Ⅲ. 研究成果の刊行物・別刷

Parasites & Vectors

BioMed Central

Research

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Loop-mediated isothermal amplification applied to filarial parasites detection in the mosquito vectors: *Dirofilaria immitis* as a study model

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Published: 15 March 2009

Parasites & Vectors 2009, **2**:15 doi:10.1186/1756-3305-2-15

Received: 4 November 2008
Accepted: 15 March 2009

This article is available from: http://www.parasitesandvectors.com/content/2/1/15

Abstract

Background: Despite recent advances in our understanding of the basic biology behind transmission of zoonotic infectious diseases harbored by arthropod vectors these diseases remain threatening public health concerns. For effective control of vector and treatment, precise sampling indicating the prevalence of such diseases is essential. With an aim to develop a quick and simple method to survey zoonotic pathogen-transmitting vectors, LAMP (loop-mediated isothermal amplification) was applied to the detection of filarial parasites using a filarial parasite-transmitting experimental model that included one of the mosquito vectors, *Aedes aegypti*, and the canine heartworm, *Dirofilaria immitis*.

Results: LAMP reactions amplifying the cytochrome oxidase subunit I gene demonstrated high sensitivity when a single purified *D. immitis* microfilaria was detected. Importantly, the robustness of the LAMP reaction was revealed upon identification of an infected mosquito carrying just a single parasite, a level easily overlooked using conventional microscopic analysis. Furthermore, successful detection of *D. immitis* in wild-caught mosquitoes demonstrated its applicability to field surveys.

Conclusion: Due to its simplicity, sensitivity, and reliability, LAMP is suggested as an appropriate diagnostic method for routine diagnosis of mosquito vectors carrying filarial parasites. This method can be applied to the survey of not only canine filariasis but also lymphatic filariasis, another major public health problem. Therefore, this method offers great promise as a useful diagnostic method for filarial parasite detection in endemic filariasis regions.

Background

For years, vector-borne zoonotic infectious diseases have had profound debilitating effects on humans. It has recently been revealed that several arthropod vectors including mosquitoes act as "bridge" vectors by transmitting pathogens from animals to humans and vice versa [1]. Human clinical cases of diseases, such as dirofilariasis, babesiosis, and leishmaniases, all caused by parasites transmitted by arthropod vectors from animals, have been reported to be on the rise [2-6]. Infection with these diseases have affects not only on humans but also on domestic and wild animals that can serve as potential reservoirs by hosting pathogens long-term despite being asymptomatic. Because eradication of animal reservoirs has been ethically rejected, surveillance and control of arthropod vectors must be central to programs aimed at elimination of vector-borne zoonotic diseases [5].

Dirofilaria species, including *D. immitis* and *D. repens*, are particularly important pathogens due to the fact that they induce serious symptoms in domestic animals, especially dogs. Adult worms in infected canines release microfilariae in their blood stream that are then ingested by mosquitoes during blood feeding. Mosquitoes then harbor these parasites until they reach the third larval stage (L3) at which point transmission back to canines is possible. Several common mosquito species are capable of transmission including, occasionally, to humans where, despite acting as a "dead end" host for the *Dirofilaria* parasites, symptoms such as coughing and chest pain are induced [2]. Human clinical cases of zoonotic infection of *Dirofilaria* are increasing in various countries [2-4] leading to frequent misdiagnosis as lung tumors or tuberculosis rather than pulmonary dirofilariasis [3,4]. Further complicating the situation is that analysis of many clinical cases involving infected humans revealed a lack of any exposure to infected domestic dogs suggesting transmission occurred through wild animals [2]. Indeed, it has been reported that wild animals including raccoons, dogs, wolves, dingoes, coyotes, and foxes are capable of infection with *Dirofilaria* species thereby serving as reservoirs [7-12]. Therefore, control of mosquitoes based on precise surveillance of *Dirofilaria* species is essential for preventing infection of both domestic animals and humans with these parasites.

Surveying of filarial parasites has long depended on microscopic examination of dissected mosquitoes or blood films. Unfortunately, this method requires a practiced-eye and oversight often occurs in specific stages where the parasite is difficult to recognize. Additionally, microscopy is laborious and time-consuming thereby impeding routine monitoring of large-scale control programs. Application of PCR (polymerase chain reaction) has been considered as a more accurate and practical diagnostic alternative leading recently to the development of several methods [13-17]. Despite its outstanding sensitivity and specificity, drawbacks remain due to the need for expensive equipment and trained technicians. Hence to reveal precisely the distribution of risk to filarial diseases novel diagnostic methods that are simple, rapid, sensitive, and reliable are required. In consideration of these points we propose a novel surveillance strategy.

Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method that allows reactions to occur under isothermal conditions [18]; the *Bst* DNA polymerase can synthesize a new strand of DNA while simultaneously displacing the complementary strand thereby enabling DNA amplification at a single temperature with a single enzyme. Four primers are required for the LAMP reaction: FIP, BIP, F3, and B3. F3 and B3 contribute to the formation of a stem-loop structure while the other two primers, FIP and BIP, designed complementary to the inner sequence of the stem-loop structure, are used to amplify the target sequence, thus providing a higher specificity to the reaction than conventional PCR methods. Another advantage using LAMP is based on the fact that the amplification from stem-loop structures leads to accumulation of large amounts of products of varying lengths ultimately making detection of amplified DNA much easier. Furthermore, the by-product of the reaction, magnesium pyrophosphate, is a white-colored precipitate easily seen by the naked eye [19].

Recently, wide applicability of LAMP in the detection of parasites such as *Trypanosoma*, *Babesia*, and *Plasmodium* has been demonstrated [20-23]. Additionally, the usefulness of LAMP has been applied to the identification of genus- and species-specific parasites [24,25]. However, application of LAMP to survey vectors has been largely neglected despite its promise. Due to the fact that a novel applicable method to survey filarial-carrying mosquitoes is still demanded, we propose the application of LAMP for detection of filarial parasites within vectors. Mosquitoes carrying one or more parasites have the ability to infect vertebrate hosts during blood feeding. This implies that all mosquitoes containing just a single parasite need to be identified unfailingly by LAMP in order to ensure a precise and practical survey. In this study, we applied LAMP in the detection of *D. immitis*, both within mosquito vectors using *Ae. aegypti* as laboratory model [26] in addition to field samples in order to demonstrate its potential as an important diagnostic tool to be coupled with ongoing vector control measures.

Method

Preparation of parasite and infected mosquitoes

Adult *Dirofilaria immitis* were isolated from an infected dog and cultured in RPMI 1640 media. Microfilariae were

paralyzed by cooling to 4°C and collected by centrifugation at 90 to 130 × g for 5 to 10 minutes. Isolated microfilariae were counted on a cytometer and stored at -20°C until DNA extraction. To evaluate usability of the LAMP method as a practical vector-diagnostic method, LAMP-based detection of *D. immitis* within mosquitoes was carried out. *Aedes aegypti* was fed with *Dirofilaria*-infected canine blood by membrane feeding. Briefly, Parafilm® (Pechiney Plastic Packaging, Inc., Illinois, USA) was fitted under the culture flask containing warm water and filled with infected canine blood. The membrane feeder was set over the netted mosquito cup and mosquitoes were allowed to feed on infected blood for 1 hour. Infected mosquitoes were kept at 27°C until 8 days post infection when *Dirofilaria* in mosquitoes reached the L2 or L3 stage. Each Malpighian tubules together with the carcass of the infected mosquito was dissected in ice-cold PBS at day 8, microscopically analyzed at 50-fold magnification to count the number of parasites, and stored at -20°C until DNA extraction. *Dirofilaria* L2 or L3 larvae removed from Malpighian tubules were also stored for use as positive control of LAMP.

Mosquito collection

Wild mosquitoes were collected in Nago, Okinawa, Japan in August 2008. Mosquitoes were caught either with CDC traps (John W. Hock Co., Florida, USA) using CO_2 or by sweeping nets at locations near domestic dogs. Collected mosquito species were identified based on keys according to description and illustrations [27].

DNA extraction

Genomic DNA of *D. immitis* larvae and infected mosquitoes was extracted as follows: *D. immitis* larvae and infected mosquitoes were collected, homogenized with a plastic homogenizer in 100 μl of Buffer A (0.1 M Tris (pH 9.0), 0.1 M EDTA, 1% SDS, and 0.5% DEPC), and incubated for 30 minutes at 70°C. 22.4 μl of 5 M KoAc was added to the mixture and incubated for 30 minutes on ice. Supernatant was collected by centrifugation at 20400 × g for 15 min at 4°C and mixed with 45 μl of isopropanol. Precipitated DNA was collected after centrifugation at 20400 × g for 20 min at 4°C, rinsed with 70% ethanol, and dried. Each DNA pellet was diluted in TE to achieve a concentration such that 1 μl of solution would contain DNA from 1 × 10^4 microfilariae (152 ng) or one-fifth of a mosquito. 1 μl of each DNA solution was then used as a template for LAMP reaction.

LAMP reactions

Specific primers for LAMP reactions were designed against the *D. immitis* cytochrome oxidase subunit I gene (GenBank: EU169124). The locus and sequence of each primer (F3, B3, FIP, BIP) in this gene are shown in Fig. 1. The LAMP reaction was performed as per manufacture's

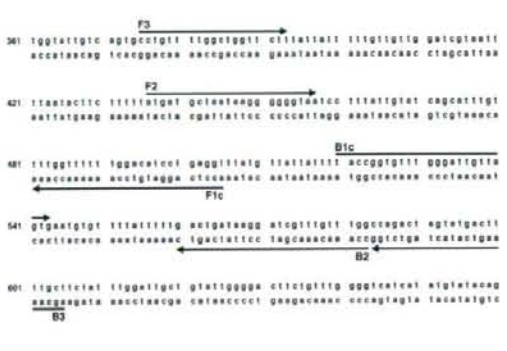

Figure 1
LAMP primer set targeting *D. immitis* cytochrome oxidase subunit I gene. (A) Partial sequence of *D. immitis* cytochrome oxidase subunit I gene and location of four primers, FIP (F1c-F2), BIP (B1c-B2), F3, and B3. Arrow indicates the direction of extension. Numbers on the left indicate the nucleotide position. (B) Sequence of primers for LAMP reaction.

instructions (Eiken Chemical Co., Ltd., Tokyo, Japan). Briefly, the reaction was performed in 12.5 μl of reaction mixture containing 1 μl of extracted DNA solution, 2.5 pmol of each F3 and B3 primers, 20 pmol of each FIP and BIP primers, 6.25 μl of 2× Reaction Mix., and 0.5 μl of *Bst* DNA polymerase. The reaction mixture was incubated at 63°C for 70 min using a Loopamp Realtime Turbidimeter (LA-200; Eiken Chemical Co., Ltd., Tokyo, Japan) and terminated by incubation at 95°C for 2 minutes.

