Inhibitions of human parainfluenza virus type 2 replication by ribavirin and mycophenolate mofetil are restored by guanosine and S-(4-nitrobenzyl)-6-thioinosine

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1. Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possesses a single-stranded, non-segmented, negative stranded RNA genome of 15,654 nucleotides (1). hPIV-2 has 7 structural proteins, NP, V, phospho (P), matrix (M), F, HN and large (L) proteins. The gene order of hPIV-2 is 3’-(leader)-NP-V/P-M-F-HN-L-(trailer)-5’. All genes of hPIV-2 were sequenced by our group (2-7). Monoclonal antibodies (mAbs) were made, and antigenic diversity of clinical isolates was investigated by Tsurudome et al. (8). The infectious hPIV-2 from cDNA clone was constructed by Kawano et al., and it was shown that its

DOI: 10.5582/ddt.2019.01084

Summary

The antiviral activities of a nucleoside analog antiviral drug (ribavirin) and a non-nucleoside drug (mycophenolate mofetil) against human parainfluenza virus type 2 (hPIV-2) were investigated, and the restoration of the inhibition by guanosine and S-(4-nitrobenzyl)-6-thioinosine (NBTI: equilibrative nucleoside transporter 1 inhibitor) were also investigated. Ribavirin (RBV) and mycophenolate mofetil (MMF) inhibited cell fusion induced by hPIV-2. Both RBV and MMF considerably reduced the number of viruses released from the cells. Virus genome synthesis was inhibited by RBV and MMF as determined by polymerase chain reaction (PCR) and real time PCR. mRNA syntheses were also reduced. An indirect immunofluorescence study showed that RBV and MMF largely inhibited viral protein syntheses. Using a recombinant green fluorescence protein (GFP)-expressing hPIV-2 without matrix protein (rhPIV-2ΔMGFP), it was found that virus entry into the cells and multinucleated giant cell formation were almost completely blocked by RBV and MMF. RBV and MMF did not disrupt actin microfilaments or microtubules. Both guanosine and NBTI completely or partially reversed the inhibition by RBV and MMF in the viral replication, syntheses of genome RNA, mRNA and protein, and multinucleated giant cell formation. NBTI caused a little damage in actin microfilaments, but had no effect on microtubules. Both RBV and MMF inhibited the replication of hPIV-2, mainly by inhibiting viral genome RNA, mRNA and protein syntheses. The inhibition was almost completely recovered by guanosine. These results indicate that the major mechanism of the inhibition is the depletion of intracellular GTP pools.

Keywords: Human respiratory tract pathogen, antiviral drug, mycophenolic acid, recovery of virus replication inhibition, a recombinant green fluorescence protein expressing hPIV-2 without matrix protein
growth property was the same as that of control natural hPIV-2 (9).

Ribavirin (RBV) is a synthesized nucleoside analog that has broad antiviral activities against many DNA and RNA viruses. RBV was reported to be effective in the treatment of hepatitis C virus in combination with interferon (10,11), and of respiratory syncytial virus (12). It was shown that its antiviral activity in vitro is mediated mainly by inhibition of inosine monophosphate dehydrogenase (IMPDH) (13), inhibition of RNA-dependent RNA polymerase, and incorporation into viral RNA and induces point mutation of RNA virus, but the precise mechanism is still not fully understood. Our previous study showed that only RBV out of eight nucleoside analog antiviral drugs inhibited replication of hPIV-2 in vitro, but mechanism of inhibition is unclear (14). Mycophenolate mofetil (MMF) has been used as an immunosuppressive agent especially in organ transplanted patients. MMF is a nonnucleoside IMPDH inhibitor. Mycophenolic acid (MPA) is a hydrolyzed material of MMF, binds to IMPDH, induces depletion of intracellular GTP pools, and inhibits replication of some viruses such as hepatitis C virus (15) and reovirus (16).

