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Brief Communication

Inhibition of Mayaro virus infection by bovine lactoferrin

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ABSTRACT

Mayaro virus (MAYV) is an arbovirus linked to several sporadic outbreaks of a highly debilitating febrile illness in many regions of South America. MAYV is on the verge of urbanization from the Amazon region and no effective antiviral intervention is available against human infections. Our aim was to investigate whether bovine lactoferrin (bLf), an iron-binding glycoprotein, could hinder MAYV infection. We show that bLf promotes a strong inhibition of virus infection with no cytotoxic effects. Monitoring the effect of bLf on different stages of infection, we observed that virus entry into the cell is the heavily compromised event. Moreover, we found that binding of bLf to the cell is highly dependent on the sulfation of glycosaminoglycans, suggesting that bLf impairs virus entry by blocking these molecules. Our findings highlight the antiviral potential of bLf and reveal an effective strategy against one of the major emerging human pathogens in the neotropics.

Introduction

Arbovirus infections are a major public health problem worldwide, affecting countless individuals each year. MAYV, a member of the genus Alphavirus in the family Togaviridae (Casals and Whitman, 1957), has gained a prominent position in recent years among those arboviruses linked to human infections. Since the first isolation of the virus in Trinidad in 1954 (Anderson et al., 1957), several cases of Mayaro fever have been described in the Amazonian rain forest regions of South America, notably in Northern Brazil (Pinheiro et al., 1981). Although Mayaro fever is highly debilitating and its urbanization from the Amazon region is imminent (Mourão et al., 2012), the disease is largely neglected, and many details of the replication cycle of the virus remain unclear. As for other arbovirus diseases, no effective antiviral intervention is available for cases of Mayaro fever.

However, a natural macromolecule may provide a potential alternative. Lactoferrin is an iron-binding globular glycoprotein with a molecular mass of approximately 80 kDa that belongs to the transferrin family of proteins (Metz-Boutigue et al., 1984). Found predominantly in milk and various mucosal secretions, such as tears, saliva and seminal and vaginal fluids, lactoferrin plays an important role in the primary defense against a broad spectrum of pathogenic microorganisms, including Gram-positive and Gram-negative bacteria, fungi and many enveloped and non-enveloped viruses (Jenssen and Hancock, 2009). This intrinsic antimicrobial activity of lactoferrin makes this protein an interesting candidate for therapeutic applications during microbial infections. Indeed, lactoferrin is currently produced on a large scale from the form found in bovine milk (bLf), and using this commercially available material has advanced scientific research on lactoferrin applications from basic studies to clinical trials (Tomita et al., 2009).

The aim of this study was to evaluate the antiviral potential of bLf during MAYV infection. Using plaque assays and imaging cells infected with fluorescently labeled virus particles, we tested the ability of bLf to inhibit MAYV infection in Vero cells and attempted to determine the stage of the infectious cycle at which the protein exerts its antiviral effects. Our results demonstrate that bLf interacts with cell-surface GAGs to inhibit the early events of virus

Abbreviations: MAYV, Mayaro virus; bLf, bovine lactoferrin; GAG, glycosaminoglycan; DMEM, Dulbecco's modified Eagle's medium; MOI, multiplicity of infection; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt; FITC, fluorescein-5-isothiocyanate; MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide; PFU, plaque-forming unit

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infection, suggesting that bLf is a potential candidate for the prevention and/or treatment of human MAYV infections.

Results

BLf toxicity in Vero cells

As a measure of the bLf toxicity in the cell lineage used for experimental infections, we tested the effect of different concentrations of the protein on the viability of Vero cells. For this experiment, the cells were incubated with different concentrations of bLf for 48 h at 37 °C and then assayed for viability. Under these conditions, all concentrations tested were non-cytotoxic (Fig. 1A). Instead, bLf tended to enhance cell viability, leading to an almost 10% increase in cell viability at a concentration of 1 mg/mL compared to the control.

