

## **A Pathway for Entry of Reoviruses into the Host through M Cells of the Respiratory Tract**

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### **Summary**

Many microorganisms gain access to the systemic circulation after entering the respiratory tract. The precise pathways used to cross the mucosal barriers of the lungs have not been clearly described. We have used the mammalian reoviruses in order to determine the pathway that a systemic virus uses to penetrate the mucosal barrier and enter the systemic circulation after entering the airways of the lungs. Reoviruses enter through pulmonary M cells, which overlie bronchus-associated lymphoid tissue, and subsequently spread to regional lymph nodes. Thus, the pathway through M cells represents a strategy by which viruses and probably other microorganisms can penetrate the mucosal surface of the respiratory tract and thereby enter the systemic circulation.

Viruses enter their hosts in a variety of ways to establish infection. A central issue in understanding the pathogenesis of the diseases that result is how entry into the host is accomplished. One important site of viral entry is the respiratory tract, where many viruses, such as measles, mumps, rubella, and varicella, to name a few, can enter the systemic circulation from the airways without causing local symptoms (1). In spite of the importance of the pulmonary pathway of entry into the host, little is understood about how viruses, as well as other microorganisms, including bacteria, cross the respiratory mucosal barrier in order to spread to distant sites.

Studies on reoviral entry into the intestinal tract have provided a possible clue for viral (and possibly microbial) entry in the respiratory tract. Reoviruses, members of the family *Reoviridae*, are nonenveloped, icosahedral viruses with a segmented double-stranded RNA genome (2). They have served as useful models for the study of viral pathogenesis, including studies on how viruses interact with the intestinal tract, central nervous system, myocardium, liver (3), and more recently, the lungs (Morin, M.J., A. Warner, and B.N. Fields, manuscript in preparation). When reovirus is introduced into the intestine, penetration of the intestinal epithelial layer takes place through intestinal M cells (4–6). M cells are part of the epithelium specialized for the sampling of antigens from the intestinal lumen that overlies Peyer's patches (7, 8). Peyer's patches are collections of lymphoid tissue that are part of the gut-associated lymphoid tissues (GALT). Studies on reoviral entry in the gut showed that reoviruses initially adhere selectively to intestinal M cells, and are subsequently transported transepithelially into Peyer's patches (4–6). Subse-

quently, reoviruses spread to the lymphatics and the systemic circulation.

M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue (BALT). Indeed, it has been hypothesized that a pathway of entry similar to the one described through intestinal M cells exist to allow entry of viruses and other microorganisms to take place in the respiratory tract (9). To date, however, no such pathway has been described. Thus, we asked, do reoviruses enter the host via pulmonary M cells? The results described in the present report indicate that, as in the gastrointestinal tract, reoviruses employ the M cell pathway in the respiratory tract.

### **Materials and Methods**

*Animals.* Adult Sprague-Dawley rats, weighing 250–300 g, were purchased from Charles River Laboratories (Wilmington, MA). 25–28-d-old juvenile Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY).

*Viruses and Inoculations.* Reovirus type 1 (Lang), derived from laboratory stocks, was used for the present study and has been described previously (10). The rats were anesthetized by exposure to vaporized halothane. The virus inoculum was instilled into the trachea of the animals with a blunt-ended, 5-cm catheter. For the adult rats, the inoculum was  $6.5 \times 10^{11}$  viral particles in 500  $\mu$ l of 0.9% sterile saline and for the juvenile rats,  $1.0 \times 10^9$  PFU in 100  $\mu$ l of 0.9% sterile saline.

*Electron Microscopy.* Lung tissues from adult rats were fixed in situ with 2.5% glutaraldehyde in cacodylate buffer 30 min and 1 h after inoculation with reovirus. The lungs were removed from the animals and examined using a dissecting microscope at low

magnification. Areas containing BALT were dissected from the lungs and embedded in epon. Ultrathin sections were made through BALT for electron microscopy and stained with lead citrate and uranyl acetate.

