Original Article

Non Susceptibility of Neonatal and Adult Rats against the Middle East Respiratory Syndrome Coronavirus

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SUMMARY: The present study examined the susceptibility of rats to the Middle East respiratory syndrome coronavirus (MERS-CoV) and determined whether this animal is a suitable model for MERS-CoV infection. Immunohistochemical analysis identified dipeptidyl peptidase 4 (DPP4), a known receptor for MERS-CoV on type I pneumocytes from infected rats. Whereas adult rats developed antibodies against MERS-CoV spike protein after intranasal inoculation, there was no evidence of viral replication in the lungs of adult, young, or neonatal rats after intranasal inoculation with MERS-CoV. In addition, human DPP4-expressing rat kidney fibroblasts, but not rat DPP4-expressing cells, were susceptible to MERS-CoV. Taken together, these results suggest that the rat is not a useful animal model for studying MERS-CoV infection.

INTRODUCTION

Middle East respiratory syndrome coronavirus (MERS-CoV) was originally isolated as a novel coronavirus from a fatal case of acute respiratory distress syndrome and renal failure (1). The receptor for MERS-CoV was recently identified as dipeptidyl peptidase 4 (DPP4) (2). As of August 2015, the virus had infected 1,413 people, 502 of whom died, and cases have been emerging weekly since September 2012 (http://www.who.int/csr/don/archive/disease/coronavirus_infections/en/). Thus, the development of effective prophylactic and therapeutic treatment strategies remains a high priority. Appropriate animal models are needed to better understand the pathogenesis of MERS-CoV and to facilitate the development of effective vaccines and drugs. A common marmoset model of MERS-CoV infection has been established, in which the marmoset develops lethal pneumonia (3); however, animal models based on monkeys present both ethical and economic problems. Therefore, there is a need for a small animal model of MERS-CoV infection. Unfortunately, MERS-CoV does not replicate or cause disease in Syrian hamsters (4) or in several strains of mice (5). Recent studies reported rodent models of MERS-CoV. One was based on mice transduced with an adenovirus expressing human DPP4 (6), whereas the other was based on transgenic mice expressing the human DPP4 receptor (7). Mice transduced with adenovirus expressing human DPP4 developed interstitial pneumonia; however, there were concerns regarding the induction of an innate immune response to the transduced adenoviral vector and

of the lack of control over the level of human DPP4 expression and/or its tissue distribution. Furthermore, the transgenic mice expressed the human DPP4 receptor in a global manner, resulting in encephalitis (which does not occur in MERS patients). Thus, there is still no suitable small animal model to study the pathogenesis of MERS.

The objective of the present study was to examine the susceptibility of rats to MERS-CoV infection. Rats can be commercially sourced, and the reagents required to study immune responses in this animal are freely available.

MATERIALS AND METHODS

Ethics: MERS-CoV infection of cultured cells and rats was performed under biosafety level 3 conditions according to the guidelines for biosafety and animal experiments at the National Institute of Infectious Diseases, Tokyo, Japan. All animal experiments involving MERS-CoV were approved by the Biosafety and the Animal Care and Use Committees at the NIID (approval No. 113033-II).

Virus and cells: MERS-CoV (strain HCoV-EMC 2012) was kindly provided by Dr Bart Haagmans and Dr Ron Fochier (Erasmus Medical Center, Rotterdam, the Netherlands). Vero E6 cells, purchased from the American Type Cell Collection (ATCC; Manassas, VA, USA), were cultured in Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum (FBS), 50 IU/mL penicillin G, and 50 µg/mL streptomycin (5% FBS-MEM). Stocks of MERS-CoV were propagated and titrated in Vero E6 cells and cryopreserved at –80°C. Viral infectivity titers were expressed as 50% of the tissue culture infectious dose (TCID₅₀)/mL in Vero E6 cells (calculated according to the Behrens-Karber method).

Rats: Lewis rats and Fischer 344 (F344) rats were used in this study. Both were inbred strains that are commonly used in medical research. Lewis rats show

Received November 13, 2015. Accepted February 12, 2016.
J-STAGE Advance Publication March 18, 2016.

