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Inactivation of vesicular stomatitis virus through inhibition of membrane fusion by chemical modification of the viral glycoprotein

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Abstract

Membrane fusion is an essential step in the entry of enveloped viruses into their host cells triggered by conformational changes in viral glycoproteins. We have demonstrated previously that modification of vesicular stomatitis virus (VSV) with diethylpyrocarbonate (DEPC) abolished conformational changes on VSV glycoprotein and the fusion reaction catalyzed by the virus. In the present study, we evaluated whether treatment with DEPC was able to inactivate the virus. Infectivity and viral replication were abolished by viral treatment with 0.5 mM DEPC. Mortality profile and inflammatory response in the central nervous system indicated that G protein modification with DEPC eliminates the ability of the virus to cause disease. In addition, DEPC treatment did not alter the conformational integrity of surface proteins of inactivated VSV as demonstrated by transmission electron microscopy and competitive ELISA. Taken together, our results suggest a potential use of histidine (His) modification to the development of a new process of viral inactivation based on fusion inhibition.

Keywords: Viral inactivation; Vesicular stomatitis virus; Membrane fusion; Diethylpyrocarbonate

1. Introduction

The development of new strategies to achieve viral inactivation is an important aim in virus research. Despite the advances in the field of immunology, molecular biology and genetics, viral inactivation remains an important procedure in basic research, since it is an easy and relatively cheap approach for producing new effective and safe vaccines against several viruses. The entry of enveloped viruses into a target cell always requires virus-mediated membrane fusion catalyzed by viral surface glycoprotein (Eckert and Kim, 2001; Hernandez et al., 1996; Skehel and Wiley, 2000). Virus-induced fusion may occur through two different general mechanisms: (i) fusion between viral envelope and host cell plasma membrane after virus interaction with its cellular receptor, or (ii) fusion with the endosomal membrane, after virus internalization by receptor-mediated endocytosis. In the latter case, the decrease in the pH of the endosomal medium triggers conformational changes in viral glycoproteins. This mechanism seems to be conserved among several viral families, which makes the fusion process a potentially attractive target for viral inactivation approaches.

Vesicular stomatitis virus (VSV), a member of Rhabdoviridae family, is composed by a helical ribonucleocapsid surrounded by a lipid bilayer covered by trimers of a single type of an integral glycoprotein, named G protein. VSV causes an acute disease that primarily affects cattle, horses and pigs. The clinical presentation of the disease is the development of vesicles and ulcers in the oral cavity and, less frequently, in the teats and coronary bands (Letchworth et al., 1999). Mortality rates are typically very low, but production suffers because affected animal lose weight and may develop lameness and mastitis. VSV enters into the cell by endocytosis followed by low-pH-induced membrane fusion (Da Poian et al., 1998; White et al., 1981). Cell recognition and fusion are mediated by the surface glycoprotein G. We have been studying the conformational changes

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in VSV G protein and its interaction with the target membrane during cellular recognition and fusion events (Carneiro et al., 2001, 2002). The observation of a dramatic reorganization in G protein structure at a very narrow pH range led us to propose a crucial role for the histidine (His) residues of VSV G protein in membrane fusion mediated by the virus (Carneiro et al., 2003). Using diethylpyrocarbonate (DEPC) to modify His residues of G protein, we showed that VSV-induced fusion was driven by His protonation at the pH range of endosomal medium. DEPC is a widely used tool in chemical modification of proteins because of the high selectivity of the reagent to histidyl residues (Miles, 1977). This compound covalently modifies histidines and makes them unable to be protonated. Modification with DEPC was successfully used in inactivation studies of various groups of enzymes, e.g., peroxidases, heparinases and ATPases (Bhattacharyya et al., 1992; Dzhandzhugazyan and Plesner, 2000; Shriver et al., 1998). However, DEPC has not been previously evaluated as a potentially useful antiviral compound.

The main aim of the present study was to evaluate whether viral treatment with DEPC was able to inactivate the virus. For this purpose, we analyzed the VSV infectivity after His modification in vitro and using an animal model. We showed that virus treatment with DEPC abolished virus replication in cell culture and eliminated the ability of the virus to cause disease in mice. Moreover, although the modified virus was shown to be completely inactivated in all the systems tested, virus structure and the antigenic domains of modified G protein were preserved. These results suggest that modification of His residues of viral fusion proteins might be used as a new process of viral inactivation.

2. Material and methods

2.1. Virus propagation and purification

VSV Indiana was propagated in monolayer cultures of BHK_{21} cells grown at 37 °C in petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 0.0085% streptomycin sulfate. When the cells reached confluence, the medium was removed, and the cell monolayer was infected with VSV at a multiplicity of infection (MOI) of 0.1. The cultures were kept at 37 °C for 16–20 h and the virus were harvested and purified by differential centrifugation followed by equilibrium sedimentation in a sucrose gradient, as described elsewhere (Da Poian et al., 1996). Purified virions were stored at −70 °C.

