Comparison of rhinovirus A infection in human primary epithelial and HeLa cells

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HeLa cells are used to study the life cycles of many different viruses, including the human rhinoviruses (HRV) in the family Picornaviridae. Although the natural targets of HRV are human bronchial epithelial cells (hBE), it is generally more difficult to obtain and maintain the relevant primary cell cultures, relative to HeLa cells. Given that the HRV are now identified as a major cause of human asthma exacerbations, it becomes important to document how much of the virus biology learned from HeLa cells is common also to natural primary cells. When compared directly in matched infections using A01a virus, the kinetics of RNA replication, the synthesis and processing of viral proteins and the general subcellular localization of key non-structural proteins were resembled in hBE and HeLa cells. Viral-induced shutoff of host cell processes (e.g. nucleo-cytoplasmic trafficking) was also comparable.

INTRODUCTION

More than 50 years after their establishment, HeLa cell lines still provide valuable models, not only for cancer research, but also for the study of different aspects of virus replication. The original cell line was adapted for cancer research (Gey et al., 1952; Scherer et al., 1953), but one of the most successful initial applications was in the study of poliovirus replication (Scherer et al., 1953; Syverton et al., 1954). Within a few years of their availability, HeLa cell infections became the standard model for virology, particularly for picornavirology. Subsequently, nearly every fundamental process that occurs during picornavirus infections was studied and described using HeLa cell lines. Among their most valuable properties, HeLa cells double quickly (about 24 h). They can be grown on plates or in suspension cultures and they have relatively stable genomes (Macville et al., 1999). Moreover, it is easy to select strains of HeLa cells with particular adaptive growth characteristics by applying selection pressure through alterations in the culture conditions, composition of the medium or serum type (Masters, 2002).

But it should not be forgotten that HeLa cells at their origin are transformed, cancer-derived cells, and the impact of their transformation could have unknown ramifications if HeLa-obtained data are overextrapolated to processes taking place in primary cells or in vivo. During the past several years this question of cell-culture relevance has became especially important to the field of human rhinovirus (HRV) research. Rhinovirus infections are the most common triggers for exacerbations of asthma and chronic obstructive pulmonary disease (Busse et al., 1997; Gern & Busse, 2002; Miller et al., 2009). Primary airway epithelial cells are frequently used to model different aspects of the rhinovirus infection cycle, particularly with regard to immune responses (Papi & Johnston, 1999; Schroth et al., 1999). However, primary cells also have some inherent experimental limitations with regard to modern molecular techniques. For example, the transfection efficiency of primary cells is usually much lower than for HeLa cells, a property which limits investigation of low-level viral protein functions inside of cells. Another disadvantage is that host immune responses, particularly those responsible for innate immunity, are more variable when measured in primary cells than in established cell lines. Moreover, the cost to grow and maintain primary cell lines, both fiscally and technically, is generally much higher than for standard cell-line cultures. HeLa cells are readily grown in suspension, for example. Primary epithelial cells are not. When using either type of system it is therefore important to define or categorise these steps in a virus life cycle, which may be influenced by the type of cell used for the experiments.

To define these parameters for rhinovirus, we studied, compared and now report our findings with key steps in the virus life cycle, including the replication rate of viral RNA (vRNA), genome translation, polyprotein processing, the localization of viral proteins and the efficiency of infectious viral particle assembly, during matched infections with human primary bronchial epithelial cells (hBE) and HeLa cells. We also investigated the ability of viral protease 2Apro to abrogate cellular nuclear-cytoplasmic trafficking during
infection of these cells, an important mechanistic phenomenon directly linked to viral, anti-host functions.

RESULTS

HRV antibody reactivity

Rabbit polyclonal antibodies (Ab) were raised to three recombinant A16 non-structural proteins (2A$^{pro}$, 3C$^{pro}$ and 3D$^{pol}$). Rhinovirus polyproteins differ (on average) by about 40% identity between species, but even within species there is considerable diversity spread widely across the genes (Palmenberg et al., 2009). To learn how far the antibody selectivity would extend, the respective sera were tested for cross-reactivity in Western assays against homologous proteins from five available strains. For the HRV-A (A01a, A02, A16 and A89) and HRV-B (B14), infected HeLa cell lysates provided the protein source. The Ab 3D (Fig. 1c) gave positive reactions against 3D$^{pol}$ from all infected extracts, but only recognized the truncated 3CD$^\prime$ protein from the cognate A16 sample. The Ab 3C serum was not as broadly reactive (Fig. 1b). It recognized 3C$^{pro}$ in all HRV-A samples, but did not react with the B14 homologue. Moreover, the 3C$^{pro}$ and 3CD$^\prime$ signals became weaker (A16$>$A01a$>$A02 or A89) as the sequence diversity increased. The Ab 2A (Fig. 1a) was also species-specific in these assays, in that the infected cell lysate from B14, again failed to react. In total, the results characterize our reagents as generally reactive with a broad range of HRV-A isolates, except for Ab 3D, they were less useful in detecting proteins from HRV-B.

