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In vivo antiviral activity of ribavirin/alpha-cyclodextrin complex: Evaluation on experimental measles virus encephalitis in mice

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Abstract

Intracranial injection of the rodent adapted CAM/RB strain of measles virus (MV) induces encephalitis in CBA/ca mice. It has already been shown that cyclodextrins can be used as carriers to increase the antiviral activity of ribavirin (RBV) against MV in cellular model. In this study, the antiviral activity of a RBV/α-cyclodextrin complex has been evaluated *in vivo* using the above model. CBA/ca mice were treated by intraperitoneal injection of free ribavirin (40 mg/kg) or a RBV/α-cyclodextrin complex (molar ratio 1:3). After 21 days, intracerebral injection of CAM/RB resulted in 100% mortality in the mock group. In contrast, mortality rates of 80% and 40%, respectively, were observed in RBV and RBV/α-CD-treated mice ($p < 0.05$ and $p = 0.06$ for distilled water and RBV, respectively). The viral load of MV in the mouse brain was monitored daily by real-time PCR until day 6 after infection, to compare virus production in treated and non-treated mice. This data shows that RBV complexation with α-cyclodextrin can increase the antiviral activity of ribavirin in a measles virus encephalitis model in mice.

Keywords: Measles virus; Encephalitis; Ribavirin; Cyclodextrin; RT-PCR; Survival rate

1. Introduction

Measles virus (MV) is responsible for a common childhood disease, which can be complicated in 10% of cases by otitis, pneumonia, diarrhea and encephalitis (Schneider-Schaulies and ter Meulen, 2002). Despite massive immunization campaigns, about 30 million measles virus infections have resulted in 530,000 deaths in 2003 (Muller et al., 2007). No chemotherapeutic agent is currently approved for the prophylactic or therapeutic treatment of measles. Ribavirin (RBV), a broad-spectrum antiviral agent, presents *in vitro* activity against numerous viruses including MV (Huffman et al., 1973). It has been reported that patients with severe measles infections may benefit from intravenous RBV injection (Forni et al., 1994). Antiviral activity of RBV against MV has already been assessed in hispid cotton rats, infected by either aerosol or intraperitoneal route (Wyde et al., 2000) and in hamsters after intracranial injection (Honda et al., 1994). However, intraperitoneal, subcutaneous or intramuscular injection of RBV was ineffective in mice inoculated intracerebrally with either rabies or Herpes Simplex Virus type 1, suggesting a failure of RBV to cross the blood–brain barrier (Bussereau et al., 1988; Sidwell et al., 1973) and to treat ribavirin-sensitive viral encephalitis.

Since cyclodextrins (CDs) have been proven to be permeation enhancers on caco-2 cell monolayer as an intestinal model (Hovgaard and Brondsted, 1995) and as caco-2 cell monolayer has already been used to predict permeability for a drug towards the blood–brain barrier (Lohmann et al., 2002), CDs could be used as permeation enhancers on the blood–brain barrier. The most common native CDs are α-, β- or γ-CD constituted by 6, 7 or 8 α-1,4-linked glucopyranose units, respectively. They are cyclic oligosaccharides with a hydrophobic central cavity which can form complexes with numerous molecules, and improve their bioavailability and their biological properties (Zhang and Rees, 1999).

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In the case of RBV, cyclodextrin interest does not focus on dissolution rate improvement because of high solubility on an aqueous medium. It focuses rather on antiviral drug access to the brain enhancement. After Grancher et al. (2005) proved the existence of a 1:1 complex between ribavirin and α-, β- and γ-CD by physicochemical studies, the authors demonstrated that RBV/α-CD and RBV/β-CD complexes increase RBV activity against MV *in vitro* (Grancher et al., 2004).

In the present work, we describe the first *in vivo* evaluation of an antiviral drug/cyclodextrin complex on central nervous system infection. After development of MV intracerebral inoculation and determination of lethal dose 50 (LD50) of free or complexed RBV and α-CD, RBV/α-CD complex was tested in an experimental measles virus encephalitis model using CBA/ca mice infected with the rodent adapted CAM/RB strain of MV (Niewiesk et al., 1993).

