High titer growth of human and avian influenza viruses in an immortalized chick embryo cell line without the need for exogenous proteases

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1. Introduction

Current influenza vaccine production technology is largely restricted to growth in embryonated hens' eggs and often requires one to two eggs per dose [1], which makes this process expensive and requires months of preparation. In addition, this scheme requires significant downstream processing to purify virus away from egg components and is subject to significant loss of product if the eggs are found to be contaminated with exogenous agents. There are also concerns that individuals who are allergic to eggs may experience adverse reactions to egg-derived vaccines [2,3].

Due to safety, time constraints, and consistency issues with egg-derived influenza vaccines, there has been a push from both regulatory agencies and major vaccine manufacturers to adopt a continuous cell culture-based influenza vaccine production system [5]. While influenza viruses generally grow efficiently on primary chick embryo kidney (CEK) cells [6,7], this system would be subject to many of the same concerns and issues surrounding egg-derived vaccines, particularly the potential presence of harmful contaminating pathogens. The existing continuous cell lines that are known to grow influenza virus, such as Madin-Darby Canine Kidney (MDCK) cells, African green monkey kidney cells (Vero) and human retinal cells (PER.C6) [8,9], require the addition of exogenous proteases, such as tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin, for propagation of the virus. Viruses grown in this manner are typically modified through reassortment and/or reverse genetics to allow high-titer growth. Thus, a well-characterized continuous cell line which could be used to establish a master cell bank that is non-tumorigenic and free of exogenous pathogens and adventitious agents is highly desirable [5].

Here, we report the use of an immortalized chick embryo cell line, termed PBS-1, which is capable of growing unmodified recent isolates of human and avian influenza A and B viruses to extremely high titers (>10^7 PFU/ml). PBS-1 cells are derived from an 11-day-old line C/CE chick embryos, which were chick helper factor negative (no env gene product) and specific pathogen-free [10]. The primary cells were passaged once, and then immortal-
ized by N-methyl-N′-nitro-N-nitrosoguanidine (MNGN) treatment [10]. There were no viruses or exogenous DNA used in the immortalization phase, and the entire process has been extensively documented internally. PBS-1 cells are free of any exogenous agents, are non-tumorigenic, and are readily adaptable to a variety of culture conditions, including growth on microcarrier beads. In addition to offering a significant improvement in vaccine production, our results demonstrate PBS-1 cells are valuable in simplifying influenza diagnostics.

2. Materials and methods

2.1. Cells

The Madin–Darby Canine Kidney (MDCK) and African green monkey kidney (Vero) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Primary chick embryo kidney (CEK) cells were purchased from Charles River Laboratories (Boston, MA). PBS-1 cells were derived and obtained as described previously [10]. All of the cell lines were cultured using Dulbecco’s Modified Eagle Medium (DMEM) (Gibco®, Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gibco®), 100 IU/ml penicillin G, and 100 μl/ml streptomycin sulfate (Gibco®).

2.2. Viruses

Human influenza virus strains A/NewCaledonia/20/1999 (H1N1), A/HongKong/26252/2006 (H1N1), A/Hiroshima/52/2005 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Florida/07/2004, and B/Malaysia/2506/2004 were generously provided by the Centers for Disease Control and Prevention (CDC, Atlanta, GA). Human influenza strains A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), Alice (H3N2), and B/HongKong/5/72 were purchased from ATCC. Avian influenza strain A/Whooper Swan/Mongolia/244/05 (H5N1) was kindly tested at and provided by the United States Department of Agriculture Endemic Poultry Viral Disease Research Unit (Athens, GA).

2.3. Virus infection and propagation

PBS-1, MDCK, Vero, and CEK cells were cultured in DMEM containing 10% FBS, 100 IU/ml penicillin G, and 100 μl/ml streptomycin sulfate in 48 well plates for 24 h (density of 3.5 × 10⁴ cells per well for PBS-1 and CEK cells, and 1 × 10⁴ cells per well for MDCK and Vero cells). Once the cells reached 80% confluency (24 h), they were washed twice with phosphate buffered saline (PBS) and inoculated with 100 μl volumes of A/NewCaledonia/20/1999, A/HongKong/26252/2006, A/Hiroshima/52/2005, A/Wisconsin/67/2005, B/Florida/07/2004, and B/Malaysia/2506/2004, giving multiplicities of infection (MOI) of 1:1 (one virus per cell). Each plate also contained an uninfected well of each cell type as control. The infection media consisted of DMEM with 5% FBS and 10 mM HEPES (Sigma–Aldrich, St. Louis, MO). Cultures were grown at 37 °C for 1 h in a 5% CO₂ incubator. After 1 h, the media was removed and 200 μl of fresh infection media was added. The infection media used for MDCK and Vero cells was also supplemented with 2 μg/ml tansyl phenylalanyl chloromethyl ketone (TPCK)-Trypsin (Gibco®). The cells were incubated until they reached 50% death, or until day 7. The supernatants were centrifuged for 5 min at 500 × g to remove cell debris. Hemagglutination (HA) assays and titer measurements were performed on all supernatants to determine virus yield as described below.

