New technologies for the control of influenza

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Suitability of PER.C6® cells to generate epidemic and pandemic influenza vaccine strains by reverse genetics

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Abstract

Reverse genetics, the generation of influenza viruses from cDNA, presents a rapid method for creating vaccine strains. The technique necessitates the use of cultured cells. Due to technical and regulatory requirements, the choice of cell lines for production of human influenza vaccines is limited. PER.C6® cells, among the most extensively characterized and documented cells, support growth of all influenza viruses tested to date, and can be grown to high densities in large bioreactors in the absence of serum or micro carriers. Here, the suitability of these cells for the generation of influenza viruses by reverse genetics was investigated. A range of viruses reflective of vaccine strains was rescued exclusively using PER.C6 cells by various transfection methods, including an animal component-free procedure. Furthermore, a whole inactivated vaccine carrying the HA and NA segments of A/HK/156/97 (H5N1) that was both rescued from and propagated on PER.C6 cells, conferred protection in a mouse model. Thus PER.C6 cells provide an attractive platform for generation of influenza vaccine strains via reverse genetics.

Introduction

Influenza A and B viruses cause annual epidemics in the human population worldwide. In addition, subtypes of influenza A viruses previously not circulating among humans occasionally cross from animals reservoirs and cause a pandemic. Vaccination is the primary means to control influenza-associated morbidity and mortality. The genomes of influenza A and B viruses consist of eight negative-sense RNA segments. When two viruses infect a single cell, viruses with new combinations of genomic segments, called reassortants, may arise. This property is used for the production of most influenza vaccines to combine the antigenic properties of target circulating strains with favorable growth characteristics and safety profile of laboratory strains. For inactivated vaccines and live attenuated vaccines respectively, the egg-adapted A/Puerto Rico/8/34 (PR8) strain and cold-adapted strains such as A/Ann Arbor/6/60 and B/Ann Arbor/1/66 are typically used to provide the RNA segments that encode the internal proteins of the virion. Traditionally, such reassortants are derived by coinfecting embryonated hens’ eggs with the circulating strain and the backbone strain, and subsequent screening of the progeny for viruses that have the haemagglutinin (HA) and neuraminidase (NA) segments of the first and at least those internal segments of the second that confer a high growth phenotype. This process is cumbersome, time consuming and cannot be done with viruses that are incapable of egg growth. Significant improvements in terms of speed, safety and applicability of the use of reassortants as vaccine seed strains are offered by the plasmid-based reverse genetics technology [1-5] which allows for the generation of influenza viruses entirely from...
cloned viral cDNA. This was initially achieved for A/WSN/33 virus by cotransfection of eukaryotic cells with 8 plasmids encoding the viral sense RNA under control of a human RNA Polymerase I (Pol I) promoter and 4 to 9 plasmids encoding mRNA for different viral proteins under the control of an RNA Polymerase II (Pol II) promoter [6, 7]. Successful recovery of recombinant viruses using plasmid-based reverse genetics relies on the transfection efficiency of the cells used, and efficient amplification of the recombinant virus. Therefore, a mixture of 293T cells that have a high transfection efficiency, and MDCK cells permissive for influenza virus infection is often used in research settings [7-11]. However, 293T cells are not suitable for the generation of reassortants intended for human use. Among the cell types that are being explored as substrates for the production of influenza vaccines are African green monkey Vero cells, Madin-Darby canine kidney (MDCK) cells, and E1-immortalized human retinal PER.C6 cells [5]. Of these, Vero cells have been used most extensively for the recovery of vaccine strains by reverse genetics [2, 4, 12-15]. However, this process is hampered by the low plasmid transfection efficiency of these cells [16-18], and recovered virus requires amplification in a more permissive substrate like eggs, MDCK cells, or chicken embryo fibroblast cells. Due to the species specificity of the human RNA polymerase I, plasmid systems using the human pol I promoter can not be used efficiently on MDCK cells, although a rescue system based on a T7 polymerase vector [19], and canine RNA Pol I-driven systems that allow for reverse genetics on MDCK cells were reported recently [16, 19, 20]. A candidate human H7N1 vaccine produced on PER.C6 cells that was based on a reverse genetics derived reassortant has been described previously [15]. However, this virus had been generated using a co-culture method with Vero cells and chicken embryonic fibroblasts. The method would be greatly simplified both from a technical and a regulatory perspective if recombinant viruses could be recovered directly from PER.C6 cells. Since PER.C6 cells are both highly permissive for influenza viruses [21] and can efficiently be transfected, we here assess the suitability of these cells for the generation of influenza viruses by reverse genetics. We demonstrate that a variety of viruses and 6:2 reassortants were successfully rescued using 12 or 10 plasmid systems. Furthermore we have established a completely animal component-free rescue procedure which eliminates the risk of transmissible spongiform encephalopathies (TSE) and allows for the generation of recombinant vaccine seed viruses and production of vaccine using cells from only one qualified Master Cell Bank. To demonstrate the utility of reverse genetics on PER.C6 cells for vaccine purposes, we set up a lethal mouse model for influenza A/HK/156/97 (H5N1) and tested the protective efficacy of a whole inactivated vaccine based on a reassortant that was both rescued from and propagated on these cells.
**Materials and methods**

