5′-TGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTC-3′ and 5′-CGGACTCGTCATACTCCTGCTTGCTGATCCACATC-3′, and 5′-GGCTGTTGAGGAAGAAGACG-3′ and 5′-CTTGGAGTCAGCAGTGACCA-3′, respectively. The FKBP8 primers are located at different exons in order to prevent the false-positive amplification of contaminated genomic DNA. The values of the HCV genome and FKBP8 mRNA were normalized with those of β-actin mRNA. Each PCR product was detected as a single band of the correct size upon agarose gel electrophoresis (data not shown).

Establishment of cell lines expressing an siRNA-resistant FKBP8 mutant and knockdown FKBP8 expression

A, G, and T at nucleotides 273, 276, and 288 from the 5′ end of the open-reading frame of human FKBP8 were replaced with G, A, and C, respectively, according to a splicing method achieved by overlap extension; these silent mutations were then cloned into pEF-Flag pGBKpuro. The resulting plasmid encoding a mutant FKBP8 resistant to knockdown by siRNA was transfected into Huh7 cells harboring the HCV RNA replicon. The culture medium was replaced with DMEM supplemented with 10% FCS and 2 μg/ml of puromycin (Nakarai Tesque, Tokyo, Japan) at 24 h post-transfection, and the cells were cultured for 7 days. The surviving cells were used for the FKBP8 knockdown experiments. The shRNAs targeted to FKBP8, the target sequences of which were 5′-GATCCGCTGGAACCTTCCA ACAAGTTCAAGAGACTTGTTGGAAGGTTCCAGCTTA-3′, and 5′-A GCTTAAGCTGGAACCTTCCAACAAGTCTCTTGAACTTGTTGGAAGG TTCCAGCG-3′, were annealed and introduced between the *Bam*HI and *Hin*dIII sites of pSilencer™ 2.1-U6 hygro (Ambion, Austin, TX) according to the manufacturer's protocol. An HCV replicon cell line cured with IFN-α was transfected with 5 μg of the plasmid by electroporation. The culture medium was replaced with DMEM supplemented with 10% FCS and 500 μg/ml of Hygromycin B (Wako, Tokyo, Japan) at 24 h post-transfection. The remaining cells were re-seeded in 98-well plates and cloned for the colony formation and transient replication assays.

Colony formation assay

The plasmid pFK-I_{389} neo/NS3-3′/NK5.1 (Pietschmann *et al*, 2002) was obtained from R Bartenschlager. The plasmid cleaved at the *Sca*I site was transcribed *in vitro* using the MEGAscript T7 kit (Ambion) according to the manufacturer's protocol. The linearized plasmid (10 μg) was introduced into Huh7 cells at 4 million cells/0.4 ml by electroporation at 270 V and 960 μF using a Gene Pulser™ (Bio-Rad, Hercules, CA). Electroporated cells were suspended at a final volume of 10 ml of culture medium. Three-milliliter aliquots of cell suspension were mixed with 7 ml of culture medium and then the cells were seeded on culture dishes (diameter: 10 cm). The culture medium was replaced with DMEM containing 10% FCS and 1 mg/ml of G418 (Nakarai Tesque) at 24 h post-transfection. The medium was exchanged weekly for fresh DMEM containing 10% FCS and 1 mg/ml G418. The remaining colonies were fixed with 4% paraformaldehyde at 4 weeks after electroporation, and the cells were stained with crystal violet.

Transient replication assay

The cDNA encoding *Renilla* luciferase was introduced between the *Asc*I and *Pme*I sites of the plasmid pFK-I_{389} neo/NS3-3′/NK5.1, in place of the *neo* gene. The resulting plasmid, pFK-I_{389} hRL/NS3-3′/NK5.1, was cleaved with *Sca*I and was transcribed *in vitro* using a MEGAscript T7 kit (Ambion). Huh7 cells were suspended at 10 million cells/ml and the suspensions were mixed with 10 μg of *in vitro*-transcribed RNA at a 400-μl volume; the cells were then electroporated at 270 V and 960 μF by a Gene Pulser™ (Bio-Rad). The electroporated cells were suspended in 25 ml of culture medium and then were seeded at 1 ml/well on 12-well culture plates. Luciferase activity was measured at 4 and 48 h post-transfection using a *Renilla* Luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. Luciferase activity at 4 h after electroporation was used to determine the transfection efficiency.

Generation of infectious HCV particles

The viral RNA of JFH1 was introduced into Huh7.5.1 according to the method of Wakita *et al* (2005). The supernatant was collected at 7 days post-transfection and used as HCV particles that are infectious in cell culture (HCVcc). The naïve Huh7.5.1 cells were transfected with siRNA of nontarget control or FKBP8-Target 1 at a concentration of 80 nM. The siRNA-treated Huh7.5.1 cells were inoculated with HCVcc at 24 h post-transfection. Infected cells and culture supernatants were harvested every day until 5 days post-infection.

Determination of FKBP8-binding proteins

MEF purification was carried out by a previously described method (Ichimura *et al*, 2005). The FKBP8 gene was amplified by PCR and introduced into pcDNA3.1 encoding the myc-TEV-Flag epitope tag (Ichimura *et al*, 2005). The resulting plasmid was transfected into 293T cells, which were then subjected to MEF purification. FKBP8-binding proteins were separated by SDS–PAGE and visualized by silver staining. The stained bands were excised, digested in gels with Lys-C, and analyzed by the direct nanoflow LC-MS/MS system (Ichimura *et al*, 2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank H Murase for secretarial work and H Miyamoto for discussion. We are also grateful to J Bukh, R Bartenschlager, and T Wakita for providing the HCV cDNAs and DCS Huang for the pEF-FLAG pGBK puro. This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the 21st Century Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

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doi:10.1038/nature04734

nature

LETTERS

Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses

Hiroki Kato[1,3]*, Osamu Takeuchi[1,3]*, Shintaro Sato[3], Mitsutoshi Yoneyama[4], Masahiro Yamamoto[1], Kosuke Matsui[1], Satoshi Uematsu[1], Andreas Jung[1], Taro Kawai[3], Ken J. Ishii[3], Osamu Yamaguchi[5], Kinya Otsu[5], Tohru Tsujimura[6], Chang-Sung Koh[7], Caetano Reis e Sousa[8], Yoshiharu Matsuura[2], Takashi Fujita[4] & Shizuo Akira[1,3]

The innate immune system senses viral infection by recognizing a variety of viral components (including double-stranded (ds)RNA) and triggers antiviral responses[1,2]. The cytoplasmic helicase proteins RIG-I (retinoic-acid-inducible protein I, also known as Ddx58) and MDA5 (melanoma-differentiation-associated gene 5, also known as Ifih1 or Helicard) have been implicated in viral dsRNA recognition[3–7]. *In vitro* studies suggest that both RIG-I and MDA5 detect RNA viruses and polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue[3]. Although a critical role for RIG-I in the recognition of several RNA viruses has been clarified[8], the functional role of MDA5 and the relationship between these dsRNA detectors *in vivo* are yet to be determined. Here we use mice deficient in MDA5 (*MDA5*$^{-/-}$) to show that MDA5 and RIG-I recognize different types of dsRNAs: MDA5 recognizes poly(I:C), and RIG-I detects *in vitro* transcribed dsRNAs. RNA viruses are also differentially recognized by RIG-I and MDA5. We find that RIG-I is essential for the production of interferons in response to RNA viruses including paramyxoviruses, influenza virus and Japanese encephalitis virus, whereas MDA5 is critical for picornavirus detection. Furthermore, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ mice are highly susceptible to infection with these respective RNA viruses compared to control mice. Together, our data show that RIG-I and MDA5 distinguish different RNA viruses and are critical for host antiviral responses.

Host pattern recognition receptors, such as Toll-like receptors (TLRs) and helicase family members, have an essential role in the recognition of molecular patterns specific for different viruses, including DNA, single-stranded (ss)RNA, dsRNA and glycoproteins[1,9,10]. dsRNA can be generated during viral infection as a replication intermediate for RNA viruses. TLR3, which localizes in the endosomal membrane, has been shown to recognize viral dsRNA as well as the synthetic dsRNA analogue poly(I:C) (refs 11, 12). The cytoplasmic proteins RIG-I and MDA5 have also been identified as dsRNA detectors[3–5,7,13]. RIG-I and MDA5 contain two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain. RIG-I recruits a CARD-containing adaptor, IPS-1 (also known as MAVS, VISA or Cardif)[14–17]. IPS-1 relays the signal to the kinases TBK1 and IKK-i, which phosphorylate interferon-regulatory factor-3 (IRF-3) and IRF-7, transcription factors essential for the expression of type-I interferons[18–22]. In contrast, TLR3 activates TBK1 and IKK-i through the TIR-domain-containing adaptor TRIF (also known as Ticam1)[12].

