Prophylactic antibody treatment and intramuscular immunization reduce infectious human rhinovirus 16 load in the lower respiratory tract of challenged cotton rats

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ABSTRACT

Human rhinoviruses (HRV) represent the single most important etiological agents of the common cold and are the most frequent cause of acute respiratory infections in humans. Currently the performance of available animal models for immunization studies using HRV challenge is very limited. The cotton rat (*Sigmodon hispidus*) is a well-recognized model for the study of human respiratory viral infections. In this work we show that, without requiring any genetic modification of either the host or the virus, intranasal infection of cotton rats with HRV16 resulted in measurable isolation of infective virus, lower respiratory tract pathology, mucus production, and expression of interferon-activated genes. Intramuscular immunization with live HRV16 generated robust protective immunity that correlated with high serum levels of neutralizing antibodies. In addition, cotton rats treated prophylactically with hyperimmune anti-HRV16 serum were protected against HRV16 intranasal challenge. Finally, protection by immunization was efficiently transferred from mothers to newborn animals resulting in a substantial reduction of infectious virus loads in the lung following intranasal challenge. Overall, our results demonstrate that the cotton rat provides valuable additional model development options for testing vaccines and prophylactic therapies against rhinovirus infection.

Introduction

Human rhinoviruses (HRV) represent the most important etiological agents of common colds and the most frequent cause of acute respiratory infections in humans [1–3]. HRV are single-stranded positive-sense RNA viruses, members of the *Picornaviridae* family, genus Enterovirus which includes the three so far recognized species rhinovirus A, B and C and also species enterovirus A–J [4,5]. The recent sequencing and analysis of all known HRV serotypes classified within species A and B and of genomic RNA corresponding to species rhinovirus C allowed a better understanding of evolutionary relations among viruses classified within the 3 species and also of some of the structural implications of genetic variability in genes encoding capsid proteins [6,7].

HRV is currently a frequently detected virus in association with hospitalizations for acute respiratory illness in young children and the elderly [8,9] and also a frequent opportunistic pathogen of transplant recipients [10]. In addition, HRV infections have been linked to exacerbation episodes in asthmatic [11], and chronic obstructive pulmonary disease (COPD) patients [12]. Due to the occurrence of more than 100 HRV serotypes with extensive sequence variability in the antigenic sites and the lack of animal models to test the efficacy of approaches to prevent or treat infection *in vivo*, no rhinovirus vaccines are available for use in humans. The lack of animal models has also limited the ability to conduct preclinical studies of antiviral compounds.

Limitations on animal modeling options for human rhinovirus infections are – at least in part – consequences of the high host species specificity of these viruses. Chimpanzees have been successfully infected with HRV14 and HRV43, and gibbons with HRV1A, HRV2, and HRV14, but no overt illness was observed in the infected animals [13,14]. Intracranial injections of virus into monkeys, hamsters, or baby mice did not result in either infection

or disease [15,16]. Various BALB/c and C57/BL mouse models have been recently developed and utilized for the study of aspects of minor- and major-group HRV-induced disease [17–21]. In both mouse models HRV infection was tested showing limited replication of the virus [17]. The recent work by Edlmyer et al., McLean et al., and Glanville et al. examined immunization of mice with recombinant rhinovirus capsid proteins [22–24] showing strong serological data suggesting cross protection. However their published data did not include proof of vaccine efficacy. Thus, no successful immunization/challenge/protection studies in any animal model have been reported. The availability of new experimental small animal model options is therefore still a major pressing need in the vaccinology field.

Over the years, the cotton rat (*Sigmodon hispidus*) has been shown to support replication of a broad spectrum of human viruses including respiratory syncytial virus (RSV) [25], non-adapted strains of human influenza [26,27], and measles [28,29], among others [30], providing modeling capabilities for the corresponding infections. In the present study, we evaluated the cotton rat as a model of human rhinovirus infection that could potentially facilitate the development and testing of vaccines and prophylactic therapies aimed against these important human viruses.

Materials and methods

Virus and cells

Stocks of HRV1B (ATCC cat# VR-1645), and HRV16 (strain 11757, ATCC cat# VR-283), were produced in HeLa Ohio cells, a generous gift of Dr. Dean Erdman (CDC, Atlanta, GA, USA). Cells were grown in Minimal Essential Medium containing Earle salts (E-MEM), 10% fetal bovine serum (FBS); 1.5 g/ml Na_2CO_3, L-glutamine and penicillin/streptomycin and maintained in the same medium with 2% FBS. Replication-deficient virus was generated by exposing the stock to ultraviolet light for 15 min on ice at 100 mJ/cm^2. Infectious virus titers were determined by plaque assay under agarose overlay in monolayers of the same cell line as described below to control for ablation of infectivity.

