Phosphatidylglycerol provides short-term prophylaxis against respiratory syncytial virus infection

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Abstract  Respiratory syncytial virus (RSV) causes respiratory tract infections in young children, and significant morbidity and mortality in the elderly, immunosuppressed, and immunocompromised patients and in patients with chronic lung diseases. Recently, we reported that the pulmonary surfactant phospholipid palmitoyl-oleoyl-phosphatidylglycerol (POPG) inhibited RSV infection in vitro and in vivo by blocking viral attachment to epithelial cells. Simultaneous application of POPG along with an RSV challenge to mice markedly attenuated infection and associated inflammatory responses. Based on these findings, we expanded our studies to determine whether POPG is effective for prophylaxis and postinfection treatment for RSV infection. In vitro application of POPG at concentrations of 0.2–1.0 mg/ml at 24 h after RSV infection of HEp-2 cells suppressed interleukin-8 production up to 80% and reduced viral plaque formation by 2–6 log units. In vivo, the turnover of POPG in mice is relatively rapid, making postinfection application impractical. Intranasal administration of POPG (0.8–3.0 mg), 45 min before RSV inoculation in mice reduced viral infection by 1 log unit, suppressed inflammatory cell appearance in the lung, and suppressed virus-elicited interferon-γ production. These findings demonstrate that POPG is effective for short-term protection of mice against subsequent RSV infection and that it has potential for application in humans.—Numata, M., Y. Nagashima, M. L. Moore, K. Z. Berry, M. Chan, P. Kandasamy, R. S. Peebles, Jr., R. C. Murphy, and D. R. Voelker. Phosphatidylglycerol provides short-term prophylaxis against respiratory syncytial virus infection. J. Lipid Res. 2013. 54: 2133–2143.

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Respiratory syncytial virus (RSV) infects nearly 90% of children under age 2. Immunity to the virus is incomplete, and reinfection of adults, especially the elderly, patients with chronic lung disease, and those with impaired immunity (1–4), can produce serious respiratory disease. The mortality rate for RSV infection in immunosuppressed populations ranges from 30% to 100% (1, 3). RSV infection is a significant cause of acute, severe exacerbations of asthma (5, 6) and chronic obstructive pulmonary disease (COPD) (7). Infection by this virus results in approximately 250,000 hospitalizations annually in the United States (1, 8). Despite decades of research, there is currently no clinically approved vaccine or effective treatment for RSV (1, 9). Current antiviral approaches (10, 11) include treatment with a monoclonal antibody, Palivizumab, directed against the viral F protein, and administration of an aerosolized nucleotide analog, ribavirin. Palivizumab treatment has restricted application, and ribavirin treatment is of questionable efficacy (1, 12–14).

The pulmonary surfactant system of the lung is a lipid and protein complex that plays an important role in regulating both the biophysical and immunological properties of the organ (15).

Numerous studies focused upon the pulmonary surfactant proteins A and D have demonstrated their contributions to innate immunity, including antiviral defense within the organ (15, 16). Recent work has now unexpectedly implicated anionic surfactant phospholipids as important regulatory molecules for controlling innate immunity within the lung and executing antiviral functions within the lung (17–19).

Phosphatidylglycerol (PG) is a minor phospholipid component of pulmonary surfactant, with the palmitoyl-oleoyl molecular species (POPG) being most abundant in humans (16, 20). Although POPG is a minor lipid within the surfactant complex, the absolute levels of this lipid...
In vitro virus infection and phospholipid treatment

To examine phospholipid inhibition of RSV propagation following viral infection, monolayers of HEp-2 cells (3 × 10^5/well) were infected using an MOI of 1 × 10^-3, and they were subsequently incubated for an additional 96 h. Lipid antagonism of RSV infection was performed simultaneously with viral challenge or 24 h following viral challenge. The lipids were prepared by bath sonication of aqueous suspensions of phospholipids to create unilamellar liposomes at concentrations of 40 mg/ml in PBS, as described previously (17, 18, 21). The lipids were subsequently diluted into tissue culture medium and filter sterilized, then added to cell cultures at concentrations ranging 0.2–1.0 mg/ml. After 96 h, culture supernatants were harvested for measurement of interleukin (IL)-8 by ELISA. For quantifying viral titers, the entire contents of infected T-25 flasks were subjected to two freeze-thaw cycles, and then the remnant cell monolayer and culture supernatant were harvested and processed for quantitative plaque measurements as previously described (17, 29). For measurement of the IC_{50} values of different molecular species of phospholipid as antagonists of IL-8 production, we utilized BEAS2B cells that were cultured in 24-well plates as previously described (17, 18) and preincubated with phospholipids for 1 h prior to the addition of virus. The cells were infected with RSV at an MOI of 0.25, and the infection continued for 48 h. IL-8 production in culture supernatants was measured by ELISA.

