INTRODUCTION

Negative-stranded RNA viruses include some of the most important human pathogens, such as the hemorrhagic fever viruses Ebola and Lassa (up to 80% mortality), rabies virus (up to 100% mortality), and influenza virus. In addition to these viruses, which are transmitted between mammalian hosts, several negative-stranded RNA viruses, called arthropod-borne viruses or arboviruses, are transmitted to humans by insect vectors. For example, Rift Valley Fever virus, a bunyavirus that is transmitted by *Aedes* and *Culex* mosquitoes, causes encephalitis and hemorrhagic fever (1). Another member of the family Bunyaviridae, Crimean-Congo hemorrhagic fever virus, which is endemic in many countries of Africa, Europe, and Asia, is transmitted by ticks and causes severe disease in humans, with 30% mortality. Therefore, continued, intensive research is necessary to define the interactions of negative-stranded RNA arboviruses with their insect hosts. Japanese encephalitis virus (JEV) is the leading cause of epidemic encephalitis worldwide, and there are approximately 70,000 cases each year (overall incidence: 1.8 per 100,000 per year) in endemic countries (2). JEV is transmitted by *C. tritaeniorhynchus* in East Asia, Southeast Asia, and South Asia.

The Toll pathway is thought to be activated during dengue virus (DENV) infection in *Ae. aegypti* (3,4). The JAK-STAT pathway is also activated by DENV infection in *Ae. aegypti* (5) and *o’nyong nyong* virus infection in *Anopheles* mosquitoes. Sindbis virus, chikungunya virus (CHIKV), and DENV activate the RNA interference (RNAi) pathway in *Aedes* mosquitoes (6–9). However, less is known about JEV suppression mechanisms in mosquitoes. Therefore, we attempted to determine the mechanism of JEV suppression in *Aedes* mosquitoes.

Argonaute 2 (AGO2) is a key molecule in the RNAi pathway of plants and animals (10). In this pathway, dsRNA is recognized by Dicer-2, and short interfering RNAs (siRNAs) of viral origin are generated from dsRNA precursors, which are produced during virus replication by the viral RNA-dependent RNA polymerase. Dicer-2 interacts with the dsRNA-binding protein R2D2, and together they load one strand of the siRNA into a multiprotein effector complex and the other strand is degraded. The protein complex is called the RNA-induced silencing complex (RISC); it targets RNA with sequences complementary to the loaded siRNA strand. AGO2 is the catalytic component of RISC, and it cleaves the target RNA.

The mammalian JAK-STAT pathway is involved in interferon-mediated signal transduction; thus, it participates in antiviral immunity (10). JEV inhibits STAT phosphorylation in *Ae. albopictus* cells (11); this finding indicates that viruses can actively suppress host signaling and suggests a possible strategy for immune evasion. The Toll pathway is crucial for defense against fungal and gram-positive bacteria that express Lys containing peptidoglycans (10). This study focused on the JAK-STAT, Toll, and RNAi pathways, and used a reverse genetics technique. In the present study, we present evidence that AGO2 mediates *Ae. aegypti* resistance against JEV infection.

SUMMARY: There are three main innate immune mechanisms against viruses in mosquitoes. Infection with the flavivirus dengue virus is controlled by RNA interference (RNAi) and the JAK-STAT and Toll signaling pathways. This study showed that another flavivirus, Japanese encephalitis virus (JEV), did not invade the salivary glands of *Aedes aegypti* and that this may be a result of the innate immune resistance to the virus. Argonaute 2 (AGO2) plays a critical role in the RNAi pathway. To understand the mechanism of JEV resistance, we focused on AGO2 as a possible target of JEV. Here, we show that the expression of *MyD88* (a mediator of Toll signaling) and AGO2 mRNAs was induced by JEV in the salivary glands of *Ae. aegypti* mosquitoes and that AGO2, JAK, and domeless (DOME) mRNAs were induced by JEV in the bodies of *Ae. aegypti* mosquitoes. Double-stranded (ds) AGO2 RNA enhanced JEV infection, and the virus was detected in salivary glands by immunofluorescence assay. In contrast, *MyD88* dsRNA had no effect on JEV infection. These data suggest that AGO2 plays a crucial role in mediating the innate immune response of *Ae. aegypti* to JEV in a manner similar to that employed by dengue virus.
MATERIALS AND METHODS

