Rhinovirus-induced barrier dysfunction in polarized airway epithelial cells is mediated by NADPH oxidase 1

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Running title: NOX1 and barrier function in rhinovirus infected cells
ABSTRACT

Previously, we showed that rhinovirus (RV), which is responsible for the majority of the common colds, disrupts airway epithelial barrier function, as evidenced by reduced transepithelial resistance ($R_T$), dissociation of zona occludins (ZO)-1 from the tight junction complex and bacterial transmigration across polarized cells. We also showed that RV replication is required for barrier function disruption. However, the underlying biochemical mechanisms are not known. In the present study, we found that a double-stranded (ds) RNA mimetic, poly I:C, induced tight junction breakdown and facilitated bacterial transmigration across the polarized airway epithelial cells similar to RV. We also found that RV and poly I:C each stimulate Rac1 activation, reactive oxygen species (ROS) generation and Rac1-dependent NADPH oxidase (NOX)-1 activity. Inhibitors of Rac1 (NSC23766), NOX (diphenylene iodonium) and NOX1 (siRNA) each blocked the disruptive effects of RV and poly I:C on $R_T$, as well as the dissociation of ZO-1 and occludin from the tight junction complex. Finally, we found that TLR3 is not required for either poly I:C or RV-induced reduction in $R_T$. Based on these results, we conclude that Rac1-dependent NOX1 activity is required for RV or poly I:C-induced ROS generation, which in turn disrupts the barrier function of polarized airway epithelia. Further, these data suggest that dsRNA generated during RV replication is sufficient to disrupt barrier function.
INTRODUCTION

Tight junctions located at the apicolateral borders of adjacent airway epithelial cells significantly contribute to epithelial barrier function. Tight junctions regulate the selective passage of ions and solutes through the paracellular space, and prevent paracellular migration of pathogens and their products from lumen to interstitium. Thus, perturbation of the barrier function may increase paracellular permeability, facilitate translocation of pathogens and their soluble products, and expose basolateral receptors.

Rhinovirus (RV), which is responsible for the majority of common colds (1), also provokes acute lower respiratory symptoms in healthy individuals (7, 18) and exacerbates airway diseases in patients with asthma, chronic obstructive pulmonary disease and cystic fibrosis (9, 32, 47, 50). In addition to stimulating production of pro-inflammatory cytokines (13, 34), RV infection may also promote secondary bacterial infections by interfering with host innate defense mechanisms or by increasing the adherence of bacteria to host mucosa (2, 20, 48). Recently, we and others demonstrated that RV infection compromises barrier function and facilitates bacterial transmigration across polarized airway epithelial cells (41, 54). Further, we showed that infectious RV is required for the impairment of barrier function in polarized airway epithelia. The impairment of barrier function caused by RV is independent of epithelial cell destruction, apoptosis or virus-stimulated pro-inflammatory cytokines, indicating other mechanisms play a role.

Oxidative stress has been implicated in the impairment of airway and colonic epithelial barrier function (6, 38, 51, 52). Treatment with hydrogen peroxide disrupted barrier function of airway epithelial cells by destabilizing the actin cytoskeleton, damaging tight junctions and inhibiting cell proliferation (53). In colonic epithelial cells, hydrogen peroxide caused tyrosine...
phosphorylation of occludin and dissociation of occludin and ZO-1, leading to decreased transepithelial resistance ($R_T$) and increased epithelial permeability (39). RV has been shown to induce oxidative stress in non-polarized airway epithelial cells by generating reactive oxygen species (ROS) (5, 23, 36), but the role of ROS in barrier function disruption has not been investigated in polarized epithelial cells.

ROS play an important role in innate immune host defense mechanisms. In phagocytes, ROS are necessary for the killing of invading microorganisms. In other cells, ROS act as a molecular switch to stimulate proinflammatory responses. The majority of intracellular ROS is generated from two sources: the mitochondrial electron transport chain complex and membrane bound NADPH oxidase (NOX) enzymes. In phagocytes, the NOX holoenzyme is a multisubunit complex composed of the membrane-bound p22$^{phox}$ and catalytic subunit gp91$^{phox}$ (now known as NOX2) and the cytoplasmic regulators p47$^{phox}$ and p67$^{phox}$. During the last decade, six functionally distinct homologs of NOX2, namely NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2, were identified in a wide variety of cells including airway epithelial cells (3, 14, 29). NOX1-5 generate superoxide, which converts to other ROS, whereas DUOX1 and DUOX2 produce hydrogen peroxide at the cell surface. NOX2 was recently shown to control respiratory syncytial virus-stimulated NF-κB activation in airway epithelial cells (14, 55). Similarly, RV-stimulated IL-8 responses were shown to be dependent on p47$^{phox}$, though the specific identity of the NOX2 homolog was not determined (5, 23, 36). The role of ROS and NOX in the RV-induced impairment of barrier function has not been investigated.

In the present study, we examined the biochemical mechanisms of RV-impaired barrier function in polarized airway epithelial cells, focusing on the generation of ROS by replicating RV and NOX enzyme. We demonstrate that RV-induced Rac1-dependent NOX1 activity is...
required to disrupt barrier function. We also show that polyinosinic-polycytidylic acid (poly I:C),
a synthetic analogue of dsRNA that has been used to examine viral dsRNA-stimulated responses
in airway epithelial cells (26, 30, 49), impairs barrier function by a mechanism similar to RV,
indicating that dsRNA generated during replication contributes to the disruption of barrier
function.
MATERIALS AND METHODS

Rhinovirus. Rhinovirus 39 (RV39) was purchased from American Type Culture Collection (Manassas, VA) and viral stocks were generated as described previously (43). Briefly, cell culture supernatants from RV-infected HeLa cells was partially purified by ultrafiltration and 50% tissue culture infectivity (TCID50) values of viral stocks were determined by the Spearman-Karber method (22). Sham was prepared similar to RV but from uninfected HeLa cell supernatant. Sham served as a control for cells infected with RV or UV-RV.

Bacteria and growth conditions. A clinical isolate of non-typeable Hemophilus influenzae (NTHi), 6P5H, obtained from a patient with chronic obstructive pulmonary disease (COPD) at the time of exacerbation, was kindly provided by Dr. T. Murphy (University of Buffalo) and has been described previously (41). Bacteria maintained as a glycerol stock at -80°C were subcultured on a chocolate agar plate and incubated overnight at 37°C/5% CO2. Bacteria were scraped off of the plate and suspended in serum and antibiotic-free cell culture medium at the required concentration.