RSV binding to phospholipids and HEp-2 cells

The interactions between RSV and phospholipids were measured using solid-phase phospholipid-binding assays coupled with ELISA detection of the virus (17, 21, 31). Different molecular species of PG and PC were coated onto 96-well microtiter plates air-drying ethanol solutions of phospholipids in the wells (1.5 nmol/well, 25 μl ethanol) followed by hydration and incubation with varying concentrations of RSV. The binding reactions were conducted at 37°C for 1 h. Virus binding was detected with HRP-conjugated anti-human RSV antibody (1:500) and orthophenylendiamine substrate using absorbance at 450 nm (17). Viral attachment to HEp-2 cells (1 × 10^5 cells/well in 24-well plates) was measured at 18°C to minimize endocytosis and viral entry. The binding reaction was performed in DMEM/F12 for 2 h using varying concentrations of RSV. Phospholipid antagonism of the binding reaction was examined by adding 0.2–1.0 μg of POPG or POPC to the cultures 1 h prior to the addition of virus. At the end of the incubation period, the cells monolayers were washed with PBS three times at 0°C, and the contents of each well were harvested in SDS-PAGE loading buffer. The samples were subjected to electrophoresis and further processed for immunoblotting (18). Viral proteins were detected using HRP-conjugated anti-human RSV antibody (1:500) (17). Quantification of viral binding used National Institutes of Health Image J software for analysis of chemiluminescence.

In vivo RSV infection and its prevention by POPG

Female, six-week-old BALB/c mice were obtained from Jackson Laboratory. All animal protocols were approved by the National Jewish Health (NJI) Institutional Animal Care and Use Committee. Mice were anesthetized by injection of 0.25 g/kg ativan given intraperitoneally (17, 18). Mice were infected by intranasal inoculation of 50 μl of PBS containing no additions, 1 × 10^6 PFU of RSV, 400 μg POPG, or a combination of RSV and POPG. For examination of POPG prophylaxis, varying concentrations of the lipid were tested (800, 1,600, 3,000 μg/mouse). Preliminary experiments

MATERIAL AND METHODS

Viruses, cell culture, and phospholipids

The human RSV A2 strain (VR-1540), was obtained from the American Type Culture Collection (ATCC). HEp-2 cells and BEAS2B cells were also obtained from ATCC and cultured as previously described (17). Virus stocks were propagated in HEp-2 cell monolayers using Dulbecco’s Modified Eagle Medium / Ham’s F12 Medium (DMEM/F12, Gibco) plus 5% bovine growth serum (BGS, Hyclone). The virus was prepared and purified using methods previously described (17, 27, 28).

Viral titers and growth were estimated using quantitative plaque assays (29). RSV surface protein expressions and plaque formation were also determined by immunostaining for viral antigens using HRP-conjugated polyclonal goat anti-human RSV antibody (1:100) (AbD, Serotec).

For patient samples, RSV antigenic subgroup A clinical isolates were obtained from the Vanderbilt Vaccine Clinic (Nashville, TN), and the viruses were propagated by 10 passages through HEp-2 cells as described (30). To evaluate the susceptibility of clinical isolates and recombinant viruses to inhibition by POPG, HEp-2 cells were infected at a multiplicity of infection (MOI) of from 10^-5 to 10^-2 in the absence of lipid, or at MOI of from 10^-1 to 10^-4 in the presence of lipid. We routinely used 3 MOIs when testing strains of unknown susceptibility to phospholipid antagonism. Phospholipids were purchased from Avanti Polar Lipids.

(>)1.7 mg/ml in the alveolar hypophase are extraordinarily high when compared with the amounts found in other tissues or at epithelial interfaces (16, 20). Recent findings reveal that POPG is a potent regulator of innate immune and respiratory viral infection (17). POPG is an effective antagonist of Toll-like receptor 4 (TLR4) activation and acts by disrupting interactions of lipopolysaccharide (LPS) with cluster of differentiation 14 (CD14) and lymphocyte antigen 96 (MD2) (21–24). The lipid also antagonizes Toll-like receptor 2 (TLR2) activation by inhibiting signaling from the cell surface (25). The antiviral actions of POPG include its activity against RSV and influenza A virus (18, 19). For both viruses, POPG acts by blocking viral attachment to epithelial cell surfaces. Similar to POPG, di-palmitoyl-PG (DPPG) also inhibits vaccinia virus infection of epithelial cells (26). In quantitative plaque assays, POPG added with RSV inhibits plaque formation by 4–5 log units. Of particular importance is the finding that POPG prevents the intercellular spreading of the virus, when applied shortly after an infection is established in vitro (17). In vivo experiments also demonstrate that POPG significantly attenuates RSV infection in mice (17) by 3 log units.