**Viruses**

Influenza viruses A/PR/8/34 (H1N1), A/Panama/2007/99 (H3N2), IVR-116 (high growth reassortant of A/New Caledonia/20/99 (H1N1)), NYMC X-161B (high growth reassortant of A/Wisconsin/67/2005 (H3N2)), and NIBRG14 (reverse genetics derived reassortant containing the HA and NA of A/Viet Nam/1194/2004 (H5N1)) were obtained from the NIBSC (Herts, UK). Influenza virus A/Hong Kong/156/97 (H5N1) was originally isolated from a 3-year-old child suffering from respiratory disease [22]. This virus has been passaged two times on MDCK cells. The viral stock used to infect mice was propagated once more in embryonated chicken eggs. The highly pathogenic H5N1 virus was handled in the BSL3 facility at the Central Veterinary Institute, Lelystad.

**Plasmid DNA**

The 12 plasmid system for the rescue of A/PR/8/34 has been described before [23]. The 10 plasmid system was constructed as follows: after eleven passages of A/PR/8/34 on PER.C6 cells, RNA was isolated using the QIAamp® Viral RNA Mini Kit (Qiagen) and cDNA of all 8 segments was made with the Superscript one-step RT-PCR with Platinum Taq kit (Invitrogen) using universal degenerate primers AGTAGAACAAGGNNNTTTTTT and AGCRAAAGCAGG. Each segment was subsequently amplified with Pwo DNA polymerase (Roche) using segment-specific primers (sequences available upon request) and cloned into a PCR®4 blunt TOPO® vector (Invitrogen). A DNA fragment containing a human Pol I promoter and a mouse terminator sequence separated by a linker containing two SapI restriction sites was synthesized by Geneart (Regensburg, Germany) and cloned in pPCR-Script vector. The SapI site in the backbone was removed by deletion of a TfiI segment, giving rise to a vector named pST which is essentially the same as plasmid pPollSapiT described by Subbarao et al., [14]. The cloned PR8 segments were amplified with specific primers containing SapI sites at the 5'-ends and directionally cloned into the pST vector (primer sequences available upon request).

The coding regions of PB2, PB1, PA, and NP of PR8 were cloned into two expression plasmids: one carrying PB1 and PB2 separated by an internal ribosomal entry site (IRES) sequence of EMCV (Clontech) and another carrying NP and PA also separated by an IRES sequence. These double constructs were cloned in pCDNA3/neo (Invitrogen) between a CMV promoter and BGH polyadenylation site.

The plasmids used for the rescue of A/Victoria/3/75, B/Beijing/87 and A/Chicken/Italy/1347/99 have been described elsewhere [15, 24, 25]. The genome segments encoding the haemagglutinin (HA) and neuraminidase (NA) of A/Panama/2007/99 (H3N2), NYMC X-161B, NIBRG14 (H5N1) and the NA segment of IVR-116, were amplified by RT-PCR and cloned into pST as above, using RNA isolated directly from the reconstituted NIBSC material. Since cloning of the HA segment of IVR-116 repeatedly failed, this sequence was made synthetically (Baseclear) and Pol I promoter and terminator sequences were fused to this segment by PCR. The resulting PCR fragment was used for transfection directly. RNA from A/HK/156/97 was isolated from allantoic fluid of infected eggs and the HA and NA segments were cloned as above, except that the HA cleavage site was modified as described previously [14]. All plasmids described here for the first time were prepared in accordance with WHO guidelines for infection control for transmissible spongiform encephalopathies (TSE).
Cells