Cell culture and infection. Immortalized human bronchial epithelial cells, 16HBE14o- were grown in Transwells (Corning, Lowell, MA) using Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA) amended with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM of L-glutamine, as described previously (41). Briefly, 8 x 10^4 or 3 x 10^5 cells were seeded in 6.5 mm or 12 mm collagen-coated transwells, respectively, and grown under submerged conditions until the transepithelial resistance (R_T) reached 800 to 1000 Ω.cm², which usually takes 4 to 5 days. Medium in both the apical and basolateral chambers was changed every other day. In nearly all experiments, transwells with pore size of 0.4 µ were used. To determine bacterial transmigration, cells were grown in transwells with a pore size 3 µ.
On the day of infection, medium in the basolateral chamber was replaced with fresh growth medium, and the apical surface was infected with 100 µl RV diluted in growth medium at a multiplicity of infection (MOI) of 1, or an equivalent volume of UV-irradiated RV (UV-RV) or sham control. After 90 min of incubation, infection medium from the apical surface was replaced with fresh growth media and the cells were further incubated for 4 to 24 h at 33°C. In inhibition studies, N-propyl gallate, diphenylene iodonium (DPI), oxypurinol or quercetin (all from Sigma-Aldrich, St Louis, MO) or the Rac1 inhibitor NSC23766 (EMD chemicals, Gibbstown, NJ) were added to both the apical and basolateral chambers 90 min after RV infection, incubated for 24 h and $R_T$ was measured with an EVOM voltmeter equipped with EndOhm 6 tissue resistance measurement chamber (World Precision Instruments, Sarasota, FL) (25, 41).

**Treatment with poly I:C.** Polarized 16HBE14o- cells were treated apically with 300 µl of media containing 500 ng/ml of high molecular weight poly I:C (1.5 to 8 kb; Invivogen) and $R_T$ was measured at the time points indicated in the Results section.

**Transmigration of bacteria from apical to basolateral chamber.** Transmigration of bacteria across the polarized cells was measured as described earlier (41). Briefly, 16HBE14o- cells grown in transwells with a pore size of 3 µ were infected with RV, UV-RV at MOI of 1 or treated with poly I:C as described above. Eight (for poly I:C treated cells) or 24 h (for RV and UV-RV-infected cells) later, 100 µl of NTHi at MOI of 10 was added to the apical chamber and further incubated for 3 h. Media from basolateral chambers was sampled at 3 h and plated on chocolate agar to determine number of bacteria transmigrated across the polarized airway epithelial cells.
**Viral load and cell infectivity.** Total RNA was extracted 90 min or 24 h after RV infection from polarized epithelial cells, and vRNA was quantified by qPCR as described (43). To quantify the percent of cells infected with virus, cells after appropriate treatment were fixed/permeabilized with 4% paraformaldehyde, blocked with normal goat serum, and incubated with AlexaFluor 488-conjugated antibody to VP2, one of the capsid proteins of RV (21) (kindly provided by Dr. Wai-Ming Lee, University of Wisconsin, Madison). Cells were analyzed by flow cytometry. Similarly-treated uninfected cells were used as negative controls.

**Expression of NOX mRNA by qPCR.** The mRNA expression of NOX genes was assessed by qPCR using gene specific primers (SA Biosciences, Frederick, MD). All PCR reactions were performed on an Eppendorf Mastercycler (Westbury, NY) using the comparative Ct method. The expression levels were normalized to reference gene glyceraldehydes-3-phosphate dehydrogenase (G3PDH) as described previously (43).

**Confocal indirect immunofluorescence.** Distribution of the tight junction proteins ZO-1 and occludin was determined by indirect immunofluorescence microscopy, as described previously (41). Briefly, after appropriate treatments, polarized 16HBE14o- cells were fixed in cold methanol, blocked with PBS containing 1% (wt/vol) bovine serum albumin (BSA), and incubated overnight at 4°C with polyclonal antibody to ZO-1 (1 µg/ml), or monoclonal antibody to occludin (both from BD Biosciences, San Jose, CA). Bound antibodies were detected by using appropriate AlexaFluor-conjugated second antibody (Invitrogen) and visualized by confocal fluorescent microscopy (Carl Zeiss, Thornwood, NY). Cells treated normal IgG isolated from non-immunized animals (Jackson ImmunoResearch Laboratories, West Grove, PA) instead of primary antibody served as negative controls.
To localize polyI:C, polarized 16HBE14o- cells were treated with rhodamine-labeled polyI:C, incubated for 4 h, fixed in methanol, immunolabeled with anti-ZO-1 and visualized by confocal microscopy.

**Determination of NOX enzymatic activity.** NOX enzymatic activity in cells treated with RV or UV-RV was determined by measuring the reduction of cytochrome c (36). Briefly, polarized 16HBE14o- cells were infected with RV or UV-RV or treated with poly I:C as described above. Cells were collected, homogenized in a Dounce homogenizer, centrifuged and supernatant was collected. An aliquot of cell supernatant containing equal amounts of total protein was mixed with NADPH and cytochrome C, incubated exactly for 10 min at 37°C in the absence or presence of an flavoprotein inhibitor, DPI, or oxypurinol (both of from Sigma Aldrich.co. St. Louis, MO), and OD$_{550}$ was measured.

