Pathogenicity of Sendai Viruses Adapted into Polarized MDCK Cells

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ABSTRACT. Apically and basally released Sendai viruses (SeV) were obtained after infection of polarized Madin-Darby canine kidney (MDCK) cells grown on permeable membrane culture inserts. After 20 passages of adaptation in MDCK cells, we compared their *in vivo* and *in vitro* pathogenicity with the parental Mol-strain of SeV. These viruses had comparable *in vitro* pathogenicity, but the *in vivo* pathogenicities were varied. The apically released MDCK-adapted virus showed comparable pathogenicity with the parental virus, in contrast with the basally released MDCK-adapted virus, which showed *in vivo* attenuation.—**KEY WORDS:** bronchopneumonia, MDCK cell, mouse, polarized epithelial cell, Sendai virus.

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Sendai virus (SeV), a member of the paramyxovirus family, is pneumotropic in rodent species and the infection has been reported in mice, hamsters, rats and guinea pigs [5, 11, 18, 22]. It has been reported that the pathology of SeV infection corresponds to that of bronchopneumonia. The replication of viruses in bronchiolar epithelium resulting in epithelial degeneration or proliferation is accompanied by an inflammatory response of variable intensity. Peribronchial lymph nodes are enlarged and densely populated with reactive lymphocytes. Interstitial pneumonia is characterized by the swelling and sloughing of type 1 and 2 alveolar epithelial cells with the occasional presence of multinucleated type 2 syncytial cells. The SeV are known to initiate damage resulting in the disorganization of alveolar parenchyma and fibrosis around terminal and alveolar ducts [5, 6, 8, 21].

Generally, all the epithelial cells, including the pulmonary bronchial epithelial cells, play a role as a barrier between the external environment or lumen and the internal environment or underlying tissue [29]. To carry out this barrier function, the epithelial cells show a high degree of structural polarization, which occurs because of the different composition of protein and lipid components between the apical and basolateral surfaces of the cells. The pores, channels, carriers, pumps and receptors present in the apical domain are entirely different from those in the basolateral domain. Such an asymmetric distribution of surface components in epithelial cells implies the existence of a molecular sorting mechanism [2, 4, 20, 24]. The two surface domains are delineated by the presence of junctional complexes between the adjacent cells, which prevent the free transport of ions or molecules across the epithelial layer

[29]. Each virus has been observed to have a specific preference for budding direction in polarized epithelial cells. Enveloped virus is usually released preferentially by budding at the apical plasma membrane and it has been previously demonstrated that the wild type SeV is released predominantly, though not exclusively from the apical surface of polarized epithelial cells [3], in contrast with the F1-R mutant SeV virus which is released from both the apical and basolateral surfaces [25, 27]. The polarized entry and release of SeV play a role in the pathogenesis of the disease. Virus that enters and is released at the apical membranes can be transmitted laterally from cell to cell in intact epithelium without ever transferring the cell layer, thus resulting in a localized infection. However viruses that are released through the basolateral surface can infect the underlying tissue and gain access to the bloodstream; thus the virus could be disseminated throughout the host's other organs [2, 25, 27, 29].

Understanding the steps of viral infection *in vitro* may provide further insight into the pathological process of infection and spread. When grown on permeable membrane culture inserts, the epithelial cells form a polarized epithelial monolayer with an apical-to-basolateral orientation, which more closely reflects the *in vivo* situation [30]. Since polarized entry and release of virus can play a role in pathogenesis of viral disease and a low level of virus was found to be released into the basal medium, we were interested to study the pathogenicity of the basally released SeV. We collected the basally released SeV and adapted them into the Madin-Darby canine kidney (MDCK) cells grown on permeable membrane culture inserts. The MDCK cell line has been widely used as a convenient model for studying the interaction of viral pathogens with polarized epithelial cells *in vitro*. In this study, we compared the *in vivo* and *in vitro* pathogenicity of the basally released MDCK-adapted viruses with the apically released viruses

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adapted into MDCK cells grown on conventional petri dishes and with their egg-grown parental virus.

