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Lung surfactant DPPG phospholipid inhibits vaccinia virus infection

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ABSTRACT

Vaccinia virus (VACV) was used as a surrogate of Variola virus (genus *Orthopoxvirus*), the causative agent of smallpox, to study orthopoxvirus infection *via* the respiratory airway. Lung surfactant, a physiological barrier to infection encountered by the virus, is predominantly composed of phospholipids whose role during orthopoxvirus infection has not been investigated. An attenuated Lister strain, derived from the traditional smallpox vaccine and the Western Reserve (WR) strain, lethal for mice infected by the respiratory route, were examined for their ability to bind various surfactant phospholipids. Dipalmitoyl phosphatidylglycerol (DPPG) was found to interact with both VACV strains. DPPG incorporated in small unilamellar vesicle (SUV-DPPG) inhibited VACV cell infection, unlike other phospholipids tested. Both pre-incubation of virus with SUV-DPPG and pretreatment of the cell with SUV-DPPG inhibited cell infection. This specific DPPG effect was shown to be concentration and time dependent and to prevent the first step of the viral cycle, i.e. virus cell attachment. Cryo-electron microscopy highlighted the interaction between the virus and SUV-DPPG. In the presence of the phospholipid, virus particles displayed a hedgehog-like appearance due to the attachment of lipid vesicles. Mice infected intranasally with VACV-WR pre-incubated with SUV-DPPG survived a lethal infection. These data suggest that DPPG in lung surfactant could reduce the amount of orthopoxvirus particles able to infect pneumocytes at the beginning of a respiratory poxvirus infection. The knowledge acquired during this study of virus–DPPG interactions may be used to develop novel chemotherapeutic strategies for smallpox.

1. Introduction

Vaccinia virus (VACV) is the prototypic member of the *Poxviridae* family and particularly of the *Orthopoxvirus* genus which includes Variola virus. VACV was used to vaccinate against smallpox for over 200 years and finally achieve complete eradication of the disease in the late 1970s through a worldwide vaccination campaign headed by the WHO. The threat of Variola virus as a bioweapon has emerged during the last years principally because of the uncovering of a large ex-USSR smallpox-militarization program and the vulnerability of a large proportion of the world population after the discontinuation of smallpox vaccination (Sherman, 2008). Variola virus, principally transmitted by the respiratory route, could easily be aerosolized and used as a bioweapon causing massive casualties in the population. The lung would be the first targeted organ in the course of infection. In this first step, virus must cross the pulmonary surfactant barrier separating the alveolar epithelium from the respiratory lumen.

Pulmonary surfactant is a complex mixture of lipids (90%) and proteins (10%), participating in reducing surface tension at the air–liquid interface and in protecting lungs against pathogens as an innate immune system (Haagsman et al., 2008; Kingma and Whitsett, 2006; Rooney et al., 1994). Phospholipids such as phosphatidylcholine (PC) or phosphatidylglycerol (PG), including their unsaturated derivatives, are major surfactant components whose physiological concentration ranges between 10 and 265 μM (Heeley et al., 2000; Mander et al., 2002). The composition is common among different animal species (Rooney et al., 1994; Veldhuizen et al., 1998). Surfactant as a natural barrier is present in organs such as the lung and the stomach (Lichtenberger, 1995). The phospholipid-rich mixture in the lung is synthesized in type II pneumocytes (alveolar epithelial cells) (Lieber et al., 1976; Stoner et al., 1975) and secreted by those cells *via* cAMP or Ca^{2+} signaling after being stored in inclusion bodies (Andreeva et al., 2007). The lipid composition differs in surfactant and in the inner compart-

Abbreviations: DPPG, dipalmitoyl-phosphatidylglycerol; DPPC, dipalmitoyl-phosphatidylcholine; PC, phosphatidylcholine; VACV, vaccinia virus; VARV, Variola virus; SUV, small unilamellar vesicle.

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ment of type II pneumocytes (Postle et al., 2006) but a substantial amount of lipids is present in both compartments. The phospholipids used in this study were chosen because of their abundance and physiological importance in the surfactant (Postle et al., 2006; Rooney et al., 1994; Veldhuizen et al., 1998).

To date only a few studies have described the role of surfactant phospholipids in virus entry into cells. Viruses infecting the respiratory epithelium have been shown to interact with surfactant before cell entry. Dipalmitoyl phosphatidylcholine (DPPC) contained in lung surfactant or expressed by lung cells facilitated respiratory adenovirus entry independently of any specific receptors (Balakireva et al., 2003). The role of pulmonary surfactant in poxvirus entry into alveolar epithelium has not been studied. Determining the contribution of virus–surfactant phospholipid interactions before cell entry is essential for gaining a better insight into the factors affecting poxvirus infectivity.

In this study, we decided to focus on PC and DPPC, both major components of lung surfactant phospholipids and also on some minor phospholipids such PG, dipalmitoyl phosphatidylglycerol (DPPG) and phosphatidylethanolamine (PE) as potential factors which might have an impact on orthopoxvirus infectivity. Two VACV strains were used: the attenuated Lister strain (VACV-List) from the traditional smallpox vaccine and the Western Reserve (VACV-WR) strain which is lethal in mice (Fenner, 1958). For studying virus infectivity *via* the respiratory tract, the most robust viral particles resistant to environmental and physical stress called intracellular mature virus (IMV), were used. IMV is thought to be responsible for the initiation of infection whereas the second distinct form of VACV called the extracellular enveloped virus (EEV) which is surrounded by an additional lipid envelope is thought to be the cause of spread within the host (Smith et al., 2002). *In vitro* overlay assays and electron spin resonance (ESR) data showed that DPPG, but not the other phospholipids, interacted with both VACV strains. Pre-treatment of VACV with small unilamellar vesicles (SUVs), enriched in DPPG (SUV-DPPG), induced inhibition of viral infection in cell culture. Cryo-electron microscopy showed viral particles surrounded by SUV-DPPG and therefore confirmed these interactions. This inhibition of infection in human epithelial lung cells was also observed in mouse epithelial lung cells, prompting us to assess the effect of SUV-DPPG in mice. Animals infected with the WR strain were completely protected from death with SUV-DPPG. Altogether these results lead us to suggest that surfactant phospholipid factors such as DPPG participate in the natural protection from poxvirus infection.

