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A rapid method for immunotitration of influenza viruses using flow cytometry

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Abstract

Reliable assays for accurate titration of influenza virus in infectious samples are pivotal to both influenza research and vaccine development. A titration assay adopted commonly for this purpose is the plaque assay on Madin–Darby canine kidney (MDCK) cells, despite it being time and labour consuming. A novel assay is described for titration of influenza viruses based on the detection of intracellular viral nucleoprotein (NP) by fluorescence-activated cell sorting (FACS). By using a panel of viruses of different type, subtype and origin, it is demonstrated that there is a mathematical correlation between titres measured by immunotitration and by classical plaque assay on MDCK cells. Moreover, the availability of NP antibodies specific for type A or type B influenza virus ensures the specificity of the assay. Based on speed, accuracy and specificity, it is concluded that the FACS-based immunotitration of influenza virus represents a valid and efficient alternative to the classical plaque assay.

Keywords: PER.C6; MDCK; Influenza virus; Plaque assay; Titre; Method

1. Introduction

Reliable and efficient quantitation of influenza virus titres is of critical importance for a variety of applications, including basic research, laboratory detection, and vaccine development and manufacture. Therefore, several titration methods have been developed that detect different biological characteristics of the virus, such as the number of virion-associated RNA molecules, the amount of a specific viral protein, or the number of cell-free virions (reviewed by Rimmelzwaan et al., 1998). A commonly adopted definition of infectious titre is the so-called plaque-forming unit (pfu), which is determined by plaque assay. The plaque assay for influenza virus was first described three decades ago (Gaush and Smith, 1968) and since then it has undergone little modification. The assay is based on the appearance of localised foci of infection denoted by cell lysis within a monolayer of cells, covered by a solid overlay immediately after infection. The overlay prevents the formation of secondary infections, thus allowing only direct cell-to-cell spread of the virus. MDCK is historically the cell line of choice for plaque assay for influenza virus, as these cells support productive infection and plaque formation of most strains of A and B influenza viruses (Gaush and Smith, 1968; Tobita, 1975; Tobita et al., 1975). Although sensitive, plaque assays are time consuming and labour intensive, requiring up to several days depending on the virus type, subtype and source. Moreover, plaque counting is done manually, which limits the throughput and increases the risk of introducing errors when handling numerous samples.

A novel assay is described for the determination of influenza virus titres based on flow cytometry and compared with the classical plaque assay in terms of specificity, accuracy and throughput. By using this FACS-based assay, accurate titres can be determined within a couple of hours at 5 h post infection, as

Abbreviations: fiu, FACS infectious units; MDCK, Madin–Darby canine kidney; m.o.i., multiplicity of infection; MVSS, master virus seed stock; NP, nucleoprotein; pfu, plaque forming units; p.i., post infection.

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opposed to several days required for the plaque assay on adherent MDCK cells. In addition, a large number of samples can be treated simultaneously as the assay is performed in micro titre plates. Finally, titres are determined by FACS rather than by visual inspection by an operator. This reduces the risk of errors due to subjective interpretation of the results and increases the reproducibility of the assay.

2. Materials and methods

2.1. Cells and viruses

PER.C6 is a human cell line generated by immortalisation of primary human retinoblasts with an E1-mini gene of human adenovirus serotype 5 (Fallaux et al., 1998). PER.C6 cells were shown previously to support the replication of influenza virus type A and B (Pau et al., 2001). PER.C6 cells were grown in suspension in serum-free AEM medium (Gibco-BRL) at 37 °C and 10% CO_2. MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% heat-inactivated fetal bovine serum (Gibco) and 2 mM L-glutamine (Gibco), and maintained at 37 °C and 10% CO_2.

All influenza viruses were obtained from the National Institute for Biological Standards and Control (NIBSC) and are produced in embryonated hen's eggs. The following viruses were passaged on suspension PER.C6 to produce master virus seed stocks (MVSS): X-127 (type A, H1N1), Resvir-17 (type A, H3N2), B/Harbin/7/94 (type B), B/Guangdong/120/2000 (type B). The following viruses were not passaged on cell culture: A/New Caledonia/20/99 (type A, H1N1) and B/Guangdong/120/2000 (type B). These are indicated in the text as NIBSC.

