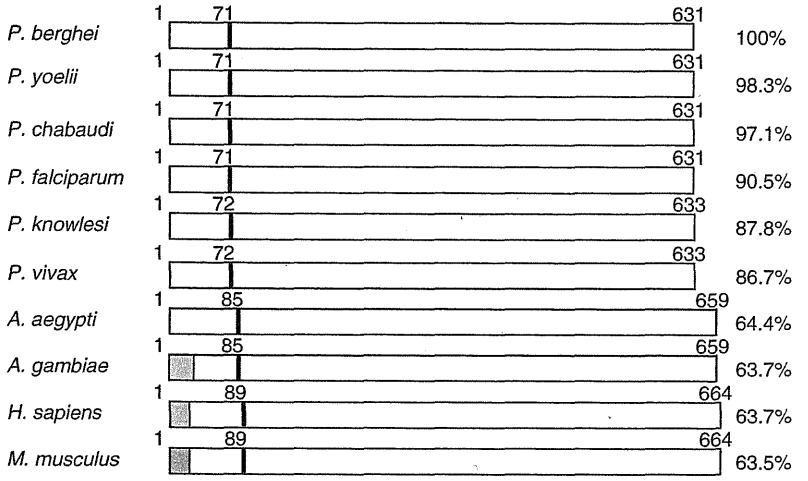

Fig. 1 Primary structure of Fp proteins from 10 species. The box indicates the full-length Fp protein. The mitochondrial sorting signal is colored in grey. The black line with number indicates the conserved His for FAD binding site. The total number of amino acid residues and the amino acid identity to PbFp are indicated on the right (%).

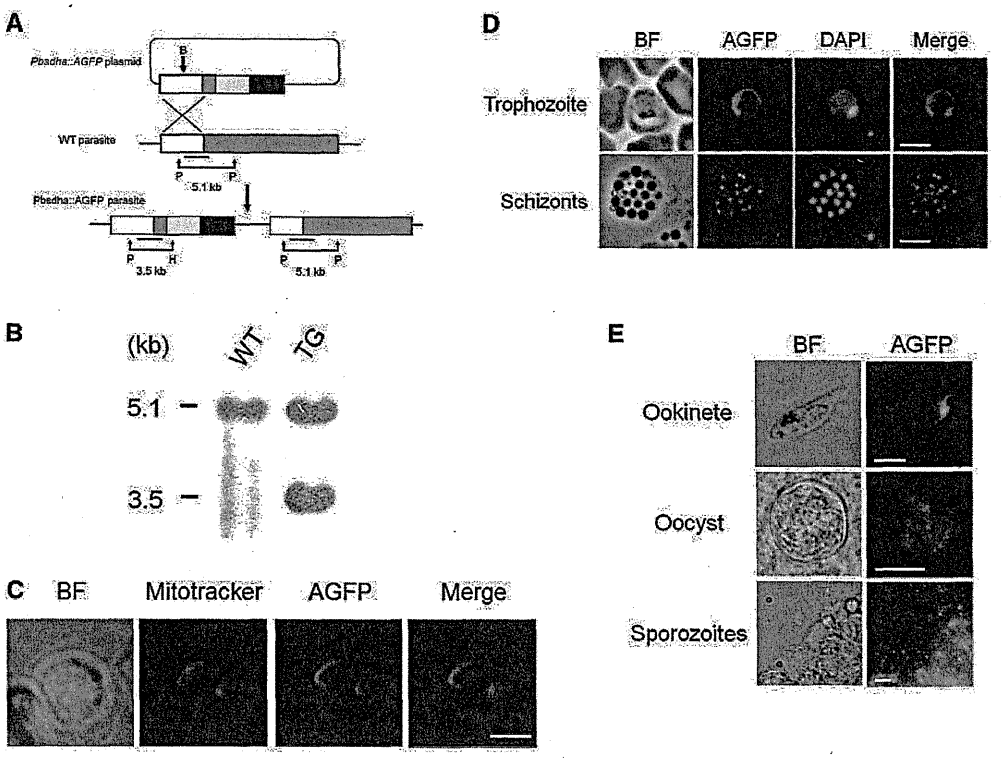

Fig. 2 Generation of the transgenic parasite line Pbsdha::AGFP. (A)Schematic representation of *AGFP* tagging of the *Pbsdha* locus using a plasmid that integrated through single crossover homologous recombination. In the *Pbsdha::AGFP* plasmid, the boxes indicate *Pbsdha* gene promoter (white), the first 60 amino acids of Pbsdha (dark gray), AGFP (light gray), 3'UTR of *Pbsdha* gene and *TgDHFR/ts* selectable marker cassette (black). In the *Pbsdha* gene locus (middle), the box with dark gray indicates full open reading frame of *Pbsdha*. B, H and P indicate *BstXI*, *HpaI* and *PacI* digestion sites, respectively. Bars represent the position of the probe used in Southern blot analysis. (B) Southern blot analysis of WT and Pbsdha::AGFP parasites. Hybridization of the probe with *PacI*- and *HpaI*-digested genomic DNA yielded a 5.1 kb for WT, and 3.5 kb and 5.1 kb for Pbsdha::AGFP parasites (TG). (C) Cellular localization of AGFP in Pbsdha::AGFP parasites. The mouse erythrocyte infected with Pbsdha::AGFP parasites was observed under bright field (BF). The signals of MitoTracker and AGFP in the same cell were detected through red (MitoTracker) and green filter (AGFP), respectively. Bar represents 5 μm. (D) Expression of *AGFP* gene in Pbsdha::AGFP parasites at blood stages. The AGFP signal was detected in the parasites synchronized at trophozoite (upper) and schizont stages (lower). Nuclei were stained with DAPI. Bars represent 5 μm. (E) Expression of the *AGFP* gene in Pbsdha::AGFP parasites at mosquito stages. The AGFP signal was detected in *in vitro* cultured-ookinetes, oocysts in the midguts and sporozoites in the salivary gland of mosquitoes. Bar represents 20 μm.

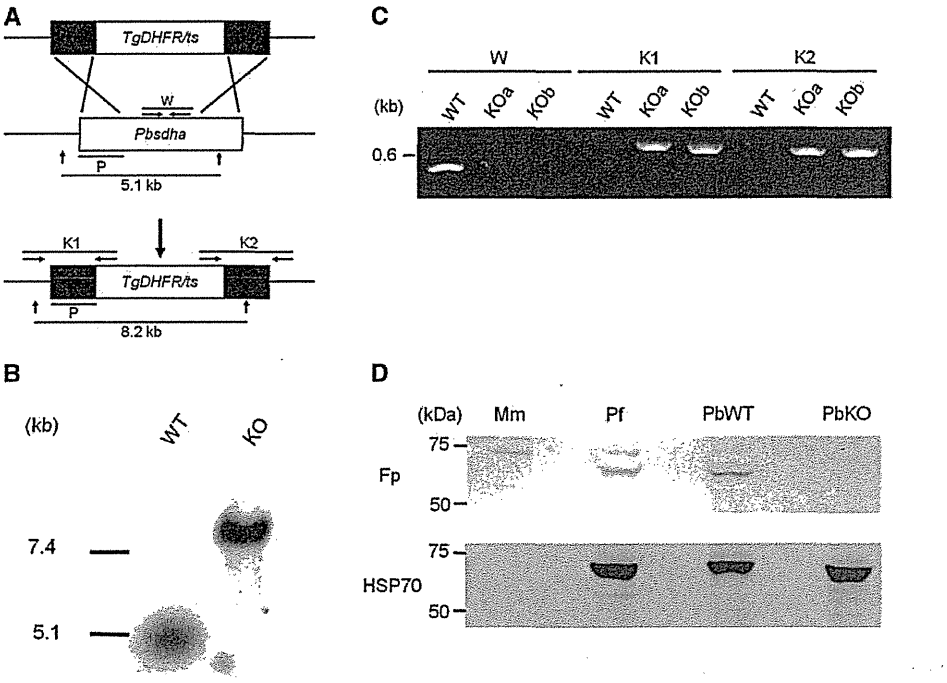

Fig. 3 Targeted disruption of the *Pbsdha* gene. (A) Schematic representation of the replacement strategy to generate Pbsdha(-) parasites. The WT *Pbsdha* gene locus is replaced with 5′ and 3′ UTRs of the *Pbsdha* gene and *TgDHFR*, a selectable marker. Vertical arrows indicate *PacI* site. P with bar indicates the probe position for Southern blot analysis. Arrows marked with W (WT specific) or K1/K2 (knockout specific) indicate the primer positions used in diagnostic PCR. (B) Southern blot genotyping confirmed integration. Hybridization of the probe with *PacI*-digested genomic DNA of WT and Pbsdha(-) parasites yielded a 5.1 kb and 8.2 kb band, respectively. (C) Confirmation of *Pbsdha* gene disruption by diagnostic PCR. Genomic DNA from WT, Pbsdha(-) clone A (KOa) and clone B (KOb) were used as templates. The positions of the PCR products in W and K1/K2 are depicted in Fig. 2A. (D) Detection of Fp peptides by Western blot analysis. The Fp peptides in mitochondrial fractions of *Mus musculus* (Mm), *P. falciparum*(Pf), *P. berghei* wild-type (PbWT) and Pbsdha(-) (PbKO) parasites were detected by anti-PfFp antiserum (upper panel). The anti-PbHSP70 antiserum was used to confirm equal loading of the protein samples in each lane (lower panel).

