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RESEARCH ARTICLE

Real-time quantitation of viral replication and inhibitor potency using a label-free optical biosensor

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Abstract

Real-time detection of viral replication inside cells remains a challenge to researchers. The Epic® System is a high-throughput, label-free optical detection platform capable of measuring molecular interaction in a biochemical assay, as well as integrated cellular response from measurement of cellular dynamic mass redistribution (DMR) in a cell-based assay. DMR has previously been used to measure cell signaling upon receptor stimulation. In this report, we present the first example of Epic® measurement of viral replication-induced cellular response and demonstrate that this system is extremely powerful not only for the sensitive and quantitative detection of viral replication inside cells but also for screening of viral inhibitors. By comparing with conventional assays used for the measurement of viral replication, we show that the Epic® response has many advantages including sensitivity, high throughput, real-time quantification and label-free detection. We propose that the Epic® system for measurement of integrated cellular response will be an excellent method for elucidating steps in viral replication as well as for the high-throughput screening of inhibitors of rhinovirus and other viruses.

Keywords: *Rhinovirus; Epic®; system; label-free detection; viral inhibitors*

Introduction

Human rhinoviruses (HRV) have been identified as the major causative agent of the common cold (1,2) and the most common acute infectious illness in humans (3). Studies suggest that both adults and children experience rhinovirus infection every 1–2 years (4). The onset of symptoms typically occurs 1–2 days after viral infection and the time to peak symptoms is generally 2–4 days (5). It is thought that virus shedding persists after the resolution of symptoms and that virus may be cultured from 10–20% of subjects 2–3 weeks after infection (6). In most cases, rhinovirus leads to a short self-limiting illness. However, for asthmatics, the elderly, and immuno-compromised patients, rhinovirus infection can lead to life-threatening complications (3). Of the 102 HRV serotypes known to date, approximately 90% use intercellular adhesion molecule-1 (ICAM)-1 as a host receptor. Due to the large number of HRV serotypes, little immunological protection is offered by prior rhinovirus exposure, rendering vaccination approaches ineffective.

In spite of the medical significance of rhinovirus infection in humans and its complications, attempts to develop effective treatments have been relatively limited and unsuccessful. Once the cognate receptor for a virus is discovered (e.g., ICAM-1 for HRV, CD4 for HIV), this paves the way for the use of soluble forms of the receptor as receptor decoys (7,8). Alternatively, antibodies and peptides against the host receptor may be utilized to block virus binding to the host receptor. Studies in volunteers with experimental colds showed that rhinovirus infection could be prevented by blocking ICAM-1 with monoclonal antibodies (9, 10). A clinical trial has

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also been carried out with nasal delivery of soluble truncated ICAM-1 consisting of domain-1 to domain-5 (D1-D5) to test whether it could neutralize the virus by blocking its attachment to the cell surface ICAM-1 (7). As an alternative to the use of soluble ICAM-1, small molecule drugs targeting viral capsid or protease were tested (10–12). The treatments listed here were found to be only marginally effective, with some reducing symptoms and viral shedding, while others helped to accelerate recovery. Although some progress has been made in the development of anti-viral treatments for rhinovirus, none of these agents have been approved as yet for treatment of the common cold.

The need to identify other inhibitors that efficiently block rhinovirus still exists, and a real-time, sensitive, high-throughput assay would greatly assist these efforts. Conventional assays such as cytopathic effect (CPE) assays or plaque-forming unit (PFU) assays assess virus replication and inhibitor potency by measuring host cell death, which takes typically 24–72 hours. Quantitative PCR may be used to directly measure viral genome copies as a measure of virus replication (13,14); however, real-time, quantitative methods for the detection of virus replication, and inhibitors thereof, have not been available.

The Epic® system is a novel high-throughput, label-free technology with great potential for screening assays. The Epic® system has been developed for both biochemical assays and cell-based assays. For the cell-based assay, the system measures the integrated cellular dynamic mass redistribution (DMR) within the sensing volume that may occur within the cell, either through increased cell adhesion due to cell proliferation, or through cell detachment from the sensor due to a toxic effect or apoptosis (15). The DMR signal is also uniquely sensitive to cellular changes that occur due to protein recruitment, endocytosis, and receptor recycling, as well as gross cell morphology changes. Previous studies have utilized this system for the study of cell signaling through G protein-coupled, EGF, and bradykinin B2 receptors (15–17) amongst others, and have yielded insights into the receptor signaling pathways and regulation. In this study, we demonstrate that the Epic® response provides a real-time measurement of viral replication inside the cells and consequently an assessment of the potency of inhibitors on viral infection of cells. The application of this technology may constitute a major breakthrough for the screening and development of novel antiviral therapeutics.