2. Materials and methods

2.1. Cells and viruses

Both A549 cells (human lung carcinoma cell line, ATCC CCL-185) and LA-4 cells (mouse lung adenoma cell line, ATCC CCL-196) were grown in F12K medium (Gibco). BHK-21 cells (hamster kidney cells, ATCC CCL 10) were grown in GMEM (Gibco) supplemented with 10% tryptose phosphate (Sigma) and 50 mM HEPES (Gibco). Vero cells (African green monkey kidney cells, ATCC CCL 81) were grown in M199 medium (Gibco). For cell culture, all media were supplemented with 10% inactivated fetal calf serum (FCS), whereas medium for titration was supplemented with 5% FCS and 1% antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin, Gibco). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. Cytotoxicity was determined by trypan blue staining and cell counting. The vaccinia virus Western Reserve strain (VACV-WR), obtained from the ATCC (ATCC VR-119), and the first-generation Lister smallpox vaccine (VACV-List, X55-33), provided by the French health authorities, were produced in BHK-21 cells, IMV were purified using sucrose density gradient centrifugation as previously described (Mackett et al., 1985; Lee et al., 2001). Virus was stored in 10 mM Tris–HCl (pH 8). Virus productions were titrated in Vero and A549 cells. Before infection, cells were rinsed with medium and infected with virus in culture medium supplemented with 0.5% FCS and 1% antibiotics. Viruses were inactivated for some experiments using a previously described protocol (Tsung et al., 1996). Briefly, 100 μL were incubated with 1 μL Psoralen (Sigma, 1 mg/mL in DMSO) and exposed for 1 h to UV light (365 nm) in a 48 well tissue culture plate.

2.2. Reagents

Phosphatidylcholine (PC, ref-P3556) from egg yolk, dipalmitoyl-phosphatidylcholine (DPPC, ref-P0763), phosphatidylethanolamine (PE, ref-P1223), phosphatidylserine (PS, ref-P5660) phosphatidylglycerol (PG, ref-P8318) and dipalmitoyl-phosphatidylglycerol (DPPG, ref-P5650) were purchased from Sigma Chemical Co. (France). Rabbit anti-vaccinia whole virus antibody was purchased from Fitzgerald-Interchim (France) (20VR-69) and peroxydase-conjugated goat anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch-Interchim (France).

2.3. Lipid–virus interaction

Freeze-dried lipids were reconstituted in a 1:1 (v/v) mixture of chloroform/methanol at 1 mM concentration as previously described (Favier et al., 2004). Replicates of 1 mM stock solutions (PC, DPPC, PE, PG, DPPG and PS) were spotted onto HybondC-extra nitrocellulose membranes (Amersham) and allowed to dry in the dark at room temperature (RT) for 1 h. Membranes were blocked for 1 h at RT in either buffer A (50 mM MES–NaOH pH 5.5, 150 mM NaCl, and 0.1% Tween 20) or buffer B (10 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 3% fatty acid-free BSA (Sigma). The lipid–protein interaction was performed either in presence or in absence of 10 mM $MgCl_2$/5 mM $CaCl_2$. The membranes were then incubated overnight at 4 °C in the same buffers containing 2 μg/mL intracellular mature virus (OD 480 determination). The membranes were washed five times for 10 min in the respective buffer and then incubated for 1 h at room temperature with the polyclonal rabbit anti-VACV serum (1/1000 dilution) containing 3% BSA. After four 20-min washes the membranes were incubated for 1 h with anti-rabbit horseradish peroxidase conjugate (1/10,000 dilution). Finally, after six 10 min washes, the interactions were detected by Enhanced ChemiLuminescence (ECL).

2.4. Preparation of small unilamellar vesicles (SUVs)

Freeze-dried phospholipids were dissolved in chloroform at the desired molar concentration. Unsaturated phospholipids (DPPC or DPPG) were added to a 2 mM PC solution in a 30% final ratio. The mixture was dried overnight under vacuum. The lipid film was hydrated with water and subjected to water bath sonication for 2 h at different temperatures depending on the fusion temperature of the lipids present in the mixture.

2.5. Virus–SUV interactions via electron spin resonance (ESR)

SUV/virus interactions were assessed by electron spin resonance (ESR) spin labelling experiments. Inactivated virus (1.5×10^8 PFU) was mixed with the 5-nitroxide stearate (5NS) probe (Sigma, France) for 5 min at room temperature. The probe was composed of a fatty acid (C16) and a stabilized-free radical. The free radical provided information of label motional freedom in the system. The free radical scavenger vitamin C reduces the spin label from a detectable

free radical to an undetectable one. The decrease of the ESR signal is related to the accessibility of the probe to vitamin C. If accessibility to the free radical increases, its reduction increases as well and this entails a decrease in the ESR signal. Each SUV solution (50 μL at 2 mM) was added to the virus–probe mix and incubated for 90 min at 37 °C. The beginning of the kinetics was triggered by vitamin C addition (L-ascorbic acid, 15 μL, 0.2 M, Sigma) and recorded 5 min later on an ESP 380 Brucker apparatus. Spectra were acquired in a time sweep mode using static field, determined at the middle lines maximum amplitude of the T0 spectrum, conducted in EPR continuous wave mode (3428 G). The instrument parameters were: microwave power 10 mW, modulation frequency at 100 kHz with a modulation amplitude of 2.53 G, receiver gain was 6.3×10^4. Each sample was scanned at controlled temperature 37 °C, with the following acquisition parameters: Time constant 163.84 ms, conversion time 163.84 ms. The recording time was 671 s. All experiments were performed in triplicate.

2.6. SUV–virus infection inhibition assay

A549 cells grown in 24 multi-well plates were seeded to obtain confluent plates after 48 h culture. VACV was incubated with 0.01–0.04 mM SUV for 1 h at 37 °C in 100 μL F12K medium 0.5% FCS and 1% antibiotics. During this time, A549 cells were rinsed with fresh F12K medium 0.5% FCS and 1% antibiotics. Then, virus–SUV mixes were added to the cell monolayers at a multiplicity of infection (MOI) of 0.0005 and the plates were incubated at 37 °C in 5% CO_2 atmosphere for 1 h. Cells were rinsed with 0.5% FCS medium, and filled with 1.5 mL fresh F12K medium 5% FCS and cultured for an additional 48 h. To count plaque forming units (PFU), cells were fixed by adding 0.75 mL of fixing and staining solution (0.2% crystal violet, 4.5% formaldehyde and 7.5% ethanol in PBS) per well for 2 h.

2.7. Infection inhibition assay after lipid pre-treatment

A549 cells grown in 24 multi-well plates were seeded to obtain confluent plates after 48 h culture. Cells were rinsed with fresh F12K medium 0.5% FCS and SUV solutions at different concentrations (0.01–0.04 mM) were added directly to the cells for 1 h at 37 °C in 5% CO_2 atmosphere (12, 25). Cells were rinsed with fresh F12K medium 0.5% FCS before VACV (MOI 0.0005) was added to the cell monolayers and the plates were incubated at 37 °C in 5% CO_2 atmosphere for 1 h. Wells were rinsed with 0.5% FCS medium and filled with 1.5 mL fresh F12K medium 5% FCS, and incubated for an additional 48 h.

