Unilateral nasal infection of cotton rats with respiratory syncytial virus allows assessment of local and systemic immunity

Susan A. Johnson,1 Martin G. Ottolini,2 Miriam E. R. Darnell,1 David D. Porter3 and Gregory A. Prince1,*

1 Virion Systems Inc., 9610 Medical Center Drive, Suite 100, Rockville, MD 20850, 2 Department of Pediatrics, Uniformed Services University of the Health Sciences, Bethesda, MD 20814 and 3 Department of Pathology and Laboratory Medicine, University of California School of Medicine, Los Angeles, CA 90024, USA

An in vivo model for the study of local and systemic effectors of immunity to respiratory syncytial virus (RSV) is described. Cotton rats (Sigmodon fulviventer) inoculated in one nostril with a small volume (2 μl) of virus suspension contracted a unilateral nasal infection which did not extend to the contralateral nasal turbinates, nor to the lungs. Immunity to subsequent RSV challenge could be induced by small priming doses (<10 p.f.u. per animal), but was dependent upon viral replication, as virus inactivated by UV light was not immunogenic. Immunity occurred in the absence of detectable neutralizing serum antibody. The onset of resistance to viral challenge occurred simultaneously in ipsilateral nasal, contralateral nasal and pulmonary tissues. However, low levels of transient viral replication occurred in contralateral nasal turbinates and in lungs following virus challenge, thus indicating that local components of immunity acting at the ipsilateral site of infection were more effective than systemic components acting at the other sites. Further evidence is provided to suggest that three types of immunological effectors – local, persistent systemic and transient systemic – participate in the immune response to RSV infection.

Introduction

Since its identification as a human pathogen in 1957, respiratory syncytial virus (RSV) has come to be recognized as the primary cause of infant pneumonia and bronchiolitis throughout the world (McIntosh & Chanock, 1990). Intensive efforts spanning three decades have failed to yield a safe and effective vaccine, and even the most optimistic investigators concede that no vaccine is likely to provide durable immunity, since natural infection induces only short-term immunity. Indeed, the failure of the host’s immune response to maintain resistance to a virus which does not undergo substantial antigenic evolution remains the most enigmatic feature of RSV disease.

In recent years some progress has been made in identifying effectors of RSV immunity. Studies using the cotton rat showed that, contrary to conventional wisdom, serum antibody with high RSV-neutralizing antibody could prevent RSV infection (Prince et al., 1983, 1985a). Recently, completed clinical studies confirmed the efficacy of IgG in preventing RSV disease in high-risk infants (Groothuis et al., 1993). Other studies have characterized immune lymphocyte populations generated in response to RSV infection (Kulkarni et al., 1993; Openshaw et al., 1990), some of which are capable of preventing infection in adoptive transfer experiments (Alwan et al., 1992; Nicholas et al., 1991).

Important though these studies have been, they have not addressed two issues crucial to understanding the ephemeral nature of RSV immunity. First, they have focused on single effectors of immunity (either purified antibody or T cells), and thus have shed no light on the interactions of two or more effectors. Second, they have examined systemic effectors, isolated from whole blood or non-respiratory lymphoid tissues, but effectors within the respiratory mucosa itself have not been studied. In as much as we have demonstrated solid immunity to RSV in the absence of detectable systemic effectors (Piazza et al., 1993; Prince et al., 1985a), we have developed an in vivo model to allow the study of local effectors of immunity in situ.
Methods

Animals. Cotton rats (Sigmodon fulviventer, SIF/Vsi) were obtained from a colony maintained by Virion Systems Inc., Rockville, Md., USA and were housed and fed as previously described (Prince et al., 1978). Cotton rats used in the studies were outbred, but were in the early stages of an inbreeding program in a closed colony. Animals were shown to be free of serum neutralizing antibody against RSV prior to inclusion in the study.

Virus and cells. The Long strain of RSV (Group A), obtained from the ATCC was used in the study. Stocks of this virus were prepared in HEp-2 cells (ATCC) grown in Eagle’s minimal essential medium supplemented with 10% fetal calf serum, antibiotics and glutamine, and contained 10^6 p.f.u./ml. Parainfluenza virus 3 (PIV3) (lot 0691) was obtained from Program Resources Inc., Rockville, Md., USA and contained 10^6 p.f.u./ml.