2.2. Virus modification with DEPC

Diethylpyrocarbonate solutions were freshly prepared by dilution of the reagent in cold ethanol. The concentration of stock DEPC solution was determined by reaction with 10 mM imidazole (Miles, 1977). VSV was diluted in PBS, pH 7.4, to a final protein concentration of 60 μg/ml and incubated for 15 min at room temperature. The final concentration of DEPC ranged from 0.01 to 0.5 mM.

2.3. Preparation of liposome and fusion assay

Phosphotidylcholine (PC) and phosphatidylserine (PS) from bovine brain (Sigma Chemical Co.) in a molar ratio of 1:3 were dissolved in chloroform and evaporated under nitrogen. The lipid film formed was resuspended in 20 mM MES, 30 mM Tris buffer (pH 6.0) at a final concentration of 1 mM. The suspension was vortexed vigorously for 5 min. Small unilamellar vesicles (SUV) were obtained by sonicating the turbid suspension using a Branson Sonifier (Sonic Power Company, Danbury, CT) equipped with a titanium microtip probe, in an ice bath, alternating cycles of 30 s at 20% full power, with 60 s resting intervals until a transparent solution was obtained (approximately 10 cycles). For fusion assays, equal amounts of unlabeled vesicles and vesicles labeled with 1 mol% of each *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl) phosphatidylethanolamine (Rh-PE) (Molecular Probes Inc., Eugene, OR) were prepared in 20 mM MES, 30 mM Tris buffer (pH 6.0), at a final phospholipid concentration of 0.1 mM. The fusion reaction was initiated by addition of the VSV (final concentration of 5 μg/ml). Fusion was followed by the fluorescence resonance energy transfer (FRET) assay as described in Struck et al. (1981). In this assay, vesicles labeled with a combination of fluorescence energy transfer donor and acceptor lipid probes, NBD-PE and Rh-PE, respectively, are mixed with unlabeled membranes. FRET, detected as rhodamine emission resulting from NBD excitation, decreases when the average spatial separation of the probes is increased upon fusion of labeled membranes with unlabeled membranes. In our experiments, the samples were excited at 465 nm and the fluorescence intensities were collected at 530 and 590 nm for NBD-PE and Rh-PE, respectively, using a Hitachi F-4500 Fluorescence Spectrophotometer. The fusion index was calculated using as the 100% value, the fluorescence ratio after addition of 0.2% Triton X-100 to the vesicles.

2.4. Infection of BHK_{21} cells with VSV

Monolayers of the BHK_{21} cells in 6- or 24-well plates were first adsorbed with unmodified VSV or VSV treated with DEPC at a MOI of 0.1 for 1 h at 37 °C. In the case of the treated samples, the inoculum was based on the titer of untreated virus, what means that all the samples contain the same number of virus particles although the number of infectious viruses was lower in the treated samples. After 1 h incubation, the unbound viruses were removed by three gentle washings with serum-free medium and fresh medium was added to each plate for further incubation at 37 °C.

2.5. Determination of virus titers by $TCID_{50}$

Infectious particles of VSV were quantified by $TCID_{50}$ on BHK_{21} cells for unmodified VSV and VSV treated with different concentrations of DEPC, or for supernatants of infected cultures of BHK_{21}. The cells were grown at 37 °C in 96-well plates until the confluence was reached. Samples were serially

diluted 10-fold in culture medium, and 100 μl was added to cells in quadruplicate. Cells were cultured at 37 °C in 5% CO_2 for 24 h and stained with 0.2% crystal violet in 20% ethanol. The $TCID_{50}$ was calculated by the method described by Reed and Muench (1938). To test the reversibility of inactivation, VSV modified with 0.5 mM DEPC was incubated with 400 or 1200 mM hydroxylamine (from a 3 M stock solution) for 30 min or 2 h, at room temperature before the quantification of the infectious particles by $TCID_{50}$.

2.6. Western blotting analysis

Cells were infected in 6-well plates and after 4, 12 and 20 h of infection, the medium was removed, the cells were washed with PBS and lysed with 100 μl of lysis buffer (20 mM Tris–HCl, 0.2 mM EDTA, pH 7.4, containing 1% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). The lysates were diluted five-fold in 30 μl of SDS-PAGE buffer containing β-mercaptoethanol and boiled for 5 min. Proteins were separated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane according to standard protocols. Blots were blocked in 5% nonfat dry milk in PBS and incubated with a mouse monoclonal antibody against VSV G protein (Alpha Diagnostic Int, San Antonio, TX), at a 1:5,000 dilution in blocking buffer. PVDF blots were washed with PBS, incubated with goat anti-mouse antibody conjugated with peroxidase (1:10,000 dilution) and revealed with ECL kit reagents (Amersham Biosciences). Molecular sizes were determined using prestained molecular weight markers (Sigma Chemical Co.). Purified VSV (100 ng) was added as positive control.