2.8. Cryo-electron microscopy

VACV-WR was incubated with a 0.4 mM SUV for 1 h at 37 °C. Samples were prepared for cryo-EM essentially as previously described (Adrian et al., 1984). For vitrification, 4–5 μl of each sample was applied to the metal-coated face of a Quantifoil grid previously submitted to a glow discharge (1 min at 20 mA). The grid was mounted on a plunger, blotted, and vitrified in liquid ethane. The samples were then transferred in an FEI Polara electron microscope equipped with a CCD cold stage 4 × 4K camera and observed at liquid nitrogen temperature at 300 kV. Pictures were taken under low-dose conditions after the detection of interesting areas by using the low-dose mode.

2.9. Virus cell-binding inhibition assay

VACV was incubated with 0.4 mM SUV for one hour at 37 °C in a final 100 μL volume. During this time cells were rinsed with cold F12K medium 0.5% FCS. Virus–SUV solutions were then added to cell monolayers (MOI 0.005) at 4 °C for 2 h to allow virus binding. Wells were rinsed three times with 1 mL cold 0.5% FCS medium and 400 μL fresh M199 medium 0.5% FCS was added. Cells cultures were frozen-thawed three times in the M199 medium. The supernatant was then titrated in confluent Vero cells. Cells were cultured for 48 h.

2.10. Virus infection during cell seeding

Due to the poor-adhering phenotype of LA-4 cells, the infection inhibition assay was adapted from a previously described assay (Jesus et al., 2009), performed during cell seeding. Viruses were incubated with SUV solutions for 1 h at 37 °C. LA-4 and A549 cell suspensions (2.5×10^5 cells) were infected at a MOI of 1.25×10^{-3} and 1.25×10^{-4}, respectively. Then the mixture of cells, virus and SUV were directly seeded in 1 mL of 0.5% FCS F12K to obtain a confluent monolayer in 24 multi-well plates after 48 h of culture. After 16 h of culture, 0.5 mL of fresh 15% FCS F12K medium was added to each well. Cells were cultured for an additional 32 h.

2.11. Animals

Female BALB/c ByJ mice (four weeks old) were purchased from Charles River Laboratories (Arbresle, France). General anesthesia was performed by intraperitoneal injection with a mix of ketamin (100 mg/kg) and atropin (1.5 mg/kg). Animals were culled when necessary by intraperitoneal injection of pentobarbital (330 mg/kg). Each group composed of 6 animals was housed in filtered boxes and maintained under specific pathogen-free conditions. Experiments were performed in accordance with national guidelines governing use of laboratory animals and were approved by the local animal care and use committee (ethical protocol number 2009/11.0 and 2009/22.0).

2.12. SUV intrapulmonary assay

VACV-WR was pre-incubated with SUV solutions (final concentration 0.5 mM or 1 mM) for 1 h at 37 °C in a final volume of 50 μL, and then each mouse was infected intranasally with 50 μL of the virus–SUV mix containing 5×10^6 PFU (determined in Vero cell monolayers) corresponding to 100 LD_{50} (Hayasaka et al., 2007; Lee et al., 1992). For both SUV pre-treatment and post-treatment, a final volume of 25 μL control SUV solution (1 mM) was intranasally administered 4 h before or after challenge, respectively.

Groups of mice were monitored for body weight and mortality daily for the first 9 days post-infection (p.i.), then every two days until 40 days p.i.

2.13. Statistical analysis

For *in vitro* studies, the two-tailed unpaired Student's *t*-test was performed. Animal survival in protection studies was analyzed with the Log-rank test comparing two groups at a time while morbidity (loss of body weight) was analyzed with one way ANOVA test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Binding of vaccinia virus to phospholipids

The interaction of vaccinia virus with phospholipids was studied using a protein-lipid overlay assay. Incubation was carried out in neutral (pH 7.5) or acidic (pH 5.5) conditions as VACV enters cells by two distinct pH dependent pathways (Townsley et al., 2006) and in the absence or presence of Mg^{2+}/Ca^{2+} ions, according to the conditions used for the investigation of lipid interactions with pleckstrin homology domains (Dowler et al., 1999; Thomas et al.,

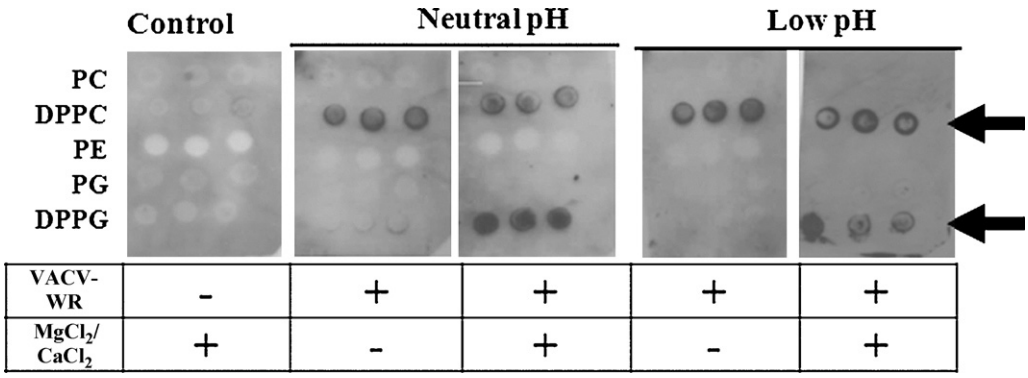

Fig. 1. Phospholipid binding properties of VACV. Replicates of PC, DPPC, PG, DPPG and PE were spotted on membranes (1 nM/spot) and interactions with VACV-WR were performed in neutral (pH 7.5) or acidic (pH 5.5) buffer in presence or absence of $CaCl_2/MgCl_2$ (5 and 10 mM, respectively). The presence of VACV-WR was detected with a specific α-VACV antibody after extensive washing.

2002; Thomas et al., 2001). Virus bound to the membranes was detected using an anti-VACV antibody and ECL. The study focused on PC, DPPC, PG, DPPG and PE, the most important components of lung surfactant. Nonspecific antibody binding was not observed after incubation of the membranes with anti-VACV. Both DPPG and DPPC interacted similarly with VACV-WR (Fig. 1) and VACV-List (data not shown). For both VACV strains, DPPG and DPPC interactions occurred at both neutral and low pH in the presence of Mg^{2+}/Ca^{2+} ions while only the DPPC interaction was observed in the absence of Mg^{2+}/Ca^{2+} ions at either pH.