Currently, there are significant gaps in our understanding of how POPG or structurally related lipid molecules might be applied as antiviral agents for humans. The purpose of this project was: 1) to determine the efficacy of POPG added after RSV infection is established in vitro, 2) to identify which molecular species of PG have the greatest potency for preventing RSV infection in vitro, 3) to test the effectiveness of POPG against clinical isolates of RSV, and 4) to define the dose, and timing, of POPG for prophylaxis against RSV in mice. The findings from this study demonstrate that POPG given prior to viral challenge in vivo can protect mice from RSV infections.
revealed that prophylaxis could not be achieved by administration of POPG at times longer than 45 min prior to viral challenge. The final method used POPG inoculation 45 min prior to RSV infection. At 5 days after the infection, mice were euthanized with 0.25 ml of Nembutal (10 mg/ml) given intraperitoneally. Bronchoalveolar lavage with 1 ml of PBS was performed prior to opening the thorax, and the recovered fluid (BALF) was used for total cell numbers, differential cell quantification, and interferon (IFN)-γ analysis (17, 18). The homogenates of left lungs were processed for RSV plaque assays, and the right lungs were used for histopathology scoring (17, 18), which was performed in a blinded protocol.

**Liquid chromatography and tandem mass spectrometry**

For LC/MS/MS analysis, 5% of the extracted lipids from each of the BALF samples, with 500 ng of d₃₁-16:0/18:1-PC added as internal standard for both PG and PC molecular species, were introduced onto the mass spectrometer. The different lipid classes present were separated using normal-phase HPLC with an Ascen- tis 5µ Si (15 cm × 2.1 mm) column (Supelco, Bellefonte, PA). A normal-phase solvent system of 30:40 hexane-2-propanol (solvent A) and 30:40:7 hexane-2-propanol-water with a final concentration of 1 mM ammonium acetate (solvent B) was used. The initial mobile phase was 25% solvent B at a flow rate of 0.2 ml/min. This initial mobile phase was held for 5 min, and then a linear gradient was started from 25% solvent B to 60% solvent B in 10 min, followed by another linear gradient from 60% solvent B to 100% solvent B in 5 min. The final step of the HPLC gradient was an isocratic elution at 100% solvent B for 15 min. Using this gradient, the PG molecular species eluted from 8–10 min and was well separated from other phospholipid classes.

The HPLC system was directly interfaced to the electrospray source of a triple quadrupole mass spectrometer (MDS Sciex API 3200; Applied Biosystems, Foster City, CA) and detection of the endogenous PG and the d₅-labeled PG lipid molecular species present in BALF was achieved using multiple reaction monitoring (MRM) analysis in negative ion mode. The specific transitions monitored during the MRM experiment were: m/z 719.6→253.2 for (16:0/16:1-PG), 724.6→253.2 for (d₅-16:0/16:1-PG), 721.6→255.2 for (16:0/16:0-PG), 726.6→255.2 for (d₅-16:0/16:0-PG), 745.6→279.2 for (16:0/18:2-PG), 750.6→279.2 for (d₅-16:0/18:2-PG), 747.6→281.2 for (16:0/18:1-PG), 752.6→281.2 for (d₅-16:0/18:1-PG), 769.6→303.2 for (16:0/20:4-PG), 774.6→303.2 for (d₅-16:0/20:4-PG), 793.6→327.2 for (16:0/22:6-PG), 798.6→327.2 for (d₅-16:0/22:6-PG) and 819.7→281.2 for (acetate adduct of d₅₅-16:0/18:1-PG). The relevant mass spectrometric experimental parameters in the negative ion mode for the MRM analysis were an electrospray voltage of ~4500 V, a declustering potential of ~55 V, and a collision energy of ~50 V. For quantitation of d₅-P POPG in the turnover experiments, a standard curve with a range of 0–5 µg of d₅-P POPG was used with d₅-16:0/18:1-PC as the internal standard. For the remodeling experiments, the area of the newly formed d₅-P PG lipid species (16:0/16:1-PG, 16:0/16:0-PG, 16:0/18:2-PG, 16:0/20:4-PG, and 16:0/22:6-PG) and the area of d₅-P POPG were used to demonstrate remodeling in this system.