PER.C6 cells [26] were cultivated either adherently in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum and 10 mM MgCl₂, or in suspension in Adeno Expression Medium (AEM) (Invitrogen) supplemented with 4 mM L-glutamine (Invitrogen) at 37°C in 10% CO₂ atmosphere. To allow for influenza replication, medium was replaced for virus growth medium, consisting for 2/3 of AEM medium supplemented with 4 mM L-glutamine and 1/3 of VP-SFM AGT™ (Invitrogen), supplemented with 3 µg/ml trypsin/EDTA (Invitrogen) or an equivalent amount of TrypLE select (Invitrogen). MDCK cells were obtained from the American Type Culture Collection and were maintained in DMEM containing 10% FBS at 37°C in 10% CO₂ atmosphere.

Generation of recombinant viruses

Transfection of 293T/MDCK co-cultures

Rescue of recombinant influenza virus from 293T cells in co-culture with MDCK cells was performed as described previously [24]. Briefly, 293T cells cultured in DMEM supplemented with 3% FCS were seeded in 12 well plates to reach 70-80% confluency the next day. Twenty µl Fugene reagent (Roche) was incubated with 200 µl DMEM at room temperature for 5 minutes before being mixed with 0.5 µg of each of the 8 Pol I plasmids and the Pol II plasmids encoding the PB1 and PB2 segments, 0.05 µg of the Pol II plasmid encoding PA and 1 µg of that encoding NP. After a further 15 minutes of incubation, this mixture was added dropwise to the cells which were subsequently incubated o/n at 37 °C, 10% CO₂. The next day, 293T cells were detached with trypsin/EDTA, mixed with a suspension of MDCK cells, seeded in a 25 cm² flask and incubated for 6 hours at 37 °C, 10% CO₂ to allow the cells to attach. After washing of the cells with DMEM, 5 ml DMEM supplemented with pyruvate and 2.5 µg/ml trypsin was added and cell were incubated at 33 °C, 10% CO₂ until cytopathic effect was observed and supernatant was harvested.

Transfection of adherent PER.C6 cells with 12 plasmid system

PER.C6 cells were seeded at a density of 1 x 10⁶ cells in a 6-well plate in DMEM supplemented with 10% FBS and 10 mM MgCl₂ and incubated o/n at 37 °C, 10% CO₂. The next day, medium was replaced with DMEM with 2% FBS, 4 mM L-glutamine and 10 mM MgCl₂. Twenty five microliters Lipofectamine 2000 (Invitrogen) was mixed with 250 µl Optimem (Invitrogen) and incubated at room temperature. After 5 minutes, a mixture of 1 µg of each of the 8 plasmids for the synthesis of viral RNAs (pPol I PB1, -PB2, PA1, HA, NP, NA, M, NS) and of the plasmids pPol II PB1 and pPol II PB2, 0.1µg of pPol_ II PA and 2 µg of pPol II NP in 250 µl Optimem (Invitrogen) was added and incubated for a further 20 minutes at room temperature. This transfection mix was added dropwise to the cells, which were subsequently incubated at 37 °C, 10% CO₂. After 3 hours, the medium was replaced with virus growth medium and cells were resuspended by pipetting and incubated on a rotating platform at 37 °C, 10% CO₂ for 7 days after which supernatant was harvested.

Transfection of adherent PER.C6 cells with 10 plasmid system

PER.C6 cells were seeded at a density of 12 x 10⁶ cells in a 75 cm² flask in DMEM supplemented with 10% FBS and 10 mM MgCl₂, and incubated o/n at 37 °C, 10% CO₂. The next day, 120 µl Lipofectamine (Invitrogen) was mixed with DMEM (end volume 300 µl) and incubated for 5 minutes at room temperature. Subsequently, 300 µl DMEM
containing 24 µg DNA (2.4 µg of each of the 10 plasmids) was added and incubated at RT for 40 minutes. After incubation, 5.9 ml DMEM was added, and the mixture was added to PER.C6 cells that had been washed once with unsupplemented DMEM. After 3 hours of incubation at 37 °C, 10% CO₂, the DNA-transfection mixture was replaced by virus growth medium and cells were incubated at 37 °C, 10% CO₂ for 7 days after which supernatant was harvested.