3.2. DPPG–VACV interaction confirmed by electronic spin resonance

To perform *in vitro* studies, phospholipids were organized in small unilamellar vesicles (SUVs) allowing their use in aqueous solution. To confirm the results from the overlay binding assay, SUV composed of PC alone, enriched with DPPC or DPPG (called SUV-PC, SUV-DPPC and SUV-DPPG, respectively) were prepared and incubated with VACV WR or VACV-List. PC was used as a backbone lipid because no interaction with it was observed *in vitro* (see Fig. 1). The ESR results showed 2 different kinds of kinetics in the drop of the ESR signal. After interaction between VACV and SUV-PC or SUV-DPPC, the ESR signal decreased quickly, while the kinetic with SUV-DPPG was significantly slower (Fig. 2). With SUV-DPPG, the probes were less accessible to vitamin C, meaning closer and stronger interactions between SUV-DPPG and virus than with the other SUVs. The virus–DPPC interactions (see Fig. 1) were not confirmed *via* ESR, hence DPPG was considered as the only phospholipid interacting strongly with VACV.

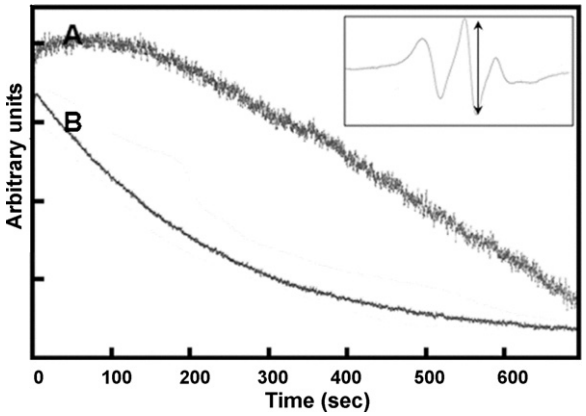

Fig. 2. ESR scheme of DPPG–VACV interaction. Peak to peak evolution measured on the central line of spectra after ascorbate addition for DPPG–VACV-WR system (A) and both other phospholipids (PC, DPPC)–VACV-WR system (B). Scale from curve (A) was magnified to compare both curves. Insert show the typical spectrum recorded on ESR system. Peak to peak height is labelled with arrows.

3.3. DPPG phospholipid blocks vaccinia virus infection in human epithelial cells

In order to examine whether the *in vitro* interactions between DPPG and VACV had physiological consequences, we studied the interactions between virus and phospholipid vesicles in a cell infection assay. VACV was pre-incubated with 0.2 mM SUV-PC, SUV-DPPC or SUV-DPPG. A549 cells were infected with VACV–SUV mixes containing a 20 μM final SUV concentration. The infectivity was estimated by the number of PFUs two days post-infection (p.i.) (Fig. 3A). No inhibition of the plaque count was observed with SUV-PC or with SUV-DPPC ($p > 0.05$, two-tailed unpaired Student's *t*-test). SUV-DPPG induced a significant inhibition of VACV infection: 40% reduction of the number of PFU was observed for VACV-WR and 45% for VACV-List (data not shown), compared with non pre-incubated virus ($p = 0.0017$ and $p < 0.0001$, respectively, two-tailed unpaired Student's *t*-test). SUV-DPPC was unable to inhibit virus infectivity in agreement with the lack of interaction in the ESR study, while SUV-DPPG interaction with VACV was confirmed through its ability to inhibit virus infection.

3.4. Dose dependent SUV-DPPG inhibiting activity

To characterize SUV-DPPG–virus interactions, increasing concentrations of SUV-DPPG (0.01 mM to 0.4 mM) were pre-incubated with the same amount of VACV and infection of A549 cells was performed under the previous conditions (Fig. 3B). For both virus strains, no inhibition of infection was observed with both SUV-PC and SUV-DPPC, even at the highest dose ($p > 0.05$, two-tailed unpaired Student's *t*-test). SUV-DPPG induced a statistically significant dose-dependent inhibition of VACV-WR (up to 80%), from 0.1 to 0.4 mM ($p = 0.003$, $p < 0.0001$ and $p < 0.0001$, respectively; two-tailed unpaired Student's *t*-test). Similar results were obtained with VACV-List (data not shown, up to 87%), with a SUV-DPPG dose ranging from 0.05 to 0.4 mM ($p = 0.0065$ for 0.05 mM and $p < 0.0001$ for 0.1 mM to 0.4 mM, two-tailed unpaired Student's *t*-test). Therefore, the activity of SUV-DPPG on VACV was confirmed and characterized as a dose dependent inhibition.

3.5. Time course for SUV inhibition activity

In the experiments reported so far inhibition of virus infection resulted from a binding interaction carried out for 1 h between SUV and VACV. The kinetics of binding was performed to esti-

