Mouse respiratory epithelial cells support efficient replication of human rhinovirus

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Human rhinoviruses (HRV) are responsible for the majority of virus infections of the upper respiratory tract. Furthermore, HRV infection is associated with acute exacerbation of asthma and other chronic respiratory diseases of the lower respiratory tract. A small animal model of HRV-induced disease is required for the development of new therapies. However, existing mouse models of HRV infection are difficult to work with and until recently mouse cell lines were thought to be generally non-permissive for HRV replication in vitro. In this report we demonstrate that a virus of the minor receptor group, HRV1B, can infect and replicate in a mouse respiratory epithelial cell line (LA-4) more efficiently than in a mouse fibroblast cell line (L). The major receptor group virus HRV16 requires human intercellular adhesion molecule-1 (ICAM-1) for cell entry and therefore cannot infect LA-4 cells. However, transfection of in vitro-transcribed HRV16 RNA resulted in the replication of viral RNA and production of infectious virus. Expression of a chimeric ICAM-1 molecule, comprising mouse ICAM-1 with extracellular domains 1 and 2 replaced by the equivalent human domains, rendered the otherwise non-permissive mouse respiratory epithelial cell line susceptible to entry and efficient replication of HRV16. These observations suggest that the development of mouse models of respiratory tract infection by major as well as minor group HRV should be pursued.

INTRODUCTION

Human rhinovirus (HRV) infections cause approximately two-thirds of upper respiratory tract infections (common colds), which are associated with significant levels of morbidity, absence from school and work, and health care costs. Lower respiratory tract HRV infections are also associated with illnesses, such as pneumonia in the immunosuppressed, and acute exacerbation of respiratory diseases, such as asthma (Johnston, 1998) and chronic obstructive pulmonary disease (COPD) (Seemungal et al., 2001). Asthma now affects up to 30% of children and 10–15% of adults in Westernised communities and HRV infection is implicated in the majority of acute exacerbations of the disease (Johnston et al., 1995). Therefore, the overall morbidity and economic burden attributable to HRV is considerable.

HRVs constitute the majority of the genus *Rhinovirus* of the family *Picornaviridae*. There are over 100 distinct serotypes of HRV and this has prevented the development of effective vaccines. A small animal model of HRV infection would be invaluable for testing anti-viral compounds already in development and for elucidating mechanisms of disease in order to select targets for novel therapies. Although a mouse model of HRV infection has been
described previously (Yin & Lomax, 1986), it is a difficult system to work with, requiring adaptation of virus in cell culture and complex techniques for detection of virus replication. As a consequence HRV research in vivo is restricted to large primate or human volunteer studies.

A major obstacle to the development of mouse models of infection is the host cell tropism of HRV. Approximately 10% of HRV serotypes make up the minor receptor group, which can use both the human and mouse forms of low-density lipoprotein receptor (LDLR) to enter cells of either species (Hofer et al., 1992; Marlovits et al., 1998; Yin & Lomax, 1983). However, the remaining 90% of HRVs belong to the major receptor group and use human intercellular adhesion molecule-1 (ICAM-1) to effect cell attachment and entry (Greve et al., 1989; Staunton et al., 1989; Tommassini et al., 1989; Uncapher et al., 1991). These viruses do not bind to mouse ICAM-1 (Register et al., 1991; Staunton et al., 1992) and therefore species-specific restriction of virus replication is implemented at the earliest stage in the infection cycle. Consequently, the provision of human ICAM-1 on the surface of mouse cells must be the first step in the development of a mouse model for major receptor group HRV infection.

ICAM-1 (CD54) is involved in inflammatory functions mediated by leukocyte adhesion (Makgoba et al., 1988). It belongs to the immunoglobulin gene superfamily and both human and mouse ICAM-1 have five homologous ‘Ig-like’ extracellular domains, a transmembrane domain and a short C-terminal cytoplasmic domain. Major group HRVs bind to the two N-terminal domains of human ICAM-1 (Bella et al., 1998; Register et al., 1991; Staunton et al., 1990) and domain-swap experiments have shown that these domains confer the ability to bind major group HRVs on mouse ICAM-1 (Staunton et al., 1990, 1992). The transmembrane and cytoplasmic domains of ICAM-1 are not required for internalization of major group HRVs (Staunton et al., 1992) but may be required for normal ICAM-1 intracellular signalling functions.