MVSSs were produced on PER.C6 cells as follows. Virus samples were diluted serially in suspension media containing 3 μg/ml trypsin/EDTA. Cells were seeded in 6-well plates (1 ml/well at a density of 2×10^6 cells/ml) and 1 ml of each virus dilution was added to each well. Plates were incubated at 35 or 34 °C (influenza A or B, respectively). At day 3, 4, and 5 p.i. (type A viruses) and day 5, 6, and 7 p.i. (type B viruses), cultures were harvested and centrifuged at 5000 rpm (Eppendorf Microfuge) for 5 min. Supernatants were aliquoted in cryotubes (500 μl/tube) and snap-frozen in liquid nitrogen. Infectious titres were measured subsequently by plaque assay. Based on the dilution and day of harvest showing the highest titre, serial dilutions corresponding to m.o.i. of 10^{-4}–10^{-6} were used to infect PER.C6 as above. This process was repeated at least two times in 6-well plate and finally scaled up to roller bottle (50–150 ml cultures). After collection and titration, samples were stored at −80 °C and used as MVSS.

For UV-inactivation, an X-127 virus supernatant was dispensed onto a 96-well plate. The plate was then irradiated with UV by exposure under a 30 W UV lamp for 15 min. The lamp was located inside a laminar flow cabinet at a distance of about 30 cm from the open plate. After irradiation, PER.C6 cells were added and the titration was carried out as described in Section 2.3.

2.2. Plaque assay

Except for few minor modifications, plaque assays on MDCK cells were carried out essentially as described previously (Tobita et al., 1975). Briefly, MDCK cells (95% confluent in 6-well plates) were washed twice with DMEM without trypsin and subsequently inoculated with serially diluted viral supernatants (1 ml). After 1 h at 35 or 34 °C (influenza A or B, respectively), cells were washed once with PBS and covered with 3 ml of an agarose overlay, containing 1.2 ml 2.5% agarose (SeaPlaque, BMA), 1.5 ml 2× MEM, 30 μl 200 mM L-glutamine, 24 μl 500 μM trypsin/EDTA, and 250 μl PBS. Cells were incubated at 35 or 34 °C (influenza A or B, respectively) for 3–5 days, until plaques became detectable by visual inspection. Cells were then fixed without removing the overlay by treatment with 1.5 ml 5% gluteraldehyde (Sigma) for 2 h at room temperature. After fixation, plates were washed under the tap to remove the overlay and stained with 1.5 ml 5% carbol fuchsin (BDH Laboratory Supplies) for 30 min at RT. After washing, the plates were dried and the plaques scored visually.

2.3. FACS titration

PER.C6 cells were resuspended at 1×10^6 cells/ml in fresh infection medium (AEM medium containing 3 μg/ml trypsin/EDTA). Cells were then plated in a 96 well U-bottomed plate, 20 μl/well corresponding to 20 000 cells. Cells were infected by addition of 200 μl virus supernatant diluted serially in infection medium. A MVSS of known titre was included as a positive control. Following addition of the virus, cells were kept for 5 h at 35 or 34 °C (type A or B viruses, respectively) and subsequently stained as follows. Cells were washed once with PBS and fixed/permeabilised in 100 μl cold Cytoperm/Cytofix (Pharmingen). After 20 min incubation at 4 °C, cells were washed with cold PBS and resuspended in cold staining solution (PBS, 1% BSA, 0.1% sodium azide) containing 1 μl FITC-labelled antibody against type A or type B NP (Imagen Kit, Dako). Cells were incubated at 4 °C for 20 min and washed subsequently once with cold PBS to remove unbound antibody. Finally, cells were fixed with Cell Fix (Becton Dickinson) and stored at 4 °C for further FACS analysis. Stained cells were analysed on a FACS

Calibur apparatus (Becton Dickinson) according to standard procedures.

3. Results

3.1. FACS-based influenza virus titration

An overview of the method is depicted schematically in Fig. 1A. The assay entails infection of PER.C6 cells in suspension by serial dilutions of the supernatant of interest. The cells are collected 5 h after infection, i.e. within the first replication round. Although positive cells can be detected also at earlier times p.i., it was found that the best results with all virus strains are obtained at this time point (not shown).