The present investigation aimed at ascertaining the inhibitory capacity of RBV and MMF for hPIV-2 replication and, furthermore, elucidating the inhibitory mechanism of the drugs. To investigate the effects of the drugs on viral genome synthesis, virus RNA was prepared and analyzed by polymerase chain reaction (PCR) and real time PCR. To elucidate the effects of the two drugs on mRNA synthesis, cDNA was synthesized using oligo dT primer, and PCR was carried out. Virus protein expression was observed by indirect immunofluorescence study using mAbs against NP, F and HN proteins of hPIV-2 (8). The inhibitory effects of RBV and MMF on cell-to-cell spreading of hPIV-2 were analyzed using a recombinant green fluorescence protein-expressing hPIV-2 without matrix protein (rhPIV-2ΔMGFP) (9,17,18). The number of viruses released from infected cells was determined. Cytoskeleton was reported to have an important role in paramyxovirus replication. Actin microfilaments are important in the human parainfluenza virus type 3 life cycle, specifically at the level of viral transport and replication (19). Tubulin also acts as a positive transcription factor for in vitro RNA synthesis by Sendai virus (20). The effects of RBV and MMF on actin microfilaments and microtubules were analyzed using rhodamine phalloidin and anti-tubulin α mAb, respectively.

2. Materials and Methods

2.1. Drugs used in this study

Antiviral drugs, RBV and mycophenolate mofetil (MMF) were purchased from Wako Chemicals (Osaka, Japan). They were dissolved at 1 mg/mL in 10 mM phosphate buffered saline, pH 7.2 (PBS), and sterilized by filtration. MMF, a produg of mycophenolic acid (MPA), is rapidly hydrolyzed to MPA in aqueous solution. Guanosine was from Wako Chemicals, and dissolved at 1 mg/mL in dimethyl sulfoxide, and S-(4-nitrobenzyl)-6-thioinosine (NBTI) also from Wako Chemicals, dissolved at 1 mg/mL in PBS and sterilized by filtration.

2.2. Virus and recombinant virus

The virus and the recombinant virus were approved by the relevant biosafety committees of Suzuka University of Medical Science. hPIV-2 (Toshiba strain) was used. rhPIV-2ΔMGFP was constructed according to the method described previously (9,17,18), and it was shown that it did not produce infectious virus particles without addition of M protein gene in trans (data not shown). The virus titer was determined using Vero cells and the titer was about 1 × 10^6 TCID_{50}/mL.

2.3. Cell line and cultivation of cells

LLCMK2 cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. Minimum essential medium α (MEMα: Wako Chemicals), supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin, was used. The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. After 3 days, when the cells became confluent (5 × 10^5 cells), the medium was changed to MEMα with 0.5% FCS and 0.1 mg/mL kanamycin. The antiviral drugs were added to the cells, and the cells were infected with hPIV-2 (3 × 10^7 TCID_{50}).

2.4. Cytotoxicity assay

Cell fusion was observed at 4 days post infection under cell culture microscope (Olympus, Tokyo, Japan).

2.5. RNA preparation, cDNA synthesis and real time PCR

RNA was extracted from the cells (2 × 10^6 cells) cultured in a flat-bottomed 6-well plate using TRIZOL
inhibitory effect by guanosine and NBTI (data not shown). RBV (20 µg/mL) and MMF (2 µg/mL, 1.48 µg/mL as MPA) completely inhibited the cell fusion, indicating that lower concentration of MMF can inhibit hPIV-2 replication. The inhibitions of cell fusion by RBV and MMF were recovered with the addition of guanosine and NBTI (data not shown).

3.2. Titration of virus released from infected cells and RBV or MMF treated cells, and restoration of the inhibitory effect by guanosine and NBTI

The titers of virus released from cells cultured with and without RBV (20 µg/mL) and MMF (2 µg/mL) at 4 days post infection were determined. Without the drugs, the virus titer was about 5 × 10^6 TCID₅₀/mL, and it was reduced to less than 10 TCID₅₀/mL by RBV and MMF dose dependently (data not shown), indicating that both drugs largely prevented the virus replication and the release of virus from the infected cells. However, the inhibitions were dose dependently recovered (more than 10⁷ TCID₅₀/mL) by guanosine and NBTI (Figure 1). Guanosine fully recovered the inhibition by RBV (Figure 1A) and MMF (Figure 1C), and NBTI could recover the inhibition by RBV to about 10⁶ TCID₅₀/mL (Figure 1B), and the inhibition by MMF only to about 10⁵ TCID₅₀/mL (Figure 1D).

We carried out the restoration experiment using 20 µg/mL of RBV, 2 µg/mL of MMF, 50 µg/mL of guanosine and 70 µg/mL of NBTI. Figures 2A and 2B are the results of 5 independent experiments, showing that guanosine fully restored the inhibition by RBV and MMF, and that NBTI partially restored the inhibition.