In vitro studies have shown that both RIG-I and MDA5 can bind to poly(I:C) and respond to poly(I:C) and RNA viruses[6]. We have generated *RIG-I*$^{-/-}$ mice, and show that RIG-I is essential eliciting the immune responses against several RNA viruses, including Newcastle disease virus (NDV), Sendai virus (SeV) and vesicular stomatitis virus (VSV), in various cells except for plasmacytoid dendritic cells (pDCs)[8]. Hepatitis C virus and Japanese encephalitis virus are also reported to be recognized by RIG-I *in vitro*[23,24].

The *in vivo* functional relationship between RIG-I and MDA5 remains to be determined. To investigate a functional role for MDA5 *in vivo*, we generated *MDA5*$^{-/-}$ mice and investigated viral recognition (Supplementary Fig. 1). In contrast to *RIG-I*$^{-/-}$ mice, which are mostly embryonic lethal, *MDA5*$^{-/-}$ mice are born in a mendelian ratio, grow healthily and do not show gross developmental abnormalities until 24 weeks of age. Flow cytometric analysis of leukocytes from the spleen and lymph nodes (staining for CD3, B220 and CD11c) revealed that the composition of lymphocytes and dendritic cells is similar in wild-type and *MDA5*$^{-/-}$ mice (data not shown).

TLR3, RIG-I and MDA5 have been implicated in the recognition of poly(I:C) and the subsequent induction of antiviral responses. However, their exact contribution to *in vivo* responses against dsRNA has yet to be clarified. We therefore examined the *in vivo* responses to poly(I:C) in mice lacking RIG-I, MDA5 or TRIF, or both MDA5 and TRIF. Administration of poly(I:C) led to rapid induction of the cytokines interferon-α (IFN-α), IFN-β, interleukin-6 (IL-6) and IL-12 in sera of both wild-type and *RIG-I*$^{-/-}$ mice (Fig. 1a and Supplementary Fig. 2a). In contrast, *MDA5*$^{-/-}$ mice failed to produce IFN-α and IFN-β in response to poly(I:C), and production of IL-6 and IL-12p40 was also significantly impaired (Fig. 1b). Although *Trif*$^{-/-}$ mice produced normal amounts of IFN-α, they also showed severely impaired production of IL-12p40 and partial impairment in IL-6 production. *MDA5*$^{-/-}$; *Trif*$^{-/-}$ double-knockout mice failed to induce IFN-α, IL-6 and IL-12p40 in response to poly(I:C). These results indicate that MDA5 is essential for poly(I:C)-induced IFN-α production and TLR3 signalling is critical for IL-12 production, whereas both MDA5 and TLR3 regulate IL-6 production.

[1]Department of Host Defense, [2]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, and [3]ERATO, Japan Science and Technology Agency, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. [4]Department of Genetics and Molecular Biology, Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. [5]Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. [6]Department of Pathology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. [7]Department of Medical Technology, Shinshu University School of Allied Medical Sciences, 3-1-1 Asahi, Matsumoto 390-8621, Japan. [8]Immunobiology Laboratory, Cancer Research UK London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.
*These authors contributed equally to this work.

When bone-marrow-derived dendritic cells generated by granulocyte–macrophage colony-stimulating factor (GMCSF) were incubated in the presence of poly(I:C), production of IFN-α and IFN-β was severely impaired in *MDA5*$^{-/-}$, but not in *RIG-I*$^{-/-}$ or *Trif*$^{-/-}$, GMCSF-DCs (Fig. 1c and Supplementary Fig. 2b). Even when poly(I:C) was transfected into GMCSF-DCs using lipofectamine, poly(I:C) induced IFN-β production in an MDA5-dependent, but not a RIG-I- or TRIF-dependent, manner (Fig. 1d). IFN-β production in response to poly(I:C) was also impaired in *MDA5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Fig. 1e), indicating that poly(I:C) is primarily recognized by MDA5, not RIG-I and TLR3, in these cells.

dsRNAs transcribed *in vitro* (Supplementary Fig. 2c) also stimulated MEFs to produce IFN-β. Unlike for poly(I:C), wild-type and *MDA5*$^{-/-}$ MEFs produced comparable amounts of IFN-β (Fig. 1e) in response to *in vitro* transcribed dsRNAs. In contrast, *RIG-I*$^{-/-}$ MEFs did not produce detectable amounts of IFN-β, indicating that RIG-I is essential for the detection of *in vitro* transcribed dsRNAs. As RIG-I, but not MDA5, is responsible for IFN-β production in response to dsRNAs of various lengths, these helicases probably distinguish nucleotide structure or sequence, but not length. Together, these results indicate that MDA5 and RIG-I are involved in the detection of poly(I:C) and *in vitro* transcribed dsRNAs, respectively.

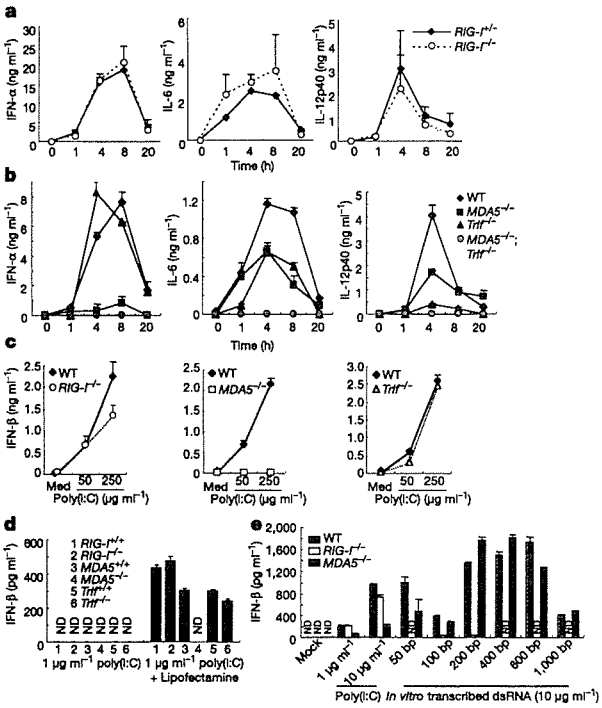

Figure 1 | Roles of MDA5, RIG-I and TRIF in the recognition of synthesized dsRNAs and dsRNA analogues. a, b, *RIG-I*$^{-/-}$ and littermate *RIG-I*$^{+/-}$ mice (**a**) or wild-type (WT), *MDA5*$^{-/-}$, *Trif*$^{-/-}$ or *MDA5*$^{-/-}$; *Trif*$^{-/-}$ double-knockout mice (**b**) were injected intravenously with 200 μg poly(I:C) for the indicated periods, and IFN-α, IL-6 and IL-12p40 production was measured in serum by ELISA. Data show mean ± s.d. **c,** GMCSF-DCs from *RIG-I*$^{-/-}$, *MDA5*$^{-/-}$, *TRIF*$^{-/-}$ and littermate control mice were incubated in the presence of 50 or 250 μg ml^{-1} poly(I:C) for 24 h. IFN-β production in the cell culture supernatants was measured by ELISA. Med, medium only. **d,** GMCSF-DCs were treated with 1 μg ml^{-1} poly(I:C) complexed with or without lipofectamine 2000 for 24 h, and IFN-β production was measured. **e,** Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ MEFs were treated with poly(I:C) or *in vitro* transcribed dsRNAs of indicated lengths complexed with lipofectamine 2000 for 12 h, and IFN-β production was measured. Error bars indicate s.d. of triplicate wells in a single experiment; data are representative of three independent experiments. ND, not detected.

This finding led us to hypothesize that RIG-I and MDA5 are involved in the detection of different RNA viruses. We have previously shown that a set of negative-sense RNA viruses are recognized by RIG-I[8]. We first examined IFN-β and IFN-α production in *MDA5*$^{-/-}$ MEFs in response to a set of negative-sense ssRNA viruses, including NDV, SeV, VSV and influenza virus. As infection with most of the wild-type viruses (except NDV) failed to induce type-I interferons in MEFs, owing to suppression of interferon responses by viral proteins (data not shown), we also used mutant viruses lacking viral interferon-inhibitory proteins. As shown in Fig. 2a and Supplementary Fig. 4b, wild-type MEFs produce IFN-β and IFN-α in response to these mutant viruses. Production of type-I interferons was severely impaired in *RIG-I*$^{-/-}$ MEFs compared to wild-type cells, but MDA5 was dispensable for the production of type-I interferons. Japanese encephalitis virus (JEV), a positive-sense ssRNA virus belonging to the flavivirus family, also required RIG-I, but not MDA5, for IFN-β production (Fig. 2b).