Analysis of LAMP products

Amplified DNA in the LAMP reaction causes turbidity due to the accumulation of magnesium pyrophosphate, a by-product of the reaction. Turbidity was monitored using Loopamp Realtime Turbidimeter in addition to the naked eye. All LAMP products were electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

DNA sequencing of LAMP products

LAMP reaction products forming structures with stem-loops at each end were collected from agarose gels after electrophoresis and purified by QIAEX II Gel extraction Kit (QIAGEN, Dusseldorf, Germany) following manufacture's instructions. Sequences were directly determined with FIP and BIP primers using ABI PRISM 3100 Genetic

Analyzer (Applied Biosystems, CA, USA) and analyzed using BLAST.

Results

Sensitivity of LAMP in the detection of Dirofilariaimmitis.

Primers for *D. immitis* detection were designed to target the *D. immitis* cytochrome oxidase subunit I gene due to availability of sequence data in GenBank (EU169124) (Fig. 1A and 1B). Initial tests examining specificity and sensitivity of this primer set were performed using serial dilutions of *D. immitis* DNA prepared from cultured microfilariae. Optimization of LAMP reaction conditions (temperature and time) using the described primer set revealed ideal settings to be 63 °C and 70 minutes (data not shown). Sequencing of these LAMP products showed that amplified DNA were of *D. immitis* cytochrome oxidase subunit I gene (data not shown).

Having determined the experimental conditions, the sensitivity of LAMP reactions in the detection of the *D. immitis* microfilariae was tested. *D. immitis* microfilariae DNA (equivalent to 1×10^0, 1×10^1, 1×10^2, 1×10^3, and 1×10^4 parasites) obtained from cultured adult worms was used as a template for LAMP reactions and yielded an amplified product approximately 30 minutes after incubation using DNA from 1×10^4 parasites (152 ng) (Fig. 2A). Importantly, a product could be detected from samples containing just 1×10^0 microfilaria while control samples remained negative (Fig. 2A). All samples containing DNA of microfilariae showed white precipitates detected by naked eye (data not shown). In agreement with turbidity analysis and eye-observation, gel electrophoresis also showed that one microfilaria is sufficient for LAMP detection (Fig. 2B). These data indicate that LAMP reactions using the primer set designed against the *D. immitis* cytochrome oxidase subunit I gene is able to identify purified parasites collected from both the blood and vector stages down to levels of a single microfilaria parasite.

Evaluation of LAMP for diagnosis of D. immitis*-carrying mosquitoes.*

One of the field mosquito vectors of *D. immitis*, *Ae. aegypti* [28], was employed as laboratory model [26]. In an experiment using 6 independent mosquito samples, the number of L2 parasites ranged from 1 to 16 (Fig. 3A–3F). After microscopic observation, each Malpighian tubules was collected together with its carcass before being subjected to DNA extraction to be used as a template for LAMP reactions. The amplified product of each reaction mixture was determined by a combination of electrophoresis and a real-time turbidimeter revealing a detection profile correlated with L2 parasite numbers (Fig. 3G and 3H). Consistent with these results, white precipitates, which indicates LAMP-dependent DNA amplification, were produced in all reaction mixtures of infected samples

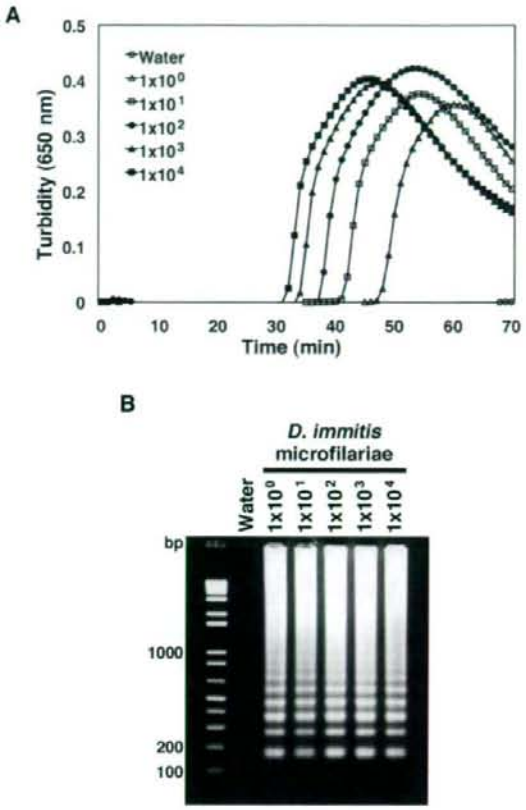

Figure 2
Kinetics of LAMP for *D. immitis* microfilariae. (A) Amplification of target sequence with primer set monitored by real-time turbidimeter (turbidity at 650 nm). (B) Detection of reaction products by LAMP with genomic DNA from *D. immitis* microfilariae. 1 μl of reaction mixture was run on a 2% agarose gel. Amplified products were detectable with any samples containing genomic DNA corresponding to the amount of one or more microfilariae. Numbers on the left indicate migration of molecular weight marker (bp).

(data not shown). It is particularly worth noting that even a single L2 parasite within a mosquito was successfully detected by the LAMP reaction (Fig. 3G and 3H).

LAMP-based identification of wild-caught D. immitis*-carrying mosquitoes.*

To evaluate whether the LAMP method is appropriate for diagnosis of *D. immitis*-carrying mosquitoes, we applied this technique to intact wild mosquitoes caught in the field. Mosquitoes were collected at several locations in Okinawa, Japan, species were determined (72 *Aedes albopictus*, 43 *Armigeres subalbatus*, 2 *Culex pipiens*, 2 *Culex*

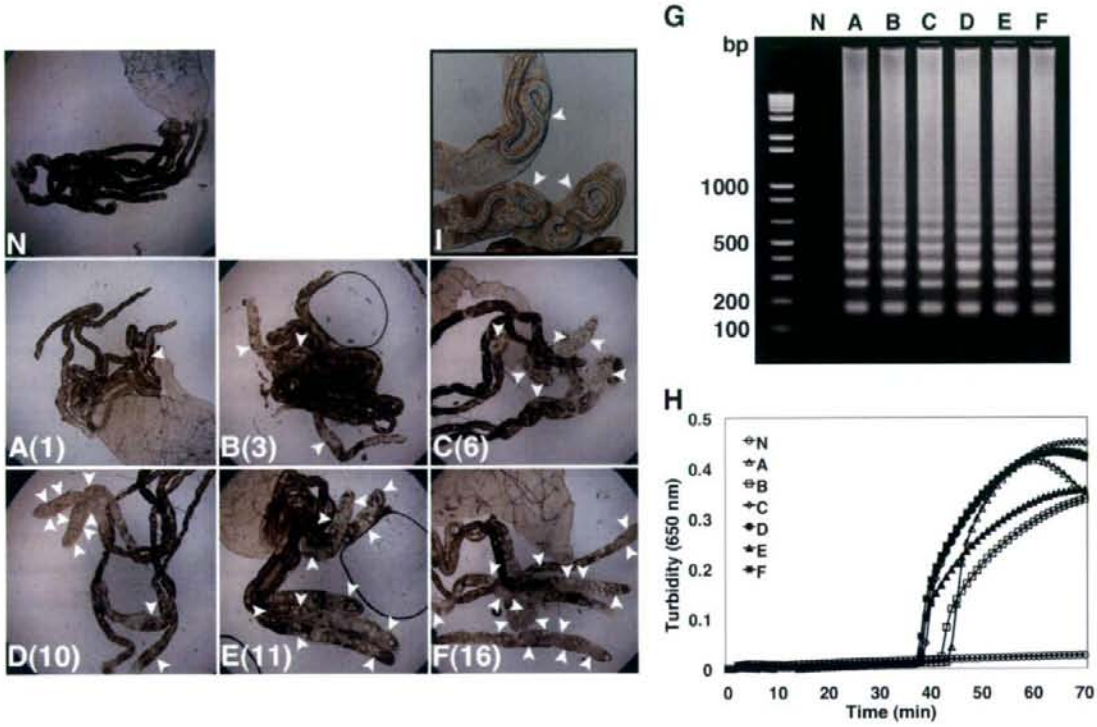

Figure 3
Evaluation of LAMP by detecting L2 stage *D. immitis* in mosquitoes. *D. immitis* in L2 stage were counted under microscopy prior to DNA extraction. (A-F) Mosquito Malpighian tubules infected with *D. immitis* in L2 stage. Figure in parentheses indicates the number of *D. immitis*. (N) Malpighian tubules from intact mosquito. White arrowheads indicate *D. immitis* in mosquito Malpighian tubules. (G and H) LAMP detection of *D. immitis* by electrophoresis (G) and real-time turbidimeter (H) with genomic DNA from mosquitoes corresponding to (N)-(F) shown left. Numbers on the left indicate migration of molecular weight marker (bp). (I) An example of mosquito Malpighian tubules infected with *D. immitis* L2 larvae at 200-fold magnification.

vishunui comp., and 1 *Aedes aureostriatus*), and DNA was extracted. For initial screening, DNA from 10 mosquitoes were pooled and used for each LAMP reaction. LAMP analysis revealed that 5 out of 12 groups reacted positively indicating one or more infected mosquitoes to be contained within those groups (data not shown). Individual mosquitoes from positive groups were then tested for the presence of *Dirofilaria* parasites. Use of LAMP revealed each positive group of 10 mosquitoes to contain 1 to 4 infected mosquitoes (totally 10 *Ae. albopictus* and 1 *Ar. subalbatus*) (Fig. 4). The amplified products of LAMP reactions were sequenced and determined to be of *D. immitis* (data not shown).

Discussion

In this study, we demonstrated the detection of filarial parasites within vector mosquitoes using the LAMP method. Elimination of zoonotic infectious diseases has been identified as crucial to public health particularly in light of the recent emergence and re-emergence of diseases such as West Nile virus, malaria, dengue fever, Japanese encephalitis and novel clinical cases of infection of humans with diseases previously unknown to infect humans. Many of these diseases require arthropod vectors, therefore, surveillance-based control of arthropods is critical in the management of such vector-borne diseases.