MATERIALS AND METHODS

Virus: SeV used in the present study was the virulent Mol-strain isolated from the lung of mice native to Japan [31, 32]. The seed stock of this strain was grown in the allantoic sac of 11-day-old embryonated chicken eggs, inoculated with 0.1 ml of a 10^{-3} dilution of 9×10^8 plaque-forming units (PFU) of virus stock and incubated at 34°C. Allantoic fluid was harvested at 48 hr and debris was removed by centrifugation at $2,000 \times g$ for 15 min.

Cells and cell culture: Polarized strain II [30] MDCK line of canine kidney cells (RIKEN Cell Bank, Tsukuba, Japan) were grown in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS). To collect the basally released virus, the MDCK cells were grown on polycarbonate membrane culture inserts (Iwaki glass, U.S.A., pore size 3 μ m). A total of 1×10^5 cells per insert were seeded and grown to confluence in 24-well plates. The integrity of the monolayer of MDCK cells was examined by measuring transepithelial electrical resistance (TER) with a Millicell-ERS apparatus (Millipore Corp., Bedford, Mass., U.S.A.). As defined in previous investigations, MDCK II cell cultures have TER values that ranged from 41 to 100 Ω/cm^2 [23]. If the net TER was greater than 50 Ω/cm^2 then the cells were considered to be sufficiently polarized.

Viral adaptation in MDCK cells: For infection, viruses at a multiplicity of infection (m.o.i.) of 10 were inoculated into the cells grown on the apical chamber of a polycarbonate membrane culture insert. After 1 hr of adsorption, the apical chamber was washed and incubated with MEM containing 10% (v/v) tryptose phosphate broth (TPB) in the presence of 4 μ g trypsin/ml medium, to support multiple cycles of replication of the virus [26], at 34°C for only 24 hr. The basally released SeV were collected and propagated on the MDCK cells grown on the conventional petri dishes until reasonable hemagglutination (HA) titers were obtained. The viruses were then used for reinfected MDCK cells in the next passage in the apical chamber, and this procedure was repeated for 20 times. For comparison, the apically released SeV were passaged serially 20 times in MDCK cells grown on conventional petri dishes. These viruses were plaque purified twice in LLC-MK₂ cells and propagated in MDCK cells before use in the experiment.

Polarity characteristic of the viruses: Polarized MDCK cells were grown on the polycarbonate membrane culture inserts in 24-well plates in MEM containing 10% (v/v) FBS. The integrity of the monolayer of MDCK cells was examined before infection and at each sampling time by measuring the TER with a Millicell-ERS apparatus. The virus (0.01 m.o.i.) was inoculated into the apical chamber of polycarbonate membrane culture inserts. After 1 hr of adsorption, the apical chamber was washed and incubated with MEM containing 10% (v/v) TPB in the presence of 4

μ g trypsin/ml medium at 34°C. The apically and basally released SeV were collected separately at predetermined intervals after inoculation and the virus titers were determined by plaque assay.

Mouse pathogenicity study: Specific-pathogen-free female ICR mice (Charles River, Japan) at 3.5 weeks of age, 12–16 g body weight, were used throughout the experiments. Groups of 36 mice each were infected intranasally under mild ether anesthesia with 25 μ l of 106 PFU of apically or basally released adapted viruses or the egg-grown parental virus. Three groups of four mice each were observed for symptoms and weighed daily for up to 14 days post infection (p.i.). One group of mock-infected mice were 'infected' with phosphate buffered saline (PBS). The effect of infection on body weight was examined because body weight change is one of SeV pathogenicity indicator. For the other three groups, six mice from each group were killed on days 1, 2, 3, 5, 7 and 10 p.i. Their lung consolidation scores were graded, and each grade was expressed as follows: score 0 signified no lesion; score 1~less than 25% of lobules affected; score 2~between 25% and 50% of lobules affected; score 3~between 50% and 75% of lobules affected; score 4~more than 75% of lobules affected; 1 point was added if the mice died beforehand [16, 26].

Lung viral titration: Lung tissue for virus titration was collected aseptically, frozen in vials and stored at -80°C until viral assays were run. Lung samples were thawed and 10% (w/v) homogenates in MEM were made in ice with a homogenizer. Homogenates were then clarified by centrifugation at $3,000 \times g$ for 15 min. The supernatants were then titrated using the plaque assay method.

