Phosphatidylglycerol Suppresses Influenza A Virus Infection

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ONLINE DATA SUPPLEMENT

Supplemental Materials and Methods

Sources of reagents:

MDCK cells and BEAS2B cells: ATCC, Manassas, Virginia, USA

Phospholipids: Avanti Polar Lipids, Alabaster, AL, USA

Goat polyclonal anti-IAV: Millipore, Billerica, MA, USA

Qiagen RN-easy kit: Qiagen, Germantown, MD, USA

qRT-PCR kit: Invitrogen, Camarillo, CA, USA

BALB/C mice: Jackson Laboratory, Bar Harbor, ME, USA

Dulbecco's Modified Eagle Medium (DMEM) and Bronchial Epithelial Cell Growth Medium (BEGM): Lonza, Walkersville, MD

AlexaFluor 568-rabbit-anti goat antibody: In Vitrogen, Carlsbad, CA, USA

Bovine Growth Serum (BGS): Hyclone, South Logan, UT, USA

Human IL-8 ELISA kit: In Vitrogen, Camarillo, CA, USA

Mouse interferon γ (IFNγ) ELISA kit: BD Biosciences, San Diego, CA, USA

Polyclonal rabbit Anti β-actin:Cell Signaling Technology, Danvers, MA, USA

Paraformaldehyde: Electron Microscopy Science, Hatfield, PA, USA

Tissue Culture Methods:

MDCK cells were cultured in DMEM plus 10% bovine growth serum, penicillin (50U/ml) and Steptomycin (50μg/ml)/. Beas2B cells were cultured in Bronchial Epithelial Cell Growth Medium (BEBM) supplemented with antibiotics.

Phospholipid Preparation

Organic solvents were removed from stock phospholipids by evaporation under a stream of N_2. The dried film was suspended in methanol and dried again under N_2 to remove traces of chloroform. The lipid films were hydrated in phosphate buffered saline (PBS) at 37°C for 1hr at phospholipid concentrations of 10-40 mg/ml. After hydration the lipid preparations were bath sonicated until the solutions clarified. The stock liposome suspensions were diluted to final concentrations in media, or PBS and filter sterilized prior to use.

Lipid antagonism of viral infection and cell binding

In tissue culture experiments phospholipid liposomes at concentrations of 0.2-1.0 mg/ml were either pre-incubated with cells, or pre-incubated with viral preparations as specified in Figures. For in vivo experiments phospholipids were preincubated with IAV for 15 min at 22°C prior to intranasal inoculation.

Two types of experiments were devised to test for a direct virucidal effect of phospholipids upon IAV. In one series of experiments IAV at an moi = 1.0 was incubated with a monolayer of MDCK in DMEM at 8°C for 4hr, to allow cell surface binding without endocytosis. Following this incubation the monolayers were washed three times at 0°C with cold PBS to remove unbound virus. Next the cells with adsorbed virus were incubated with 0.2-1.0 mg/ml phospholipid (POPC, or POPG) and incubated for another 4 hours at 8°C in DMEM. Subsequently the phospholipid was removed by washing three times with cold PBS. The cultures were shifted to 37°C and the viral infection was allowed to proceed for 36 hr. Progress of the viral infection was monitored by

immunoblotting of cell extracts separated by gel electrophoresis using β-actin for standardization. Quantification of NA, MP and β-actin was performed using NIH Image J1-34 software.

In a second type of experiment we examined the reversal of POG antagonism of viral infection. Aliquots of IAV ($1x10^8$ pfu/ml) were incubated in either the absence or presence of 1mg/ml POPG for 1h at 37°C. Subsequently, the viral preparations were diluted 10^3 fold in either the absence or presence of POPG, and used to infect monolayers of MDCK cells either without or with POPG in the culture medium. After 1h of viral adsorption the unbound virus was removed by washing three times with PBS and then culturing in DMEM, either without, or with POPG. The cells were further incubated for 6h and then washed with PBS and fixed in 3.2% paraformaldehyde. The cells were washed five times with PBS and permeabilized with PBS/0.2% triton X-100 for 15 min. The cells were next washed with PBS five times and blocked overnight at 4°C with 10% BGS. After blocking, the cells were washed five times with PBS and then incubated with goat anti-IAV antibody (1:200) for 2 hrs at 37°C. The unbound primary antibody was removed by PBS washing and Alexa Fluor 568 conjugated rabbit anti goat antibody (1:200) was incubated with the samples for 1 hr at room temperature. Finally the cells were washed with PBS and analyzed by quantitative immunofluorescence using a Mariana's microscope and Slide book software. Single cell fluorescent foci were scored for each sample at 10x magnification. The time window for the anti-viral action of POPG was examined by quantifying cell viability 36 hrs after IAV infection (moi=0.5) conducted in the absences or presence of 1 mg/ml POPG. The POPG was added to

MDCK monolayers either 0.5 hr prior to virus or simultaneously with the virus, or 1 hr following virus addition, 2 hr following virus addition. Both adherent and floating cells were harvested from the wells and 0.02% trypan blue dye exclusion was used to score cell viability.

Histopathology Scoring

The histopathology scoring used the methods described by Cimolai et al (27) that apply weighted evaluation of: A, the percentage of perbronchiolar and bronchial infiltrates (scored 0-3); B, the quality of peribronchiolar/bronchial infiltrates (scored 0-3); C, bronchiolar/bronchial luminal exudate (scored 0-2); D, perivascular infiltrate (scored 0-3) and E, parenchymal pneumonia (scored 0-5). The weighting is described by the formula: $A + 3(B + C) + D + E$. The final numeric score by this method ranges from 0-26. The scoring was performed for each group of animals (saline control, + RSV, + RSV + POPG, and + POPG alone) in 3 independent experiments.