Dose-dependent effect of bLf on MAYV infection in Vero cells

Because bLf was non-cytotoxic in the range of 0.2–1 mg/mL, we then assayed the ability of these bLf concentrations to inhibit MAYV cytopathic effect. Vero cells were incubated with different concentrations of bLf throughout the infection procedure (during and after virus inoculation), including a pretreatment step for 1 h at 37 °C, and the effect on virus plaque formation was assessed. Under these experimental conditions, bLf showed a dose-dependent inhibitory activity, preventing virus infection by approximately 85% at a concentration of 1 mg/mL (Fig. 1B).

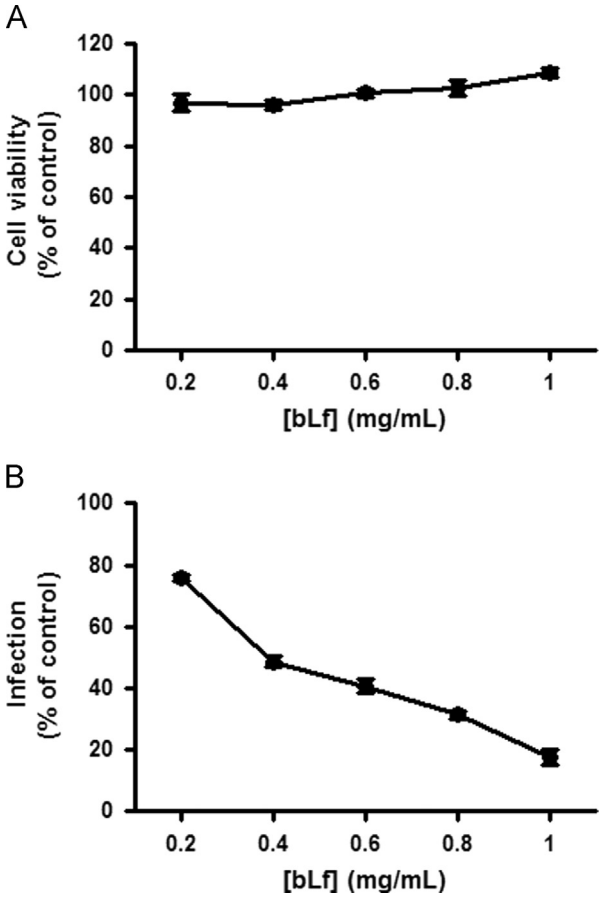

Fig. 1. Cytotoxicity and antiviral activity of bLf. (A) Vero cell monolayers were treated with the indicated concentrations of bLf for 48 h at 37 °C or incubated with culture medium as a control and then subjected to an MTT reduction assay to determine cell viability. (B) Vero cell monolayers were pretreated with bLf at the indicated concentrations for 1 h at 37 °C and then inoculated with MAYV under the same MOI. The bLf protein was maintained at the same pretreatment concentrations during and after the virus inoculation step. After 48 h of infection, cells were stained, and the virus plaques were counted to determine the efficiency of infection.

Effects of bLf on different stages of MAYV infection in Vero cells

After establishing the inhibitory profile for increasing bLf concentrations, we performed a time-of-addition assay to investigate the step in virus infection that was affected by the protein. Our result showed that 1 mg/mL bLf was able to inhibit MAYV plaque formation when added to Vero cells at different time-points during virus infection (Fig. 2). However, the antiviral effect was more pronounced when bLf was added during virus inoculation, inhibiting plaque formation by approximately 85%, and when the protein was present throughout the infection. A slight inhibition of infection was observed when the protein was present before (approximately 35%) or after (approximately 30%) virus inoculation. When bLf was added for 1 h after virus inoculation – to prevent its effects on the entry of newly synthesized virus particles into neighboring cells – a minimal inhibition of plaque formation was observed (approximately 25%). These results suggest that bLf acts primarily on MAYV entry into host cells and, to a lesser extent, on virus replication and egress.

No significant change in virus titer was observed when MAYV was separately incubated with 1 mg/mL bLf for 1 h at 4 °C, indicating that the antiviral effect of bLf was not due to virucidal activity of the protein through direct interactions with the virus particles (Suppl. Fig. 2).