For our experimental purposes, this cross-reactivity was important to define. Among the HRV-A and HRV-B, 88 of 99 serotypes, comprising the so-called ‘major’ group of viruses, use ICAM-1 as their cellular receptor (Greve et al., 1989). Our strains of A16 (from cDNA), A89 and B14 (from cDNA) belong to this group. All other HRV-A and HRV-B viruses (‘minor’ group) including A01a and A02, use LDLR receptors. It is generally acknowledged that the minor viruses are more infectious to primary cell cultures than major group viruses (Mosser et al., 2005). Therefore, A16 Ab cross-reactivity with A01a proteins allowed us to track both types of infections in hBE and HeLa cells, equivalently.

Comparative vRNA synthesis

HeLa and hBE monolayers were infected with A01a [m.o.i. of 0.1, 1 (three wells per condition, primary cells isolated from one donor) and 10 p.f.u. per cell (3–6 wells per condition, three different donors] then assayed by quantitative RT-PCR to measure the relative rates of vRNA synthesis in the cells and the comparative release of vRNA into the media. Within experimental error the cell-associated 0-time points were similar for both cell types (Fig. 2a), confirming that virus attachment was reasonably equivalent. As the infections progressed, the cell-associated (Fig. 2a) and media-released (Fig. 2b) curves also rose, reaching nearly the same levels by 9–12 h post-infection (p.i.). Statistically significant differences were only documented at later time points in cell-associated and media-released samples (15 and 18 h p.i.) collected from cells with m.o.i. of 0.1 and 1 p.f.u. per cell (P<0.001), but not from 10 p.f.u. per cell-infected samples (P=0.386). The observed differences were similar or less than 1 log$_{10}$ of p.f.u. equivalent units. Based on these results we conclude that virus attachment, penetration and the overall rate and extent of vRNA synthesis were similar for both cell types at earlier time points and comparable later during infection.

The plaque titres from the final media and cell-associated samples (m.o.i. of 10 p.f.u. per cell, three independent experiments) determined by viral plaque assay gave $1.2(\pm 0.2) \times 10^6$ p.f.u. ml$^{-1}$ for HeLa, and $1.8(\pm 0.4) \times 10^7$ p.f.u. ml$^{-1}$ for hBE.

Comparative viral protein synthesis

The viral protein processing profile, especially for precursors derived from the P3 (COOH) region of the
polyprotein, is another indicator of picornavirus translation and replication efficiency. Lysates from A01a-infected HeLa and hBE cells, collected at different time points, were monitored for the processing of 3C-containing proteins by using Ab 3C in Western analyses (Fig. 3). Although the profiles of non-specific Ab reactivity ('M' lanes, panels a and b), were somewhat different for each cell type, the processing of viral proteins could be clearly followed, and was similar for both infections. At the early times p.i. (e.g. 4–8 h p.i.), there was detection of high molecular mass protein precursors (2BCP3 and 3CD) followed by the expected spectrum of mature 3C-containing proteins (3Cpro and 3CD). Therefore, the same P3-region precursors were produced by both cells in roughly the same time frame. A few differences were noted. In primary cells, free 3Cpro protein band prevailed over other precursors at later time points, while in HeLa cells the continued accumulation of precursors was observed for up to 20 h. In addition, the appearance of free 3Cpro band started earlier in primary cells, than in HeLa cells, but by 16 h the intensity of the specific 3Cpro band was similar in both cell types. Overall we can conclude that hBE infections did not induce an alternative processing scheme, or significantly alter the rate at which viral proteins were made available (i.e. for replication).

**Localization of viral proteins**

The fates of viral proteins for which we had available Abs were also monitored comparatively. The hBE cells are only half to one-third the size of HeLa cells, so care was taken to ensure that all imaging was at equivalent magnification. As reported previously for A16 in HeLa cells (Amineva et al., 2004), Abs against 3Cpro and 3Dpro localize these proteins to densely staining nucleoli, as well as to cytoplasmic locales. Fig. 4 shows a similar distribution for our new Ab.