Transfection of suspension PER.C6 cells with 10 plasmid system
5 x 10⁶ suspension PER.C6 cells cultured in AEM medium supplemented with 4 mM L-glutamine were centrifuged for 5 minutes at 300g and resuspended in 100 µl solution T (Amaxa). A mixture of 1 µg of each of the 10 plasmids was added to the cells and nucleofection was performed using a Nucleofector apparatus (Amaxa), according to manufacturer’s instructions. Cells were subsequently resuspended in 10 ml virus growth medium, seeded in a 25 ml shaker flask and incubated on a rotating platform at 37 °C, 10% CO₂ for 7 days after which supernatant was harvested.

Vaccine preparation for use in mice
Inactivated vaccines were prepared by infecting suspension PER.C6 cells in virus growth medium with wild type A/HK/156/97 and reassortant rgPR8-H5N1-HK/97 viruses at an MOI of 0.001. Five days post-infection, 10 U/ml Benzonase (Sigma) was added together with 2 mM MgCl₂ (final concentration). After incubation for 30 minutes at 37 °C, the cell suspension was centrifuged for 20 minutes at 3500 RPM and supernatant was harvested. Upon one freeze-thaw cycle, supernatant was buffered with HEPES (final concentration of 50 mM) and β−propiolactone (BPL; Ferak Berlin) was added to a final concentration of 0.1%. After incubation for 44 hours at 4 °C, BPL was inactivated by incubation at 37 °C for 2 hours. Inactivation was confirmed by the absence of detectable infectivity after two blind passages of the treated supernatant in embryonated eggs. Inactivated viruses in the supernatant were clarified by centrifugation (30 minutes at 4000 RPM at 4 °C), filtered through 0.45 µm and 0.2 µm filters and concentrated by crossflowfiltration using a Sartorius Sartoflow Slice2000 300kD filter. Aliquots were stored at -70 °C. The haemagglutinin content was standardized by the single radial immunodiffusion technique [27].

As adjuvant, double oil emulsion (DOE) was used. The oil phase of the vaccine was made by adding 9 parts of Marcol-52 to 1 part of Montanide-80. The oil phase was emulsified with the HA antigen (first aqueous phase) and subsequently with 2% Montanox-80 (second aqueous phase) to achieve a 1:2 ratio of oil to water.

Chicken pathogenicity test
The standardized OIE chicken pathogenicity test was conducted at the Central Veterinary Institute, Lelystad, at BSL 3. Ten 6-week-old chickens were inoculated intravenously with 0.1 ml of a 1/10 dilution of the egg-grown A/HK/156/97 virus, or an equal dose (determined by TCID₅₀) of the reassortant rgPR8-H5N1-HK97 virus, and observed over a period of 10 days. The in vitro pathogenicity index (IVPI) was determined according to the OIE’s manual of diagnostic tests and vaccines for terrestrial animals.
Influenza vaccine strain generation via reverse genetics on PER.C6 cells

Infection of mice and challenge experiment

Female 7-week-old SPF BALB/c mice (Charles River Laboratories) were used in all experiments. For infections, animals were anesthetized with ketamin/xylazin intraperitoneally and inoculated intranasally with 50 µl infectious virus diluted in PBS. The LD50 of A/HK/156/97 was determined by inoculation groups of 20 mice with 2, 2.5, 3, 3.5, 4, 4.5, or 5 log TCID50 of egg-grown A/HK/156/97 virus diluted in 50 µl PBS. One group of 10 mice received an equal volume of PBS. The mice were weighed and observed daily for signs of disease and mortality for 14 days. To evaluate the degree of protection from lethal challenge, vaccinated mice were infected as above 4 weeks after the second vaccination with 25 LD50 of A/HK/156/97 virus and weighed and observed daily for signs of disease and mortality for 14 days.

Viral titration

Virus titres in allantoic fluids and culture supernatants were determined by either plaque assay or TCID50 on MDCK cells as described previously [21, 24].

Serologic testing

Immune sera from blood samples were treated with receptor destroying enzyme from Vibrio Cholera, heat-inactivated at 56 °C for 30 minutes, preadsorbed with 5% turkey red blood cells (TRBCs), and tested by HI assay with 1% TRBCs and 4 HAU of A/HK/156/97, according to standard methods [28]. Individual mice were considered to have responded to vaccination if serum HI titres were ≥ 40.

