Epithelial cells of the gastrointestinal (GI) tract react to the presence of pathogenic microbes by producing inflammatory cytokines. One such group of cytokines is the interferons (IFNs). The IFN system is a vital part of the innate immune response, mediating pleiotropic antiviral effects in tissues. In the main, IFNs exert their effects on cells by interacting with cell-surface receptors, resulting in activation of the Janus tyrosine kinase (JAK)–signal-transducing activators of transcription (STAT) signalling pathway (Randall & Goodbourn, 2008). There are ten IFN isoforms in mammals that are divided into three distinct classes (IFN types I–III), depending on receptor–complex interactions. IFNs upregulate hundreds of IFN-stimulated genes (ISGs) and it is the induction of some of these ISGs that confers antiviral properties to cells (Randall & Goodbourn, 2008). Viruses, including the calciviruses (especially norovirus), rotaviruses, astroviruses and adenoviruses, are a major cause of gastroenteritis (Wilhelmi et al., 2003). Species F human adenovirus types 40 and 41 (HAdV-40 and -41, respectively) are common aetiological agents of gastroenteritis. In the main, IFNs exert their effects on cells by interacting with cell-surface receptors, resulting in activation of the Janus tyrosine kinase (JAK)–signal-transducing activators of transcription (STAT) signalling pathway (Randall & Goodbourn, 2008). There are ten IFN isoforms in mammals that are divided into three distinct classes (IFN types I–III), depending on receptor–complex interactions. IFNs upregulate hundreds of IFN-stimulated genes (ISGs) and it is the induction of some of these ISGs that confers antiviral properties to cells (Randall & Goodbourn, 2008).

Virology, including the calciviruses (especially norovirus), rotaviruses, astroviruses and adenoviruses, are a major cause of gastroenteritis (Wilhelmi et al., 2003). Species F human adenovirus types 40 and 41 (HAdV-40 and -41, respectively) are common aetiological agents of gastroenteritis (Lin et al., 2000; Saderi et al., 2000). These viruses preferentially infect the enterocytes of the small intestine (Phillips, 1981; Whitelaw et al., 1977). Disease is most common in children of <2 years of age, resulting in prolonged watery diarrhoea that, if left untreated, can lead to severe dehydration (Uhnoo et al., 1984). Many enteric viruses, including species F human adenoviruses, grow poorly or not at all in cell culture (Brown et al., 1992; Duizer et al., 2004).

The fastidious growth characteristics of HAdV-40 and -41 in culture have prompted our group and others to develop modified cell lines that are permissible to species F human adenovirus infection (Sherwood et al., 2007; Yamasaki et al., 2010). Our strategy was to impede IFN signalling, which resulted in increased HAdV-40 infection (Sherwood et al., 2007). This suggests that IFN sensitivity could at least in part be responsible for the fastidious growth of species F human adenoviruses. Experiments in conjunctival cells suggest that these viruses are defective in their ability to circumvent the antiviral actions induced by IFN (Tiemessen & Kidd, 1993). These observations were limited, however, because the cell systems employed were not GI in origin, and it could be that susceptibility to IFN is actually circumvented in GI cells.

To investigate the effects of IFN signalling on the growth of species F adenoviruses, we first established a cell system that more closely resembles the natural tropism of the virus, the small intestinal enterocyte. Caco2 cells, which are derived from a colonic adenocarcinoma, exhibit enterocyte-like differentiation (Hidalgo et al., 1989) and are often used as a GI cell-culture model (Halleux & Schneider, 1994; Martin-Latil et al., 2004; Richard et al., 2002). In the GI tract, enterocytes exhibit classical epithelial apical-to-basolateral polarity that facilitates barrier function. Many GI cells in culture are able to differentiate to form this polarity. A marker of this is the ability to transport water/
ions across the polarized monolayer, resulting in the formation of domes or hemicysts (Friedman et al., 1981).