We then examined the interferon responses of MEFs to encephalomyocarditis virus (EMCV), a positive-sense ssRNA virus belonging to the picornavirus family. EMCV-induced IFN-β production was abrogated in *MDA5*$^{-/-}$ MEFs (Fig. 2c). In contrast, wild-type and *RIG-I*$^{-/-}$ MEFs produced comparable amounts of IFN-β, indicating that EMCV is specifically recognized by MDA5. The induction of genes encoding IFN-β, IP-10 and IL-6 in response to EMCV was abrogated in *MDA5*$^{-/-}$ macrophages (Supplementary Fig. 3d). The synthesis of cellular proteins in *MDA5*$^{-/-}$ MEFs was progressively inhibited during EMCV infection, to an extent and with kinetics similar to wild-type MEFs (Supplementary Fig. 5), indicating that the EMCV infection was established in wild-type and *MDA5*$^{-/-}$ MEFs in a similar manner. Moreover, other viruses belonging to the picornavirus family (Theiler's and Mengo viruses) also induced IFN-α through MDA5 (Supplementary Fig. 4d). Furthermore, the production of IFN-β in response to SeV and EMCV was impaired in *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ GMCSF-DCs, respectively (Fig. 2d, e), indicating that conventional dendritic cells (cDCs) also use these helicases for the differential recognition of viruses. EMCV-induced production of IL-6 was also abrogated in *MDA5*$^{-/-}$, but not *RIG-I*$^{-/-}$, cDCs (Supplementary Fig. 4c). Therefore, MDA5 is critical for the regulation of pro-inflammatory cytokines as well as type-I interferons in response to EMCV.

We next examined whether viral RNAs derived from VSV and EMCV recapitulate the production of interferons through MDA5 and RIG-I. When transfected into GMCSF-DCs by lipofection, RNAs prepared from VSV or EMCV induced production of IFN-α in a RIG-I- or MDA5-dependent manner, respectively (Fig. 2f). We also performed reconstitution experiments by transfecting RIG-I or MDA5 expression vectors into *RIG-I*$^{-/-}$; *MDA5*$^{-/-}$ MEFs, in which IFN-β induction was completely abrogated in response to infection with EMCV or SeV Cm (SeV with a mutated C protein) (Fig. 2g). The ectopic expression of human RIG-I, but not MDA5, activated the *Ifnb* promoter in response to SeV Cm. Reciprocally, cells expressing human MDA5, but not RIG-I, activated the *Ifnb* promoter in response to EMCV in a dose-dependent manner (Fig. 2h). These results indicate that human RIG-I and MDA5 recognize different RNA viruses by recognizing viral RNAs.

Previous studies have shown that pDCs use mainly the TLR system instead of RIG-I in the recognition of several RNA viruses[8]. MyD88 is an adaptor protein essential for TLR signalling (except through TLR3). We purified B220^{+} pDCs from Flt3L-generated bone-marrow-derived dendritic cells (Flt3L-DCs) and infected them with EMCV. pDCs from *Myd88*$^{-/-}$, but not *MDA5*$^{-/-}$, mice showed a profound defect in IFN-α production (Supplementary Fig. 6). Reciprocally, MDA5, but not MyD88, is required for the production of IFN-α in B220^{-} cDCs purified from Flt3L-DCs (Supplementary Fig. 6). These results indicate that both MDA5 and RIG-I are

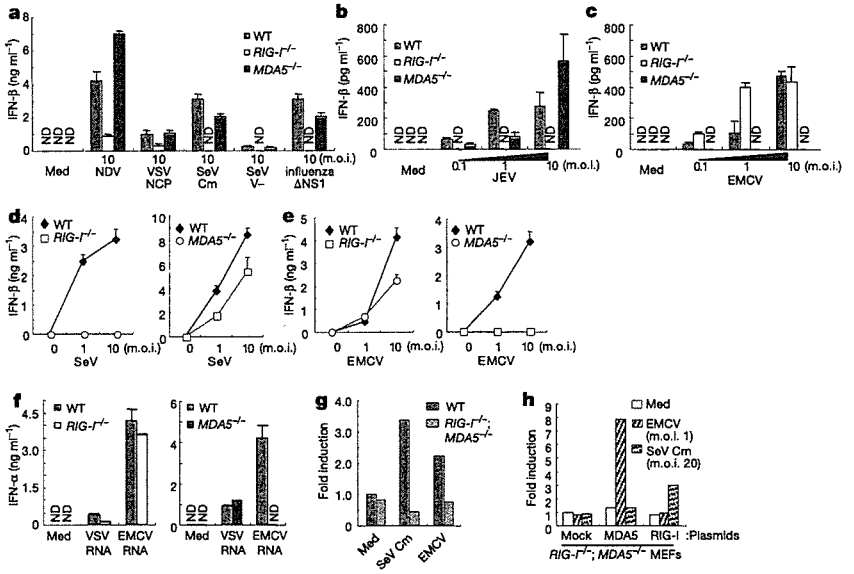

Figure 2 | Differential viral recognition by RIG-I and MDA5. **a**, Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ MEFs were exposed to negative-sense ssRNA viruses, including NDV, VSV lacking a variant of M protein (NCP), SeV with a mutated C protein (Cm), SeV lacking V protein (V^{-}), and influenza virus lacking the NS1 protein (ΔNS1) for 24 h. IFN-β production in the culture supernatants was measured by ELISA. **b**, **c**, Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ MEFs were exposed to the positive-sense ssRNA viruses JEV (**b**) and EMCV (**c**), and IFN-β production was measured. **d**, **e**, GMCSF-DCs from *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ mice and their littermate wild-type mice were infected with an increasing m.o.i. of SeV V^{-} (**d**) or EMCV (**e**) for 24 h, and IFN-β production was measured. **f**, Wild-type, *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ GMCSF-DCs were treated with RNAs directly prepared from VSV and EMCV (complexed with lipofectamine 2000) for 24 h, and IFN-α production was measured. **g**, Wild-type and *RIG-I*$^{-/-}$; *MDA5*$^{-/-}$ MEFs were transiently transfected with a reporter construct containing the *Ifnb* promoter and exposed to SeV Cm or EMCV for 24 h. Cell lysates were then prepared and subjected to a luciferase assay. **h**, RIG-I$^{-/-}$; MDA5$^{-/-}$ MEFs were transiently transfected with the *Ifnb* promoter construct together with expression plasmids encoding human RIG-I or MDA5. The cells were then infected with EMCV or SeV Cm for 24 h and were subjected to a luciferase assay. Error bars in **a**–**g** indicate s.d. of triplicate wells in a single experiment; data are representative of three independent experiments. ND, not detected.

dispensable for the viral induction of IFN-α in pDCs.

We next examined the *in vivo* roles of MDA5 and RIG-I in host defence against viral infection. Although most *RIG-I*$^{-/-}$ mice are embryonic lethal[8], we could efficiently obtain live adult mice by intercrossing the *RIG-I*$^{+/-}$ mice obtained after *RIG-I*$^{+/-}$ × ICR crosses (Supplementary Table 1). When the mice were infected with JEV, serum IFN-α levels were markedly decreased in *RIG-I*$^{-/-}$ mice compared to littermate *RIG-I*$^{+/-}$ mice. In contrast, *MDA5*$^{-/-}$ mice did not show a defect in JEV-induced systemic IFN-α production (Fig. 3a). IFN-α production was partially impaired in *Myd88*$^{-/-}$ mice compared to wild-type mice, but the extent of this impairment was far less than in *RIG-I*$^{-/-}$ mice (Fig. 3a). These data suggest that the TLR system is not critical for the induction of serum IFN-α *in vivo* in response to JEV. Consistent with this finding, *RIG-I*$^{-/-}$ mice, but not *MDA5*$^{-/-}$ or *Myd88*$^{-/-}$ mice, were more susceptible to JEV infection than control mice (Fig. 3b). Furthermore, *RIG-I*$^{-/-}$ mice, but not *MDA5*$^{-/-}$ mice, succumbed to VSV infection, consistent with abrogated interferon responses (Supplementary Fig. 7). Thus, RIG-I-mediated recognition of a specific set of viruses has a critical role in antiviral host defence *in vivo*.

We next challenged the mice with EMCV as a model virus that is recognized by MDA5. Induction of IFN-β, IFN-α, RANTES and IL-6 was severely impaired in the sera of *MDA5*$^{-/-}$ mice (Fig. 4a and Supplementary Fig. 8). *MDA5*$^{-/-}$ mice and mice null for the IFN-α/β receptor (*Ifnar1*$^{-/-}$) were highly susceptible to EMCV infection (viral titre of 1×10^2 plaque-forming units (p.f.u.)) compared to littermate controls ($P < 0.01$) (Fig. 4b). In contrast, deficiency of neither RIG-I nor TLR3 affected the survival of mice infected with EMCV. Consistent with a previous report[22], *Myd88*$^{-/-}$ mice were modestly susceptible to EMCV infection compared to wild-type mice, implying that pDC-mediated responses are not critical for eliminating EMCV (Fig. 4b).