70.5 kDa (WT *P. berghei*) and 73.0 kDa (mice), as well as 70.7 kDa (*P. falciparum*). In contrast, no band was detected in Pbsdha(-) (PbKO) except for the high molecular weight band, which is speculated as contaminated mice Fp because the molecular weight of this faint band is similar to that of the band on Mm lane (Fig. 3D). The equal loading of each sample on the gel was confirmed by anti-PbHSP70 antiserum (lower panel in Fig. 3D). These results demonstrated that the PbFp protein was surely deleted from Pbsdha(-) parasites. Next, we investigated the SQR activity in Pbsdha(-) parasites by using the same samples used for Western blot analysis. While WT parasites showed an SQR activity of 4.38 ± 0.96 nmol/min/mg ($N = 3$), Pbsdha(-) did not show any detectable SQR activity (Table I). These results demonstrated that the PbFp protein and the SQR activity were completely eliminated from the Pbsdha(-) parasites.

Phenotypic analysis of Pbsdha(-) parasites

As the disruption of the *Pbsdha* gene was confirmed, phenotypic effect of the gene disruption on the parasite was analyzed. In erythrocytic stages, Pbsdha(-) parasites underwent normal development and differentiation into gametocytes, which were not significantly different from those of WT parasites ($P > 0.1$, Fig. 4 and Table II). Successful *Pbsdha* gene deletion indicates that the SQR enzyme is not essential for the survival of the parasite at asexual stages and sexual differentiation. The mitochondria of Pbsdha(-) parasites were stained with Mitotracker, indicating that disruption of *Pbsdha* did not affect the mitochondrial membrane potential (Fig. 5).

Next, we investigated the parasite development at mosquito stages. In *P. berghei*, an *in vitro* assay has been established that mimics the gametogenesis and fertilization taking place in the mosquito body (*22*). Using this system, we confirmed that the efficiency of male gametogenesis in Pbsdha(-) parasites was comparable with that of WT parasites (data not shown). However, Pbsdha(-) parasites showed severe defects in ookinete formation, the stage next to fertilization. The conversion rate of female gametes to ookinetes in Pbsdha(-) was significantly reduced to 17% of WT parasite ($P < 0.05$ ($N = 3$), Fig. 6A). We further investigated the infectivity of Pbsdha(-) parasites to mosquitoes and the subsequent transmission to mice. The mosquitoes were fed on mice carrying either Pbsdha(-) or WT parasites, and then these mosquitoes were dissected for the evaluation of parasite development at day 16 post-feeding. Interestingly, several independent experiments using two clones showed that no oocysts were detected in the midguts of mosquitoes fed on mice carrying Pbsdha(-) parasites, while oocysts

Table I. SQR activity of the mitochondrial fraction in WT and Pbsdha(-) parasites.

	Exp 1	Exp 2	Exp 3
WT	5.31	4.44	3.40
KO	0.00	0.00	0.00

The value represents SQR activity in each independent assay (nmol/min/mg protein)

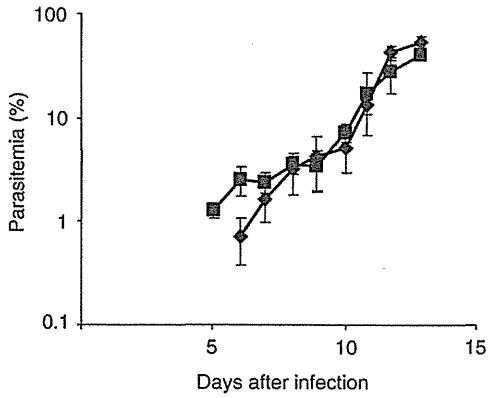

Fig. 4 The growth rate of intraerythrocytic stages of WT and Pbsdha(-) parasites. The parasitemia of WT and Pbsdha(-) parasites are indicated by closed square and diamond, respectively. Bar represents SD (N=4).

Table II. The gametocytemia of WT and Pbsdha(-) parasites.

Parasites	Macrogametocytemia/ parasitemia	Microgametocytemia/ parasitemia
WT	6.5±2.72	1.7±0.45
KO	5.0±1.11	1.5±0.64

were detected in the mosquitoes fed on mice carrying WT parasites (Fig. 6B). Moreover, no transmission was observed in the mice challenged by the mosquitoes carrying Pbsdha(-) parasites, while WT parasites were transmitted to mice (Table III). Taken together, these results indicated that the development of Pbsdha(-) parasites was completely halted at the stage of oocyst formation.

Discussion

It is known that asexual stages parasites possess a single acristate mitochondria, while gametocytes, mosquito stages and preerythrocytic stage parasites possess five to six cristate mitochondria (*9*, *23*). This morphological maturation of mitochondria in the sexual stage parasites may correlate with the increased need of mitochondrial metabolism in the insect stage parasite development. Once malaria parasites are introduced to mosquitoes, they encounter to drastic environmental changes where the main sugar source is changed from glucose to torehalose, and they need to adapt to this (*24*). Our present work clearly shows that complex II has a critical role in parasite adaptation to the insect body. Namely, the parasite lacking complex II activity failed to form oocysts in mosquitoes, while the development of blood stage parasites in mice was not affected by *Pbsdha* gene disruption at all.

Developmental expression and targeting disruption of Pbsdha gene

By using Pbsdha::AGFP parasites as a reporter, we followed the AGFP expression during the whole parasite life cycle except for liver stage. Except for the ring stage, AGFP signals were detected in all developmental stages. It was reported that the size and morphology of mitochondria spectacularly changes during the life cycle and that the mitochondrial size is much smaller in the ring stage than in any other stage (*25*). Thus, the failure of signal detection in ring stage could be attributed to lower signal strength than the detection limit. According to gene expression data in PlasmoDB, the *P. falciparum* orthologous gene of *Pbsdha*, PF10_0334 is substantially expressed in all developmental stages including the ring form. Taken together, it can therefore be speculated that the *Pbsdha* gene is also expressed in all developmental stages.

In a global gene expression analysis, it was reported that several lines of *in vitro*-cultured *P. falciparum* showed very similar pattern in gene expression. In contrast, it was recently demonstrated that parasites derived directly from infected patients showed three distinct gene expression states (*26*). One of these states showed that the expression levels of *sdha* and other TCA cycle- or ETC-related genes are increased. Because such expression state has never been detected in *in vitro* studies and parasitic mitochondria are immature acristates, it had been considered that the blood-stage parasites mainly use cytosolic glycolysis and mitochondrial oxidative phosphorylation only marginally (Fig. 7). The discrepancy between the *in vivo* and *in vitro* studies above suggests that the parasites may use oxidative phosphorylation in *in vivo* in case that the patient would be hypoglycemic, which is reflected in the upregulation of the genes involved in oxidative phosphorylation. In the *in vitro* system, on the other hand, glucose is continuously supplied in the medium and the environmental condition is artificially controlled, therefore the parasites may exclusively undergo glycolysis and the enzymes of oxidative phosphorylation may be marginally expressed. Thus, it is important to undertake experiments using both *in vitro* and *in vivo* systems in order to understand how parasites respond to various environmental stimuli. In this sense, the rodent malaria model could be an ideal tool to investigate the changes in parasite metabolism *in vivo*.