Mosquitoes and virus: Ae. aegypti mosquitoes (Surabaya strain) (12) were maintained on a 10% sucrose solution at 28°C and 65% humidity with a 16-h light and 8-h dark photoperiod. JEV strain 8-37 was isolated in 2005 from Nagasaki Prefecture, Japan (manuscript in preparation). C. tritaeniorhynchus mosquitoes (Izumo strain) were colonized in 2008 from live specimens collected in Shimane Prefecture, Japan. The C6/36 Ae. albopictus cell line (Health Science Research Resources Bank [HSRRB], Osaka, Japan) was maintained in minimal essential medium (MEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), l-glutamine, 2% non-essential amino acids, and 100 U/mL penicillin. The Vero African green monkey kidney cell line (HSRRB) was cultured in MEM (Gibco) supplemented with 10% FBS, l-glutamine, and 2% non-essential amino acids.

JEV infection: Mosquitoes were infected with JEV as described previously (13). C6/36 cells or Vero cells were infected with JEV-1 strain 8–37 at a multiplicity of infection of 2 plaque-forming units (PFU)/cell and was propagated in C6/36 cells for 4 days at 28°C in 5% CO2 or in Vero cells for 4 days at 37°C in 5% CO2. Culture supernatants were used as virus stocks and were mixed with an equal volume of commercial defibrinated rabbit blood (Nippon Biotest Laboratories Inc., Tokyo, Japan). The blood meal (105 PFU/mL) was maintained at 37°C for 30 min and then offered to mosquitoes using an artificial membrane feeding system (Discovery Workshops, Accrington, UK). Mosquitoes fed with blood that was not mixed with virus were used as a control.

Gene silencing assays: dsRNA was synthesized using the T7 system (Ambion, Austin, TX, USA). RNAi-mediated candidate gene silencing in mosquitoes was performed as described previously (14). For the gene silencing assays, salivary glands, midguts, and carcasses of 4-day-old female mosquitoes fed JEV-supplemented blood were analyzed. The mosquitoes were anesthetized with CO2 and injected with 207 ng of dsRNA per mosquito (14). Mosquitoes injected with dsRNA specific for green fluorescent protein (GFP) were used as a control.

Indirect immunofluorescence assay: Salivary glands were dissected from JEV-infected mosquitoes 14 dpi, squashed on Matsunami adhesive silane–treated glass slides (Matsunami Glass, Tokyo, Japan), and fixed in paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, the methylcellulose overlay was removed, and the slides were stained with methylene blue solution (16).

RESULTS

JEV infection of Ae. aegypti: C. tritaeniorhynchus is the primary vector for JEV, and JEV infects C. tritaeniorhynchus (Fig. 1A). Ae. aegypti cannot transmit JEV for unknown reasons. In our study, we used Ae. aegypti for JEV experiments. Salivary glands are important for the transmission of JEV, and JEV is transmitted from salivary glands. The JEV load was the highest in salivary glands (approximately 16,000 copies), whereas there were approximately 6,000 copies of JEV in midguts, and approximately 1,000 copies in carcasses at 22 dpi in C. tritaeniorhynchus. In this study, JEV replicated in the salivary glands, midguts, and bodies of Ae. aegypti until 25 dpi (Fig. 1B). Although a high level of JEV replication was observed in salivary glands of C. tritaeniorhynchus at 22 dpi, JEV replicated at a low level in the salivary glands of Ae. aegypti at 25 dpi.
Fig. 1. Japanese encephalitis virus (JEV) infection of *Culex tritaeniorhynchus* (A) and *Aedes aegypti* (B). *A. aegypti* were infected orally with JEV. On day 1 and 22 days post-infection in *C. tritaeniorhynchus* and on days 4, 11, and 25 dpi in *A. aegypti*, 5–20 tissues per sample were harvested, and JEV was measured using quantitative reverse transcription polymerase chain reaction ([q]RT-PCR). SG, salivary glands; MG, midguts; CA, carcasses. Error bars: mean ± SD (n = 3).