There is little information available on the ability of HRVs to replicate in small animal cell culture and information regarding mouse cell culture relates to fibroblast rather than respiratory epithelial cell lines. In addition to the receptor restriction of major group HRVs, previous investigators identified intracellular blocks to HRV replication in both mouse fibroblast (L) cells and baby hamster kidney (BHK) cells (Grunert et al., 1997; Lomax & Yin, 1989; Yin & Lomax, 1983). In both cell lines a related picornavirus, coxsackie A21 virus, which also uses human ICAM-1 as a receptor, productively infected cells which were transfected to express ICAM-1 (Grunert et al., 1997; Shafren et al., 1997). However, replication of both major and minor receptor group viruses in mouse L cells was only demonstrated after adaptation by alternate passage in mouse (L) and human (HeLa) cells (Lomax & Yin, 1989; Yin & Lomax, 1983). In these experiments, the lack of a receptor on the mouse cells for the major group virus (HRV39) was overcome by transfecting the cells with viral RNA.

Against this uncertain background, we set out to re-assess the feasibility of developing both minor and major group mouse models of rhinovirus infection. First, we evaluated the ability of minor group HRV to replicate in mouse lower respiratory epithelial cells and then we investigated the replication of major group HRV in mouse respiratory epithelial cells following transfection of in vitro-transcribed RNA. Finally, we tested the ability of major group HRV to infect the same mouse epithelial cells expressing a chimeric form of ICAM-1, comprising human extracellular domains 1 and 2 and mouse domains 3, 4 and 5 and transmembrane and cytoplasmic domains.

**METHODS**

**Cells and viruses.** Ohio HeLa cells were originally obtained from the Medical Research Council (MRC) Common Cold Unit (Salisbury, UK) and were used for the routine propagation of HRV. Mouse (Mus musculus) lower respiratory epithelial cell lines LA-4 and Mad-C3 were obtained from ECACC. The origin of these mouse cell lines was confirmed by karyotyping. L cells were obtained from G. E. Blair (University of Leeds, UK). HRV1B and HRV16 were generated from infectious cDNA clones as described below. The identities of both viruses were confirmed by neutralization with serotype-specific antiserum (ATCC).

**Infection of mouse cells.** Subconfluent (≥60%) cell monolayers were infected with virus for 1–2 h at room temperature with gentle rocking of the inoculum. Cells were washed twice and maintained at 33 °C in media with reduced serum content (2%) and supplemented with 20 mM MgCl₂.

**Virus CPE assay.** Subconfluent Ohio HeLa cells in 96-well plates were exposed to serial dilutions of infectious samples. Development of a cytopathic effect (CPE) was visualized after 7 days by fixing and staining for damage to the cell monolayer with 5% formaldehyde/5% ethanol/1% crystal violet in PBS. Assays were performed in quadruplicate wells and end point titres were defined by the highest dilution at which CPE was observed in 50% of the wells (TCID₅₀).

**Virus plaque assay.** Confluent Ohio HeLa cells in 6-well plates were exposed to serial dilutions of infected samples for 1 h and overlaid with 0.6% agarose in medium. After 5 days, monolayers were stained with neutral red to facilitate the counting of plaques. Endpoint titres were expressed as p.f.u. ml⁻¹.

**Virus radio-labelling and immunoprecipitation.** Cell cultures were labelled 24 h post-infection with [³⁵S]methionine for 6–24 h and lysed by freeze-thawing. Polyclonal HRV-specific antibodies (ATCC) were added to the cell lysates and immuno-complexed virus was purified using protein A-Sepharose (Sigma). Labelled proteins were separated by SDS-PAGE and visualized by autoradiography.

**Rhinovirus RT-PCR.** Semi-quantitative RT-PCR for detection of HRV RNA was carried out as previously described (Papadopoulos et al., 2000; Johnston et al., 1993). In brief, RNA purified from cell lysates was reverse-transcribed using random primers and a 380 bp cDNA fragment amplified from the 5' untranslated region of the virus genome by PCR with primers OL27 (5'-CGG ACA CCC AAA GTA G-3') and OL26 (5'-GCC CTG CTT CCC C-3') and 32 thermal cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min. After agarose gel electrophoresis and ethidium bromide staining, the intensity of the RT-PCR signal was measured by densitometry.
Using these conditions, there was an approximately linear relationship between RT-PCR signal and input RNA, as determined by a standard curve derived from serial dilutions of viral RNA.