**Viral Titration of Rat Tissues.** Bronchial lymph nodes, mesenteric lymph nodes, and spleens were removed from juvenile rats immediately after (time 0), and 2, 4, and 8 h after inoculation, and transferred to gelatin-saline. Because of time lapses between inoculation, euthanasia, and tissue dissection, time 0 was actually 5–10 min after inoculation. After freezing and thawing the tissue samples three times, then sonicating, the tissue suspensions were titered by standard plaque assay on mouse L cells (11). Titers of the regional and mesenteric lymph nodes were expressed in the results as an average titer: the nodes were pooled before titration and the titer was divided by the number of nodes dissected, in order to determine the average.

## Results and Discussion

BALT is similar to GALT (i.e., Peyer's patches) in that it is an organized aggregate of B and T lymphocytes, macrophages, and other immunocompetent cells covered by a specialized epithelium containing M cells (12). M cells overlaying BALT are distinct from typical ciliated cells of the airway epithelium in that they are flattened with irregular microfolds at the apical surface (13, 14). Previously, it has been shown that ferritin and horseradish peroxidase (HRP) can be taken up by pulmonary M cells and transported into BALT (15, 16).

To test whether the M cells overlaying BALT can serve as sites of entry for virus, reovirus type 1 (Lang) was inoculated intratracheally into adult laboratory rats. Adult rats were used because BALT becomes more developed as an animal ages (17). We used a large inoculum ( $6.5 \times 10^{11}$  viral particles per rat) to facilitate detection of virus by electron microscopy. Within 30 min after the inoculation, we found reovirus adhering to the apical surface of M cells in the epithelium overlaying BALT (Fig. 1 A). The binding appeared to be preferential for pulmonary M cells; rarely were reovirus particles adherent to adjacent ciliated cells.

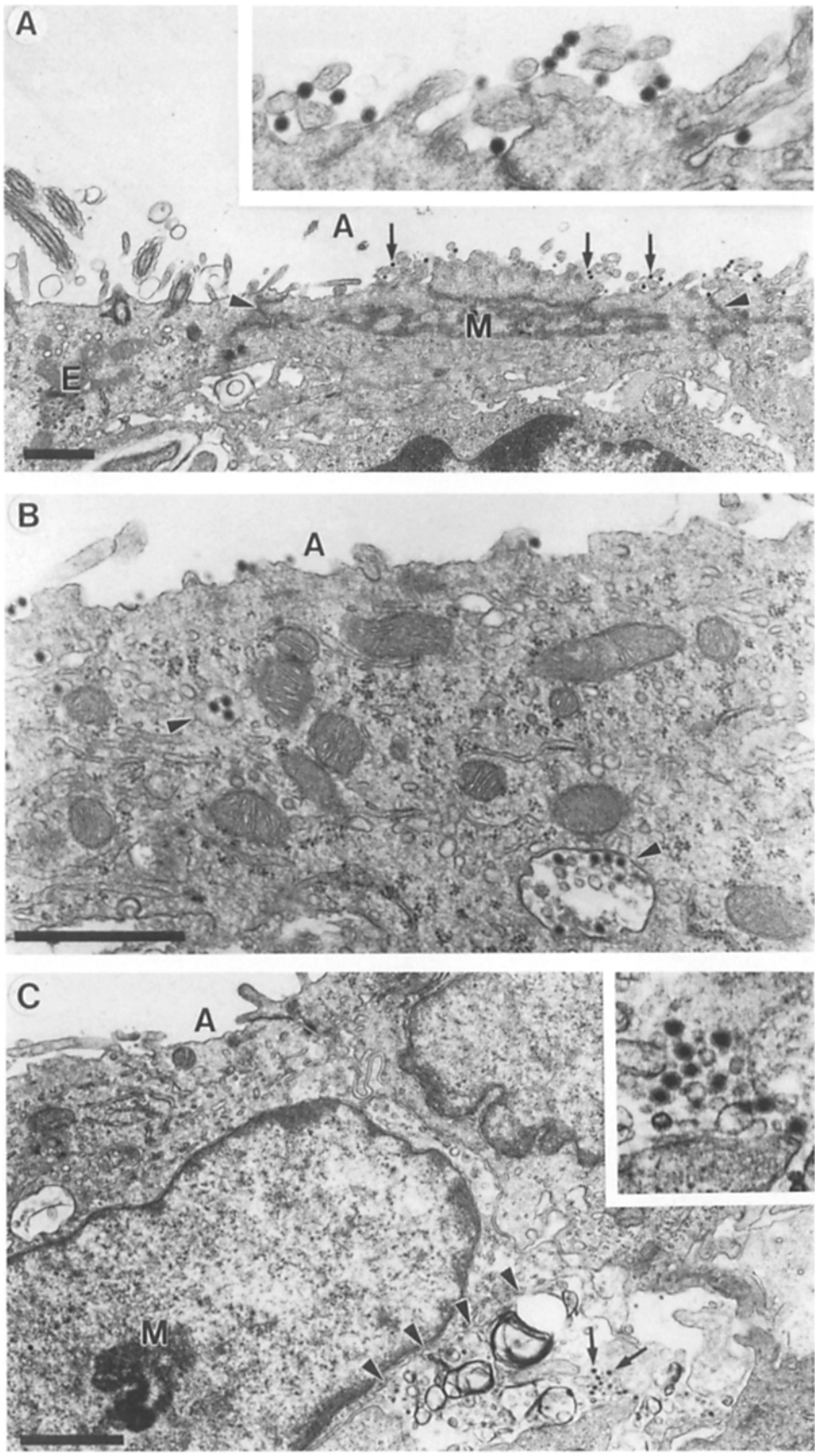
Vesicles containing viral particles were seen as early as 30 min after inoculation (Fig. 1 B), and regularly seen at 1 h. Also by 1 h, in the intercellular space on the basal side of the BALT epithelium, "pools" of virus appeared (Fig. 1 C). The presence of virus particles beneath the epithelium suggested that virus was shuttled in vesicles through the M cells from the apical to the basal side, where virus was released into the intercellular space within BALT.