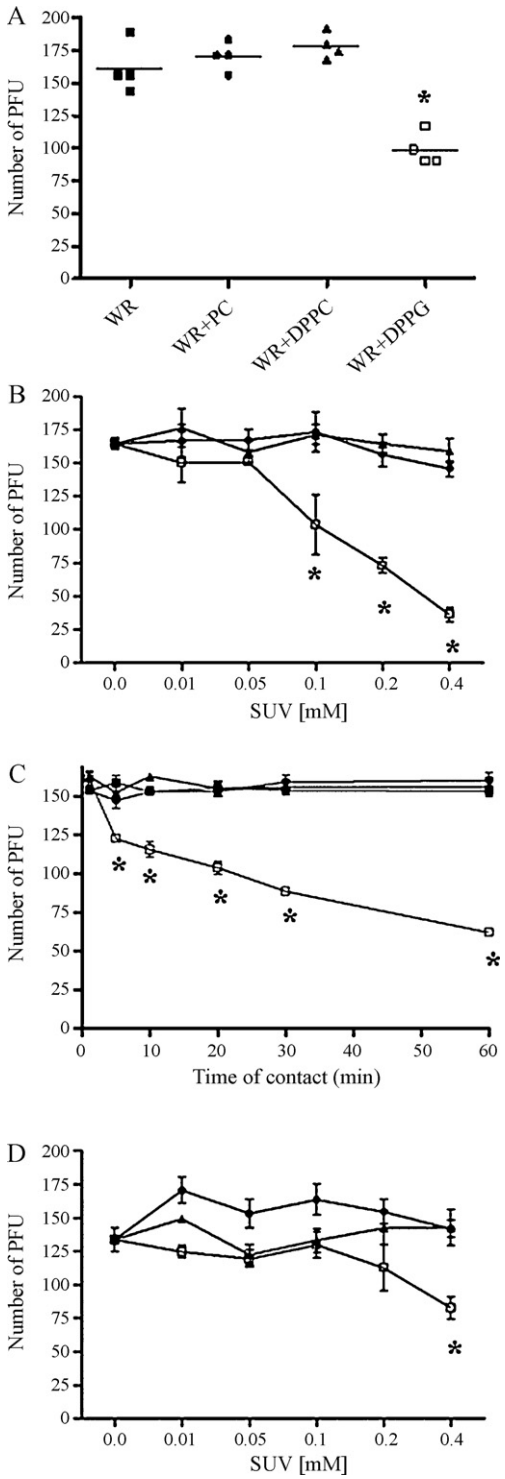

Fig. 3. (A) Replication of VACV after virus–SUV interaction. VACV-WR was incubated with SUV (0.2 mM final) for 1 h. A549 cells were infected at a MOI of 0.0005 and virus replication was measured by a PFU assay. PFU were revealed using crystal violet. SUV pre-treated groups were compared to the VACV-WR group (WR) using the two-tailed unpaired Student's *t*-test. An asterisk indicates a statistically significant difference ($p < 0.05$). Symbols: ■, WR; ●, WR + PC; ▲, WR + DPPC; □, WR + DPPG. (B) Dose dependence of SUV viral inhibition. Increasing SUV doses were pre-incubated with VACV-WR for 1 h. A549 cells were infected at a MOI of 0.0005 and the inhibition of virus infection was measured by a PFU assay. SUV pre-treated groups were compared to the VACV-WR group using the two-tailed unpaired Student's *t*-test. An asterisk indicates a statistically significant difference ($p < 0.05$). Symbols: ●, WR + PC; ▲, WR + DPPC; □, WR + DPPG. (C) Time-course for SUV inhibition. SUV (0.4 mM final) was pre-incubated with VACV-WR from 1 min to 1 h. A549 cells were infected at a MOI of 0.0005 and the inhibition of virus infection was measured by a PFU assay. SUV pre-treated groups were compared to VACV-WR group using the two-tailed unpaired Student's *t*-test. An asterisk indicates a statistically significant difference ($p < 0.05$). Symbols: ■, WR; ●, WR + PC; ▲, WR + DPPC; □, WR + DPPG. (D) Effect of phospholipid pre-treatment of A549 cells on VACV infection. A549 cells were pre-treated with increasing concentrations of phospholipids then rinsed before being infected at a MOI of 0.0005. The inhibition of VACV-WR infection was measured by a PFU assay. SUV-enriched cells were compared to the untreated cell group using the two-tailed unpaired Student's *t*-test. An asterisk indicates a statistically significant difference ($p < 0.05$). Symbols: ●, WR + PC; ▲, WR + DPPC; □, WR + DPPG.

mate the minimum time of contact between SUV and VACV to allow efficient inhibition of virus infection (Fig. 3C). SUVs were pre-incubated for increasing periods from 1 min to 1 h and infection was carried out. No virus inhibition was observed with both SUV-PC and SUV-DPPC at any time point ($p > 0.05$, two-tailed unpaired Student's *t*-test, compared to the VACV-WR control). SUV-DPPG induced a time-dependent inhibition of VACV-WR, statistically significant as soon as 5 min of incubation ($p = 0.0043$, from 5 to 60 min, two-tailed unpaired Student's *t*-test). These data indicate that the inhibitory activity of SUV-DPPG on VACV is dependent on the time of interaction between the two partners and needs at least 5 min contact.

3.6. Treatment of the A549 cells with SUV-DPPG results in a decrease of VACV infection

As the surfactant phospholipids under study are also present in the plasma cell membrane, we decided to treat A549 cells with increasing concentrations of SUV solutions (0.01–0.4 mM) without any pre-incubation of the virus with SUV. Under these conditions phospholipid uptake by cells is relatively efficient and a large part of the incorporated lipids are rapidly directed to intracellular compartments (Huang and Pagano, 1975; Pagano and Huang, 1975; Struck and Pagano, 1980; Wirtz, 1974). Passive transfer and accumulation of phospholipids in cell membranes was performed for 1 h, then cells were washed with medium and infected with virus and the number of PFU was estimated 48 h p.i. Under these conditions, the SUV suspensions did not induce any cytotoxicity up to 0.8 mM (data not shown). No inhibition was observed with both SUV-PC and SUV-DPPC ($p > 0.05$, two-tailed unpaired Student's *t*-test) (Fig. 3D) whereas SUV-DPPG inhibited virus infection at 0.4 mM for VACV-WR ($p = 0.0018$, two-tailed unpaired Student's *t*-test) and both 0.4 mM and 0.2 mM for VACV-List ($p = 0.0004$ and $p = 0.0086$, respectively, data not shown) when compared to the untreated control cells. The inhibition of VACV infection after cell SUV-DPPG treatment was weaker than that observed in the direct interaction assay.

3.7. Cryo-electron microscopy allows visualization of SUV binding to VACV

Initial electron microscopy observations of purified VACV incubated with SUV and prepared by conventional negative staining and air drying suggested that numerous SUVs were attached to the virus particles (data not shown). To further study these interactions; samples were examined by cryo-electron microscopy because this technique allows the visualization of intact phospholipid vesicles together with virus in a defined aqueous ionic environment and without any heavy metal staining. Virus particles in amorphous ice without SUV displayed their characteristic rectangular shape in a two dimensional projection with a smooth well defined surface (Fig. 4A) as originally described by Dubochet et al. (1994). A 30 nm thick surface domain could be readily distinguished from the more electron dense core region surrounded by a faintly visible palisade layer. In contrast, when virus was incubated with SUV-DPPG it was more difficult to distinguish the surface layer from the