Inhibition of MAYV entry into Vero cells by bLf

To directly access the effect of bLf on MAYV entry into Vero cells, we imaged this early event of infection by fluorescence microscopy using virus particles whose lipid envelopes were labeled with the lipophilic probe DiD, and compared the efficiency of virus entry in the presence of bLf with that in the absence of the protein. It is important to highlight that no change in virus titer due to fluorescent labeling was detected (data not shown). Fifteen minutes after virus inoculation, we could observe several fluorescent signals in the cells that were not exposed to bLf, while only a few fluorescent spots were observed in the cells that were exposed to 1 mg/mL bLf during virus inoculation (Fig. 3A). As expected, mock-infected cells showed no fluorescent signal. By counting the number of fluorescent spots in individual cells, we determined that bLf inhibited virus entry by approximately 60% (Fig. 3B).

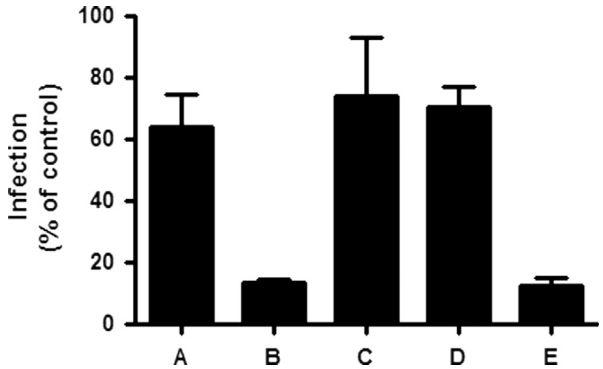

Fig. 2. Anti-MAYV effect of bLf on different stages of the infectious cycle. Monolayers of Vero cells infected with MAYV under the same MOI were treated with 1 mg/mL bLf at different phases of infection: (A) before virus inoculation, (B) during virus inoculation, (C) after virus inoculation for 1 h, (D) after virus inoculation for 48 h and (E) throughout infection. After 48 h of infection, cells were stained, and the virus plaques were counted to determine the efficiency of infection.