Statistical analyses

The LD50 of A/HK/156/97 in BALB/c mice was determined from the serial dilutions by Probit analysis and found to be 3.1 log TCID50 (95% CI 2.7-3.5). The log transformed HI titres in serum samples from mice that received vaccines based on HK/97 or PR8-HK/97 were compared with an independent t-test. After challenge the vaccinated mouse groups were compared to the control group for differences in survival proportions using Chi-square tests. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., USA)
Results

Establishment of reverse genetics on PER.C6 cells

To assess if PER.C6 cells can be used for the generation of influenza virus by reverse genetics, cells grown adherently in static culture were transfected with 12 plasmids routinely used for the rescue of PR8 virus from 293T/MDCK co-culture. Daily harvests of cell supernatant were tested for the presence of virus by plaque assay on MDCK cells. In parallel, a virus rescue using the same plasmids and an established method of 293T/MDCK coculture [24] was performed. Figure 1 illustrates a typical rescue performed in PER.C6 cells or by the co-culture method. Recombinant PR8 virus was detected at day 2 post-transfection using the co-culture method and similar titres of rescued virus were obtained at day 3 after transfection using PER.C6 cells. Peak recovery of virus was obtained 6 days after transfection. These results demonstrate that PER.C6 cells can be transfected with a sufficiently high efficiency to be used for the generation of influenza viruses from 12 plasmids.

In parallel, a 10 plasmid system for the rescue of a PER.C6-adapted PR8 virus was constructed in which the coding regions of PB1 and PB2 and those of NP and PA are separated by an IRES sequence and cloned between a CMV promoter and BGH polyadenylation site. Transfection of adherent PER.C6 cells with these 10 plasmids consistently resulted in high viral yields after seven days. The geometric mean of the titres obtained in 6 independent experiments was $2.5 \times 10^7$ pfu/ml (Figure 2).

Figure 1. Comparison of rescue of recombinant A/PR/8/34 virus from 293-T cells in co-culture with MDCK cells (CC) and PER.C6 cells (PER.C6). Cells were transfected with 12 plasmids for the rescue of A/PR/8/34 and supernatants were harvested 1 to 7 days after transfection. Virus titres were determined by plaque assay on MDCK cells. The dotted line indicates the limit of detection.

Rescue of a range of recombinant influenza viruses from PER.C6 cells

Using the established protocols, we next attempted to generate influenza viruses with genome compositions reflective of vaccine strains (Table 1). All viruses attempted were successfully recovered. The viral titres in supernatants of transfected cultures obtained in individual rescues varied from $2.3 \times 10^8$ pfu/ml for PR8, to less than 10 (below the detection limit of the plaque assay) for PR8-HK/97, a reassortant with the internal segments of
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A/PR/8/34 and HA and NA segments of A/Hong Kong/156/97. Nevertheless, this virus was recovered after inoculating a fresh culture of PER.C6 cells with this supernatant. Sequencing of the HA RNA of PR8-HK/97 reassortant virus confirmed the sequence to be identical to that of the transfected plasmid cDNA, illustrating that the human PER.C6 cells can be used to faithfully amplify avian influenza viruses whose receptor binding preference is for the α2–3 linked sialic acid receptor.

Table 1. Viruses rescued from adherent PER.C6 cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>Virus titre (pfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34</td>
<td>H1N1</td>
<td>5 x 10^6-2 x 10^8</td>
</tr>
<tr>
<td>A/Victoria/3/75</td>
<td>H3N2</td>
<td>8 x 10^3</td>
</tr>
<tr>
<td>B/Beijing/87</td>
<td>B</td>
<td>1 x 10^4</td>
</tr>
<tr>
<td>6:2 A/PR/8/34 + A/New Caledonia/20/99 HA &amp; NA</td>
<td>H1N1</td>
<td>1 x 10^2</td>
</tr>
<tr>
<td>6:2 A/PR/8/34 + A/Victoria/3/75 HA &amp; NA</td>
<td>H3N2</td>
<td>2 x 10^5</td>
</tr>
<tr>
<td>6:2 A/PR/8/34 + A/Panama/2007/99 HA &amp; NA</td>
<td>H3N2</td>
<td>19</td>
</tr>
<tr>
<td>6:2 A/PR/8/34 + A/Chicken/Italy/1347/99 HA &amp; NA</td>
<td>H7N1</td>
<td>5 x 10^7</td>
</tr>
<tr>
<td>6:2 A/PR/8/34 + A/Viet Nam/1194/2004 HA &amp; NA</td>
<td>H5N1</td>
<td>9 x 10^3</td>
</tr>
<tr>
<td>6:2 A/PR/8/34 + A/Hong Kong/156/97 HA &amp; NA</td>
<td>H5N1</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