**RESULTS**

**POPG inhibits IL-8 production after RSV infection is established**

We previously reported that simultaneous addition of POPG and RSV to human bronchial epithelial cells (either BEAS2B cells or primary cultures of human bronchial epithelial cells) attenuates the virus-elicited production of the inflammatory mediators IL-6 and IL-8 (17). In experiments described in Fig. 1, we examined IL-8 production by HEp-2 cells challenged with RSV at an MOI of 1×10⁻³ in the absence or presence of POPG or with POPG added 24 h after the viral challenge to evaluate the efficacy of the lipid treatment after the establishment of a viral infection for a significant period of time. At 4 days after the viral infection, the culture medium was harvested and IL-8 production was measured. The results demonstrate that sham infection elicits a negligible response, whereas the viral challenge elicits secretion of up to 18 ng/ml of IL-8. The inclusion of increasing concentrations of POPG along with the virus suppresses IL-8 production to levels of sham-infected cultures. In contrast to POPG, the control lipid POPC is without effect. The hydrophobic portion of POPC is identical to that of POPG, but the polar head groups of the two lipids (phosphocholine and phosphoglycerol) are different. The addition of increasing concentrations of POPG at 24 h after viral infection shows a concentration-dependent suppression of IL-8 production. These data demonstrate that, even after an RSV infection has progressed for 24 h, addition of POPG can act to suppress inflammatory mediator production from epithelial cells. In several previous studies (17, 18, 21, 25), we established that the levels of POPG applied to these cells do not exert any cytotoxic effects and do not act as nonspecific inhibitors of the NFκB signaling pathways that induce IL-8 gene transcription.

**POPG attenuates RSV propagation when added 24 h after a viral infection is established**

One potential mechanism by which POPG could suppress IL-8 production from HEp-2 cells is by preventing further rounds of viral infection. We evaluated viral propagation by either simultaneous or 24 h postinfection addition of POPG to cell cultures, followed by plaque assay...
after 4 days (17, 29). Simultaneous treatment with POPG (200 μg/ml) reduced the RSV plaque titer by 4 log units, but simultaneous addition of 1 mg/ml of POPC was ineffective (Fig. 2A). The addition of increasing concentrations of POPG introduced 24 h after the initial infections occurred reduced the viral titers from 1.5 to 6.5 log units in a concentration-dependent manner (Fig. 2A). Examination of viral antigens on cell monolayers by immunostaining demonstrated markedly reduced numbers and sizes of viral foci when POPG was added concomitantly with RSV or 24 h after the addition of POPG (Fig. 2B). Immunostaining of cultures treated with RSV and POPC was no different from that of cultures infected with RSV alone. From these data, we conclude that POPG effectively prevents viral propagation from infected to uninfected cells, even when the lipid is added 24 h after the RSV infection is established. These data demonstrate that maintenance of high levels of POPG is sufficient to suppress the spread of an established infection.

Multiple molecular species of PG bind to RSV with high affinity

Previous studies have reported that the anionic phospholipid antagonism of LPS activation of macrophages through CD14/MD2/TLR4 and the anionic phospholipid antagonism of TLR2 activation are specific to the molecular class and species of phospholipid used as an antagonist (21–25). Pulmonary surfactant contains several molecular species of PG in addition to POPG (16, 19). Next, we compared the potency of six molecular species of PG [16:0/16:0-PG (DPPG), 16:0/18:1-PG (POPG), 16:0/18:2-PG (PLPG), 18:0/18:1-PG (SOPG), 18:1/18:1-PG (DOPG), and 18:2/18:2 (DLPG)] against RSV infection using our in vitro system. Human bronchial epithelial cells (BEAS2B) were infected with RSV at an MOI of 0.25, and IL-8 secretion was measured 48 h later. We examined the molecular specificity of the PG effects upon RSV-induced IL-8 production by comparing various concentrations of phospholipids. In Fig. 3A, the actions that we observe for POPG are specific to this class of phospholipid compared with POPC. This result parallels the differential actions of POPG and POPC upon the CD14/MD2/TLR4 complex (21). Fig. 3A also shows the concentration-dependent antagonism of virus-induced IL-8 production by PLPG and demonstrates that this lipid has reduced potency compared with POPG. Expansion of these latter experiments to encompass other molecular species of PG and calculation of IC50 values from the dose-response curves produces the data shown in Fig. 3B. The data indicate that multiple molecular species of PG have similar potency as antagonists of RSV-induced inflammation. The IC50 values range 2–8 μg/ml for all the PG molecular species tested except for PLPG, which has an IC50 value of 25 μg/ml. There is no simple structure-based explanation that accounts for the 3- to 12-fold difference in potency of PLPG compared with the other PG molecular species.