Animals

Four to eight weeks old cotton rats of both genders were obtained from the inbred colony maintained at Sigmovir Biosystems, Inc. (SBI). Sera from sentinel cotton rats in the colony during the time period of all experiments tested seronegative for rhinovirus by neutralization assay, and seronegative by ELISA to adventitious respiratory viruses (e.g. Pneumonia Virus of Mice, Rat parvovirus, Rat coronavirus, Sendai virus), and *Mycoplasma* sp. Oropharyngeal and fecal cultures tested negative for *Salmonella* sp., *Klebsiella* sp., *Pseudomonas* sp., and *Citrobacter* sp.). Pups were obtained from mothers in the same colony. Animals were housed in large polycarbonate cages, and fed a diet of standard rodent chow and water *ad libitum*. All experiments were performed following federal guidelines and protocols approved by SBI's Institutional Animal Care and Use Committee (IACUC). Cotton rats were infected intranasally (i.n.) or immunized intramuscularly (i.m.) with HRV16 under isoflurane anesthesia by application of 100 μl of solution (10^6–10^7 PFUs) per rat. I.m. immunization with 10^7 PFUs of live HRV1B, UV-inactivated HRV16 (10^7 PFU), or lPOL® polio vaccine was carried out under the same conditions. Serum was obtained by retro-orbital blood collection under isoflurane anesthesia. Pups (3–5 days old) were infected i.n. with 20 μl of virus

($\sim$5 × 10^6 PFUs). Adult animals were euthanized by carbon dioxide asphyxiation. Pups were euthanized by decapitation.

Virus load titration assay

Tissue samples (left lung lobe, entire nose, and trachea) from adult animals were homogenized in 3 ml of infection medium (E-MEM, 2% fetal calf serum, 1.5 g/ml sodium bicarbonate, 25 mM HEPES, penicillin and streptomycin), whereas left lung lobes from pups were homogenized in 1 ml. Infectious virus titers were determined by standard plaque assay and expressed as plaque forming units (PFU)/g of tissue. Briefly 100 μl of pure or tenfold serial dilutions of tissue homogenate supernatant or virus stocks were plated in triplicate onto confluent monolayers of HeLa OH cells in 6-well plates. After 1 h adsorption with rocking every 10 min, monolayers were overlayed with 2 ml of 0.7% low melt agarose in E-MEM containing 2% FBS. Following incubation for 3 days at 33 °C, monolayers were fixed with buffered formaldehyde and stained with crystal violet.

Antibody assay

Neutralizing antibody titers were determined in a plaque-reduction assay. Serial fourfold dilutions of serum were incubated for 1 h at 37 °C with $\sim$50 PFU of HRV16. Virus incubated with PBS was used as control. Neutralization mixes were then plated in quadruplicate onto confluent HeLa cell monolayers in 24-well plates, incubated at 33 °C for 1 h and overlayed with 0.7% Low Melt Agarose in MEM. Cells were incubated at 33 °C and 5% CO_2 for 72 h and then fixed with 1% buffered formaldehyde and stained with crystal violet for reading.

RNA isolation and RT-PCR analysis

RNA was isolated from the lung lingular lobe using the RNeasy Kit (Qiagen Sciences). Bronchoalveolar lavage (BAL) was collected for total RNA isolation from a dedicated group of infected animals by lavaging 3 times with a total 2 ml of PBS each. For mRNA assays, cDNA was prepared by using QuantiTect Reverse Transcription Kit (Qiagen Sciences). Each cDNA reaction was diluted to match 1 μg of the initial RNA per 100 μl of the final cDNA volume. Three microliters of diluted cDNA were used for each qPCR reaction. A HRV16-specific qPCR protocol was developed using primers that target the 5′UTR of HRV-16 (GenBank Accession# L24917). For a quantitative assessment of viral replication, cDNA for the negative stranded viral RNA (as an indicator of active RNA transcription), was synthesized by priming with 5′-CCCTTTCCCAAATGTAACTTAGAAGC-3′. Real-time PCR quantification of both strands was performed using the forward primer 5′-TCAAGCACTTCTGTTTCCCCGGT-3′ and the reverse primer 5′-TCCCATCCCGCAATTGCTCATTAC-3′, generating a fragment of 370 bp. A plasmid containing the 5′UTR of HRV16 was diluted to generate a copy number standard curve that allowed for the quantification of copies/g tissue of the negative replication intermediates. The assessment of cotton rat Mx-1 and Mx-2 mRNA expression was carried out as previously described [30,31].

Histopathology analysis

The right lobes of the lung were dissected with the caudal 1/3 of the trachea attached. Lung lobes were inflated to their normal volume with 10% neutral buffered formalin, immersed in the same fixative, and then processed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Additional sections were stained with Alcian Blue-Periodic acid-Schiff (AB-PAS) for visualization of mucus and mucus-bearing cells. Lung sections were evaluated for several indices of inflammation and cell

changes in the conducting airway and parenchymal (alveolar) compartments (described more fully in the results). Changes were assessed subjectively and scored blindly on a 0 to 4 scale for severity (absent, minimal, mild, moderate, marked), with validation of scoring done by two pathologists experienced in respiratory viral pathogenesis. Graded findings included peribronchiolar inflammatory cell infiltrates, bronchiolitis, bronchiolar epithelial degeneration, mucous cell hyperplasia /hypertrophy, airway luminal mucous /other exudate, perivascular inflammatory cell infiltrates, alveolar septal infiltrates, and alveolar luminal inflammatory cell exudates. Tracheas and nasal turbinates from selected, infected animals were also evaluated.