2.7. Immunofluorescence

BHK_{21} cells were grown on sterile coverslips in 24-well tissue culture dishes. At 12 h after infection, they were fixed and permeabilized with 100% methanol for 20 min at −20 °C. Cells were blocked at room temperature using 4% goat serum in PBS for 2 h and incubated with mouse monoclonal anti-VSV G protein antibody (Alpha Diagnostic Int) diluted 1:200 in PBS containing 1% goat serum. As secondary antibody, Cy3-labeled goat anti-mouse immunoglobulin G conjugate was used at a 1:400 dilution (Sigma Chemical Co.). Finally, slides were mounted with *N*-propyl-galacto and examined with a Nikon epi-fluorescence microscope equipped for photomicroscopy.

2.8. Infection of mice

Four- to 5-weeks-old BALB/c male mice were slightly anesthesized and inoculated intranasally with 2×10^6 infectious units of unmodified VSV or VSV treated with different concentrations of DEPC in 5 μl of sterile PBS. In the case of the treated samples, the inoculum was based on the titer of untreated virus, which means that all the samples contain the same number of virus particles although the number of infectious viruses was lower in the treated samples. Control mice received only PBS. After infection, mice were caged, maintained with free access to food and observed daily for survival for at least 35 days. Animals were housed under pathogen-free conditions.

2.9. Intracerebral inflammatory response

BALB/c mice were inoculated intranasally with VSV or VSV treated with 0.5 mM DEPC as described above. At day 5, the animals were perfused with PBS followed by 4% paraformaldehyde solution in phosphate buffer. The brains were removed and post-fixed for 2 h in the same paraformaldehyde solution. After serial dehydration in ethanol and xylol solutions the whole brain was embedded in paraffin. Serial sections of each brain were mounted in glass slides for staining with hematoxylin and eosin.

2.10. Electron microscopy

Unmodified VSV or VSV treated with 0.5 mM DEPC were visualized by transmission electron microscopy (TEM) after negative staining. Briefly, a drop (5 μl) of each sample was placed to a copper 300 mesh grid coated with a thin formvar film (20 nm). The liquid was partially removed with a filter paper after 45 s. The wet grids were stained with 5% phosphotungstic acid solution during 45 s. The grids were completely dried and observed in a JEOL 1200 EX TEM, operated at 80 kV.

2.11. Competitive ELISA

The antigenic properties of VSV G protein after virus treatment with DEPC were evaluated by competitive ELISA. ELISA microplates were coated with 0.1 μg VSV overnight at 4 °C. The plates were washed with PBS/0.05% Tween-20 and blocked with 2% milk powder in PBS/0.05% Tween-20 for 2 h at room temperature. After blocking, the plates were washed as before. Subsequently, immune sera collected from mice 7 days after intranasal inoculation of 2×10^6 infectious units of unmodified VSV (at a fixed dilution) were incubated at 37 °C for 1 h together with serial dilutions of VSV modified with 0.5 mM DEPC or virus denatured by boiling for 5 min. As positive control, the immune serum was incubated with unmodified virus in the same dilutions of treated viruses, and as negative control albumin at the same protein concentrations was used. After incubation, the samples were transferred to coated ELISA plates and incubated for 1 h at room temperature. Plates were washed as before and bound IgG was detected by addition of an anti-mouse horseradish peroxidase conjugate (1:5,000 dilution) (Santa Cruz Biotechnology) followed by *o*-phenylenediamine (OPD) substrate as indicated by the manufacturer (Sigma Chemical Co.). The reaction was stopped with 3 M sulfuric acid, and the optical density at 492 nm was determined in an ELISA reader.

3. Results

3.1. Treatment with DEPC decreases VSV infectivity by inhibiting membrane fusion

During VSV infection, the pH-induced membrane fusion is a crucial step for the viral RNA evasion from the endosome