In additional experiments, we examined the affinity of RSV for different molecular species of PG by using a solid-phase binding assay (17, 21); these data are summarized in Fig. 3C, D. Detailed RSV-binding data are shown in Fig. 3C for a nonspecific interaction (POPC), for a high-affinity reaction (POPG), and for an intermediate-affinity (DPPG) reaction. Data summarizing the results with the aforementioned lipids and SOPG, DOPG, DLPG, and SLPG are shown in Fig. 3D. The data in Fig. 3D are expressed as the concentration of virus required to achieve one-half maximal binding (Bmax/2) to a fixed quantity of phospholipid solid phase. The data demonstrate that a mono-unsaturated fatty acid in the sn-2 position of PG produces higher-affinity virus binding than either a diunsaturated or saturated fatty acid in the same position. In conjunction with the sn-2 position preference, the RSV-binding affinity is higher with a saturated or mono-unsaturated fatty acid in the sn-1 position of the PG.

**Fig. 3.** POPG suppresses RSV propagation after an RSV infection is established in vitro. (A) HEp-2 cells (10 × 10⁶ cells/ T25 flask) were either uninfected (CONL) or challenged with virus (RSV) at an MOI = 1 × 10⁻⁷ for 96 h, in either the absence or presence of 200, 500, or 1,000 μg/ml of POPG or 1,000 μg/ml of POPC, with the lipid added 1 h before or 24 h after RSV infection as indicated. Values shown are means ± SE for three independent experiments. *P < 0.001; **P < 0.0001. (B) Result of immunostaining for RSV against RSV. The 3 × 10⁶ cells/well were infected with RSV under the same conditions as in (A). RSV-infected cells were detected with HRP-conjugated polyclonal antibody directed against RSV.

**Fig. 2.** POPG suppresses RSV propagation after an RSV infection is established in vitro. (A) HEp-2 cells (10 × 10⁶ cells/ T25 flask) were either uninfected (CONL) or challenged with virus (RSV) at an MOI = 1 × 10⁻⁷ for 96 h, in either the absence or presence of 200, 500, or 1,000 μg/ml of POPG or 1,000 μg/ml of POPC, with the lipid added 1 h before or 24 h after RSV infection as indicated. Values shown are means ± SE for three independent experiments. *P < 0.001; **P < 0.0001. (B) Result of immunostaining for RSV against RSV. The 3 × 10⁶ cells/well were infected with RSV under the same conditions as in (A). RSV-infected cells were detected with HRP-conjugated polyclonal antibody directed against RSV.
concentrations of RSV incubated at 18°C to minimize viral entry. The specific binding interactions between RSV and epithelial cells were measured by performing quantitative western blotting of the virus attachment glycoprotein (GP) (18, 31). Fig. 4A shows the concentration-dependent binding of RSV to HEp-2 cell monolayers in the absence and presence of phospholipid. In the absence of phospholipid, RSV binding to cells is concentration dependent, and the addition of 200 μg/ml, or 1000 μg/ml of POPG markedly suppresses the viral attachment to the monolayer. In contrast, POPC has little effect on RSV attachment to the cells. Fig. 4B summarizes data from 3 experiments with the RSV GP levels normalized to cellular β-actin content. The data demonstrate that POPG markedly inhibits virus attachment to the HEp-2 cells and that increasing virus concentrations tends to overcome the lipid inhibition, consistent with the conclusion that PG molecular species competitively inhibit the process.

POPG suppresses infection by clinical isolates of RSV and strains with enhanced virulence

Previous work demonstrated that POPG inhibits infection by the A2 laboratory strain of RSV in vitro and in vivo (17). We examined the effect of POPG against recent clinical isolates of RSV and strains genetically engineered for enhanced virulence in mice (33), using plaque assays. We examined five RSV strains from patients (strain designations are by GenBank numbers) and a recombinant strain that induced increased airway hypersensitivity and goblet cell hyperplasia (rA2-19F). Additional control recombinant strains rA2-A2F and rA2-LongF were also examined (33, 34), along with a standard RSV-A2 strain. In these experiments, the different strains of RSV were incubated with a HEp-2 cell monolayer in the absence or presence of phospholipid for 2 h at 37°C. Subsequently, nonadherent virus and phospholipids were removed, and the monolayers were overlaid with 0.3% agar in DMEM/F12. After 6 days, the monolayers were examined for plaque formation. The results are shown in Table 1. The inclusion of POPG with the viral inoculum reduced infection by 4–7 log units, whereas POPC failed to inhibit plaque formation by as much as 1 log unit. These findings demonstrate that POPG is a potent inhibitor of circulating strains of RSV and recombinant strains of the virus that exhibit enhanced pathogenesis in mice (33).