**Transcription and translation of infectious RNA.** Infectious cDNA clones of HRV16 and HRV1B in T7-transcription plasmids were obtained from W. M. Lee (University of Wisconsin, WI, USA) and G. Stanway (University of Essex, UK), respectively. Plasmids were linearized downstream of the HRV genome and RNA was transcribed in vitro using standard protocols. Hela cells were electroporated as described by McKnight & Lemmon (1996), except that only one pulse was used. LA-4 and Mad/C3 cells were transfected using DOTAP (Roche). Both procedures resulted in transfection of 75–85% of cells, as determined using a plasmid which expressed β-galactosidase (data not shown).

**Construction and transfection of chimeric human/mouse ICAM-1 cDNA.** Plasmids pCDM8-HuICAM-1 and pBS-Mo-ICAM-1 (Siu et al., 1989) containing the full-length cDNAs of human and mouse ICAM-1, respectively, were obtained from A. Craig (University of Oxford, UK) and D. Haskard (Royal Postgraduate Medical School, London, UK) respectively. HindIII–NotI fragments for each ORF were subcloned into pCDNA3.1(+) (Invitrogen). The HindIII–BglI fragment containing mouse ICAM-1 domains 1 and 2 was replaced by a HindIII–BglI fragment containing human ICAM-1 domains 1 and 2 to create pHu/MuICAM-1. Cell monolayers were transfected with pHu/MuICAM-1 DNA using Lipofectin (Life Technologies) and selected in the presence of G418 (600 μg ml⁻¹).

**Flow cytometry.** 2 x 10⁵ cells were washed in PBS supplemented with 2% FCS and 0.01% NaN₃ and incubated with 10 μl of anti-ICAM-1 R-phycocerythrin-conjugated monoclonal antibody HA58 (specific for domain 1 of human ICAM-1) or its isotype control (Becton Dickinson) at 4°C (specific for domain 1 of human ICAM-1) or its isotype control at 4°C for 30 min. Cells were washed three times, resuspended in PBS and analysed for fluorescence by single colour flow cytometry using a Beckman Coulter Epics Elite.

**Western blot.** Cell lysates were subjected to SDS-PAGE, proteins were transferred to nitrocellulose membrane (Amersham) by semi-dry blotting and the resulting membranes were blocked with 5% dried skimmed milk in PBS. The membranes were incubated with purified rabbit immunoglobulins raised against a species-specific peptide sequence from domain 1 of human ICAM-1 at a concentration of 5 μg ml⁻¹ in 5% dried skimmed milk/0.1% Tween 20 in PBS. Bound antibodies were revealed using anti-rabbit immunoglobulin HRP conjugate (Dako) followed by enhanced chemiluminescence detection.

**Sucrose density gradients.** Radiolabelled cell lysates were prepared as described above and loaded onto gradients of 15–45% (w/v) sucrose in PBS. After centrifugation at 25000 r.p.m. (81500 g average force) for 3.5 h at 4°C in a Sorvall AH629 rotor, gradients were fractionated and the presence of radioactive material measured by liquid scintillation counting (TriCarb, Perkin Elmer).

**RESULTS**

**Minor group HRV produces CPE in mouse respiratory epithelial cells**

Subconfluent LA-4 cells were challenged with minor group HRV1B. At an m.o.i. of 5 or more, a CPE typical of HRV infection was seen by 48 h post-inoculation (Fig. 1a) and a high proportion of the cells were dead by 96 h. Similar results were observed with Mad/C3 cells (data not shown). CPE was most evident in relatively sparsely seeded cells whereas inoculation of confluent or nearly confluent monolayers produced a less severe CPE. Exposure of cells to a 50-fold reduction in m.o.i. (0·1) did not give rise to noticeable CPE.

In parallel experiments, the major group virus HRV16 had no effect on the appearance of the cells. This was consistent with the requirement for human ICAM-1 for entry of major group virus.