Experimental protocol. Cotton rats were anaesthetized with methoxyflurane (Mallinckrodt Veterinary) inhalation for virus inoculation. Priming inoculation was performed with a microsyringe (1700 series gastight syringe with removable needle, 225 gauge, 100 µl capacity, and Chaney adapter, Hamilton Co.) attached to tubing (Intramedic medical/surgical tubing, 0.03 inch i.d. ×0.048 inch o.d., Becton Dickinson) with a small catheter (Intramedic medical/surgical tubing, 0.011 inch i.d. ×0.024 inch o.d.) attachment. Once anaesthetized, animals were placed in the supine position and the inoculating catheter was inserted 5–8 mm into one nare. Priming virus suspension was delivered in a 2 µl volume. Challenge inoculation was performed intranasally with 100 µl of RSV (10^6 p.f.u. per animal). Animals were sacrificed by carbon dioxide asphyxiation at intervals following inoculation.

For preparation of nasal tissues, the skin was removed from the head with a size #21 scalpel blade and the skulls were split sagittally in the midline using a single edge razor blade. Using a small scalpel blade (size #15), the nasal septum was carefully removed, exposing the nasal turbinates. The primed (ipsilateral) and unprimed (contralateral) turbinates were removed using separate sterile size #15 scalpel blades and processed separately in porcelain mortars and pestles containing sterile sand and Hanks’ balanced salt solution (supplemented as described below). Precise, exact technique in removal of the turbinates is essential in this model to avoid cross-contamination.

For virus titration, nasal turbinates and lungs were homogenized in 10 parts (w/v) of Hanks’ balanced salt solution supplemented with 0.218 M-sucrose, 4.9mM-glutamate, 3.8mM-KH2PO4 and 7.2mM-K2HPO4. Following homogenization, samples were centrifuged at 770 g, 4 °C for 10 min. Resulting suspensions were stored at −70 °C until assayed.

Animals were bled from the retro-orbital venous plexus, and serum was stored at −20 °C until assayed for neutralizing antibody.

Virus assay. Virus titres of RSV were determined by plaque assay on HEp-2 cell monolayers as previously described (Prince et al., 1978). Virus titres were expressed as the geometric mean of individual titres (p.f.u./g tissue) for all animals at a given time point. The sensitivity of this assay is 10^6 p.f.u/g for pulmonary tissues, and 10^6 p.f.u/g for nasal turbinates.

Antibody assay. Serum neutralizing antibody against RSV was measured by a plaque reduction neutralization assay on HEp-2 cell monolayers, using a 60% reduction end-point, as previously described (Prince et al., 1978). Antibody titres were expressed as the geometric mean of individual titres for each animal group at a given time.

Histopathology. Skulls for histopathological analysis of nasal turbinates were fixed with 10% neutral buffered formalin, acid decalcified, and embedded in paraffin for sectioning. Histological sections were stained with haematoxylin and eosin.

UV inactivation. Virus inactivation was performed by exposing a glass culture dish containing RSV 3 inches from a double bulb UV light source for 60 s (Prince et al., 1979). Inactivation was confirmed via plaque assay on HEp-2 monolayers (data not shown).

Statistical analysis. Geometric means of virus titres of experimental groups were compared with those of control groups by using the two-tailed Student’s t-test of summary data.

Results

Growth of RSV following unilateral priming

Cotton rats were inoculated with 2 µl (10^3 p.f.u.) of RSV in the ipsilateral nostril and sacrificed at 1 through 8 days for virus titration (Fig. 1). The ipsilateral nasal turbinates replicated virus to a peak titre of 10^7 p.f.u./g at 4 days (Fig. 1 a). In contrast, virus replication was not detected in the contralateral nose or the lungs at any time interval (Fig. 1 b, c).