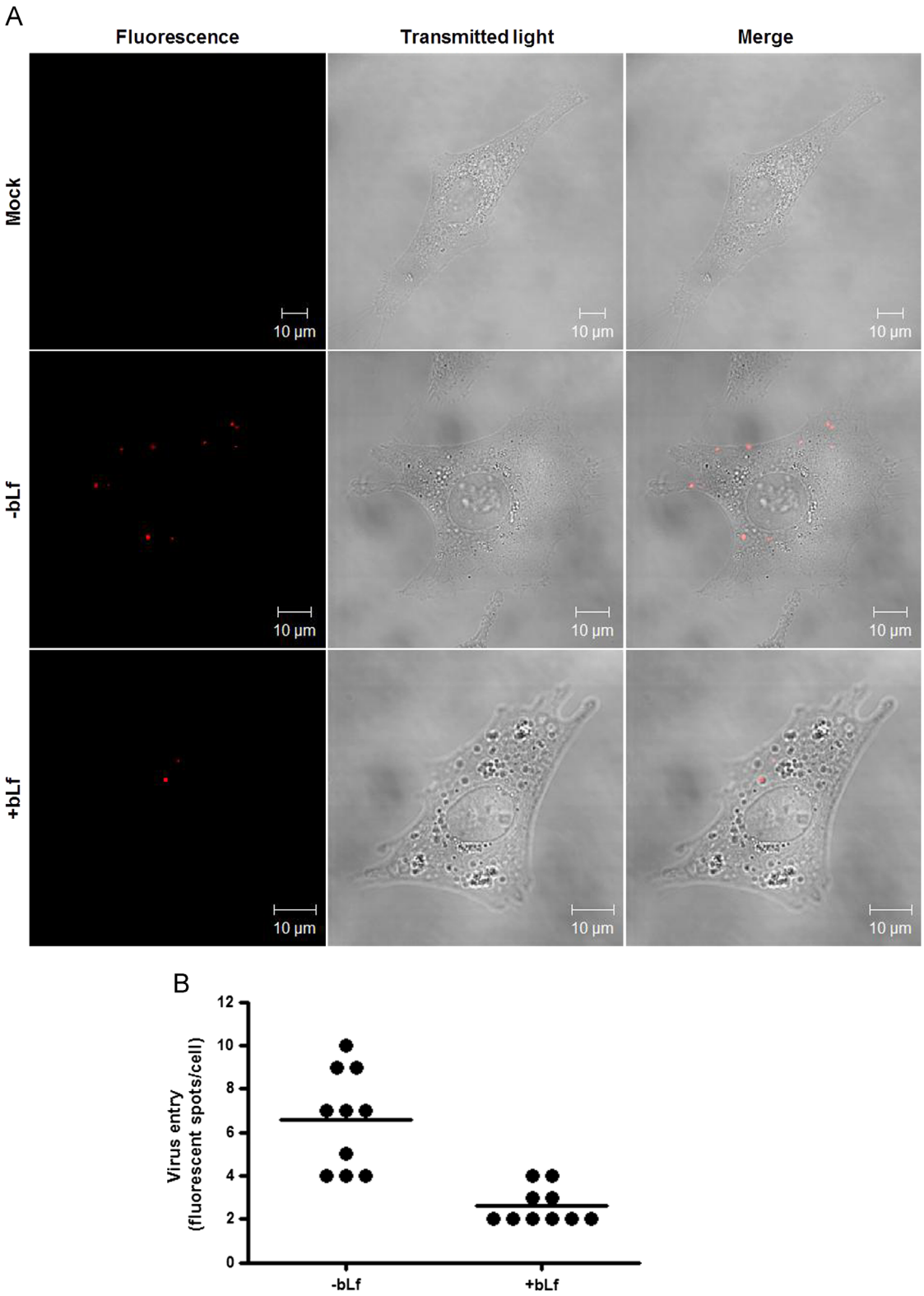

Fig. 3. Effect of bLf on MAYV entry into host cells. (A) Subconfluent Vero cells were inoculated with DiD-labeled MAYV at an MOI of 10 in the presence (+bLf) or absence (−bLf) of 1 mg/mL bLf. Infection was synchronized by allowing virus binding to the cells for 15 min at 4 °C, and then cells were incubated at 37 °C to allow the infection to progress. At 15 min post-heating, the samples were fixed and visualized by fluorescence microscopy. The images of individual cells are representative of their respective visual fields. Mock: uninfected cells. (B) Fluorescent spots in 10 random cells from each experimental condition above were counted, and the data were expressed as the dispersion and mean of the number of fluorescent spots per cell.

Role of GAG sulfation in bLf binding to the surface of Vero cells

Due to its positive net charge, bLf is likely to interact with the sulfate groups of GAGs on the cell surface, and this could be the mechanism underlying the observed interference with MAYV entry. To test this hypothesis, we first checked whether the ability of MAYV to infect Vero cells is dependent on the sulfate groups of GAGs. Using $NaClO_3$, a specific inhibitor of GAG sulfation, we