Complex II is essential for the parasite survival at mosquito stages

We successfully generated Pbsdha(-) parasites, confirmed by Southern blot, diagnostic PCR and SQR activity assay. It is anticipated that the mitochondrial membrane potential was decreased in Pbsdha(-) parasites as a result of the knockout. Unexpectedly, the

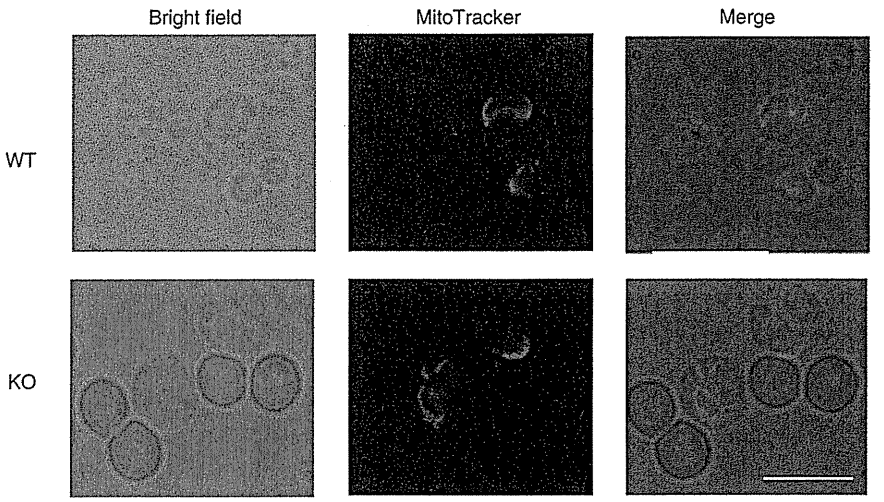

Fig. 5 MitoTracker staining of WT and Pbsdha(-) parasites. The erythrocytes infected with WT or Pbsdha(-) (KO) parasites was stained with MitoTracker to assess the integrity of the mitochondrial membrane potential. Bar represents 5 μm.

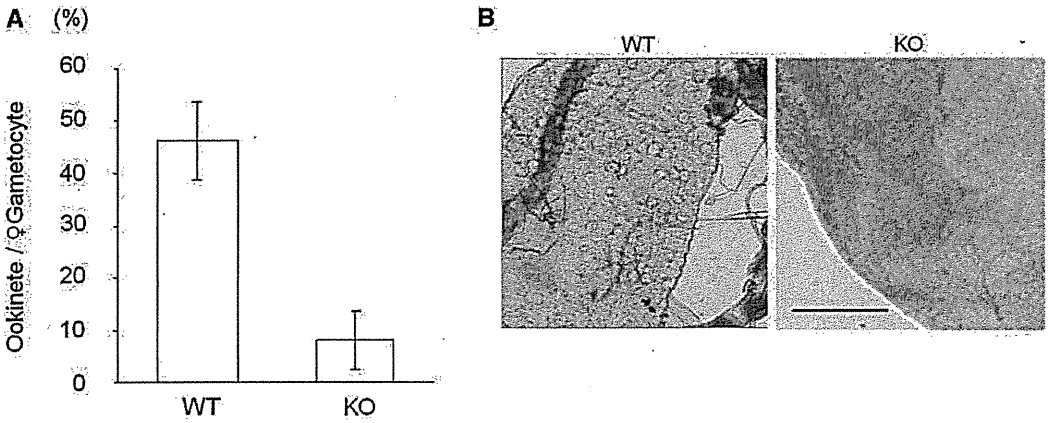

Fig. 6 Phenotypic analysis of Pbsdha(-) parasites. (A) Ookinete formation rate of WT and Pbsdha(-) (KO) parasites. The mouse blood infected with either WT or Pbsdha(-) parasites was incubated in fertilization medium to induce fertilization and differentiation into ookinetes. The ookinete formation rate was calculated by the percentage of female gametocytes, which converted into ookinetes. Error bars represents mean ± SD (n = 3). (B) Mosquito midguts infected with WT and Pbsdha(-) (KO) parasites. The latter carries no oocyst. Bars represent 200 μm.

Table III. Infectivity of WT and Pbsdha(-) parasites.

Parasite		Oocyst-positive mosquitoes	Mean oocysts/ midgut ± SD	Mouse infection
WT	Exp1	7/21	5.4 ± 11.3	+
	2	6/20	7.3 ± 6.4	+
	3	5/20	18.0 ± 30.3	+
	4	13/20	25.1 ± 30.5	+
	5	15/32	51.8 ± 63.4	+
Koa	Exp1	0/20	0	−
	2	0/20	0	−
	3	0/20	0	−
	4	0/20	0	−
	5	0/20	0	−
	6	0/20	0	−
Kob	Exp1	0/26	0	−

mitochondrial membrane potential seemed to be maintained in blood-stage parasites in the absence of complex II, which was demonstrated by the positive signal in MitoTracker staining. In addition to complex II, there are three other enzymes involved in electron flux that contribute to mitochondrial membrane potential; MQO (malate–quinone oxidoreductase), DHOD (dihydroorotate dehydrogenase) and NDH2 (type2 NADH–ubiquinone oxidoreductase), which is a single peptide dehydrogenase different from multi-subunit complex I (Fig. 7). It is therefore conceivable that any of these three enzymes may be a functional complement for the absence of complex II. Recently, it was reported that *P. berghei* lacking NDH2 (NDH2(-)) did keep mitochondrial membrane potential (*27*), supporting the hypothesis of mutual complementation among ETC enzymes.

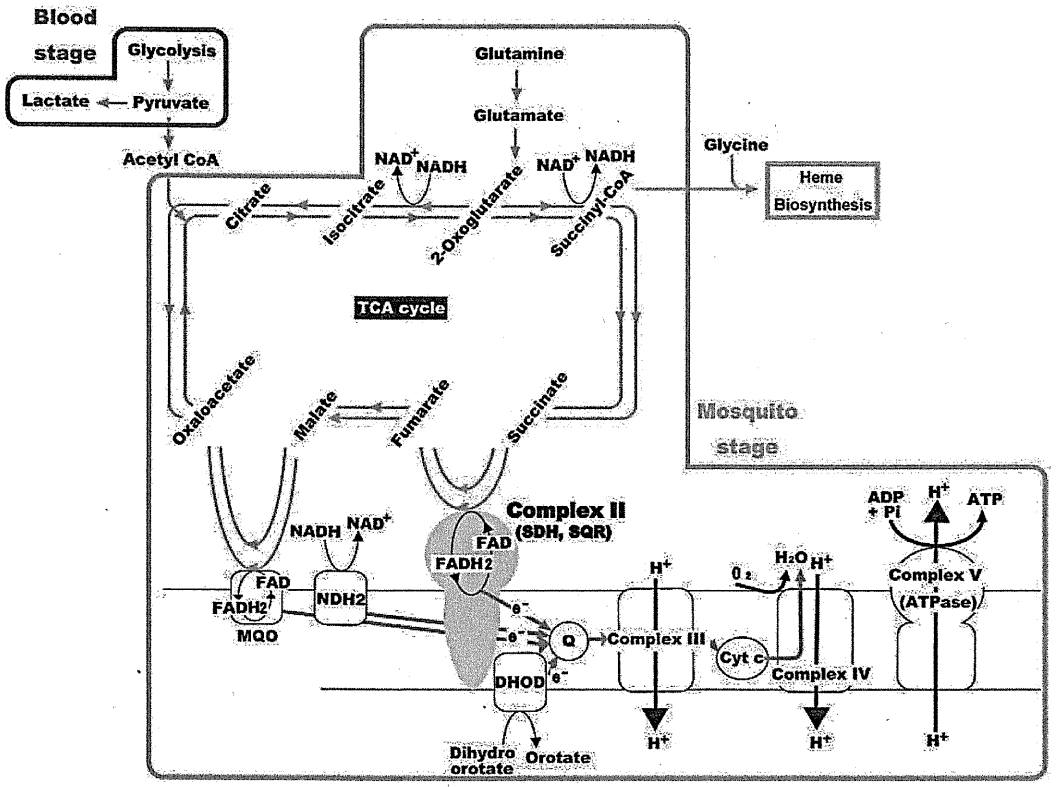

Fig. 7 Hypothetical Model of Plasmodial energy metabolism. Blue arrows represent canonical flow of glycolysis and TCA cycle. Green arrows represent *Plasmodium* TCA metabolism pathway. Unlike other eukaryotes, glycolysis (enclosed by red) does not link to TCA cycle. *Plasmodim* TCA cycle is initiated by uptaking of glutamine into mitochondria. Mitochondrial oxidative phosphorylation (enclosed by green) is essential for the parasite survival at mosquito stage because the Pbsdha(-) parasite is lethal at this stage.

Our phenotypic study revealed that Pbsdha(-) parasites proliferated and produced gametocytes with a similar rate to WT parasites in mice. Recently, we observed similar results in the blood-stage *P. falciparum*, of which Fp subunit was disrupted (Tanaka *et al.*, submitted for publication). *In vitro*, Pbsdha(-) male gametocytes differentiated to gametes (exflagellated) as WT ones, suggesting that complex II is not required for those developmental stages. It is known that mammalian sperm requires ATP for flagellum movement, which is supplied mainly from mitochondrial oxidative phosphorylation (*28*). While male gametes of malaria parasites show similar motility to that of mammalian sperm, it is obvious that parasite male gametes do not rely on oxidative phosphorylation because the male gametes do not possess mitochondria (*24*). This indicates that the driving force for male motility is exclusively supplied from glycolysis. The *in vitro* fertilization assay showed that Pbsdha(-) parasites were defect in ookinete formation. At this moment, it is not clear whether complex II is critical at female gametogenesis, fertilization or ookinete formation itself. Nevertheless, we speculate that the impact of *Pbsdha* gene disruption gave adverse influence on female, probably the stages after gametocytogenesis.