The ability of LAMP to detect just a single microfilaria suggests the applicability of this method for clinical cases with extremely low microfilaremia. Complicating their detection at the vector stage is potential contamination with mosquito debris, an issue of particular concern for PCR based detection methods. However, this appears to be overcome by LAMP as shown by sensitivity of detection

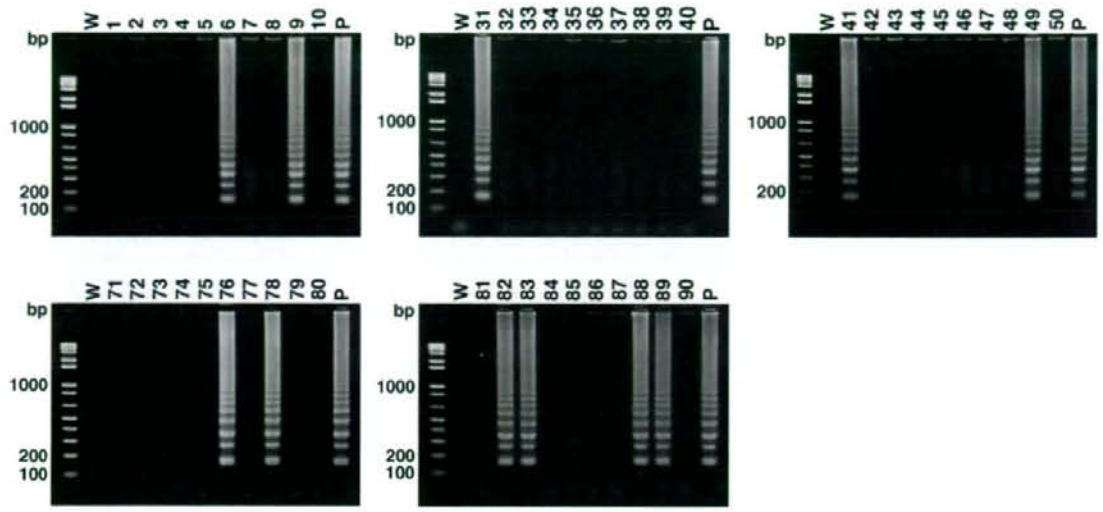

Figure 4
LAMP-based identification of wild-caught *D. immitis*-carrying mosquitoes. Detection of *D. immitis* by LAMP with genomic DNA from individually wild-caught mosquitoes within 5 positive groups screened initially. 11 among 120 mosquitoes showed positive signal indicating carrying *D. immitis*. Water served as a negative control (W) and pure DNA from *D. immitis*L3 served as a positive control (P).

down to levels of a single parasite within a whole mosquito. Furthermore, sequencing of LAMP products showed specific amplification of *D. immitis* DNA, indicating the high specificity of this method. Considering infected mosquitoes typically carry anywhere from 1 to 40 parasites it appears this method is sufficient to address the need for a reliable and sensitive diagnostic method that is completed within 60 minutes.

Human lymphatic filariasis remains a major public health problem reported to put more than a billion people in 83 endemic countries at risk of infection [29] with *Wuchereria bancrofti* and *Brugia malayi* or *B. timori* being the most common parasites [30]. In 1997, WHO called for the elimination of lymphatic filariasis [31] and The Global Programme to Eliminate Lymphatic Filariasis (GPELF) has been given the task of eliminating filariasis by 2020 [32]. To achieve this elimination-goal, emphasis was placed on the use of the newest, most cost-effective diagnostic and mapping tools available [30]. These tools should also have the added characteristics of being sensitive and specific. The cost of LAMP reaction is comparative to PCR reaction, and it can be carried out with lower cost due to no requirement for a thermal cycler but only warm water. Additionally, we have already confirmed that LAMP can be well performed using reduced reaction mixture to half volume with detection by naked eye and electrophoresis (data not shown), suggesting the possibility of further reduction of cost. The applicability of LAMP to field-surveys, and hence as a diagnostic and mapping tool, was demonstrated by performing diagnosis of wild-caught mosquitoes. Specifically, *Dirofilaria*-carrying mosquitoes collected from an endemic area were identified from a pool of infected and non-infected mosquitoes via LAMP reactions. Taken together, the LAMP method appears ideal to fit the criteria laid out for the eradication of filariasis through careful and reliable monitoring of parasites, yet further studies to test this method for the detection of the parasites species responsible of lymphatic filariasis are needed.

Conclusion

Our successful detection of parasites demonstrated the applicability of LAMP in the diagnosis of filarial parasites-carrying mosquitoes using not only a laboratory model but also field samples. Due to its isothermal reaction conditions and simple diagnostic output, LAMP can be easily combined with typical field collections of vectors to survey pathogens *in situ*; indeed, only warm water is required to perform this assay. Though we used *D. immitis* as a model, our method is also applicable to diagnosis of other vector-borne filarial diseases such as *W. bancrofti*, *B. malayi*, and *D. repens*. With these features this method

offers great promise to achieve a useful method for surveying filarial parasites in regions where filariasis is endemic.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HA conceived the study, performed the experiments, and wrote the manuscript. AY prepared filarial parasites and infected mosquitoes. NP assisted with determination of reaction conditions. NS and HB helped collection of wild mosquitoes. SO arranged collection of mosquito. BN clarified the manuscript. SF conceived and supervised the study. HK supervised the study. All authors read and approved the final manuscript.

Acknowledgements

We thank Yoshio Tsuda and Mutsuo Kobayashi for advice on mosquito collection and identification. We also thank Hiroshi Iseki for primer design. We are also grateful to Tokiyasu Teramoto, Kazutaka Yamada and Ryuichiro Maeda for filaria and mosquito rearing. This study was supported in part by grants from Health Sciences Research Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare to H.K. and S.F., Grants-in-Aid for Scientific Research from Japanese Ministry of Education, Science, Sports, Culture and Technology to H.K. and S.F., and the Program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) to H.K. N.S., H.B., and B.N. are research fellows of the Japan Society for the Promotion of Science.

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Veterinary Parasitology 159 (2009) 97–104

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Isolation and pathogenic characterization of an OB1 variant of *Babesia rodhaini* which has a glycophorin A-independent pathway to murine red blood cells

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ARTICLE INFO

Article history:
Received 7 February 2008
Received in revised form 15 September 2008
Accepted 7 October 2008

Keywords:
Babesia
Red blood cell
Invasion
Glycophorin A
Autoantibody

ABSTRACT

Recent studies using several *Babesia* spp. have demonstrated that these species commonly recognize host sialic acids of red blood cells (RBCs) for their invasion. Glycophorin A (GPA), which is a major carrier of the sialic acids on RBCs, is a possible invasive receptor for *Babesia* parasites. In the present study, a variant of *Babesia rodhaini* was successfully isolated from a GPA homozygous knockout ($GPA^{-/-}$) mouse infected with an Australian strain of *B. rodhaini* which had originally been unable to replicate in $GPA^{-/-}$ mice. The isolated parasite (designated as an OB1 variant) caused lethal infection to wild-type mice, as in the case of the parent Australian strain. However, although the growth of the OB1 variant in $GPA^{-/-}$ mice was comparable with that in wild-type mice at 1–4 days after infection, the growth was significantly inhibited from day 5 onward, leading to the eventual survival of the $GPA^{-/-}$ mice. Resistance of $GPA^{-/-}$ mice against OB1 infection was lost by splenectomy, although the cytokine responses to the infection in the sera of $GPA^{-/-}$ mice were similar to those of wild-type mice. The autoantibody levels to GPA-defective RBCs in the sera of $GPA^{-/-}$ mice were depressed at a lower level at 0–2 days after infection than those of wild-type mice, while the levels of $GPA^{-/-}$ mice progressively increased and reached comparable levels to those of wild-type mice at day 3 or later. These results indicate that the isolated OB1 variant has a GPA-independent invasion pathway into murine RBCs and suggest that the resistance of $GPA^{-/-}$ mice against infection with the OB1 variant may be attributed to the effective clearance of the parasitized RBCs lacking GPA in the spleen, possibly mediated by preferential autoantibody binding to the RBC membrane.

1. Introduction

Babesiosis, caused by hemoprotozoan parasites of the genus *Babesia*, is one of the most common infections of pastured animals and is responsible for remarkable economic losses to the livestock industry worldwide (Brown and Palmer, 1999). The disease has also received increased attention as a worldwide-distributed zoonosis in humans (Homer et al., 2000). The intraerythrocytic asexual development of *Babesia* spp., which is a critical part of their life cycle and is responsible for the majority of the clinical signs relating to babesiosis, is initiated by parasite invasion of host red blood cells (RBCs) (Homer et al., 2000). Therefore, a better understanding of the molecular mechanism of parasite invasion can contribute to the successful development of effective strategies against babesiosis.

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0304-4017/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.vetpar.2008.10.022

Recent studies have reported that the *in vitro* invasion or growth of several *Babesia* spp. is compromised when the host RBCs are treated with neuraminidase (Gaffar et al., 2003; Lobo, 2005; Okamura et al., 2005; Takabatake et al., 2007b), indicating that these species commonly use the host sialic acids on RBCs as their invasive receptor. Glycophorin A (GPA) is an erythroid-specific membrane protein conserved in mammals and is also a major carrier of sialic acids on RBCs (Matsui et al., 1989; Murayama et al., 1989). In our previous study, GPA-homozygous knockout ($^{-/-}$) mice were shown to be completely resistant against infection with *B. rodhaini* that causes lethal infection in wild-type mice (Takabatake et al., 2007a). Taken together, these results have suggested that *B. rodhaini* is dependent on the sialic acids of GPA for the invasion of murine RBCs. On the other hand, *in vitro* growth of *B. divergens* is only mildly inhibited when the host RBCs lack the GPA (Lobo, 2005), suggesting that *B. divergens* has an alternative GPA-independent invasion pathway. This implies the possibility that *B. rodhaini* also has an alternative GPA-independent invasion pathway.

In the present study, a variant of *B. rodhaini* that can proliferate in GPA$^{-/-}$ mice was successfully isolated. The pathogenicity of the cloned parasite (designated as the OB1 variant) to both wild-type and GPA-knockout mice was examined. The present findings indicate that *B. rodhaini* also has a GPA-independent invasion pathway into murine RBCs.

2. Materials and methods

2.1. Parasites

An Australian strain of *B. rodhaini* (kindly provided by the Kyusyu Branch of the National Institute of Animal Health, Japan) and a Munich strain of *B. microti* (kindly provided by the Free University of Berlin, Germany) have been maintained by intraperitoneal passage with the parasitized RBCs in mice at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan. Parasitemia was determined by counting the percentage of parasitized RBCs in a total of 300–10,000 RBCs at the center of Giemsa-stained thin blood smears (Igarashi et al., 2000).

2.2. Mice

Eight- to 12-week-old female mice were used for all infectious experiments. GPA-homozygous knockout ($^{-/-}$) mice (Arimitsu et al., 2003) had been back-crossed into C57BL/6J mice (Nihon CLEA, Tokyo, Japan) for 12 generations at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan (Takabatake et al., 2007a). C57BL/6J mice were used as the control wild-type mice. Genotyping of the mice was performed by PCR as previously described (Arimitsu et al., 2003).

