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Cellular impedance measurement as a new tool for poxvirus titration, antibody neutralization testing and evaluation of antiviral substances

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ABSTRACT

Impedance-based biosensing known as real-time cell electronic sensing (RT-CES) belongs to an emerging technology for analyzing the status of cells *in vitro*. In the present study protocols were developed for an RT-CES-based system (xCELLigence™, Roche Applied Science, ACEA Biosciences Inc.) to supplement conventional techniques in pox virology. First, proliferation of cells susceptible to orthopoxviruses was monitored. For virus titration cells were infected with vaccinia virus and cell status, represented by the dimensionless impedance-based cell index (CI), was monitored. A virus-dose dependent decrease in electrical impedance could be shown. Calculation of calibration curves at a suitable CI covering a dynamic range of 4 log enabled the quantification of virus titers in unknown samples. Similarly, antiviral effects could be determined as shown for anti-poxviral agents ST-246 and Cidofovir. Published values for the *in vitro* concentration that inhibited virus replication by 50% (IC_{50}) could be confirmed while cytotoxicity in effective concentrations was excluded in long-term incubation experiments. Finally, an RT-CES-based virus neutralization test was established. Various poxvirus-specific antibodies were examined for their neutralizing activity and a calculation mode for the neutralizing antibody titer was introduced. In summary, the presented RT-CES-based methods outmatch end-point assays by observing the cell population throughout the entire experiment while workload and time to result are reduced.

1. Introduction

Virological standard procedures include virus titration for estimating the number of infectious particles [1], plaque reduction neutralization tests (PRNT) for determining neutralizing antibody (Ab) titers [2] and characterization of antiviral agents by means of cytotoxicity and antiviral effects [3,4]. Although these techniques are laborious, they are irreplaceable for (orthopox) virus research. Hence, simplified and time-saving approaches might be beneficial.

Orthopoxvirus (OPV) titration is usually performed on a cell layer within microtiter vessels by infection of cells with serial dilutions of a viral stock solution. After a 3–5 day incubation period followed by washing and staining procedures, virally induced plaques are quantified to calculate the number of infectious particles (or plaque-forming units [PFU]) within the viral stock solution. Based on this quantification, antiviral substances and virus neutralizing Abs can be evaluated. For the evaluation of a compounds' antiviral activity determination of its cytotoxicity is a necessity and commonly performed by colorimetric assays using WST-1 or MTT [5]. Ab neutralizing activities are conventionally determined by preincubation of virus particles and Abs before being used in a quantitative plaque titration assay. These simple and potent techniques are laborious and, as end-point assays, allow assessment only after a defined time span, but not during the time course of the experiment.

In order to simplify these techniques by reducing work load and hands-on time the xCELLigence™ RT-CES system was employed for routine virological standard methods.

The utilized RT-CES system is equipped with 96-cavity plates that contain microelectronic impedance sensor arrays capable of measuring distinct changes in interactions between cells and vessel ground (see [6] for a detailed description). Changes in cell number, morphology and interplay with the cavity ground lead to measurable impedance shifts. These were recorded over time and represented by a dimensionless parameter called cell index (CI).

Abbreviations: CI, cell index; IC_{50}, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPV, orthopoxvirus; RT-CES, real-time cell electronic sensing; WST, water soluble tetrazolium salt.

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Thus, the system offers the possibility to non-invasively quantify cell status in real-time.

The general benefit of using RT-CES in cell culture experiments has been shown for live monitoring of cell growth and quality control [7–9], cytotoxicity assays [6,10,11], cell adhesion and spreading [12] immune cell activation [13], receptor tyrosine kinase activation [14], G protein-coupled receptor activation [15] as well as cell motion [16]. Preliminary tests presented in an application note by Roche Applied Science showed that impedance-based methods allow the monitoring of cell infection with vesicular stomatitis virus [17].

In this study, we systematically evaluated the RT-CES xCELLigence™ system for accurate poxvirus titration, antiviral compound testing including drug-related cytotoxicity and as a screening tool for OPV neutralizing Abs.

2. Materials and methods

2.1. Viruses and cell culture

HEK293 (ATCC ID: CRL-1573) and HEp-2 (ATCC ID: CCL-23) cells were both cultured in standard D-MEM medium containing 5% FCS and 2 mM L-glutamine, while VERO E6 (ATCC ID: CRL-1586) cells required an FCS concentration of 10%.

The following OPV were used: vaccinia virus (VACV) IHD-W (ATCC ID: VR-1441), VACV Western Reserve (WR, ATCC ID: VR-1354), VACV New York City Board of Health (NYCBOH, ATCC ID: VR-1536), VACV Copenhagen (COP, kindly provided by Dr. Albert Zimmermann), VACV Lister Elstree (LE, kindly provided by Bavarian Nordic), cowpox virus (CPXV) Brighton Red (BR, ATCC ID: VR-302), camelpox virus (CMLV) CP-19 (kindly provided by Dr. Hermann Meyer). High-titer viral stocks were produced on HEp-2 cells according to standard procedures [18].