* Sequence of the HA cleavage site modified to remove pathogenic traits

Reverse genetics on suspension PER.C6 cells

The rescues described above have all been performed on adherent cells cultured in the presence of serum. However, PER.C6 cells can grow in suspension in the absence of serum and such cells from qualified Master Cell Banks are used for the propagation of influenza viruses. From practical and regulatory points of view, it would be beneficial to be able to generate vaccine seed viruses and propagate these using cells from a single cell bank. This was achieved firstly by culturing suspension PER.C6 cells statically in the presence of 10% serum, thereby adapting them to adherent growth. Subsequent transfection of these
cells using Lipofectamine as above resulted in the rescue of recombinant viruses (data not shown). In a second approach, suspension PER.C6 cells were transfected directly. Since transfection of PER.C6 cells grown in AEM medium using Lipofectamine was inefficient (data not shown), we attempted to rescue influenza viruses using a Nucleofector device (Amaxa). Transfection of 5 x 10^6 suspension cells with 1µg of each of the 10 plasmids resulted in the presence 3.1 x 10^7 pfu/ml of PR8 in the culture supernatant after seven days. Using this method, reassortant PR8-HK/97 was recovered with a titre of 3.4 x 10^4 pfu/ml. An extra benefit of this approach is that it allows for the serum-free generation of vaccine seed viruses.

Protective efficacy of an inactivated vaccine based on PR8-HK/97

The PR8-HK/97 virus was used as a model to study the protective efficacy of a vaccine based on a reassortant that was both rescued from and propagated on PER.C6 cells. For safety reasons, the HA cleavage site had been modified to attenuate the virus. Indeed, unlike the wild type A/HK/156/97 virus, PR8-HK/97 was unable to form plaques in a monolayer of MDCK cells in the absence of trypsin. Furthermore, the pathogenicity of both viruses was compared using the standardized OIE chicken pathogenicity test. The IVPI of A/HK/156/97 is 2.9 out of a possible maximum of 3.0, indicating that this virus is highly virulent in chickens. In contrast, the IVPI of PR8-HK/97 is 0.0 as inoculation with this virus did not result in any signs of infection, indicating that this virus is completely apathogenic in chickens. The reassortant PR8-HK/97 virus was expanded on PER.C6 cells, inactivated with BPL, and concentrated by tangential flow filtration. To assess the protective efficacy of this whole virus vaccine, a group of 8 mice was immunized twice (on day 0 and 21) subcutaneously with 1.6 µg HA of PR8-HK/97 adjuvanted with double oil emulsion (DOE) and challenged four weeks later (day 49) by intranasal inoculation of 25 LD_{50} of A/HK/156/97. Clinical signs and weights were recorded daily until 14 days after infection. As a positive control, a group of 8 mice was immunized as above with 1.6 µg HA of a similarly prepared vaccine based on wild type A/HK/156/97. As challenge control, mice received DOE alone. Figure 3A shows the HI antibody titres in individual serum samples collected immediately prior to challenge. Mock vaccinated mice all had a titre below the limit of detection and were assigned a value of 10. The geometric means of the HI titres in serum of mice that received vaccines based on wtHK/97 or PR8-HK/97 were 698 and 453, respectively. These titres were not significantly different (p=0.15). The percentages of mice surviving infection and median clinical scores per group are shown in figures 3B and C, respectively. All vaccinated mice survived challenge whereas 7 animals (87.5%) of the control group died within 14 days after infection (p<0.001). Onset of respiratory distress was observed on day 6 post infection in all mock-vaccinated animals, and continued until the animal died or, in one case, until the end of the study. In contrast, no signs indicative of disease and no weight loss were recorded in any of the
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Figure 3. Protective efficacy of inactivated vaccine based on a reassortant virus rescued from and produced on PER.C6 cells. Groups of mice (n=8) were vaccinated s.c. twice (day 0 and 21) with 1.6 µg DOE-adjuvanted inactivated vaccine based on wild type A/HK/156/97 (HK/97) or reassortant A/PR/8/34 virus with the HA and NA segments of A/HK/156/97 (PR8-HK/97). As a challenge control, a group of 8 mice was mock vaccinated with DOE. Four weeks after the second vaccination (day 49), blood samples were collected and mice were challenged intranasally with 25 LD50 of A/HK/156/97 and observed daily for clinical signs and weighed daily until 14 days after infection. Clinical signs were scored with a scoring system (0 = no clinical signs; 1 = rough coat, 2 = rough coat, less reactive, passive during handling; 3 = rough coat, rolled up, laboured breathing, passive during handling; 4 = rough coat, rolled up, laboured breathing, unresponsive). Graphed are HI titres (A), Kaplan-Meier survival curves (B), and median clinical scores (C) for each group. Each circle in A represents the geometric mean of duplicate HI measurements per animal. Horizontal bars indicate geometric mean titres per group. The dotted line indicates the limit of detection.
Discussion