Histopathology of unilateral infection

Histopathological changes in the nasal epithelium of ipsilateral and contralateral nasal turbinates were assessed 4 days after unilateral priming with 2 µl of RSV in the ipsilateral nostril (Fig. 2). The ipsilateral nasal turbinates showed severe damage to the ciliated epithelium and marked dilation of the submucosal blood vessels (Fig. 2 a). In contrast, the contralateral nasal turbinates showed normal ciliated epithelium and submucosal blood vessels (Fig. 2 c).
Fig. 2. Cotton rat nasal epithelium. (a) Infected with RSV 4 days earlier, showing severe damage to the ciliated epithelium and marked dilation of the submucosal blood vessels. Magnification 510 ×. (b) Low power view of the midline of the nose showing infected epithelium on the left (ipsilateral nasal turbinate) and uninfected epithelium on the right (contralateral nasal turbinate). Magnification 129 ×. (c) Uninfected (contralateral) side with normal ciliated epithelium and submucosal blood vessels. Magnification 510 ×. All stained with haematoxylin and eosin.
Table 1. Onset of immunity following unilateral priming

<table>
<thead>
<tr>
<th>Day of sacrifice</th>
<th>Virus titre 2 days after RSV challenge (geometric mean, log10 p.f.u./g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ipsilateral nose</td>
</tr>
<tr>
<td>4</td>
<td>Primed</td>
</tr>
<tr>
<td></td>
<td>5.53</td>
</tr>
<tr>
<td></td>
<td>4.41</td>
</tr>
<tr>
<td>5</td>
<td>Primed</td>
</tr>
<tr>
<td></td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>4.93</td>
</tr>
<tr>
<td>6</td>
<td>Primed</td>
</tr>
<tr>
<td></td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>4.09</td>
</tr>
<tr>
<td>8</td>
<td>Primed</td>
</tr>
<tr>
<td></td>
<td>4.62</td>
</tr>
<tr>
<td>10</td>
<td>Primed</td>
</tr>
<tr>
<td></td>
<td>3.42‡</td>
</tr>
<tr>
<td>14</td>
<td>Primed</td>
</tr>
<tr>
<td></td>
<td>≤ 3.30‡</td>
</tr>
</tbody>
</table>

* P < 0.05. † P < 0.005. ‡ P < 0.001.

Onset of immunity following unilateral priming

Cotton rats were inoculated with 2 μl (10³⁸ p.f.u.) of RSV or left unprimed. Groups of eight animals per time period were bilaterally challenged intranasally with 100 μl of RSV (10⁴ p.f.u.) at 2, 3, 4, 6, 8 and 12 days post-priming and sacrificed 2 days post-challenge. Virus titres were determined in ipsilateral and contralateral nasal turbinates and lungs (Table 1).

Ipsilateral and contralateral nasal turbinates displayed significant protection against RSV challenge as soon as day 8 post-priming. Lung tissues remained susceptible to RSV challenge for a slightly longer period of time, and were never completely free of virus at any time period tested.

Effect of unilateral priming with UV-inactivated virus

To determine if a protective immune response could be induced with non-replicating virus, unilateral priming was performed after exposure of the priming virus to a UV light source. Cotton rats were divided into three groups: the first group received 2 μl (10³⁸ p.f.u.) of live RSV, the second group received 2 μl of RSV exposed to a UV light source for 60 s (non-replicating virus), and the third group served as an unprimed control. Two weeks post-priming, all groups were bilaterally challenged with 10⁵ p.f.u. of RSV in a 100 μl volume and sacrificed 4 days post-challenge. Virus titration was determined in ipsilateral and contralateral nasal turbinates and lungs for all groups (Table 1).

Unilateral priming with 2 μl (10³⁸ p.f.u.) of live, replicating RSV significantly protected animals from subsequent RSV challenge (P < 0.001) when compared to controls. These tissues were virus free (ipsilateral and contralateral nasal turbinates) or nearly virus free (lungs) following RSV challenge. In contrast, unilateral priming with UV-irradiated (non-replicating) virus did not confer immunity in any of the tissues when compared to those of unprimed control animals.