The most drastic phenotypic change in Pbsdha(-) parasites was the complete failure of oocyst formation. In malaria parasites, the ookinetes traverse midgut cell and arrive to the basal lamina where they transform into oocysts. In a single oocyst, mitosis occurs and several hundreds of sporozoites are generated inside. To accomplish such task, the parasites may need more ATP, which could be generated by oxidative phosphorylation. The complex II activity deletion may therefore adversely affect the oocyst formation. Recently, it was reported that NDH2(-) parasites developed normally in asexual stages but transformed into aberrant immature oocysts (*27*). Our present study together with this finding suggests that the ETC enzymes are essential in insect stages. However, there are several phenotypic differences between Pbsdha(-) and NDH2(-) parasites; 1) Pbsdha(-) parasites differentiated into ookinetes with low efficiency, while NDH2(-) ookinete formation is similar to WT parasites. This indicates that the Pbsdha deletion affects an earlier parasite stage compared with that of NDH2 deletion; 2) Pbsdha(-) parasites failed completely in oocyst formation, while NDH2(-) parasites formed immature oocysts with smaller size, demonstrating that the absence of complex II gives more severe defects in parasite development than NDH2 does. In other eukaryotes, it is known that mitochondrial complex II converts succinate to fumarate and reducing equivalents are transferred to quinone. In addition, complex II is one of the TCA cycle members

generating NADH, which is a substrate of NDH2. Thus, the deletion of complex II activity may render NDH2 unable to function in ETC. Severe phenotypic changes in Pbsdha(-) parasites could be therefore attributed to the aberration of NDH2 as well as complex II itself.

Conclusion

In the present work, we show that complex II has a critical role in insect-stage parasites. ETC is not only involved in ATP metabolism, but as well in heme biosynthesis (Fig. 7), which is also crucial for parasite survival (*29*). In addition to the ETC, complex II functions as the TCA cycle enzyme. Further studies are required to determine whether lack of any of these functions are the cause for the developmental arrest in Pbsdha(-) parasites. In any case, our study demonstrates that malaria parasite drastically switches energy metabolism when the parasites initiate sexual maturation and is subsequently introduced into the mosquito (Fig. 7).

The importance of complex II in insect-stage parasites suggests the possibility that complex II could be a novel target for transmission blocking (*30*). Previously, we reported that the amino acid sequences of the membrane anchor subunits CybL and CybS of *Plasmodium* complex II show exceptionally low homology to that of any other organism including human (*12*). In addition, our previous work revealed that atopenin A5 is a potent inhibitor against mammalian complex II with IC_{50} values of three-order of magnitudes lower than that of *Plasmodium* complex II (*13*). This indicates that 3D structure of ubiquinone-binding site in the parasitic complex II is quite distinct from those of mammalian complex II. Therefore, it is conceivable that the development of a parasitic complex II-specific inhibitor would be feasible by structure-based drug design targeting to the mosquito-stages parasite. Currently, further ATP metabolic gene knockout experiment using mouse malaria models are now in progress to draw general view of parasite energy metabolism in response to various environmental stimuli that may have been overlooked in *in vitro* culture systems as pointed out by Daily (*31*).

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Conflict of interest

None declared.

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Original article

One Injection of DsRed Followed by Bites from Transgenic Mosquitoes Producing DsRed in the Saliva Elicits a High Titer of Antibody in Mice

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Abstract: It has been proposed that transgenic mosquitoes can be used as a "flying syringe" for infectious disease control. We succeeded in generating a transgenic (TG) mosquito, *Anopheles stephensi*, excreting and discharging DsRed in saliva. DsRed was deposited on the membrane where the TG mosquito probed with its proboscis. Repeated feeding by the TG mosquitoes induced anti-DeRed as well as anti-SG antibodies in mice. This indicates that the TG mosquitoes can immunize the animal. Moreover, in this report, we employed a pre-immunization method before exposing mice to the TG mosquitoes. We injected DsRed to mice to prepare memory B cells and exposed the mice to bites by the TG mosquitoes excreting DsRed. The mice produced a higher titer of antibody to DsRed, suggesting that the bites from TG mosquitoes act as a booster and that primary immunization with a vaccine protein and exposure to TG mosquitoes excreting the vaccine protein in the saliva produces a synergistic effect.
Key words: *Anopheles stephensi*, DsRed, flying syringe, salivary gland, transgenic mosquito

INTRODUCTION

Transgenic techniques have been applied in recent years not only to bacteria but also to animals. Transgenic mice, sheep, dogs, zebra fish, flies and silkworms have appeared in laboratories. Transgenic mosquitoes have also appeared. Anopheline mosquitoes, which transmit malaria parasites, were targeted for the insertion of transgenes to reduce their ability to spread disease [1–3]. Some laboratories, including ours, have succeeded in producing transgenic mosquitoes with a lower level of malaria parasites in the digestive tract after blood meals on malaria-infected animals [4, 5]. The goal of these endeavors is to control disease transmission through genetic modification of the mosquitoes [6].

We next considered producing a useful protein in the mosquito SG by adding a new gene to a mosquito chromosome, because mosquitoes secrete saliva into the host while taking a bloodmeal. Our idea is to insert a gene encoding a useful protein into the mosquito chromosome, causing the mosquito to produce the protein in its saliva and inject it, via the saliva, into animals or humans upon blood feeding. The host can be expected to develop antibodies to the recombinant protein as a reaction. If TG mosquitoes with saliva containing a vaccine protein against a disease are spread in an area where the disease is prevalent, it follows that people who are bitten daily by the TG mosquitoes will develop antibodies to the vaccine protein, ultimately vaccinating the community against the disease. In this situation, mosquitoes would play the role of vaccine deliverers [7].

We previously discovered a platelet inhibitor in the salivary gland (SG) of the mosquito *Anopheles stephensi*, cloned its gene, and named the molecule "anopheline antiplatelet protein" (AAPP) [8]. AAPP is expressed only in the female SG. We used an upstream region of the AAPP gene (*aapp*) as a promoter for a vaccine protein. We have already produced three TG strains of mosquitoes expressing an alien protein in the SG [9–11]. One strain produced a candidate malarial vaccine protein, circumsporozoite protein (CSP), in the SG. However, mice bitten by these mosquitoes did not develop anti-CSP antibodies, because CSP was not discharged outside the SG [9]. The second strain produced SP15, a leishmanial protein, which was discharged into the saliva. Anti-SP15 antibody production was induced in mice

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bitten 20 times with 50 of these mosquitoes. It took 20 weeks to detect the antibody [10]. Recently we succeeded in producing a transgenic mosquito expressing a part of CSP in the saliva. The mice bitten by the TG mosquitoes produced anti-CSP antibody, but 20 feedings by 100 TG mosquitoes [11].

We need a more efficient TG mosquito and a more suitable way to elicit the development of antibodies. In the present study, we employed pre-immunization methods before exposing mice to the TG mosquitoes. We injected an immunogen to prepare memory B cells in the mice, and exposed the mice to bites by TG mosquitoes in order to induce a high titer of antibody.

MATERIALS AND METHODS

Ethics statement

All care and handling of the animals was conducted in accordance with the Guidelines for Animal Care and Use prepared by Jichi Medical University, following approval (ID: 09-192) by the Jichi Medical University Ethical Review Board.

Mosquitoes and mice

Adult mosquitoes, *Anopheles stephensi* SDA 500, were reared in 30 × 30 × 30 cm cages with stainless steel frames and a white cloth covering. The rearing conditions were: 26°C, 50–70% relative humidity, 13 hours of light and 11 hours of darkness. Adult females could draw blood through the cloth when a mouse was anesthetized and laid on the top of the cage. Female ICR mice were purchased from SLC (Shizuoka, Japan).

DsRed protein, antibody and DNA

A recombinant DsRed-monomer protein and a rabbit antibody to DsRed-monomer were purchased from Clontech Laboratories (Mountain View, CA, USA). We also purchased DsRed monomer vector (Clontech Laboratories) for the preparation of template DNA.