Reverse genetics is already the method of choice for the generation of seed viruses for vaccines against potentially pandemic strains and may be used for seasonal vaccines in the future. A prerequisite for the use of reverse genetics in vaccine production is the use of a suitable cell line. The PER.C6 cell line has been extensively documented and cell banks were prepared and characterized according to FDA and European Medicines Agency (EMEA) guidelines.

Here we have demonstrated that these cells can readily be used for the generation of recombinant influenza viruses. Rescued viruses include PR8-based reassortants containing the HA and NA segments of avian influenza subtypes H5N1 and H7 (identified by the WHO as most likely to cause a pandemic [29]), A/Panama/2007/99 (H3N2), A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 and also an influenza B virus, B/Beijing/87. Transfections were performed with unidirectional plasmid systems comprising of 12 and 10 plasmids, and 10 out of 10 viruses were obtained directly in supernatant fluids. In attempts to improve the efficiency of influenza virus rescue from Vero cells, variants of the original 12 plasmid system requiring reduced numbers of plasmids [10, 11, 18] or using adenoviral vectors instead of plasmids [17] have been described. Although these systems can undoubtedly be used on PER.C6 cells, the transfection efficiency is not a limiting factor for viral rescue from 12 plasmids on these cells. Unlike Vero cells [2, 4, 12-15], PER.C6 cells are both sufficiently transfectable and permissive for influenza facilitating rescue of recombinant viruses in this substrate alone.

In these studies we rescued recombinant A/PR/8/34 virus from PER.C6 cells using both a previously described set of 12 plasmids cloned from the PR8 stock provided by the NIBSC, UK (15) (figure 1), and a newly generated 10 plasmid system based on a PER.C6-passaged PR8 virus (figure 2). Although A/PR/8/34, which is traditionally used to confer a high-growth phenotype in eggs [30], replicates well on PER.C6 cells, other viruses may have even more favorable growth characteristics on this substrate and could be used as backbone strain. The generation of recombinant A/Victoria/3/75 and B/Beijing/87 viruses demonstrates that reverse genetics on PER.C6 cells is not limited to A/PR/8/34 and reassortants based on this strain. The rapid generation of safe reassortants that is not reliant on supply of chicken eggs is particularly crucial for the production of vaccines against potentially pandemic influenza viruses. In this study, as a model to obtain proof of concept, we generated an inactivated vaccine based on a 6:2 reassortant rescued from, and propagated on PER.C6 cells that protected against a stringent lethal challenge with a highly pathogenic avian H5N1 strain.

We have shown that recombinant influenza viruses can be generated in adherent PER.C6 cells using a transfection reagent appropriate for production of vaccines approved for human use (Lipofectamine 2000 [4, 15]). Furthermore, we have established
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a cGMP compliant method for the rescue of influenza viruses from suspension PER.C6 cells cultured in serum-free chemically defined growth medium, resulting in a procedure devoid of any animal-derived components. This improved methodology offers advantages over existing methods in terms of process simplicity (only cells from one qualified Master Cell Bank required for both the generation of the vaccine seed strain and vaccine production) and compliance to regulatory guidelines.

Together, our results demonstrate that PER.C6 cells are a suitable substrate for the generation of recombinant influenza virus vaccines by reverse genetics.

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PER.C6® is a registered trademark owned by Crucell Holland B.V.

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