3.3. Viral genome RNA synthesis and mRNA synthesis

RNA was prepared from the infected cells at 4 days post infection, and the viral genome RNA was analyzed by real time PCR. Figures 2C and 2D show that RBV and MMF inhibited viral genome RNA synthesis, and guanosine and NBTI almost fully restored the inhibitions by RBV and MMF. There are some discrepancies between the results of virus titers and the result of real time PCR.
In the following experiment, genome RNA synthesis was analyzed by PCR. PCR was carried out using hPIV-2 specific primers for NP, F and HN genes (Figures 3A and 4A). The number of base pairs between forward and reverse primers of NP, F and HN genes was about 400, 860 and 760, respectively.

To detect mRNAs of the three viral proteins, cDNA was synthesized using oligo dT primer, and PCR was carried out using hPIV-2 specific primers for NP, F and HN genes (Figures 3B and 4B). Figure 3A shows that NP (lane 4), F (lane 5) and HN (lane 6) gene were detected in the virus-infected cells, but not in the RBV-treated infected cells (NP: lane 7, F: lane 8 and HN: lane 9). Guanosine (NP: lane 10, F: lane 11, HN: lane 12), and NBTI (NP: lane 13, F: lane 14, HN: lane 15) restored the genome RNA syntheses. Figure 3B shows that viral mRNA was detected in the infected cells, but not in RBV-treated cells, and that the inhibition was restored by guanosine and NBTI.

MMF inhibited both genome RNA and mRNA syntheses, and the inhibitions were also restored by guanosine, but not by NBTI (Figures 4A and 4B).

3.4. Viral protein synthesis

Indirect immunofluorescence study was performed to investigate the effects of the two antiviral drugs on hPIV-2 protein synthesis, and restoration by guanosine and NBTI. The drugs were added to the cells and they were infected with hPIV-2. At 4 days post infection, the cells were fixed and stained with the mAbs against NP, F and HN proteins of hPIV-2.
In Figure 5, Figures 5A, 5B, and 5C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 infected cells, NP, F and HN proteins were observed in almost all the cells: NP protein was observed in many strong fluorescent dots mainly in the cytoplasm, while F and HN proteins were in small dots in the cytoplasm and on the cell surface. RBV almost completely inhibited the expression of NP (Figure 5D), F (Figure 5E), and HN (Figure 5F) proteins. However, the expressions of the NP, F and HN proteins were recovered by guanosine (Figures 5G, H and I, respectively) and by NBTI (Figures 5J, K and L, respectively). MMF also inhibited the syntheses of viral proteins (NP: Figure 5M, F: 5N, and HN: 5O) and restoration of the syntheses by guanosine (NP: Figure 5P, F: 5Q, and HN: 5R) and partial restoration by NBTI (NP: 5S, F: 5T, and HN: 5U). Bars in A and M: 100 µm.

3.5. Cell-to-cell spreading of hPIV-2

The above results showed that RBV and MMF inhibited viral genome RNA, mRNA and protein syntheses, and the inhibitions were almost completely or partially recovered by guanosine and NBTI. In the following experiment, we determined the effect of the drugs on the cell-to-cell spreading of hPIV-2 using rhPIV-2ΔMGP (Figure 6).

Figure 6A is a positive control: there are multinucleated giant cells with strong fluorescence. Figures 6B, 6C, and 6D show the infected cells cultured with RBV, RBV plus guanosine, and RBV plus NBTI, respectively. In Figure 6B, RBV inhibited multinucleated giant cell formation. In Figure 6C, guanosine restored the multinucleated giant cell formation, and in the cells with RBV plus NBTI, relatively small sized fused cells with strong fluorescence were observed (Figure 6D).
3.6. The effects of the drugs on actin microfilaments and microtubules

The drugs were added to the cells, and actin microfilaments and microtubules were observed under a fluorescence microscope at 4 days of cultivation without virus infection.

Figure 7A shows actin microfilaments in LLCMK2 cells. As shown in Figures 7B and 7C, RBV and RBV plus guanosine did not disrupt actin microfilaments. However, RBV plus NBTI caused slight damage (Figure 7D). MMF (Figure 7E) and MMF plus guanosine (Figure 7F) did not cause actin microfilament damage, but MMF plus NBTI (Figure 7G) caused damage in actin microfilaments. These results indicated that one of the causes of the partial restoration by NBTI was due to the actin microfilament damage.