2.4. Hemagglutination assay

The HA assay was performed by serially diluting 25 μl of culture supernatants 2-fold with PBS in V-bottom plates. Subsequently, 25 μl of 1% chicken red blood cells (Innovative Research, Inc., Southfield, MI) were added to each well. The plates were incubated for 1 h at 4 °C and the HA patterns were determined visually.

2.5. Virus infectivity assay

Titration of infectious virus was performed on CEK cells. CEK cells were seeded in 96 well plates at a density of 1.0 × 10⁴ cells/well in DMEM containing 10% FBS, 100 IU/ml penicillin G, and 100 μl/ml streptomycin sulfate and incubated at 37 °C until the cells reached 80% confluency (24 h). Culture supernatants were diluted 10-fold starting at 10⁻¹ and diluted down to 10⁻⁷ in infection media consisting of DMEM with 5% FBS (GIBCO) and 10 mM HEPES (Sigma). The CEK cells were inoculated in sextuplicate with 100 μl diluted virus sample. After 2 days of incubation at 37 °C, immunostaining was performed as described below for detection of infected cells. The 50% tissue culture infective dose (TCID₅₀) was determined via the Reed and Muench method [11].

2.6. Immunostaining assay

Supernatants of infected cells were removed and the monolayers were washed 2 times with PBS. They were then fixed by incubating with Bouin’s solution (Sigma) for 1 min, washed 2 times with PBS, and permeated with a 1:1 solution of methanol:acetone. After rinsing 3 times with Tris-buffered saline (TBS), monolayers were blocked with 5% horse serum (Gibco) and washed 2 more times with TBS. Monoclonal mouse anti-influenza virus primary antibodies specific for hemagglutinin of H1N1 (HyTest, Turku, Finland), H3 (Chemicon® International, Temecula, CA), and nucleoprotein (Chemicon®) antigens were used for H1N1, H3N2, and type B strains, respectively. The primary antibodies were diluted 1:1000 with TBS containing 1% horse serum. Following 30 min incubation of the primary antibodies, the monolayers were washed 3 times with TBS. A secondary goat anti-mouse IgG antibody conjugated to alkaline phosphatase (BioLegend, San Diego, CA) and an alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) were used to detect primary antibody binding.

2.7. Isolation and identification of influenza virus from respiratory samples

The samples were sent in transport media and were not previously isolated in cell culture or embryonated chicken eggs. Each sample was diluted 1:4:1 with DMEM containing 5% FBS and 10 mM HEPES used to infect 80% confluent PBS-1 cells in triplicate. Following 96 h cultivation, the monolayers were immunostained using H1, H3, and B antibodies as described in Section 2.

3. Results

3.1. PBS-1 cells are susceptible to human and avian influenza virus

PBS-1 cells were shown to be susceptible to human and avian influenza when they were infected with influenza strains for two days at a MOI of 1 (Fig. 1). The cells were immunostained using a monoclonal antibody against the specific influenza subtype and a secondary antibody conjugated to alkaline phosphatase as described in Section 2. The uninfected PBS-1 cells remained con-
Fig. 1. Susceptibility of PBS-1 cells to human and avian influenza virus as detected by immunostaining. PBS-1 cells were infected with influenza strains at a MOI of 1. After two days of incubation, PBS-1 cells were immunostained using a monoclonal mouse anti-influenza antibody against the specific influenza subtype and an alkaline phosphatase goat anti-mouse IgG antibody as described in Section 2.

fluent throughout the experiment; however, all of the infected cells experienced 25-50% cell death.