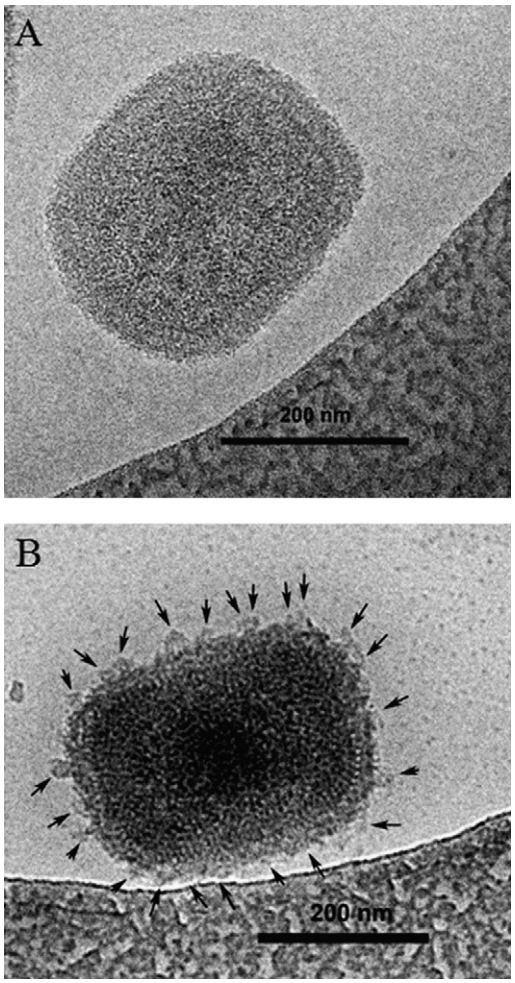

Fig. 4. Cryo-electron microscopy of vaccinia virus particles in the presence of SUV-DPPG. VACV-WR was incubated with 0.4 mM SUV-DPPG for 1 h at 37 °C. Cryo-electron microscopy was performed on untreated VACV-WR (A) and VACV-WR incubated with SUV-DPPG (B). Arrows point to SUV particles.

core region most likely on account of the SUV bound at the virus surface. Indeed, numerous small vesicles were clearly identified on the periphery of the particles which had a spiky, bubbly appearance (Fig. 4B).

3.8. SUV-DPPG diminishes virus infection by inhibiting cell attachment

Entry of VACV involves at least three steps: virus attachment, fusion protein activation, and membrane fusion (Moss, 2006). In order to determine the mechanism of DPPG action on VACV infection, we first focused on VACV binding to the cell membrane. VACV was pre-incubated with SUV for one hour at 37 °C then infection was performed at 4 °C for 2 h to allow virus attachment without entry, then cells were rinsed to remove unattached virus. The remaining virus attached onto the A549 monolayer was titrated in Vero cells (Fig. 5A). In these conditions, SUV-DPPG drastically reduced virus attachment to A549 cells compared to an untreated VACV-WR control (99%, $p < 0.0001$ two-tailed unpaired Student's *t*-test) while no effect was observed with both SUV-PC and SUV-DPPC compared to untreated VACV-WR control ($p > 0.05$ two-tailed unpaired Student's *t*-test). A parallel experiment was performed to assess the inhibition of virus infection by incubating virus with SUV-DPPG and then immediately carrying out titration at 37 °C (Fig. 5B). The capacity of SUV-DPPG to inhibit virus infection (50.9%, $p < 0.0001$, two-tailed unpaired Student's *t*-test, compared to VACV-WR control) was confirmed whereas, as expected, no inhibition of virus infection occurred with SUV-PC and SUV-DPPC ($p < 0.5$, two-tailed unpaired Student's *t*-test). Based on these data, SUV-DPPG appears to selectively block the adsorption of VACV to A549 cells at 4 °C more drastically than the direct inhibitory effect it displays on VACV after pre-incubation and subsequent infection of cells at 37 °C.

3.9. Comparison of mouse and human cell models

The ability of DPPG to inhibit virus infection of human epithelial lung cells has been established. We therefore wondered whether this inhibition could also be observed on mouse lung cells before evaluating its activity in an animal model. The inhibitory activity of SUV on VACV-WR was assessed in both A549 human and LA-4 mouse cells (Lieber et al., 1976; Stoner et al., 1975) (Fig. 6). Instead of infection of cell monolayers, infection was performed during cell seeding, as LA-4 cells adhered poorly in cell culture plates. Then, infectivity was estimated by the number of PFU two days post-infection. The experiment was not performed with VACV-List as this strain was ineffective in replicating in mouse lung (Abdalrhman et al., 2006).

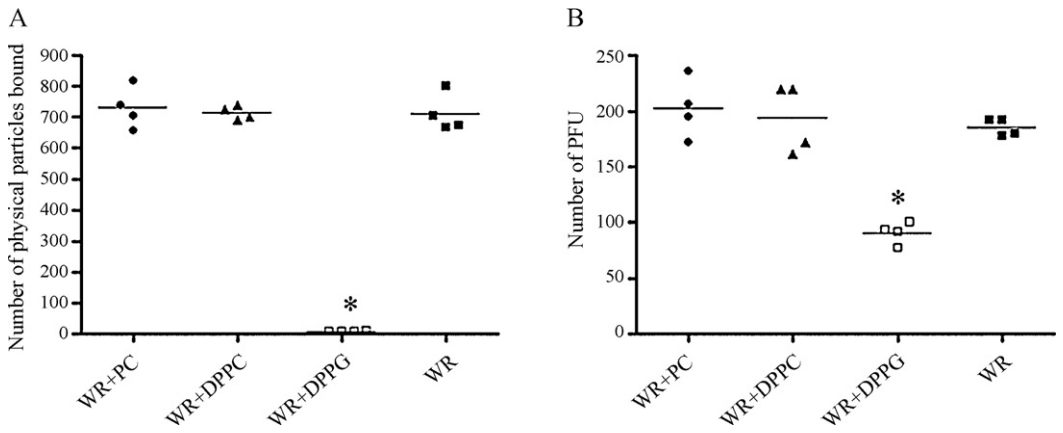

Fig. 5. Virus cell-attachment. SUV were pre-incubated with VACV-WR at 0.4 mM for 1 h. (A) For virus attachment, A549 cells were infected at a MOI of 0.005 at 4 °C for 2 h and freeze–thawed immediately. Inhibition of virus binding was measured by titrating virus associated with cell lysates on Vero cells. Titers obtained corresponded to the amount of attached virus harvested from each A549 well. (B) For virus infection, SUV were pre-incubated with VACV-WR for 1 h. A549 cells were infected at a MOI of 0.0005 at 37 °C for 1 h. Plates were then maintained at 37 °C for 2 days. In both experiments plaques were revealed using crystal violet. An asterisk indicates a statistically significant difference compared to the VACV-WR control ($p < 0.05$; two-tailed unpaired Student's *t*-test). Both experiments were performed simultaneously. Symbols: ■, WR; ●, WR + PC; ▲, WR + DPPC; □, WR + DPPG.