Figure 8A shows microtubules in the cells. As shown in Figures 8B, 8C and 8D, respectively, RBV, RBV plus guanosine, and RBV plus NBTI, did not induce damage in microtubules. MMF (Figure 8F), MMF plus guanosine (Figure 8G), and MMF plus NBTI (Figure 8G) also caused no damage.

4. Discussion

The present study showed that RBV and MMF had inhibitory effects on hPIV-2 replication in LLCMK2 cells and that the inhibition was restored by guanosine or NBTI. Almost no virus was found in the culture medium of RBV- or MMF-treated cells infected with hPIV-2. Viral genome RNA and mRNA were not detected in the RBV- or MMF-treated infected cells. In addition, viral proteins were not observed in the drug-treated infected cells. Using rhPIV-2∆MGFP, it was shown that both RBV and MMF completely inhibited
multinucleated giant cell formation. The inhibitory effects of RBV and MMF were not due to the disruption of actin microfilaments or microtubules. Guanosine and NBTI almost fully restored the inhibition of genome RNA, mRNA and protein syntheses by the two drugs. Multinucleated giant cell formation was also restored by guanosine or NBTI. However, guanosine is more effective than NBTI, one reason being that NBTI caused some damage in actin microfilaments. The discrepancy between the result of virus titer and the result of real-time PCR were in part due to a little disruption of actin microfilaments, resulting in the partial inhibition of virus budding.

RBV is a synthesized nucleoside analog that has broad antiviral activities against many RNA viruses. RBV has been reported to be effective in the treatments of hepatitis C virus in combination with interferon (10,11), of respiratory syncytial virus (12) and of Lassa fever virus (21), yellow fever virus (22), Andes virus (23), flaviviruses and paramyxoviruses (13). Shah et al. examined the ability of RBV against vesicular stomatitis virus and Sendai virus using different cell types, and they found RBV exhibited its inhibitory action only on the cells that could uptake RBV (24). Upon uptake, RBV is phosphorylated into mono-, di- and tri-phosphate by cellular kinases. Several mechanisms are proposed (13,21,24), but the mechanism of RBV action on the viruses is still not fully elucidated. RBV inhibits the host enzyme inosine monophosphate dehydrogenases (IMPDH) essential for the de novo synthesis of GTP. Phosphorylated RBV directly inhibits viral RNA polymerase. RNA chain elongation is terminated by the incorporation of ribavirin tri-phosphate into viral RNA. Ribavirin tri-phosphate incorporation as a substitute for guanosine or adenosine leads to lethal mutagenesis. In this experiment, a sufficient amount of guanosine might competitively block RBV tri-phosphate incorporation into viral RNA, resulting the restoration from the inhibition. NBTI (a nucleoside transporter inhibitor) could block the incorporation of RBV tri-phosphate into viral RNA, and also partly recovered the inhibition. However, NBTI caused partial disruption of actin microfilaments, so the restoration may be partial.

MMF has been used as an immunosuppressive agent (10 µg/mL or greater), and inhibits replication of some viruses such as Dengue virus (25), hepatitis B virus (26), human immunodeficiency virus (27), and Japanese encephalitis virus (28). In the present study, 2 µg/mL of MMF (1.48 µg/mL as MPA) had an inhibitory effect on hPIV-2 replication, and Hermann et al. reported that 3 µg/mL MMF inhibited Type 2 Winnipeg, a cerebrospinal fluid-derived reovirus isolate (16). In addition, MMF had an inhibitory effect at 1/10 the dose of RBV (present experiment). From these results, MMF may be a promising agent for the treatment of human parainfluenza viruses which cause recurrent infection in infants and children.

MMF is a nonnucleoside inhibitor of IMPDH. Its hydrolytic product MPA binds to IMPDH and finally causes depletion of the GTP pool. Therefore, it is reasonable that a sufficient amount of guanosine could recover the inhibition by MMF. Though NBTI is a nucleoside transporter inhibitor, it also could partly restore the inhibition by MMF: viral RNA was fully restored and virus titer and viral protein were partly restored. The mechanism of restoration by NBTI remains to be elucidated.

References

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(Received November 8, 2019; Revised December 23, 2019; Accepted December 28, 2019)