**De novo synthesis of minor group HRV proteins in mouse respiratory epithelial cells**

LA-4 cells were infected with HRV1B and labelled with L-[³⁵S]methionine. Proteins corresponding to the sizes of HRV capsid proteins VP1 and VP3 were subsequently immunoprecipitated from the infected cell lysates by anti-HRV1B specific antisera (Fig. 1b, lanes 1 and 2). Parallel experiments using HeLa cells produced the same profile of viral proteins (Fig. 1b, lanes 3 and 4).

**Replication of minor group HRV RNA in mouse respiratory epithelial cells**

LA-4 or Mad/C3 cells were infected with HRV1B and the level of viral RNA in the cells was monitored by semi-quantitative RT-PCR using an established protocol (Johnston et al., 1993). The viral RNA titre increased with time in both cell lines, but higher levels were seen in LA-4 cells than in Mad/C3 cells (Fig. 1c).

**Production of infectious minor group virus in mouse respiratory epithelial cells**

LA-4 cells were exposed to HRV1B and the infectious titres of the cell extracts or culture media were assayed on HeLa cells at various times post-infection. The resulting growth curve showed significant increases in virus titres post-infection and elevated levels were observed for at least 6 days (Fig. 1d). Furthermore, the increased HRV1B titres observed in the clarified culture medium alone indicated that infectious particles were released from the cells, thus confirming their ability to support the entire replication cycle of minor group HRV.

**Efficiency of HRV replication in mouse cells is tissue-type specific**

As the post-entry block to HRV replication previously reported in L cells was not observed in LA-4 cells, we proposed that the intracellular restriction of HRV replication may be tissue-specific, rather than species-specific, and is encountered in mouse fibroblasts but not respiratory epithelial cells. To test this hypothesis, we compared the growth of HRV1B after inoculation of mouse LA-4, mouse L or human HeLa cells. In contrast to earlier reports (Lomax & Yin, 1989; Yin & Lomax, 1983) which did not detect virus replication in L cells but in agreement with two recent studies (Reithmayer et al., 2002; Harris & Racaniello, 2003), we observed replication of HRV1B in L cells, in addition to...
LA-4 and HeLa cells. However, the efficiency of replication followed a descending hierarchy from HeLa to LA-4 to L cells with an approximately 10-fold reduction in replication between LA-4 and L cells (Fig. 1e) that was consistent with the proposed tissue-specific restriction. Furthermore, while replication in LA-4 cells resulted in elevated virus titres for up to 6 days, replication in L cells was significantly less prolonged.

**Major group HRV RNA is infectious after transfection into mouse respiratory epithelial cells**

Having demonstrated that minor group HRV1B was able to replicate effectively in two different mouse respiratory epithelial cell lines, we investigated the ability of major group viral RNA to replicate in these cells after transfection of virus-specific RNA, thereby bypassing receptor restriction. Recombinant major group HRV16 RNA was introduced into HeLa, LA-4 and Mad/C3 cells and the production of virus measured by plaque assay of the culture medium 2 days after transfection. Production of infectious virus was observed in all three cell lines (HeLa, $3 \times 10^4$; LA-4, $7 \times 10^3$; Mad/C3, $3 \times 10^4$ p.f.u. µg⁻¹ RNA) and furthermore, the level of replication in the mouse cell lines appeared to be comparable to that seen in the human cells. This indicated that there was no block to major group virus replication once viral RNA was transported into the cytoplasmic compartment of mouse respiratory epithelial...
cells and encouraged us to attempt to overcome the receptor restriction in these cells by expression of the HRV major group receptor human ICAM-1.

**Mouse respiratory epithelial cells expressing chimeric ICAM-1 are susceptible to major group HRV infection**

The substantial literature on the structure and function of ICAM-1 suggested that domains 1 and 2 of mouse ICAM-1 could be replaced with the equivalent human domains with little effect on the integrity of the remainder of the mouse molecule. Domain replacement was facilitated by a BglII restriction site between domains 2 and 3 (aa 184) of both the human and mouse ORFs that was used to generate a chimeric plasmid construct, pHu/MuICAM-1. There was no difference in the apparent size of the chimeric protein when compared with both human and mouse forms of ICAM-1 by *in vitro* translation and SDS-PAGE (data not shown). The use of this chimeric molecule was intended to preserve the potential role in infection of mouse ICAM-1 mediated signalling.