HA assay: Titration of HA activity was done using a two-fold dilution of the samples and a 1% (v/v) suspension of chicken red blood cells at 4°C.

Plaque assay: A rhesus monkey kidney cell line (LLC-MK₂) grown in 35-mm petri dishes was infected each with 0.2 ml of a ten-fold dilution of virus in MEM and 0.1% (w/v) bovine serum albumin (BSA). After 1 hr of adsorption at 34°C, the inoculum was removed, the cells were washed twice with PBS and covered with 2 ml agarose at a final concentration of 0.8% (w/v) in MEM containing 0.1% (w/v) BSA, 0.01% (w/v) sodium bicarbonate and 7 μ g/ml trypsin. After 7 days of incubation at 34°C, the cells were additionally overlaid with 2 ml agarose which contained 0.01% (w/v) neutral red and 5% (v/v) FBS. Plaques were counted 2 days later.

Histopathological examination: All tissues, including lung, liver, heart, spleen, kidney and brain, that were collected for histological examination and immunohistochemical observation were fixed in neutral buffered 10% (v/v) formalin. Tissues were processed in a routine manner, embedded in paraffin, sectioned at 4 μ m, stained with hematoxylin and eosin (HE) and prepared for immunohistochemical assessment. Five transverse sections of lung were chosen, analyzed and scored. The histopathological scores for lung were expressed as follows: score 0 signified no lesion; score 1 ~ hyperplastic bronchi

and bronchiole with proliferation of the epithelium, degeneration or necrosis; score 2 ~ similar to score 1, with peribronchial lymphocytic accumulation; score 3 ~ similar to score 2, with infiltration of lymphocytes into adjacent alveoli, which caused multifocal or diffuse interstitial pneumonia; score 4 ~ similar to score 3, with infiltration of an admixture of various amounts of neutrophils, cell debris, mucus, fibrin and macrophages in the lumen of the alveoli which causes pneumonic lesion. For detection of the viral antigens, immunohistochemical staining method was performed using anti-SeV polyclonal rabbit serum (1:4000) and a Histofine SAB-PO(R) Kit (Nichirei Co., Tokyo, Japan). The immunohistochemical analysis was scored according to the following four grades: (-) ~ negative; (+) ~ mildly positive; (++) ~ moderately positive; (+++) ~ strongly positive. Few bronchi-bronchiole epithelial cells showed positive reactions for SeV antigen in those scored as "mildly positive score"; for those scored as "moderately positive", positive reactions were observed in almost all bronchi-bronchiole epithelia. When the SeV antigen was also observed in the alveolar wall and the macrophages inside the alveolar lumen, we considered the response to be "strongly positive".

RESULTS

In vitro replication, pathogenicity and polarity properties: After 20 passages of adaptation in MDCK cells, we compared the titers of the apically and basally released MDCK-adapted viruses with their egg-grown parental virus in polarized MDCK cells after infection with 0.01 m.o.i. of virus. Their HA titers were 2^8 and 2^6 HA units and their PFU were 7.5×10^6 and 3.5×10^6 , respectively, while their egg-grown parental virus had a titer of 2^{12} HA units and 4×10^9 PFU. In the MDCK cells, the apically and basally released virus showed almost the same plating efficiency as their parental strain, although the basally released MDCK-adapted virus reached higher titer than the apically released MDCK-adapted virus and the egg-grown parental virus (Fig. 1). It has been shown previously that the SeV is released from epithelial cells in a polarized manner, predominantly at the apical surface [3]. The polarity properties of the adapted virus released from polarized MDCK cells were examined by infecting cells grown on inserts and collecting and determining the virus yields in both the apical and the basal media after predetermined intervals, in triplicate experiments. The apically released MDCK-adapted virus exhibited slightly faster kinetics than the other two viruses, but its final titer again was comparable with that of the basally released MDCK-adapted and the parental strain virus, as shown in Fig. 1. The result also indicated that the release pattern of the viruses was similar to that of their parental strain, which showed preferential release into the apical surface.