Subsequently, cells are stained with a FITC-labelled antibody specific for the nucleoprotein (NP) of influenza virus type A or B. Cells are then analysed by flow cytometry, plotting fluorescence (FLI-H) versus forward scatter (FSC-H), an indicator of cell size. Infected cells are identified by FACS due to the fluorescence conferred by the NP-specific antibody, and they appear in the top right quadrant of the plot. Results of a representative FACS analysis are depicted in Fig. 1B, showing that the percentage of NP-positive cells is dependent inversely on the virus dilution used for the infection. The percentage of NP-positive cells is then plotted against the dilutions of virus supernatant used (Fig. 1C). The titre is expressed as FACS infectious units/ml (fiu/ml) and is calculated using the formula given in Fig. 1D. In this mathematical equation, C represents the number of cells used for the initial infection, D the dilution at which 50% of the cells are NP-positive by FACS, and V the volume (ml) of virus used for the infection.

3.2. FACS titres are equivalent to titres obtained by plaque assay

It was first determined whether and how closely FACS titres reflect titres obtained by plaque assay. A broad panel of influenza viruses was selected containing different strains of type A and B viruses produced on different platforms. These included wild type as well as reassortant viruses produced on cultured cells (MVSS) and in eggs (NIBSC). The titre of each sample was determined by FACS and plaque assay, and the results were plotted as a head-to-head comparison (Fig. 2A). It was found that for all viruses the titres determined by