Materials and methods

Cells and media

HeLa cells were propagated in DMEM with 5% FBS (Atlanta Biologicals, Lawrenceville, GA) containing 2 mM L-glutamine, Pen-strep (Gibco) and 50 µg/mL Gentamycin (Gibco). ICAM-1 D1-D5 was produced from a CHO stable cell line, as described previously (18). R6.5 monoclonal antibody was produced from hybridoma (19), and purified by Protein A column followed by gel filtration chromatography. Unless otherwise noted, HeLa cells were maintained at 37°C in a 5% CO_2 humidified incubator.

Purification of HRV

Two different HRV serotypes were used in this study: HRV14 and HRV16. The rhinoviruses were purified based on a protocol developed by Rossmann and co-workers (20). Briefly, HeLa cells were grown to confluency, and infected with HRV at a multiplicity of infection (MOI) of 5 PFU/cell. Cells were harvested and subjected to three freeze-thaw cycles followed by homogenization and centrifugation. Virus was precipitated overnight at 4°C in 0.5 M NaCl and polyethylene glycol (PEG 8000). The resulting precipitate was then collected by centrifugation, resuspended in HEPES buffer, pH 7.5 containing 0.25 M NaCl, and treated with DNAse, RNAse, and Trypsin. The virus was then spun at 45,000 rpm for 2 hours in an SW41 rotor on a sucrose cushion of 30%, followed by a spin at 36,000 rpm for 90 minutes in an SW41 rotor on a 0–40% sucrose gradient. Virus bands were collected and quantified with a spectrophotometer using an extinction coefficient of 7.7 at 260 nm for 1 mg/mL solution. A final spin at 45,000 rpm for 2 hours in an SW41 rotor was carried out to pellet the virus.

PFU assay

The PFU assay was adapted from a method of Rueckert and Pallansch (21). HeLa cells were seeded in a 12-well plate at 50% confluency in culture media. Next day, the cells were washed once in DMEM and 250 µL of diluted virus was added to each well and allowed to incubate at room temperature for 30 minutes. Following incubation, the solution was removed from the wells and the cells were overlayed with a 1.0% solution of low melting point agarose (Invitrogen) diluted in media with 25 mM $MgCl_2$. The culture plate was left at room temperature for 5 min until the agarose solidified. The agarose was overlayed with 1 mL of culture media. The 12-well plate was incubated at 35°C for 60–72 hours. The plaques were observed by staining the monolayer with a 0.25% solution of crystal violet in 20% methanol and washing extensively with water.

CPE assay

HeLa cells were seeded at 50% confluency in each well of a 96-well plate or 384-well Epic® plate. On the day of the assay, culture medium was removed from the wells,

and dilutions of virus (diluted in media) were added to the wells. Inhibitors (diluted in media), where present, were added by premixing with the virus at 37°C for 30 minutes with a final volume of 10 µL. The mixture of virus and inhibitor was then added to culture wells in a total volume of 100 µL (30 µL for 384-well plate). Plates were incubated at 35°C for the times specified. At the end-points indicated, the remaining viable cells were stained with 0.25% crystal violet prepared in 20% methanol and washed extensively with water. 1% SDS was added to solubilize the dye, which was then quantified by measuring the absorbance at 570 nm.

The Epic® system and measurement of cellular response

The Corning Epic® system used in the this study consists of an Epic® microplate reader that has a temperature-controlled environment, an internal liquid handling system as previously described, and the SBS-standard Epic microplate with the integrated optical resonant waveguide biosensor (16,22). Figure 1 illustrates the principle of operation of the Epic® biosensor. The Epic®system measures the resonant coupled wavelength reflected by the sensor. The wavelength measured is a function of the optical refractive index of the sensing zone. The magnitude of this wavelength shift is proportional to the changes of DMR as a result of an integrated cellular response to the change in cellular response. The mass redistribution of cellular contents towards the sensor surface is thought to contribute positively to the overall response, while redistribution of mass away from the sensor results in a net negative response. The Epic response signal is an integrated cellular response to compound stimulation/inhibition, cell morphology, and cell death as shown in this study. A temperature control system minimizes interference associated with temperature fluctuations and is set at 28°C.