Fig. 2. Analysis of the expression of genes encoding mediators of innate immunity. Five–20 tissues per sample were harvested on 4, 11, and 25 days post-infection, and MyD88 (A), Argonaute 2 (B), JAK (C), and domeless (DOME) (D) mRNA were measured using (q)RT-PCR, and were normalized to RpS7. SG, salivary glands; MG, midguts; CA, carcasses. Data were analyzed using Student’s t-test. Error bars: mean ± SD (n = 3).
Analysis of the expression of genes encoding mediators of innate immunity in JEV-infected *Ae. aegypti*: We investigated whether JEV infection induced the expression of *Ae. aegypti* genes that encode innate immune mediators using qRT-PCR (Fig. 2). We focused on *MyD88*, *Ago2*, and *JAK* and *DOME*, which are essential components of the Toll, RNAi, and JAK-STAT pathways, respectively. JEV-infected mosquitoes expressed the Toll pathway components as follows: *MyD88* mRNA was expressed in the salivary glands at 11 and 25 dpi; mRNA of the RNAi pathway component *Ago2* was expressed in salivary glands at 11 dpi, with an approximately 3-fold difference between 4 and 11 dpi, and in carcasses at 25 dpi, with an approximately 3-fold difference between 4 and 25 dpi; and mRNAs of the JAK-STAT pathway components *JAK* and *DOME*, were expressed in carcasses at 25 dpi, with an approximately 4-fold difference between 4 and 25 dpi and an approximately 1.5-fold difference between 11 and 25 dpi. However, *JAK* and *DOME* mRNAs were downregulated in carcasses at 11 dpi, with an approximately 2-fold difference between 4 and 11 dpi, and an approximately 1.5-fold difference between 11 and 25 dpi.

*Ago2 suppresses JEV infection in *Ae. aegypti*: Injecting mosquitoes with *Ago2* dsRNA induced JEV genomic RNA expression in salivary glands, midguts, and bodies at 9 dpi (Fig. 3). Furthermore, the nuclei of secretory cells in salivary glands were stained with DAPI (Fig. 4A, C, and E). JEV was not detected by immunofluorescence assay in the salivary glands of non-JEV-infected *Ae. aegypti* at 14 dpi (Fig. 4B); however, it was detected at a low level in the salivary glands of *Ae. aegypti* fed a JEV blood meal at 14 dpi (Fig. 4D). JEV was detected at a high level in the salivary glands of *Ae. aegypti* treated with *Ago2* dsRNA at 14 dpi (Fig. 4F). As shown in Fig. 5, the level of JEV in *Ae. aegypti* treated with *MyD88* dsRNA in the salivary glands, the midguts, and carcasses was the same as that of the control. However, JEV in *Ae. aegypti* treated with *Ago2* dsRNA increased in the salivary glands, midguts, and carcasses compared to that of the controls. Plaque assays confirmed the presence of infectious virus in each of these three tissues at 10 dpi (Fig. 5).
Fig. 4. The effect of Argonaute 2 (Ago2) dsRNA on Japanese encephalitis virus (JEV) replication in the salivary glands of Aedes aegypti. Double-stranded (ds)RNA was injected 4 days before infection. (A) and (B), uninfected mosquitoes; (C) and (D), JEV-infected mosquitoes 14 days post-infection; and (E) and (F), JEV-infected mosquitoes inoculated with Ago2 dsRNA 14 dpi. DAPI staining (A, C, and E), and JEV staining (B, D, and F).

Injecting mosquitoes with dsRNA of MyD88 (Fig. 5) or JAK or DOME (data not shown) had no effect on virus replication in the three tissues.