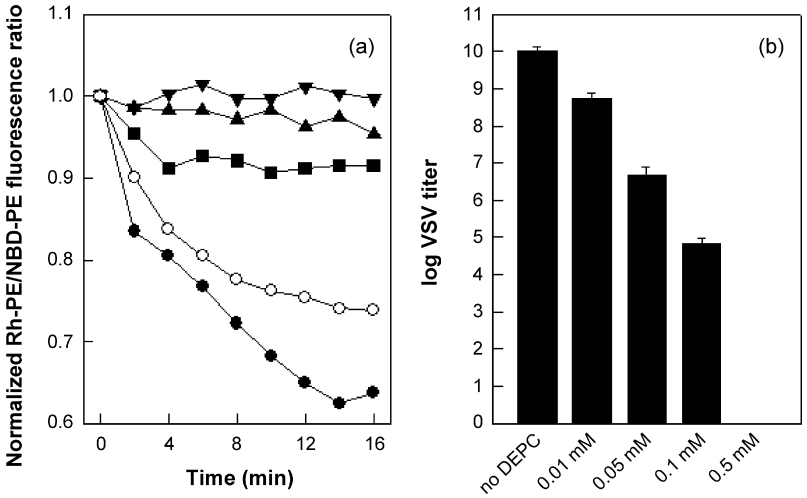

Fig. 1. DEPC treatment decreases the viral infectivity by inhibiting membrane fusion. (a) Equal amounts of unlabeled vesicles and vesicles labeled with NBD-PE and Rh-PE were incubated with purified VSV (●), or VSV pre-incubated with 0.01 mM (◯), 0.05 mM (■), 0.1 mM (▲) and 0.5 mM (▼) DEPC. The vesicles were composed of PC:PS (1:3) and were prepared in 20 mM MES, 30 mM Tris buffer, pH 6.0, at a final phospholipid concentration of 0.1 mM. VSV-induced membrane fusion was measured by the decrease in the Rh-PE/NBD-PE fluorescence ratio after addition of the virus in a final protein concentration of 5 μg/ml. The samples were excited at 465 nm and the fluorescence intensities were collected at 530 and 590 nm for NDB-PE and Rh-PE, respectively. (b) Titers of unmodified VSV or VSV treated with different concentrations of DEPC were measured in BHK_{21} cells by $TCID_{50}$. Data shown correspond to the results of four independent experiments.

into the cytoplasm. To quantify the VSV-induced membrane fusion after virus modification with DEPC, we used a liposome fusion assay based on NBD-PE/Rh-PE energy transfer (Struck et al., 1981). The addition of the untreated virus to the vesicles at pH 6.0 induced a decrease in the Rh-PE/NBD-PE fluorescence ratio that indicates an effective membrane fusion process (Fig. 1a). In contrast, DEPC treatment inhibited membrane fusion mediated by the virus in a concentration-dependent manner. To address whether DEPC treatment also decreases viral infectivity, we measured viral titers after treatment with different DEPC concentrations. Although the effect on viral titers was less pronounced than on membrane fusion after treatment with 0.05 and 0.1 mM DEPC, the treatment with 0.5 mM was sufficient to completely abolish VSV infectivity (Fig. 1b). Virus treated with the vehicle alone showed no change on the viral titer, excluding the possibility that the viral manipulation during the DEPC treatment decreased the viral titer (not shown).

To investigate the effect of DEPC treatment on viral replication, the viral protein synthesis and the viral titers recovered in the culture medium at different times after infection were measured (Fig. 2). When VSV was added to the culture medium at a MOI of 0.1, the release of viral progeny from BHK_{21} cells was maximal at 15–20 h after infection (Fig. 2a). Virus treatment with 0.1 and 0.5 mM DEPC abolished completely the production of virus progeny in the cultured cells, while treatment with 0.05 mM DEPC did not affect viral replication (Fig. 2b). Although virus growth was not observed for the sample treated with 0.1 mM DEPC, this sample did give an infectious titer of approximately 10^5 $TCID_{50}$/ml (Fig. 1b). This fact might be explained by the absence of infectious virus in the inoculum after the dilution required to normalize the number of total particles to that corresponding to the MOI of 0.1 used for the untreated sample inoculum (see Section 2). To monitor viral protein synthesis we performed an immunoblotting using anti-G protein antibody (Fig. 2c). G protein expression correlates to the appearance of viral progeny in the culture medium of the infected cells. It was maximal at 20 h after the infection and it was inhibited when the virus was treated with 0.1 and 0.5 mM DEPC. Treatment with 0.05 mM DEPC led to lower levels of G protein expression after 20 h of infection. The effect of VSV treatment with DEPC on the viral protein synthesis was confirmed by immunofluorescence (Fig. 2d). In cells incubated with the unmodified virus, G protein synthesis was clearly detected 12 h after infection, with the fluorescence staining particularly concentrated around the nucleus. On the other hand, when the virus was pretreated with 0.5 mM DEPC, no staining was observed in the cells.

3.2. DEPC is a safe approach for inactivating viruses

To be considered a safe method for viral inactivation, it is imperative that the infectivity is not restored in any condition. Hydroxylamine is a compound that specifically reverses DEPC modification of His residues by removing the carbethoxy group from imidazole group (Miles, 1977). Although the inhibition of VSV-induced fusion by virus treatment with 0.02 mM DEPC was reverted by incubation of the modified virus with hydroxylamine at a final concentration of 400 mM (Carneiro et al., 2003), fusion and infectivity were not restored after hydroxylamine treatment, even when higher concentrations of hydroxylamine or longer incubation periods were tested (Table 1).

Another concern is the possible cytotoxicity of DEPC. However, it is important to point out that free DEPC is very unstable in aqueous solution, being rapidly hydrolyzed (Berger, 1975; Miles, 1977), which suggests that no free DEPC would be present in the inactivated virus preparation. Indeed, time course of DEPC hydrolysis in PBS, pH 7.4, the buffer solution used to perform viral inactivation, revealed a half-time of 5 min (data not shown).