**Reactive oxygen species (ROS) production.** ROS production was measured by using carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA, Invitrogen) as described previously (40). Briefly, after appropriate treatment, cells were infected with either RV or UV-RV as above. Six hours before the measurement of ROS, medium was removed, cells were rinsed with HBSS and then incubated in HBSS containing 5 µM carboxy-H$_2$DCFDA without phenol red for 6 h. At this concentration, carboxy-H$_2$DCFDA, had no effect on the cells or RV replication, as measured by LDH release and vRNA copy number. For poly I:C treatment, cells were preloaded with 5 µM carboxy-H$_2$DCFDA for 1 h and then incubated with poly I:C for the predetermined time. Cells were then detached from the wells, washed and resuspended in PBS. Fluorescent emission was quantified by flow cytometry (FACS caliber, BD) and the results were analyzed using WinMDI software.
Transfection of 16HBE14o- cells growing in transwells. 16HBE14o- cells (8 x 10^4 cells/well) were seeded in 6.5 mm transwells and grown for two days. At this time, cells were partially polarized and showed an R_τ ranging between 60 to 100 Ω.cm^2. A pool of ds siRNA specific to NOX1 (antisense sequence of siRNA pool 5’ GGUUAGGGCUGAAUGUUUU 3’, 5’CUGCUCAUAACACGUAAUU3’, 5’GACAAAUACUACACAA3’ and 5’UGAGAAAGCAAUUGGAUA3’), TLR3 (antisense sequence of siRNA pool 5’GAACUAAAGAGUUGGUAUU3’, 5’CAGCAUCUGCUUUUAUAA3’, 5’AGACCAUCUCUCAAAUUU3’ and 5’UCACGCAAUUGGAAGAUUA3’) and non-targeting (NT) siRNA (sense 5′-CGAAGCAGCCUGGUGCCU dTdT-3′ and antisense, 5′-GGUCAGACCAGUGAGUUCG dTdT-3′) were purchased from Dharmacon (Lafayette, CO). siRNA (5 to 10 pmoles/well) was incubated with 1 μl of Lipofectamine™ RNAiMAX (Invitrogen) in 100 μl OptiMEM (Invitrogen) for 20 min at room temperature. Medium in the basolateral chamber was replaced with fresh medium, apical chamber medium was removed, and 100 μl of transfecting reaction mix was added. After 6 h of incubation, volume in the apical chamber was made up to 300 μl with fresh media and incubation continued for another 18 h. Media in both apical and basolateral chamber were changed and incubated for another 24 h. Forty eight hours after transfection, R_τ of cells was measured to confirm that cells were polarized similar to non-transfected cells.

Western blot analysis. After relevant treatment, cells were lysed in RIPA buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 1% Nonidet P (NP) -40, 0.25% sodium deoxycholate, 0.1% SDS) containing complete protease inhibitors (Roche Diagnostics, Indianapolis, IN), centrifuged and supernatant collected. In some experiments, cells were lysed in NP-40 solubilization buffer [25 mM HEPES (pH 7.4), 150 mM NaCl, 1% (vol/vol) NP-40 and complete protease inhibitor] and
centrifuged (10,000 g for 30 min at 4°C). The pellet containing NP-40 insoluble material was collected and suspended in Laemmli reducing buffer and heated for 10 min at 100°C. Equal amounts of protein (for total cell lysates) or equal volumes (for NP40-insoluble fractions) were subjected to SDS-PAGE and proteins transferred to nitrocellulose membranes by wet transfer for 1 h (for determination of occludin and NOX1) and 90 min (for ZO-1). The membranes were blocked with either 5% bovine serum albumin or fat-free milk and probed with antibody to ZO-1 (BD Biosciences, San Diego, CA; cat #610967; dilution; 1:1000), occludin (BD Biosciences; dilution, cat #611091; 1:500, NOX1 (Abcam, Cambridge, MA; cat #ab780161; 1 µg/ml) or β-actin (Sigma-Aldrich; cat#A5441; dilution 1:10000). The bound antibody was detected with appropriate second antibody conjugated with horse radish peroxidase (BIO-RAD, Hercules, CA) and chemiluminescent substrate (Pierce, Rockford, IL). Specific bands were quantified by densitometry using NIH imageJ.

Determination of TLR3 expression by flow cytometry. After relevant treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin in blocking buffer (1% BSA in PBS) on ice for 15 min. Cells were washed with blocking buffer, incubated with 1:10 diluted antibody to TLR3 (Abcam, cat#AB12085) or normal mouse IgG (Jackson Immuno Laboratories) for 1 h, washed and incubated with 1:1000 diluted Alexafluor 488 conjugated antimouse IgG. Cells were then analyzed by flow cytometry.

Rac1 activation assay. Activation of Rac1 in 16HBE14o- cells was determined by using Rac1 activation assay kit following the manufacturer’s instructions (Millipore, Temecula, CA).

Briefly, after relevant treatment, cells were lysed in the presence of aprotinin and leupeptin, lysates were incubated with glutathione agarose for 10 min and centrifuged, and total protein determined in the supernatant. A volume equivalent to 500 µg of total protein was incubated
with PAK-1 PBD agarose for 60 min at 4°C. Agarose beads were collected by brief centrifugation, and washed with lysis buffer. Bound Rac1-GTP were resolved by 15% SDS-PAGE and Rac1 was detected by immunobloting using monoclonal antibody to Rac1. Clarified cell lysates incubated with GTP\textsubscript{\gamma}S or GDP followed by incubation with PAK-1 PBD agarose were used as positive and negative controls, respectively. An aliquot of clarified cell lysate equivalent to 5 \(\mu\)g of total protein was used as a loading control. Quantification of ECL signals was performed by densitometry using NIH imageJ.

Data analysis. Statistical significance was assessed by analysis of variance (ANOVA) or ANOVA on Ranks as appropriate. Differences identified by ANOVA or ANOVA on ranks were pinpointed by Tukey-Kramer or Dunn’s test respectively.
RESULTS.

**Poly I:C treatment decreases TER of polarized airway epithelial cells.** Previously, we demonstrated that RV replication is required for disruption of barrier function. RV generates double-stranded RNA (dsRNA) during replication. To examine whether dsRNA is sufficient to disrupt barrier function, we treated polarized 16HBE14o- cells with 500 ng/well poly I:C and monitored $R_T$ up to 48 h. Poly I:C decreased $R_T$, showing a significant reduction starting at 6 h and reaching a maximum at 8 h. $R_T$ returned to normal by 48 h (Figure 1A). As observed earlier, cell cultures infected with infectious RV also showed a significant reduction in $R_T$, compared to cells treated with media or replication-deficient UV-irradiated RV (UV-RV) (Figure 1B).

Next, we examined distribution of the tight junction proteins ZO-1 and occludin in poly I:C-treated (8 h post treatment) and RV-infected (24 h post infection) polarized 16HBE14o- cells by indirect immunofluorescence confocal microscopy. While media treated control cells showed normal distribution of both ZO-1 and occludin, poly I:C-treated and RV-infected cell cultures each showed dissociation of these tight junction proteins from the periphery of cells (Figure 1C). These results indicate that dsRNA generated during RV replication may play a role in reducing $R_T$.