We considered the possibility that BALT may serve as a site from where virus disseminates throughout the host, as is thought to occur in Peyer's patches in the gastrointestinal tract. Indeed, there are lymphatic vessels within BALT that virus could use to spread from the lungs (15, 18–21). Therefore, in order to determine whether virus disseminates from the lungs, bronchial lymph nodes were assayed for virus at various times after inoculation. Infectious reovirus, as determined by plaque assay, was recovered in bronchial lymph nodes within 10 min after intratracheal inoculation, shown in Table 1 as time 0. Increased amounts of virus were seen at 2, 4, and 8 h. The spleen and mesenteric lymph nodes were titered to determine if systemic spread occurred hematogenously.

The amount of virus in these organs between time 0 and 2 h was, with one exception, below that detectable by the assay (Table 1). At later times, however, reovirus was detected in the mesenteric nodes and spleen. The titers of the mesenteric nodes and spleen at all times were lower than those seen in the bronchial nodes, implying that the initial spread to the bronchial nodes occurred from the lungs via the lymphatic system. Detection of virus in the mesenteric nodes and spleen at later times indicated that, in addition to spread to bronchial nodes, virus subsequently entered the circulation. The lymph nodes may then have served to further disseminate the virus into the bloodstream.

We observed, in addition to entering pulmonary M cells, reovirus entering the type 1 epithelial cells that line alveolar spaces. Although we did not see transcytosis of virus by the alveolar cells, we cannot exclude an alternate pathway of viral entry and spread through the alveoli. A potential pathway for uptake of foreign particles through the alveolar cells, rather than M cells, into lymphatics was proposed in a study using ferritin and carbon particles (22). However, the authors (22) did not examine the contribution of other routes of entry into the lymphatics from the airways, therefore, their data do not rule out M cell entry.