Specificity of protection from RSV challenge

To determine if protection of tissues from RSV challenge was specific, unilateral priming was performed with PIV3. Cotton rats were unilaterally inoculated in the right nostril with 2 μl (10³⁸ p.f.u.) of PIV3 or left unprimed. Two or four weeks later, groups of animals were bilaterally challenged with 10⁵ p.f.u. of RSV in a 100 μl volume and sacrificed 4 days post- RSV challenge.

In contrast to unilateral priming with RSV, animals primed with PIV3 showed no protection in contralateral nasal turbinates and lungs upon RSV challenge at 2 or 4 weeks post-unilateral priming (data not shown). The ipsilateral nasal turbinates of animals primed with PIV3 showed a slight reduction in titre (from 10⁴ p.f.u./g to 10³ p.f.u./g) upon challenge with RSV which was statistically significant (P < 0.005) at 2 weeks, although this protection was not complete. Similar animals challenged with RSV at 4 weeks were not significantly protected in the ipsilateral nasal turbinates.

Immunogenicity of varied priming doses

The effect upon subsequent challenge of unilateral priming with various doses of RSV was assessed by inoculating cotton rats in the right nostril with graded doses of RSV (2000, 500, 125, 31, 2 or 0.5 p.f.u.), each in a volume of 2 μl. Actual doses were confirmed at the time of delivery via plaque assay on HEp-2 monolayers, although two doses (2 and 0.5 p.f.u.) were below the sensitivity of the plaque assay (data not shown). Two
weeks later, all animals were bled and bilaterally challenged with $10^5$ p.f.u. of RSV delivered intranasally in a 100 µl volume and sacrificed 4 days post-challenge (Fig. 3).

The serum neutralizing antibody titre against RSV at the time of challenge was undetectable ($< 1:20$) for all groups of unilaterally primed animals. Despite the lack of serum neutralizing antibody, unilateral priming from 2000 p.f.u. through the 8 p.f.u. per animal dose conferred significant protection in ipsilateral (Fig. 3a) and contralateral (Fig. 3b) nasal turbinates when compared to unprimed controls ($P < 0.005$ and $P < 0.001$, respectively).

---

### Table 2. Effect of unilateral priming with UV-inactivated virus on RSV challenge

<table>
<thead>
<tr>
<th>Virus titre 4 days after RSV challenge (geometric mean, log$_{10}$ p.f.u./g)</th>
<th>Ipsilateral nose</th>
<th>Contralateral nose</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unilateral priming, RSV ($10^5$ p.f.u.)</td>
<td>$&lt; 3.30^*$</td>
<td>$&lt; 3.30^*$</td>
<td>2.00$^*$</td>
</tr>
<tr>
<td>Unilateral priming, UV-inactivated RSV</td>
<td>5.50</td>
<td>5.56</td>
<td>4.49</td>
</tr>
<tr>
<td>Unprimed</td>
<td>5.15</td>
<td>5.28</td>
<td>4.38</td>
</tr>
</tbody>
</table>

*$^* P < 0.001.$

---

Fig. 3. Immunogenicity of various priming doses (2000, 500, 125, 31, 8, 2 or 0.5 p.f.u.). (a) Ipsilateral nasal turbinates; (b) contralateral nasal turbinates; (c) lungs. The geometric mean virus titres (log$_{10}$) and standard errors are shown. Virus titres significantly lower than control values are marked with asterisks ($^* P < 0.001$, ** $P < 0.005$). Serum neutralizing antibody titres at the time of challenge (2 weeks post-priming) were $< 1:20$ (undetectable) for all groups.

Fig. 4. Kinetics of virus clearance upon RSV challenge 2 weeks after unilateral priming. (a) Ipsilateral nasal turbinates; (b) contralateral nasal turbinates; (c) lungs. ■ Unprimed animals; ▲ animals primed with 2 µl of RSV. Geometric mean virus titres (log$_{10}$) and standard errors are shown.
tively). Significant protection was observed in lungs (Fig. 3c) through the 2 p.f.u. per animal priming dose when compared to unprimed control animals ($P < 0.001$).

