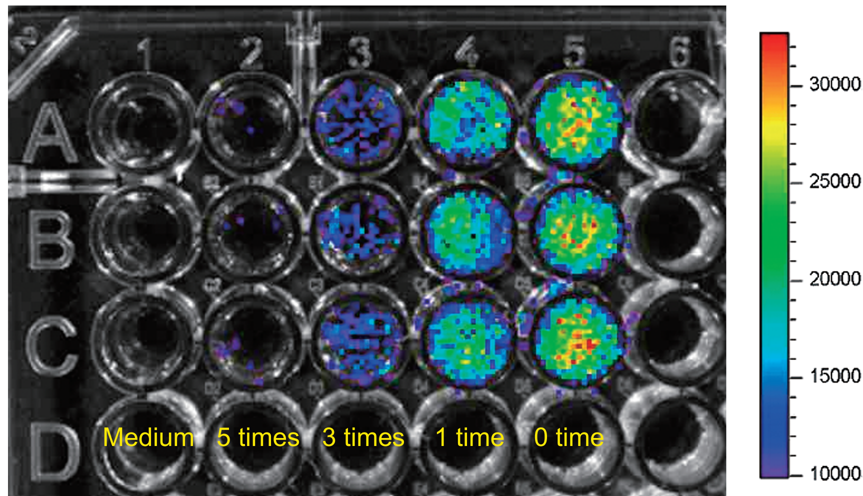

Fig. 5. Sonication effects for luciferase activity. After sonication for the indicated times, 1,000 sporozoites were placed in each well. Bioluminescence was measured after the addition of 75 µg luciferin to each well.

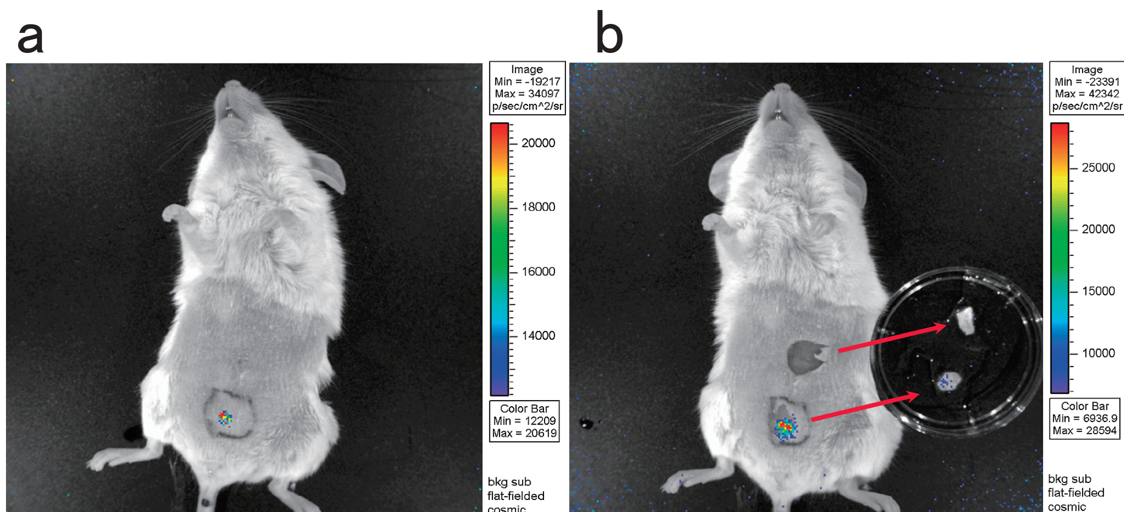

Fig. 6. Sporozoites injected by an infective mosquito reached the peritoneal membrane. (a) One mouse was probed by a mosquito and the deposition of sporozoites was confirmed. (b) The skin at the biting site was removed. Bioluminescence was detected in both the skin and peritoneal membrane. Upper dissected skin was a control without probing by an infective mosquito.

the injected site by 24 h, which is inconsistent with our results. This may have been because of differences in sporozoite-injection methods. The above authors used an artificial syringe injection, while we used an infective mosquito from which sporozoites were injected via the saliva of the mosquito. Although further studies are needed to clarify this issue, we confirmed that sporozoites stayed at the skin site for more than 24 hours when deposited by infective mosquitoes.

The whole body of the mouse was blue with bioluminescence 72 h after probing because blood stage parasites had started circulating throughout the body. The biting site could no longer be observed at this stage, and parasites could hardly be detected using the Giemsa staining method. Parasitemia in this stage was estimated to be between 0.0001 and 0.001%.