LA-4 cells were stably transfected with the chimeric ICAM-1 DNA and expression confirmed by immunofluorescence (not shown) and Western blots with species-specific antibodies against human ICAM-1 (Fig. 2a). Flow cytometry using a species-specific antibody against human ICAM-1 was used to compare the expression of ICAM-1 in the stably transfected cells (LA-4-Hu/MuICAM-1), the LA-4 parental cell line, a human bronchial epithelial cell line (16HBE) and the human cell line (Ohio Hela) used for routine propagation of HRVs (Fig. 2b). These analyses indicated that the chimeric ICAM-1 in LA-4-Hu/MuICAM-1 cells was expressed at similar levels to human ICAM-1 in 16HBE and Ohio Hela cells.

In experiments similar to those described above, LA-4 and LA-4-Hu/MuICAM-1 cells were challenged with either minor group HRV1B or major group HRV16 at a high (5) and a low (0.1) m.o.i. The appearance of the cells and virus titres of cell extracts were recorded at various time points post-infection.

As expected, both cell lines became infected with HRV1B, as evinced by the appearance of CPE following infection at a high m.o.i. (Fig. 3a, panels ii and v) and rising virus titres post-inoculation (Fig. 3b, panels i and iii).

As in earlier experiments, challenge with HRV16 induced no CPE in the LA-4 cells (Fig. 3a, panel iii). Furthermore, after inoculation of these cells with HRV16, virus titres declined (Fig. 3b, panel ii) at the same rate as that observed for the decay of virus in medium lacking cells (data not shown). This further confirmed the inability of these cells to support the replication of major group HRV.

However, after inoculation of LA-4-Hu/MuICAM-1 cells with HRV16, the cells developed CPE (Fig. 3a, panel vi) and supported virus replication as judged by rising virus titres (Fig. 3b, panel iv). Furthermore, the data indicate that the rate of replication of HRV16 in these cells may be more rapid than that of HRV1B, with the major group virus titre reaching maximum levels after 24 h and producing full CPE after 48 h. Major group HRV16 replicated only in those mouse respiratory epithelial cells expressing the chimeric Hu/MuICAM-1, suggesting that expression of the correct ICAM-1 molecule capable of virus binding is both necessary and sufficient for efficient HRV16 infection of these cells.
DISCUSSION

Until recently, studies of HRV replication in mouse cell culture used fibroblast (L) cell lines and did not produce encouraging results. In earlier studies, replication of HRV in L cells was detected only after adaptation by serial passage (Lomax & Yin, 1989; Yin & Lomax, 1983), implicating an intracellular block to replication that was circumvented by mutations in non-structural proteins of the adapted virus (Lomax & Yin, 1989). Subsequently, it was reported that the expression of human ICAM-1 in L cells rendered them permissive for infection with the related picornavirus coxsackie A21 but did not permit replication of major group HRV (Shafren et al., 1997).

Furthermore, in studies using monkey (Staunton et al., 1992) and hamster (Grunert et al., 1997) cell lines, a post-entry block to replication prevented virus growth in hamster cells even after provision of the receptor by transfection with human ICAM-1. Therefore, replication of HRV appeared to be restricted at the intracellular level in cells of mouse and other non-primate species.

As these early studies indicated that fibroblast cell lines were unable to support replication, we considered whether alternative mouse cell lines would be more likely candidates for supporting growth of HRV. In the course of natural infection, virus replication is restricted to the human respiratory epithelium and cell lines derived from these cells have been shown to support replication of HRV in vitro (Papadopoulos et al., 2000). It therefore seemed logical to test mouse cell lines such as LA-4, which are also derived from the respiratory epithelium.

In contrast to earlier reports, we have now demonstrated that a mouse cell line (LA-4) is capable of supporting replication of both minor (HRV1B) and major (HRV16) group viruses. HRV1B was capable of effective replication from cell-entry to release of infectious virus (Fig. 1). Although HRV16 virions were not able to infect these cells (which lack human ICAM-1), transfection of in vitro-transcribed viral RNA resulted in the production and release of infectious virus. When stably transfected to express a chimeric human/mouse ICAM-1, LA-4 cells (LA-4-Hu/MuICAM-1, Fig. 2) became permissive for the entry and replication of HRV16 (Fig. 3). The level of HRV replication in these cells appeared to be broadly comparable to that in human respiratory epithelial cell lines (Johnston et al., 1998; Papadopoulos et al., 2000; Papi et al., 2000).