DOI: 10.7883/yoken.JJID.2015.589

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good reproductive performance and growth; thus, the newborn rats are larger and can be more easily treated by intranasal inoculation than F344 rats. In contrast, F344 rats are docile and moderately easy to handle, and historically have been used for experimental models of infection. We have previously used these rats in a SARS-CoV infection study (8).

Pregnant Lewis rats were purchased from Japan SLC (LEW/SsN Slc, inbred; Japan SLC, Inc., Shizuoka, Japan) and housed at the animal facility at the NIID under biosafety level 3 conditions. Pregnant rats were closely monitored, and the date and approximate time of delivery were noted. F344 rats (5-weeks-old or 6-months-old) were purchased from Japan SLC (F344/N Slc, inbred, Japan SLC, Inc.) and housed at the animal facility until experimental infection.

Histopathological and immunohistochemical analysis: Animals were sacrificed through the delivery of an excess dose of isoflurane and then perfused with 5 mL of 10% phosphate-buffered formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The lung, liver, spleen, kidney, heart, gastrointestinal tract, salivary glands, and brain tissues were obtained and routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical analysis of the rat DPP4 antigen was performed in paraffin-embedded sections. Antigen retrieval was achieved by hydrolytic autoclaving in retrieval solution (pH 6) (Nichirei Biosciences Inc., Tokyo, Japan) for 10 min at 121°C. Rabbit polyclonal anti-CD26 (Cat# ab28340; 1:800; Abcam, Cambridge, UK) was used as the primary antibody (specific for human, mouse, and rat DPP4) and Histofine Simple Stain Rat MAX-PO (Nichirei Biosciences Inc.) was used to detect DPP4 antigens. Sections stained with non-immune rabbit serum (Dako Corp., Glostrup, Denmark), rather than with the primary antibody, served as a negative control.

Quantitative real-time reverse-transcription (RT)-PCR: To measure the expression of rat DPP4 mRNA and MERS-CoV open reading frame (ORF1 and ORF5) mRNA in tissues, RNA was first extracted from paraffin-embedded tissues using the PureLink™ FFPE Total RNA Isolation Kit (Life Technologies, CA, USA), according to the manufacturer's instructions. β -actin was used as an internal control. In addition, total RNA was extracted from the supernatants of lung homogenates generated from the lungs of F344 rats using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) to measure the actual copy number of rat DPP4 mRNA/g of tissue. Real-time one-step quantitative RT-PCR assays were performed using the QuantiTect Probe RT-PCR kit (Qiagen) and an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). TaqMan probes and primers are listed in the Table 1. The reaction conditions have been described previously (9). The expression of each gene was normalized relative to that of β -actin mRNA.

Animal experiments: Neonatal Lewis rats (3-days-old) were anesthetized with diethyl ether (Wako), which has a wider margin of safety than isoflurane, and inoculated intranasally with 10 μ L of MERS-CoV (1×10^5 TCID₅₀/mL) in MEM supplemented with 2% FBS, 50 IU/mL penicillin G, and 50 μ g/mL streptomycin

Table 1. Primers and probes used for quantitative real-time RT-PCR

Target	Sequence (5' to 3')
MERS-CoV ORF1	
Forward	GAATAGCTTGGCTATCTCAC
Reverse	CACAATCCCACCAGACAA
Probe	GCCCTGTTGTTTAGCGTATCGTTGC
MERS-CoV ORF5	
Forward	GAATAGCTTGGCTATCTCAC
Reverse	ACTAGCTGGACGGGTTTA
Probe	ACGAACTATGGCTTCTCGGCGT
rat DPP4	
Forward	CTACTTGTGTGACGTGGCCT
Reverse	TAGTCGCAGATCGCCATCAC
Probe	TCGTTGCAGTGGCTCAGGAGGA
β -actin	
Forward	ACGCCAGGTCATCACTATTG
Reverse	CAAGAAGGAAGGCTGGAAAAGA
Probe	CAACGAGCGGTTCCGATGCC

(referred to as 2% FBS-MEM). Body weight was then measured daily for 14 days. Animals were sacrificed on days 3, 7, or 17–19 post-inoculation (p.i.) ($n = 3$ per group), and virus replication and pathology were analyzed.