**Effect of unilateral priming on kinetics of virus clearance upon challenge**

Cotton rats were unilaterally inoculated in the right nostril with $2 \mu l (10^{3.8} \text{ p.f.u.)}$ of RSV or left unprimed. Two weeks later, all animals were bilaterally challenged intranasally with $10^5 \text{ p.f.u.}$ of RSV delivered in a 100 $\mu l$ volume. Groups of animals were sacrificed on days 1 through 5 and 7 post-challenge for virus titration (Fig. 4).

Viral replication was not observed in ipsilateral nasal turbinates at any time after challenge (Fig. 4a). In contralateral nasal turbinates and lungs, levels of replication were reduced, but still detectable for several days after challenge (Fig. 4b, c). The virus titres of unprimed control animals peaked at day 4 post-challenge and reached undetectable or nearly undetectable levels by day 7. In all tissues, the RSV titres of unprimed control animals were significantly higher than those of unilaterally primed animals.

**Discussion**

The described model of unilateral RSV infection takes advantage of three characteristics of the ‘fulvous’ cotton rat, *Sigmodon fulviventer*. First, there is no opening between the right and left nasal passages. Second, RSV infection does not cause rhinorrhoea. Third, the neutralizing antibody response of *S. fulviventer* to RSV is approximately one order of magnitude lower than that of the ‘hispid’ cotton rat, *Sigmodon hispidus* (Prince *et al.*, 1978), and at the low priming doses used in the current experiments, elicited no detectable serum neutralizing antibody.

The first two of these characteristics allow for a model in which infection does not extend beyond the site of inoculation. Figs 1 and 2 confirm that infection was restricted to the ipsilateral nasal tissues. Because there was no direct antigenic stimulation of contralateral nasal turbinates nor of pulmonary tissues, a different immunological status existed among the various tissues. That is, immunological effectors in ipsilateral nasal turbinates consisted of those which were stimulated by infection and which did not migrate to distant tissues (‘local’), as well as those which did migrate (‘systemic’). By contrast, the immunological factors in contralateral nasal turbinates and in pulmonary tissues consisted only of those which migrated from the infected ipsilateral nasal turbinates (‘systemic’).

The time of onset of immunity did not differ in the three tissues (Table 1), suggesting either that systemic immunity developed prior to local, or that they developed simultaneously. However, when cotton rats primed 14 days earlier were challenged bilaterally and then examined daily for 7 days, clear differences were seen. Whereas no infectious virus could be recovered from ipsilateral nasal tissues at any time, low levels of viral replication were found in contralateral nasal turbinates for 3 days, and in lungs for 4 days (Fig. 4). These data highlight the ability of this model to differentiate between local and systemic effectors of immunity, and suggest that, in the short-term, the local are the more potent. The identity of such effectors remains to be determined. While local antibody may have been the primary mediator of resistance in ipsilateral nasal turbinates, other local factors such as cellular infiltrates, interferons or other cytokines may have been involved. Similarly, although the lack of detectable serum neutralizing antibody in these experiments suggests that cellular immunity was prominent in the contralateral nasal turbinates and lungs, it is possible that serum antibodies other than those which neutralize RSV *in vitro* might have been important. Indeed, preliminary work in our laboratory has shown that some monoclonal antibodies against RSV are capable of conferring limited resistance in animals, in spite of their inability to neutralize the virus *in vitro*. Further definition of effectors awaits the development of immunological reagents specific for cotton rat immunoglobulins and T cell antigens, the development of which currently proceeds in our laboratory.

Earlier experiments from our laboratory suggested that ‘systemic’ immunity to RSV might be further subdivided into ‘persistent’ and ‘transient’ categories. In ‘persistent systemic’ immunity, immunological effectors continue to pass through the blood and lymphatic circulation; in contrast, in ‘transient systemic’ immunity, effectors circulate for a brief period of time, and then deposit in mucosal tissues, a phenomenon referred to as ‘trafficking’. Our first use of the technique of parabiosis, in which two animals were surgically linked 3 weeks after RSV infection of the ‘immune’ partner so as to effect cross-circulation of blood (Prince *et al.*, 1983), showed that pulmonary, but not nasal immunity could be transferred to the naive partner. Subsequent experiments involving adaptive transfer of blood components showed that immunoglobulin, but not white blood cells, accounted for the observed pulmonary immunity. Thus, these earlier experiments suggest that only serum antibody was a ‘persistent systemic’ effector of immunity to RSV.