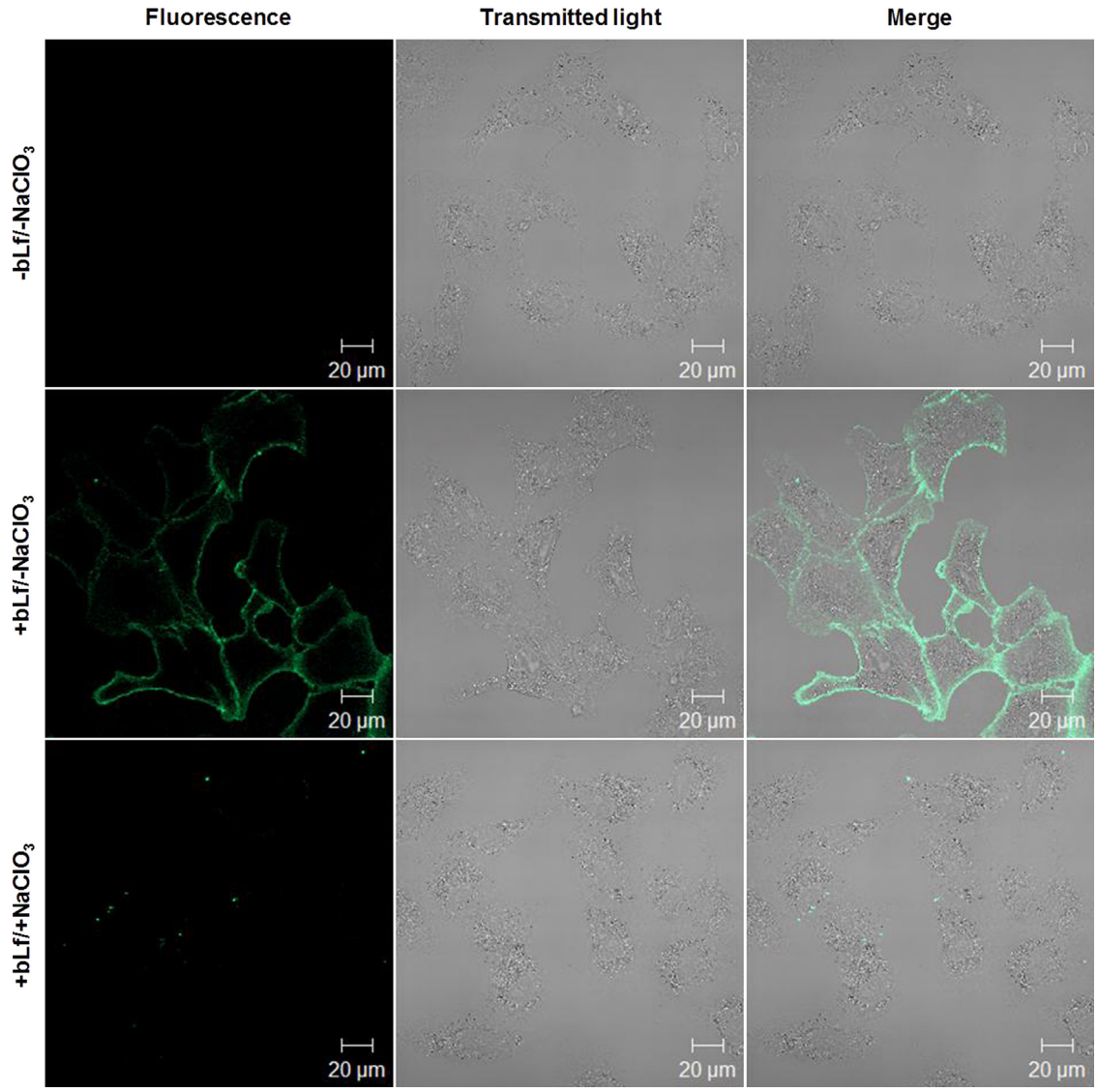

Fig. 4. Requirement for sulfated GAGs for bLf binding to the cell surface. Subconfluent Vero cells previously cultured in the presence (+$NaClO_3$) or absence (−$NaClO_3$) of 50 mM $NaClO_3$ for 24 h were incubated with 1 mg/mL FITC-labeled bLf in PBS (+bLf) or with PBS alone (−bLf) for 15 min at 4 °C. Following incubation, samples were fixed and visualized by fluorescence microscopy. The images of cell clusters are representative of their respective visual fields.

aimed to exchange cell surface GAGs for non-sulfated versions, and observed that cell treatment with the compound for 24 h prior to infection inhibited virus plaque formation by 65% (Suppl. Fig. 3).

We next assayed whether binding of bLf to the cell surface would also be affected by inhibition of GAG sulfation. For this experiment, FITC-labeled bLf molecules were allowed to bind to control or $NaClO_3$-treated Vero cells, and the fluorescent signals were imaged by fluorescence microscopy. We found that treatment of Vero cells with $NaClO_3$ dramatically prevented the interaction of bLf with the plasma membrane (Fig. 4).