To localize poly I:C, polarized cells were treated with rhodamine-labeled poly I:C and visualized in context with ZO-1. In X-Z section we observed that poly I:C was located at and below the apical surface and also loss of ZO-1 from the tight junctions of the cells that contained poly I:C (Figure 1D). The X-Y optical section taken in the middle of the cells showed poly I:C within the cells and ZO-1 in the periphery of the cells (Figure 1E).

**RV and poly I:C treatment each induce ROS generation.** RV infection may generate ROS (5, 23, 36). Therefore, we measured ROS production in 16HBE14o- polarized cells infected with
either infectious RV or UV-RV for 8, 12, 16 or 24 h. Cells were also treated with media or poly I:C for 2, 4, 6, 8 or 16 h. Compared to media or UV-RV-treated cells, RV-infected cells showed a significant increase in ROS production starting at 12 h, which sustained up to 24 h (Figure 2A and 2B). Similarly, poly I:C treated cells showed an increase in ROS production starting at 4 h and reaching a maximum at 6 h (Figure 2C and 2D). The increase in ROS production in both RV-infected cells and poly I:C-treated cells preceded the reduction in R\textsubscript{T}.

**DPI inhibits RV- or poly I:C-induced reduction in TER in polarized epithelial cells.** To determine the contribution of ROS in reduction of R\textsubscript{T} caused by RV, polarized 16HBE14o- cells were infected with RV. Ninety minutes later, the ROS scavenger N-propyl gallate or inhibitor of ROS-generating enzymes DPI (a NOX flavoprotein inhibitor) or oxypurinol (a xanthine oxidase inhibitor) were added and R\textsubscript{T} was measured 8 or 24 h later. Propyl gallate and DPI, inhibited the RV-induced reduction in R\textsubscript{T} (Figure 3A and 3B). This was not due to a reduction in RV load, as there was no significant difference in vRNA levels between untreated and antioxidant-treated cells (Figure 3C), nor the number of cells infected with RV, as determined by flow cytometry (Figure 3D). Propyl gallate and DPI also inhibited poly I:C-induced reductions in R\textsubscript{T} (Figure 3E). Oxypurinol had no effect on either RV- or poly I:C- induced reductions in R\textsubscript{T} (data not shown).

**DPI inhibits transmigration of bacteria in RV-infected polarized airway epithelial cells.**

Next, we examined the effect of DPI on the transmigration of bacteria across the polarized 16HBE14o- cells infected with RV. As previously observed (41), we recovered bacteria from the basolateral media of RV-infected cells (Figure 3F). On the other hand, cells infected with RV and incubated in the presence of DPI did not show bacteria in the basolateral chamber, indicating that DPI abrogates RV-induced changes in the paracellular permeability of polarized airway.
epithelial cells. A similar abrogation in the transmigration of bacteria was observed in cells treated with poly I:C in the presence of DPI. Together, these results indicate that ROS generation mediated by NOX is required for RV- or poly I:C-induced barrier function disruption.

**RV and poly I:C stimulate NOX 1 expression and activity.** So far, at least seven NOXs have been described. We examined the effects of RV infection on the expression of all 7 NOXs in 16HBE14o- cells by qPCR. NOX3 and NOX4 were undetectable in our cell culture model at baseline (untreated cells) as well as after RV or poly I:C treatment (Data not shown). Baseline expression of DUOX1 and DUOX2 was higher than NOX1, NOX2 and NOX5 (Figure 4A). DUOX2 expression further increased by 4 fold in RV-infected cells (Figure 4B). On the other hand, NOX1 increased by almost 30 folds in RV-infected cells compared to sham or UV-RV infected cells. RV-infection did not alter expression of either NOX2 or NOX5. These results indicate that DUOX2 and NOX1 may contribute to RV-induced ROS generation. Next, we determined the kinetics of NOX1 and DUOX2 expression in cells treated with infectious RV or UV- RV (Figure 4C and 4D). In cells incubated with intact RV, but not UV-RV, NOX1 expression increased as early as 8 h and reached a maximum at 16 h after infection and this coincided with the increased ROS generation caused by RV infection (Figure 2B). In contrast, DUOX2 did not show a significant increase until 16 h post infection, and further increased 24 h post infection. Expression of NOX1 was much higher than the DUOX2 at all time points examined. Poly I:C-treated cells also showed increased mRNA expression of both NOX1 and DUOX2, and again NOX1 expression was higher than DUOX2 expression reaching maximum at 6 h (Figure 4E and 4F), which again coincided with poly I:C-induced ROS generation (Figure 2D).
We then examined the total NOX activity in RV- or poly I:C-treated cells by measuring the reduction of cytochrome c (36). This assay measures the activities of both NOX and xanthine oxidase. Therefore, we measured the cytochrome c reduction in the presence or absence of DPI (an inhibitor of NOX) or oxypurinol (an inhibitor of xanthine oxidase). Compared to cells treated with UV-RV, cells infected with RV showed a significant increase in the reduction of cytochrome c at 8, 12 and 16 h post-infection (Figure 4G) coinciding with expression of NOX1 and ROS generation. DPI, but not oxypurinol, abrogated the reduction of cytochrome c in RV-infected cells, indicating that cytochrome c reduction is due to the activity of NOX, not xanthine oxidase. Poly I:C-treated cells also showed a time-dependent increase in NOX activity which peaked at 6 h (Figure 4H), coinciding with NOX1 expression and ROS production.