POPG prophylaxis provides short-term protection of mice from RSV

We previously reported that simultaneous treatment of mice with RSV and POPG was very effective for suppressing the viral infection in vivo (17). To extend these studies, we examined the effect of POPG prophylaxis against RSV infection in vivo. Mice were inoculated with various concentrations of POPG (800, 1,600, and 3,000 μg/mouse) 45 min prior to RSV inoculation (1 × 10⁷ PFU/mouse) and compared with either untreated mice or animals receiving simultaneous treatment of POPG (400 μg/mouse) and virus. After 5 days, the mice were euthanized, and the lungs were processed for quantitative plaque assays and histopathological scoring. Lavage was performed on each animal, and the BALF was processed to measure total cell numbers, inflammatory cell populations, and IFN-γ production. The results in Fig. 5A show that RSV infection increased total cell numbers in BALF by 3-fold and that the prophylaxis treatments (RSV + pPG) suppressed the influx of inflammatory cells with the highest dose of POPG.
induced a robust IFN-γ response (5.0 ± 0.5 ng/ml) in infected mice, and there was no detectable IFN-γ production in either sham-infected animals or those simultaneously treated with RSV and POPG (Fig. 5C). In animals pretreated with POPG, there was a concentration-dependent suppression of IFN-γ production, and doses of 1,600–3,000 g of phospholipid were not significantly different from either sham-infected animals or animals receiving simultaneous exposure to RSV and POPG. We also examined the effect of POPG on BALF recovery and cell types, with the aim of understanding the mechanisms behind RSV infection.

### TABLE 1. POPG inhibits infection by clinical isolates of RSV and recombinant variants of RSV

| RSV Strain       | Titer (PFU/ml) | Plaques with POPG | Plaques with POPC |
|------------------|----------------|-------------------|-------------------|
| JX069798.1       | 8.3 ± 4.7 × 10^5 | 0                 | 3.0 ± 3.5 × 10^7  |
| JX069803.1       | 4.8 ± 0.4 × 10^6 | 0                 | 0.5 ± 0.1 × 10^6  |
| JX069801.1       | 1.1 ± 0.2 × 10^7 | 2.6 ± 3.6 × 10^2  | 0.4 ± 0.2 × 10^2  |
| JX069800.1       | 1.1 ± 0.1 × 10^7 | 3.0 ± 4.2 × 10^2  | 0.5 ± 0.2 × 10^2  |
| JX069799.1       | 5.3 ± 0.5 × 10^4 | 0                 | 0.7 ± 0.5 × 10^4  |
| rA2-A2 F         | 1.2 ± 0.4 × 10^7 | 0.5 ± 0.01 × 10^2 | 0.8 ± 0.02 × 10^7 |
| rA2-line19F      | 3.0 ± 0.7 × 10^6 | 0                 | 3.0 ± 0.2 × 10^6  |
| rA2 Long F       | 6.2 ± 0.2 × 10^6 | 0                 | 1.6 ± 0.6 × 10^6  |
| wtRSV A2         | 2.7 ± 1.1 × 10^7 | 0                 | 1.3 ± 0.5 × 10^7  |

Five different clinical isolates of RSV (designated by their GenBank accession numbers) were passaged 10 times through HEp-2 cells to establish stocks for experimental analysis. The clinical isolates and three different recombinant strains (rA2-A2F, rA2-19F, and rA2 LongF) were compared with RSV-A2. The titer of each sample is shown as PFU/ml, and the effects of 200 µg/ml POPG and 200 µg/ml POPC upon the viral titers quantified in plaque assays are shown. Data are means ± SD from two independent experiments. For each strain examined, three MOIs were tested in the range from 10^-1 to 10^-7 in the absence of POPG and in the range from 10^-1 to 10^-4 in the presence of POPG.
Phosphatidylglycerol prophylaxis against RSV

Phospholipid concentration-dependent protection of the mice from RSV infection. The maximum level of protection attained with the highest dose of POPG pretreatment was indistinguishable from that obtained by simultaneous treatment with POPG. From these data, we conclude that POPG provides significant short-term prophylaxis against RSV infection and accompanying inflammation.

We also measured changes in the surfactant protein A (SP-A) content of BALF in response to infection and POPG treatment. With RSV challenge, SP-A levels after 5 days of infection were reduced by 41%, and this reduction was not as pronounced with inclusion of POPG in the infecting inoculum. Pretreatment of mice with 800 μg of POPG before RSV infection prevented the reduction in SP-A caused by virus. However, pretreatment with the 3,000 μg of POPG prior to infection did not prevent a reduction in SP-A levels. The treatment of mice with 3,000 μg of POPG alone modestly reduced the amount of BALF SP-A recovered after 5 days.