We used mammalian reoviruses for our study because they have served as excellent models for studies of viral pathogenesis and because reoviruses were used to describe the pathway of viral and bacterial entry through the intestinal M cells into the systemic circulation. We report in our study that reoviruses are also capable of entering the host through pulmonary M cells. Do other viruses and microorganisms which gain entry into the host via the respiratory system also use M cell transport? A few observations support the hypothesis that the pulmonary M cell pathway may be a general one that is used by other viruses and microorganisms. First, in the intestine, the M cell transport pathway is a general one that is used by a wide variety of microorganisms (23–27). After the initial demonstration of intestinal M cell entry by reoviruses, entry through intestinal M cells into the host was also demonstrated with bacteria such as *Vibrio cholerae* (23) and *Escherichia coli* RDEC-1 (24), as well as other viruses such as transmissible gastroenteritis virus (25), polio (26), and HIV (27). Second, many viruses (e.g., measles and mumps) and bacteria (e.g., meningococcus) which use the respiratory tract as a portal of entry into the host do so without initially causing overt pathology within the lung itself, but rather appear first in lymphoid tissue, such as BALT. M cells provide the most direct pathway of entry into BALT. The M cell-initiated pathway appears to function by sampling airway proteins, such as HRP, and viruses, such as reovirus, and by subsequently transporting them into BALT where further dissemination may occur via the lymphatics. We therefore propose that the pulmonary M cell pathway may provide a general pathway used by many viruses and bacteria to penetrate the mucosal lining of the airways in order to enter the host. The pathway may function to initiate an immune response to the invading microorganisms as they encounter immune cells in BALT and lymph nodes, but in some cases, the pathway may be exploited by pathogens to spread disease within the host.



**Figure 1.** Entry of reovirus into M cells overlying BALT. An inoculum of  $6.5 \times 10^{11}$  particles of reovirus type 1 Lang was instilled into the tracheas of anesthetized, adult Sprague-Dawley rats. Electron micrographs are shown demonstrating BALT from rats 30 and 60 min after inoculation. (A) Virus particles (arrows) at the surface of BALT, bound to M cells (M) within 30 min of inoculation. (Arrowheads) The boundaries of one M cell. The adjacent cell on the left is a ciliated epithelial cell (E) and no virus particles were bound here. (A) Airspace. Bar, 1.0  $\mu\text{m}$ . (Inset) M cell with virus particles bound to the surface at higher magnification,  $\times 40,000$ . (B). Reovirus particles within membrane-bound vesicles (arrowheads) inside M cells within 1 h of inoculation. (A) Airspace. Bar, 1.0  $\mu\text{m}$ . (C) "Pool" of virus particles in the intercellular space beneath an M cell (M) within 1 h of inoculation. (Arrowheads) Basal side of the M cell; (arrows) virus particles. (A) Airspace. Bar, 1.0  $\mu\text{m}$ . (Inset) Higher magnification of virus particle pool on the basal side of an M cell.  $\times 49,000$ .

**Table 1.** Spread of Reovirus from the Lungs

	Animal number	Regional lymph nodes (avg. PFU/node)	Mesenteric lymph nodes (avg. PFU/node)	Spleen (PFU/spleen)
time 0	1	$6.3 \times 10^2$	$<2 \times 10^0$	$<2.5 \times 10^1$
	2	$1.0 \times 10^2$	$<2 \times 10^0$	$<2.5 \times 10^1$
	3	$3.4 \times 10^1$	$<2 \times 10^0$	$<2.5 \times 10^1$
	4	$2.2 \times 10^2$	$<2 \times 10^0$	$<2.5 \times 10^1$
	5	$1.9 \times 10^1$	$<2 \times 10^0$	$<2.5 \times 10^1$
2 h	1	$2.8 \times 10^3$	$<2 \times 10^0$	$<2.5 \times 10^1$
	2	$2.0 \times 10^3$	$7.5 \times 10^0$	$<2.5 \times 10^1$
	3	$1.3 \times 10^3$	$<2 \times 10^0$	$<2.5 \times 10^1$
4 h	1	$1.6 \times 10^3$	$<2 \times 10^0$	$3.2 \times 10^2$
	2	$9.4 \times 10^2$	$1.2 \times 10^1$	$3.7 \times 10^2$
	3	$1.6 \times 10^2$	$<2 \times 10^0$	$<2.5 \times 10^1$
8 h	1	$3.5 \times 10^3$	$1.5 \times 10^1$	$<2.5 \times 10^1$
	2	$7.5 \times 10^3$	$<2 \times 10^0$	$2.2 \times 10^2$
	3	$4.0 \times 10^3$	$6.0 \times 10^0$	$1.0 \times 10^2$

\* Time 0 is actually 5–10 min because of time lapses between inoculation, euthanasia, and dissection.

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