Female F344 rats (6-weeks-old or 6-months-old) were anesthetized with diethyl ether and inoculated intranasally with 1×10^6 TCID₅₀ (100 μ L or 150 μ L, respectively) of MERS-CoV. Body weight was then measured daily for 10 days. Animals were sacrificed on days 1, 3, 6, 10, or 29 p.i. ($n = 3$ –4 per group) and virus replication, cytokine expression, and pathology were analyzed. Mock-infected rats (inoculated with 2% FBS-MEM alone) were used as controls for body weight measurements and histopathology.

To examine seroconversion in rats after MERS-CoV inoculation, 6-week-old and 6-month-old female F344 rats were administered 2 intranasal inoculations of 1×10^6 TCID₅₀ MERS-CoV at 14-day intervals. Blood was taken from the tail vein on days 12 and 26 after the first inoculation for use in neutralizing assays.

Virus titration: Lung tissue was homogenized and diluted in 2% FBS-MEM to yield 20% (w/v) homogenates. Before this, the lungs were washed out as previously described (8), and fluid was collected for the measurement of infectious virus titers. The homogenates were centrifuged at $740 \times g$ for 10 min, and the supernatants were titrated in VeroE6 cells. Inoculated cells were assessed for cytopathic effects (CPE) at 5 days p.i. The detection limit of the assay was $10^{1.5}$ TCID₅₀/mL of tissue.

Infection of rat DPP4-expressing kidney cells with MERS-CoV: NRK-49F cells (derived from rat kidney fibroblasts) were purchased from ATCC and transiently transfected with plasmids expressing human or rat DPP4 proteins as previously described (10). At 48 hours post-transfection, the cells were infected with MERS-CoV at a multiplicity of infection (MOI) of 1. Cell culture medium was harvested at days 0, 1, 2, and 3 p.i. for virus titration in Vero E6 cells (TCID₅₀ assay).

MERS-CoV neutralizing assay: Blood was obtained from the tail vein of each rat and allowed to clot. Se-

rum was collected by centrifugation and inactivated by heating to 56°C for 30 min. One hundred TCID₅₀ aliquots of MARS-CoV were incubated for 1 hour in the presence or absence of rat serum (serially diluted 2-fold) and then added to confluent Vero E6 cell cultures in 96-well microtiter plates. The presence of viral CPE was determined on day 5, and neutralizing antibody titers were determined based on the reciprocal of the highest dilution at which no CPE was observed. The lowest and highest serum dilutions tested were 1:2 and 1:512, respectively.

In vitro expression of MERS-CoV nucleocapsid or spike proteins and indirect immunofluorescence staining: BHK cells were transiently transfected with plasmids expressing the MERS-CoV nucleocapsid (N) or spike (S) proteins. At 48 hours post-transfection, the cells were washed 3 times with phosphate buffered saline (PBS) and then fixed in 10% formalin followed by methanol/acetone (1:1). The cells were then incubated

with serum from rats infected with MERS-CoV, followed by a FITC-labeled anti-rat IgG antibody (Invitrogen Corp., Carlsbad, CA, USA; 1:100).

MERS-CoV S protein-specific IgG ELISA: IgG ELISA was performed as previously described, except for antigen preparation (11). Briefly, microtiter plates were coated overnight at 4°C with lysates of MERS-CoV-infected or mock-infected Vero cell lysates that had been treated with 1% NP-40. The plates were then washed 3 times with PBS containing 0.05% Tween-20 (PBS-T). PBS-T containing 5% skim milk (M-PBS-T) was then added to each well, and the plate was incubated for 2 hours at 37 °C. Rat sera were diluted 1:100, 1:400, 1:1,600, or 1:6,400 in M-PBS-T. Hyper-immune rabbit serum was serially diluted (4-fold) from 1:1,000 to 1:64,000 in M-PBS-T and used as a positive control. One hundred microliters of each dilution were then added to the plates and incubated for 1 hour at 37°C. After washing 3 times with PBS-T, the cells were fur-