We conducted a heat treatment on the biting site after the deposition of sporozoites had been confirmed. We placed Kyu on the biting site. The temperature on the skin surface increased to more than 65°C for at least 30 seconds. This procedure may have killed sporozoites in the skin. The heat-treated mice were mostly intact after this

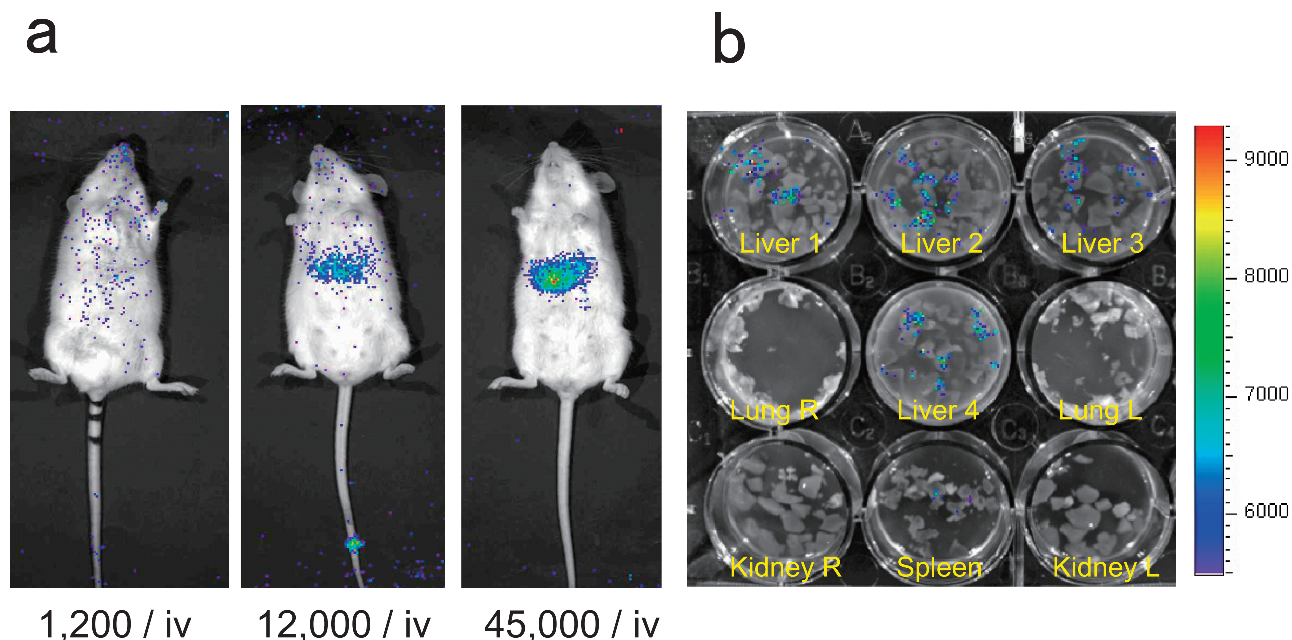

Fig. 7. Sporozoites reached the liver 15 min after the intravenous injection. PbLuc sporozoites were obtained from the salivary glands of infective mosquitoes. (a) Different numbers of PbLuc sporozoites (1,200, 12,000 and 48,000) were prepared and injected into mice intravenously. Bioluminescence appeared 10 to 15 minutes after mice were injected with 12,000 or 48,000 sporozoites. (b) The organs of the mouse injected intravenously with 48,000 sporozoites. After confirming the accumulation of sporozoites in the liver, the mouse was killed and the liver, lung, kidney and spleen were removed. Each organ was cut into pieces and placed in each well with 0.8 ml of RPMI 1640 medium. Bioluminescence was recorded after the addition of 300 μg luciferin to each well. Only the liver (four wells) emitted bioluminescence. The lung (R: right; L: left), kidney (R: right; L: left) and spleen did not.

treatment: 9 out of 10 mice did not develop malaria. Another group of mice that were also probed with infective mosquitoes underwent the Kyu treatment, but at a site 10 mm from the biting site. Five out of six mice developed malaria. This difference was significant ($P < 0.01$). These results were consistent with our previous findings [10]. Therefore, we confirmed that almost all sporozoites stayed in the skin for the first 15 minutes following probing.

The information on the skin stage of malaria parasites presents new opportunities to prevent malaria because it may be possible to kill or neutralize malaria parasites once itching develops following a mosquito bite. If we can identify an effective medicine for killing sporozoites and a method to deliver it to the bottom of the skin, we can apply it to the irritated site together with anti-itching medicine. This may represent a new malaria preventive method.

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Conflicts of Interest

All authors have no conflict of interest to declare.

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