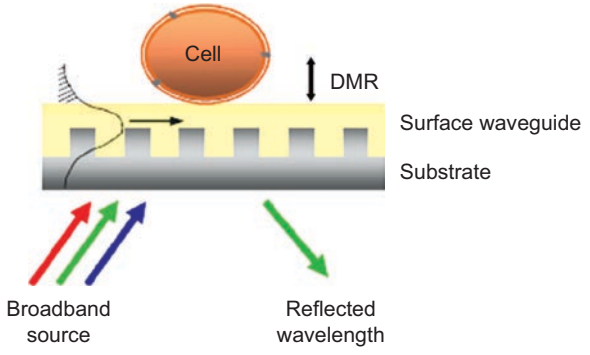

Figure 1. A schematic view of the Epic® biosensor. When the glass substrate is illuminated with broadband light, only a 'single' wavelength that is resonant with the waveguide grating structure is strongly reflected. The Epic® system measures the wavelength reflected by the sensor which is determined by the optical properties of the sensing zone within approximately 150 nm of the sensor. The magnitude of this wavelength shift is proportional to the amount of DMR.

The day before the assay, HeLa cells were seeded onto a 384-well Epic® sensor microplate (Corning) at a density of 15,000 cells/well and cultured for 20–24 h (37°C, 5% CO_2) to obtain confluent monolayers. After the removal of medium, cells were washed once with fresh media and 20 µL of media was added to each well. The sensor plate and the compound source plate were maintained for 1 hour in the Epic® reader at a constant temperature of 28°C. The sensor plate was then scanned, and a baseline optical signature was recorded. Stock solutions of virus were diluted in media, and buffer additions were limited to less than 1% of total volume in the well. 40 µL of the compound solutions were prepared in the source plate, of which 20 µL was added to the sensor plate. Inhibitors, where present, were added with the virus in the source plate. The cellular response from the sensor plate was monitored for a period of 20–24 hours.

Results

Quantitative measurement of HRV replication using the Epic® system

The Epic® platform developed by Corning is a novel system that measures refractive index changes as a result of dynamic mass redistribution (DMR) changes that occur in cells within approximately 150 nm of the 384-well sensor surface (Figure 1) (16). It was used in this study to determine if it would be a good method for tracking virus replication inside cells, specifically human rhinovirus (HRV). For these studies, HRV14 and HRV16 were used as they belong to the major group of rhinoviruses (2), and have been used in previous studies to test the effects of antiviral compounds (13). To assess the DMR response of HRV on a monolayer of HeLa cells, different concentrations of virus were added to a confluent monolayer and were incubated inside the Epic® instrument where measurements were taken continually over a 22-hour period. The virus was titrated from 10 PFU/cell to 0.1 PFU/cell (Figure 2a), with no virus as reference control. An MOI of 10 PFU/cell is enough to amplify HRV after a period of approximately 15 hours (23); therefore, it was reasoned that large cellular changes would occur even at this low concentration, causing major changes in the DMR response. A dose–response effect was observed with 10 PFU/cell of HRV14 leading to 50% of the maximum decrease in DMR signal as early as 11 hours, while the virus dose as low as 0.1 PFU/cell showed approximately 30% decrease in DMR response after 22 hours (Figure 2a). A similar dose–response effect was seen with HRV16 (Figure 2b).