Plasmid construction (Fig. 1)

A DNA fragment (1,746 bp) covering the anopheline anti-platelet protein gene (*aapp*) promoter region and signal peptide coding region of *An. stephensi* (GeneBank accession no. AB212871.1) was amplified with the primers AAPP-attB4F (5'-*GGGGACAACTTTGTATAGAAAAGTTG* TTATAAGACGGAGCTCATTGTCGCTCGTC-3') and AAPP-attB1R (5'-*GGGGACTGCTTTTTTGTACAAACTTG* CGGCCGTGCGGATACGATCAGCGCAAGGC-3'), then cloned into the vector pDONR™P4-P1R (Invitrogen, Carlsbad, CA, USA) (plasmid a). The dsRed monomer gene (Invitrogen)

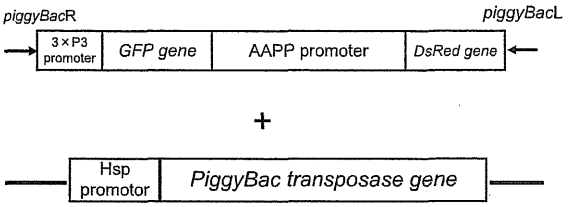

Fig. 1. DNA injected into mosquito eggs. Two plasmids were injected. One contained GFP and DsRed genes with piggyBac arms. The other contained the PiggyBac transposase gene. 3xP3: a protein expressed in the eyes (fragment size 41 bp). GFP: Green fluorescent protein as a marker of eyes (717 bp). AAPP: Anopheline anti-platelet protein (1746 bp). DsRed: a marker of saliva from the salivary gland expected to be secreted in the saliva (675 bp). Hsp: Heat shock protein (393 bp).

was amplified with dsRed-attB1F (5'-*GGGGACAAGTTTG TACAAAAAAGCAGGCT*ATGAAGCTTGCCTCCTCCGA GAACGTC-3') and dsRed-attB2R (5'-*GGGGACCACTTTG TACAAGAAAGCTGGGT*TTAGTGGTGGTGGTGGTGGTG-3'), then cloned into the vector pDONR™221 (plasmid b). The 3' non-coding region of the AAPP gene was amplified with 3UTR-attB2F (5'-*GGGGACAGCTTTCTTGTACAAAGTGG* GAAACACACCGTTAACGACAC-3') and 3UTR-attB3R (5'-*GGGGACAACTTTGTATAATAAAGTTG*TATTCAAAG GTCCACAAATGTC-3'), and cloned into the vector pDONRP2R-P3™ (plasmid c). The pBac [3xP3-EGFP] vector and helper plasmid were kindly provided by Professor A. S. Raikhel (Department of Entomology, University of California at Riverside, USA) [12]. The pBac [3xP3-EGFP] vector was digested with *AscI*, blunted with a Klenow fragment, and dephosphorylated. The reading frame cassette A (Invitrogen) was then cloned into the vector (destination vector). The inserts of plasmids a, b and c were serially ligated in destination vector using the MultiSite Gateway® Three Fragment Vector Construction Kit (Invitrogen).

The resulting plasmid (pBac-AAPP-DsRed) was expected to drive expression of the dsRed gene under the control of the AAPP promoter. The pBac-AAPP-dsRed plasmid was mixed with piggyBac helper (Fig. 1) and microinjected into the eggs of *A. stephensi.*

Microinjection of the plasmids into mosquito eggs

The microinjection was performed as described elsewhere [13]. In brief, blood-fed *An. stephensi* mosquitoes were allowed to oviposit on a wet filter sheet 72–84 h after a blood meal. Eggs were injected within 120 minutes of oviposition. The injection was done using glass needles (Eppendorf, Hamburg, Germany) with a mixture of pBac-

AAPP-DsRed (400 ng/μl) and piggyBac helper (100 ng/μl) in injection buffer (5 mM KCl, 0.1 mM Na_2HPO_4, pH 6.8). The eggs were then placed in water and observed for hatching. The hatched larvae were analyzed on a fluorescence microscope at a wavelength of 488 nm to detect GFP expression.

Selection of transgenic mosquitoes

We reared the hatched larvae and allowed them to emerge as adults. One adult mosquito was placed in a cage containing five wild-type adult mosquitoes of the opposite sex. After mating and blood feeding, each female was allowed to lay eggs individually. The hatched larvae were observed under a fluorescence microscope, and GFP-expressing larvae were isolated as a G1 strain. Among the same G1 batch, mosquitoes were allowed to mate, feed, and lay eggs. The hatched larvae were observed, and the GFP-expressing larvae were isolated as a G2 strain.

Western blotting

Three to four days after emergence, ten pairs of SG were collected from both TG females and wild-type females in ten micro-liters of phosphate-buffered saline (PBS) in an Eppendorf tube. The tube was frozen at –80°C and thawed at room temperature. It was then centrifuged at 8,000 rpm for 3 min. The supernatant was used as the SG antigen assuming that one pair of SG protein was contained in one micro-liter. Half of the samples (five pairs of SG) were separated on a 10% SDS-polyacrylamide gel under reducing conditions (with 2% 2-mercaptoethanol) and transferred to a nitrocellulose (NC) sheet. The sheet was soaked in 1% skim milk for ten minutes, and probed with a rabbit anti-DsRed antibody (Clontech) diluted 1,000 fold. The sheet was washed with 0.05% Tween20 in PBS four times and then incubated with anti-rabbit IgG conjugated with HRP (Bio-Rad Lab.) diluted 3,000 fold. The sheet was washed with 0.05% Tween20 in PBS four times and reacted with Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). Positive bands were visualized with a Lumino Image Analyzer (LAS-1000) (Fuji Film, Tokyo, Japan).

Observation of the SG

An adult female mosquito was dissected three days after emergence. A pair of SG was isolated and examined under a confocal microscope, FV1000 (Olympus Co., Tokyo, Japan) to observe the red color of DsRed in the SG.

Discharge of recombinant DsRed from TG mosquitoes

To observe the discharge of DsRed from the proboscis of the TG mosquitoes, we employed the method of Billingsley *et al.* [14] with some modifications. We put a 30 × 40 mm piece of NC sheet on top of the mosquito cage and placed a 30-ml trianglular flask containing 40–45°C of water on the NC sheet. The mosquitoes sensed the higher temperature at the top of the cage, gathered at the location on the sheet, and started probing for blood capillaries. In 10 minutes, they deposited saliva on the NC sheet. We cut the sheet into four pieces and incubated each with the rabbit anti-DsRed antibody (Clontech), normal rabbit serum, the mouse anti-SG antibody [9] and normal mouse serum. These sheets were reacted with HRP-conjugated anti-rabbit IgG or anti-mouse IgG, then probed with substrate solution. Positive spots were visualized with the LAS-1000.

TG mosquito biting on naïve mice

To produce anti-DsRed antibodies, two naïve ICR mice (No. 1 and No. 2) were each bitten by 50 female DsRed-TG mosquitoes every two weeks for ten weeks (five times). Anti-DsRed antibody production was monitored by ELISA as shown below. As a negative control, two ICR mice (No. 3 and No. 4) were each bitten by 50 female wild-type mosquitoes every two weeks for ten weeks. Anti-SG antibody production was monitored by ELISA.

Pre-immunization of mice with DsRed

Six ICR mice were peritoneally injected with 1 μg of DsRed plus Alum. Two weeks later, two of the mice (No. 5 and No. 6) were bitten by 50 DsRed-TG mosquitoes. The biting was repeated five times at two-week intervals. Two other mice (No. 7 and No. 8) were each bitten by 50 wild-type mosquitoes at two-week intervals. Two more mice (No. 9 and No. 10) were injected with 1 μg of DsRed plus Alum one more time but not bitten by mosquitoes.

Evaluation of antibody concentrations by ELISA

For the evaluation of anti-DsRed antibody production, 30 wells of a 96-well assay plate were coated with 100 μl of DsRed (1 μg/ml) (Clontech) in 0.05 M carbonate buffer (pH 9.6). To evaluate anti-SG antibody production, 40 pairs of SG were collected from wild-type female mosquitoes, disrupted in 4.0 ml of carbonate buffer by sonication (1 sec × 5 times), and centrifuged at 8,000 rpm for 3 min. The supernatant was used as SG antigen. Thirty wells were coated with 100 μl of SG antigen. Another 30 wells were filled with 100 μl of carbonate buffer without antigen. The plate was incubated at 4°C overnight.

Five micro-liters of blood was collected from the tail of each mouse 7 days after the final mosquito biting. The blood was mixed with 1.0 ml of phosphate-buffered saline containing 1% bovine serum albumin (BSA/PBS), and centrifuged at 8,000 rpm for 3 min. The supernatant was used

as 400-fold diluted serum.