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**Fig. 2. vRNA accumulation.** Primary hBE and HeLa cells were infected as in Methods. At the indicated times, the total amount (per well) of cell-associated vRNA (a) and media-released vRNA (b) was determined by quantitative real-time PCR. Parallel calibration of these reactions for each experiment used plaque titrated A01a vRNA dilutions. Error bars show the range of values for duplicate wells in separate experiments.
The cytoplasmic 3D\textsuperscript{pol} is presumably involved in vRNA synthesis. The nuclear 3D\textsuperscript{pol} participates as 3CD or 3CD\textsuperscript{'} within protease complexes involved in anti-host activities, such as the cleavage of nuclear transcription factors [Amineva et al., 2004]. The 2A\textsuperscript{pro} protein has a variety of additional anti-host activities, including the cleavage of eIF4G translation factors (cytoplasmic) and cleavage of a cohort of nucleoporins (Nups) regulating nucleo-cytoplasmic trafficking through the nuclear pore complexes (NPC). Unique to the hBE cells, 2A\textsuperscript{pro} was visualized in the cytoplasm and also as distinct sharp circles around the nucleus. In HeLa cells, 2A\textsuperscript{pro} was perinuclear, but not nearly as distinctly localized.

**Nuclear efflux assays**

A key consequence of 2A\textsuperscript{pro} cleavage of Nups is the cessation of active nuclear import as evidenced by unregulated efflux (via diffusion) of untethered nuclear proteins back into the cytoplasm. The common assay for this activity monitors the redistribution of a nuclear-targeted reporter protein [e.g. glutathione S-transferase (GST)–nuclear localization signal motifs (NLS)] after infection (Gustin & Sarnow, 2002). Lipofectamine and FuGENE were used to introduce a GFP–NLS expressing cDNA into HeLa and hBE cells, respectively. After 24 h, both cell types had ‘green’ nuclei (Fig. 5, mock), albeit the transfection efficiency for the hBE was only about half of that of the HeLa cells. When plates containing these cells were subsequently infected with A01a (10 p.f.u. per cell), the GFP effluxed from the nuclei, progressively staining the cytoplasm over a time-course of 9–15 h p.i. Fig. 5 (+A01a) shows examples of this redistribution at 12 h p.i. Such multiple observations did not detect overt kinetic differences in the redistribution of the reporter from the nucleus into the cytoplasm for ‘green’ cells, of either type (data not shown).

**Cleavage of Nups**

Nuclear efflux is caused by 2A\textsuperscript{pro} cleavage Nups, thereby opening the central channel of NPC to unregulated diffusion. For A02 and B14, channel proteins, Nup62, Nup98 and Nup153 are among the known 2A\textsuperscript{pro} substrates. When these Nups are cleaved, their signals in Western assays, diminishes accordingly (Gustin & Sarnow, 2002; Park et al., 2008). To examine the comparative fates of these proteins, HeLa and hBE cells were infected with A01a then harvested 0–20 h later for Western analyses (Fig. 6). In the starting samples, the total amount of Nup153 and Nup62 was found to be significantly higher in HeLa than in primary cells, probably reflecting a different density of nuclear pores in each cell type. Therefore, although virus-induced cleavage of Nup153, Nup98 and Nup62 was clearly apparent in both types of infected cells, the changes appeared more dramatic in HeLa cells. For hBE cells, the amount of remaining Nup protein at 20 h p.i. was 28 (Nup153), 30 (Nup98) and 29% (Nup62), compared with 5, 18 and 13%, respectively, in HeLa cells.

**DISCUSSION**

The HeLa lines used in most virus laboratories have been selected over hundreds if not thousands of cell divisions, for populations which are easy to grow and/or amplify preferred viruses to reproducibly high titres. For the medically important picornaviruses, much of what we know about these agents and their life cycles was generated with HeLa cell systems. With HRV in particular, we have assumed, but never tested the assumption, that HeLa data readily modelled natural infection processes as they occur in humans. Reassuringly, when we used molecular reagents to directly compare HeLa infections with those in human primary airway epithelial cells, the overall replication patterns were remarkably similar.