In contrast to these findings, however, were earlier experiments which suggested a non-antibody, systemic component to RSV immunity (Prince *et al.*, 1979). We
found that cotton rats immunized in the gluteal muscles with live RSV developed complete pulmonary and nasal immunity in spite of the fact that infectious virus did not appear beyond the injection site. Since there was no direct stimulation of nasal tissues by RSV, we concluded that the observed 'systemic' immunity – immunological effectors which trafficked via the blood and lymphatic circulation for a brief period – deposited in mucosal tissues, and thus disappeared from the circulation. Such an hypothesis was consistent with our parabiosis findings, which had involved animals whose cross-circulation was not established until 4 weeks after priming of the immune partner. Therefore, had trafficking occurred at any time prior to 4 weeks, it would not have been detected.

Subsequent experiments in which a parabiotic union was established prior to RSV priming of the immune partner allowed us to test the hypothesis of trafficking (Piazza et al., 1995). These experiments demonstrated the transfer of nasal as well as pulmonary immunity, thus providing direct evidence of 'transient systemic' immune effectors. In as much as serum antibody has been shown not to confer nasal immunity at physiological levels (Prince et al., 1985b), we concluded that the transient systemic effectors were cellular. The experiments described in the current report provide two additional pieces of evidence for cellular, transient systemic effectors. First, we were unable to detect serum neutralizing antibody in these animals, and were unable to induce immunity in naive recipients of serum from simultaneously primed animals (data not shown), thereby lending indirect evidence for cellular effectors. Second, the kinetics of viral clearance in contralateral nasal turbinates and pulmonary tissues (Fig. 4), which required 3–4 days to completely clear the challenge virus, are suggestive of cellular effectors. Further definition of the role of such effectors will require experiments involving passive transfer of immune cells. While we anticipate performing such experiments, they await the inbreeding programme for the past 5 years, and within the next 3 years anticipate having a fully inbred strain of S. fulviventer. We have been pursuing an inbreeding programme for the past 5 years, and within the next 3 years anticipate having a fully inbred strain of S. fulviventer which will then allow such studies.

The fact that priming with PIV3 2 weeks prior to RSV challenge provided slight protection to ipsilateral nasal tissues, but none to contralateral nasal nor pulmonary tissues, suggests a minor role for non-specific resistance factors (presumably cytokines, such as interferon) in infected tissues. However, the disappearance of non-specific resistance 4 weeks after PIV3 priming compared to the maintenance of solid immunity 4 weeks after RSV priming (data not shown) confirms that the role of such non-specific factors is insignificant in the overall scenario of immunity.

The demonstration in this model of highly effective immunization of pulmonary tissues by an infection restricted to the nasal tissues is important in the context of current interest in mucosal vaccination. It is important to note that our data contrast with those of Graham et al. (1995), who recently showed that nasal infection of mice did not protect the lungs against a very high-titre RSV challenge. The mouse is approximately 100-fold less sensitive to RSV than is the cotton rat – in other words, to achieve a given titre of RSV replication in the lungs of mice requires a challenge dose 100-fold higher than in the cotton rat. It is likely that resistance in the mouse lung was thus overwhelmed by the magnitude of the viral challenge.

The unilateral nasal infection model provides an in vivo system which, without surgical manipulation or externally modulated immunological depletion, allows for the study of local versus systemic effectors of immunity to RSV. The remarkable sensitivity of the cotton rat to RSV, wherein immunity is stimulated by less than 10 p.f.u. of virus, underscores the utility of this model. Ongoing work in our laboratory to develop monoclonal antibodies against cotton rat IgG, IgA, IgM and T cell surface markers will ultimately allow more precise definition of these effectors, and perhaps will lead to an understanding of the ephemeral nature of RSV immunity.

This work was presented in part at the American Pediatric Society/Society for Pediatric Research Annual Meeting, May 3–6, 1993, Washington, DC, USA.

References


(Received 20 June 1995; Accepted 5 September 1995)