Plaque Formation Assay for Human Parainfluenza Virus Type 1

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Human parainfluenza virus type 1 (hPIV1) generally does not show visible plaques in common cell lines, including Lewis lung carcinoma-monkey kidney (LLC-MK₂) cells, by plaque formation assays for human parainfluenza virus type 3 (hPIV3) and Sendai virus. In several conditions of the plaque formation assay, complete elimination of serum proteins in the overlay medium was necessary for visualization of hPIV1-induced plaque formation in LLC-MK₂ cells. We developed a plaque formation assay for hPIV1 isolation and titration in LLC-MK₂ cells using an initial overlay medium of bovine serum albumin-free Eagle's minimum essential medium containing agarose and acetylated trypsin for 4–6 d followed by a second overlay staining medium containing agarose and neutral red. The assay allowed both laboratory and clinical hPIV1 strains to form large plaques. The plaque reduction assay was also performed with rabbit anti-hPIV1 antibody as a general evaluation model of viral inhibitors to decrease both the plaque number and size. The results indicate that the plaque formation assay is useful for hPIV1 isolation, titration, evaluation of antiviral reagents and epidemiologic research.

Key words human parainfluenza virus type 1; plaque assay; isolation; Lewis lung carcinoma-monkey kidney cell; antiviral reagent

Human parainfluenza virus type 1 (hPIV1; genus *Respirovirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales*) possesses a hemagglutinin-neuraminidase (HN) glycoprotein and a fusion glycoprotein on the viral envelope. After trypsin-like proteases cleave the fusion glycoprotein into a large F1 subunit and a small F2 subunit, the virus can fuse with the cell membrane and invade cells. hPIV1 often causes severe respiratory tract illness that can lead to hospitalization of infants and young children^{1,2)} and occasionally causes sudden death of newborns and infants.³⁾ Since there is no clinical therapy for or vaccine against hPIV1, an effective strategy for prevention of hPIV1 infection, especially in developed countries with a low birthrate, is needed. For development of hPIV1 vaccines and antiviral reagents, basic techniques for virus isolation such as a plaque assay are necessary. An immunofluorescent antibody assay,⁴⁾ the hemadsorption assay⁴⁾ and reverse transcription real-time polymerase chain reactions (PCR)⁵⁾ have generally been used for hPIV1 isolation and detection. These methods are useful for accurate and sensitive detection of hPIV1, but not effective to isolate the virus. On the other hand, the plaque assay can be used to purify a clonal population of virus or to determine viral titer as plaque-forming units. Additionally, the assay could be applied to estimate antiviral reagents or select mutant strains because a plaque size reflects virus propagations.

Human parainfluenza virus type 3 (hPIV3; genus *Respirovirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales*) has been reported to form large plaques in human epidermoid cancer (HEp-2) cells in Dulbecco's modified Eagle's medium (DMEM) with 0.3% agarose.⁶⁾ Sendai virus (SeV; genus *Respirovirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales*) has been reported to form large plaques in several

cell lines with neutral red staining,^{7–9)} and recombinant SeV possessing HN genes from hPIV1 or hPIV3 has also been reported to form plaques in Lewis lung carcinoma-monkey kidney (LLC-MK₂) cells in Eagle's minimum essential medium (MEM) with 2% bovine serum albumin (BSA) or 5% fetal bovine serum (FBS).^{10–12)} In contrast, no clear plaque formation of hPIV1 was observed by these methods reported for hPIV3 or recombinant SeV possessing hPIV1 HN glycoprotein. Plaque formation of hPIV1 has been difficult to visualize because the virus does not show obvious cytotoxicity and syncytium formation activity, in comparison with that of hPIV3 and SeV.^{13,14)} It is necessary to select and optimize appropriate media and cell lines in order to realize visible plaque formation of hPIV1.