3.2. PBS-1 cells support replication of human influenza strains at greater titers than CEK cells

Once PBS-1 cells were proven to be susceptible to influenza virus, we wished to determine the titer of infectious virus produced from CEK and PBS-1 cells. PBS-1 cells had comparable, and sometimes greater, virus titers compared to CEK cells (Table 1), with the exception of B/Malaysia/2506/2004. Virus production was determined through virus infectivity and HA assays. The log10 TCID50 titer varied depending on the virus strain, but A/NewCaledonia/20/1999 and A/HongKong/2654/2006 influenza strains reached a titer of >7.5 when grown on CEK and PBS-1 cells. A/Wisconsin/67/2005, A/Hiroshima/52/2005, and B/Florida/07/2004 strains also grew more effectively on PBS-1 cells than on CEK cells. The fold increases of titers for PBS-1 cells compared to CEK cells were 10^3.6 for A/Wisconsin/67/2005, 10^0.7 for A/Hiroshima/52/2005 and 10^2.2 for A/Hiroshima/52/2005. Strain B/Malaysia/2506/2004, however, grew better on CEK cells with nearly an 8-fold increase compared to PBS-1 cells. The number of viral particles present, determined via the HA assay, generally followed the same patterns as the TCID50 results. The maximum HA titer of 320 was reached for A/NewCaledonia/20/1999 and A/HongKong/2654/2006 in PBS-1 cells. The virus supernatants of A/NewCaledonia/20/1999, A/Wisconsin/67/2005 and A/HongKong/2654/2006 from PBS-1 cells were also tested for virus infectivity using TCID50 and HA titers. All three strains were shown to have successfully replicated on PBS-1 cells (TCID50 > 7.5). Strain A/Wisconsin/67/2005 did not agglutinate chicken red blood cells even though high titers were reached. It has previously been shown that some recent H3N2

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>CEK infection</th>
<th>PBS-1 infection</th>
<th>PBS supernatant infection on CEK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log10 TCID50</td>
<td>HAU/ml</td>
<td>log10 TCID50</td>
</tr>
<tr>
<td>A/NewCaledonia/20/1999</td>
<td>&gt;7.5</td>
<td>320</td>
<td>&gt;7.5</td>
</tr>
<tr>
<td>A/Wisconsin/67/2005</td>
<td>2.9</td>
<td>0</td>
<td>&gt;6.5</td>
</tr>
<tr>
<td>A/HongKong/2654/2006</td>
<td>&gt;7.5</td>
<td>160</td>
<td>&gt;7.5</td>
</tr>
<tr>
<td>A/Hiroshima/52/2005</td>
<td>2.2</td>
<td>0</td>
<td>2.9</td>
</tr>
<tr>
<td>B/Florida/07/2004</td>
<td>4.0</td>
<td>80</td>
<td>6.2</td>
</tr>
<tr>
<td>B/Malaysia/2506/2004</td>
<td>3.4</td>
<td>80</td>
<td>2.5</td>
</tr>
</tbody>
</table>

CEK and PBS-1 cells were infected with human influenza strains at a MOI of 1. Supernatants from the PBS-1 infection were reinfected on 80% confluent CEK cells for growth comparison. TCID50 titers were performed by immunostaining and HA activity was determined for all supernatants through an HA assay.
strains lose the ability to agglutinate chicken red blood cells [12,13], and may be what is occurring.

3.3. Human influenza replication comparison with CEK, PBS-1, Vero, and MDCK cell supernatants

The TCID50 titers of CEK cell supernatants were compared with supernatants from PBS-1, MDCK, and Vero cells (Table 2). The supernatants from all cell lines were used to infect 80% confluent CEK cells for direct growth comparison. Only MDCK and Vero cells contained media supplemented with 2 µg/ml of TPCK-trypsin. All cells were infected with influenza virus strains at an MOI of 1. With the exception of A/NewCaledonia/20/1999, the supernatants from PBS-1 cells exhibited higher titers than those of CEK, MDCK, and Vero cells. In fact, there was no observable virus replication from the Vero cell supernatants. Only one of the five strains, A/NewCaledonia/20/1999, was able to grow on MDCK cells. Importantly, none of these virus strains were previously adapted to any of the cell lines tested.

3.4. PBS-1 cells can be used for the isolation and identification of influenza virus from respiratory samples for diagnostic testing

Nineteen blind influenza samples consisting of throat swab samples were sent from the Michigan Department of Community Health (MDCH) (Lansing, MI). These samples were independently tested by the MDCH before being tested by us and their results remained concealed until our results were obtained. All of the samples were successfully identified as type A influenza using PBS-1 cells and, more specifically, consisted of 21% H3N2 and 79% H1N1 subtypes through immunostaining procedures. These results were identical to those sent from the MDCH.

4. Discussion

Due to increased demand for seasonal influenza vaccination, reduced and variable vaccine supply, and the threat of pandemic influenza [5], cell culture-derived influenza vaccines are being considered in addition to, or in replacement of, egg-based production. Some potential advantages of cell-derived influenza vaccines are uniform characterization of the production cells, reduced risk of exogenous or endogenous adventitious agents, more consistent means of vaccine production by using a closed bioreactor process, and the ability to grow avian influenza without genetic modification [5]. Our results demonstrate that PBS-1 cells can support replication of influenza strains to sufficient titer for vaccine production and decreased downstream production methods by not requiring trypsin, thereby providing ideal characteristics of cell lines that could be utilized for vaccine production. Additionally, this cell line has previously been shown to be non-tumorigenic [10].