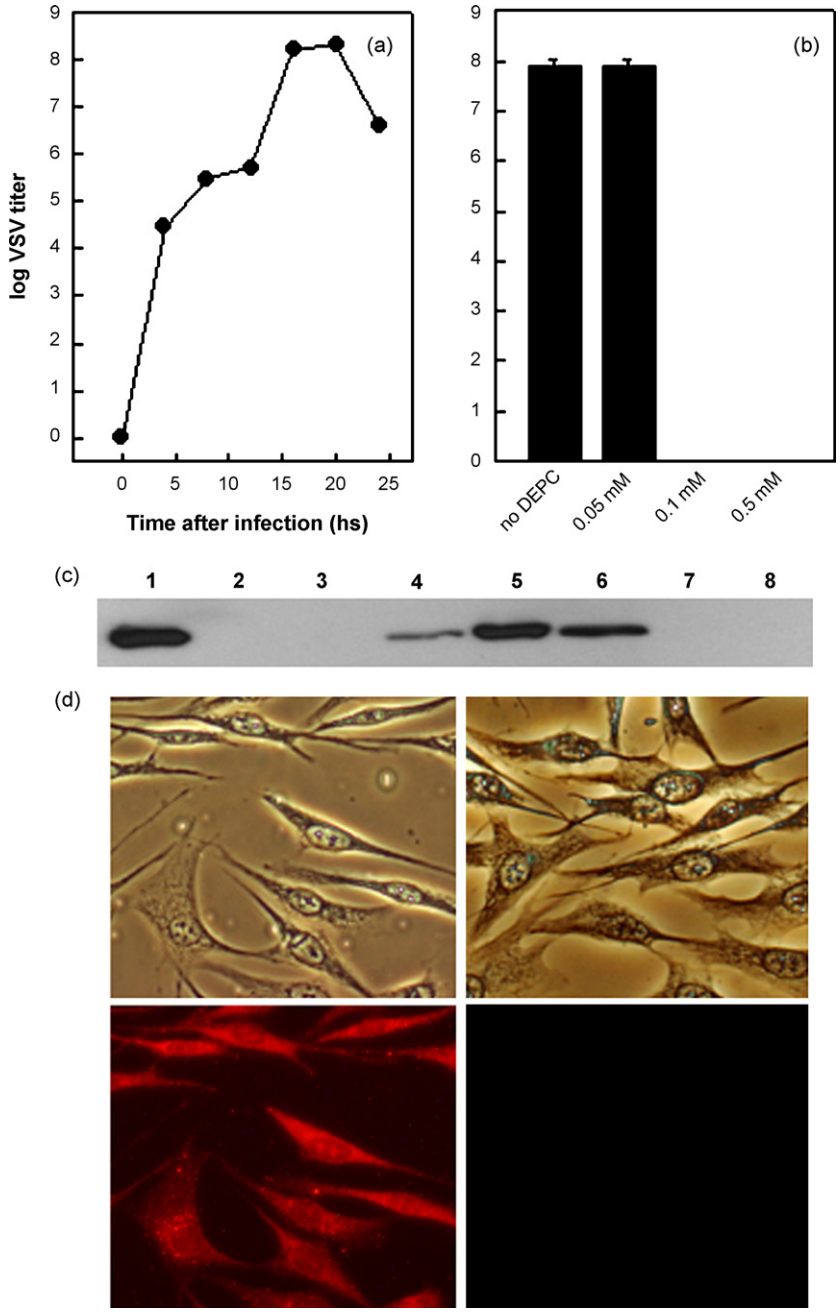

Fig. 2. Viral replication is inhibited by virus modification with DEPC. (a) Virus progeny released from BHK_{21} infected with unmodified VSV was followed by titrating the culture supernatants. (b) Virus release in supernatants of BHK_{21} cultures 20 h after incubation with control VSV or VSV treated with 0.05, 0.1 and 0.5 mM DEPC were compared. Data shown correspond to the results of four independent experiments. (c) Lysates from BHK_{21} cells were prepared for immunoblotting with monoclonal antibody against G protein. Purified VSV (100 ng) was used as positive control (lane 1). Lysates were prepared from non-infected cells (lane 2); from cells infected with unmodified VSV after 4 h (lane 3), 12 h (lane 4) and 20 h (lane 5); and from cells after 20 h of incubation with VSV treated with 0.05 mM DEPC (lane 6), 0.1 mM DEPC (lane 7) and 0.5 mM DEPC (lane 8). (d) BHK_{21} cells were infected with unmodified VSV (left) or VSV treated with 0.5 mM DEPC (right) and were visualized by phase-contrast (top) or by immunofluorescence using a VSV G protein monoclonal antibody (bottom) after 12 h. Magnification was 400×.