POPG efficacy window and turnover rate in mice

In the initial phase of this study, we tested multiple time windows for POPG prophylaxis against RSV infection. Pretreatment time periods of 12, 8, or 4 h were all ineffective, but at 2 h, POPG reduced RSV plaque numbers by 50%. In contrast, the 45 min interval, described in Fig. 5, provided...
and 16:0/18:2-d<sub>5</sub>-PG increase significantly. In addition, at 60 min, 16:0/16:0-d<sub>5</sub>-PG, 18:0/20:4-d<sub>5</sub>-PG, and 18:0/22:6-d<sub>5</sub>-PG appear and then increase significantly through 360 min. Phospholipid remodeling via deacylation-reacylation reactions is an intracellular process (35, 36). This remodeling of PG indicates that the d<sub>5</sub>-POPG is taken up by the epithelium and metabolized to new molecular species, which are subsequently secreted into the bronchoalveolar space. Although the mass spectrometry data enable us to detect the uptake and remodeling of d<sub>5</sub>-POPG, they do not allow us to determine how much of this lipid is taken up by the epithelium and secreted without remodeling (i.e., directly recycled). However the second, slower phase of the apparent turnover of d<sub>5</sub>-POPG is most likely due to some recycling of this lipid.

**DISCUSSION**

POPG is a minor lipid of pulmonary surfactant that is synthesized and stored within alveolar type 2 cells and secreted at the air-tissue interface of the alveolar compartment (16, 37). The phospholipid concentration within the microscopic fluid layer (alveolar hypophase) that overlays the alveolar epithelium is extraordinarily high and estimated to be ~50 mg/ml (16, 19, 38). The concentration of the PG phospholipid class within the alveolar hypophase is approximately 5 mg/ml, with POPG being the most abundant molecular species. The high local concentrations of PG in the alveolar compartment are unique to the lung; no other epithelial surface or secretion has been reported to contain PG.
Phosphatidylglycerol prophylaxis against RSV

Lung in vivo, the lipid will be an effective therapeutic for established infections. Our recent work has focused upon POPG because it is the most abundant molecular species of PG present in human pulmonary surfactant. To examine whether the interaction of POPG with RSV is unique, we investigated the interaction of other PG molecular species with the virus by quantifying the antagonism of IL-8 production and the direct binding interactions to solid-phase phospholipids. These studies reveal that both unsaturated PG molecular species (POPG, SOPG, DOPG, DLPG, and DPPG) and saturated PG molecular species (DPPG) antagonize RSV-induced IL-8 production with similar IC$_{50}$ values (3-8 μg/ml). The PLPG molecular species was unusual because its IC$_{50}$ for antagonizing IL-8 production was 25 μg/ml. Examination of direct physical interactions between RSV and solid-phase phospholipids showed variation in virus-binding affinity, with comparable Bmax/2 values for POPG, SOPG, and DOPG and significantly higher Bmax/2 values for DLPG < PLPG < DPPG. The greatest difference in affinity was by a factor of 4 and occurred between SOPG and DPPG. These data demonstrate that there is no absolute structural specificity to the PG recognition but variability over a range of several fold depending upon the fatty acid composition of the phospholipid.

We also conducted experiments examining the direct binding of RSV to HEp-2 and its inhibition by phospholipids. In these studies, we observed concentration-dependent and saturable binding of the RSV to the surface of epithelial cells at 18°C. POPC failed to disrupt the interaction between virus and the cell, whereas POPG at either 200 or 1,000 μg/ml inhibited the binding by greater than 90%.

A growing body of evidence identifies POPG as a potent negative regulator of TLR2 and TLR4 activation and an inhibitor of RSV and influenza A (IAV) infection in the lung (17, 18). The antagonistic actions of the lipid against TLR activation are consistent with an innate immunosuppressive function that acts to prevent engagement of inflammatory cascades upon casual exposure to microbial ligands, such as LPS, that are routinely present in inspired air. This immunosuppressive function of POPG appears to be part of a homeostatic process for preventing chronic inflammation of the lung. In addition to activating the innate immune system in response to LPS, TLR4 and CD14 have been implicated in the innate immune response to RSV (39), although the mechanism by which this occurs is not clear.

Previous studies established that simultaneous treatment of mice with RSV and POPG almost completely attenuates viral infection. These studies raise the possibility that POPG could be useful in both the short-term prevention and treatment for recently acquired RSV infection in humans. In this article, we demonstrate that the addition of POPG at 24 h following in vitro viral infection of cultured cells potently suppresses IL-8 production. In addition, POPG introduced 24 h after RSV infection suppresses the recovery of infectious particles measured in plaque assays by 2–6 log units in a POPG concentration-dependent manner. Histochemical staining for RSV demonstrates that the POPG reduces both the number and size of viral foci. These data demonstrate that, when POPG concentrations are maintained at sufficiently high levels, established infections can be arrested. These in vitro results suggest that if high POPG levels can be sustained within the lung in vivo, the lipid will be an effective therapeutic for established infections.