We observed post-confluent hemicyst formation in Caco2 monolayers grown under normal growth conditions [Dulbecco’s modified Eagle medium (DMEM) containing 4.5 g glucose l⁻¹, 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 1% non-essential amino acids; all from Gibco; Fig. 1a], suggesting that these cells were able to differentiate to form a functional barrier. The hemicysts were pronounced and numerous, reaching approximately 50 cm⁻² at 9–12 days post-confluence. This suggests that Caco2 cells can differentiate spontaneously when confluent, which is an indication of their ability to exhibit an epithelial enterocyte-like phenotype. Subconfluent, undifferentiated Caco2 cells are permissive to enteric adenovirus infection (Pinto et al., 1994). To test whether the differentiated Caco2 cells were also permissive to HAd-40 infection, we used HAdV-40 Dugan strain (National Collection of Pathogenic Viruses, UK), cultured using 293-SV5V cells as described previously (Sherwood et al., 2007). In response to HAdV-40 infection, differentiated Caco2 cell monolayers produced characteristic adenovirus cytopathic effect (CPE) at 6–10 days post-infection (p.i.), with clusters of rounded cells evident against a healthy background monolayer (Fig. 1b). These data demonstrate that differentiated Caco2 cells are indeed permissive to HAdV-40 infection.

Fig. 1. Highly differentiated Caco2 monolayers as a model for species F adenovirus infection. (a) Individual hemicysts formed in an 8 day, post-confluent Caco2 monolayer. Left, focused on the top of the hemicyst (Hemi); right, focused on the surrounding monolayer (Mono). (b) CPE in highly differentiated monolayers at 10 days p.i.; mock- (left panel) and HAdV-40- (right panel) infected cells. Extensive areas of CPE are highlighted (*). (c) Percentage of HAdV-40-infected cells (m.o.i. = 10) in subconfluent (low differentiation; low) and highly differentiated (high) monolayers. Low-differentiated monolayers were infected when 50–70% confluent and infection rates were calculated as the percentage of hexon-expressing cells. (d) Time-course of HAdV-40 infection in highly differentiated Caco2 monolayers as determined by FFA. White bars, m.o.i. = 1; black bars, m.o.i. = 10. (e) TER traces in confluent Caco2 monolayers using normal DMEM glucose levels (4.5 g l⁻¹; □) or reduced glucose DMEM (1 g l⁻¹; ■). Readings were taken for 47 days and corrected for background. (f) TER traces following mock or HAdV-40 (m.o.i. = 10) infection. For each panel, representative experiments are shown from a minimum of n=3. Error bars represent SD. Images, ×10 magnification.
We postulated that highly differentiated Caco2 monolayers probably lead to an enterocyte-like phenotype (Hidalgo et al., 1989) and therefore may be able to improve the efficiency of HAdV-40 infection. Cell dissociation is difficult for highly confluent, differentiated Caco2 monolayers; therefore, we monitored the production of HAdV-40 infectious virus by a fluorescent focus assay (FFA) in intact monolayers (Albinsson & Kidd, 1999), using an antibody directed against adenovirus hexon protein (Novoceastra). The virus was absorbed apically onto the cell sheet for 1 h at 37 °C in 5% CO2. For infection of highly differentiated monolayers, the cells were grown for 10 days post-confluence prior to infection, where high monolayer differentiation was determined by hemicyst formation. Poorly differentiated Caco2 monolayers were defined as those that lacked hemicyst formation and were subconfluent (approx. 60% confluent). The infection of highly differentiated monolayers compared with poorly differentiated monolayers improved the infection rate from 0.8±0.2 to 6.6±2.1% of cells, a difference of approximately 8-fold (Fig. 1c). A time-course revealed that, between 2 and 10 days p.i., an m.o.i. of 10 gave higher percentages of infected cells, compared with a lower m.o.i. (Fig. 1d). Indeed, elongated infectious cycles are typical of HAdV-40; therefore, our time-course probably represents a single life cycle of the virus, rather than multiple cycles (Witt & Bousquet, 1988). Whilst these rates are low compared with those for other human adenovirus species, they are comparable to those described previously in vivo for porcine enteric adenoviruses (Ducatelle et al., 1982).