Statistical analysis

Viral titers, expression of Mx genes, and production of the HRV16 replicative intermediate were calculated as geometric means ± standard error for all animals in a group at a given time post infection. Student t-test was applied to determine statistically significant differences between two groups, using an unpaired, two-tailed test set at $p < 0.05$. Pulmonary pathology scores were expressed as the arithmetic mean ± standard error for all animals in a group. Significance of score differences between uninfected and infected groups at different days post-infection for each histological parameter was analyzed by one way Kruskal–Wallis ANOVA and set at a value of $p < 0.05$.

Results

HRV16 infection in cotton rats

Adult cotton rats were infected i.n. with 10^7 PFU of HRV16. Groups of 5 animals were euthanized at 30 min, 2, 4, 6, 8, and 10 h, and at day 1, 2, and 4 post-infection (p.i.) (Fig. 1A). Non-infected animals or animals inoculated with UV-inactivated HRV16 were used as controls. Infectious HRV16 was recovered from the
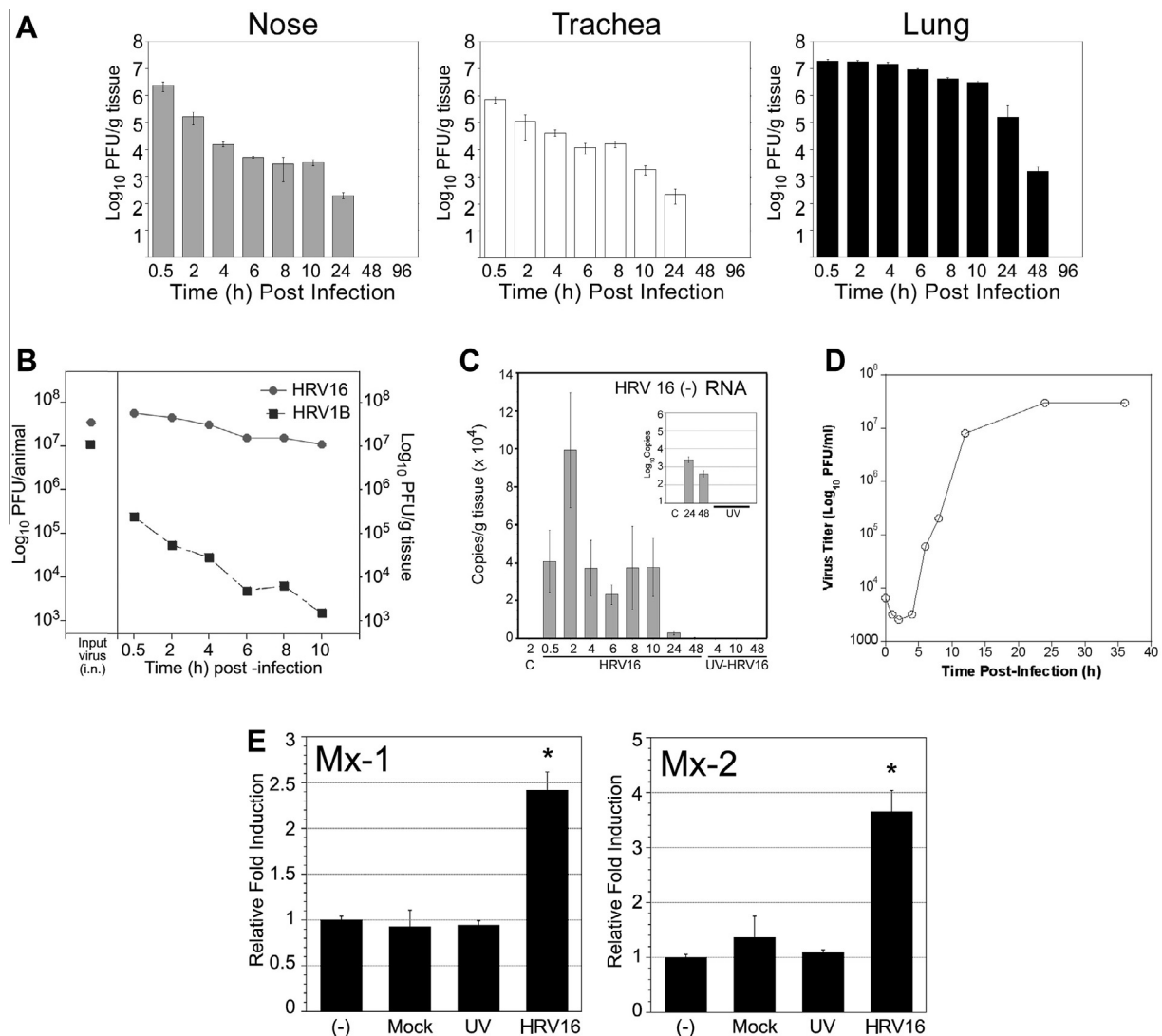