In vivo replication and pathogenicity: SeV causes bronchopneumonia in mice. To determine the ability of the apically and basally released MDCK-adapted viruses to

Fig. 1. Released pattern of viruses in the apical and basal medium. The circles represent basally released MDCK-adapted virus, the squares represent apically released MDCK-adapted virus and the triangles represent the egg-grown parental virus. The clear shapes represent the virus yields in the apical medium, while the solid shapes represent the yields in the basal medium.

replicate and produce lesion in mouse organs, the mice were infected with 10^6 PFU of virus. The effect of infection on body weight was examined and is summarized in Fig. 2. The mice infected with the parental Mol strain virus showed a marked decrease in body weight, in contrast to those infected with apically and basally released MDCK-adapted viruses, which only showed slight a decrease in body weight compared with the mock-infected mice from 8 days p.i. The mice infected with apically released MDCK-adapted virus showed more variable body weight gain than those with the basally released MDCK-adapted virus. None of the mice infected with apically or basally released MDCK-adapted virus died during the 14-day infection period, but two of the mice infected with egg-grown parental virus died at 9 days p.i.

Macroscopically, lungs showed variable size areas that appeared as dark red discoloration or consolidation in one third of the viruses infected mice (Fig. 3a). During the 10 days of infection, both mice infected with apically and basally released MDCK-adapted viruses showed minimal consolidation scores from day 5 p.i., in contrast with the mice infected with the egg-grown parental virus, which had moderate to maximum consolidation scores.

Fig. 2. The effect of viruses infection on mice body weight. The solid circles represent basally released MDCK-adapted virus, the clear squares represent apically released MDCK-adapted virus and the solid triangles represent the egg-grown parental virus. The average body weight of the mock infected mice were represented as line in each graphs. The x marks represent the death mice.

The histopathological examination of the mice infected with the apically or basally released MDCK-adapted virus or with egg-grown parental virus were confined to the previous study within various degrees of the pulmonary lesion [5–8, 15, 21]. The lesion was restricted to the lung and there were no remarkable histopathological changes in the liver, heart, spleen, kidney and brain. To understand the development process of the lung lesion, each histopathological event was defined and given a score. Minimal microscopic changes in the lungs were noted in the form of hyperplasia of the cell lining of the bronchiole (Fig. 4a). This change was followed by peribronchial edema and lymphocytic infiltration (Fig. 4b). Occasionally, inflammatory cells and cellular debris were present in some bronchioles, with sloughing of the epithelial cells. The next change noted was a thickening of the interalveolar septa

Fig. 3. Mice pathogenicity. (a) The lung consolidation score; (b) The lung histopathological score; (c) The lung immunohistochemical staining score; (d) Lung viral titration. The solid circles represent basally released MDCK-adapted virus, the clear squares represent apically released MDCK-adapted virus and the solid triangles represent the egg-grown parental virus.

Fig. 4. The lung histopathological lesion. H.E. (a) Hyperplasia of the cell lining of the bronchiole ($\times 145$); (b) Peribronchial edema and lymphocytic infiltration ($\times 75$); (c) Thickening of the interalveolar septa associated with edema and mobilization of macrophages, neutrophils and lymphocytes ($\times 75$); (d) Hyperplastic of type II alveolar cell and infiltration of macrophages, lymphocytes and neutrophils into alveolar spaces ($\times 75$).

associated with edema and the mobilization of macrophages, neutrophils and lymphocytes (Fig. 4c). The type II alveolar cells became hyperplastic, and macrophages, lymphocytes and neutrophils infiltrated the alveolar spaces, which caused the pneumonic lesion (Fig. 4d). This chronology of

histopathological events was scored as described in Materials and Methods. Five transverse sections of the lung of mice infected with apically and basally released MDCK-adapted viruses were chosen and scored. The mean score of five areas from four mice each infected with

Fig. 5. The lung immunohistochemical staining result. SAB method. (a) The viral antigen were observed focally within limited area of bronchial epithelium (arrow head) of a few mice infected with basally released MDCK-adapted virus, day 1 p.i. ($\times 105$); (b) From the day 2 p.i., the apically released MDCK-adapted virus could detected diffusely in almost bronchiole of areas observed ($\times 120$); (c) The positive cells were also detected in the alveolar wall and macrophages inside the alveolar lumen of mice infected with apically released MDCK-adapted virus, day 5 p.i. ($\times 105$).

apically and basally released MDCK-adapted viruses and their parental strain are summarized in Fig. 3b. The minimal scores were already observed at day 1 p.i. for all virus-infected mice. Although they showed different mean lung histopathological scores, the scores for all virus-infected mice gradually increased up to 7 days p.i. and decreased at day 10 p.i. The apically released MDCK-adapted virus-infected mice had scores that were similar to the egg-grown parental virus, while the basally released MDCK-adapted virus had the lowest scores.