Discussion

In this study, we investigated the activity of bLf against MAYV infection in Vero cells. Our results showed that bLf provides a strong antiviral activity on MAYV infection, acting primarily on events leading to virus entry into the cell. This is the first study showing the efficient prevention of MAYV infection by a natural non-cytotoxic molecule. In this study we tested bLf only in its apo form. There is a striking number of studies in the literature showing that the iron saturation of lactoferrin does not play an important role in its antiviral effect. However, apolactoferrin remains more potent than its metal-saturated isoforms against some virus species (Van der Strate et al., 2001). Moreover, apolactoferrin has a 600 times higher selectivity index, due to its lack of toxicity (Superti et al., 1997).

Regarding its effects on Vero cells, we observed that bLf promoted a slight increase in cell viability that has to be further investigated. Indeed, it has previously been shown that bLf can sustain cell survival by protecting some cell lineages from apoptosis (Grey et al., 2006; Huang et al., 2008; Pietrantoni et al., 2010; Tang et al., 2010; Francis et al., 2011; Jiang and Lönnerdal, 2012). Thus, we speculate that the observed increase in viability promoted by bLf could be due to a reduction in cell death by apoptosis that occurs as a consequence of stress from cell confluence in culture.

Our results, showing that bLf prevents infection of the host cell rather than inhibiting virus replication after the target cell becomes infected, are in accordance with the mode of action of bLf previously shown for other alpha and unrelated viruses (Waarts et al., 2005; Van der Strate et al., 2001). Because we

excluded the possibility of bLf directly binding to virus particles, bLf could prevent MAYV infection of the host cell by either interfering with the docking of virus to cells by binding to cell surface proteoglycans or by directly binding to viral receptors on the host cell that the virus uses for cell entry. Our observation that binding of bLf to the cell surface is dependent on the presence of sulfated GAGs on the plasma membrane suggests that the inhibition of virus entry promoted by the bLf protein is primarily due to its interaction with the sulfate groups of these molecules. The blockage of GAGs by bLf would impair their use by virus particles as initial adhesion molecules. Because the specific receptor for Mayaro virus remains unknown, we were unable to test whether its interaction with bLf may also play a role in the inhibition of virus entry promoted by the protein.

Because MAYV urbanization is imminent and the virus has a real potential to adapt to new vectors and spread to non-endemic areas (Receveur et al., 2010; Hassing et al., 2010; Long et al., 2011; Neumayr et al., 2012), the findings of this study, highlighting the antiviral activity of bLf, are of utmost importance and may contribute to the development of an effective strategy against MAYV infection, which is one of the major emerging diseases in the neotropics (Vasconcelos et al., 2001).

Materials and methods

Virus and cell cultures

MAYV (VR-1277) was obtained from the American Type Culture Collection (Manassas, VA, USA). Baby hamster kidney (BHK-21) and African green monkey kidney (Vero) cells were cultured as monolayers at 37 °C in a humidified atmosphere with 5% CO_2 in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 5 μg/mL gentamicin sulfate (Invitrogen, Carlsbad, CA, USA).

Virus propagation and purification

MAYV was propagated and purified as previously described (Mezencio and Rebello, 1993) with several modifications. After propagation in BHK-21 cells under an MOI of 0.1 for 48 h, the culture supernatant was collected and cleared of cell debris by centrifuging at 8000 rpm for 20 min at 4 °C in an RPR 12-2 rotor (Hitachi, Tokyo, Japan). The supernatant was applied to a 30% sucrose cushion and centrifuged in a Type 45 Ti rotor (Beckman Coulter, Brea, CA, USA) at 32,000 rpm for 1 h 40 min at 4 °C. The pellet was suspended in PBS, layered onto a discontinuous 5–50% sucrose density gradient and centrifuged at 30,000 rpm for 1 h 30 min at 4 °C in an SW 40 Ti rotor (Beckman Coulter). Fractions were collected, and the fraction containing the virus was identified by reading the optical density at 260 and 280 nm. Purified virions were aliquoted and stored at −80 °C until further use.

BLf preparation

Apolactoferrin from bovine whey (Life Extension, Fort Lauderdale, FL, USA) was dissolved to a concentration of 100 mg/mL in PBS and centrifuged at 6000 rpm for 5 min at 4 °C to remove the cellulose excipient. The supernatant was passed through a 0.22-μm syringe-driven filter unit (Millipore, Billerica, MA, USA), aliquoted and stored as a stock solution at −20 °C until further use. This procedure eliminated all excipients described in the formulation provided by the manufacturer. Protein purity was 95%, as stated by the manufacturer and confirmed by SDS-PAGE (Suppl. Fig. 1).