2.3. Parasite cloning

The limited dilution cloning of OB1 was conducted as described below. The OB1 variant-infected blood was diluted with phosphate-buffered saline (PBS), pH 7.4, to 5 parasitized RBCs/ml. Into a splenectomized GPA$^{-/-}$ mouse, 0.2 ml of the diluted blood containing one parasitized RBC was inoculated intravascularly (I.V.). After the inoculation, parasitized blood was obtained from the mouse. The cloning procedure described above was repeated twice more. Parent Australian strain was also cloned as indicated above using splenectomized wild-type mice.

2.4. Experimental infections of mice

GPA$^{-/-}$ and wild-type mice were respectively inoculated intraperitoneally (I.P.) with 1×10^7 parasitized RBCs with the OB1 variant and the Australian strain cloned as above. After the infection, the parasitemia dynamics and survival rate of each group were monitored until the mouse died or the parasitemia reached an undetectable level. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan.

2.5. DNA sequencing of the parasites' 18S ribosomal RNA (rRNA) gene

Blood from the OB1 variant, the cloned Australian strain, or *B. microti*-infected mice was obtained by cardiac puncture and anticoagulated with citrate-phosphate-dextrose with adenine (Sigma, St. Louis, MO, USA). RBCs were collected by centrifugation at $500 \times g$ for 10 min at 4 °C and washed three times in PBS. The RBCs were lysed with an RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) for 10 min at room temperature and then centrifuged at $15{,}000 \times g$ for 10 min at 4 °C. Parasite DNAs were extracted from the pellet using proteinase K and phenol-chloroform-isoamyl alcohol (Sigma) as previously described (Dalrymple, 1990). The DNA sequences of 18S rRNA from *B. rodhaini* (Genebank accession number AB049999) and *B. microti* (Genebank accession numbers AB190459 and AB119446) were aligned with the CLUSTAL W Multiple Sequence Alignment Program (version 1.83, http://align.genome.jp/clustalw/) (Thompson et al., 1994). Based on the alignment, Buni1 (forward primer, 5′-gtcatatgcttgtcttaaagattaagcc-3′) and Buni2 (reverse primer, 5′-gccaataccctaccgtcca-3′) were designed on the conserved regions between *B. rodhaini* and *B. microti*. Partial 18S rRNA genes were amplified from each parasite DNA using Buni1 and Buni2 with a high-fidelity DNA polymerase (PrimeSTAR HS DNA Polymerase; Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The thermal conditions of the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) were 30 cycles at 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 1 min. The amplified DNA products were purified with a DNA fragment purification kit (MagExtractor; Toyobo, Osaka, Japan), sequenced directly using the primers above with an ABI PRISM BigDye TM Terminator Cycle Sequencing Kit (Applied Biosystems), and then analyzed on an ABI 3100 genetic analyzer (Applied Biosystems) according to the

manufacturer's instructions. These DNA sequences were aligned with the CLUSTAL W Multiple Sequence Alignment Program.

2.6. Cytokine profiling

Serum samples were collected from the GPA$^{-/-}$ and wild-type mice at day 0 just before inoculation of 1×10^7 OB1-parasitized RBCs and, subsequently, at days 1–4 (every 24 h) and day 25 after the inoculation. Sera were stored at −80 °C until use. Gamma-interferon (IFN-γ), interleukin (IL)-2, IL-4, and IL-10 were quantified in the collected sera using mice Th1/Th2 cytokine and inflammatory cytometric bead array kits (BD Bioscience Pharmingen, San Diego, CA, USA) with a FACSCalibur flow cytometer (BD Bioscience Pharmingen) according to the manufacturer's instructions.

2.7. Preparation of RBC membrane extracts

The RBCs obtained from uninfected GPA$^{-/-}$ mice were treated with the RBC lysis buffer as above, centrifuged at 15,000 × *g* for 10 min at 4 °C, and then washed three times with PBS. The pellet was extracted with the same volume of PBS containing 1% (w/v) Triton X-100 for 10 min on ice. The suspension was centrifuged at 15,000 × *g* for 20 min at 4 °C, and the obtained supernatant was regarded as an RBC membrane extract. A parasitized RBC membrane extract was also prepared from OB1-infected GPA$^{-/-}$ mice (parasitemia, about 30%) in the same way. The protein concentrations of the extracts were determined with a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. These membrane extracts were stored at −80 °C until use.

2.8. Detection of antibody response by enzyme-linked immunosorbent assay (ELISA)

The serum samples described above were also examined to detect antibody binding to the RBC membrane extracts obtained from OB1-infected or uninfected GPA$^{-/-}$ mice by ELISA. Briefly, wells were coated with 50 μg of the indicated RBC membrane extract as above in a sodium carbonate buffer, pH 9.6, at 4 °C overnight and then incubated with a blocking solution in PBS for the immunoassay, pH 7.2 (Nacalai Tesque, Kyoto, Japan), for 1 h at 37 °C. The antigen-coated wells were incubated for 1 h at 37 °C with 100 μl of the indicated serum samples diluted in the blocking solution. Sera were tested over a range of serial twofold dilutions from 1:50. All sera were tested in duplicate, and the results were averaged. After washing with PBS containing 0.05% (w/v) Tween 20 (Sigma), 100 μl of goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark) diluted at 1:1000 was added to each well and then incubated for 1 h at 37 °C. The enzyme reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) (TBNP-0100-01; BioFX, Owings Mills, MD, USA). The reaction was terminated with 0.1 N HCl, and the absorbance was recorded at OD450 nm using an ELISA microplate reader (MTP-500; Corona, Hitachinaka, Japan). To detect the antibody response to parasite antigens, sera were incubated with the uninfected RBC membrane extract (1 μl of sera/50 μg of extract) for 1 h at 37 °C in the blocking solution and then subjected to ELISA as above.

2.9. Statistical analyses

The significance of differences between values in parasitemia, cytokine levels, and autoantibody levels was examined by the Mann–Whitney *U*-test. Survival estimates were calculated by the Kaplan–Meier method, and the significance of differences between values in survival rate was analyzed by the log-rank test. Differences giving $P < 0.05$ were regarded as significant.

3. Results

3.1. Isolation of the OB1 variant from a GPA$^{-/-}$ mouse infected with the parent Australian strain of *B. rodhaini*

To examine whether *B. rodhaini* has an alternative GPA-independent invasion pathway to murine RBCs, we repeated infectious experiments in which GPA$^{-/-}$ mice were inoculated I.P. with 1×10^7 RBCs parasitized with an Australian strain of *B. rodhaini*. From a total of 26 GPA$^{-/-}$ mice, only one mouse showed proliferation of the *Babesia* parasite in the blood circulation. The parasite was initially detected at day 11 after infection, and the parasitemia subsequently increased and peaked (27.9%) at day 14. It gradually decreased and reached an undetectable level on day 28 (Fig. 1). Blood was collected from the tail vein of the infected mouse and serially passaged I.P. in other GPA$^{-/-}$ mice seven times. Eventually, the parasite grew well in all of the inoculated GPA$^{-/-}$ mice. For further characterization, we cloned the parasite and the parent Australian strain by limiting dilution of the infected blood and subsequent inoculation into mice. The cloned parasite capable of proliferating in GPA$^{-/-}$ mice was designated as the OB1 variant. There was no morphological difference between the OB1 variant and the cloned Australian strain

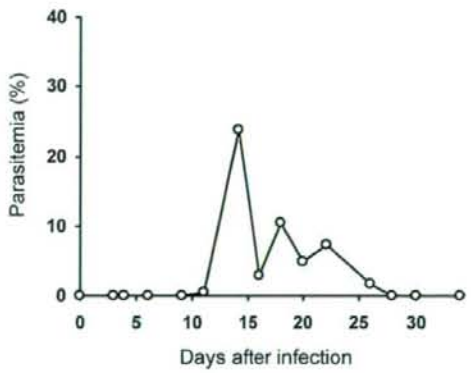

Fig. 1. Isolation of a *Babesia* parasite that grows in GPA$^{-/-}$ mice. From a total of 26 GPA$^{-/-}$ mice inoculated with 1×10^7 Australian strain of *B. rodhaini*-parasitized RBCs, only one showed proliferation of *Babesia* parasites in the blood circulation. The data indicate the dynamics of the parasitemia (%) in this mouse.

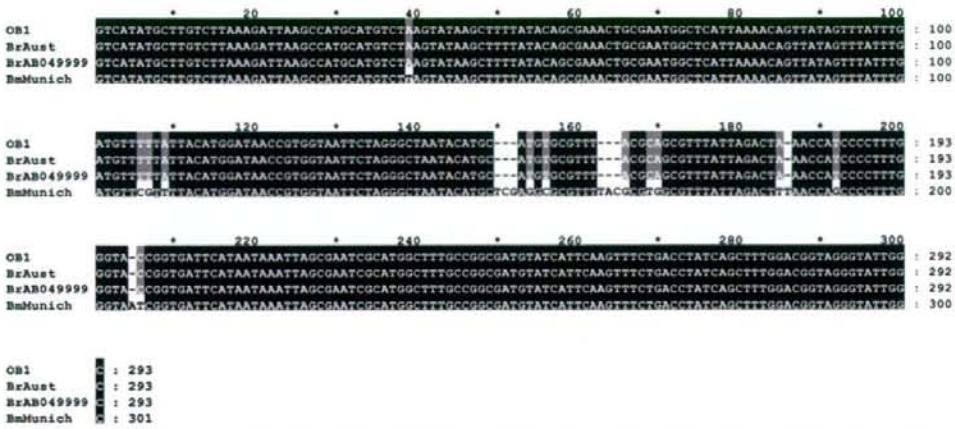

Fig. 2. Comparison of the partial DNA sequences of 18S rRNA genes from the OB1 variant, *B. rodhaini*, and *B. microti*. The multiple sequence alignment was generated using the ClustalW program. BrAust, the Australian strain of *B. rodhaini* cloned in our laboratory; Br AB04999, *B. rodhaini* (GenBank accession no. AB04999); BmMunich, the Munich strain of *B. microti* maintained in our laboratory; dashes, gaps introduced for optimal alignment. Residues shaded in black and gray are the conserved regions in four and three sequences, respectively. The determined sequences of BmMunich, BrAust, and OB1 were registered in the DDBJ database under accession numbers AB366158, AB366159, and AB366160, respectively.

(data not shown). To confirm that the OB1 variant was *B. rodhaini*, the partial DNA sequences of 18S rRNA genes from the OB1 variant, the cloned Australian strain of *B. rodhaini*, and a Munich strain of *B. microti* were determined and then aligned (Fig. 2). The aligned scores of the sequence of OB1 versus those of the cloned Australian strain of *B. rodhaini*, *B. rodhaini* (GenBank accession number AB049999), and the Munich strain of *B. microti* were 100, 100, and 96, respectively (Fig. 2). The data showed that the OB1 variant is *B. rodhaini* but not a contaminant derived from another rodent *Babesia* parasite.