Fig. 1. (Color online) Expression of dipeptidyl peptidase 4 (DPP4) in rat lung and small intestine. Lung and small intestine tissues from Lewis and Fisher 344 rats were stained with an anti-DPP4 antibody (A). DPP4 antigen was present in the cytoplasm of type I pneumocytes (arrows) in the lung (A, upper panels) and in epithelial cells (arrows) in the small intestine (A, lower panels) of neonatal Lewis rats and 6-week-old and 6-month-old Fisher 344 rats. Inset, magnified image of the area indicated by the arrows. Original magnification, $\times 40$; inset, $\times 100$. Quantitative real-time RT-PCR analysis of DPP4 mRNA expression in the lungs of Fisher 344 rats inoculated with MERS-CoV (B). DPP4 mRNA levels were normalized against β -actin (endogenous control).

ther incubated with HRP-conjugated anti-rat IgG or anti-rabbit IgG (Invitrogen) antibodies (diluted 1:1,000 in M-PBS-T). After washing 3 times with PBS-T, ABTS substrate (Roche Diagnostics, Basel, Switzerland) was added to the wells, and the plates were incubated for 30 min at 37°C. The OD value for each well was measured at 405 nm. Adjusted OD values were cal-

culated by subtracting the OD value of wells coated with mock-infected Vero cells from that of wells coated with MERS-CoV-infected Vero cells.

The mean OD value plus 3 standard deviations (mean + 3SD) was calculated using serum samples from uninfected rats and used as the cut-off for the IgG ELISA.

Fig. 2. (Color online) Middle East Respiratory Syndrome coronavirus (MERS-CoV) infection to rats. Body weight was measured once daily for 14 (neonatal animals) or 10 days (others) post-MERS-CoV infection. Data derived from neonatal Lewis rats and 6-week-old and 6-month-old Fisher 344 rats are shown (A). Some neonatal animals were sacrificed on Days 3 and 7 post-inoculation (p.i.) for virus or pathological assays (initial number of animals: MEM, $n = 15$; MERS-CoV, $n = 18$). Histological analysis (H&E staining) of lung tissues from neonatal Lewis rats and from 6-week-old and 6-month-old Fisher 344 rats on Day 3 p.i. (B). Original magnification, $\times 40$. No pathological lesions were observed in the lungs of neonatal Lewis rats or 6-week-old Fisher 344 rats; however, focal lesions were present in the lungs of 6-month-old Fisher 344 rats. Quantitative real-time RT-PCR analysis of MERS-CoV viral RNA and subgenomic RNA in the lungs of Fisher 344 rats inoculated with MERS-CoV (C). Transcripts for both ORF1 and ORF5 were normalized against β -actin (endogenous control).

Statistical analysis: Data are expressed as the mean and standard error of the mean. Statistical analysis was performed using Graph Pad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Inter-group comparisons were performed using the Mann-Whitney U test. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Rat DPP4 localizes to the lungs and small intestine of neonatal and adult rats: First, to confirm whether DPP4 (the receptor for MERS-CoV) was present in rat organs, we performed polymer-based immunohistochemical analysis for DPP4 expression in formalin-fixed paraffin-embedded lung, liver, spleen, kidney, heart, gastrointestinal, salivary gland, and brain tissues obtained from MEM-inoculated neonatal Lewis rats and F344 rats aged 6 weeks (hereafter referred to as “young”) and 6 months (hereafter referred to as “adult”). DPP4 antigens were detected in type I pneumocytes of the lungs and in epithelial cells of the small intestine in neonatal Lewis rats and in both young and adult F344 rats (Fig. 1A). No DPP4 antigens were detected in the liver, spleen, kidney, heart, salivary glands, or brain tissues. According to an epidemiological study (12), age (>45 years) is a risk factor for MERS-CoV infection. Thus, we hypothesized that DPP4 expression would be higher in adult rat lungs than in those of young rats. However, there was no difference in DPP4 mRNA expression in paraffin-embedded lung tissues from young and adult rats (Fig. 1B). The copy numbers of rat DPP4 mRNA were $1.4 \times 10^8 \pm 1.4 \times 10^7$ copies/g of tissue for young and $8.2 \times 10^7 \pm 1.2 \times 10^7$ copies/g of tissue for adult rats, using 20% (w/v) lung homogenates.