It is known that EMCV preferentially infects cardiomyocytes and causes myocarditis. Consistent with increased susceptibility to EMCV, viral titre in the heart was much higher in *MDA5*$^{-/-}$ mice compared to control mice (Fig. 4c). Histological analysis of hearts two days after EMCV infection revealed that focal necrosis of

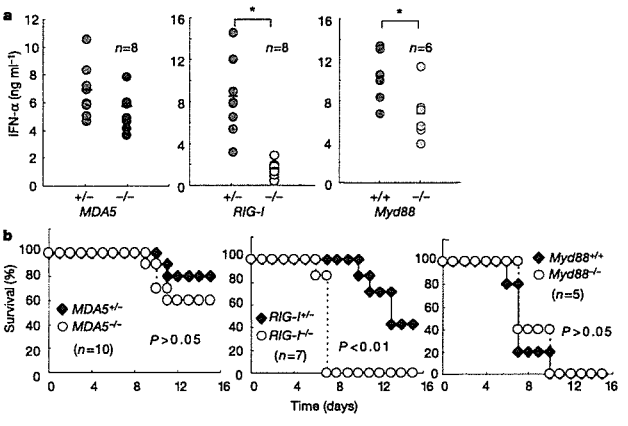

Figure 3 | Susceptibility of *RIG-I*$^{-/-}$ and *MDA5*$^{-/-}$ mice to JEV infection. **a**, *RIG-I*$^{+/-}$, *RIG-I*$^{-/-}$, *MDA5*$^{+/-}$ and *MDA5*$^{-/-}$ mice ($n = 8$), and *Myd88*$^{+/+}$ or *Myd88*$^{-/-}$ mice ($n = 6$), were injected intravenously with 2×10^7 p.f.u. JEV. Sera were collected 24 h after injection, and IFN-α production levels measured by ELISA. Circles represent individual mice, bars indicate mean values. Asterisk, $P < 0.05$ versus controls (*t*-test). **b**, The survival of 6-week-old mice (genotypes as indicated) infected intravenously with 2×10^7 p.f.u. JEV. Mice were monitored for 15 days ($P < 0.01$ between *RIG-I*$^{-/-}$ mice and their littermate controls, generalized Wilcoxon test).

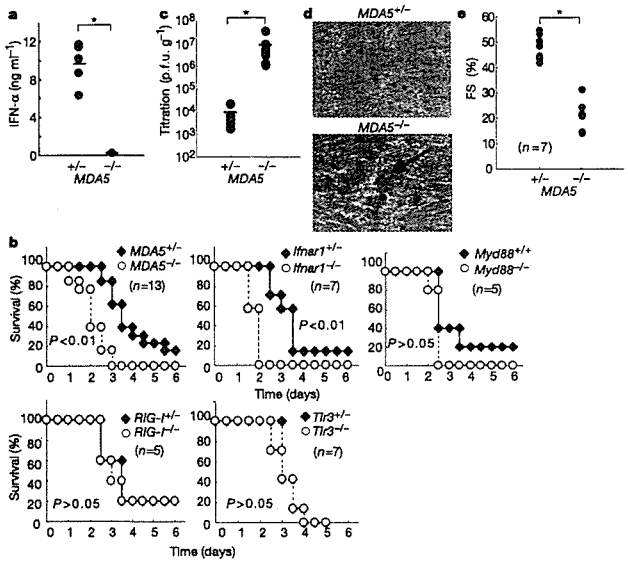

Figure 4 | Role of MDA5 in host defence against EMCV infection. **a**, $MDA5^{+/-}$ and $MDA5^{-/-}$ mice ($n = 5$) were inoculated intravenously with 1×10^7 p.f.u. EMCV. Sera were prepared 4 h after injection and IFN-α production levels determined by ELISA. **b**, The survival of 6-week-old mice (genotypes as indicated) infected with 1×10^2 p.f.u. EMCV intraperitoneally was monitored every 12 h for six days ($P < 0.01$ between $MDA5^{-/-}$ or $Ifnar1^{-/-}$ mice and their littermate controls, generalized Wilcoxon test). **c**, $MDA5^{+/-}$ and $MDA5^{-/-}$ mice were infected intraperitoneally with 1×10^2 p.f.u. EMCV. After 48 h, mice were killed and virus titres in hearts were determined by plaque assay. **d**, Heart sections of $MDA5^{+/-}$ and $MDA5^{-/-}$ mice, two days after infection, were assessed for histological changes using haematoxylin and eosin staining. Arrow indicates the focal necrosis of cardiomyocytes. **e**, Cardiac function of mice 48 h after EMCV infection was assessed by echocardiography (see Supplementary Fig. 8b). The fractional shortening (FS) after infection determined by transthoracic M-mode echocardiographic tracings is shown. Asterisk, $P < 0.05$ versus $MDA5^{+/-}$ mice (*t*-test).

cardiomyocytes had developed in $MDA5^{-/-}$ mice, but wild-type hearts showed no histological abnormalities at this time point (Fig. 4d). Notably, no infiltration of immune cells was observed in either wild-type or $MDA5^{-/-}$ heart sections at this time point. However, when cardiac performance was analysed by echocardiography two days after infection (Fig. 4e), cardiac contractility was severely depressed in $MDA5^{-/-}$ mice (fractional shortening 48.2 ± 4.9% in $MDA5^{+/-}$ mice, 21.2 ± 5.8% in $MDA5^{-/-}$ mice), indicating that $MDA5^{-/-}$ mice developed severe heart failure due to virus-induced cardiomyopathy. Thus, MDA5-mediated recognition of EMCV is a prerequisite for triggering antiviral responses as well as for prevention of myocardial dysfunction.

Together, our results demonstrate that RIG-I and MDA5 have essential roles in the recognition of different groups of RNA viruses, as well as in the subsequent production of type-I interferons and pro-inflammatory cytokines. We have found that poly(I:C) and *in vitro* transcribed dsRNA are recognized by MDA5 and RIG-I, respectively; this is in contrast to results from previous *in vitro* studies. RIG-I probably recognizes dsRNA generated over the course of RNA virus replication, as all *in vitro* transcribed dsRNAs tested except for poly(I:C) induced type-I interferons through RIG-I. In contrast, the endogenous ligand of MDA5 remains enigmatic. Moreover, how RIG-I and MDA5 differentially recognize natural dsRNAs is undetermined. Given that the helicase domains of RIG-I and MDA5 bind to dsRNA, analyses of the crystal structures of these domains should to help achieve a better understanding of the molecular mechanisms underlying this differential recognition. Furthermore, it is still possible that unknown dsRNA-binding proteins also function as direct receptors for viral RNAs.

Finally, the picornavirus family contains several viruses that are pathogenic for humans, including poliovirus, rhinovirus and the virus causing foot-and-mouth-disease. Our studies suggest that human MDA5 and RIG-I also recognize RNA viruses. Thus, identification of therapeutic agents that modify RIG-I or MDA5 may lead to antiviral strategies against selected viruses.

METHODS

Mice, cells and reagents. The generation of $MDA5^{-/-}$ mice is described in the Supplementary Information. $Myd88^{-/-}$, $Tlr3^{-/-}$ and $Trif^{-/-}$ mice have been described previously[12]. $Ifnar1^{-/-}$ mice have also been described previously[25]. $RIG\text{-}I^{+/-}$ mice in a 129Sv × C57BL/6 background were crossed with ICR mice, and the resulting $RIG\text{-}I^{+/-}$ mice were further intercrossed. Interbreeding of these $RIG\text{-}I^{+/-}$ mice produced healthy and fertile $RIG\text{-}I^{-/-}$ offspring, although their number was less than half that of $RIG\text{-}I^{+/+}$ progeny (Supplementary Table 1). $RIG\text{-}I^{-/-}$ and $RIG\text{-}I^{+/-}$ littermate mice were used for *in vivo* experiments. $RIG\text{-}I^{-/-}$; $MDA5^{-/-}$ mice in a 129Sv × C57BL/6 background were lethal at embryonic day 12.5. Additional details regarding cells, reagents and the preparation of *in vitro* transcribed dsRNA are provided in the Supplementary Information.

Viruses. NDV (ref. 3), VSV, VSV lacking a variant of M protein (NCP) (ref. 8), influenza virus lacking the NS1 protein (ΔNS1) (ref. 26), JEV (ref. 27) and EMCV (ref. 3) have been described previously. SeV and SeV lacking the V protein (V^-) or with mutated C proteins (Cm) were provided by A. Kato[28].