3.2. Pathogeneses of the OB1 variant and the cloned Australian strain to wild-type and GPA$^{-/-}$ mice

The OB1 variant and the cloned Australian strain infections in wild-type mice were examined by comparing the parasitemia dynamics and survival rate in the two groups (5 mice in each group). The parasitemia rapidly increased in the mice infected with Australian strain, eventually leading to the death of hosts at day 6 after infection. The mice infected with OB1 variant also showed a rapid elevation of parasitemia and finally died at day 6 or 7 after infection (Fig. 3). No significant difference in parasitemia ($P > 0.347$) and survival rate ($P = 0.518$) was found between the OB1 variant and the cloned Australian strain in wild-type mice.

Next, the infectious course of the OB1 variant in GPA$^{-/-}$ infected mice was examined by comparing the parasitemia dynamics and survival rate with those of wild-type mice (5 mice in each group). In GPA$^{-/-}$ mice, although the parasitemia increased at a similar pace to that in wild-type mice by day 4 after infection, it peaked (40.7 ± 9.3%, mean ± standard deviation [SD]) at day 5, and the peak was significantly lower ($P = 0.037$) than that in wild-type mice (69.6 ± 19.4%) (Fig. 4). It subsequently decreased and reached an undetectable level at day 22, and all OB1-infected GPA$^{-/-}$ mice eventually survived (Fig. 4). In contrast, the growth of the cloned Australian strain was completely inhibited in the inoculated GPA$^{-/-}$ mice as reported before (Takabatake et al., 2007a). These data indicated that invasion and development of the OB1 variant

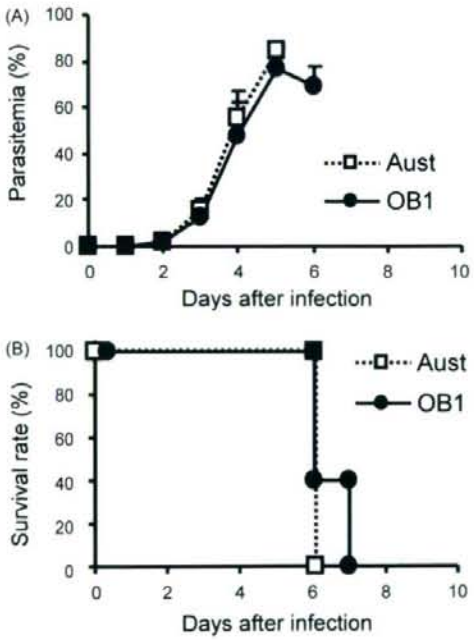

Fig. 3. Comparison of the pathogeneses of the cloned OB1 variant and the cloned Australian strain against wild-type mice. Wild-type mice ($n = 5$ in each group) were inoculated I.P. with 1×10^7 OB1 variant- or Australian strain-parasitized RBCs. (A) Dynamics of the parasitemia. Aust, Australian strain; Bar, SD. (B) Survival rate. Data are representative of two separate experiments.

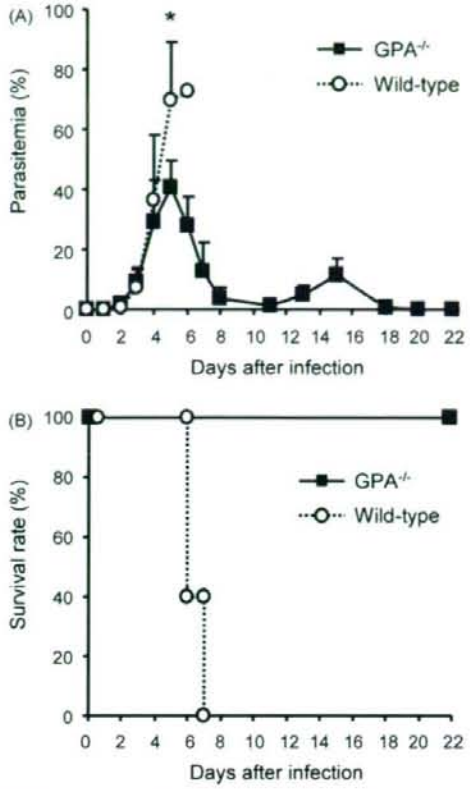

Fig. 4. Susceptibility of GPA$^{-/-}$ and wild-type mice to infection with the OB1 variant. GPA$^{-/-}$ and wild-type mice ($n = 5$ in each group) were inoculated I.P. with 1×10^7 of the parasitized RBCs. (A) Dynamics of the parasitemia. Bar, SD; $^*P = 0.007$. (B) Survival rate. Data are representative of three separate experiments.

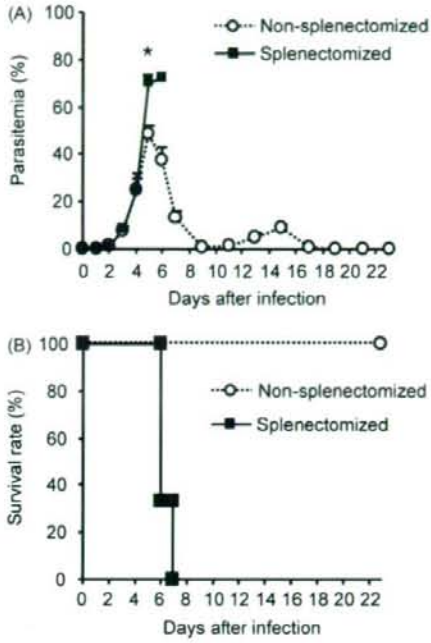

Fig. 5. Effect of splenectomy on the resistance of GPA$^{-/-}$ mice against infection with the OB1 variant. Non-splenectomized and splenectomized GPA$^{-/-}$ mice ($n = 3$ in each group) were inoculated I.P. with 1×10^7 parasitized RBCs. (A) Dynamics of the parasitemia. Bar, SD; $^*P = 0.049$. (B) Survival rate. Data are representative of two separate experiments.

within the GPA-defective RBCs were equal to those within wild-type RBCs, but GPA$^{-/-}$ mice inhibited the growth of the OB1 variant from day 5 onward.

3.3. Roles of the spleen and cytokine responses in the resistance of GPA$^{-/-}$ mice against OB1 infection

To assess the contribution of the spleen to the resistance of GPA$^{-/-}$ mice against OB1 infection, the parasitemia dynamics and survival rate of splenectomized GPA$^{-/-}$ mice were compared with those of non-splenectomized GPA$^{-/-}$ mice (3 mice in each group). The parasitemia in splenectomized GPA$^{-/-}$ mice acutely increased at a similar rate to that in wild-type mice (Figs. 3 and 4) at days 1–4, and all splenectomized mice were dead by day 6 or 7 (Fig. 5). Next, to evaluate the role of cytokine responses in the resistance of GPA$^{-/-}$ mice against OB1 infection (5 mice in each group), serum cytokine levels in GPA$^{-/-}$ and wild-type mice were monitored from day 0–4 after infection (Fig. 6). A spike in IFN-γ secretion at day 1 after infection and progressive increases of IL-2, IL-4, and IL-10 secretions were observed in GPA$^{-/-}$ and wild-type mice. There was no significant difference ($P > 0.066$) between the two groups (Fig. 6).

3.4. Antibody responses to RBC membrane extracts

To evaluate the contribution of the antibody responses to the resistance of GPA$^{-/-}$ mice against the OB1 variant infection, the levels of antibody recognizing the OB1-parasitized (Fig. 7A) or uninfected RBC membrane extract (Fig. 7B) were compared between serum samples periodically collected from GPA$^{-/-}$ and wild-type mice (5 mice for each group). Progressive increases in the antibody responses were observed in the groups against the parasitized and uninfected RBC membrane extracts, but the levels of GPA$^{-/-}$ mice were significantly lower ($P < 0.017$) than those of wild-type mice at 0–2 days after infection (Fig. 7A and B). There was no significant difference in antibody levels to the parasite antigens between the two groups by 4 days after infection (Fig. 7A and B). These results show that (1) the autoantibody level to host RBC membrane proteins was depressed in uninfected GPA$^{-/-}$ mice, (2) the level increased progressively and became comparable with that of wild-type mice after infection, and (3) specific antibody responses to the parasite antigens were not different between the GPA$^{-/-}$ and wild-type mice by 4 days after infection.

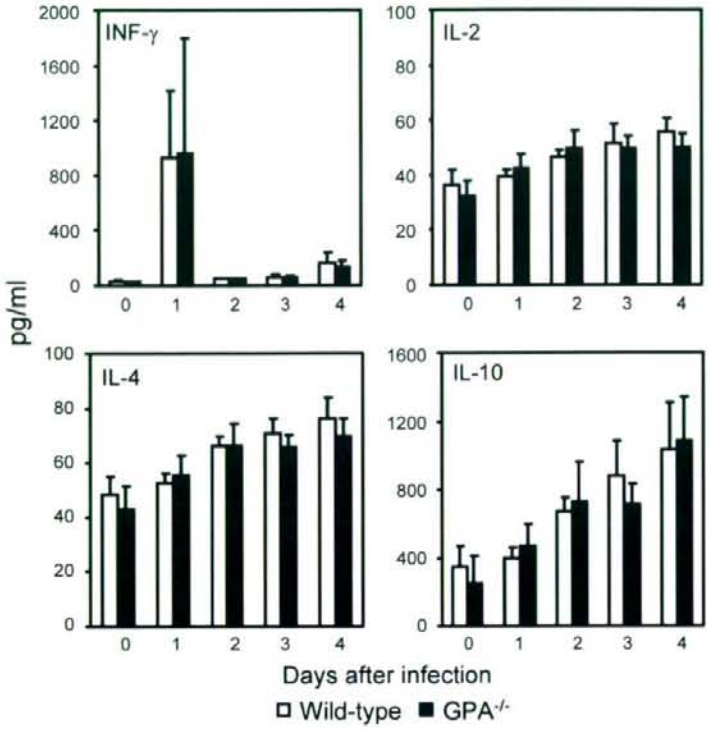

Fig. 6. Serum cytokine profiles in GPA$^{-/-}$ and wild-type mice after OB1 infection. Serum cytokine levels in GPA$^{-/-}$ and wild-type mice ($n = 5$ in each group) following inoculation of 1×10^7 OB1 variant-parasitized RBCs are presented as the mean ± SD at each sampling point. Data are representative of three separate experiments.

4. Discussion

The results in the present study showed that the growth of the isolated OB1 variant in GPA$^{-/-}$ mice was comparable to that in wild-type mice in the early phase of infection, indicating that the asexual growth cycle of the OB1 variant in the blood stage is independent of GPA on murine RBCs. While our previous study suggested the inability of the parent Australian strain of *B. rodhaini* to invade GPA-defective RBCs (Takabatake et al., 2007a), the results of the present study demonstrate that the OB1 variant of *B. rodhaini* has an alternative invasion pathway that is independent of GPA.