After removal of the antigen solution and two washes, the wells of the ELISA plate were blocked with 150 µl of 1% BSA/PBS for 30 min. The blocking solution was removed, and 100 µl of mouse serum diluted 400-fold was added to three DsRed antigen-containing wells, three SG antigen-containing wells and three antigen-free wells. After 2 h of incubation and five washes, a secondary antibody, rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Bio-Rad) diluted 3,000-fold, was added. After 1 h of incubation and five washes, a substrate solution, a mixture of 0.04% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, St. Louis, MO, USA), 0.05% H_2O_2 in 0.05 M phosphate and 0.1 M citrate buffer (pH 4.5), was added. After 20–60 min, a green color appeared. Absorption was measured at a wavelength of 405 nm. The mean absorbance of antigen-containing wells minus that of antigen-free wells was used as the OD value.

RESULTS

Isolation of TG mosquitoes

Two recombinant plasmids (Fig. 1) were injected into 117 eggs of wild-type mosquitoes. Forty-four larvae hatched and 38 of these grew and emerged. Adult females were mated with wild-type males, and adult males were mated with wild-type females, and then eggs were obtained from each female individually. After hatching, the GFP-expressing larval group was considered to be a transgenic mosquito strain. In the end, four strains were established.

Molecular size of recombinant DsRed produced in the SG

Three to five days after emergence, ten pairs of SG were collected from female TG mosquitoes in each colony. Half of the samples were separated on a SDS-polyacrylamide (10%) gel under reducing conditions and transferred to a NC sheet. Western blotting produced bands corresponding to a molecular size of 28 kDa. Among the four colonies, #4 expressed DsRed most strongly (data not shown). A comparison of the density of the 28 kDa band with that of the positive control bands for a series of different amounts of DsRed monomer revealed that the amount of DsRed in the #4 lanes (5 and 10 pairs, respectively) was equivalent (in reactivity with the anti-DsRed antibody) to 125 and 250 ng of DsRed monomer (Fig. 2). This, in turn, indicated that approximately 25 ng of DsRed was expressed in one pair of SG in the #4 TG mosquitoes. We chose colony #4 for the subsequent experiments.

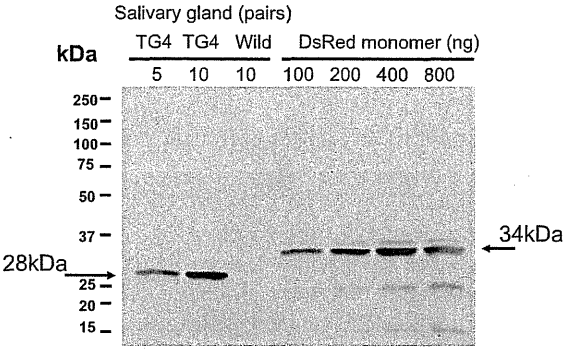

Fig. 2. Estimation of DsRed amount produced in the SG of #4 DsRed-TG mosquitoes. Five and ten pairs of SG of DsRed-TG mosquitoes were separated, and compared with a series of different amounts of DsRed monomer (34 kDa).

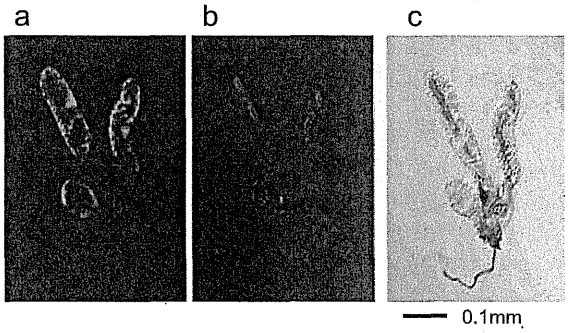

Fig. 3. Salivary gland of TG mosquitoes expressing GFP and DsRed. a: GFP expressed in salivary gland cells. b: DsRed in the lumen of the salivary gland. c: combined photograph.

Observation of the SG

An adult female TG mosquito was dissected three days after emergence, and the SG was isolated and observed under a confocal microscope. As shown in Fig. 3, the green color of GFP was observed in cellular areas where saliva proteins were produced. The red color of DsRed was observed in the lumen, the cavity for storing saliva, in the central part of the SG. This indicates that an alien protein, DsRed, was secreted into the cavity of the SG.

Discharge of recombinant DsRed from the SG

As shown in Fig. 4, mosquito saliva was deposited on the NC sheets. Spots of DsRed were detected on the sheet probed by the DsRed-TG mosquitoes. This is direct evidence that an alien protein, DsRed, was discharged on the NC sheet from the TG mosquitoes.

Development of anti-DsRed antibody through TG mosquito bites

After being bitten repeatedly by the DsRed-TG mosquitoes, ICR mice developed anti-DsRed as well as anti-SG antibodies in their serum (Fig. 5). This was indirect evidence that the TG mosquitoes deposited DsRed together with saliva into the mouse skin when they drew blood. In the next experiment, we injected 1 μg of DsRed monomer into ICR mice with Alum before any mosquito biting. The pre-immunized mice (No. 5 and 6) produced anti-DsRed antibody after repeated biting by the TG mosquitoes. Compared with mice No. 1 and No. 2, mice No. 5 and No. 6 produced more anti-DsRed antibody. However, No. 9 and No. 10, which were injected with DsRed two times, produced more anti-DsRed antibody than No. 5 and No. 6.

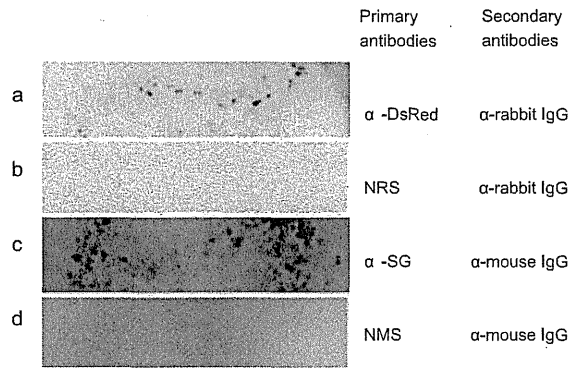

Fig. 4. Discharge of recombinant DsRed from TG mosquitoes. Spots on NC sheets are saliva deposited by DsRed-TG mosquitoes. NRS: normal rabbit serum. NMS: normal mouse serum.

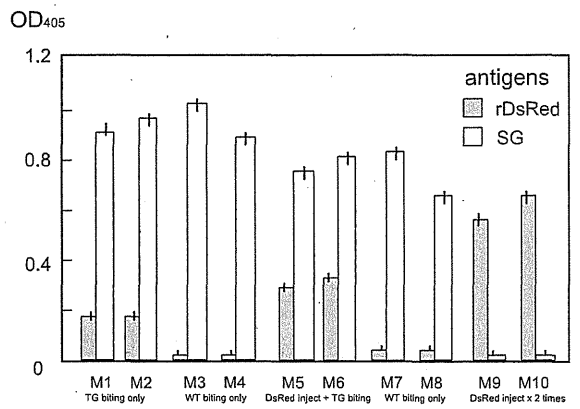

Fig. 5. Development of anti-DsRed antibody by DsRed-TG mosquito bites. Mice No. 1 and No. 2 were each bitten by 50 DsRed-TG mosquitoes five times at two-week intervals. Anti-DsRed antibody was produced in the two mice. Mice No. 3 and No. 4 were each bitten by 50 wild-type mosquitoes five times. Mice No. 5–10 were injected with 1 μg of DsRed. Then mice No. 5 and No. 6 were each bitten by 50 DsRed-TG mosquitoes five times at two-week intervals. Much anti-DsRed antibody was produced in the two mice. Mice No. 7 and No. 8 were each bitten by 50 wild-type mosquitoes five times. Mice No. 9 and No. 10 were injected with 1 μg of DsRed one more time.

DISCUSSION

Mosquitoes deposit saliva in the skin when they take a bloodmeal, causing a variety of physiological responses in the host [15]. Repeated biting leads to the development of an anti-saliva antibody [16]. In areas where malaria is hyper-endemic, more than 100 anopheline mosquitoes can attack a person in a single hour during the night [17]. In these areas, therefore, most people have high levels of anti-saliva antibodies [18–20]. It follows that if transgenic (TG) mosquitoes expressing a vaccine protein in saliva could be produced and released by the millions into the field, people would be supplied with the protein when they were bitten, produce antibodies to the protein, and develop immunity to the disease.

In 2010, we succeeded in producing a TG mosquito expressing GFP in the eyes and producing a recombinant CSP in the SG [9]. We allowed a hundred of the CSP-expressing TG mosquitoes (CSP-TG mosquitoes) to feed on mice several times, but the mice only developed anti-saliva antibodies. The reason for this might be that the TG mosquito produced CSP in the salivary gland cells but did not excrete it in the saliva and so did not inject it into the mice. In this study, we employed a colored protein, DsRed, in order to observe the location of the alien protein under a fluorescence microscope. As expected, we observed that the TG mosquitoes excreted DsRed in the cavity of the SG (Fig. 3). Moreover, the TG mosquitoes discharged DsRed via their proboscis (Fig. 4).