Our recent work has focused upon POPG because it is the most abundant molecular species of PG present in human pulmonary surfactant. To examine whether the interaction of POPG with RSV is unique, we investigated the interaction of other PG molecular species with the virus by quantifying the antagonism of IL-8 production and the direct binding interactions to solid-phase phospholipids. These studies reveal that both unsaturated PG molecular species (POPG, SOPG, DOPG, DLPG, and DPPG) and saturated PG molecular species (DPPG) antagonize RSV-induced IL-8 production with similar IC$_{50}$ values (3-8 μg/ml). The PLPG molecular species was unusual because its IC$_{50}$ for antagonizing IL-8 production was 25 μg/ml. Examination of direct physical interactions between RSV and solid-phase phospholipids showed variation in virus-binding affinity, with comparable Bmax/2 values for POPG, SOPG, and DOPG and significantly higher Bmax/2 values for DLPG < PLPG < DPPG. The greatest difference in affinity was by a factor of 4 and occurred between SOPG and DPPG. These data demonstrate that there is no absolute structural specificity to the PG recognition but variability over a range of several fold depending upon the fatty acid composition of the phospholipid.

We also conducted experiments examining the direct binding of RSV to HEp-2 and its inhibition by phospholipids. In these studies, we observed concentration-dependent and saturable binding of the RSV to the surface of epithelial cells at 18°C. POPC failed to disrupt the interaction between virus and the cell, whereas POPG at either 200 or 1,000 μg/ml inhibited the binding by greater than 90%.
These data provide clear evidence that POPG, but not POPC, prevents RSV attachment to the cell surface. Experiments were also conducted to determine whether the activity of POPG against RSV was relevant to circulating strains of the virus. Samples from five patients infected with antigenic subgroup A RSV were collected and propagated in HEP-2 cells, and the POPG and POPC antagonism of viral infection was measured using in vitro plaque assays. These studies demonstrate that POPG suppresses the infection by strains recovered from patients. POPG was also highly effective against recombinant strains of RSV engineered to have increased pathogenesis in mice (33). These studies provide good evidence that PG antagonism of RSV infection is likely to be highly relevant to human infections.

We conducted several lines of experimentation to test for long-term prophylaxis and postinfection treatment for RSV with single doses of POPG, but these studies were unsuccessful. We reasoned that short-term prophylaxis studies were necessary to define the time period of efficacy for a single dose of POPG. Using intranasal inoculation with POPG 45 min prior to challenge with RSV, we were able to achieve a significant level of protection from the virus. The POPG pretreatment effectively reduced the number of lymphocytes and neutrophils entering the lungs to basal levels, suppressed the host IFN-γ response to undetectable levels, and reduced the number of infectious particles recovered from the lung by a factor of 10. The effects of POPG were concentration-dependent in the range of 800–3,000 μg/mL and the quantitative histopathology was the most sensitive indicator of the dose dependence of lipophilic action.

The data indicate that the effective time window for POPG action in mice is relatively short (~45 min) and requires high doses of the lipid for maximum effect. This finding is not entirely unanticipated in mice for several reasons. The turnover of pulmonary surfactant constituents is regulated by the respiratory rate. In humans, the respiratory rate is ~18/2/min in adults and ~30/min in newborns. In mice, the resting respiratory rate can be 300/min, and a rapid turnover of surfactant lipids is expected. Interestingly, PG reconstituted in artificial pulmonary surfactant given to human neonates has been estimated to have a half-life of 30 h (20). These data suggest that PG given to human subjects may remain effective against RSV for a period of 12–24 h. When the above factors are considered in the context of the results in Fig. 2, the data indicate that POPG has significant potential for use in humans even after an RSV infection is established.

The short efficacy window of POPG is accompanied by evidence for uptake, metabolism, and recycling of PG by the lung epithelium. At the earliest time point of BALF recovery following the POPG inoculation, there is evidence for low levels of conversion of this lipid to 16:0/16:0-PG, 16:0/16:1-PG, and 16:0/18:2-PG. At longer postinoculation times, these molecular species continue to increase in amount, and the 18:0/20:4-PG and 18:0/22:6-PG molecular species begin to appear. The major known routes of phospholipid remodeling are intracellular and involve deacylation-reacylation reactions (35, 36). Alveolar type 2 cells are the only demonstrated source of surfactant PG (16, 37, 40), and it is most likely that the POPG uptake, remodeling, and secretion occur via these cells.

In summary, our data provide clear evidence that POPG is effective in preventing the infection of epithelial cells by RSV and arresting the progression of established RSV infections in vitro. POPG is also effective in vivo in mice, although the efficacy window for the lipid is brief. These findings suggest that POPG could be beneficial in humans for short-term prophylaxis against RSV and that POPG has significant potential for therapeutic treatment of individuals with established RSV infections.

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