The parent stock of an Australian strain of *B. rodhaini* may contain a mixture of multiple parasite variants that have GPA-dependent or -independent invasion pathways. Because *B. rodhaini* undergoes an antigenic variation under host immune pressure (Allred, 2001), the parent population of the Australian strain maintained in mice for many years in our laboratory may have achieved multiple invasion pathways by antigenic variation. Consequently, GPA-deficient RBCs possibly selected the OB1 variant from the mixture of parasites that exhibit multiple invasion pathways. In *Plasmodium falciparum*, the polymorphism of the RBC binding ligand, BAEBL/EBA-140, leads to changes in its receptor specificity on human RBCs (Mayer et al., 2004). Alternatively, *B. rodhaini* may be able to originally vary in its dependency on GPA for invasion, as *P. falciparum* is known to have the ability to switch the receptor used for invasion from sialic acid-dependent to acid-independent pathways by differential expression of the ligand genes (Stubbs et al., 2005). By the present time, however, no parasite growth has been detected in the GPA$^{-/-}$ mice infected with the cloned Australian strain, not even in the splenectomized mice (data not shown).

We previously reported that intracellular parasites of *B. equi* cannot grow in neuraminidase-treated RBCs (Okamura et al., 2005) and that intracellular *B. bovis* incorporates the host sialic acids during asexual development, suggesting that the host sialic acids on RBCs are required not only for RBC invasion but also for the intraerythrocytic maturation of *Babesia* spp. (Okamura et al., 2007). In the present study, *B. rodhaini* could not be detected to incorporate the host GPA within the RBCs of infected wild-type mice as assessed by confocal microscopy with antiserum against mouse GPA as previously reported (Takabatake et al., 2007a), and no morphological abnormality was observed in the intracellular development of the OB1 variant. Further study is required to understand the involvement of the host sialic acids and GPA in the asexual growth cycle of *B. rodhaini*.

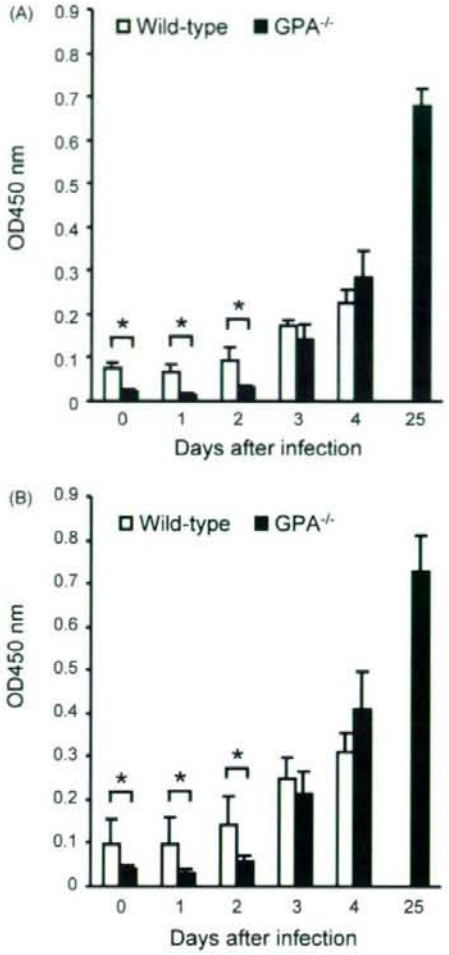

Fig. 7. Antibody responses in GPA$^{-/-}$ and wild-type mice against OB1-parasitized (A) or uninfected RBC membrane extract (B) after infection with the OB1 variant. Antibody levels in GPA$^{-/-}$ and wild-type mice ($n = 5$ in each group) following inoculation of 1×10^7 OB1 variant-parasitized RBCs against extract from the OB1 variant-infected or uninfected GPA-defective RBCs are presented as the mean ± SD at each sampling point. *$P < 0.016$ (wild-type vs. GPA$^{-/-}$ mice). The dilution fold of sample sera is 1:100. Data are representative of three separate experiments.

The present study also showed that GPA$^{-/-}$ mice could inhibit the growth of the OB1 variant in the later phase and eventually survived the lethal infection. The spleen is central to the innate and acquired immune responses to babesiosis (Brown et al., 2006). The spleen was indeed shown to be essential for growth inhibition in the present study, although the cytokine responses of GPA$^{-/-}$ mice were similar to those of wild-type mice after infection. The binding of naturally occurring autoantibodies to the host RBC membranes is known to mediate the clearance of aging RBCs by macrophage phagocytosis in the spleen (Ensinck et al., 2006), and the loss of RBC membrane constituents, such as sialic acids, enhances spleen clearance (Biondi et al., 2002). GPA$^{-/-}$ mice originally lose a large quantity of sialic acids from the surface of RBCs but never present with anemia (Arimitsu et al., 2003; Takabatake et al., 2007a). The lower level of autoantibody against the uninfected-RBC in GPA$^{-/-}$ mice should reflect the depression of the excessive activation of RBC clearance. After OB1 infection, the amount of the autoantibody progressively increased in GPA$^{-/-}$ mice and reached a comparable level with that of wild-type mice. Therefore, GPA-defective RBCs may become more susceptible to the clearance than wild-type RBCs under the increased appearance of autoantibodies following OB1 infection.

The presence of anti-RBC autoantibodies has been reported in infected mice with *B. rodhaini* (Matsuda et al., 1987), dogs with *B. gibsoni* (Adachi and Makimura, 1992), and cattle with *B. bigemina* (Goes et al., 2007). Although we agree that the presence of autoantibodies in the infected animals may be relevant to hemolytic anemia caused by the incidental destruction of uninfected RBCs under low parasitemia (Adachi and Makimura, 1992; Goes et al., 2007), the results in the present study suggest that the autoantibodies also contribute to the resistance of animals against *Babesia* infection. In particular, we postulate that an unknown alteration in the parasitized RBC membrane enhances the preferential binding of the autoantibodies to the parasitized RBC and induces the systematic clearance of the parasitized RBCs in the spleen. *P. falciparum* promotes oxidative alterations in the RBC membrane, which lead to the exposure of antigenic sites recognized by anti-band 3 autoantibodies and enhance the host phagocytosis of parasitized RBCs (Giribaldi et al., 2001; Turrini et al., 2003). Interestingly, the levels of parasite-induced oxidative modification and phagocytosis induction are remarkably higher in humans with mutant RBCs, which are widespread in geographic regions with a high incidence of *P. falciparum* malaria (Ayi et al., 2004).

In summary, the isolated OB1 variant of *B. rodhaini* has a GPA-independent invasion pathway to murine RBCs, and the resistance of GPA$^{-/-}$ mice against infection with the OB1 variant, which causes lethal infection in wild-type mice, may be attributed to the effective clearance of the parasitized RBCs lacking GPA in the spleen, possibly mediated by autoantibody binding to the RBC membrane. The OB1 variant and GPA will provide considerable insight into the molecular functions involved in the parasite invasion of host RBCs and the pathological mechanisms of babesiosis in the future.

Acknowledgments

This research was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), the Program of Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), the Program of Founding Research Centers for Emerging and Re-emerging Infectious Diseases, MEXT Japan, Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), the Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, and The 21st

Century COE Program (A-1), Ministry of Education, Culture, Sports, Science, and Technology, Japan. N.T. was supported by a research fellowship from JSPS.

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Biochemical and Biophysical Research Communications 379 (2009) 6–10

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Rapid recruitment of innate immunity regulates variation of intracellular pathogen resistance in *Drosophila*

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ARTICLE INFO

Article history:
Received 8 November 2008
Available online 4 December 2008

Keywords:
Infection
Bacteria
Innate immunity
Drosophila

ABSTRACT

Genetic variation in susceptibility to pathogens is a central concern both to medicine and agriculture and to the evolution of animals. Here, we have investigated the link between such natural genetic variation and the immune response in wild-type *Drosophila melanogaster*, a major model organism for immunological research. We found that within nine wild-type strains, different *Drosophila* genotypes show wide-ranging variation in their ability to survive infection from the pathogenic bacteria *Listeria monocytogenes*. *Canton-S*, a resistant strain, showed increased capacity to induce stronger innate immune activities (antimicrobial peptides (AMPs), phenol oxidase activity, and phagocytosis) compared to the susceptible strain (*white*) at early time points during bacterial infection. Moreover, PGRP-LE-induced innate immune activation immediately after infection greatly improves survival of the susceptible strain strongly suggesting a mechanism behind the natural genetic variation of these two strains. Taken together we provide the first experimental evidence to suggest that differences in innate immune activity at early time points during infection likely mediates infection susceptibility in *Drosophila*.

There is a well-documented genetic variation in resistance to infection in the animal kingdom including humans [1]. Pathogens are capable of causing considerable morbidity in host populations therefore these invaders exert potent selective forces thereby creating the potential to drive the evolution of various genetic traits in their hosts. Included among these traits, the efficacy of the immune response is a critical determinant of fitness, subsequently higher eukaryotes have evolved elaborate mechanisms for eradicating pathogen infection. For invertebrates, this immune response is mediated by highly conserved innate immune pathways that control both cellular and humoral responses including phagocytosis by scavenging plasmatocytes (*Drosophila* macrophages) and the secretion of antimicrobial peptides (AMPs) [2,3]. The Toll pathway responds to Gram-positive and fungal infections while the Imd pathway is required for proper immune responses to Gram-negative bacteria; mutation to either pathway results in severely immunocompromised flies [2].

The fruit fly *Drosophila melanogaster* is an interesting model to study genetic variation of resistance to infection due to the relative ease in assessing functional variation within natural populations. Studies of population-based variation suggest that immunity genes in *Drosophila* evolve under positive natural selection [4] however, phenotypic effects of naturally occurring genetic variation in the ability to activate innate immune mechanisms in invertebrates (e.g., production of AMPs) remain largely unknown. *Drosophila* rely largely on their hardwired host defense mechanisms that are constructed along broadly similar lines to the innate immune systems of humans and mice [5]. Furthermore, *Drosophila* has been well characterized in terms of response to human pathogenic organisms, especially *Listeria monocytogenes*, a Gram-positive, intracellular bacterial pathogen especially in food-borne infections in immunocompromised individuals and pregnant women. Making it of particular interest, *L. monocytogenes* infection of *Drosophila* shares numerous features with mammalian infection [6].

In this study, nine wild-type *Drosophila* strains were examined for their susceptibility to *L. monocytogenes* infection. Consistent with antimicrobial peptide (AMPs) expression, *Canton-S* was found to be most resistant while *white* was particularly susceptible; *Canton-S* showed high AMP expression at early time points (~24 h) but was level with the susceptible *white* by later time points (1 day~). Taken together, our results indicate that variation in the immune-activating ability of flies during the early phase post-infection could drive variation in the susceptibility of host invertebrates.