**DISCUSSION**

In the present study, we showed that JEV infects Aedes aegypti at a low level and that injection of Ago2 dsRNA enhances replication of the virus. These findings indicate that Ago2 suppresses the growth of JEV in Aedes aegypti by a mechanism similar to that employed by dengue virus. Dengue virus replication in Aedes aegypti is also suppressed through the JAK-STAT and the Toll pathways (3–5). However, our data indicate that these other pathways may not be important to suppressing JEV in this mosquito species. Therefore, the RNAi pathway may be critical in mediating Aedes aegypti resistance against JEV infection and may contribute to the low susceptibility of Aedes aegypti for JEV. Although the midgut barrier is known to be important for the suppression of flavivirus transmission, we detected a large number of JEV particles in carcasses (fat body, head, and legs) of Aedes aegypti. Invasion of the salivary glands in mosquitoes may be crucial for transmission of JEV. The viral titer (Fig. 5) was much higher than the RNA copy number (Fig. 3) in mosquitoes, particularly in the midgut. Although the experimental conditions were not exactly the same, the difference was >1,000 fold. The difference at 9 dpi (Fig. 3) and 10 dpi (Fig. 5) may be factors. JEV replicated and invaded salivary glands, and a large number of JEV particles were detected in these organs. Although Ago2 mRNA was not induced in midguts and bodies (Fig. 2B), knockdown of Ago2 enhanced JEV replication (Fig. 5). Constitutive expression of Ago2, therefore, may be sufficient to defend against JEV in the midgut and body.

JEV infection reduces tyrosine phosphorylation of STAT proteins in Aedes albopictus C6/36 cells (11). Here, we show that the expression of JAK and DOME mRNAs decreased at 11 dpi and then increased at 25 dpi in Aedes aegypti bodies. However, the JAK-STAT pathway may be affected by JEV infection. Whereas the expression of JAK and DOME mRNAs increased in bodies with JEV infection, the expression of MyD88 and Ago2 mRNAs increased in salivary glands. This suggests that the Toll and RNAi pathways are active in the salivary glands, whereas the JAK-STAT signaling pathway is active in the fat body. However, dsMyD88 showed no effect in the salivary glands of JEV-infected Aedes aegypti. This suggests that the Toll pathway is not related to Aedes aegypti immunity to JEV. The interaction between mosquitoes and virus may be specific to mosquito immunity.

The secreted antiviral protein Vago restricts West Nile virus infection in Culex mosquito cells by activating the JAK-STAT pathway (17). Whether Vago inhibits JEV replication in Aedes aegypti is unknown. DEXD/H-box helicase Dicer-2 mediates the induction of antiviral activity in Drosophila (18). However, it remains to be determined whether Vago is required.

The phenol oxidase cascade acts as a defense system against malarial parasites in Anopheles mosquitoes (19). Phenol oxidase also mediates the innate immune response against the arbovirus Semliki Forest virus (Togaviridae: Alphavirus) (20) and may be similarly involved in inhibiting Aedes aegypti infection by JEV. C-type lectin collaborates with a CD45 phosphatase homolog to facilitate West Nile virus infection of mosquitoes (21), and Ebola virus infection is enhanced by soluble and transmembrane C-type lectin receptors (22). In addition, RNAi-mediated immunity provides strong protection against infection by the negative-stranded RNA vesicular stomatitis virus in Drosophila (23).

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Fig. 5. The effect of Argonaute 2 (Ago2) and MyD88 dsRNAs on the growth of Japanese encephalitis virus (JEV) in the salivary glands. Double-stranded (ds) RNA was injected 4 days before infection. Five–20 tissues per sample were harvested on 10 dpi, and JEV was measured using a plaque assay. (A) salivary glands (SG); (B) midguts (MG); and (C) carcasses (CA). PFU, plaque forming units. Data were analyzed using Student’s t-test. Error bars: mean ± SD (n = 3).

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