Luciferase assay. Wild-type or $RIG\text{-}I^{-/-}$; $MDA5^{-/-}$ MEFs were transiently transfected with a reporter construct containing the *Ifnb* promoter together with an empty vector (mock), or *RIG-I* or *MDA5* expression vectors. As an internal control, a *Renilla* luciferase construct was transfected. Transfected cells were untreated or infected with EMCV or SeV Cm (m.o.i. 20) for 24 h. The cells were lysed and subjected to a luciferase assay using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Analysis of mice after EMCV infection. Methods for plaque assays, histological analysis and echocardiography are described in the Supplementary Information.

Measurement of cytokine production. Cell culture supernatants were collected and analysed for IFN-β, IFN-α, IL-6 or IL-12p40 production using enzyme-linked immunosorbent assays (ELISAs). ELISA kits for mouse IFN-α and IFN-β were purchased from PBL Biomedical Laboratories, and those for IL-6, IL-12p40 and RANTES were obtained from R&D Systems.

Statistical analysis. Kaplan–Meier plots were constructed and a generalized Wilcoxon test was used to test for differences in survival between control and mutant mice after viral infection. Statistical significance of any differences in cytokine concentration and ECMV titres was determined using Student's *t*-tests.

Received 30 January; accepted 20 March 2006.
Published online 9 April 2006.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank all colleagues in our laboratory, K. Takeda, T. Shioda, E. Nakayama and K. Kiyotani for helpful discussions, A. Kato, T. Abe, Y. Mori, B. S. Kim and A. Palmenberg for viruses and plasmids, M. Hashimoto for secretarial assistance, and Y. Fujiwara, M. Shiokawa, N. Kitagaki and A. Shibano for technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan, and from the 21st Century Center of Excellence Program of Japan.

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Am. J. Trop. Med. Hyg., 74(5), 2006, pp. 932–936

SEROLOGIC EVIDENCE FOR HEPATITIS E VIRUS INFECTION IN MONGOOSE

TIAN-CHENG LI,* MIKA SAITO, GO OGURA, OSAMU ISHIBASHI, TATSUO MIYAMURA, AND NAOKAZU TAKEDA

Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan; Division of Molecular Virology and Oncology, Department of Microbiology, Graduate School of Medicine, University of the Ryukyus, Okinawa; Laboratory of Subtropical Zoology, Faculty of Agriculture, University of the Ryukyus, Okinawa

Abstract. Although pig and wild boar are considered to be the reservoirs of hepatitis E virus (HEV) in Japan, the spread of HEV to other animals is unknown. Serum samples from 84 mongooses (Small Asian mongoose; *Herpestes javanicus*) collected in Okinawa, Japan were examined for antibodies to HEV by enzyme-linked immunosorbent assay and RNA was analyzed by reverse transcription–polymerase chain reaction. Seven (8.3%) of 84 mongooses were positive for IgG antibodies to HEV, and the antibody-positive rate increased with body weight and size, whereas HEV RNA was not detected in these samples. These results are consistent with the possibility that mongooses in Okinawa are occasionally infected with HEV; however, they are not considered the major zoonotic reservoir of HEV.

INTRODUCTION

Hepatitis E virus (HEV) is the most important cause of acute hepatitis in many developing countries in Asia, the Middle East and north Africa,[1] and was recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae*.[2] Hepatitis E was first recognized when a large waterborne hepatitis outbreak occurred in India in 1955, where the antibody-positive rate of hepatitis A virus is extremely high in all age groups.[3] Because HEV is transmitted via an oral-fecal route, contaminated drinking water and food are the primary source of the infection. Although hepatitis E is self-limiting and neither chronic nor persistent infection is observed in the adult population in general, a high mortality rate of 15–20% is reported in pregnant women.[4]

Hepatitis E virus is a nonenveloped, single-stranded positive-sense RNA virus.[5] Phylogenetic analysis has identified at least four major genotypes of HEV.[6] Genotype 1 (G1) HEV was isolated from Asia and Africa,[1,4] genotype 2 (G2) from Mexico,[7] and genotypes 3 (G3) and 4 (G4) from the United states, European countries, China, Japan, and Vietnam.[8–13] These viruses are believed to compose a single serotype.[14]

Although most cases were imported into developed countries, recent studies have showed that hepatitis E occurred in patients who had never been abroad.[9,10] Genetically similar G3 and G4 HEVs isolated from pigs, deer, and wild boars,[8,15,16] and serum antibodies to HEV in a variety of animals including pigs, deer, wild boars, wild rats, dogs, cats, and cows[17,18] suggest that hepatitis E is a zoonosis. Recently, direct evidence of G3 HEV transmission from deer and wild boar meats to humans was clearly provided in Japan, suggesting that wild animals are the zoonotic reservoir of HEV in this country.[15,16] Transmission from visceral organs of pigs to humans has also been suspected.[19]

Okinawa is located southwest of Japan, where mongoose (Small Asian mongoose; *Herpestes javanicus*), an exotic animal, was introduced from India in 1910 for the control of a poisonous snake (habu) and rats. The number of mongooses and their living area increased quickly because there was no natural predator of this animal in Okinawa. This caused a disruption of the ecology. Since wild boars also live on this island, mongooses have an opportunity to be exposed to HEV from infected boars.

* Address correspondence to Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan. E-mail: litc@nih.go.jp

To find HEV reservoirs other than pigs, deer, and wild boars, and to determine whether HEV spreads to other wild animals, we analyzed serum samples from 84 mongoose captured in Okinawa for antibodies to HEV and viral RNA. IgG antibodies to HEV were found in 8.3% of the mongooses, suggesting that although HEV infection may occur among mongooses, they are not the major zoonotic reservoir of HEV.

MATERIALS AND METHODS

Mongoose sera. Wild *H. javanicus* (54 males and 30 females) were captured between July 2004 and May 2005 in Okinawa. The mongooses were transferred to the laboratory and anesthetized. Their sex was identified, and their body weights and head and body length (body size) were measured. Blood was collected by cardiac puncture under anesthesia, and allowed to clot at room temperature for one hour. The serum fraction was collected by centrifugation and stored at −20°C until use.

Preparation of recombinant virus-like particles. A recombinant baculovirus, Ac5480/7126, that harbors the G1 HEV capsid protein gene with a 111-amino-acid deletion at the N-terminal was constructed as previously described.[20] Briefly, Tn5 cells (High Five™; Invitrogen, Carlsbad, CA) were infected with Ac5480/7126 at a multiplicity of infection of 10 and incubated at 26.5°C for 7 days. The intact cells and cell debris were removed from the culture medium, and the recombinant virus-like particles (VLPs) with a molecular mass of 53 kD were concentrated by centrifugation at 100,000 × *g* for 2 hours in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). The VLPs were further purified by isopycnic binding in a CsCl gradient.[21] Recombinant baculoviruses that express N-terminal truncated capsid proteins of G3 and G4 HEV were similarly prepared, and the 53-kD VLPs were also prepared (Li T-C and others, unpublished data).

Detection of antibodies to HEV in mongoose. Flat-bottom 96-well polystyrene microplates (Immulon 2; Dynex Technologies, Inc., Chantilly, VA) were coated with the purified VLPs (1 μg/mL, 100 μL/well). The plates were incubated overnight at 4°C. Unbound VLPs were removed, and the wells were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and blocked at 37°C for 1 hour with 200 μL of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. After the plates were washed four times with PBS-T, mongoose serum (100 μL/well) was added in duplicate at a dilution of 1:200 in PBS-T containing 1%

skim milk. The plates were incubated at 37°C for 1 hour and washed four times as described above. The wells were incubated with 100 μL of peroxidase-conjugated goat anti-cat IgG (heavy plus light chain) (1:4,000 dilution) (Kirkegaard and Perry Laboratories, KPL, Guildford, United Kingdom) or anti-cat IgM (1:1,000 dilution) (Kirkegaard and Perry Laboratories) in PBS-T containing 1% skim milk. The plates were incubated at 37°C for 1 hour and washed four times with PBS-T. One hundred microliters of substrate (*o*-phenylenediamine; Sigma Chemical Co., St. Louis, MO) and H_2O_2 was added to each well. The plates were incubated in the dark at room temperature for 30 minutes and 50 μL of 4 N H_2SO_4 was added to each well. After the plates were incubated at room temperature for 10 minutes, the absorbance at 492 nm was measured.

Western blot assay. Approximately 1 μg of VLPs was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 50 mM Tris–HCl, pH 7.4, and 150 mM NaCl and incubated with mongoose serum (1:500 dilution), followed by horseradish peroxidase (HRP)–conjugated goat anti-cat IgG (heavy plus light chain) (1:1,000 dilution). The membrane was treated with electrogenerated chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction and exposed to FP-3000B45 film (Fuji, Tokyo, Japan).