Fig. 1. Cotton rat infection with HRV16. Cotton rats were infected i.n. with 10^7 PFU of HRV16. (A) Infectious virus titers in nose, trachea, and lung homogenates from infected animals at the indicated times (h) post-infection. Groups of 5–10 animals were euthanized at each time in a total of 3 independent experiments. (B) Lung viral loads in rats infected with HRV16 compared to those of rats infected with HRV1B. Left axis shows input virus. (C) Quantification of (−) vRNA by RT-PCR in lung tissue at the indicated times post-infection. Animals inoculated with UV-inactivated HRV16 were used as control. Insert is a blow-out of the 24 h and 48 h time points from HRV16-infected and UV-HRV16-inoculated animals using logarithmic scale ($n = 5$ per time point). (D) One-cycle replication of HRV16 in HeLa Ohio cells. (E) qPCR quantification of Mx-1 and Mx-2 transcripts in BAL cells from uninfected or mock-inoculated rats, and rats instilled with UV-inactivated HRV16 or live HRV16 at 6 h after challenge. $n = 8$ per group. $^*p < 0.05$ in Student t-test analysis between HRV16-infected group and each of the control groups.

nose and trachea until day 1 p.i., and from the lung until day 2 p.i. No virus was detected in any of the tissues from animals euthanized on day 4 p.i. or in any of the tissues from uninfected controls (not shown). The highest virus titers were detected in the lungs (>10^7 PFU/g of tissue), followed by the nose and the trachea ($\sim 10^6$ PFU/g of tissue). A brief plateau of viral loads was detectable in the nose and trachea between 6–10 h p.i. followed by a decrease and clearance of virus. Infection with HRV16 at a lower dose (10^6 PFU/animal) resulted in lung viral titers of $5.9 \times 10^5 \pm 8.6 \times 10^4$ PFU/g at 8 h p.i. ($n = 16$), with no detectable infectious virus at 48 h p.i. (data not shown). We compared the viral load in the lung, between rats infected with the major group HRV16 and rats infected with the minor group HRV1B and found that the course of infection with the 2 viruses was very different (Fig. 1B). Higher infectious virus titers were recovered from the lungs of animals infected with HRV16 than from animals infected with a similar dose of hRV1B, suggesting that the cotton rat is significantly more permissive to infection by HRV16. Based on these results we only used HRV16 in all the subsequent experiments.

The lung load of virus was also examined by determining production of the replication intermediate, negative strand viral RNA [(−)vRNA] by PCR (Fig. 1C). (−)vRNA strand was detected in lungs of infected animals up to 48 h p.i. (Fig. 1C, see also inset). Animals inoculated with UV-inactivated HRV16 (10^7 PFU) showed undetectable negative strand HRV-16 RNA. The apparent rapid kinetics of viral replication observed *in vivo* were consistent with data from one-step growth curves carried out in HeLa Ohio cells showing that a complete replication cycle of HRV16 occurs in 6–10 h (Fig. 1D).

We measured the expression of cotton rat Mx1 and Mx-2 genes in the lungs in response to HRV16 infection as evidence of presence of type I IFNs. Mx1 and Mx2 are two IFN-inducible genes that mediate antiviral activity [31–33]. The activation of expression of Mx-1 and Mx-2 was detected in BAL cells of HRV16-infected cotton rats at 6 h p.i. (Fig. 1E) but not in either of the two subsequent time points (12 h and 24 h – data not shown), indicating that the induction of IFN was transient.

Histopathology in HRV16-infected cotton rats

Analysis of the pathology associated with HRV16 infection was performed in the nose, trachea, and lung. No significant lesions were observed in the nasal turbinate sections. Epithelial degeneration was present in the trachea and large pulmonary airways of HRV16-infected rats. Infection was associated with direct and pro-
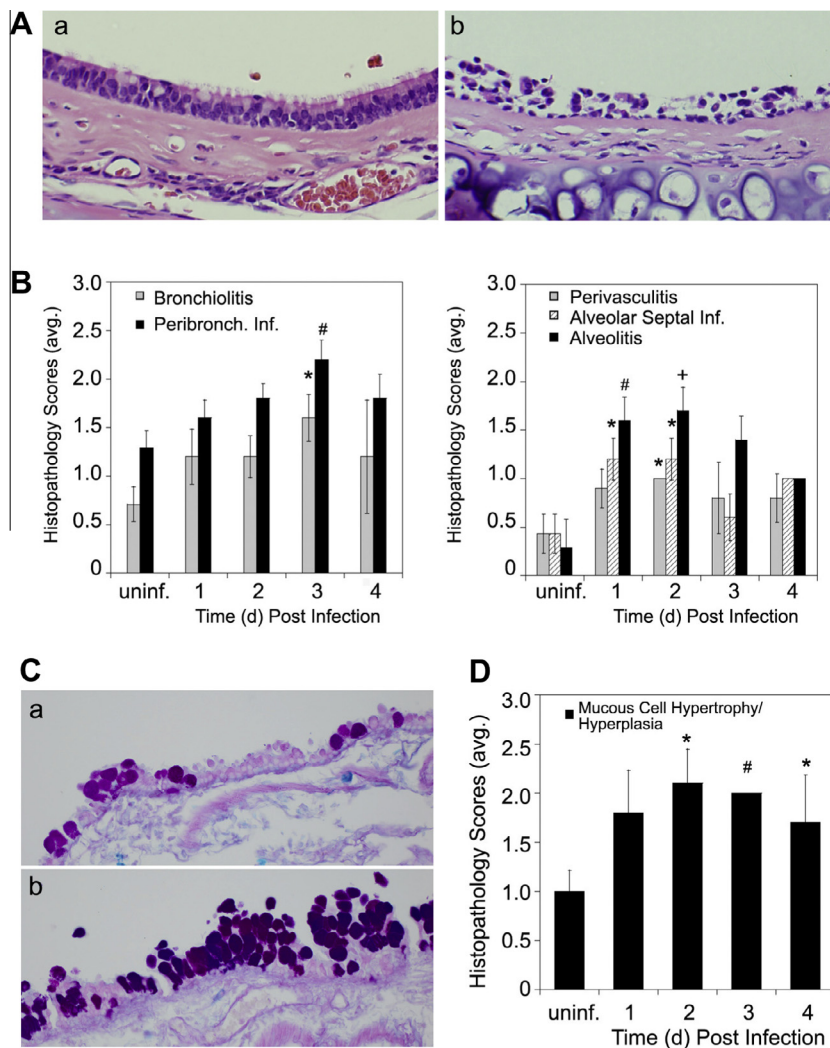