It should be noted that the HeLa cell line is not the only one used to study rhinoviruses. Other cell lines including BEAS-2B (Hudy et al., 2010; Wang, Q. et al., 2009), H292 (Bianco et al., 1998) and A549 cells (Jang et al., 2006; Konno et al., 2002; Peng et al., 2007; Wang, J. H. et al., 2009) are also utilized to investigate rhinoviruses. But all of them, similar to HeLa cells, are transformed cell lines. One common feature of these cell lines is that they have originated from airway epithelium. BEAS-2B cells were isolated from normal hBE, then transformed with adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned (Reddel et al., 1989). A549 cell line was initiated from lung carcinomaotous tissue (Giard et al., 1973). H292 cells
were derived from a pulmonary mucoepidermoid carcinoma metastasis (Banks-Schlegel et al., 1985). Several authors compared results documented for different aspects of rhinovirus pathogenesis in HeLa and A549 cells (Peng et al., 2007) or BEAS-2B and hBE (Zaheer & Proud, 2010). But it was very important to compare the cycle of rhinovirus infection in HeLa cells, which have non-airway epithelium origin, and primary cells isolated from donor’s bronchi to confirm the biological relevance of findings observed in HeLa cells to rhinovirus pathogenesis.

Preliminary visual observation of cytopathic effects during rhinovirus infection in both cell types initially suggested that the course of hBE infection might be slower than in HeLa cells (not shown). However, a direct comparison of vRNA signals in HeLa and hBE demonstrated that the dynamics of RNA synthesis was similar and moreover, the total amount of newly synthesized vRNA was reasonably equivalent in both cell types, as was the distribution of vRNA, between cell-associated and media-released material (Fig. 2). The fact that a significant amount of RNA signal remained cell-associated, even up to 18 h p.i., correlates with data collected previously (Anne Mosser, personal communication) measuring A16 infections of HeLa cells. This high level of continued cell association is the reason HRV growth protocols commonly advocate multiple freeze–thaw cycles on the cells, prior to harvest (Medappa et al., 1971).

The media-released RNA signal largely represents packaged virions freed by cell lysis or intact-cell excretion of particles. Our PCR protocols were calibrated with titrated aliquots (p.f.u.) of A01a virus grown in HeLa cells. Both tested cell types released (roughly) the same $10^7$ p.f.u.
equivalents of vRNA per ml of media, consistent with the ~10^7 p.f.u. ml^-1 actually plaqued from the media at 20 h p.i. (m.o.i. of 10 p.f.u. per cell). The only statistical significant difference observed was at a lower m.o.i. (0.1 and 1 p.f.u. per cell) when cells from one donor were used. Including cells from different donors (m.o.i. of 10 p.f.u. per cell) flattened out the differences between two cell lines. Yet over many experiments of this type, despite equivalent PCR signals, the virus released from HeLa cells usually titrated 10–20-fold lower in subsequent plaque assays than virus released from the hBE cells. The rates of viral protein synthesis and polyprotein processing were similar in both cell types (Fig. 3), so we suspect the titre disparity is qualitative rather than quantitative, perhaps representing cell-type differences in virus-specific infectivity (particle:p.f.u.), or in the ratio of ‘mature’ to ‘immature’ particles (i.e. maturation cleavage of the capsid) released by the respective cells. In other words, the hBE cells seemed more adept than HeLa at converting equivalent vRNA and protein resources into transmittable infectivity, and/or releasing the same into the media. Essentially, the primary cells make better virus, but not more of it.

Within cells, our panel of Ab to non-structural proteins allowed comparative tracking of replication complexes (3D^pol) and the viral proteases (2A^pro and 3C^pro) that control many aspects of host shutoff. The 3C^pro and 3D^pol signals did not distinguish themselves between cell types, a result not unexpected given the comparable replication fecundities. For 2A^pro, shortly after infection, this protease attacks translation factor eIF4G in the cytoplasm to shutoff cap-dependent (host) translation. It then cleaves at least four different Phe/Gly-containing Nup proteins with the NPC to shutoff active nucleo-cytoplasmic trafficking of old/new cellular proteins and mRNA (Gustin & Sarnow, 2002). The efflux of NLS-targeted GFP observed in both cell types (Fig. 5) showed this protease was doing its job quite effectively after infection, and within the same time frame. Rather, differences between the cells manifest in the specific pattern of 2A^pro localization as the job was accomplished (Fig. 4), and in the voraciousness with which the protease depleted the more abundant NPC in HeLa cells (Fig. 6). HeLa cell genomes are hyper-triploid (3n1) (Macville et al., 1999), a factor that may contribute to a higher density of NPC per nucleus. Certainly, on a per cell basis, mAb 414, a reagent reactive with multiple Phe/Gly Nups gave a much stronger Nup153 and Nup62 signal from the HeLa cells relative to hBE. Nonetheless, in both cases, the relevant Nup signals diminished significantly during A01a infections, causing the non-retention (efflux) of those cell proteins/reporters requiring continued active uptake (e.g. with NLS) for apparent nuclear accumulation.