From tests of 4 cell lines, 3 kinds of medium and different concentrations of additives and cell culture times, we developed a visible-plaque formation assay for hPIV1 by using an overlay medium (MEM containing 0.8% agarose and 3 µg/ml acetylated trypsin without FBS and BSA) and an overlay staining medium (MEM containing 0.8% agarose and 0.25 mg/ml neutral red) for hPIV1 isolation and titration in LLC-MK₂ cells. We found that serum proteins such as BSA strongly inhibited visualization of hPIV1-induced plaques. The assay allowed both laboratory and clinical hPIV1 strains to form large plaques without cell fixation enabling isolation of the live virus clones. The plaque assay could also be applied to a plaque reduction assay, which has been widely used to evaluate efficiency or resistance for antiviral reagents.^{15,16)} Establishment of the hPIV1 plaque assay will accelerate basic research on the virus, especially phylogenetic study from clinical isolates and selection of drug candidates against hPIV1.

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MATERIALS AND METHODS

Cells, Viruses and Antibodies LLC-MK₂ cells were maintained in MEM supplemented with 5% heat-inactivated FBS. The hPIV1 strain C35, the hPIV3 strain C243 and the hPIV1 clinical isolate CL-5 were kindly provided by Allen Portner (St. Jude Children's Research Hospital). C35 and C243 were propagated in LLC-MK₂ cells. CL-5, which was utilized as clinical strain,¹⁷⁾ was isolated in 1973 from infected children¹⁸⁾ and passaged totally seven times in LLC-MK₂ cells after isolation. The SeV strain Enders was propagated in 10-d-old embryonated hen's eggs for 2 d at 34 °C. Rabbit anti-hPIV1 antibody was prepared by immunization with C35 as described previously¹⁹⁾ and purified on a Protein G column HiTrapTM Protein G HP (CE Healthcare Biosciences Corp., Priscataway, NJ, U.S.A.).

Plaque Formation Assay LLC-MK₂ cells were grown to complete confluence with MEM containing 5% FBS in a 6-well tissue culture plate (FALCON, Franklin Lakes, NJ, U.S.A.) at 37 °C under 5% CO₂. The growth medium was removed and the cell monolayers were washed once with phosphate-buffered saline (PBS; pH 7.2, 131 mM NaCl, 14 mM Na₂HPO₄, 1.5 mM KH₂PO₄ and 2.7 mM KCl). Ten-fold serial dilutions of hPIV1 C35 or CL-5 in a serum-free medium, SFM (Hybridoma-SFM; Invitrogen Corp., Carlsbad, CA, U.S.A.), were inoculated and incubated for 30 min at room temperature. During the virus adsorption, the overlay medium was prepared as follows: 2× concentrated MEM containing 6 μg/ml acetylated trypsin was warmed to 37 °C in a water bath, and 1.6% agarose (Bacto Agar; Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) in ultrapure water was melted at 92 °C for 15 min and then kept at 60 °C. The 2× concentrated MEM was mixed with the agarose to prepare the overlay medium (1× MEM, 0.8% agarose and 3 μg/ml acetylated trypsin). After removing the virus inoculum, the cell monolayers were washed once with PBS. Four milliliters of the overlay medium was added to each well. After the overlay medium had set, the plate was incubated in an upside-down position for 4 or 6 d (for hPIV1 strain C35, 4 d; for hPIV1 isolate CL-5, 6 d) at 37 °C under 5% CO₂. The overlay staining medium was prepared as follows: 2× concentrated MEM containing 0.5 mg/ml neutral red (Polysciences, Inc., Warrington, PA, U.S.A.) was warmed to 37 °C in a water bath and 1.6% agarose in ultrapure water was melted at 92 °C for 15 min and then kept at 60 °C. The 2× concentrated MEM was mixed with the agarose to prepare the overlay staining medium (1× MEM, 0.8% agarose and 0.25 mg/ml neutral red). On days 4–6, 2 ml of the overlay staining medium was added to the overlay medium of each well. After the overlay staining medium had set, the plate was again incubated in an upside-down position for an additional 3 d at 37 °C under 5% CO₂. When plaques were not clear, the plate was left for an additional 1 d or 2 d. For a total of 6–10 d, the plaques were visualized as white circles.

The assay was tested by selection of several media for LLC-MK₂ cells or HEp-2 cells. Overlay medium consisting of 1× MEM, DMEM or SFM, 0.8% agarose, 3 or 5 μg/ml acetylated trypsin without or with 2% BSA and without or with 5% FBS and overlay staining medium consisting of 1× MEM, DMEM or SFM, 0.8% agarose, 0.25, 0.16 or 0.08 mg/ml neutral red without or with 5% FBS were used

for hPIV1 and SeV plaque formation assay in LLC-MK₂ cells. Overlay medium consisting of 1× DMEM, 0.8% agarose and 3 μg/ml acetylated trypsin and overlay staining medium consisting of 1× DMEM, 0.8% agarose and 0.25 mg/ml neutral red were used for hPIV1 and hPIV3 plaque formation assay in HEp-2 cells.