Caco2 cells form highly differentiated and polarized monolayers post-confluence by the formation of cell–cell junction complexes, exhibiting high levels of transepithelial electric resistance (TER) comparable to in vivo observations (Vachon & Beaulieu, 1992). To test whether the monolayers that we were using were truly differentiated once post-confluent, we grew the cells on 0.4 μM Transwell membrane inserts (Corning) and measured TER using an EVOM voltohmmeter (World Precision Instruments). TER readings for the Caco2 monolayers were in excess of 2000 Ω cm-2 in standard growth medium and were also maintained in reduced-glucose medium (Fig. 1e). Furthermore, the monolayers could maintain this differentiated state for up to 47 days post-confluence. This is in contrast to other GI cell lines tested, including INT407 and HCA7 cells, which failed either to reach comparable TER readings or to maintain these readings for prolonged periods of time (data not shown). HAdV-40 also affected Caco2 TER readings, causing a distinct drop in resistance measurements for the first few hours of infection, but later recovering (Fig. 1f). This transient effect is probably caused by localized foci of infection, owing to the relatively low infection rates that we observe compared with those of other human adenovirus species, where infection rates are close to 100% of differentiated cell monolayers, providing complete loss of TER (Walters et al., 2002). Our data suggest that highly differentiated Caco2 monolayers provide a permissive cell-culture system for the replication of HAdV-40 under conditions that more closely resemble the natural tropism of the virus, compared with other cell systems used previously for culturing HAdV-40. A recent study investigating species F human adenovirus cell entry identified a block in uptake of HAdV-41 virions into HEK-293 and A549 cells (Leung & Brown, 2011). Comparison of uptake mechanisms between highly differentiated Caco2 and other cell-line systems may identify enterocyte-specific infection mechanisms utilized by species F adenoviruses.

We further investigated the response of highly differentiated Caco2 cells to IFN type I (IFN-α) and type II (IFN-γ) signalling. The induction of many ISGs in response to IFN signalling is STAT1-dependent. Tyrosine phosphorylation of STAT1 in response to cytokine activation of the IFN-mediated signalling pathway results in its activation and subsequent expression of ISGs. Therefore, we investigated STAT1 phosphorylation (p-STAT1) status in Caco2 cells following IFN treatment using a previously described immunoblotting protocol (Mellits et al., 1993). We used antibodies directed against the STAT1 N terminus (BD Biosciences), Y701 p-STAT1 (Cell Signaling Technology) and IFN-α receptors (RIG-I) (Novoceastra). Western blotting was performed as described in (a). (c) The induction of many ISGs in response to cytokine activation of the IFN-mediated signalling pathway results in its activation and subsequent expression of ISGs. Therefore, we investigated STAT1 phosphorylation (p-STAT1) status in Caco2 cells following IFN treatment using a previously described immunoblotting protocol (Mellits et al., 1993). We used antibodies directed against the STAT1 N terminus (BD Biosciences), Y701 p-STAT1 (Cell Signaling Technology) and IFN-α receptors (RIG-I) (Novoceastra). Western blotting was performed as described in (a). (c) Similar dose-response experiment as described for (b), using IFN-γ (Centralised Facility for AIDS Research, NIBSC, UK). Representative experiments are shown from a minimum of n=3.
14-3-3β (as a loading control; Santa Cruz Biotechnology). Highly differentiated Caco2 monolayers were treated with IFN-α (Wellferon) and p-STAT1 levels were monitored. The cells responded rapidly to IFN-α treatment (100 IU ml⁻¹), resulting in activated STAT1 within 10 min of treatment that lasted for up to 24 h (Fig. 2a). Activated p-STAT1 was detectable in response to ≥1 IU IFN-α ml⁻¹ (Fig. 2b). Similar responses were observed with IFN-γ (Fig. 2c), although the response was 10-fold less sensitive than with IFN-α. These findings suggest that highly differentiated Caco2 cells are IFN-sensitive and can mount a rapid and sustainable signalling response to IFN stimulation. To investigate whether HAdV-40 infection leads directly to IFN production from Caco2 cells, we used an ELISA for human IFN-β (type I IFN) production (VeriKine-HS; PBL). We measured IFN-β secretion in response to mock infection, HAdV-40 infection (m.o.i. = 10, 18 h) or transfection with a positive control (a dsRNA synthetic analogue, poly I:C). HAdV-40 infection induced IFN-β secretion by 9.7-fold (242.3 ± 37.8 pg ml⁻¹) compared with mock-infected cells, reaching a level comparable to that produced by dsRNA transfection (9.8-fold; 244.6 ± 26.9 pg ml⁻¹). Caco2 cells clearly produce a modest level of type I IFN in response to HAdV-40 infection.