Fig. 2. Airway pathology in HRV16-infected cotton rats. (A) Tracheal epithelial cell defoliation in HRV16-infected animals. (a) Trachea from an uninfected, or (b) HRV16-infected cotton rat. H&E staining, magnification 100×. (B) Histopathology scores obtained from lungs of uninfected (uninf) or HRV16-infected animals and euthanized at the indicated days post-infection. Graphs represent the extent of peribronchiolar infiltration, bronchiolitis, and epithelial degeneration (left); perivascular infiltration, alveolar septal infiltration and alveolar exudates (right). $n = 3$–9. *$p < 0.05$, #$p < 0.01$, +$p < 0.005$ in one way Kruskal–Wallis ANOVA. (C) Alcian blue-PAS staining of a bifurcation of the main axial airway of an uninfected (a), or a HRV16-infected animal (b). (D) Scores for mucous cell hypertrophy/hyperplasia. *$p < 0.05$, #$p < 0.01$ in one way Kruskal–Wallis ANOVA.

Table 1
Serum neutralizing activity.

Prior exposure to HRV16[a]	No of rats tested	Percentage plaque reduction (SE)[b]	
		1:16	1:1280
None	5	<10	<10
10^6 i.m	8	100	91 (9)
10^7 i.n.	8	25 (19)	<10

[a] Animals exposed to HRV16 on day 0 and 21.
[b] Plaque reduction assay using serum obtained at day 42 post-first exposure in the indicated dilutions.

gressive damage of the ciliated columnar epithelium of the trachea that peaked on day 4 p.i. and often exposed the basal membrane (Fig. 2A).

Lung pathology demonstrated mild but significant alveolitis (neutrophilic and histiocytic), and peribronchiolar infiltrates of neutrophils, macrophages, and lymphocytes (Fig. 2B). Peak damage of the lung parenchyma (perivasculitis, alveolar septal infiltrates, and alveolitis) was recorded on day 1–2 p.i., whereas airway damage was predominantly seen on day 3 p.i. Mucous cell hypertrophy/hyperplasia was evident in H&E- and AB-PAS-stained lung sections as early as 1 day p.i. but continue elevated by day 4 p.i. (Fig. 2C). Thus, HRV16 infection in the cotton rat reproduces aspects of human disease in the URT with detectable inflammation in the lower airways and lung parenchyma. In contrast, infection with HRV1B did not result in significant pathology.

Antibody production in response to HRV16

Intramuscular immunization of adult rats with live HRV16 at a dose of 10^6 PFUs in a priming (day 0) and boosting (day 21) schedule resulted in high serum levels of neutralizing antibodies at 42 days after the first immunization. Surprisingly, that was not the case when the same amount of virus was instilled i.n. following an identical schedule. As shown in Table 1, all animals immunized intramuscularly showed neutralizing antibody titers >1280, whereas animals that underwent i.n. infection or re-infection with HRV16 showed low neutralizing antibody titers (<16). Furthermore, when animals were immunized i.m. once with 10^7 PFUs and challenged i.n. 21 days later with HRV16 (10^7 PFUs), infectious virus was not detectable in the nasal turbinates or in the trachea, and a reduction (>3$\log_{10}$) in infectious virus titers was detected in the lung (Fig. 3A). As expected, intramuscular immunization with live HRV1B, or UV-inactivated HRV16 (10^7 PFU), or with a current polio vaccine (Ipol) failed to confer measurable protection upon i.n. HRV16 challenge (Fig. 3B).