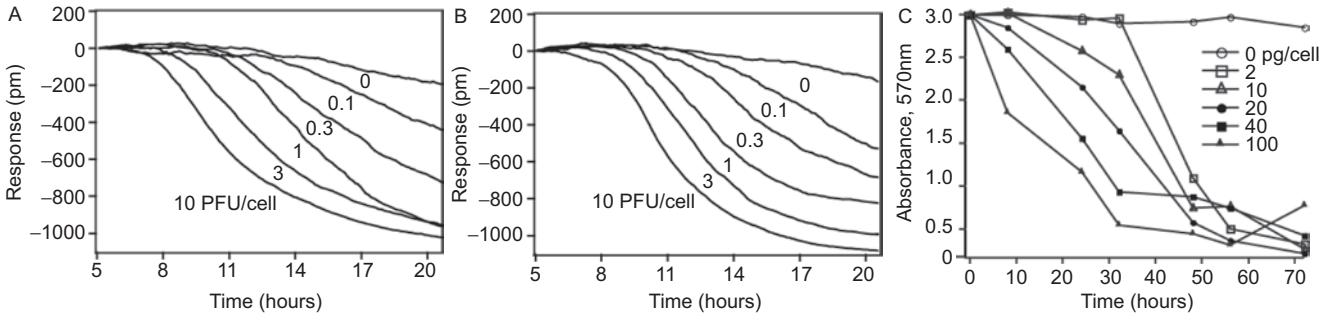

Figure 2. The DMR response (expressed as wavelength shift in picometer (pm)) measured upon the addition of increasing concentrations of HRV14 (A) and HRV16 (B) to a HeLa cell monolayer cultured in the 384-well Epic® microplate. Virus concentration is expressed in PFU/cell assuming 15,000 cells per well. Virus stock concentration was at 10^8 PFU/ml. Traces shown are representative traces of duplicate experiments. (C) The CPE assay measuring the effects of the addition of increasing amounts of HRV14 to HeLa cell monolayers in a 96-well plate. Virus concentration is expressed in pg/cell assuming 50,000 cells per well.

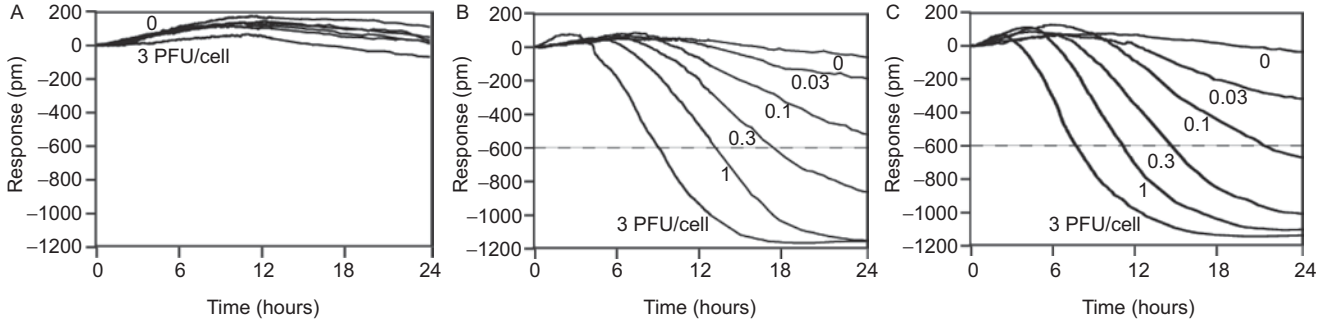

Figure 3. The DMR response measured upon addition of fixed concentrations of monoclonal antibody R6.5 (A), ICAM-1 D1-D5 (B), and BSA (C) to a titration of HRV14 from 3 PFU/cell to 0.03 PFU/cell in HeLa cell monolayers. The concentration of R6.5 added was 8 μg/ml, while ICAM-1 D1-D5 and BSA were at 2 μg/ml.

An initial test confirmed that cytopathic effects were observed in a 384-well Epic® plate at 28°C without a marked difference from CPE assays carried out at 35°C (data not shown). A titration of virus showed that at very high concentrations of virus (100 pg/cell), cell detachment from the monolayer occurred as early as 8 hours, while at the lowest concentration (2 pg/cell) approximately 48 hours were required for cells to detach (Figure 2c). Allowing for batch-to-batch variation, 2 pg/cell of HRV14 is equal to approximately 20–200 PFU/cell as measured in a PFU assay (data not shown). It should be stressed that while the cells in the monolayer are lysed after viral infection and round up, they may not fully detach from the surface until significantly later (48–72 hours). In our hands, it has not been possible to detect complete detachment of cells until 72 hours as measured in the CPE assay at concentrations as low as 10 PFU/cell (data not shown).

Detection of HRV inhibitors using the Epic® system

Given the success of the Epic® system in quantifying viral replication, we hypothesized that it would be an excellent method for assessing viral inhibitors. Two inhibitors, R6.5 and ICAM-1 D1-D5, with different modes of action were chosen. R6.5 is a monoclonal antibody that binds to domain 1-2 of ICAM-1 (24), thereby sterically hindering virus attachment to ICAM-1. ICAM-1 D1-D5 is a soluble form of the ICAM-1 receptor (CD54) consisting of the five extracellular domains of ICAM-1. ICAM-1 D1-D5 inhibits infection by HRV major group serotypes, and is thought to act as a 'decoy' by blocking and neutralizing virus particles before they can bind to cellular receptors and gain entry to the cell (25).