Immunological Detection of Virus-Infected Cells

After plaque formation, the cell monolayers in a well were fixed with 3 ml of ethanol–acetic acid mixture (5 : 1) for 2 d at 4 °C. The overlay media were removed carefully and the cell monolayers were washed twice with PBS. hPIV1-infected cells were detected by probing with a rabbit anti-hPIV1 antibody diluted 1 : 200 with PBS at room temperature for 30 min, followed by horseradish peroxidase (HRP)-conjugated Protein A (Sigma-Aldrich, St. Louis, MO, U.S.A.) at room temperature for 30 min as described previously.¹⁹⁾ Each step was preceded by washing wells three times with PBS. Finally, the virus-infected cells were stained by addition of an immunostaining reagent containing H₂O₂, *N,N*-diethyl-*p*-phenylenediamine sulfate and 4-chloro-1-naphthol in 100 mM citrate buffer (pH 6.0) as described previously.^{20,21)} The cells were washed with deionized water and dried.

Plaque Reduction Assay To examine inhibition of virus growth, rabbit anti-hPIV1 antibody dilutions (1 : 100, 1 : 200 or 1 : 400) as an inhibitor were mixed with the overlay medium. As a control for complete inhibition of hPIV1 growth, the overlay medium without acetylated trypsin was used. The overlay staining medium was the same as that described for the plaque formation assay.

RESULTS

Plaque Formation Assay for the hPIV1 Laboratory Strain C35 and the Clinical Isolate CL-5

Several parameters including cell lines, types of overlay medium, staining reagents, various brands of agarose and carboxy methyl cellulose, and culture conditions were tested to establish a plaque assay for hPIV1 isolation. Plaque assay conditions we tried for the establishment were summarized in Table 1. As a result, we found that hPIV1 produced large plaques in LLC-MK₂ cells, which were detected by the addition of a second overlay staining medium (1× MEM, 0.8% agarose and 0.25 mg/ml neutral red) after incubation with the initial overlay medium (1× MEM, 0.8% agarose and 3 μg/ml acetylated trypsin) for 4 d without cell fixation. On day 4 after virus infection, plaques were barely visible. After staining with neutral red for an additional 3 d, plaques were clearly visualized as white circles in a virus dilution-dependent manner (Fig. 1A). By immunological detection of the virus-infected cells after fixing with an ethanol–acetic acid mixture (5 : 1) for 2 d at 4 °C, all plaques were clearly stained dark-gray as foci. Some foci that were not visible in a plaque assay with neutral red were also detected as small foci (Fig. 1B). Plaques of the clinical isolate CL-5 were also visualized similar to the laboratory strain C35 (Fig. 1C). Therefore, the assay was also useful for detection and isolation of the hPIV1 clinical strain as well as the laboratory strain.

Additionally, we demonstrated that hPIV3 also formed visible plaques in LLC-MK₂ cells under the same conditions as those for the hPIV1 virus. We also tried to isolate respective

viruses from the mixture of hPIV1 strain C35 and hPIV3 strain C243, and hPIV1 and hPIV3 could be separated from each other by picking individual plaques (data not shown). This result confirmed that the assay is useful for isolation of

the hPIV1 strain.

Selection of Appropriate Media and Cell Lines Recombinant SeV and hPIV3 have been reported to form plaques respectively in LLC-MK₂ cells^{10–12)} and HEp-2 cells.⁶⁾ We compared our established method to the reported methods by using an overlay medium consisting of 2% BSA or 5% FBS and an overlay staining medium consisting of 0.08 mg/ml neutral red and 5% FBS (Fig. 2A). Both SeV and hPIV1 plaques were clearly visible by our method (Method