Detection of HEV RNA by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted with RNAzol-LS reagent (Tel-Test Inc., Friendswood, TX) using 200 μL of the mongoose serum and resuspended in 20 μL of DNase-, RNase-, and proteinase-free water. Reverse transcription was performed at 42°C for 50 minutes and 70°C for 15 minutes with 1 μL of oligo (dT) primer, 1 μL of superscript II reverse transcriptase (Gibco-Bethesda Research Laboratories, Gaithersburg, MD), 0.5 μL of 0.1 M dithiothreitol, 4 μL of 5× reverse transcription buffer, and 1 μL of 10 mM deoxynucleoside triphosphates. Two microliters of the resulting cDNA were amplified in a 50-μL nested PCR with ExTaq DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) with an external sense primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′, nucleotide residues 5903-5922 of the G1 Myanmar strain D10330) and an antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′, nucleotide residues 6486-6467 of the G1 Myanmar strain, GenBank accession no. D10330) using the GeneAmp PCR System 9700 (Perkin Elmer Biosystems, Foster City, CA). Each cycle consisted of the denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes. The nested PCR was conducted with the internal sense primer HEV-F2 (5′-TAYCGHAAYCAAG-GHTGGCG-3′, nucleotide residues 5939-5958) and the internal antisense primer HEV-R1 (5′-CGACGAAATYAAT-TCTGTCG-3′, nucleotide residues 6316-6297) using the same conditions.[16]

RESULTS

Detection of mongoose IgG and IgM with anti-cat IgG and IgM. Because no peroxidase-conjugated antibody to mongoose IgG or IgM is commercially available, we explored the cross-reactivity of mongoose IgG and IgM with those of other animals. Since the mongoose is in the family *Herpestiade* order *Carnivora*, the reactivity between mongoose IgG or IgM and anti-cat IgG or IgM was evaluated.

Two-fold dilutions of pooled mongoose sera were used to coat the microplate. After blocking, peroxidase-conjugated goat anti-cat IgG or IgM was added to determine whether anti-cat antibodies are capable of binding to the mongoose antibodies. Peroxidase-conjugated goat anti-rabbit IgG or IgM was added to the control wells. As shown in Figure 1, the mongoose serum reacted with both HRP-goat anti-cat IgG and IgM. The control well did not show any reactivity with these antibodies. These results indicated that the anti-cat antibodies cross-reacted with mongoose IgG and IgM, and that the HRP-goat anti-cat IgG and IgM are useful as the second antibody in detecting mongoose IgG and IgM by enzyme-linked immunosorbent assay (ELISA).

Detection of IgG and IgM antibodies to HEV in mongoose sera. The mongoose serum samples were tested for IgG and IgM antibodies to HEV at a dilution of 1:200 by ELISA. The distribution of optical density (OD) values is shown in Figure 2. The OD values for IgM antibodies to HEV ranged from 0.09 to 0.321, and one serum sample with a titer of 200 had an OD value greater than 0.20. The OD values for IgG antibodies to HEV ranged from 0.011 to 3.751, and 7 sera whose titers ranged from 200 to 12,800 had OD values greater than 0.20 (Table 1).

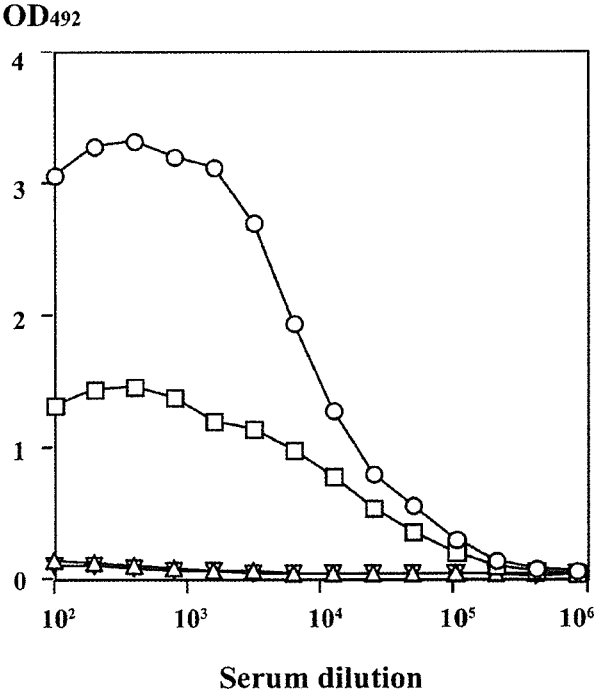

FIGURE 1. Detection of mongoose IgG and IgM with anti-cat IgG and IgM by enzyme-linked immunosorbent assay. Two-fold dilutions of pooled sera from 10 mongooses were used to coat a 96-well microplate. The reactivity of peroxidase-conjugated goat anti-cat IgG (○) and IgM (□) or horseradish peroxidase–conjugated goat anti-rabbit IgG (△) and IgM (▽) was measured. OD_{492} = optical density at 492 nm.

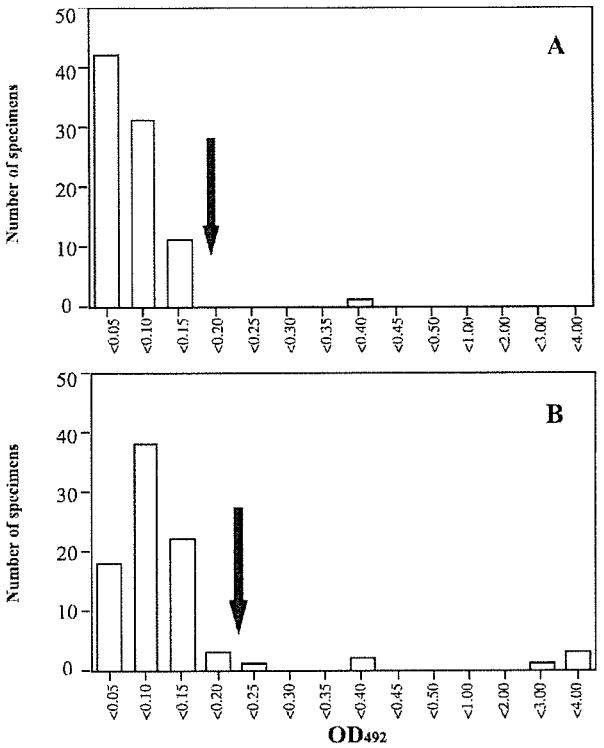

FIGURE 2. Distribution of mean optical density values at 492 nm (OD_{492}) of mongoose IgM (**A**) and IgG (**B**) antibodies. Serum samples from 84 mongooses were tested, and the values were plotted as a frequency distribution. The arrows indicate the cut-off values.

Specificity of IgG antibody in mongoose sera. To determine whether the IgG antibody detected in mongoose sera was specific for HEV, nine serum samples were selected and examined by Western blot assay. The G1, G3, and G4 VLPs were separated by SDS–PAGE and electrophoretically transferred onto a nitrocellulose membrane. A serum dilution of 1:500 was used for the assay. As shown in Figure 3, strong bands with a molecular mass of 53 kD corresponding to G1, G3, and G4 VLPs were detected with in serum samples 8, 39, 58, and 66. The OD values of these sera ranged from 2.529 to 3.751, as determined by ELISA. Weak bands were detected with in serum samples 52 and 65 whose OD values were 0.383 and 0.387, respectively. No bands were detected in serum samples 60, 41, and 10, which had low OD values, 0.204, 0.175, and 0.065, respectively, as determined by ELISA. These results indicated that the IgG antibodies to HEV detected in mongoose serum by ELISA were specific for HEV.

TABLE 1
OD values and antibody titers in mongoose sera*

Serum sample	OD values (IgG/IgM)	Antibody titers (IgG/IgM)
No. 8	3.751/0.321	12,800/200
No. 39	3.268/0.065	6,400/(< 200)
No. 52	0.383/0.047	400/(< 200)
No. 58	3.268/0.103	12,800/(< 200)
No. 60	0.204/0.049	200/(< 200)
No. 65	0.387/0.067	400/(< 200)
No. 66	2.529/0.098	3,200/(< 200)

* OD = optical density.

Prevalence of IgG and IgM antibodies to HEV in mongoose sera. The cutoff value of IgG for the ELISA was determined with 78 antibody-negative serum samples. The OD values for IgG antibodies to HEV in these serum samples ranged from 0.011 to 0.204 (mean ± SD value = 0.086 ± 0.037). The cutoff value (mean + 3SD) was 0.196 (Figure 2). When this value was used, the prevalence of IgG antibodies to HEV was 8.3% (7 of 84). The antibody-positive rate was 3.3% in females and 11.1% in males; however, the difference between sexes was not statistically significant ($P > 0.05$). The average body weight and body size were 565.3 grams (range = 182.5–1,037.9) and 553.8 mm (range = 402–654), respectively.