**Inhibition of NOX1 abrogates RV-induced NOX activity and ROS generation.** To determine the contribution of NOX1 to RV-induced NOX activity, 16HBE14o- cells transfected with NOX1-specific (siNOX1) or non-targeting siRNA (siNT) were infected with intact or UV- RV. After 16 h incubation, expression of NOX1, NOX activity and ROS generation were determined. Both untransfected cells and cells transfected with siNT followed by RV infection showed increased NOX1 expression (Figure 5A), NOX activity (Figure 5B) and ROS generation (Figure 5C) compared to cells infected with UV-RV or sham. In contrast, cells transfected with siNOX1 prior to RV infection showed no increase in NOX1 expression, as well as reduced NOX activity and ROS generation. This was not due to decreased RV infection or replication in siNOX1-transfected cells (Figure 5D and 5E). These results indicate that RV-induced NOX1 activity contributes to total NOX activity and ROS generation. Poly I:C-induced NOX activity and ROS generation were also found to be partially dependent on NOX1 (Figure 5F-5H).
RV infection stimulates Rac1 activation in 16HBE14o- cells. Rac1 activation is required for assembly of holoenzyme and maximal activity of NOX1 (8). Therefore, we investigated whether RV stimulates Rac1 activity in 16HBE14o- cells by pull-down assay. Compared to cells infected with UV-RV, cells infected with infectious RV showed increased Rac1 activity at 8 h after infection, which further increased at 16 h (Figure 6A). In contrast, poly I:C-treated cells showed the highest activity 2 h after treatment, which slowly returned to normal by 8 h (Figure 6B).

We then examined the requirement of Rac1 activation for RV-induced NOX1 activity. The 16HBE14o- cells were infected with intact or UV-irradiated RV and incubated for 1 h. Infection medium was replaced with fresh medium containing 50 µM NSC23766, a chemical inhibitor of Rac1 (17), and NOX activity determined 16 h after incubation. NSC23766 (50 µM) reduced RV-induced NOX activity by 71%, and ROS production by 51% (Figure 6C and 6E). NSC23766 also partially inhibited poly I:C-induced NOX activity in 16HBE14o- cells (Figure 6D and 6F). Sixteen h after RV infection, there was no difference in levels of vRNA or the percent of cells infected between untreated and NSC23766-treated cells (Figure 6G and 6H).

Together, these results indicate that both RV and poly I:C stimulate Rac1 activation, and that Rac1 activity is required for maximal NOX activity and ROS production induced by these agents.

RV-induced disruptions in barrier function require Rac1 and NOX1. To determine the requirement of Rac1 activation for the observed reductions in RT, polarized 16HBE14o- cells were infected with RV for 90 minutes to allow endocytosis, media replaced with fresh media containing NSC23766, and RT measured at the end of 24 h. The Rac1 inhibitor attenuated the RV-induced reduction in RT (Figure 7A). Cells transfected with siRNA specific to NOX1 were
resistant to RV-induced reductions in $R_T$ compared to cells transfected with siNT (Figure 7B). These results suggest that both Rac1 and NOX1 are required for RV-induced reduction in $R_T$.

In cells with intact tight junctions, most of the cellular ZO-1 and occludin partitions to the cytoskeletal fraction. We have previously shown that RV infection reduces the amount of ZO-1 in the cytoskeletal fraction (41). To assess whether inhibition of Rac1 and NOX1 improve $R_T$ by increasing the retention of ZO-1 and occludin with the cytoskeleton, we treated cells with NSC23766 and siNOX1, and subjected cytoskeletal fractions to Western blot analysis. RV-infected cells showed decreased amounts of both ZO-1 and occludin in the cytoskeletal fraction compared to cells infected with UV-RV (Figure 7C and 7D). The Rac1 inhibitor partially inhibited the loss of both ZO-1 and occludin from the cytoskeletal fraction of RV-infected cells. RV-infected cells transfected with siNOX1 also showed increased amounts of ZO-1 and occludin in cytoskeletal fraction compared to cells transfected with siNT. Consistent with these findings, confocal microscopy revealed increased retention of ZO-1 and occludin in tight junctions of cells infected with RV in the presence of Rac1 inhibitor, and also in RV-infected siNOX1-transfected cells (data not shown). These results imply that inhibition of Rac1 activity and NOX1 expression improve $R_T$ in RV-infected cells by reducing dissociation of ZO-1 and occludin from the cytoskeleton.

Next, we examined whether inhibition of Rac1 and NOX1 improves the barrier function in RV-infected cells. As observed earlier, RV-infected cells showed increased transmigration of bacteria. Transmigration was significantly inhibited by NSC23766 and siNOX1, but not siNT (Figure 7E).

Rac1 and NOX1 are also required for disruption of barrier function induced by poly I:C. Polarized 16HBE14o- cells were treated with poly I:C in the presence or absence of NSC23766,
and $R_T$, and association of ZO-1 and occludin with the cytoskeleton were determined. The Rac1 inhibitor attenuated poly I:C-induced reductions in $R_T$, and this was associated with increased retention of both ZO-1 and occludin with cytoskeleton fraction (Figure 8A, 8C and 8D). Consistent with this, transmigration of bacteria from the apical to basolateral surface was decreased in the cells treated with poly I:C in the presence of the Rac1 inhibitor (Figure 8E).

Similarly, cells transfected with siNOX1 and then treated with poly I:C showed higher $R_T$, more ZO-1 and occludin in the cytoskeletal fraction, and less bacteria in the basolateral chamber than similarly-treated cells transfected with siNT (Figure 8B-8E). Collectively, these results suggest that Rac1-dependent NOX1 activity is required for RV- and poly I:C-induced reductions in tight junction function in polarized airway epithelial cells.

**TLR3 is not required for either RV or poly I:C-induced reduction in $R_T$.** TLR3 recognizes both poly I:C and RV dsRNA (31, 49). To determine the role of TLR3 in RV- and poly I:C-induced reductions in $R_T$, polarized cells transfected with siNT or TLR3-specific siRNA (siTLR3) were either infected with RV or treated with poly I:C as above, and $R_T$ measured 24 h after RV infection or 8 h after poly I:C treatment. Both RV and poly I:C caused reductions in $R_T$ in siTLR3-transfected cells similar to those observed in cells transfected with siNT (Figure 9A and 9B). Flow cytometry indicated reduced TLR3 expression in cells transfected with siTLR3 (Figure 9C). Together these results suggest that TLR3 is not required for either RV- or poly I:C-induced reductions in $R_T$.