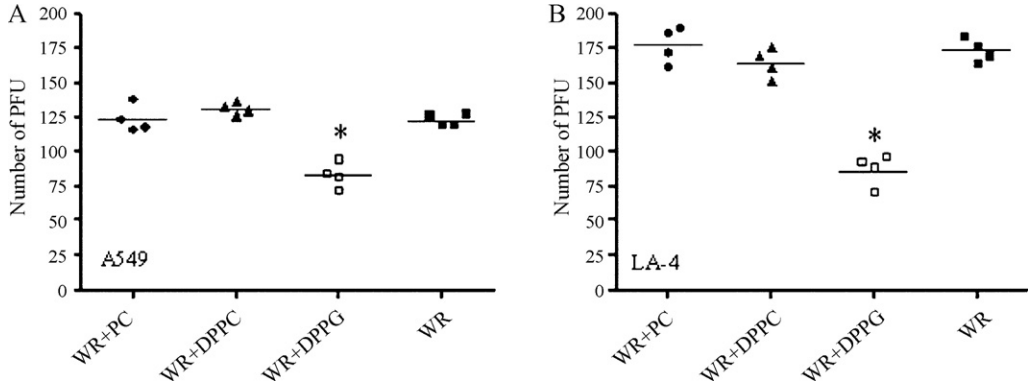

Fig. 6. Comparison of SUV inhibition in A549 and LA-4 cells. SUV (0.2 mM) were pre-incubated with VACV-WR for 1 h. For both A549 (A) and LA-4 (B) cell lines, infection occurred during cell seeding at a MOI of 1.25×10^{-4} and 1.25×10^{-3}, respectively. Inhibition of virus infection was measured by a PFU assay. SUV pre-treated groups were compared to the VACV-WR group using the two-tailed unpaired Student's *t*-test. An asterisk indicates a statistically significant difference ($p < 0.05$). Symbols: ■, WR; ●, WR + PC; ▲, WR + DPPC; □, WR + DPPG.

In both A549 and LA-4 cell lines, no inhibition of VACV infection was observed with SUV-PC or with SUV-DPPC compared to a VACV-WR control ($p > 0.05$, two-tailed unpaired Student's *t*-test). As previously observed in monolayer A549 cells, SUV-DPPG induced a significant inhibition of infection during cell seeding compared to a VACV-WR control ($p = 0.0002$, two-tailed unpaired Student's *t*-test) (Fig. 6A). Virus inhibition was also observed with SUV-DPPG in LA-4 mouse cells compared to VACV-WR control ($p < 0.0001$, two-tailed unpaired Student's *t*-test) (Fig. 6B), inducing an inhibition of infection of about 50%.

3.10. SUV treatment protects mice from lethal VACV challenge

The WR strain of VACV provides a model for evaluating the pathogenicity of SUV-DPPG in mice. The VACV-WR strain was chosen because of its ability to induce death in contrast to VACV-List. The effect of the different phospholipid-enriched SUVs was observed on morbidity and mortality (Fig. 7). Intranasal administration of all SUV solutions tested in uninfected mice did not induce morbidity or mortality of mice compared to the control group (untreated, uninfected mice) (data not shown). At 4 days p.i., all infected mice underwent a drastic weight loss (14–24%) compared to the control group ($p < 0.01$, one way ANOVA test). The treatment of VACV-WR with the different phospholipid-enriched SUVs and particularly with SUV-DPPG at 0.5 mM did not entail any change in morbidity as measured by body weight loss in the course of infection (Fig. 7A). All mice belonging to VACV-WR, VACV-WR + PC and VACV-WR + DPPC groups died with a mean survival of 6, 7.5 and 8 days, respectively (except one mouse in the VACV-WR + PC group which survived) (Fig. 7B). Thus no significant effect was observed with these phospholipids ($p < 0.0003$, compared to control group, Log rank test). In contrast, when used at 0.5 mM, all mice of the VACV-WR + DPPG group survived ($p = 1$, compared to control group, Log rank test). Using a 1 mM SUV dose, survival

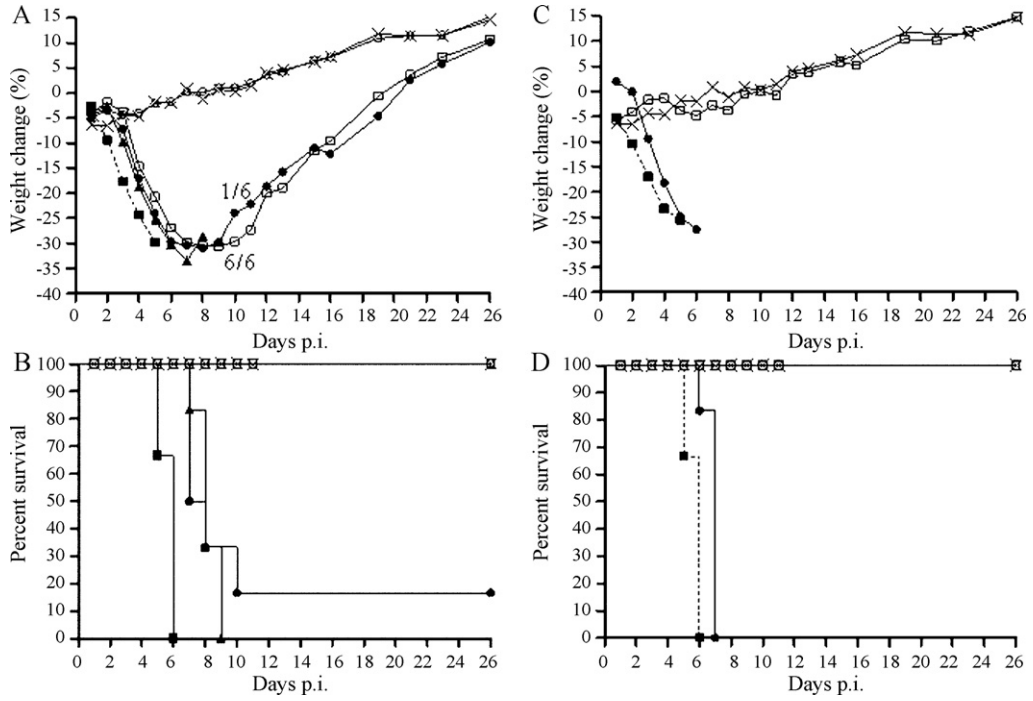

Fig. 7. Survival and weight loss of mice infected with VACV-WR pre-incubated with SUV. Four week old Balb/c Byj mice were infected intranasally with 100 LD_{50} of VACV-WR. VACV-WR was pre-incubated with 0.5 mM (A and B) or 1 mM (C and D) of SUV. Survival and weight loss were monitored for 26 days. Each group was compared to a VACV-WR and a mock infected group (ANOVA and Log rank test). Symbols: ×, Control; ○, SUV; ■, WR; ●, WR + PC; ▲, WR + DPPC; □, WR + DPPG.

was also observed in VACV-WR + DPPG group contrary to VACV-WR and VACV-WR + PC (Fig. 7D) ($p = 1$, $p < 0.0001$ and $p < 0.0001$, respectively, compared to control group, Log rank test). Using the 1 mM dose, the SUV-DPPG pre-treatment of virus protected mice from morbidity as no weight loss was observed compared to the control group (Fig. 7C) ($p > 0.05$, one way ANOVA test). On the other hand, no protection was observed when SUV-DPPG was administered to mice three hours pre-infection or post-infection (supplementary data 1). Taken together the results demonstrated that the interaction of DPPG with VACV prevented mice from being lethally infected after a VACV challenge but failed to be effective upon pre-treatment of mice or after infection had begun.