Finally, highly differentiated Caco2 cells were used as a model to determine whether HAdV-40 is sensitive to the effects of type I and type II IFNs. Monolayers were either mock-treated with carrier or pre-treated with IFN (100 IU ml⁻¹; 22 h prior to virus infection). Furthermore, we conducted a post-treatment test, where the cells were treated with IFN directly following HAdV-40 infection.
(post-treatment). Cells were monitored for virus titre by measurement of the late gene hexon (by FFA). We observed a striking reduction in hexon-expressing cells in the monolayers that had been pre-treated with either IFN type (Fig. 3a). Infection counts showed that HAdV-40 infection rates were reduced significantly when the cells were pre-treated with IFN-α, compared with those either mock-treated or treated with IFN following infection (post-treated; Fig. 3b). Similarly, pre-treatment with IFN-γ also reduced the percentage of HAdV-40-infected cells significantly (Fig. 3c). Post-treatment of the Caco2 monolayers with IFN-γ also appeared to reduce the virus infection rate, although this was not significant. Bile acid treatment of cells has previously been shown to reduce p-STAT1 levels and to improve the growth of porcine enteric calicivirus (Chang et al., 2004). We examined whether bile acid treatment of highly differentiated Caco2 cells could alleviate the inhibitory effect of IFN on HAdV-40 infection. A number of bile acids were tested, but none were able to improve HAdV-40 infection rates (Fig. 3d). Moreover, bile acid treatment was insufficient to block IFN-mediated activation of STAT1 in Caco2 cells (Fig. 3e). Taken together, our findings show that HAdV-40 is sensitive to type I and type II IFN treatment in GI-like cells; this phenotype cannot be alleviated by bile acid treatment.

We have described the use of highly differentiated Caco2 cells as a relevant model to investigate the poorly understood process of species F adenovirus infection. We have used this model to investigate the susceptibility of HAdV-40 to the antiviral effect of IFN during infection. Consistent with the investigations of Tiemessen & Kidd (1993), we demonstrate that HAdV-40 is indeed sensitive to the effects of IFN in a cell system that more closely resembles its natural tropism. IFNs provide cells with immediate defence against invading viruses. It is not surprising, therefore, that many viruses (including many adenovirus species) have evolved mechanisms to evade the antiviral effects of IFN. Respiratory adenoviruses are relatively insensitive to the effects of IFN (Anderson & Fennie, 1987; Gallagher & Khoobayanian, 1972), due to the expression of virus-encoded gene products that can circumvent the antiviral effects of IFN, such as the adenovirus E1A protein and the VA (virus-associated) RNAs (Randall & Goodbourn, 2008). Indeed, E1A of HAdV-5 (adenovirus species C) can rescue growth of the IFN-sensitive vesicular stomatitis virus from IFN-mediated inhibition in culture (Anderson & Fennie, 1987).

The question remains as to why species F adenoviruses do not display the capacity to block IFN signalling exhibited by other adenovirus species. Clearly, species F adenoviruses propagate successfully in nature and are endemic in the global population (Slatter et al., 2005). One plausible explanation is that, in vivo, the enterocyte provides enteric adenoviruses with an environment that does not respond readily to IFN treatment. The tight control of pro-inflammatory responses in the GI tract is necessary to prevent inappropriate responses in an environment challenged by microbes and, in doing so, prevents responses such as lymphocyte infiltration that are a characteristic feature of inflammatory bowel disease (Breese et al., 1994). Species F adenoviruses may have adapted to tissues that are restricted in their ability to mount an inflammatory cytokine response.

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