Table 1. Summary of hPIV1 Plaque Assay Parameters and Results

Cell	Medium	Additive	Day	Result
HEp-2	DMEM	0.5 mg/ml neutral red*	7	—
MDCK	MEM	0.5 mg/ml neutral red*	7	—
HeLa	DMEM	0.5 mg/ml neutral red*	7	—
HEp-2	DMEM		15<	—
LLC-MK ₂	SFM		15<	—
LLC-MK ₂	MEM		15<	+
LLC-MK ₂	MEM	0.25 mg/ml neutral red	7–9	++
LLC-MK ₂	MEM	0.08 mg/ml neutral red, 2% BSA	9<	—
LLC-MK ₂	MEM	0.08 mg/ml neutral red, 5% FBS	9<	—
LLC-MK ₂	MEM	0.08 mg/ml neutral red	9<	+
LLC-MK ₂	MEM	0.25 mg/ml neutral red, 2% BSA	9<	+
LLC-MK ₂	DMEM	0.25 mg/ml neutral red	9<	+
LLC-MK ₂	SFM	0.25 mg/ml neutral red	9<	+
HEp-2	DMEM	0.25 mg/ml neutral red	9<	—

—, No plaques were observed. +, Few plaques were observed but they were too small and unclear. ++, Clear and large plaques were observed. *Neutral red was added as an aqueous solution after fixing the cells.

Fig. 1. Plaque Formation by hPIV1 Laboratory Strain C35 (A, B) and Clinical Isolate CL-5 (C) in LLC-MK₂ Cells

(A, C) Plaques were visualized by staining under agarose overlay containing neutral red. (B) Cell monolayers shown in panel A were fixed, and virus-infected cells were immunostained using rabbit anti-hPIV1 antibody and HRP-conjugated protein A.

Fig. 2. Selection of Media and Cell Lines for Plaque Assay

(A) Comparison of the hPIV1 plaque formation assay and reported methods for recombinant SeV in LLC-MK₂ cells. Method 1 was performed in the same way as in Fig. 1A. In accordance with a previous report on recombinant SeV, method 2 was performed using an overlay medium consisting of 1× MEM, 0.8% agarose, 5 μg/ml acetylated trypsin and 2% BSA and an overlay staining medium consisting of 1× MEM, 0.8% agarose, 0.08 mg/ml neutral red and 5% FBS. Method 3 was performed using an overlay medium consisting of 1× MEM, 0.8% agarose, 5 μg/ml acetylated trypsin and 5% FBS and an overlay staining medium of the same composition as that used in Method 2. (B) Comparison of the concentration of BSA and neutral red. hPIV1 and SeV plaque formation assay in LLC-MK₂ cells was performed using an overlay medium consisting of 1× MEM, 0.8% agarose, 3 μg/ml acetylated trypsin and without or with 2% BSA and an overlay staining medium consisting of 1× MEM, 0.8% agarose and 0.25, 0.16 or 0.08 mg/ml neutral red. (C) Comparison of media. The hPIV1 plaque formation assay was performed using MEM, SFM and DMEM in LLC-MK₂ cells. Concentrations of agarose, acetylated trypsin and neutral red and other conditions were the same as those in as Fig. 1A, method 1. (D) Comparison of cell lines. The hPIV1 and hPIV3 plaque formation assay was performed with DMEM in HEp-2 cells. Concentrations of agarose, acetylated trypsin and neutral red and other conditions were the same as those in Fig. 1A, Method 1.

Fig. 3. Plaque Reduction Assay of hPIV1 Strain C35

The assay was performed as described in Materials and Methods in the presence of indicated dilutions of rabbit anti-hPIV1 antibodies, without antibodies (control) and without addition of acetylated trypsin (without AT).