The possibility that the observed reduction in viral titers in the lungs of animals vaccinated i.m. was caused by neutralization *in vitro* was evaluated by mixing an equal amount of HRV16-infected lung homogenate from a naïve animal with lung homogenates (h1, h2, h3) of individual vaccinated animals, all euthanized at 10 h p.i. As shown in Fig. 3D, homogenate mixes yielded the amount of virus in the lung suspension predicted from dilution in the indicated proportions (1:2 and 1:5 respectively) without evidence of *in vitro* neutralization. This result indicated
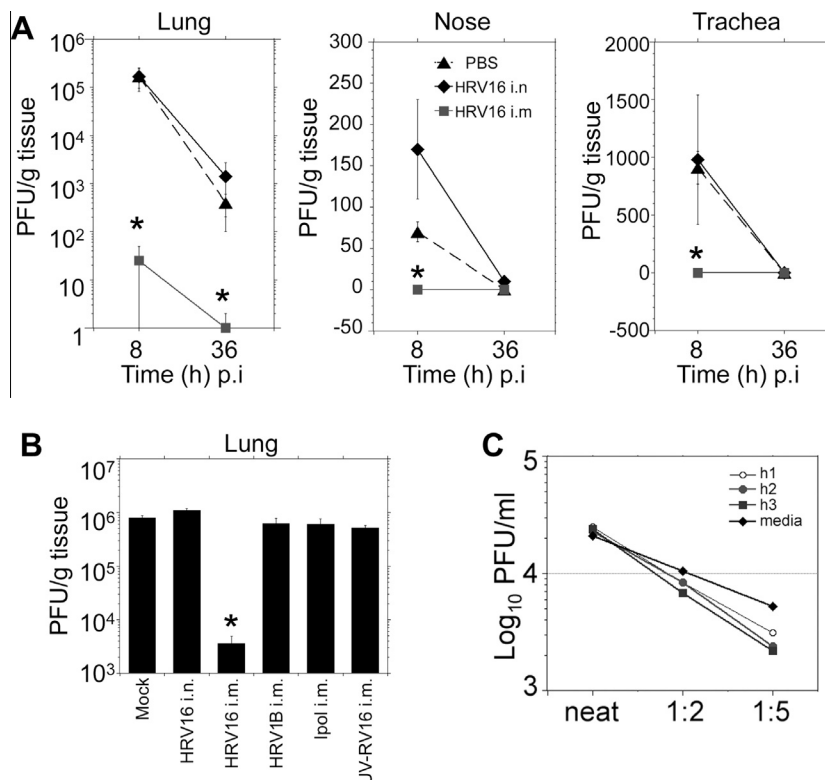

Fig. 3. Immunogenicity and efficacy of immunization with live HRV16. (A) Viral titers in the lung, nose, and trachea of animals challenged with HRV16 (10^7 PFUs) 21 days post i.m. injection of PBS, post i.n. infection with 10^7 PFUs of HRV16, or post i.m. immunization with 10^7 PFUs HRV16. $n = 5$ per group. *$p < 0.05$ in Student t-test analysis between HRV16-i.n. immunized group and the PBS-injected control group. (B) Immunization with different viral preparations showed that protection by immunization was specific for HRV16 and the intramuscular route. (C) Neutralization of viral infection occurs *in vivo*. Lung homogenates from 3 animals previously immunized with HRV16 (neutralization titers > 1280), subsequently challenged and sacrificed 10 h after challenge (h1, h2, h3) were mixed with homogenates of a naïve, challenged animal (PBS i.m.) also sacrificed 10 h p.i. Mixed homogenates in the indicated dilution (immune:control) were then titrated for determination of viral load. A control curve (media) was performed by diluting a control homogenate with titration media. The reduction of resulting titers in all mixes was consistent with the dilution factor and not consistent with neutralization occurring *ex vivo*.

that the protection observed was due to *bona fide* immunity and not to neutralization of the virus *ex vivo* after homogenization of the tissue.

Passive transfer of anti-HRV16 immune serum confers protection against HRV16 challenge

We tested the efficacy of the prophylactic administration of immune (neutralizing antibody titer of 1280 against HRV16) or normal cotton rat serum to protect against HRV16 challenge. The neutralizing antibody titers detected in treated animals prior to challenge are shown in Table 2. All animals that received 500 µl of undiluted sera (neat) intraperitoneally or serum diluted 1:4, 24 h prior to challenge showed a reduction of lung viral titers of 2 and 1 $\log_{10}$, respectively, whereas animals that received more diluted immune serum (1:8), naïve/control cotton rat serum, or PBS, remained unprotected (Fig. 4). These data indicate that passive transfer of antibodies can be an effective prophylactic therapy against HRV infection.

Maternal immunization results in transfer of protection to pups

Young female cotton rats that were immunized twice i.m. with live HRV16 (10^6 PFUs) were bred to unimmunized males. Newborn pups delivered from immunized or non-immunized females (the gestation time for *S. hispidus* is 4 weeks) were challenged at 3–5 days of age with HRV16 i.n. and euthanized at 10, 24, and 48 h p.i. Pups from non-immunized mothers showed lung HRV16 titers consistent with those of naïve adult cotton rats challenged with HRV16 (Fig. 5A). Pups from immunized mothers showed a statistically significant reduction in infectious virus titers (>3$\log_{10}$) and also a significant reduction in the viral load measured as copies of HRV16 (−)vRNA (Fig. 5B). Neutralizing antibody titers in the pups correlated with the level of lung protection. Fully protected pups (all animals euthanized at 10 h, Fig. 5) showed neutralization titers >8192, whereas pups euthanized at 24 or 48 h post infection harboring low but detectable virus titers showed neutralizing antibody titers of ~256, suggesting a correlation of protection and serum neutralizing antibody titers as seen in the adults. These data indicate that maternal immunity conferred by immunization is transferable to newborn pups and sufficient for protection of offspring from viral infection and possible replication in the lung.