**Fig. 5.** GFP efflux. HeLa and hBE were transfected with plasmid p3NLS–GFP and then 36 h later the cells were infected with A01a. Bright-field images of live cells showing GFP localization were recorded at 12 h p.i. Bars, 10 μm.

**Fig. 6.** Nup cleavage. Equivalent samples of HeLa (0.5×10^6 cells per well) and hBE cells (0.5×10^6 cells per well) were infected with A01a (10 p.f.u. per cell) and then harvested between 0 and 20 h p.i. The resulting cell lysates were fractionated by SDS-PAGE and then probed in Western blot analyses using the indicated mAb or Ab reagents as described in Methods. The relative band intensities were normalized to the 0-time (mock-infected) samples and the amount of actin in each line.
In hBE cells, the 2Apro formed clear, sharp rim-like patterns around the nuclei, a distribution we have never seen with other HRV proteins. The 2Apro in HeLa cells was also perinuclear, but did not have the same focused halo effects as infections with hBE. Possibly, the protease has additional targets or binding partners in these respective cells contributing to these patterns.

Based on our total results and on the overall yield of virus, we conclude that HRV infections of HeLa and hBE cells are readily analogous with regard to vRNA synthesis, protein translation, protein processing, the general intracellular localization of viral proteins and the ability to disrupt host-cell functions like nucleo-cytoplasmic trafficking. The fact that the virus synthesized in hBE consistently had higher specific infectivity, may help explain the extraordinarily effective human-to-human transmission of the ‘common cold’. HeLa cells still provide a very good, relevant model for the virus life cycle and its effects on cellular metabolism.

**METHODS**

**Cells and viruses.** HeLa cells (ATCC® CRL-1958) were grown in suspension cultures (Medapa et al., 1971) using minimal essential medium (Gibco Invitrogen) supplemented with Earle’s salts, l-glutamine, penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and 10 % FCS (Sigma). Sucrose-purified rhinovirus stocks were kindly provided by Wai-Ming Lee (A01a) and Anne Mosser (A02, B14, A16 and A89) of the University of Wisconsin-Madison. Virus nomenclature follows the ICTV Picornavirus Study Group recommendation, using the HRV species designation (‘A’ or ‘B’) followed by the isolate type (e.g. 01a, 02, etc.). hBE were isolated from post-mortem lung transplant tracheal or bronchial tissues, cultured and passaged as described previously (Schroth et al., 1999). The second passage hBE cells were used for infection experiments. Subconfluent HeLa monolayers or hBE monolayers were washed with PBS three times, then inoculated with 5 x 10⁶ p.f.u. of virus (typically A01a) in PBS. After 1 h at room temperature (to allow adsorption), the cells were washed with PBS, overlaid with serum-free medium, then moved to an incubator (34 °C, 5 % CO₂). For kinetic studies, this was considered the ‘zero time point’.

**Recombinant viral proteins.** Recombinant A16 3CPro was described previously (Aminieva et al., 2004). The genes for A16 2APro and 3Dpol were isolated by PCRIs templated by pHHRV-16 cDNA (Lee et al., 1995). For the 2A reactions, one primer encoded an engineered NdeI site, an ATG codon and a site, an ATG codon and 19 nt 5’ to the end of the gene, a TAG codon, and a BamHI site. The amplicon was subcloned into the expression vector PET-41b (Novagen) using NdeI and BamHI sites, then transformed into Escherichia coli strain BL21(DE3)-pLysS; Novagen. In a similar manner, the A16 3CPro gene was subcloned into vector PET-26, using a protocol described for poliovirus 3DPro (Gohara et al., 2000). The resulting plasmid (pPET-26-3Dpol) expressed 3DPro, without an NHI-terminal methionine. Bacterial transformations, protein induction by IPTG and recombinant protein isolation was as described previously (Aminieva et al., 2003).