When the antibody-positive rate was analyzed according to body weight, the antibody-positive rate for IgG to HEV was 11.1% in animals with body weights of 500–599 grams, 12.5% in animals with body weights of 600–699 grams, 11.1% in animals with body weights of 700–799 grams, 20% in animals with body weights of 800–899 grams, and 33.3% in animals with body weights greater than 900 grams. IgG antibody to HEV was not detected in animals with body weights less than 500 grams. Thus, the antibody-positive rate increased with body weight.

The antibody-positive rate for IgG to HEV was 11.1% in animals with a body size of 550–599 mm and 21.2% in animals with a body size of 600–654 mm. IgG antibody to HEV was not detected in animals with body sizes less than 500 mm, which shows that antibody-positive rate also increased with the body size. The mean ± SD OD value of IgM antibody to HEV in these 78 sera was 0.062 ± 0.031. The cutoff value was 0.155 (0.062 + 3 × 0.031). When analyzed with this cutoff value, one mongoose serum sample (no. 8) was positive for IgM antibody; the prevalence rate was 1.2% (1 of 84).

Detection of HEV RNA by RT-PCR. All 84 mongoose serum samples tested by RT-PCR were negative for HEV RNA.

DISCUSSION

The mongoose is a small, cat-like carnivore that is a member of the family *Herpestidae*. It is between one and four feet in length, and inhabits in Asia, the Caribbean, and southern Europe, comprising more than 30 species. Although the small Asian mongoose was introduced into Okinawa for the control of habu snakes and rats, this animal eats not only habu snakes and rats, but also other small animals.

An ELISA with recombinant HEV VLPs was used to detect IgG and IgM antibodies to HEV in the mongoose. This assay was capable of detecting antibodies to HEV in human sera with high sensitivity and specificity.[22] Although only one serotype was recognized and four genotypes were identified in HEV, we used G1, G3, and G4 VLPs as antigens to compare the reactivity. No significant difference was found between the genotypes. The specificity of the ELISA was confirmed by Western blot assay. Among seven samples positive for IgG antibodies to HEV by ELISA, one sample (no. 60) was be negative by Western blot assay, a result that was probably due to a low antibody titer in the ELISA (OD value of the 1:200 serum dilution = 0.204 and antibody titer = 200) and the lower sensitivity of the Western blot assay.

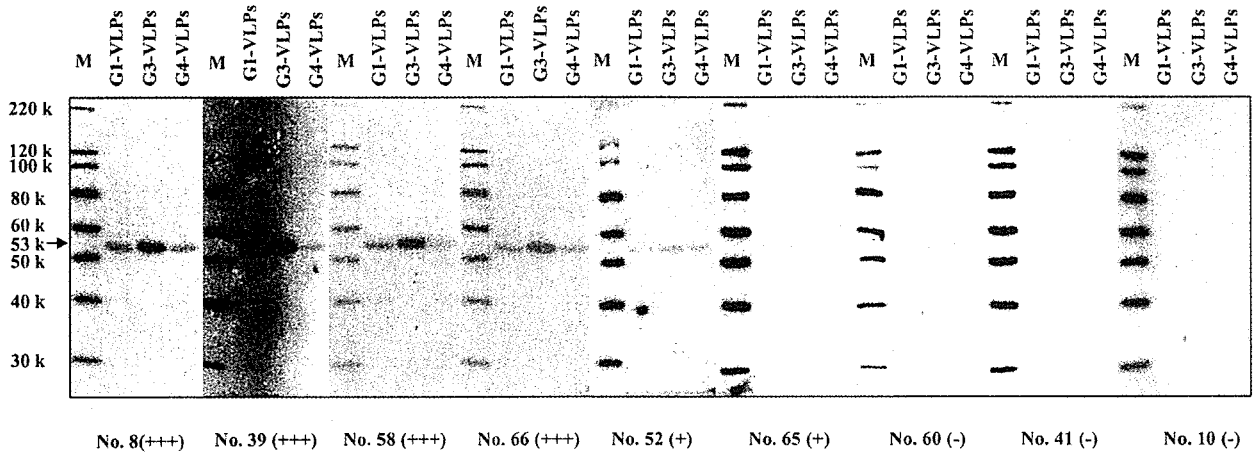

FIGURE 3. Specificity of the IgG antibody determined by Western blot assay. The G1, G3, and G4 virus-like particles (VLPs) were used as the antigens, and nine mongoose sera with different optical density values were evaluated. Strong bands (+++), weak bands (+), and no bands (−) by Western blot assay are indicated. Lane M = molecular weight marker.

In this study, only one mongoose was positive for IgM antibodies to HEV and the titer was low (200). We were not able to amplify any HEV sequence in these 84 mongoose serum samples, including the sample positive for IgM antibodies to HEV. Therefore, the genotype of the HEV-infected mongoose in Okinawa is unknown. In experimentally infected monkeys, the period of the viremia was very short, and HEV RNA was detected in serum only 1–2 weeks after seroconversion.[23] This could be one of the reasons why the HEV genome was not detected in serum.

The prevalence of antibodies to HEV was high in domestic pigs and wild boar; they are considered to be possible reservoirs of HEV in Japan. In contrast, the prevalence of IgG antibodies to HEV in mongooses (8.3%) was lower than that in pigs (58%), wild rats (44–94%), or wild boars (44%)[24,25] (Li T-C and others, unpublished data). This finding suggests that mongooses may not be the major reservoir of HEV in Okinawa, but may occasionally be infected with HEV. The current source and route of infection are not clear. Since many wild boars inhabit Okinawa and were eventually infected with HEV, the virus might spread to the surrounding environment by means of animals' stool. Mongooses also inhabit the region that wild boars inhabit, and might thus be exposed to HEV. Because mongooses catch insects, crabs, worms, lizards, and other small creatures for food, transmission of HEV may occur if these creatures are infected. The positive rate for IgG antibody to HEV increases with body weight and size, indicating that the exposure to HEV increases with age.

It has been reported that HEV is excreted in low concentrations by humans,[26] and that a small amount of HEV is excreted in experimentally infected monkeys. This may explain the lower efficiency of the transmission of HEV, and may result in the lower prevalence of antibodies to HEV in the mongoose.

In summary, mongooses in Okinawa were infected with HEV; however, the source of infection, the routes of transmission, and their genotype are still unknown. Further serologic and genetic investigations with larger number of specimens are needed.

Received November 14, 2005. Accepted for publication January 23, 2006.

Acknowledgments: We thank Tomoko Mizoguchi for secretarial work.

Financial support: This study was supported in part by grants for Research on Emerging and Re-Emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labor and Welfare, Japan.

Authors' addresses: Tian-Cheng Li, Tatsuo Miyamura, and Naokazu Takeda, Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan, Telephone: 81-42-561-0771, Fax: 81-42-561-4729, E-mails: litc@nih.go.jp, tmiyam@nih.go.jp, and ntakeda@nih.go.jp. Mika Saito, Division of Molecular Virology and Oncology, Department of Microbiology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan, Telephone: 81-98-895-1132, Fax: 81-98-895-1410, E-mail: mikas@med.u-ryukyu.ac.jp. Go Ogura and Osamu Ishibashi, Laboratory of Subtropical Zoology, Faculty of Agriculture, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan, Telephone and Fax: 81-98-895-8779, E-mail: ogurago@agr.u-ryukyu.ac.jp.

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Am. J. Trop. Med. Hyg., 76(1), 2007, pp. 170–172

SHORT REPORT: DETECTION OF HEPATITIS E VIRUS RNA FROM THE BIVALVE YAMATO-SHIJIMI (*CORBICULA JAPONICA*) IN JAPAN

TIAN-CHENG LI,* TATSUO MIYAMURA, AND NAOKAZU TAKEDA
Department of Virology II, National Institute of Infectious Diseases, Tokyo Japan

Abstract. To evaluate whether bivalves are contaminated with hepatitis E virus (HEV), samples of a bivalve called Yamato-Shijimi (*Corbicula japonica*) were examined for HEV by reverse transcription–polymerase chain reaction. Genotype 3 HEV was detected from 2 of 32 packages of Yamato-Shijimi obtained from Japanese rivers, which indicated that HEV contaminates river water in Japan.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family Hepeviridae.[1,2] This virus causes human hepatitis E and is transmitted primarily by the fecal-oral route through contaminated drinking water.[3,4] However, recent studies have demonstrated that various animal species have serum antibodies to HEV, and its viral genome has been detected in swine, wild deer, wild boar, and mongoose, which suggests that hepatitis E is a zoonotic disease.[5–10] Because HEV is excreted into feces,[11,12] there is a risk of HEV contamination in environmental water. In fact, HEV has been detected in sewage from industrialized countries, including Spain, the United States, and France.[13–15] However, HEV contamination of river water has not been examined. In the present study we detected the HEV genome from a bivalve called Yamato-Shijimi (*Corbicula japonica*), which suggested that river water in Japan is contaminated with HEV.