3.3. Treatment with DEPC abolishes VSV pathogenesis in mice

To determine the effect of treatment with DEPC on the pathogenesis of VSV, the survival rate of mice inoculated intranasally with VSV unmodified or treated with different concentrations of DEPC (Fig. 3) and the intracerebral inflammatory response to the virus (Fig. 4) were analyzed. It has been shown that when VSV is applied to the nasal neuroepithelium, it initially replicates in olfactory receptor neurons, and then it is transmitted along the olfactory nerve to the central nervous system (CNS) within 12 h (Reiss et al., 1998). In the olfactory bulb, the virus replicates invasively through the layers of the olfactory bulb, reaching the cerebral ventricles by days 4–5 post-infection, and the hindbrain by day 8 post-infection. In mice, infection causes encephalitis and may result in a 50% mortality rate when 2×10^6

Table 1
Effect of hydroxylamine on VSV inactivation and fusion

Treatment	Titer ($TCID_{50}$/ml)	Fusion index
Unmodified	1.70×10^{11}	17.2
DEPC 0.5 mM	$<10^{2}$[a]	0.69
DEPC 0.5 mM + NH_2OH 0.4 M, 30 min	$<10^{2}$[a]	0.89
DEPC 0.5 mM + NH_2OH 0.4 M, 2 h	$<10^{2}$[a]	ND[b]
DEPC 0.5 mM + NH_2OH 1.2 M, 2 h	$<10^{2}$[a]	ND[b]
Unmodified + NH_2OH 0.4 M	2.15×10^{9}	ND[b]
Unmodified + NH_2OH 1.2 M	$<10^{2}$[a]	ND[b]

[a] 10^2 was the initial dilution required for avoiding hydroxylamine toxicity to cells.
[b] ND: not determined.

infectious units of VSV are inoculated intranasally (Reiss et al., 1998). Fig. 3 shows that we observed a survival rate of approximately 45% 7 days after mice inoculation with unmodified VSV, a result compatible with the literature. A similar survival profile was obtained when VSV was treated with 0.05 mM DEPC, while a mortality of 35% was observed for mice inoculated with the 0.1 mM treated sample. Such mortality rate shows that the mouse model is really sensitive to VSV infection since in this case the number of infectious particles inoculated was very low (the amount of infectious virus injected in the 0.1 mM treated sample was approximately 20 virions assuming an approximate 5log drop in titer due to treatment with this DEPC concentration, as shown in Fig. 1b). In contrast, for the groups inoculated with VSV treated with 0.2 and 0.5 mM DEPC the mortality rate was null. To confirm the viral inactivation in vivo, we performed pathological analysis of the brains of the infected animals with hematoxylin and eosin stain. In mice infected with

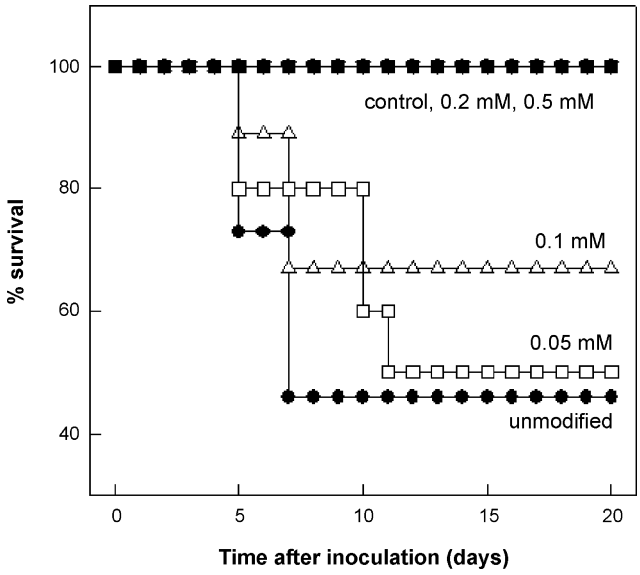

Fig. 3. Survival of BALB/c mice. BALB/c mice (10 per group) were inoculated intranasally with unmodified VSV (●) or VSV treated with 0.05 mM (□), 0.1 mM (△), 0.2 mM (▲) and 0.5 mM (▼) DEPC. The mortality rate was evaluated daily during 3 weeks. The control group was inoculated with PBS containing 0.5 mM DEPC (■).

unmodified VSV, we observed CNS inflammation, with complete destruction of the normal olfactory bulb parenchyma, and the presence of numerous neutrophiles at subpial region and in the lateral ventricles, characterizing viral meningitis and ventriculitis (Fig. 4a and c). However, brain from mice inoculated with VSV modified with 0.5 mM DEPC showed no inflammation (Fig. 4b and d).