Discussion

The morbidity and mortality attributable to rhinovirus infection are considerable and result in billions of dollars of health care costs every year. Despite the significance of the problem, no effective prevention of HRV infection or treatment of HRV-associated disease is currently available due in part to the lack of a reliable animal model suitable for protection studies.

HRV16 is one of the 91 serotypes classified within the major group that use the well-characterized ICAM-1/CD56 receptor for attachment and entry [34], and has been a model virus for studying transmission of rhinoviruses [35], pathogenesis of the common cold [35,36], virus-induced asthma and COPD [37–39], and for the evaluation of anti-rhinovirus drugs in human volunteers because it reproducibly induces severe symptoms in human subjects. HRV16 has been recently used for *in vitro* studies aiming to define the molecular mechanisms of rhinovirus-induced inflammatory responses in airway epithelial cells [40,41].

Transgenic BALB/C mice expressing a human/mouse ICAM-1 chimeric receptor show human disease-relevant outcomes including pulmonary inflammation and mucin production when infected i.n. with HRV16, despite the low levels of viral replication

Table 2
Prophylactic effect of anti-HRV16 serum.

Serum inoculated i.p	No. of rats	Serum neutralizing antibodies at the time of challenge (geometric mean, reciprocal ± S.E.)[a]	Lung titer of virus 12 h after challenge with 10^7 PFU of HRV16 (geometric mean $\log_{10}$ PFU/g ± S.E.)[b]
Control serum (neutralizing antibody titer < 1:20)	10	<20	6.1 ± 0.12[c,d]
Immune serum (neutralizing antibody titer 1:1280)	4	340 ± 96	3.9 ± 0.12[c,d]
(neutralizing antibody titer 1:320)	3	104 ± 52	4.9 ± 0.22[d]
(neutralizing antibody titer 1:160)	3	55 ± 32	6.1 ± 0.23

[a] Measured by 60% plaque reduction assay against HRV16 and expressed as reciprocal of the geometric mean. Serum was collected one day post-treatment.
[b] Measured by plaque assay.
[c] $p<0.005$.
[d] $p<0.01$.

detectable by RT-PCR [17]. Our data show that in contrast to the mouse, which requires genetic manipulation, the cotton rat appears to host infection and possibly replication of HRV16 in the URT and LRT. Although the data from examination of viral load in the respiratory tract over time is certainly compatible with a non-permissive model of infection and clearance of the inoculum over time, the differences observed between the course of infection by HRV16 and HRV1B together with the detection of (−)vRNA in infected tissues suggest that HRV16 may be actually replicating in the cotton rat. The refinement of our real time qRT-PCR protocols to increase specificity for the detection of the (−) strand viral RNA will provide the ability to address this important issue and better describe the model.

In contrast to what has been described for other respiratory viral infections, such as influenza [26,27], HRV infection of cotton rats resulted only in mild epithelial damage (vacuolar degeneration,
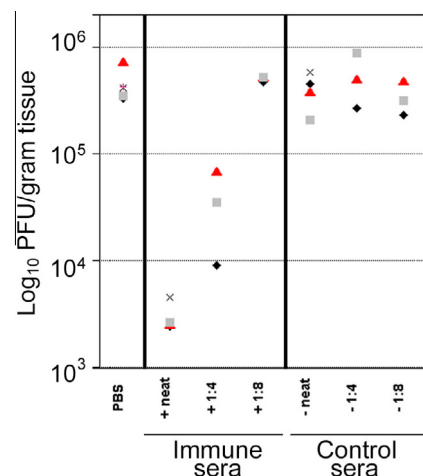

Fig. 4. Passive HRV16 antibody transfer protects animals from HRV16 challenge. Animals were treated intraperitoneally with control cotton rat serum (anti-HRV16 neutralization titers < 20) or with different serum dilutions from animals immunized i.m. with HRV16 (1×10^6 PFU). At 1 day post-treatment, animals were challenged i.n. with 10^7 PFUs of HRV16, and euthanized 8 h later to determine lung viral titers. Each symbol corresponds to one animal.
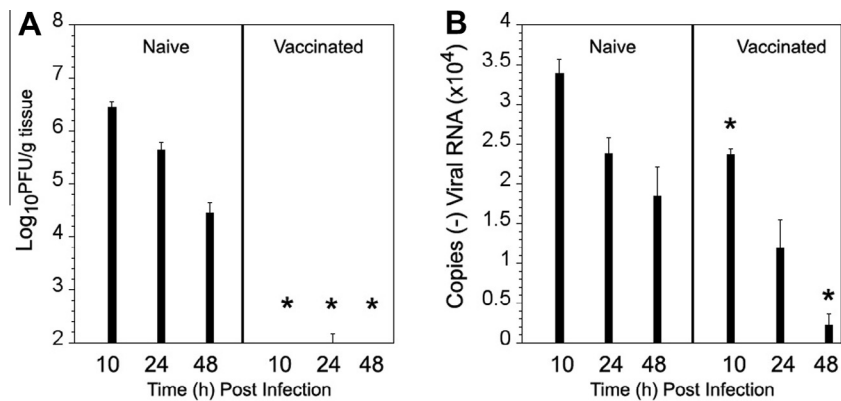
Fig. 5. Maternal immunity confers protection to pups. (A) Newborns from naïve or HRV16-immunized females were challenged i.n. with ∼5 × 10⁶ PFUs of HRV16 in 20 µl. Infectious virus titers in the lung were determined by plaque assay at the indicated times p.i. (B) HRV16 (−) viral RNA detection in lungs of newborn cotton rats. $n = 6–8$ animals/group where each group consisted of pups from 2 different mothers. *$p < 0.05$ in Student t-test comparison between group of pups whose mothers were immunized *vs* groups of pups of the same age from unimmunized, naïve mothers.

apical blebbing in the bronchioles, sloughing in the trachea), consistent with previous observations in humans [42,43].