The estimated amount of DsRed in the salivary grand was 25 ng per individual female mosquito (Fig. 2). In our previous experiments, the estimated amount of transgenic protein in the salivary gland was 40 ng of CSP [9], 20 ng of SP15 [10], and 10 ng of DsRed-CSP [11]. These amounts were not significantly different.

The mice bitten by DsRed-TG mosquitoes produced anti-DsRed antibodies as expected (Fig. 5), but the titer was not as high as that of the anti-SG antibody. In the next experiment, we injected 1 μg of DsRed intraperitoneally once and allowed the mice to be fed upon by DsRed-TG mosquitoes. After several rounds of biting, the mice developed a

higher concentration of anti-DsRed antibody than did the mice only bitten by DsRed-TG mosquitoes (Fig. 5). Memory B cells might be produced in the mice in response to the first injection of DsRed. The DsRed-TG mosquitoes' biting might then act as a booster. This suggests that the primary immunization of babies with a malarial vaccine protein and then exposure to TG mosquitoes excreting the malarial vaccine protein in saliva would produce a synergistic effect in endemic areas.

In our previous report, we allowed mice to be bitten 20 times by 100 TG mosquitoes. Over four months and about 1,500 bites were needed for the suitable antibody titer to inhibit the invasion of malaria parasites to hepatic cells [11]. However, pre-immunizing methods may reduce the number of mosquito bites. With one pre-immunization in a mouse, biting by 50 TG mosquitoes five times should be enough to induce a similar titer of anti-malarial antibody in the mouse. Further experiments are needed to confirm this possibility.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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弱いといわれている．

3. **ブタ回虫**　養豚業者などで時にみられることが知られているが，肝好酸球肉芽腫と血清 IgE 値の高値，末梢血好酸球増多がみられる．

4. **アライグマ回虫**　その幼虫移行症は近年米国などで報告され，上述の他の動物由来回虫の幼虫移行症と異なり，脳障害を起こし，時として死に至る疾患として注目する必要がある．

Ⓑ 診断

確定診断はいずれも幼虫の摘出による病理組織学的診断であるが，寄生虫学的血清診断が最も有用な補助診断である．また，画像診断では，CT，MRI で肝好酸球肉芽腫が多発性の占拠性病変を認める．眼症の場合，眼底の腫瘤病変を認め，これらが診断の糸口となる場合がある．

治療方針

℞ 処方例

エスカゾール錠　10－15 mg/kg　分3　4－8週間　保外

網膜腫瘤の場合，内服治療によるアレルギー反応のため失明することがあり，禁忌である．外科的摘出が望ましい．

Ⅲ 顎口虫症

病態と診断

Ⓐ 病態

わが国でみられる顎口虫症の原因寄生虫は，有棘顎口虫，剛棘顎口虫，ドロレス顎口虫，日本顎口虫が知られ，成虫はネコやイヌ（有棘顎口虫，剛棘顎口虫），イノシシ（ドロレス顎口虫），イタチ（日本顎口虫）などの胃を本来の寄生部位とし，ヒトへの感染は第2中間宿主のドジョウ，ライギョ，カエルなどやヘビなどの待機宿主の筋肉内に含まれる幼虫を生食することによる．

近年メキシコなどに渡航して，風土料理であるセビーチェなどを食し，皮膚爬行疹や移動性腫瘤を呈した二核顎口虫症疑診例も報告され，注意を要する．

Ⓑ 診断

本幼虫移行症は遊走性限局性浮腫や皮膚爬行疹として症状を呈する場合が多い．末梢血好酸球増多や血清 IgE 値高値が認められる．

確定診断は病巣からの虫体の摘出によるが，実際は困難なことが多く，寄生虫学的血清診断が補助診断として有用である．

治療方針

確定診断と治療目的で，病変部の摘出を行う．

摘出できない場合は内服治療の適応となるが，効果は不確実なことがあり，長期の経過観察が必要である．

℞ 処方例

エスカゾール錠　10－15 mg/kg　分3　4－8週間　保外

Ⅳ 旋尾線虫症

病態と診断

Ⓐ 病態

旋尾線虫幼虫は，富山県を中心とした北陸近郊の日本海沿岸で収穫されるホタルイカに寄生している．生活史はまだ完全に解明されていないが，ヒトへの感染はホタルイカの生食による．

本寄生虫による幼虫移行症は大きく2つの病態に分かれ，皮膚爬行症を呈するものと，小腸壁に肉芽腫を形成し腸閉塞症状を呈するものとがある．

Ⓑ 診断

確定診断はいずれも病変の摘出による病理学的診断である．補助診断として寄生虫学的血清診断が有用であるが実施機関は限られている．日本寄生虫学会に問い合わせるとよい．

治療方針

確定診断と治療目的で，病変部の摘出を行う．腸管寄生の場合，摘出困難なことも多く，血清診断などで，本疾患であると判明した場合，対症療法で経過観察し，虫体の死滅するのを待つこともできる．

アニサキス症
anisakiasis

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病態と診断

Ⓐ 病態

アニサキス症は，新鮮な海産魚介類を刺身などで食べて，その魚介類に寄生している生きたアニサキス幼虫を摂取して感染，発症する．幼虫が死滅していれば感染が成立しないので，－20℃で一晩以上凍結した食材を用いた料理や，60℃以上で加熱調理した料理であればアニサキス症の心配はない．

わが国でアニサキス症を起こすのは，*Anisakis simplex*，*Pseudoterranova decipiens*，*Anisakis physeteris* である．成虫は海生哺乳類（クジラ類やアザラシ類）の胃にもぐり込んで寄生している．宿主の糞便とともに海中へ放出された虫卵から幼虫が孵化し，中間宿主である海産甲殻類（オキアミなど）に寄生する．幼虫は，この甲殻類を摂取した魚類やイカ類の体内で幼虫のまま寄生し続ける．われ

われ人間への感染源は，これら幼虫に寄生された海産魚介類であり，サバ，タラ，オヒョウ，スルメイカ，イワシ，サケなどが知られている．アニサキス症の多い時期は，感染源となる海産魚介類の漁期である12-3月の寒い時期が多く，7-9月の夏場は少ない．

アニサキス症の病型を大きく分けると，胃アニサキス症，腸アニサキス症，そして消化管外アニサキス症に分けられる．

1. 胃アニサキス症　アニサキス症のほとんどはこの病型である．典型的には，感染源の魚介類を摂取して数時間後から遅くとも24時間以内に，心窩部痛や上腹部痛で発症する．悪心・嘔吐を伴うこともあるが下痢はしない．内視鏡検査により，体長2-3cmの透明感を伴う乳白色の虫体が胃粘膜に刺入しているのを認めれば，診断が確定する．内視鏡検査前に，前日に食べたものをよく聞き出すことが重要である．幼虫の摘出により症状はすぐに改善する．

2. 腸アニサキス症　頻度は少ないが，幼虫が腸管粘膜に刺入して腸アニサキス症となる．胃アニサキス症よりも発症が遅く，魚介類の摂取後1-5日で発症する．虫体の直接証明が難しく，ほとんどは原因不明の腹痛として対応されていると考えられるが，イレウス症状を起こして開腹手術に至り，摘出小腸から虫体がみいだされた例もある．

3. 消化管外アニサキス症　幼虫が消化管壁を通り抜けてほかの臓器に迷入し，肉芽腫を形成する．手術標本に死滅したアニサキス幼虫が偶然みつかるような場合から，胸膜炎などの病型をとることもある．食歴の注意深い聴取とともに，胸水や血清などの抗アニサキス抗体を検出して総合的に診断する．

治療方針

胃や十二指腸であれば内視鏡を用いて虫体を摘出する．いわゆる駆虫薬とよべるものは存在しないが，アニサキスの幼虫は人体内で長期間生存することはできず，1週間程度で死滅するので，その間は鎮痛薬や抗アレルギー薬などの内科的な処置を行い経過を観察する．

患者説明のポイント

・アニサキスは人体内で長期間生存できないこと，−20℃で一晩以上凍結した食材を用いた料理や60℃以上で加熱調理した料理であれば，アニサキスに感染するリスクはないことを説明する．

消化管寄生条虫症

cestodiasis in gastrointestinal tract

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病態と診断

消化管寄生条虫症には多くの種類があるが，日常臨床上遭遇する機会が多いものは日本海裂頭条虫症である．そのほかにはアジア条虫症，無鉤条虫症，有鉤条虫症，大複殖門条虫症，マンソン裂頭条虫症，瓜実条虫症などがある．ヒトの小腸に寄生するこれらの条虫の成虫は，腸管組織に侵入せず，さらに消化管をもたずに虫体表面から栄養を吸収している．一般的に成虫のヒトに対する病害性は少ない．