To determine the time course and demonstrate the distribution of viral antigen in the lung and other organs, we stained the tissues immunohistochemically using polyclonal anti-SeV antibody. The immunohistochemical

examination revealed that viral growth in lung was restricted to the bronchiolar epithelium, alveolar wall and alveolar macrophages. Within the infected cells, SeV antigens were distributed throughout the entire cytoplasm; they showed no selective localization near the apical surface, from which they were preferentially budding. No apically and basally released MDCK-adapted viral antigen was detected either in the bronchial sub-epithelial cells or in the infiltrating mononuclear cells, or in any organ other than lung, which means that all the adapted viruses were exclusively pneumotropic. Viral antigen was not detected in liver, heart, spleen, kidney or brain. Five transverse sections of the lungs of mice infected with apically and basally released MDCK-adapted viruses were chosen and scored as described

in Materials and Methods. The mean score of five areas from four mice each infected with apically and basally released MDCK-adapted viruses are summarized in Fig. 3c. One day after inhalation, the viral antigen could be observed focally within limited areas of bronchial epithelium of a few mice in all infected groups, similar as Fig. 5a. From day 2 p.i., the apically released MDCK-adapted virus could be detected diffusely in most bronchioles of the areas observed (Fig. 5b), and from day 5 p.i., some positive cells in the alveolar wall and macrophages were detected inside the alveolar lumen (Fig. 5c). In contrast, only a few mice infected with basally released MDCK-adapted virus had lesions as shown in Fig. 5a. The antigens were rarely detected diffusely in the bronchi-bronchiole epithelium, and never seen in the alveolar wall; by day 7 p.i., no antigen became to be observed in the tissue. The egg-grown parental virus showed maximum antigen distribution by day 2 p.i. and gradually decreased until day 10 p.i.

The virus titer in each lung of mice infected with apically and basally released MDCK-adapted viruses and their egg-grown parental virus were determined by plaque assay of their lung homogenates. The mean titers of each group are presented in Fig. 3d. The apically released MDCK-adapted virus generally had higher titers than the basally released MDCK-adapted virus and the egg-grown parental virus. The apically released MDCK-adapted virus grew rapidly in the lung, and reached a peak titer of 2×10^6 PFU on day 3 p.i. and then began to clear gradually on day 10 p.i. The basally released MDCK-adapted virus grew slower than the apically released MDCK-adapted virus in the lung, and reached a peak titer of 5×10^3 PFU on day 5 p.i. and cleared gradually by day 10 p.i. Although at a slightly lower titer, the egg-grown parental virus showed similar lung viral titer pattern to that of the apically released MDCK-adapted virus, which reached its peak titer of 1×10^5 PFU on day 3 p.i., before gradually clearing by day 10 p.i.

DISCUSSION

Even though we found differences in growth kinetics, our *in vitro* studies showed that the apically and basally released adapted viruses had comparable cytopathogenicity and polarity properties in the polarized MDCK cells to those of their parental virus. The pathogenicities for mice were clinically likely to follow the general rule of adaptation [14], which predicts attenuation of the virulence against the natural host animal. The mice infected with apically and basally released MDCK-adapted viruses showed only slight body weight decrease compared with those of mice infected with their parental virus. Their gross lesions of the lung also reflected a similar phenomenon. Mice infected with either type of MDCK-adapted virus showed low consolidation, while the mice infected with parental virus showed high consolidation from day 3 p.i. These findings were also reflected in the microscopic lesion of the basally released MDCK-adapted virus, which showed the lowest scores. However, the mice infected with the apically

released MDCK-adapted virus had similar lesions and antigen distributions to those infected with the parental virus, especially during the later stages of infection. The titer of replicated viruses within the lung was also detected. The result appears to indicate that the apically released MDCK-adapted virus was able to replicate better than the parental virus, while the basally released MDCK-adapted virus showed the lowest viral titer. The results of the microscopic examinations and the virus titer in lung suggest that the apically and basally released MDCK-adapted viruses have different pathogenicities even though both were much less virulent than their parental virus.