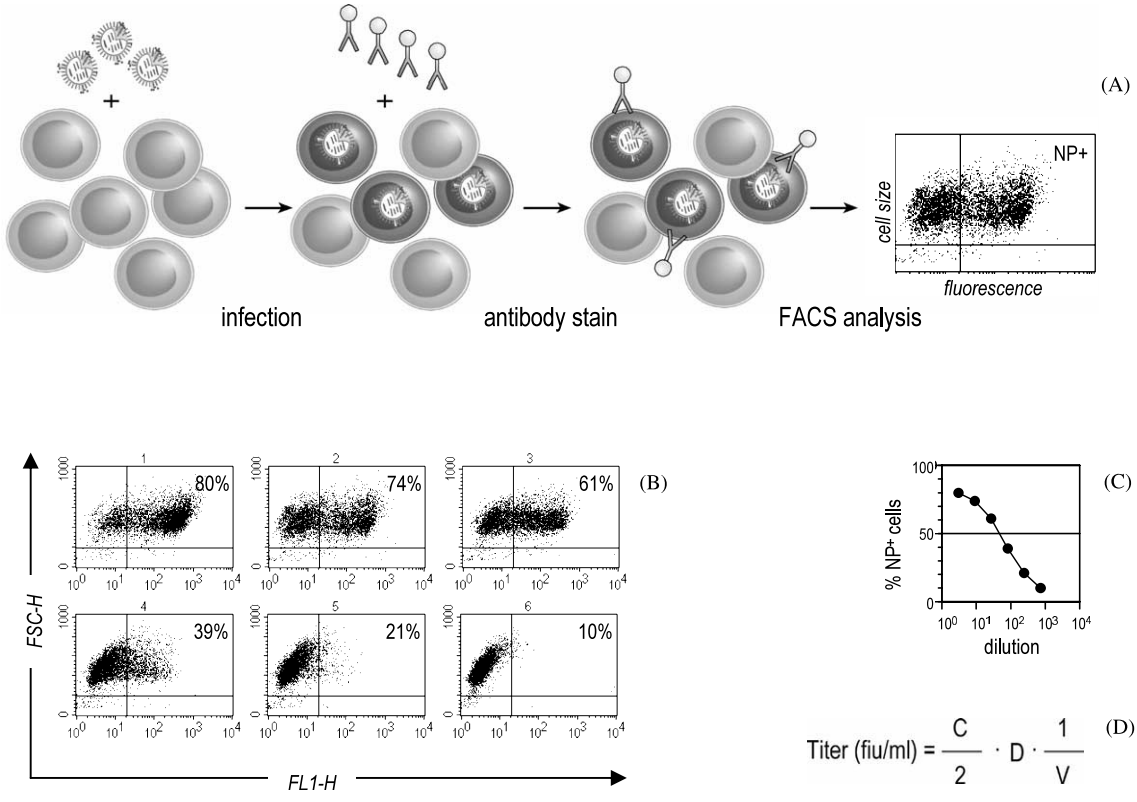

Fig. 1. FACS titration on PER.C6 cells. (A) Schematic representation of the procedure. (B) FACS plots resulting from infection of PER.C6 with serial viral dilutions (graph 1–6). The upper right quadrant of each graph represents the NP^+ cells. (C) The number of infected cells (percent) is plotted against the dilutions used. The dilution that infects 50% of the cells is calculated. (D) Formula for the calculation of FACS titres expressed in fiu/ml. C is the number of cells used for the initial infection, D the dilution at which 50% of the cells are infected, and V the volume (ml) of virus used for infection.

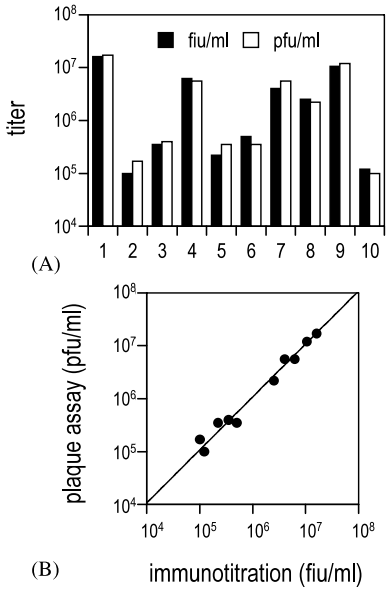

Fig. 2. FACS titres correspond to titres determined by plaque assay. (A) A panel of type A and type B viruses of different strains and origin were titrated by FACS and plaque assay. Viruses indicated as MVSS and NIBSC derive from PER.C6 cultures and eggs, respectively. Lanes 1, 2, 3, X-127 MVSSs (type A, H1N1); lane 4, Resvir-17 MVSS (type A, H3N2); lanes 5 and 6, B/Harbin/7/94 (type B); lane 7 and 8, B/Guangdong/120/2000 MVSSs (type B); lane 9, NIBSC A/New Caledonia/20/99 (type A, H1N1); lane 10, NIBSC B/Guangdong/120/2000 (type B). (B) Correlation between titres obtained by immunotitration and by plaque assay of the viruses described in panel A.

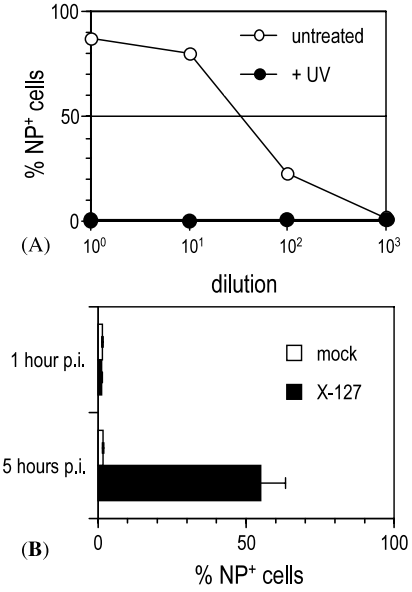

Fig. 3. FACS titres reflect the number of infectious particles. (A) Infection of PER.C6 cells with X-127 MVSS inactivated by UV treatment. The titration curve is compared with that of the same sample without UV treatment. (B) Detection of NP at 1 and 5 h p.i. using 0.5 m.o.i. of X-127 MVSS.

plaque assay (pfu/ml) corresponded closely with the FACS titres (fiu/ml). Moreover, no difference in behaviour was observed between viruses produced on eggs and viruses produced in PER.C6 cultures. When the fiu/ml and the pfu/ml values were plotted against each other (Fig. 2B), a good correlation was evident ($R^2 = 0.988$).