Unlike the laboratory mouse, which either lacks or has defective Mx genes, the cotton rat has a set of fully functional Mx genes. As described for the human Mx counterparts [31,32,44,45], cotton rat Mx genes are expressed in response to infection with influenza and RSV. As shown by the present study, transient Mx gene expression was detected in infected coton rat BAL cells 6 h post HRV16 infection. This result parallels the detection of Mx1 (MxA) and Mx2 expression in nasal epithelial scrapings obtained 8 h after experimental HRV16 infection of humans [46].

The results of the described experimental work show that HRV16 infection in the cotton rat reproduces aspects of HRV-associated human disease in the respiratory tract, causing detectable inflammation in the lower airways and lung parenchyma and mucus production, and inducing a transient expression of interferon-stimulated genes that merits further investigation. In our model of i.n. infection, the pulmonary infectious viral load is measurable within the 48 h following instillation and thus provides a powerful readout for assessment of protection by immunization and passive transfer of neutralizing antibodies. We found that i.m. inoculation of live virus but not UV-inactivated resulted in a significant decrease of viral loads in nasal turbinates, trachea, and lung of challenged animals suggesting that replication at the site of injection played a role in the resulting. A measurable protection against challenge was achieved with only one immunization (Fig. 3). The immunogenicity of the inoculum was likely dependent upon viral replication in the area of immunization since UV-inactivation of the virus completely abolished protection. The protection achieved with live virus was specific for HRV16 since no protection was conferred by immunization with live HRV1B. These results could be due to the apparent lack of replication of HRV1B in the cotton rat respiratory tract (data not shown), but are most likely the result of the lack of shared neutralizing epitopes between HRV16 and HRV1B. The spectrum of cross-reactivities of cotton rat anti-HRV16 neutralizing antibodies should be further investigated in view of the recent reports regarding the induction of *in vitro* cross-neutralizing antibodies after immunization with recombinant HRV14 and HRV89 VP1 [22], and the weak induction of HRV1B binding antibodies but not neutralizing antibodies in the mouse by immunization with HRV16 VP0 [23].

Previous studies conducted in cotton rats have demonstrated the passive transfer of maternal immunity against RSV to offspring [47]. In this study, we showed that the anti-HRV16 immunity elicited in mothers through i.m. immunization also conferred complete protection of their 3–5 days old pups against i.n.

challenge with HRV. Importantly, data from this experiment strongly suggest that newborn cotton rats also support viral replication in the LRT, setting the stage for future testing of potential antibody-based and other therapies for infants.

The cotton rat is well-recognized for its pivotal role in the development of the immunoglobulin prophylactic treatments for RSV-associated disease, RespiGam® and Synagis® [48–51]. Data from the present study demonstrate that passive transfer of cotton rat hyper-immune serum is also effective and that a neutralizing antibody titer of 320 was sufficient for a 2 log₁₀ reduction of viral load in the lung. Without a doubt, the cotton rat model will be useful for determining the value of different arrays of anti-rhinovirus antibodies in an *in vivo* challenge experimental design.

Although the high diversity of serotypes that characterize this group of viruses remains a major challenge for the development of pan-crossreactive antibody-based intervention, the work conducted using our cotton rat model of HRV infection generated strong evidence that the parenteral route of immunization can be effective and is therefore disserving of additional investigation given the important questions that have been raised in the vaccinology field regarding the design strategies that will be required for effective multivalent HRV vaccines or therapeutic antibodies. Importantly, our data highlight the large potential of the cotton rat to provide an experimental platform complementary to that offered by the available mouse models to further evaluate the immunogenicity of individual HRV capsid proteins [22,23,47], some of the already identified anti-HRV antibodies with documented neutralizing activities *in vitro* [52], antibodies targeting select domains of the virus receptor [53], as well as other alternative antibody-based strategies under consideration [54] to support new developments in this area.

Conclusions

In this work we show that the cotton rat hosts infection with HRV16 with infectious virus (and negative stranded vRNA) loads detectable in the lung of infected animals within 48 h following intranasal infection. Infection results in measurable pathology, mucus production, and expression of inflammatory mediators.

While intranasal infection with HRV16 resulted in the production of low levels of neutralizing antibodies that conferred reduced protection after re-challenge, intramuscular immunization with live HRV16 resulted in strong type-specific protection that correlated with high levels of systemic neutralizing antibodies. In addition we demonstrated that passive transfer of antibodies generated in vaccinated cotton rats can protect naïve animals from

pulmonary infection. Thus, these results demonstrate that the cotton rat is a suitable animal model for challenge studies to explore the development of anti-rhinovirus vaccines and anti-viral therapies.

Acknowledgments

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