Our studies describe the use of PBS-1 cells for the isolation and replication of human and avian influenza viruses. The data shows that a wide range of influenza subtypes are capable of infecting and replicating on PBS-1 cells (Fig. 1). Importantly, all of the strains tested propagated in PBS-1 cells without being previously adapted to this cell line. Once we determined influenza strains could propagate in PBS-1 cells, we wished to determine the quantity of influenza virus produced from PBS-1 cells.

Two commonly used methods for monitoring influenza virus replication in vitro were used: HA assay and the measurement of virus infectivity titers in culture supernatants [14,15]. The titer and HA assays were used to demonstrate all of the strains replicate to greater titers, with the exception of B/Malaysia/2506/2004, on PBS-1 cells than on CEK cells. Data in Table 2 compared influenza replication in CEK cell supernatants with replication in PBS-1, Vero, and MDCK supernatants. Once again, PBS-1 cells developed higher influenza titers than CEK cells, with the exception of A/NewCaledonia/20/1999. Although MDCK and Vero cells were infected with culture media containing 2 µg/ml of TPCK-trypsin, which extracellularly cleaves the precursor protein of hemagglutinin (HA0) into active hemagglutinin (HA1 and HA2) for adsorption of the influenza viruses on the cells [16], they were unable to replicate virus at results comparable to CEK and PBS-1 cells. One possible explanation for this is that the TPCK treated trypsin was inhibited with the addition of FBS. Since PBS-1 cells did not require TPCK-trypsin, the problem of inhibiting virus replication with FBS is eliminated. Another explanation for reduced virus replication in our study using MDCK and Vero cells could be due to the virus strains not being previously adapted to these cell lines. It has been demonstrated that most influenza strains need to be adapted to Vero cells for efficient growth [17,18].

Previous studies have also shown that Vero cells only occasionally yield detectable HA following infection with influenza virus [19,20]. Vero cells rapidly destroy exogenous trypsin [21], which limits the replication of influenza viruses with a noncleavable HA to a single cycle. With repeated addition of trypsin to the culture medium, however, replication is possible in this cell line with high virus growth yields [18]. This time consuming process, which also increases the risk of contamination, can be eliminated in PBS-1 cells.

The isolation and identification of influenza virus results from the 19 blind respiratory samples within our study showed that PBS-1 cells were a useful tool for typing unknown influenza strains in diagnostic samples. Studies examining the usefulness of PBS-1 cells for diagnostics need to be performed in the future. PBS-1 cells will be compared with the MDCK cells, which is one of the most recommendable cell lines for diagnostic samples [22].

In summary, our novel results show that PBS-1 cells offer a viable alternative system to produce human and avian influenza viruses. PBS-1 cells are susceptible to human and avian influenza virus

### Table 2

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>CEK supernatant infection (%)</th>
<th>PBS-1 supernatant infection (%)</th>
<th>Vero supernatant infection</th>
<th>MDCK supernatant infection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/NewCaledonia/20/1999</td>
<td>100</td>
<td>71</td>
<td>***</td>
<td>100</td>
</tr>
<tr>
<td>A/Wisconsin/67/2005</td>
<td>100</td>
<td>1122</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>A/Hiroshima/52/2005</td>
<td>100</td>
<td>447</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>B/Florida/7/2004</td>
<td>100</td>
<td>100</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>B/Malaysia/2506/2004</td>
<td>100</td>
<td>126</td>
<td>a</td>
<td>a</td>
</tr>
</tbody>
</table>

All cell types were infected with influenza virus at an MOI of 1 and incubated until 50% cell death or 7 days. Only MDCK and Vero cells contained media supplemented with 2 µg/ml of TPCK-trypsin. Supernatants from all cell lines were reinfected on 80% confluent CEK cells for growth comparison. TCID50 titer measurements were performed by immunostaining. Virus titers of CEK cells were standardized to 100% and used to compare with virus titers of supernatants from all other cell lines.

* No observable virus replication.
growth, can grow influenza virus to high titers, and can be used for the isolation and identification of influenza virus from respiratory samples for diagnostic testing. Unlike Vero and MDCK cells, PBS-1 cells do not require TPCK-treated trypsin for adsorption of the influenza viruses on the cells. In addition to PBS-1 cells being less prone to contaminating pathogens compared to primary CEK cells, they are a continuous cell line that can survive hundreds of passages while remaining non-tumorigenic [10].

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References