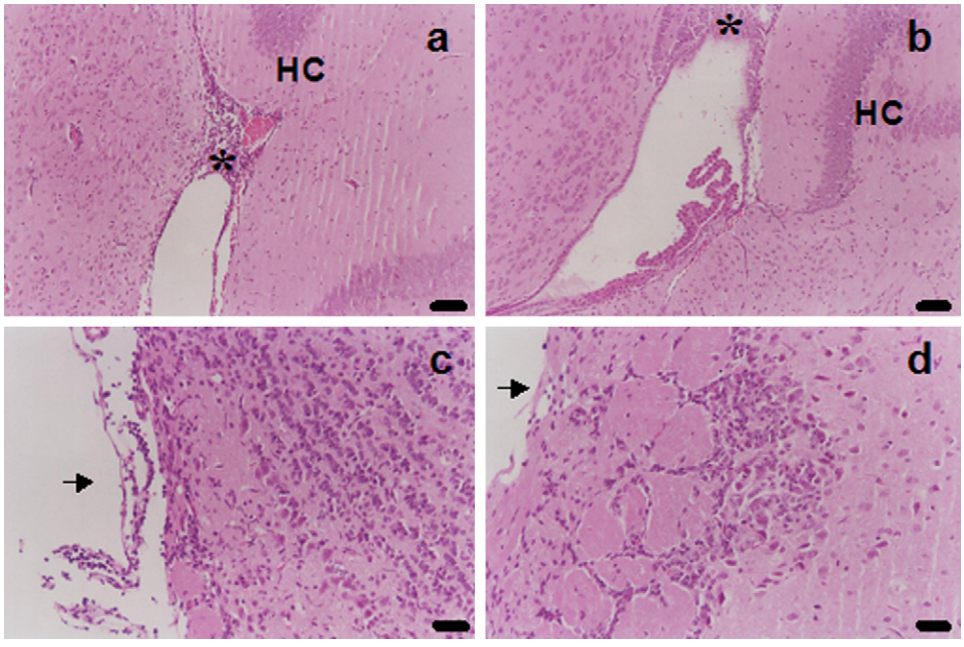

Fig. 4. DEPC-treated VSV does not cause viral encephalitis. Sections of brains showing the lateral ventricle (a and b) and the olfactory bulb (c and d) of mice infected with VSV (a and c) or VSV treated with 0.5 mM DEPC (b and d). In panels (a) and (c), there is a typical inflammatory infiltrate in the lateral ventricle, characterizing ventriculitis (*), and in subpial region of olfactory bulb (arrow), which is not observed in equivalent regions at panels (b) (*) and (d) (arrow). HC: hippocampus (A/B line = 50 mm); (C/D line = 12.5 mm).

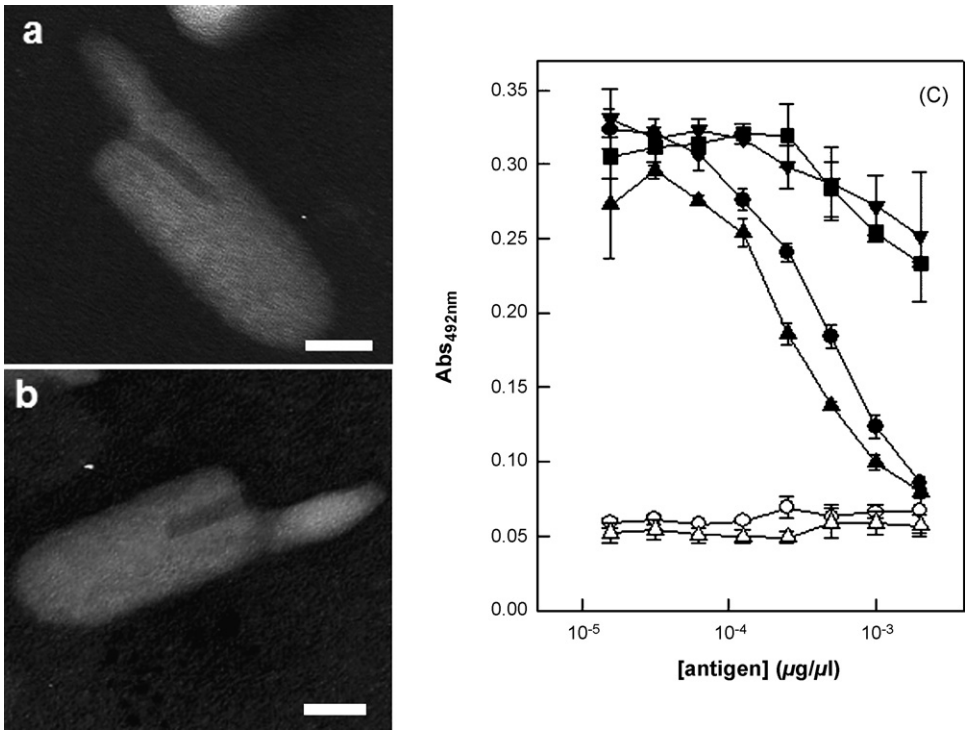

Fig. 5. Structural preservation of VSV after modification with DEPC. Transmission electron micrographs of (a) unmodified VSV, and (b) VSV treated with 0.5 mM of DEPC (bar = 50 nm). (c) Inhibition of binding of an immune serum against untreated VSV to plates coated with VSV by VSV modified with 0.5 mM DEPC (▲), or boiled (denaturated) VSV (■), assessed in a competitive ELISA. The maximal binding inhibition profile was determined using the untreated virus (●) as competition antigen, and bovine albumin (▼) was used as negative control. Unspecific binding of VSV (◯) or VSV treated with 0.5 mM DEPC (△) to sera was discarded using pre-immune sera.