To assess the effects of these inhibitors on the DMR changes caused by HRV, a fixed concentration of R6.5 (8 μg/mL)(Figure 3a) and ICAM-1 D1-D5 (2 μg/mL) (Figure 3b) were added to the varying doses of virus (3–0.03 PFU/cell) using BSA (2 μg/mL) as a control (Figure 3c). R6.5 dramatically inhibited the infection of the cells irrespective of the dose of virus. This is to be expected, as the antibody does not inhibit by binding to the virus, but rather by binding to the cellular receptor, rendering the concentration of virus irrelevant. ICAM-1 D1-D5, on the other hand, showed only low levels of inhibition, manifested by a delay in the DMR decrease with increased delays shown in lower virus concentrations.

In this case the inhibitor efficacy is directly dependent on virus concentration, as ICAM-1 D1-D5 inhibits by binding to and neutralizing the virus outside the cell.

Quantitative assessment of potency of HRV inhibitors using the Epic® system

To more fully characterize the effect of the HRV inhibitors, the virus was kept at a fixed concentration (5 PFU/cell) and the inhibitors R6.5 and ICAM-1 D1-D5 were varied with three-fold serial dilutions (Figures 4a, 4b). In the case of R6.5, complete inhibition was observed at 8.5 µg/mL, while 0.9 µg/mL appeared to produce a significant inhibitory effect (Figure 4a). The concentration of ICAM-1 D1-D5 required to cause complete inhibition of HRV infection was 7 µg/mL, while D1-D5 below 2.3 µg/mL failed to produce any inhibitory effect.

The ability of ICAM-1 D1-D5 to inhibit HRV infection was also assessed by CPE assay (Figure 4c). We chose the lowest concentration of virus shown in Figure 2c to result in cell detachment within 48 hours (2 pg/cell) so that an excess of inhibitors could be added to inhibit virus infection. The amount of cell detachment was consistent with that seen in Figure 2c, where 2 pg/cell of virus resulted in a dramatic decrease in cell attachment after 48 hours. Addition of increasing amounts of the inhibitor ICAM-1 D1-D5 (0.05–10 µg/mL), but not BSA as a control, resulted in inhibition of infection in a dose-dependent manner.

Discussion

In this report, we present the first example of Epic® measurement of viral replication. The method traditionally used to measure viral replication is a CPE or PFU assay, which has many drawbacks, the major one being a lack of sensitivity. Depending on the serotype of HRV and the concentration, a typical round of replication in HeLa cells is thought to occur in approximately 10–15 hours at 35°C (26). However, the conventional CPE or PFU assays rely on complete detachment of the cell from the surface of the tissue culture plate, an event which may not occur until 48–72 hours depending on the concentration of the virus and stringency of any wash steps. Other studies have reported using CPE assays (after 48 hours with an MOI of 10 PFU/cell) and quantitative PCR to screen inhibitors but not in a high throughput format and with a lack of sensitivity (13,14). Using the Epic® system for measurement of DMR, we were able to detect the cellular changes caused by very low concentrations of virus as early as a few hours after infection. Given that the Epic® assay was carried out at 28°C, it might be expected that the viral replication would proceed somewhat more slowly; however, close to maximum decreases in the Epic® signal were visible as early as 12 hours, which correlates very well with the data available on the kinetics of virus replication (26).

The Epic® measurement of cellular response has been described as a target or complex of a certain mass moving away from (negative response) or towards (positive response) the sensor (22). The Epic® response was further found to be sensitive to cell status, e.g. proliferation, quiescent state, the degree of adhesion, etc (15). Therefore, this system is ideally suited for the measurement of rhinovirus replication, as rhinovirus replication causes rounding up and eventual detachment of HeLa cells from the culture surface. It may also be postulated that the DMR response can be particularly useful for measuring such gross changes as occur from a virus replicating inside a cell, and eventual lysis of the cell. Both of these events would be accompanied by dramatic relocation of cellular contents within 150 nm from the biosensor surface where the cell is attached.

Picornaviruses such as poliovirus and rhinovirus enter host cells within the first 10–30 minutes after attachment, followed by a rapid release of RNA and