3.3. FACS titres correspond with the number of infectious particles

For the validity of the assay, it is critical to exclude that the NP detected by immunotitration is either introduced into the cells by replication-deficient virions or produced in cells infected by defective particles. It was first determined whether the NP^+ FACS signal is derived solely by replication-competent particles. A sample of X-127 MVSS was split in two and either UV-irradiated for 15 min or left untreated. Subsequently both samples were titrated independently on PER.C6 cells by FACS and the titration curves were compared. As shown in Fig. 3A, inactivation of the virus by UV irradiation completely abrogated detection of NP, as compared with the untreated control.

It was next investigated whether the NP detected truly represents protein produced intracellularly by the replicating virus. Hereto, cells were infected and FACS-analysed at 1 or 5 h p.i., a time at which one replication cycle is almost completed (Fig. 3B). No signal could be detected at 1 h p.i. in either the uninfected control (mock) or the infected culture. In contrast, at 5 h p.i. the expected percent of cells (about 50%) were clearly positive as compared with the negative control. Finally, in the absence of the permeabilising agent no NP signal could be detected in any sample (not shown). These results indicate that the NP signal measured by flow cytometry is generated by protein that is produced in the cells by infectious virions.

3.4. Specificity and sample stability of FACS-based immunotitration

In order to determine the specificity of the assay, a panel of influenza viruses containing two type A MVSSs (X-127, an H1N1 virus, and Resvir-17, an H3N2 virus), and one type B MVSS (B/Guangdong/120/2000) was used to infect PER.C6. Samples of infected cells were collected and stained using antibodies specific for either type A or type B NP. The percentage of NP^+ cells was determined by FACS and is plotted in Fig. 4. No cross-reactivity was observed between type A and B viruses, as type-mismatched antibodies produced only background signal.

Finally, the stability of the samples was determined at the end of the staining procedure. After infection with X-127 and antibody staining, cells were fixed and FACS-analysed at different times after fixation. No difference in titre was detected over time, demonstrating the stability of the samples at 4 °C. Identical results were

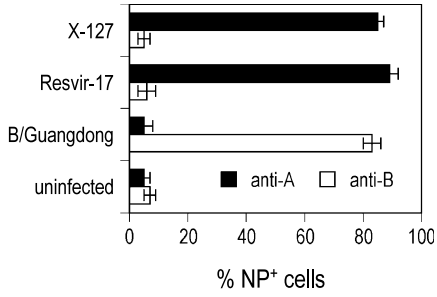

Fig. 4. FACS titration is type-specific. MVSS of X-127 (type A, H1N1), Resvir-17 (type A, H3N2), and B/Guangdong/120/2000 (type B) were used to infect PER.C6 cells. Subsequently, cells were stained using both type specific anti-NP antibodies and FACS analysed.

obtained for all strains tested of both type A and type B viruses (not shown).

4. Discussion

A novel assay is described for titration of influenza viruses that relies on the detection of the influenza NP protein in the human PER.C6 cell line by flow cytometry. NP, a key protein in the life cycle of influenza viruses, represents a suitable marker of on-going viral replication. Expression of NP in infected cells occurs at an advanced step of the virus life cycle, being dependent on efficient virus adsorption, internalisation, uncoating, and mRNA synthesis (Lamb, 1989). Moreover, NP is involved in the regulation of the switch between mRNA and genomic RNA synthesis, the assembly of the viral riboNP complex, and its nuclear export (reviewed by Portela and Digard, 2002).

Due to its high degree of conservation within viruses of the same type and low inter-type homology (less than 50%), NP is also an ideal target for antibody-based detection (Walls et al., 1986). In fact, antibodies specific for type A or type B NP are commercially available and routinely used for virus detection in infected samples. FACS immunotitration combines the specificity of the anti-NP antibodies with the precision and rapidity of flow cytometry. Consequently, accurate titres of many virus samples can be obtained in 1 day, whereas plaque assays require several days and are labour intensive. Most importantly, titres obtained by immunotitration correspond with those obtained by standard plaque assay on MDCK cells. Owing to its rapidity, reliability and ease of execution, immunotitration by FACS represents a valid and efficient alternative to the plaque assay.

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