3.4. Treatment with DEPC does not alter VSV structure

Transmission electron microscopy revealed that virus treated with 0.5 mM DEPC showed the same morphology of the unmodified VSV, suggesting that the structure of the inactivated virus was preserved (Fig. 5a and b). The preservation of G protein antigenic domains after DEPC treatment was analyzed by a competition ELISA (Fig. 5c). VSV modified with 0.5 mM DEPC bound to an immune serum against unmodified VSV with the same avidity as the unmodified virus. In contrast, virus denatured by boiling presented the same competitive activity as bovine albumin. These results show a clear difference between the antigenic properties of the DEPC-treated and heat-denatured viruses, although they are still preliminary to assure that the antigenic properties are completely preserved after DEPC treatment.

4. Discussion

In this study, we showed that treatment of VSV with DEPC results in a complete virus inactivation. We observed in cell culture that infectivity and viral replication were abolished by viral treatment with 0.5 mM DEPC and were partially inhibited at lower concentrations. Moreover, DEPC treatment also abolished VSV lethality in mice. The fact that DEPC is very hydrophilic (Miles, 1977) suggests that it should not be readily transported across the membrane. This was supported experimentally by Spires and Begenisich (1990), who showed different effects of DEPC on neurons when it was added to the intra- or to the extracellular media. Thus viral treatment with DEPC would not modify the enzymes located inside the virus particle, and the main mechanism of VSV inactivation by DEPC may reside in the reaction of this compound with the viral glycoprotein. In a previous study, we showed that 0.02 mM DEPC specifically modified G protein His residues, inhibiting pH-induced conformational changes on G protein and the fusion reaction catalyzed by the virus (Carneiro et al., 2003). However, we cannot exclude the possibility that other residues besides His could be modified after virus treatment with the higher DEPC concentration used here, since fusion and inactivation were not restored by incubation of the modified virus with hydroxylamine.

The membrane of some enveloped viruses fuses directly with the host cell plasma membrane through a pH-independent reaction, which is driven by virus binding to their cell receptor. On the other hand, many other enveloped viruses enter into the cells by the endocytic pathway and, for them, the membrane fusion reaction depends on the acidification of the endosomal medium. In this case, the low pH triggers conformational changes in the viral glycoproteins necessary for their conversion to the fusogenic state. For many viruses studied so far, the pH thresholds for fusion range between 5.0 and 6.5 (Chan and Kim, 1998; Gaudin et al., 1991; White et al., 1981), suggesting that His protonation (pKa ∼ 6.0) might be a more general requirement for the conversion of viral glycoprotein from the non-fusogenic to the fusogenic conformation. However, it could not be discarded that His residues of any viral protein (even from viruses that fuse directly to plasma membrane or non-enveloped viruses) may be

necessary for other events during the infection cycle, and then their modification with DEPC could inhibit infectivity through a mechanism different from fusion inhibition.

Other three compounds described previously promote viral inactivation by inhibiting membrane fusion: cyanovirin-N (Dey et al., 2000), hypericin and rose bengal (Lenard et al., 1993; Lenard and Vanderoef, 1993). Fusion inhibition due to the treatment of virus particles with hypericin or rose bengal results from the cross-linking of viral membrane proteins. The molecular mechanism of fusion inhibition promoted by cyanovirin-N involves physical interactions with gp120 envelope proteins (Boyd et al., 1997). Further studies on this mechanism revealed that cyanovirin-N binds to high-mannose oligosaccharides on gp120 (Bewley and Otero-Quintero, 2001; Bolmstedt et al., 2001; Shenoy et al., 2001). Cyanovirin-N was also shown to be potently active against other enveloped viruses containing similar oligosaccharides (Barrientos et al., 2003; Dey et al., 2000; O'Keefe et al., 2003). The immunogenic properties of virus inactivated by these compounds have not yet been evaluated.

In the past, the basic mechanism of enveloped viruses inactivation was to inhibit the virus entry in host cells at the adsorption stage, e.g., formalin (Bachmann et al., 1993), detergent (Seitz et al., 2002) and UV light (Bay and Reichmann, 1979). However, these inactivation procedures drastically impair induction of neutralizing IgG responses for most viruses (Bachmann et al., 1994), since these treatments led to the denaturation of viral surface proteins.

The use of DEPC for virus inactivation opens new possibilities for the development of safe vaccines. First, the inactivation by this compound is stable, since it covalently modifies histidines. Second, our present data indicate that DEPC treatment seems to preserve VSV structure. Ultrastructural morphology analyses showed that VSV treated with DEPC is similar to unmodified VSV. Furthermore, in marked contrast to virus inactivated by heat, DEPC-treated virus was recognized by antibodies against the unmodified VSV with the same avidity. However, although this data is suggestive that inactivated virus preserves the antigenic properties of the surface G proteins, further work is needed to unequivocally show that virus antigenicity is not altered, including studies on its reactivity against a panel of monoclonal antibodies. Nevertheless, the maintenance of conformation integrity in DEPC-inactivated virions suggests that such virions may be a useful in the pursuit of a potential vaccine antigen.

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