1). On the other hand, in the reported methods using an overlay medium consisting 2% BSA (Method 2) or 5% FBS (Method 3) and an overlay staining medium containing 0.08 mg/ml neutral red and 5% FBS, SeV plaques were barely visible (unable to be scanned) and hPIV1 plaques were not observed. To confirm whether concentrations of neutral red or BSA were effective for hPIV1 plaque formation and visualization, we checked these concentrations (Fig. 2B). The hPIV1 plaques were more clearly visualized with 0.25 mg/ml neutral red than with 0.08 mg/ml neutral red without BSA. With 2% BSA, plaques were barely observed with 0.25 mg/ml but they were unclear, and no hPIV1 plaques were observed with 0.08 mg/ml. While BSA had little effect on SeV plaque formation, hPIV1 plaque formation, probably plaque visualization by neutral red staining, was strongly inhibited under the condition of 2% BSA. Neutral red was reported to bind to BSA with high affinity,²²⁾ and shift in the emission peak to shorter wavelengths on binding to BSA.²³⁾ BSA probably inhibited neutral red staining by preventing neutral red transport or pH-dependent color change. Wild-type hPIV1 formed no plaques in the same conditions as those reported by Alymova *et al.*^{10,11)} and Watanabe *et al.*¹²⁾ for the assay of recombinant SeV possessing HN of hPIV1. Recombinant SeV, which possesses the fusion protein of wild-type SeV, may easily form plaques because of the strong fusion activity and propagation.

Tests using three media in the assay showed that MEM was a better medium for clear plaque formation of hPIV1 than was SFM or DMEM (Fig. 2C). The assay was also performed by using HEp-2 cells, which had been used for hPIV3 plaque assay in a previous study,⁶⁾ instead of LLC-MK₂ cells. Although hPIV3 did show clear plaque formation as reported previously, no plaque of hPIV1 was observed in HEp-2 cells (Fig. 2D). Plaques of hPIV1 were also not observed in HeLa cells and Madin–Darby canine kidney cells (data not shown). Plaque assay conditions we tried in Fig. 2 were also summarized in Table 1.

Plaque Reduction Assay We examined inhibition of hPIV1 replication in the plaque reduction assay by addition of rabbit anti-hPIV1 antibody as an inhibitor to the overlay medium. Anti-hPIV1 antibody, which blocks attachment between the virus and the receptor on the host cell surface, significantly inhibited the number and size of plaques in a dose-dependent manner, compared to the overlay medium without the antibody (Fig. 3). No plaque was observed in the absence of acetylated trypsin, which activated the fusion glycoprotein on the virus membrane, as a control for complete inhibition. This result confirms that acetylated trypsin is essential for plaque formation of the virus (Fig. 3) and indi-

cates that our plaque reduction assay will be useful for screening inhibitors of hPIV replication.

DISCUSSION

We established a plaque formation assay in LLC-MK₂ cells for hPIV1 using an agarose overlay with neutral red, which easily visualized plaques. Normal live cells actively uptake neutral red and turn red. Although hPIV1-infected cells retain a normal shape when observed under an optical microscope because of low cytotoxicity, they actually show dysfunction of membrane transport processes such as endocytosis. The loss of neutral red uptake results in visualization of the virus-infected plaques as clear white circles. BSA inhibited visualization of hPIV1-induced plaques. BSA probably affected neutral red transportation or pH-dependent color change, which is critical for hPIV1 plaque visualization. hPIV1 is a noncytopathic virus whereas hPIV3 and SeV readily produce extensive cytopathic effects (CPE).^{13,14)} Therefore SeV induces megascopic plaques without neutral red. In contrast, hPIV1 plaques were only observed by neutral red uptake.

Our established plaque assay of hPIV1 is applicable to virus titration, virus isolation and evaluation of virus inhibitors, since the plaques in the assay are visible and alive. It is important to visualize plaques of clinical hPIV1 strains. Since the limiting dilution method has been the only way to isolate hPIV1 from clinical specimens, only a few laboratory strains were available for the present study. The limiting dilution method is useful for isolation of the viruses. However, the absence of CPE in many of the cell lines used commonly makes it difficult to confirm hPIV1 growth. Our results confirmed applicability of the assay to a clinical hPIV1 strain. The assay will be suitable for isolation of hPIV1 from clinical specimens and for increasing virus strains available for basic research of hPIV1. In addition, the isolation would greatly advance phylogenetic epidemiological analysis of clinical isolates and mutation analysis of variant viruses including inhibitor-resistant viruses.

The number of plaques is widely utilized as a general virological index of virus infection titer (often expressed as plaque-forming units). The plaque reduction assay is also widely utilized to evaluate efficiency of or resistance to antiviral reagents.^{15,16)} Our plaque assay will facilitate virus isolation, virus titration and evaluation of antiviral efficacy and will thus contribute greatly to the development of antiviral therapies, elucidation of infection mechanisms and epidemiology of hPIV1 prevalence.

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