Rat DPP4 does not support MERS-CoV replication either in vivo or in vitro: After intranasal inoculation with MERS-CoV, neonatal Lewis rats and both young and adult F344 rats showed no clinical signs or changes in body weight during the observation period when compared to mock-infected rats (Fig. 2A). No virus was detected in the lung lavage fluid from young and adult F344 rats obtained on days 1, 3, 6, and 10 p.i. Virus titers in 20% (w/v) lung tissue homogenates from neonatal Lewis rats and young F344 rats were below the detection limit ($10^{1.5}$ TCID₅₀/mL) on days 1 and/or 3 p.i. However, we detected virus ($10^{1.75}$ TCID₅₀/mL) in the lung tissues of 1 of 3 adult rats on day 1 p.i. In addition, whereas the histopathological findings on days 3, 7, and 17–19 p.i. were unremarkable in neonatal Lewis rats and young F344 rats, adult F344 rats (*n* = 2) exhibited marginal focal inflammatory infiltration on day 3 p.i. This was characterized by a partial thickening of the alveolar wall and by the presence of a few alveoli containing foamy macrophages (Fig. 2B). We next tried to detect MERS-CoV replication in these lesions using real-time RT-PCR. We used 2 primer combinations to detect ORF1 transcripts (viral RNA and mRNA) and ORF5 subgenomic mRNA (13). This assay can detect at least 100 copies of the specific target per reaction. A small amount of viral RNA was detected in paraffin-embedded lung tissues from adult rats on day 1 p.i. (*n* = 3); however, no viral subgenomic RNA

Fig. 3. Rat kidney fibroblasts expressing human DPP4 support the replication of MERS-CoV. NRK-49F cells were transfected with rat or human DPP4 and infected with MERS-CoV at a MOI of 1 TCID₅₀/cell (A). Supernatants were harvested at 0, 1, 2, and 3 days post-infection and viral titers determined in a TCID₅₀ assay on VeroE6 cells. MERS-CoV growth curve shown (representative of 2 independent experiments). The dotted line indicates the detection limit ($10^{1.5}$ TCID₅₀/mL).

was detected in any of the rats on days 1 and 3 p.i. (Fig. 2C). Thus, we concluded that the viral RNA detected in the lungs of adult rats represented that of the residual virus inoculum, and that the lung lesion was not related to viral replication. In addition, whereas human DPP4-expressing NRK-49F cells (derived from rat kidney fibroblasts) can support virus replication, rat DPP4-expressing NRK-49F cells could not (Fig. 3).

Adult rats seroconvert and generate antibodies to the MERS-CoV S protein after intranasal inoculation: Interestingly, serum from adult rats, obtained on day 10 p.i. (all four tested animals from the first experiment) and day 29 p.i. (1 of 4 tested animals from the second experiment), showed low levels of antibodies against MERS-CoV S protein, suggesting partial inhibition of CPE by MERS-CoV in Vero E6 cells on day 3 or 4 p.i. (2 experiments were performed). Sera from mock-infected rats did not show partial inhibition of CPE by MERS-CoV from day 2 p.i. Thus, we speculated that adult rats underwent very weak seroconversion after inoculation with MERS-CoV. We next examined seroconversion in young and adult rats after reinfection with MERS-CoV. Briefly, young and adult rats received 2 intranasal inoculations of 1×10^6 TCID₅₀ MERS-CoV at 14-day intervals. One of 6 serum samples from adult rats showed very low levels of anti-MERS-CoV antibodies at day 12 after the first inoculation, and 4 of 6 rats had detectable antibody titers (1:2 to 1:16) on day 12 after the second inoculation (Fig. 4A). We also performed indirect fluorescence analysis of BHK cells using serum from adult rats to detect antibodies against MERS-CoV proteins. Rat serum obtained after the initial round of infection (on day 12 p.i.) reacted very weakly with MERS-CoV S protein expressed by BHK cells (Fig. 4B, first column). However, rat sera with a neutralizing antibody titer of 1:16 after the second round of infection (on day 12 p.i.) reacted more strongly with MERS-CoV S protein expressed by BHK cells, but not with the N protein (Fig. 4B, second column). Furthermore, the MERS-CoV S protein-specific IgG ELISA confirmed the presence of