Fluorescent labeling of virus particles and bLf

Approximately 10^{10} MAYV particles were incubated with 2 nmol of DiD (Molecular Probes, Eugene, OR, USA) in PBS for 10 min at room temperature. The unincorporated dye was removed by centrifuging through an Amicon Ultra filter unit with a 100-kDa molecular weight cut-off (Millipore). Labeled virus particles were suspended in PBS, passed through a syringe-driven filter unit with 0.22-μm pore size to remove virus aggregates and immediately used for experiments. DiD labeling was confirmed by scanning the light absorption spectrum of the virus sample.

BLf was incubated with FITC (Molecular Probes) at a molar ratio of 1:10 in basic phosphate buffer (2.5% $Na_2HPO_4 \cdot 7H_2O$ and 0.082% NaH_2PO_4, pH 8.0) for 1 h at 4 °C. The unincorporated dye was removed by centrifuging through an Amicon Ultra filter unit with a 30-kDa molecular weight cut-off (Millipore). Labeled protein molecules were suspended in PBS, passed through a syringe-driven filter unit with 0.22-μm pore size and immediately used for experiments. FITC labeling was confirmed by subjecting the protein sample to SDS-PAGE followed by the ultraviolet transillumination of the gel.

Cytotoxicity assay

Vero cell monolayers seeded in 96-well plates (TPP, Trasadingen, Switzerland) were incubated with different concentrations of bLf at 37 °C for 48 h and then assayed for MTT reduction as previously described (Mosmann, 1983).

Antiviral assays

All assays for assessing the activity of bLf against MAYV infection were conducted in Vero cell monolayers seeded in 12-well plates (TPP). The dose–response activity of bLf was evaluated by incubating cells with the indicated concentrations of the protein at 37 °C throughout the course of infection, including immediately before (for 1 h), during (for 1 h) and immediately after (for 48 h) virus inoculation, at a density of 100 PFUs per well. For the time-of-addition assays, the experimental procedure was the same, except bLf was present at a single concentration (1 mg/mL) separately for each stage of infection, in addition to immediately after virus inoculation for 1 h and the remainder of the infection. At 48 h post-infection, cells were stained with 1% crystal violet, and the virus plaques were counted to determine the efficiency of infection.

Imaging of virus entry and bLf binding

Subconfluent Vero cells seeded in 35-mm glass-bottom dishes (MatTek, Ashland, MA, USA) were used for both imaging procedures. To image virus entry, cells were incubated with DiD-labeled MAYV under an MOI of 10 for 15 min at 4 °C in the presence or absence of 1 mg/mL bLf. After this virus binding synchronization step, unbound virus particles were washed away with PBS, and cells were incubated in DMEM supplemented with 2% fetal bovine serum and 5 μg/mL gentamicin sulfate at 37 °C to allow for virus entry. At 15 min post-heating, cells were washed again with PBS and fixed with 3.7% formaldehyde for 15 min. To image bLf binding, cells previously cultured in the presence or absence of 50 mM $NaClO_3$ for 24 h were incubated with FITC-labeled bLf for 15 min at 4 °C, washed with PBS to remove unbound bLf molecules and fixed as above. Samples were visualized on an LSM 510 META laser-scanning confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with excitation by a helium-neon laser at 633 nm and emission collected from 650 to 710 nm for imaging

DiD-labeled MAYV or with excitation by an argon ion laser at 488 nm and emission collected from 500 to 550 nm for imaging FITC-labeled bLf.

Statistical analyses

Statistical analyses were performed using an unpaired *t*-test with a two-tailed *P*-value and a one-way ANOVA with the Dunnett's post-test. Data were expressed as the mean ± SD, and *P*-values less than 0.05 were considered statistically significant. Given these criteria, all differences observed were significant, except when otherwise stated.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.01.022.

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