2.2. Deployment of RT-CES in cell culture

The xCELLigence™ system (RTCA-SP; Roche Applied Science, Mannheim, Germany) used in this work consists of single-use E-plates inserted into an RTCA single-plate (SP) station which is located within the incubator. Moreover, an analyzer unit is placed outside the incubator and links the SP station with a computer. The RT-CES monitors the impedance of each distinct cavity of the E-plate and delivers CI values at time points specified by the user. The CI is calculated by the software according to Eq. (1):

$$\mathrm{CI} = \max_{i=1,\ldots,N}\left[\frac{R_{\mathrm{cell}}(f_i)}{R_{\mathrm{b}}(f_i)} - 1\right] \quad (1)$$

where R_{cell} stands for resistance of the electrode with attached cells and R_{b} stands for resistance of the electrode without attached cells.

The equation defines the CI as a dimensionless parameter which increases with higher cell numbers (or larger cells or stronger cell adhesion) and decreases with lower cell numbers (or smaller cells or weaker cell adhesion).

For evaluation of CI background, which is the CI signal without cells, E-plates were filled with 50 μl of pre-warmed culture medium. Thereafter cells were seeded into the E-plate cavities in pre-warmed culture medium and E-plates were placed within the plate station for monitoring of CI. To visualize the cell line-specific unique proliferation characteristics, growth curves of different cell numbers of each cell line used were recorded initially and cell numbers were optimized.

2.3. Virus titration

The conventional plaque titration test was performed on VERO E6 cells in 24-cavity plates by using tenfold serial sample dilutions from 10^{-1} to 10^{-6} according to standard procedures [18].

In order to perform virus titration with the xCELLigence™ system HEK293 cells were seeded at 7500 cells per cavity (as determined with a Neubauer chamber) into the E-plates as described above. After initial growth data collection for up to 16 h, cells were infected with VACV IHD-W (50 μl per cavity in pre-warmed culture medium) in concentrations ranging from 12.5 to 125,000 PFU/cavity. Finally, four VACV samples (LE, WR, NYCBOH, COP) of unknown titer were subjected to the adhered cells in three tenfold dilutions ($n = 3$). The CI value for all samples was measured for up to 100 h post infection (p.i.) in comparison to uninfected cells. The same samples were quantified by plaque titration in parallel.

2.4. Determination of antiviral effects and cytotoxicity of ST-246 and Cidofovir

ST-246 [19,20] and Cidofovir (CDV, Vistide® [21]) are known to display anti-OPV activity and were used for the evaluation of the RT-CES system's potential in characterizing antiviral compounds. Therefore, cytotoxicity and the *in vitro* concentration that inhibited virus replication by 50% (IC_{50}) of ST-246 and CDV were determined by RT-CES. Once background CI was recorded, 7500 HEp-2 cells/cavity were seeded into the E-Plate in a volume of 90 μl. After allowing the cells to adhere for 24 h, cytotoxicity of both compounds was determined by addition of ST-246 at final concentrations of 1–500 μM and CDV at final concentrations of 100–1000 μM in a volume of 10 μl and in the absence of virus. CI was recorded for 170 h in triplicate. For determination of dose-dependent antiviral effects, infection of adhered cells with VACV NYCBOH (multiplicity of infection (MOI) 0.15; 1125 PFU/cavity) and simultaneous addition of antiviral compounds were performed in a volume of 10 μl. ST-246 and CDV were added in triplicate at final concentrations of 10–1000 nM and 50–1000 μM, respectively. CI values were recorded and after 90 h the IC_{50} was calculated either by the spreadsheet software Graph Pad Prism version 5.01 (Graph Pad Software, San Diego, California, USA), using the log (agonist) vs. response equation, or by xCELLigence™ software version 1.2, selecting the dose–response curve (CI at time point vs. concentration) and the sigmoidal dose–response formula. For comparison, cytotoxicity of ST-246 and CDV was conventionally determined by a colorimetric assay using the cell proliferation agent WST-1 according to the manufacturer's instructions (Roche Applied Science). Calculated values for IC_{50} were compared to the literature.

2.5. NT of OPV neutralizing Abs

The conventional virus PRNT was based on the plaque titration test (see Section 2.3). A selection of sera and Ab preparations from different species was used for PRNT (Table 2). Abs and sera were treated at 56 °C for 1 h in order to inactivate complement factors and centrifuged shortly to remove any possible precipitates.

Formation of virus–Ab complexes was allowed by pre-incubating VACV IHD-W (MOI 0.1; 2.5×10^4 PFU) with serial Ab dilutions for 1 h at 37 °C. The Ab-virus mixture was applied to 2.5×10^5 VERO E6 cells. The neutralizing titer (which is defined as the dilution of Ab that induces a 50% plaque reduction compared to the non-inhibited control sample, NT_{50}) was calculated after 5 days for each Ab.

For the RT-CES based NT test of Abs from different species, 50 μl of medium were used for initial background measurement followed by seeding of 5000 HEK293 cells in a volume of 50 μl and monitoring of CI change for 4 h. A virus dilution of VACV IHD-W (MOI 0.1; 500 PFU) was prepared in 50 μl of medium per cavity. Abs were diluted serially twofold from 1:10 to 1:2560 in 50 μl aliquots of medium. Abs and virus were combined to the final dilution of 1:20 to 1:5120 in a volume of 100 μl and incubated for 1 h at 37 °C in a separate 96-well plate. Finally the 100 μl of