During the preparation of this manuscript, two reports have described the replication of several serotypes of HRV in mouse fibroblast cell lines. In one study, HRV1A was found...
to be the only serotype, of seven minor group serotypes tested, capable of replication in a fibroblast cell line (Reithmayer et al., 2002). In another study, HRV14 and HRV16, but not HRV2 or HRV39, were shown to replicate in L cells engineered to express human ICAM-1 (Harris & Racaniello, 2003). Both these studies demonstrate that replication in mouse fibroblast cell lines is serotype-specific. This is also likely to be a feature of replication in mouse respiratory epithelial cell lines, such as those used in the current study.

Earlier reports describe the requirement for adaptation of HRV for growth in mouse cell culture (Lomax & Yin, 1989; Yin & Lomax, 1983) and recent reports have confirmed such adaptation in fibroblast cell lines (Reithmayer et al., 2002; Harris & Racaniello, 2003). In the current study we have shown that in the absence of selective adaptation, HRV1B replicates more efficiently in mouse epithelial cells than in mouse fibroblasts (Fig. 1e).

A further recent report described the selection of a major group HRV adapted to growth in cells which express only a very low level of ICAM-1, suggesting that major group HRV can use alternative receptors for entry to cells (Reischl et al., 2001). However, in the current study, the primary receptor (chimeric ICAM-1) was expressed on the surface of the cells and further adaptation to mouse epithelial cell culture did not appear to be essential for replication of major group virus HRV16.

It is interesting that infection of LA-4 cells at a low m.o.i. produced no CPE, indicating that although able to replicate (as judged by rising titre), the virus did not propagate through the cell culture. However, this pattern of HRV infection was also observed in studies using human respiratory epithelial cell lines (Johnston et al., 1998) or primary human respiratory epithelial cells (Mosser et al., 2002; Papadopoulos et al., 2000). In those studies, replication of HRV was demonstrated using techniques similar to those employed here and the characteristics of viral translation, RNA replication and viral growth were very similar to our findings in mouse respiratory epithelial cell lines. As with mouse respiratory epithelial cells, it has also not been possible to serially passage HRV in human respiratory epithelial cell lines or primary cells in vitro (Johnston et al., 1998; Papadopoulos et al., 2000; S. J. Johnston, unpublished data).

A further feature of HRV replication in cell culture is that the majority of progeny virus particles remain associated with the cells or cell debris after cell death and for this reason virus purification protocols require freeze-thawing to release particles from cells. This accounts for the low level of virus released unaided into the medium from infected cells (Fig. 1d) in the current study. Interestingly, a low rate of spread of virus from cell to cell may be a natural phenomenon of in vivo infection, perhaps evolved to limit damage to airway epithelium. Reports describing the in situ-staining of infected airway sections have indeed demonstrated that only a small proportion of cells become infected in vivo (Arruda et al., 1995; Mosser et al., 2002; Papadopoulos et al., 2000).

However, in order to confirm the production of virus particles in the current study, LA-4 cells infected with HRV were radiolabelled and particles were detected that had sedimentation characteristics identical to those of HeLa cell-derived particles (Fig. 4). Furthermore, after replication resulting in at least 100-fold increases in titre, virus derived from infected mouse or HeLa cells was efficiently neutralized (99%) with serotype-specific antisera.

The close parallels between our present observations of HRV replication in mouse cells and previous observations of HRV replication in human cells (Johnston et al., 1998; Mosser et al., 2002; Papadopoulos et al., 2000) indicate that respiratory epithelial cells of both species have similar abilities to support replication of HRV.

The evidence presented here for HRV replication in mouse respiratory epithelial cells suggests that the development of a transgenic mouse model for HRV infection, like that for the closely related poliovirus (Koike et al., 1991; Ren et al., 1990), is feasible. The increasing incidence of childhood asthma and the influence of HRV infection on this disease, coupled with the sensitive techniques now available for the detection of virus replication, make such a development most timely.
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