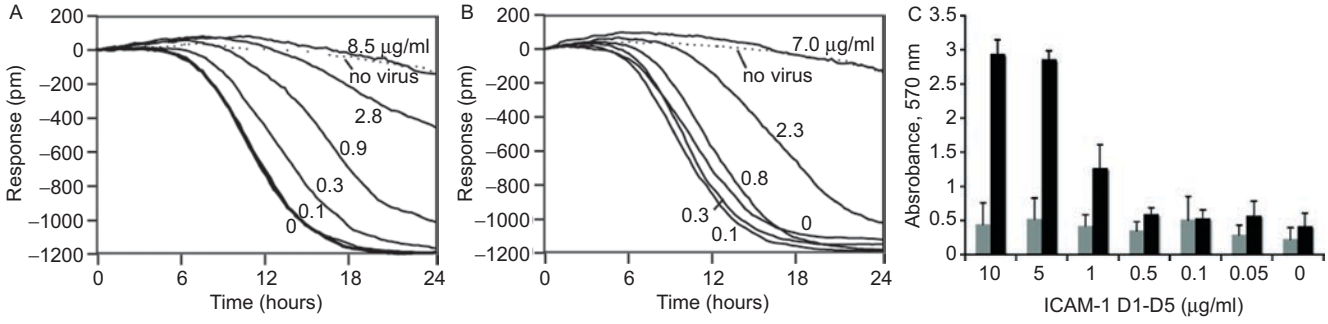

Figure 4. The DMR response measured upon addition of increasing concentrations of monoclonal antibody R6.5 (A) and ICAM-1 D1-D5 (B) to a fixed concentration of HRV16 (5 PFU/cell) in HeLa cell monolayers. Traces shown are representative traces of duplicate experiments. (C) A CPE assay measuring the effects of increasing amounts of ICAM-1 D1-D5 (black bars) or BSA as a control (gray bars) with a fixed concentration of HRV16 (2 pg/cell) in HeLa cell monolayers in a 96-well plate**.** Virus concentration is expressed in pg/cell assuming 50,000 cells per well. Results represent the average values of the CPE assay carried out in duplicate, with the standard deviation from the average as error bars.

subsequent delivery into the cytosol (27). Viral replication, packaging, and assembly appear to occur between 2 and 8 hours after attachment, with the release of intact progeny occurring as early as 8 hours depending on the cell type (28). Due to the complex nature of the DMR response, it may not be possible to point to individual steps in the viral life cycle such as RNA/protein synthesis or virus assembly. Through the careful use of inhibitors to protein synthesis or microtubule formation, however, it may be possible to dissect the various steps that may contribute to the DMR signal. The ability to directly assess integrated cellular events without recourse to labeling strategies or molecular engineering of cells is of great utility, especially as such invasive methodologies may alter cellular physiology, thereby potentially interfering with the identification of inhibitor compounds. When assessing potency of antiviral inhibitors, it is useful to be able to see the effects resulting from an impaired or delayed ability of the virus to infect cells, which may be difficult to do in a conventional CPE assay. For a real-time, large-scale screening of rhinovirus inhibitors, we sought to develop a sensitive, higher throughput assay. Results presented here demonstrate that the Epic® system for the measurement of DMR is not only highly sensitive and quantitative but allows the development of inhibitors that may at first glance not appear to show much promise. In the case of ICAM-1 D1-D5, the Epic® assay allowed us to detect low levels of inhibition at lower concentrations of virus. We then followed this by titrating the ICAM-1 D1-D5 using a lower concentration of virus and found that complete inhibition was possible at high concentrations of ICAM-1 D1-D5. Both inhibitors used in this study have been shown to block major group HRV infection although with different mechanisms of action (2,25). R6.5 is an antibody that binds to the receptor for HRV thus blocking entry to the cell, while the soluble form of ICAM-1 prevents binding to the cell by competing with the cellular receptor. The DMR response measured neatly illustrates these differences, as R6.5 at concentrations high enough to block ICAM-1 on host cells efficiently inhibited viral replication even at the higher virus concentrations.

Although the Epic® system is a relatively new technology that has been available commercially for only a couple of years, a number of studies on receptor signaling highlight the capabilities and the potential and wide application of this system (15–17). The Epic® system has been successfully demonstrated to have great potential for the high throughput screening of inhibitors of G-protein coupled receptors (17). Indeed the 384-well microplate format in combination with high-throughput liquid handling and multi-plate incubator will allow for the screening of extremely large compound libraries. One ideal potential application of this technique would be for the discovery and characterization of antivirals for viruses such as HIV, as inhibitors of viral entry and replication are being sought and novel therapies based on mechanisms of the viral life cycle are currently being developed and tested in clinical trials. One limitation of this label-free system is that due to a multitude of intracellular events caused by binding of an agonist or a virus to a cell, DMR measurements of cellular response is an integrated signal and cannot readily discriminate between individual events. However, with careful use of inhibitors or blocking agents, it may be possible to use this technology to elucidate steps in signaling pathways. In the case of viral replication, it may be possible to use inhibitors to block different stages in the replication process to get a better picture of how exactly the infection proceeds.

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