4. Discussion and conclusion

The respiratory tract is the usual entry point for Variola virus (VARV) into human beings during a smallpox epidemic. Virus entry in lungs results in an acute infection followed by dissemination of the virus to different organs (Hayasaka et al., 2007; Ramirez et al., 2003), death occurring in 30% of cases. VACV, like VARV, also replicates in lungs, as described for the Western Reserve strain (WR) which causes death in mice (Hayasaka et al., 2007; Ramirez et al., 2003). The lung surfactant is a natural barrier which must be crossed by the virus to infect lung epithelial cells. This surfactant is composed mainly of phospholipids, but few studies have investigated the involvement of these phospholipids in virus entry (Balakireva et al., 2003), and no reports have addressed the role of the lung surfactant barrier in poxvirus infection.

In this study, we have shown that VACV interacts with DPPG, one of the phospholipids of lung surfactant. The evidence for this interaction came from several experimental approaches. First, both VACV strains (WR and Lister) bound strongly to spot dotted phospholipids (DPPC and DPPG) in a pH independent manner (low and neutral pH). Phospholipids were then organized into small unilamellar vesicles to enable studies in a soluble aqueous environment. Using a biophysical method (ESR), only DPPG interactions were confirmed as only this lipid probe was protected against signal quenching. Most significantly, the data illustrated that virus–DPPG interactions resulted in the inhibition of VACV infection in cell culture (40–85%). Pre-incubation of VACV-WR with DPPG-enriched SUV inhibited virus infection in both human and mouse epithelial type II cell models (A549 and LA-4 cells, respectively). DPPG activity was also observed in an assay (Huang and Pagano, 1975; Pagano and Huang, 1975; Struck and Pagano, 1980; Wirtz, 1974) in which DPPG pre-treatment of A549 cells was performed. Moreover, we showed that inhibition of VACV infection was time and concentration dependent. Finally, inhibition of virus attachment to cells appeared to be the mechanism of action involved. Surprisingly, a more marked inhibition of virus adsorption was observed with SUV-DPPG at 4 °C than after direct incubation of virus with SUV-DPPG at 37 °C followed by titration (compare Fig. 5A and B). This may be explained by the occurrence of multiple early virus-cell interactions at 37 °C that do not occur at 4 °C. For instance passive attachment of virus that does not imply strong binding to cell receptors and subsequent penetration by endocytosis that requires 37 °C may be steps that are insensitive to DPPG. In fact, it has previously been reported that only 1.6–2% of input IMV binds to cells at 4 °C after 1 h adsorption and that binding increases with increasing temperatures (Locker et al., 2000). Cryo-electron microscopy allowed direct visualization of DPPG-enriched SUV bound to viral particles suggesting that occupancy of viral ligands or indirect steric hindrance of such ligands could interfere with virus binding to cell surface receptors.

Although this study has focused on DPPG, other components of lung surfactant particularly proteins belonging to the collectin family, may also play a role in protection of the lung from infection. In fact preliminary data indicate that at least one collectin binds VACV and inhibits viral infection in a similar manner to DPPG (manuscript in preparation).

To date, one phospholipid, namely phosphatidylserine (PS), has been shown to be important for vaccinia virus entry (Laliberte and Moss, 2009; Mercer and Helenius, 2008). After viral infection, PS is exposed at the plasma membrane of the cell where it may mimic apoptotic debris and induce macropinocytosis of virus by neighboring cells. This strategy is thought to be used by VACV to enter cells upon cell-to-cell interactions (Laliberte and Moss, 2009; Mercer and Helenius, 2008). Phospholipids are the main units composing surfactant in different mucosa (Bernhard et al., 1995; Veldhuizen et al., 1998), hence lining multiple epithelial cell types. Their presence in numerous organs might be a supplementary clue for their physiological importance. Phospholipid recycling and turnover in lung surfactant may also be used by viruses to more efficiently enter alveolar epithelium as for DPPC, in a novel adenovirus entry pathway (Balakireva et al., 2003) and for phosphatidylserine recognized by different enveloped viruses (Coil and Miller, 2005). This leads us to propose that phospholipids and particularly DPPG play an *in vivo* role in protection against virus infection *via* the respiratory route. It is unlikely that inhibition of infection by SUV-DPPG is restricted to VACV and future studies should be undertaken to determine the range of viruses that can affected. Additionally, interaction with the EEV form of VACV should be investigate however such studies are particularly difficult because EEV are only produced in small amounts during infection and are easily damaged.

The results obtained from *in vitro* studies prompted us to evaluate the activity of DPPG on VACV infection of mice. Viral infection performed with 100 LD_{50} mixed with a 0.5 mM dose of SUV-DPPG was ineffective at inducing death while a 1 mM dose of SUV-DPPG was entailed no weight loss or death of the animals. Using, the "gold-standard" of evaluating morbidity in murine models of viral disease we observed no weight loss when a control SUV-DPPG preparation was administered to uninfected mice suggesting no toxicity due to this treatment. However, one cannot exclude that interference with lung functions would not be detected if more sensitive methods such as measurement of blood oxygen levels were employed (Sidwell et al., 1992; Smee et al., 2004; Verhoeven et al., 2009). Despite this uncertainty it may be pointed out that the amount of SUV instilled into mice in our experiments was 2000 fold lower than that used in other studies which failed to induce any significant deterioration in alveolar oxygenation (Poelma et al., 2005).

Pretreatment or post-treatment of mice with DPPG were ineffective, possibly because of rapid phospholipid turn-over occurring in the lungs. Chemical modifications of DPPG to improve its *in vivo* stability could possibly display post-exposure efficacy of poxvirus respiratory infection.

This study on the DPPG–VACV interaction is only the beginning of an attempt to understand the role of phospholipids contained in lung surfactant which may ultimately lead to the development of new protective strategies against orthopoxviruses and/or respiratory viruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.11.009.

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