**Quercetin, but not N-acetyl cysteine, blocks the disruptive effects of RV- or poly I:C on airway epithelial cell barrier function.** We examined the effects of N-acetylcysteine, an antioxidant that has been used in human trials (10, 27, 46) and quercetin, a flavonoid with both antioxidant and antiinflammatory properties that may slow the progression of inflammatory lung
disease (16, 33, 37), on RV- and poly I:C-induced responses. N-acetylcysteine had no effect on either RV- or poly I:C induced reductions in $R_T$ (Figure 10A and 10B) and did not affect the viral load (data not shown). In contrast, quercetin blocked the effects of RV and poly I:C in a dose-dependent manner, showing maximum effects at 10 µM (Figure 10C and 10D). However, quercetin also decreased vRNA copy number (Figure 10E). These results suggest that quercetin may block RV-induced effects, not only by scavenging ROS, but also by decreasing RV load.
DISCUSSION

Unlike influenza virus, RV does not extensively damage the airway epithelium. But, RV compromises barrier function (41, 54), potentially increasing permeability of the airway epithelium to allergens and susceptibility to secondary bacterial infections. However, the mechanisms by which RV compromises barrier function are not well-understood. In the present study, we demonstrate that ROS generation is required for RV-induced disruptions of barrier function in polarized airway epithelial cells. We also show that RV-induced Rac1-dependent NOX1 activity is required for maximal ROS generation. Finally, we demonstrate for the first time that poly I:C, a synthetic dsRNA also disrupts barrier function by inducing ROS generation via activation of NOX1, similar to RV. Together, these results imply that dsRNA, an intermediate formed during RV replication, contributes to ROS generation and the disruption of barrier function.

Previously, we had demonstrated that replication of RV is required for the disruption of barrier function (41). dsRNA is one of the intermediates that accumulates during RV replication. A synthetic dsRNA, poly I:C has been extensively used as a surrogate for viral dsRNA to study host responses. It provokes inflammatory and IFN responses in airway epithelial cells similar to viral dsRNA (4, 30, 49). Recently, dsRNA was also shown to decrease $R_{T}$ in immortalized nasal epithelial cells (35), but the underlying mechanism was not elucidated. Therefore, to investigate whether dsRNA generated during RV replication contributes to the RV-induced disruption of barrier function, we used poly I:C (1.5 to 8 kb in length) as a surrogate for RV dsRNA. We found that Poly I:C disrupts the barrier function of polarized airway epithelial cells similar to RV. We also noted presence of poly I:C within the cells. Since the complete RV replication cycle occurs in the cytoplasm (45), it is reasonable to speculate that dsRNA generated during RV
replication will also be in the cytoplasm similar to poly I:C. Therefore, it is conceivable that
dsRNA generated during RV replication is sufficient to cause disruption of barrier function.
However, one cannot rule out the involvement of structural or non-structural proteins of RV.

Ligation of dsRNA to specific cellular proteins is required for stimulation of IFN or pro-
inflammatory responses. The pattern recognition receptor TLR3 is located primarily in the
endosomal membrane and to some extent in the plasma membrane. The RNA helicases retinoic
acid inducible gene (RIG)-I, melanoma differentiation-associated gene (MDA)5 and LGP2, and
the serine threonine kinase protein kinase R (PKR) are located within the cytoplasm. Previously,
we demonstrated in cultured airway epithelial cells that both TLR3 and MDA5 are required for
maximum RV-stimulated IFN responses (49), and that TLR3 is partially required for RV-
induced IL-8 expression (42). On the other hand, poly I:C-induced IFN and cytokine responses
were found to be dependent on TLR3 (31). Together these observations suggest that TLR3
recognizes both poly I:C and RV dsRNA, and therefore it is reasonable to postulate that TLR3
may be required for RV or poly I:C-induced disruption of barrier function. Surprisingly,
knockdown of TLR3 had no effect on either RV or poly I:C effect on R\textsubscript{T}, suggesting the
involvement of dsRNA recognition receptor other than TLR3 in this process. Further studies to
identify the receptor for dsRNA responsible for barrier disruption is under investigation.

Since our previous studies indicated that cytokines produced in response to RV-infection
do not contribute to RV-induced disruption of barrier function, we investigated other
mechanisms, in particular the role of reactive oxygen. ROS have previously been implicated in
the disruption of barrier function in polarized epithelial cells. For instance, ROS generation
stimulated by exogenous H\textsubscript{2}O\textsubscript{2} impaired the barrier function of both airway and colonic
epithelial cells by causing reorganization of the actin cytoskeleton and dissociation of occludin
and ZO-1 from the tight junction complex (6, 38). Since RV has been shown to stimulate ROS generation (5, 23, 36), we reasoned that ROS contribute to RV-induced disruption of tight junctions in polarized airway epithelial cell cultures. Consistent with this notion, we found that the antioxidants DPI and propyl gallate each blocked the disruptive effects of RV on barrier function.

DPI is an inhibitor of ROS-generating flavoenzymes, including NOX enzymes (24), suggesting that one or more NOX isoforms contribute to the RV-induced disruption of barrier function. Previously, RV and dsRNA have each been shown to stimulate mRNA expression of DUOX2, an isoform of NOX, in mucociliary-differentiated airway epithelial cells (19). We also observed increased expression of DUOX2 in both RV- and poly I:C treated polarized airway epithelial cells. DUOX2, which is located on the plasma membrane, secretes H$_2$O$_2$ into extracellular milieu (15); hence, we expected treatment with catalase would block RV- and poly I:C-induced reductions in $R_T$. However, catalase had no effect (data not shown). On the other hand, both RV and poly I:C induced robust mRNA expression of NOX1 compared to other isoforms. Furthermore, inhibition of NOX1 using gene-specific siRNA blocked the reductions in $R_T$ by more than 50%. In addition, inhibition of Rac1 also blocked RV- and poly I:C-induced disruptions of barrier function. Rac1 is required for the assembly of NOX1 holoenzyme and maximal NOX1 activity (8, 29). Finally, inhibition of NOX1 and Rac1 each partially attenuated the generation of ROS stimulated by RV and poly I:C. Together, our results suggest that both RV infection or poly I:C treatment increases expression of NOX1 and assembly of NOX1 holoenzyme which requires Rac1 activity, and this is responsible at least in part, for RV- and poly I:C-induced disruptions of barrier function in polarized airway epithelial cell cultures.
In addition to NOX, ROS is also produced by other enzymes such as the mitochondrial respiratory chain and xanthine oxidase. In non-polarized airway epithelial cells, RV was shown to stimulate ROS by activating xanthine oxidase (36). In the latter study, ROS increased within 20 min of infection and reached a maximum by 60 min, suggesting that binding of virus was sufficient for ROS generation. This effect was completely inhibited by pretreatment of cells with oxypurinol, an inhibitor of xanthine oxidase, but not DPI. In the present study, a significant increase in ROS production was not observed in polarized airway epithelial cells until 8 h after RV infection. Furthermore, UV-RV, which stimulates host responses associated with binding and endocytosis similar to intact RV, did not stimulate ROS generation. In addition, oxypurinol had no effect on either RV-induced ROS generation or the observed reduction in $R_T$. These results suggest that RV may not stimulate the activity of xanthine oxidase in polarized airway epithelial cells. Finally, it should be noted that DPI not only inhibits the activity of NOX, but also enzymes comprising the mitochondrial respiratory chain (28). Since DPI completely blocks both ROS generation and disruption of barrier function caused by RV, it is possible that ROS generated by the mitochondrial respiratory chain also contributes to the effects of RV on barrier function.