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doi:10.1016/j.bbrc.2008.11.097

Materials and methods

Fly stocks: Flies were raised on standard *Drosophila* medium at indicated temperatures. *white* (w^{1118}), *da-GAL4*, *UAS-GFP*, and *tub-GAL80^{ts}* were obtained from the Bloomington Stock Center. The *UAS-PGRP-LE* line was a gift from Sho-ichiro Kurata [7]. *Amherst*, *Canton-S*, *Harwich*, *Hikone*, *Oregon-R*, *Samarkand*, *Sevelen*, and *Swedish-C* strains were gifts from Daisuke Kageyama and Takema Fukatsu.

Bacterial strains. *Salmonella typhimurium* (SL1344) and *L. monocytogenes* (10403S) were gifts from David Schneider [8,9]. *Staphylococcus aureus* was provided from Hiroshi Hamamoto and Kazuhisa Sekimizu [10]. SL1344 was cultured at 37 °C in the dark without agitation in LB broth medium with 100 μg/ml streptomycin. 10403S was cultured at 37 °C in the dark without agitation in BHI broth medium with 100 μg/ml streptomycin. *S. aureus* was cultured at 37 °C in the dark with agitation in LB broth medium.

Fly infection. Bacteria-containing medium was adjusted to appropriate concentrations using Gene Quant *pro* (Amersham) (SL1344; 0.1 OD, 10403S; 0.01 OD, and *S. aureus*; 0.005 OD). Flies were anesthetized with CO_2 and injected in the abdomen, close to the junction with the thorax ventral to the junction between the ventral and dorsal cuticles with each strain of bacteria in 65 nl of medium. Injection was carried out by using an individually calibrated pulled glass needle attached to an IM-300 microinjector (Narishige). Injected flies were transferred to fresh vials once a week [11].

Bacterial load. Bacterial colony forming units (CFU) within infected flies were determined by grinding 30 living infected flies in 1.5 ml tubes with 100 μl of 10 mM $MgSO_4$ with a pestle as previously described [6]. Serial dilutions were prepared from each fly homogenate and aliquots were plated onto BHI agar with streptomycin and incubated overnight before counting within 24 h.

Northern blot analysis. Total RNAs were isolated from flies using TRIzol reagent (Invitrogen). mRNA expression of antimicrobial peptide (AMP) genes (*diptericin*, *attacin*, *cecropin A1*, *drosocin*, and *drosomycin*) and *rp49* was analyzed by RNA blot as previously described [12]. AMP expressions were analyzed with the STORM Bioimager (Amersham) and quantified using ImageJ (NIH) after normalizing lane loading differences using *rp49* as a control.

Phenoloxidase activity assay. Phenoloxidase (PO) assays were performed as previously described [14]. Briefly, adults were dissected in 100 mM Tris–HCl (pH 7.2) to collect hemolymph that was subsequently incubated at room temperature for 30 min. Twenty microliters of aliquots of the supernatant was added to the PO assay mixture (1 ml of 100 mM phosphate buffer (pH 6.0), 200 μl of 50 mM 4-hydroxy proline ethyl ester, and 50 μl of 100 mM 4-methyl catechol as substrate), incubated for 10 min at room temperature before reaction termination by addition of 20 μl of 1 M thiourea. Phenoloxidase activity was quantified by measuring absorbency at 520 nm.

In vivo phagocytosis assay. Phagocytosis assays were performed essentially as previously reported [9] with minor modifications. Briefly, flies were injected with 65 nl of FITC-labeled dead *L. monocytogenes* [13] and incubated for 1 h at 25 °C to permit phagocytosis of bacteria. After 1 h 130 nl of trypan blue (0.2%) was injected to quench fluorescence of extracellular FITC-labeled bacteria while leaving phagocytosed bacteria unaffected.

Control of GAL4 induction. The *GAL80^{ts}/GAL4* system [15] provides a general method for temporal gene expression using the conventional GAL4-upstream activator sequence (UAS) system and a temperature-sensitive *GAL80* molecule, a repressor of *GAL4* transcriptional activity at permissive temperatures. For combined expression of *GAL80* and *GAL4* by ubiquitous promoters, *da-GAL4* and *tub-GAL80^{ts}* transgenes were located within the same line and then crossed to the *UAS-PGRP-LE* line. Development was allowed to proceed at 18 °C until adulthood. At the permissive temperature (18 °C), the *GAL80* protein is active and represses induction of *PGRP-LE* expression by inhibiting *GAL4* (Fig. 3A, 18 °C). At the restrictive temperature (29 °C), *GAL80* is inactivated, permitting expression of *PGRP-LE* (Fig. 3A, 29 °C).

Table 1
Survival rate of wild-type *Drosophila*. Survival of wild-type flies infected with *L. monocytogenes*, *S. aureus* and *S. typhimurium* was estimated. The mean day to 50% deaths indicates the time (days) required to reach 50% mortality as calculated from survival curves (Fig. 1A and Supplementary Fig. 1). The results of *Canton-S* and *white* are highlighted as bold and underlined.

Rank order of resistance	Wild-type *D. melanogaster* (mean day to 50% death)		
	L. monocytogenes	*S. aureus*	*S. typhimurium*
1	***Canton-S*** (8.12)	*Oregon-R* (4.00)	*Harwich* (14.2)
2	*Oregon-R* (7.36)	***white*** (3.58)	*Samarkand* (13.0)
3	*Swedish-C* (7.26)	*Swedish-C* (2.83)	*Oregon-R* (12.8)
4	*Harwich* (7.11)	*Harwich* (2.68)	*Swedish-C* (12.6)
5	*Samarkand* (7.09)	*Hikone* (2.59)	*Amherst* (10.9)
6	*Hikone* (7.00)	*Amherst* (2.55)	*Hikone* (11.0)
7	*Amherst* (6.66)	***Canton-S*** (2.38)	*Sevelen* (9.05)
8	*Sevelen* (6.34)	*Samarkand* (2.10)	***Canton-S*** (9.00)
9	***white*** (5.08)	*Sevelen* (2.05)	***white*** (8.97)

Results and discussions

Different susceptibility of wild-type Drosophila strains in infection of intracellular bacteria, L. monocytogenes

A set of nine wild-type *D. melanogaster* strains (*Amherst*, *Canton-S*, *Harwich*, *Hikone*, *Oregon-R*, *Samarkand*, *Sevelen*, *Swedish-C*, and *white*) was examined for their responses to the bacterial pathogens, *L. monocytogenes*, *S. aureus*, and *S. typhimurium*. Given that genetic variation in wild-type lines should be the result of fixation of particular genetic variants occurring among natural populations, these wild-type lines represent practical models for studying the genetic basis for resistance to bacterial infection. Importantly, these bacterial pathogens can infect both humans and *Drosophila* [6,9,16]. Susceptibilities of wild-type lines of *Drosophila* to the pathogenicity of each bacterial species were markedly varied (Table 1 and Supplementary Fig. 1). Canton-S was the most resistant line (8.12 days to 50% death) while white was especially susceptible to infection with *L. monocytogenes* (5.08 days) (Table 1 and Fig. 1A) while in the case of *S. aureus* infection, the phenotypes of these two strains were reversed (Canton-S; 2.38 days, white; 3.58 days). Moreover, these lines showed the same susceptibility to *S. typhimurium* infection (Canton-S; 9.00 days, white; 8.97 days) (Table 1 and Supplementary Fig. 1), suggesting that fluctuation of susceptibility likely reflects genetic variation in biological characteristics of each host–pathogen interaction rather than in core components of the Toll- or Imd-mediated innate immunity pathways. To further investigate genetic variation-mediated infection resistance in detail, we decided to focus on infection of Canton-S and white wild-type strains with *L. monocytogenes*.

In order to determine if the increased survival of *Canton-S* lines was a result of reduced pathogen load, suspected to be related to resistance, or due to tolerance, more directly related to overall host fitness in the presence of infection (Shinzawa et al., manuscript in preparation), the bacterial load of *L. monocytogenes* was determined. Growth curves of *L. monocytogenes* in *Canton-S* and *white* showed that numbers of bacteria in *Canton-S* increased more slowly than in *white* in 1 day after infection (Fig. 1B), indicating that resistant of *Canton-S* depends on differences in the number of virulent bacteria within their bodies. While there is not always a positive relationship between survival and bacterial load [17] in this study there was a significant correlation between those

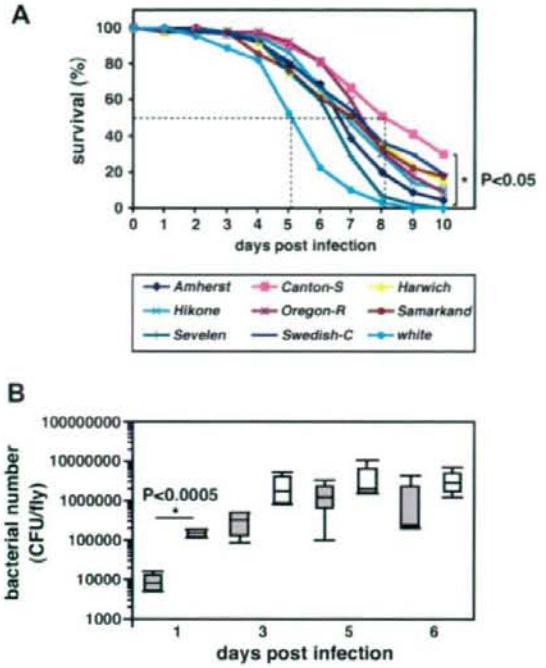

Fig. 1. Different susceptibility of wild-type *Drosophila* strains to infection of intracellular bacteria, *Listeria monocytogenes*. (A) Survival of wild-type flies injected with *L. monocytogenes*. Groups of 30-adults (aged 0–7 days) from each wild-type *Drosophila* strain (*Amherst*, *Canton-S*, *Harwich*, *Hikone*, *Oregon-R*, *Samarkand*, *Sevelen*, *Swedish-C*, and *white*) were injected with *L. monocytogenes* (OD = 0.01). The values are the averages of three independent experiments. The dotted line represents the day required to reach 50% mortality in *Canton-S* and *white*. Asterisk, $P < 0.05$ (Wilcoxon–Mann–Whitney test), for *Canton-S* versus *white*. (B) *Canton-S* inhibits the growth of *L. monocytogenes* compare with *white*. The number of *L. monocytogenes* was measured in *Canton-S* (resistant strain) and *white* (susceptible strain). Data are plotted as box plots with whiskers. Gray bars indicate the *Canton-S*. White bars indicate the *white*. Asterisk, $P < 0.0005$ (Student's *t*-test), for *Canton-S* versus *white*.

two parameters. This suggests two possible components contributing to suppression of bacteria load in resistant strains: environmental factors (e.g., nutrition) within the host fly that could affect proliferation of bacteria and/or immune components in the Toll or Imd cascades that work effectively to eradicate invading bacteria.