A total of 32 packages of Yamato-Shijimi were obtained at a fish market in December 2005 and March 2006. All samples were harvested at areas A, B, C, D, E, F, G, and H in western Japan. The package numbers and collection days are shown in Table 1. The samples were shucked, and the digestive diverticulum were removed by dissection and weighed. One gram of digestive diverticulum obtained from 10–15 Yamato-Shijimi was homogenized with an Omni-mixer (OCI Instruments, Waterbury, CT) in 10 mL of phosphate-buffered saline, pH 7.4, for two 30-second intervals at a maximum speed of 18,000 rpm. After centrifugation at 10,000 × *g* for 30 minutes at 4°C, the supernatant was centrifuged at 100,000 × *g* for 2 hours in an SW41 rotor (Beckman Instruments, Inc., Fullerton, CA). The pellet was resuspended in 140 μL of distilled water and stored at -80°C until use.

Total RNA was extracted with the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) and resuspended in 20 μL of DNase-, RNase-, and proteinase-free water. Reverse transcription (RT) was performed at 42°C for 50 minutes, followed by 70°C for 15 minutes in a 20-μL reaction mixture containing 1 μL of Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA), 1 μL of oligo (dT) primer, 1 μL of RNaseOUT™ (Invitrogen), 2 μL of 0.1 M dithiothreitol, 4 μL of 5× RT buffer, 1 μL of 10 mM deoxynucleoside triphosphates, 5 μL of RNA, and 5 μL of distilled water. An RT–polymerase chain reaction was performed to amplify part of the open reading frame 2 (ORF2) as described previously.[8,10] Two microliters of the cDNA was used for the first PCR in a 50-μL reaction mixture with external forward primer HEV-F1 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and external reverse primer HEV-R2 (5′-TGYTGGTTRT-CRTARTCCTG-3′), which corresponded to nucleotide residues 5903–5922 and 6486–6467, respectively, of the G1 Myanmar strain (D10330). Each cycle consisted of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by final extension at 72°C for 7 minutes. Two microliters of the first PCR product was used for a nested PCR with internal forward primer HEV-F2 (5′-TAYCGHAAYCAAGGHTG-GCG-3′; nucleotide residues 5939–5958) and internal reverse primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′, nucleotide residues 6316–6297) under the same conditions.

Two packages, B4 and B6, collected in area B on February 7, 2006, and March 1, 2006, were positive for HEV RNA by

TABLE 1
Detection of hepatitis E virus (HEV) in *Corbicula japonica*

Package no.	Collection day	HEV RNA
A1	12/08/05	–
A2	12/22/05	–
A3	1/14/06	–
A4	1/22/06	–
A5	1/22/06	–
A6	2/05/06	–
A7	2/17/06	–
A8	3/02/06	–
A9	3/14/06	–
A10	3/15/06	–
B1	12/10/05	–
B2	12/17/05	–
B3	1/24/06	–
B4	2/07/06	+
B5	2/19/06	–
B6	3/01/06	+
B7	3/17/06	–
B8	3/18/06	–
C1	1/10/06	–
D1	1/16/06	–
D2	1/20/06	–
D3	3/14/06	–
E1	1/21/06	–
E2	1/26/06	–
E3	2/25/06	–
E4	3/10/06	–
F1	3/13/06	–
F2	3/14/06	–
F3	3/18/06	–
F4	3/18/06	–
G	3/18/06	–
H	3/18/06	–

* Address correspondence to Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. E-mail: litc@nih.go.jp

RT-PCR. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into TA cloning vector pCR2.1 (Invitrogen). The nucleotide sequence of each of 10 clones was determined. Most of the sequences formed a single genotype 3 cluster. The exceptions (B4-13) formed a different cluster along with Sakai-9 detected from a wild boar in 2004 in Japan (Figure 1). We found large numbers of small different nucleotide sequences among the clones with 88.9–100% identity, even when they were derived from the same package, which indicated that multiple HEV strains were accumulated in the digestive diverticulum of Yamato-Shijimi.

To further analyze the HEV RNA detected in the Yamato-Shijimi, the entire ORF2 of B4 RNA was amplified as overlapping segments, and the nucleotide sequences were determined. The full-length ORF2 consisted of 1,980 basepairs and were phylogenetically classified into genotype 3. High amino acid identities (97.57–98.87%) were observed with HEV strains detected from hepatitis E patients, swine, wild boar, and wild deer in Japan. This is the first report on the detection of HEV from a bivalve.

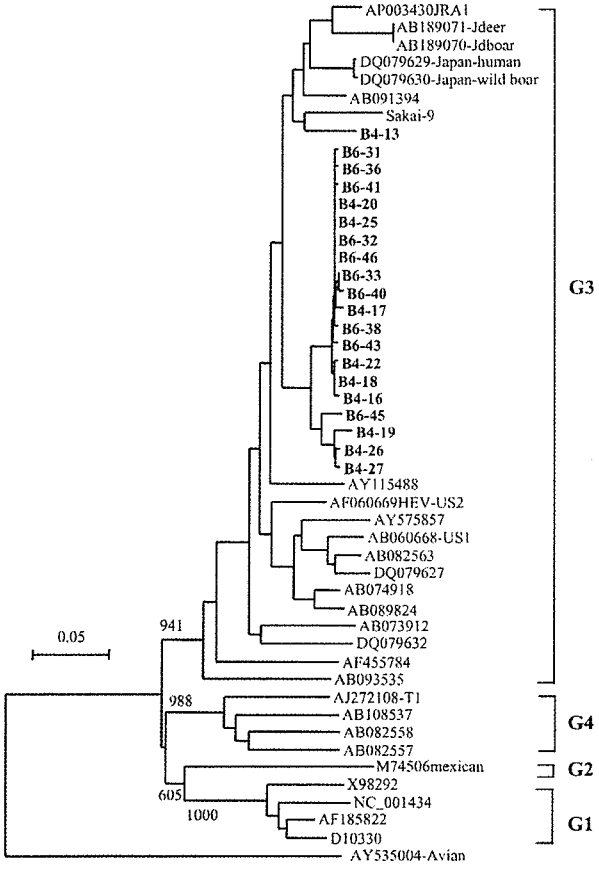

FIGURE 1. Phylogenetic trees of hepatitis E virus (HEV) constructed with avian HEV as an outgroup. A partial open reading frame 2 ORF2 (338 basepairs) of each of 10 clones of B4 (B4-13, B4-16, B4-17, B4-18, B4-19, B4-20, B4-22, B4-25, B4-26, and B4-27) and B6 (B6-31, B6-32, B6-33, B6-36, B6-38, B6-40, B6-41, B6-43, B6-45, and B6-46) were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. All nucleotide sequences determined in this study are shown in bold. Other HEV sequences were obtained from GenBank.

The HEV sequences were detected from Yamato-Shijimi (*Corbicula japonica*) harvested on February 7, 2006, and March 1, 2006, in western Japan. The Yamato-Shijimi, a brackish-water bivalve, grows in sandy mud in or near rivers, ponds, and lakes. During breathing and feeding, this bivalve filters a large amount of water. When the water is contaminated with HEV, the virus is ultimately concentrated in the digestive diverticula of the bivalves.

The source of HEV in this organism is not known. Since Japan was considered not to be endemic for this virus, and disposal of sewage is in this country is efficient, the risk of HEV contamination from human stool was believed to be low. There have been no outbreaks of hepatitis E in Japan from drinking water. However, HEV has been detected in wild deer, wild boar, and mongoose in Japan, and HEV shed in the feces of these animals may pollute environmental water. Wild deer and wild boar are controlled in Japan to eliminate their ability to damage agriculture and forestry; hunting is the main control strategy. Hunters usually wash killed animals in river water and this would increase the risk of HEV contamination in river water. These wild animals presumably play an important role in the contamination of environmental water.

In Japan, many outbreaks caused by bivalves contaminated with hepatitis A virus and noroviruses have been reported. Fortunately, Yamato-Shijimi is generally eaten as an ingredient in hot miso soup in Japan, and the heat, usually at 100°C for nearly 10 minutes, decreases the risk of HEV transmission from Yamato-Shijimi to humans. However, more efforts are needed to determine the infectivity and stability of HEV in the natural environment, including that in Yamato-Shijimi.

Received June 19, 2006. Accepted for publication September 10, 2006.

Acknowledgments: We thank Tomoko Mizoguchi and Satoko Ogawa for secretarial work.

Financial support: This study was supported in part by grants for Research on Emerging and Re-emerging Infectious Diseases, Research on Hepatitis, and Research on Food Safety from the Ministry of Health, Labor and Welfare, Japan.

Authors' address: Tian-Cheng Li, Tatsuo Miyamura, and Naokazu Takeda, Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan, Telephone: 81-42-561-0771, Fax: 81-42-561-4729, E-mails: litc@nih.go.jp, tmiyam@nih.go.jp, and ntakeda@nih.go.jp.

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