**Ab and Western blot analyses.** Rabbit polyclonal Ab raised against purified A16 proteins 2APro, 3CPro and 3Dpol were contracted from Harlan. Briefly, New Zealand white rabbits were immunized with 200 μg antigen (recombinant viral proteins in adjuvant), then boosted with more antigen after 21, 42 and 63 days. The final bleed was 73 days after the first injection. Clarified sera (Abs) were tested by indirect ELISA against the corresponding recombinant antigens, and in a Western blot assay against native viral proteins from HRV-infected HeLa cells. Murine mAb 414 was commercial (catalogue no. MMS-120P; Covance). It recognizes common FXFG sequences in HRV (Mosser et al., 2003). When appropriate, the bands were scanned for mean pixel density (Image Quant software) and compared for relative intensity.

**Confocal microscopy.** HeLa or hBE monolayers were infected with virus (10 p.f.u. per cell). At appropriate time points (5 h p.i. for HeLa and 8 h p.i. for hBE), the cells were washed with cold PBS, fixed in paraformaldehyde (4 % in PBS) and then permeabilized (0.3 % Triton X-100). The cells were then incubated with the appropriate primary and secondary Ab or DAPI stain as described previously (Aminieva et al., 2003). Images were visualized with a Nikon Eclipse TE2000U confocal microscope, taking care to use equivalent filters and settings for comparative experiments.

**Nuclear efflux assays.** Eukaryotic expression plasmid pAC-GFP–NLS (Novagen), encodes a cytoplasmic transcriptional promoter and the gene for GFP fused to three, tandem-linked SV40 NLS. Transfection of HeLa cells (Lipofectamine 2000; Invitrogen) or hBE cells (FuGENE 6; Roche Applied Science) was according to the manufacturers’ protocols. Briefly, the reagents were diluted (Opti-MEM; Gibco), mixed with DNA (4 μg per 1 well in six-well plate) for 10 min (20 °C), then added to the cell monolayers. The cells were moved to 37 °C (under 5 % CO₂) for 24–48 h to allow for GFP expression and then infected with A01a at 10 p.f.u. per cell.

**RNA detection protocols.** Twenty-four well plates with subconfluent monolayers of cells were infected with A01a (0.1, 1 and 10 p.f.u. per cell) in three independent experiments. As required by the experimental time-course, the medium was removed (0.5 ml per well), clarified (1000 g, 5 min) and saved. The plates were washed with PBS and the cells in each well were lysed (Qiagen lysis buffer) after being reconstituted with non-adherent cells and/or cell debris removed from the medium during the clarification step. Total RNA was isolated from media and cell samples (RNeasy Plus Mini kits; Qiagen) and then resuspended in water (50 μl per sample). Reverse-transcription real-time PCR (RT-PCR) techniques to quantify vRNA used SuperScript II enzyme (Invitrogen) as described previously (Brooks et al., 2006). The PCR amplifications included TaqMan Universal PCR Master Mix (1 ×) and rhinovirus-specific primers designed to identify A01a. The samples were then analysed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) as described previously (Mosser et al., 2005). Standard curves relied on parallel reactions programmed with purified virion RNA (A01a) extracted from titrated aliquots of virus (1 to 1 x 10⁵ p.f.u.). New curves were generated for each assay series to control for signal linearity and primer–probe efficiencies. The output values (p.f.u. equivalents) were calculated (Applied Biosystems software) using a selected cycle threshold within the geometric phase of the PCR amplification. Two way ANOVA was performed on the vRNA data selected cycle threshold within the geometric phase of the PCR amplification. Equivalents were calculated (Applied Biosystems software) using a selected cycle threshold within the geometric phase of the PCR amplification. Equivalent fold changes were calculated (Applied Biosystems software) using a selected cycle threshold within the geometric phase of the PCR amplification.

**Viral plaque assay.** Viral titres in supernatants of infected cells were determined by performing plaque assays described by Mosser et al.
(2002). Briefly, HeLa cell monolayers were inoculated with 10-fold dilutions of analysed samples, incubated for 48 h at 34 °C and then stained with 10% formalin. Viral plaques were visualized by staining with crystal violet, counted and viral titre was expressed as p.f.u. ml⁻¹.

ACKNOWLEDGEMENTS

The authors thank Drs Wei-Ming Lee and Ann Mosser for their generous gifts of rhinovirus stocks and Dr Louis Rosenthal for the help with statistical analysis of the data. This work was supported by NIH grant U19 AI070503. The author(s) declare that they have no competing interests.

REFERENCES