ROS generated by professional phagocytes in response to infection is beneficial for the host, because they participate in direct killing of infecting microbes. However in non-phagocytic cells, ROS regulate a variety of physiological responses, including cell proliferation, apoptosis, immune and pro-inflammatory responses (reviewed in (12)). ROS generation may not always be beneficial to the host, particularly during viral infections. Here, we show that ROS produced by NOX1 disrupts the barrier function of RV-infected airway epithelial cells. On a similar note, ROS production via NOX2 was proposed to cause excessive inflammation during RSV infection.
In influenza-infected mice, an absence of NOX2 led to improved viral clearance, an increased Th1 response and decreased airway inflammation (44). ROS also inhibit IFN-α-induced antiviral gene expression in human hepatoma cells infected with hepatitis C virus (11).

In light of these observations, we speculate that increased ROS generation during viral infection may compromise innate immune defense mechanisms, and therefore treatment with antioxidants may prevent oxidative damage and reduce susceptibility to secondary bacterial infections and permeability to allergens. Indeed, our preliminary studies showed that quercetin, a potent free radical scavenger, blocked the disruptive effect of RV on airway epithelial barrier function. On the other hand, N-acetylcysteine, a precursor of glutathione that has been used in clinical trials to inhibit influenza flu symptoms and treat chronic bronchitis (10, 27, 46) had no effect on RV-induced reductions in barrier function. Further studies are required to assess the potential beneficial effects of antioxidants in vivo.
REFERENCES


FIGURE LEGENDS

Figure 1. Poly I:C decreases $R_T$ by dissociating ZO-1 and occludin from tight junction complex similar to RV. Polarized airway epithelial cells grown in transwells were treated with 500ng/ml poly I:C (A) and incubated up to 48h or infected apically with RV or UV-RV (B) for 90 min, infection media replaced with fresh media and incubated up to 24 h. $R_T$ was measured at various time points. Results represent average ± SEM calculated from three independent experiments carried out in triplicates (*different from media or UV-RV or sham treated cells, $p \leq 0.05$, ANOVA). In some experiments, cells were treated with poly I:C or infected with RV or UV-RV (C) or treated with rhodamine-labeled poly I:C (D and E) as above, fixed in methanol and immunostained with antibody to ZO-1 or occludin (green, C, D and E). Nuclei were stained with DAPI (blue). * in panel C represent dissociation of ZO-1 or occludin from the periphery of cells. Arrow in panels D and E represents rhodamine labeled poly I:C below the apical surface and within the cells respectively. Images are representative of three independent experiments.

Figure 2. RV infection or poly I:C treatment stimulate ROS generation in polarized airway epithelial cells. Polarized 16HBE14o- cells were infected with RV or UV-RV (A and B), or treated with poly I:C (C and D). Generation of ROS was measured by flow cytometry using carboxy-$\text{H}_2\text{DCFDA}$ 24h after RV infection (A) or 8 h after poly I:C treatment (C). Kinetics of ROS generation was measured in RV-infected (B) or poly I:C treated (D) by flow cytometry. Histograms are representative of four independent experiments. Data in B and D represents average ± SEM from four independent experiments carried out in duplicates (*different from media or UV-RV-treated cells, $p \leq 0.05$, ANOVA).
Figure 3. Propyl gallate and DPI inhibit RV or poly I:C induced barrier disruption in polarized airway epithelial cells. Polarized 16HBE14o- cells were infected with RV or UV-RV as before for 90 min and infection media replaced with media containing propyl gallate (A) or DPI (B). R_T was measured after 24 h and expressed as % of UV-RV-treated controls. RNA was extracted from selected groups of RV-infected cells 24 h post infection and vRNA copies were determined (C). Percent of cells infected with RV was assessed by flow cytometry (D). For poly I:C treated cells (E), propyl gallate and DPI were added 30 min after adding poly I:C, R_T was measured after 8 h and expressed as % of similarly treated media controls. Data represents average ± SEM. The polarized cells treated with RV, UV-RV or poly I:C as above in the presence or absence of 10 µM DPI for 8 (for poly I:C treated cells) or 24 h (for RV-infected cells), NTHi was added to the apical chamber and bacteria in the basolateral media after 3 h was determined by plating (F). Data represents range and median. (*different from respective UV-RV or media treated controls, p ≤ 0.05; †different from RV-infected or poly I:C-treated cells in the absence of propyl gallate or DPI, p ≤ 0.05, ANOVA (A to E) or ANOVA on ranks (F)).