As for *in vitro* replication, our apically released MDCK-adapted virus was found to interact as well as their parental virus with the respiratory epithelial cells, although during the early phase of infection they spread more slowly than the parental virus. The viruses replicated very well within the respiratory epithelial cells; they produced higher titer than the parental virus and brought about the gradual increase from a moderate to a high score for the tissue inflammatory reaction. The gradual increase in tissue inflammatory reaction was caused mild bronchial obstruction during the early phase of infection, as reflected by the lung consolidation score. Although the replication of parental virus was slightly lower, they spread faster and caused higher inflammatory host response than the apically released MDCK-adapted virus. This suggests that the rapid virus spread and high host response of parental viral infection caused severe bronchial obstruction, which was reflected in the high lung consolidation score.

In striking contrast with their *in vitro* replication, our basally released MDCK-adapted virus did not interact well with the mouse bronchial epithelium. The immunohistochemical staining showed that the basally released MDCK-adapted virus antigens were rarely detected diffusely within the bronchi-bronchiole epithelium and were not seen in the alveolar wall of the mouse lung; while the apically released MDCK-adapted virus could be detected diffusely in the bronchi-bronchiole epithelium, alveolar epithelial cells and alveolar macrophages at levels similar to those of the parental virus. The minimum host cell inflammatory reaction and the minimum area of consolidation induced by the basally released MDCK-adapted virus were considered to be consequences of the low level of virus production in the lung.

Although the mechanism of attenuation of adaptation have not been determined, some previous study are proposed to assume such condition [9, 10, 13, 14, 16, 17, 19, 28]. We tried western-immunoblot analyses, but our apically and basally released MDCK-adapted virus had patterns similar to their parental virus (data not shown). Even though our viral polypeptide pattern provided no clear explanation, it seemed that there were mutations within the viral protein component of the apically and basally released MDCK-adapted viruses that were responsible for the viral attenuation. Further analyses will be necessary at a molecular level to explain the mechanism of adaptation

described in this study.

The adaptation of measles virus to polarized epithelia described in previous studies results in a changed virus release pattern. The adaptation increased the basally released virus yields after multiple replications, which correlated with the lack of ability of the virus to down-regulate the cell receptor CD46. After one or two passages, the basally released virus yields are increased and the virus could encounter the cells under the epithelial cells [1]. This phenomenon was not observed in our apically and basally released MDCK-adapted viruses. The adaptation of the polarized epithelial cells did not change the release pattern of our viruses. Our apically and basally released MDCK-adapted viruses were still likely to bud through the apical surface. Envelope viruses such as SeV have been reported to mature at the apical membrane, while the F1-R mutant of SeV has bipolarity budding, and causes some virus to go to the basal membrane, invade subepithelial tissue, reach the blood or lymphatic vessels and caused a systemic infection [27]. The mutated M protein of F1-R mutant SeV has been studied to disrupt the host's cell microtubules and alter the budding polarity of the viruses when they replicate in the cells [28]. In this study, although a few viruses were released through the basal membrane, our MDCK-adapted virus still lacked the capability to interact well with the bronchi-bronchiole epithelium and to enhance lymphocytes, monocytes or any other cells under the epithelium as the site of replication. Our immunohistochemical examination, revealed no evidence of SeV antigen within the subepithelial tissue under the bronchial epithelium (including the infiltrating mononuclear lymphocytes) of mice infected with apically and basally released MDCK-adapted virus, which suggests that our adapted virus produced only localized infection as did their parental viruses.

In conclusion, our study showed that all the tested viruses had comparable *in vitro* pathogenicity although vary in their *in vivo* pathogenicities. The apically released MDCK-adapted virus showed comparable pathogenicity with the parental virus; in contrast, the basally released MDCK-adapted virus showed *in vivo* attenuation.

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