診断は，自然に排出した片節や駆虫した虫体を観察して行う．

治療方針

原則として，抗寄生虫薬であるビルトリシドの経口投与で治療する．ただし，有鉤条虫症では，ビルトリシドにより腸管内で虫体が破壊される危険性があると考えられている．虫体が破壊された場合には有鉤嚢虫症の発生が懸念されるとの理由で，消化管造影剤であるガストログラフインを用いた療法を行うことが望ましいとされている（ガストログラフインには虫体を破壊する作用がないと考えられている）．

なお，ビルトリシドで治療に失敗した場合には，再度ビルトリシドで駆虫するか，ガストログラフインで駆虫する．

1. ビルトリシド療法　治療前日に下剤を服用し，当日の朝は禁食とする．さらに，ビルトリシド内服後にも下剤を服用すると虫体の回収が容易で，頭節の確認も行いやすい．われわれ（都立墨東病院感染症科）が行っている，成人患者に対する具体的な方法を以下に記述する．

ビルトリシド投与前日の20時頃に，1）を服用し，さらに21時頃に2）を内服する．当日の朝は禁食とし，3）を服用する．その約2時間後に，4）を服用する．

℞ 処方例

1）マグコロールP散（50mg/包）　1包　水150mLに溶かして服用（保外）
2）プルゼニド錠（12mg）　2錠　水500mLとともに服用
3）ビルトリシド錠（600mg）　1回10mg/kg　1回服用（保外）
4）マグコロールP散（50mg/包）　1包　水300mLに溶かして服用（保外）

Identification of a Bacteria-like Ferrochelatase in *Strongyloides venezuelensis*, an Animal Parasitic Nematode

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Abstract

Heme is an essential molecule for vast majority of organisms serving as a prosthetic group for various hemoproteins. Although most organisms synthesize heme from 5-aminolevulinic acid through a conserved heme biosynthetic pathway composed of seven consecutive enzymatic reactions, nematodes are known to be natural heme auxotrophs. The completely sequenced *Caenorhabditis elegans* genome, for example, lacks all seven genes for heme biosynthesis. However, genome/transcriptome sequencing of *Strongyloides venezuelensis*, an important model nematode species for studying human strongyloidiasis, indicated the presence of a gene for ferrochelatase (FeCH), which catalyzes the terminal step of heme biosynthesis, whereas the other six heme biosynthesis genes are apparently missing.

Phylogenetic analyses indicated that nematode FeCH genes, including that of *S. venezuelensis* (SvFeCH) have a fundamentally different evolutionally origin from the FeCH genes of non-nematode metazoa. Although all non-nematode metazoan FeCH genes appear to be inherited vertically from an ancestral opisthokont, nematode FeCH may have been acquired from an alpha-proteobacterium, horizontally. The identified *Sv*FeCH sequence was found to function as FeCH as expected based on both *in vitro* chelatase assays using recombinant *Sv*FeCH and *in vivo* complementation experiments using an FeCH-deficient strain of *Escherichia coli*. Messenger RNA expression levels during the *S. venezuelensis* lifecycle were examined by real-time RT-PCR. *Sv*FeCH mRNA was expressed at all the stages examined with a marked reduction at the infective third-stage larvae.

Our study demonstrates the presence of a bacteria-like FeCH gene in the *S. venezuelensis* genome. It appeared that *S. venezuelensis* and some other animal parasitic nematodes reacquired the once-lost FeCH gene. Although the underlying evolutionary pressures that necessitated this reacquisition remain to be investigated, it is interesting that the presence of FeCH genes in the absence of other heme biosynthesis genes has been reported only for animal pathogens, and this finding may be related to nutritional availability in animal hosts.

Introduction

Heme is essential for the vast majority of life serving as a prosthetic group for many hemoproteins such as catalase, cytochrome, hemoglobin, myoglobin, and peroxidase [1]. Although most aerobic organisms possess a complete biosynthetic pathway for this compound [2], certain organisms are deficient in heme biosynthesis, lacking some or all genes for the hemebiosynthetic pathway. Some anaerobic protists, such as *Giardia intestinalis*, *Trichomonas vaginalis*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Blastocystis hominis*, and *Encephalitozoon cuniculi* do not possess any heme biosynthetic genes [3]. Members of the family Trypanosomatidae lost some or the entire set of heme biosynthesis genes. They acquire heme or heme precursors from their diet [3,4]. In Trypanosomatidae, members of the genus *Trypanosoma* lack all the heme biosynthesis genes [3,5,6,7], whereas other members such as *Leishmania* spp. possess the genes for the last three steps which were horizontally acquired from a gamma–proteobacterium [3]. Insect trypanosomatid species (*Blastocrithidia culicis* and *Crithidia oncopelti*) cannot synthesize heme by themselves but harbor bacterial endosymbionts that generate and donate heme or heme precursors to the host (trypanosomatid) cells [4,8]. More peculiar is the case of *Phytomonas serpens*, a plant kinetoplastid [9]. This organism lacks most of the known hemoproteins including respiratory cytochromes and does not require heme for viability despite its dependence on oxidative metabolism [9]. The draft genome of *P. serpens* does not appear to contain heme biosynthesis genes other than ferrochelatase (FeCH, EC 4.99.1.1) [9].

Another important and interesting group of organisms that lack the ability to synthesize heme is the nematodes. Nematodes, or roundworms, are typically small, diverse, and highly abundant metazoan organisms [10]. Although free-living species are found in nearly all habitats (marine, freshwater, and soil), nematodes are also parasites of vertebrate and invertebrate animals as well as plants. Molecular phylogenetics have defined five major nematode clades (I through V), within which parasitism has arisen multiple times [11]. The genome of *Caenorhabditis elegans*, which was the first metazoan genome to be completely sequenced [12], appears to lack all seven genes necessary to synthesize heme from 5-aminolevulinic acid [13].

Some hemoproteins of animal parasitic nematodes are particularly well studied because of the interests in their roles in low-oxygen environment (host intestine). One such protein is perienteric hemoglobin of *Ascaris lumbricoides* (parasitic nematode of humans), which has an extraordinary

high oxygen affinity, approximately 10,000-fold higher than that of the host's globin [14]. The proposed functions of this oxygen-avid hemoglobin include oxygen detoxification by a reaction driven by nitric oxide [15] and maintenance of body wall O_2 tension by creating an inward-decreasing O_2 gradient that is considered important for oxygen unloading from body wall myoglobin, another heme-containing protein [16]. Another example of well-studied nematode hemoproteins is cytochrome *b* in the mitochondrial respiratory complex II of *Ascaris suum* (swine parasitic nematode). *A. suum* larvae utilize classic mammalian-type respiration, expressing a small subunit of larval cytochrome *b* ($CybS^L$) [17]. In contrast, adult worms live in the host small intestine, where oxygen tension is low and utilize an anaerobic NADH-fumarate reductase system expressing a different small subunit of cytochrome *b* ($CybS^A$) instead of $CybS^L$ [17]. Given the important roles played by the hemoproteins in animal parasitic nematodes, it is interesting to know how heme molecules are synthesized or acquired from the animal hosts.

Strongyloides is a genus of obligate gastrointestinal parasites of vertebrates that belong to nematode clade IV [18]. Among more than 50 documented species, two are known to cause human infections, namely *Strongyloides stercoralis* and *Strongyloides fuelleborni* [18]. It is estimated that 30–100 million individuals are infected with *Strongyloides* worldwide primarily in tropic and subtropic regions [19]. Symptoms are usually absent or mild in immunocompetent hosts. However, in impaired host immunity, severe manifestations can develop, and fatalities may ensue [20].

To study strongyloidiasis, *Strongyloides venezuelensis*, which is native to rats but can also infect mice, has been widely used as a model [21]. In a transcriptome sequencing project of this *Strongyloides* species, we identified a partial cDNA sequence that most likely encodes a gene for FeCH [22]. FeCH catalyzes the terminal step of heme biosynthesis [23]. The existence of FeCH sequences was noticed in the genomes of *Brugia malayi* (another animal parasitic nematode belonging to nematode clade III) and its bacterial endosymbiont, (*Wolbachia*). However, further analysis was conducted only on the FeCH gene in the endosymbiont genome [24,25].

In the present study, we cloned the entire cDNA sequence of the FeCH gene from *S. venezuelensis* (*Sv*FeCH). Our BLAST search on publically available databases revealed that only a fraction of nematode species possesses the FeCH gene. Interestingly, all these species were parasites of mammals. Surprisingly, in our phylogenetic analysis, nematode FeCH formed a distinctive clade, and it was placed distantly from the clade that contains non-nematode metazoan FeCH, suggesting that the origin of nematode FeCH genes are different from those of non-nematode metazoan FeCH.