Figure 4. RV and poly I:C increases mRNA expression of NOX1 and DUOX2, and enhances NOX activity. Total RNA was isolated from media (A), sham, RV or UV-RV (B) infected cells and expression of NOX1, NOX2, NOX5, DUOX1 and DUOX2 was measured by qPCR. Kinetics of NOX1 and DUOX2 mRNA expression in RV-infected cells (C and D) or in poly I:C treated cells (E and F). Kinetics of NOX enzymatic activity was measured at various time points after RV infection (G) or poly I:C treatment (H) by cytochrome c reduction assay in the presence or absence of DPI. Data represents average ± SEM calculated from 3 to 4 independent experiments carried out in duplicates (*different from sham or media treated controls, p ≤ 0.05; †different from NOX enzymatic activity determined in the presence of DPI p ≤ 0.05, ANOVA).
Figure 5. RV or poly I:C induced NOX1 expression contributes to increased total NOX activity and ROS generation. The 16HBE14o- cells were transfected siNT or siNOX1. Two days later, cells were infected with RV or treated with poly I:C as before. Cells were harvested 16 h (for RV infected cells) or 8h (for poly I:C treated cells) after treatment and assessed for expression of NOX1 by western blot analysis (A and F), total NOX activity by measuring the cytochrome c reduction (B and G) or ROS generation by flow cytometry using caroboxy-H$_2$DCFDA (C and H). vRNA copy number and % of RV-infected cells were determined in siNT or siNOX-transfected cells 24h after RV infection (D and E). Data represents average ± SEM calculated from 3 to 4 independent experiments carried out in duplicates (*different from respective controls, $p \leq 0.05$; †different from respective siNT treated controls, $p \leq 0.05$, ANOVA).

Figure 6. RV or poly I:C-induced Rac1 activity partially contributes to total NOX activity and ROS generation. The 16HBE14o- cells were infected with RV (A) or poly I:C (B) and Rac1 activity was determined by pulldown assay at various time points. Media or UV-RV treated cells were used as controls. +ve and –ve controls represents cell lysates from media treated cells incubated with GTP$_{\gamma}$S or GDP respectively. Images are representative of 3 experiments. Cells were infected with RV or UV-RV (C and E), or treated with media or poly I:C (D and F) and incubated in the presence or absence of NSC27366, cells harvested after 8h (for poly I:C treated cells) or 24 h (RV infected cells), and examined for NOX activity (C and D) and ROS generation (E and F). vRNA copy number and % of RV-infected cells were determined in siNT or siNOX-transfected cells 24h after RV infection (G and H). Data represents average ± SEM calculated from 3 to 4 independent experiments carried out in duplicates (*different from respective controls, $p \leq 0.05$; †different from RV-infected or poly I:C-treated cells in the absence of NSC23766, $p \leq 0.05$, ANOVA).
Figure 7. Rac1 and NOX1 are required for RV induced disruption of barrier function. A. Polarized airway epithelial cells grown in transwells were infected with RV or UV-RV as above and incubated for 24h in the presence or absence of varying concentrations of NSC23766, $R_T$ was expressed as % of UV-RV- infected controls. B. Cells growing in transwells were transfected with NT or NOX1 siRNA, and 2 days later cells were infected with RV or UV-RV, $R_T$ was measured after 24 h and expressed as percent of respective UV-RV controls. C. Cytoskeleton fraction was subjected to Western blot analysis with antibody to ZO-1 or occludin and the image is a representative of 3 independent experiments. D. Quantification of the ratio of occludin or ZO-1 to $\beta$-actin. E. Twenty four hours after RV or UV-RV infection, non-typeable *H. influenzae* was added to apical chamber and bacteria in the basolateral chamber were quantified to assess bacterial transmigration across polarized airway epithelial cells. Data represents average ± SEM (A, B, and D) or range with median (E) calculated from 3 to 4 independent experiments carried out in duplicates (*different from respective controls, $p \leq 0.05$; †different from RV-infected cells in the absence of NSC23766 or NT siRNA transfected cells infected with RV, $p \leq 0.05$, ANOVA or ANOVA on ranks).

Figure 8. Poly I:C induced barrier disruption requires both Rac1 and NOX1. A. Polarized epithelial cells were treated with media or poly I:C for 8 h in the presence or absence of NSC23766 and $R_T$ measured. B. Cells transfected with NT or NOX1 siRNA were treated with poly I:C, measured after 8 h. $R_T$ is expressed as percent of respective media controls. C and D. ZO-1 and occludin protein levels in cytoskeleton fraction was determined by Western blot analysis followed by densitometry and expressed as a ratio of ZO-1 or occludin to $\beta$-actin Western blot image (C) is a representative of 3 experiments. E. Eight hours after poly I:C treatment, bacterial transmigration across polarized airway epithelial cells was measured as
described above. Data represents average ± SEM (A, B, and D) or range with median (E) calculated from 3 to 4 independent experiments carried out in duplicates (*different from respective controls, p ≤ 0.05; †different from RV-infected cells in the absence of NSC23766 or NT siRNA transfected cells infected with RV, p ≤ 0.05, ANOVA or ANOVA on ranks).

Figure 9: TLR3 is not required for either RV- or poly I:C-induced reduction in $R_T$. Cells transfected with NT or TLR3 siRNA were treated with RV (A) or poly I:C (B), and $R_T$ was measured after 24 or 8 h respectively. Data are expressed as percent of respective UV-RV or media treated controls and represents average ± SEM calculated from 3 to 4 independent experiments carried out in duplicates (*different from respective controls, p ≤ 0.05, ANOVA). C, expression of TLR3 in siNT or siTLR3 transfected cells was determined by flow cytometry. Histogram shown is a representative of 3 experiments.

Figure 10. Quercetin, but not N-acetylcysteine blocks disruptive effect of RV and poly I:C on $R_T$. Polarized 16HBE14o- cells were infected with RV or UV-RV as before for 90 min and infection media replaced with media containing N-acetylcysteine (A) or quercetin (C) and $R_T$ was measured 24 h later. Cells were treated with poly I:C in the presence of N-acetyl cysteine (B) or quercetin (D) and $R_T$ was measured after 8 h. Data are expressed as % of media or UV-RV-treated controls as appropriate. Total RNA was isolated from cells infected with RV in the presence of quercetin and copies of vRNA determined by q-PCR (E). Data represents average ± SEM calculated from 2 to 3 independent experiments carried out in duplicates (*different from respective UV-RV or media treated controls, p ≤ 0.05; †different from RV-infected or poly I:C-treated cells in the absence of quercetin, p ≤ 0.05, ANOVA).
Figure 7

A

\[ R_T (\%) \text{ of control} \]

<table>
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<tr>
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B

\[ R_T (\%) \text{ of control} \]

<table>
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<th>siNOX</th>
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C

<table>
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<td>100</td>
<td>NOX1</td>
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ZO-1

Occludin

\(\beta\)-actin

D

\[ \text{ZO-1 expression (Fold change over } \beta\text{-actin)} \]

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E

\[ \text{Bacteria recovered (CFU/ml)} \]

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