Fig. 4. (Color online) Seroconversion of rats infected with Middle East Respiratory Syndrome coronavirus (MERS-CoV). Titer of MERS-CoV-specific neutralizing antibodies in rat serum 12 days after the second round of inoculation with MERS-CoV (A). The dotted line denotes the detection limit of the assay. Error bars represent the standard deviation. Indirect immunofluorescence images showing expression of MERS-CoV N or S proteins in BHK cells. Cells were incubated with serum from 6-month-old MERS-CoV-infected Fisher 344 rats (B). “1st infection” denotes the sample obtained on Day 12 after the first inoculation with MERS-CoV; “2nd infection” indicates the sample obtained on Day 12 after the second inoculation (first and second columns, respectively). Hyper-immune rabbit serum was used as a positive control (third column). Mock-infection; cells incubated with mock-infected Fisher 344 rat serum (right-hand column). The white arrows indicate cells positive for the S protein. Scattergram showing the absorbance measured in an ELISA. IgG antibodies against MERS-CoV S antigens were detected in the sera of Fisher 344 rats 12 days after a second mock or MERS-CoV inoculation (C). The dotted line indicates the cut-off OD value. Error bars represent the standard deviation.

antibodies against the MERS-CoV S protein in rat sera obtained on day 12 after the second round of infection. Rat sera that were positive for neutralizing antibodies were also positive for anti-MERS-CoV S protein IgG

antibodies (Fig. 4C).

DISCUSSION

Here, we examined the susceptibility of rats at different ages to infection by MERS-CoV. Unfortunately, the results of both in vivo and in vitro studies suggest that rats, similar to mice, hamsters, and ferrets (4,5,14), are not susceptible to MERS-CoV. Van Doremalen et al. (15) reported that DPP4 plays an important role in the host species tropism of MERS-CoV. The receptor binding domain-contacting residues in human DPP4 are only 64% identical to those of rat DPP4 (14). Therefore, the lack of infectivity shown by MERS-CoV in rats might be due to a lack of affinity between rat DPP4 and the MERS-CoV S protein, rather than to a particular host factor that inhibits MERS-CoV replication in rats. We found no evidence of MERS-CoV replication in the lungs, although adult rats did generate neutralizing antibodies, especially against the MERS-CoV S protein. In the absence of detectable viral replication, it is suspected that such a weak response is derived from the normal host immune response to MERS-CoV antigens. Therefore, the rat model might prove to be a good source of antibodies for use in both research and diagnosis of MERS-CoV. Rats have been used to test the efficacy of some vaccines (16,17); thus, this animal might be a suitable model for examining the efficacy of vaccines against MERS-CoV S protein.

Taken together, the results of the present study suggest that neither neonatal nor adult rats are susceptible to infection by MERS-CoV. In addition, in vitro data show that rat DPP4 cannot support MERS-CoV infection. However, transgenic mice expressing human DPP4 are susceptible to infection by MERS-CoV (7), although these mice unexpectedly developed encephalitis and died. Therefore, future transgenic rodent models for MERS-CoV infection should express human DPP4.

Acknowledgments We thank Dr Ron A. M. Fouchier and Dr Bart L. Haagmans of Erasmus Medical Center for providing MERS-CoV; Dr Shutoku Matsuyama (National Institute of Infectious Diseases) for valuable discussion. We also thank our colleagues at the Institute, especially Ms. Ayako Harashima and Ms. Moeko Aida, for technical assistance. This work was supported by a Grant-in Aid for research on H25-Shinko-Wakate-004 from the Ministry of Health, Labour, and Welfare, Japan, and by a Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development, AMED.

Conflict of interest None to declare.

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