Different abilities of wild-type Drosophila strains in the induction of innate immune responses

Invertebrates possess a potent innate immune system, functioning via an integrated response of both humoral and cellular components. *Drosophila* relies almost entirely on innate immunity to combat specific pathogen classes [18] including the well-studied production of circulating antimicrobial peptides (AMP). These peptides are produced rapidly (within a few hours of infection) in response to activation of one of three NF-κB-related transcription factors (Dif, Dorsal, and Relish) [11]. In order to examine AMP production in response to *L. monocytogenes* infection, we monitored a time course of AMP expression (*attacin*, *cecropin A1*, *diptericin*, *drosocin*, and *drosomycin*) via Northern blotting. All examined AMPs were induced in both *Canton-S* and *white* in response to infection with *L. monocytogenes*, however, *Canton-S* showed increased AMP expression at early time points (6 and 9 h post-infection) (Fig. 2A).

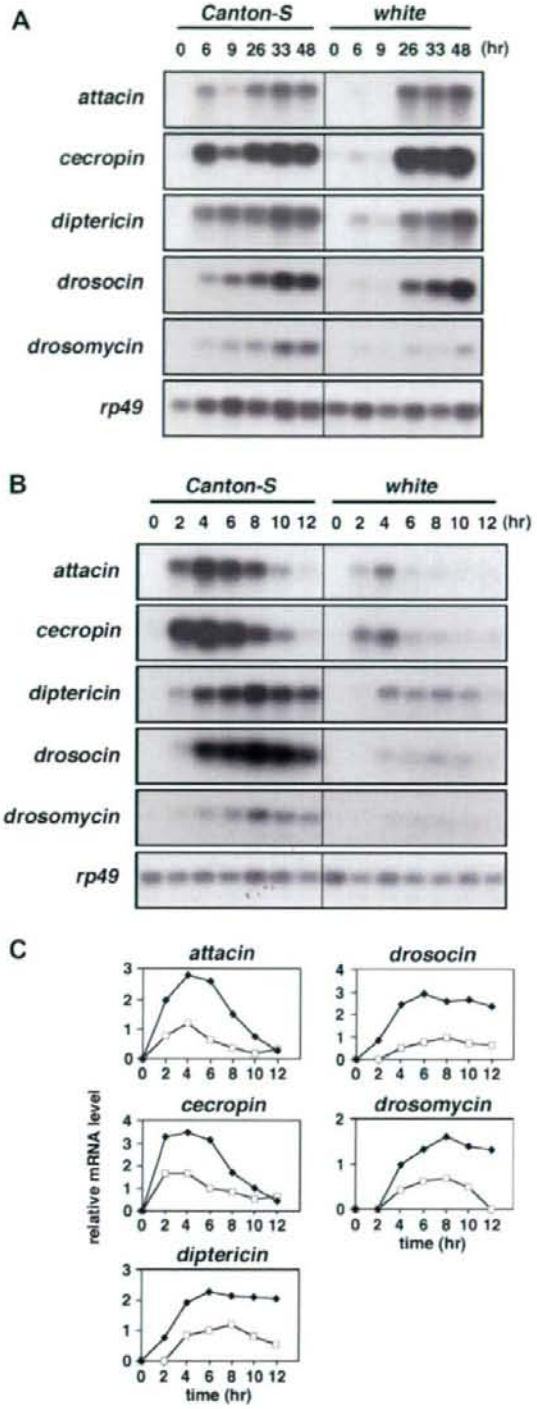

Fig. 2. *Canton-S* induces AMP expression earlier and higher than *white*. *Attacin*, *cecropin A1*, *diptericin*, *drosocin*, and *drosomycin* levels were determined by northern blot analysis from 0 to 48 h (A) and from 0 to 12 h (B) post-infection. (C) The relative induction levels of AMP mRNA in *Canton-S* (filled diamond) and *white* (open square). Expression levels shown in (B) were quantified. The levels of AMP gene expression were normalized using *rp49* as a control.

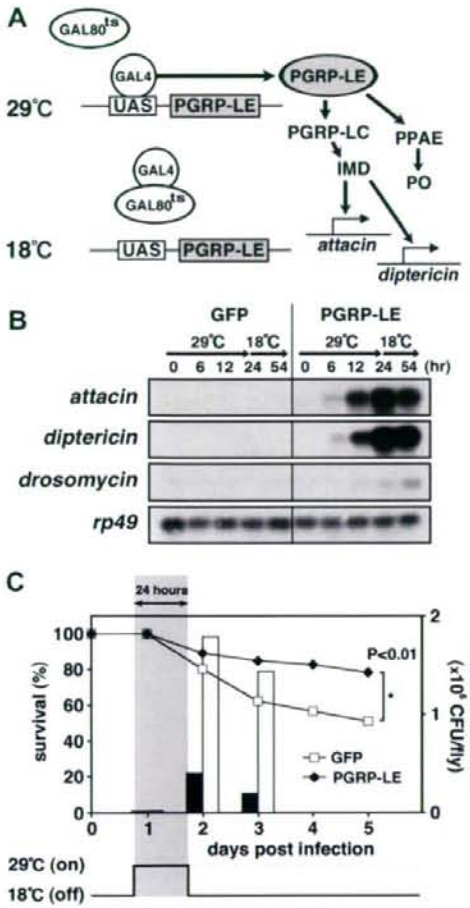

Fig. 3. Activation of innate immunity during the early stages of infection confers resistance to susceptible lines. (A) The *GAL80*^{ts}/*GAL4* system was used to temporally control induction of innate immunity. The *GAL80* repressor is active thereby inhibiting expression of *PGRP-LE* at 18 °C. Upon temperature shift to 29 °C the repressor is inactivated allowing GAL4-driven expression of *PGRP-LE*. (B) Expression of *PGRP-LE* induces *attacin* and *diptericin* at 29 °C in the absence of microbial challenge. (C) Survival rate of susceptible lines is improved and growth of *L. monocytogenes* is inhibited in flies artificially expressing *PGRP-LE* at early time points of infection. Adult flies (aged 10–20 days at 18 °C) were injected with *L. monocytogenes* (OD = 0.04). At 18 h post-infection flies were transferred to 29 °C for 24 h. The survival rate (line chart) and number of bacteria (bar graph) were examined in *PGRP-LE*-expressing flies (filled columns) and control flies (open columns). Asterisk, $P < 0.01$ (Wilcoxon–Mann–Whitney test), for *GFP* versus *PGRP-LE*.

Because differences were seen at early time points we more closely examined AMP expression during that phase of infection. Consistently, *Canton-S* showed increased AMP expression during the early phase of infection (Fig. 2B and C). In addition to AMP production, the humoral response also includes the melanization reaction whose activity can be monitored through measurement of phenoloxidase (PO) activity [18,19]. Cellular responses to infection come in the form of phagocytosis of microbes and were also measured in the two wild-type lines [20]. As seen with AMP production, both melanization and phagocytosis were both highly evoked in the resistant *Canton-S* (Supplementary Figs. 2 and 3), consistent with the notion that differences in general innate immune activity correlate to diversity of susceptibility to *L. monocytogenes* infection.

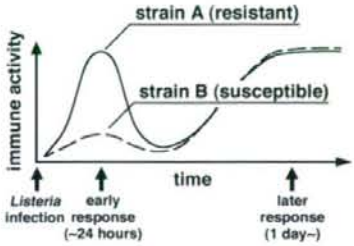

Fig. 4. A model for driving variability of resistance among wild-type *Drosophila* strains. Immune activity induced by infection of *L. monocytogenes* could be divided into early (~24 h) and later (1 day~) response in *Drosophila*. Strain A (resistant) can activate stronger early immune reactions than strain B (susceptible).

From this, an attractive hypothesis emerged whereby early activation of innate immunity alters the susceptibility of *Drosophila* to *L. monocytogenes* infection.

Activation of innate immunity at early time point of infection results in resistance in Drosophila

Since the resistant line reduced pathogen load while highly expressing AMPs we hypothesized that early activation of innate immunity during infection might be responsible for the resistance seen in the resistant *Canton-S*. If this were true then resistance of the *white* line, susceptible to *L. monocytogenes* infection and lacking a strong early immune response, might be improved by ectopic activation of the immune response. AMP gene expression is regulated through the Imd and Toll pathways when they become activated by bacterial peptidoglycans that trigger these pathways through peptidoglycan-recognition proteins (PGRPs). Despite being Gram-positive, *L. monocytogenes* contain DAP-type peptidoglycans capable of activating the Imd pathway via PGRP-LE [21]. We therefore employed PGRP-LE to stimulate an innate immune response using the GAL80^{ts}/GAL4 system capable of temporal control of gene expression and consequently temporal control over generation of an immune response (see Materials and methods). *UAS-PGRP-LE* transgenic flies in the *white* genetic background expressing both *GAL80*^{ts} and *GAL4* proteins were allowed to develop at 18 °C. At this temperature the *GAL80*^{ts} repressor is active and expression of *PGRP-LE* is subsequently inhibited (Fig. 3A). Flies were then shifted to 29 °C, resulting in inactivation of the *GAL80*^{ts} repressor and consequent *GAL4*-driven expression of *PGRP-LE* (Fig. 3A). Consistent with previous reports transient activation of *PGRP-LE* induced *attacin* and *diptericin* at 29 °C without induction of *drosomycin*, a Toll pathway reporter (Fig. 3B) [7,22] confirming activation of an Imd pathway-mediated immune response through expression of *PGRP-LE*. In order to determine if resistance of *white* to *L. monocytogenes* could simultaneously be raised *PGRP-LE* was expressed for 24 h 18 h post-infection. Indeed, survival rate was significantly improved in *PGRP-LE*-expressing flies compared with control flies (Fig. 3C). Furthermore, bacterial load was remarkably reduced after temporal expression of *PGRP-LE* (Fig. 3C). Taken together our results clearly demonstrate that transient activation of innate immunity at early time points during infection improves resistance of flies.

The expression levels of *attacin* and *cecropin* increased immediately after infection before decreasing by 12 h with maximum expression levels 4 h post-infection while *diptericin*, *drosocin*, and *drosomycin* diverged showing maximum expression levels by 6–8 h post-infection (Fig. 2A–C). From this it appears that immune activity induced by infection with *L. monocytognes* can be divided into early (~24 h) and later (1 day~) responses both in *Canton-S* and *white* (Fig. 4). The early response, conventionally referred to