2. Materials and methods

2.1. Virus

A working stock of the rodent brain-adapted CAM/RB strain (kindly provided by Prof Liebert, Leipzig, Germany) was prepared in phosphate-buffered saline (PBS), from the brain homogenates of 5-day-old CBA/ca mice, 3 days after intracerebral injection. The same homogenate was used for all *in vivo* experiments. The virus titer (7.40 $\log_{10}$ Eq. PFU/ml) was determined by limit dilution (Wyshak and Detre, 1972).

2.2. Animals

CBA/ca mice of either sex (weight range 10–14.5 g at experimental onset) from B&K Universal (East Yorkshire, England) were maintained in plastic cages under natural light cycle at 19 °C and 44–45% humidity. Food and water were provided *ad libitum*. All local legal and ethical requirements were observed. MV infected and control animals were housed in separate rooms.

2.3. Intracerebral MV challenge

On day 0, 3–4-week-old CBA/ca mice were anesthetized by intraperitoneal administration of 50 μl of a solution containing 6.25 mg/ml ketamine, 6.25 mg/ml xylazine and 0.25 mg/ml atropine. Afterwards, 1×10^3 PFU of MV CAM/RB (5× LD50) in 30 μl PBS were injected into the right temporal fontanel using an insulin syringe and a 0.33 mm × 12.7 mm needle. A rubber septum threaded on the needle was used as a stopper to standardize the depth of penetration at 5 mm in the right hemisphere (confirmed by an i.c. methylene blue injection). Weight and clinical symptoms were registered daily for 15 days.

2.4. Lethal dose 50 of MV CAM/RB strain

The LD50 of MV CAM/RB was determined using 4 groups of eight 3–4-week-old mice inoculated with 10 to 10,000 PFU of MV CAM/RB in 30 μl of PBS. The LD50 was determined by interpolation after checking the mortality rate daily during 21 days. At the end of the experiment all mice were sacrificed.

2.5. Preparation of RBV/α-CD complexes

Both RBV (MP Biomedicals) and native α-CD (Wacker-Chemie GmbH) were dissolved in distilled water and sterilized by filtration through a 0.22 μm membrane. The RBV/α-CD complex was prepared at a molar ratio of 1:3 in distilled water as previously described (Grancher et al., 2004). Concentrations of RBV/α-CD solutions are expressed as RBV concentrations.

2.6. LD50 of RBV and RBV/α-CD complex

The LD50 of free or complexed RBV and α-CD were determined using groups of five to seven 3–4-week-old mice. Each animal received a single dose per treatment. Tested doses ranged between 486 and 2916 mg/kg, 20 and 480 mg/kg and 30 to 80 mg/kg, respectively, for α-CD, RBV and RBV/α-CD (1:3). The highest dose of RBV corresponded to its limit of solubility. LD50 was calculated as described above.

2.7. Treatment

Mice were weighed and treated once a day during 10 days by intraperitoneal injection of RBV (40 mg/kg), RBV/α-CD (1:3) complex (corresponding to 40 mg/kg of RBV) or distilled water from the day before challenge until the end of the experiment. Each treatment group contained 15 mice for mortality evaluation. The benefit of treatments was first estimated by checking the mortality and then by determining the viral load as described below.

2.8. RNA isolation from the brain

Groups of 29, 31 and 29 mice treated with RBV, RBV/α-CD and distilled water, respectively, were infected to monitor the viral load between day 2 and day 6. From days 2 to 6 after challenge, 3 to 10 mice per treatment group were sacrificed each day. Total RNA was isolated from whole brain using 2 ml TRIzol® reagent (Invitrogen) following the manufacturer's instructions. Purity and concentration of total RNA were measured by UV spectrophotometry (GeneQuant II, Pharmacia Biotech) and its concentration was adjusted to 0.5 μg/μl.

2.9. RNA quantification

MV-nucleoprotein (MV-NP) mRNA was detected by a two-step quantitative reverse transcription-polymerase chain reaction (RT-PCR) using the primers and TaqMan probe® as described by Uhlmann et al. (2002). Minus strand synthesis of total cDNA was carried out at 48 °C for 30 min using 10 μl of RNA, 50 U multiscribe RT, 40 U RNAse inhibitor and 0.8 μM gene specific primer in a 25 μl reaction containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 0.6 mM dNTPs. Amplification of MV-NP cDNA by PCR was performed using PCR TaqMan Master Mix 2×, on 10 μl RT

product in the presence of 0.2 μM forward and reverse primers and 0.1 μM TaqMan probe. Cycling conditions were activation (95 °C, 10 min), 40 cycles of annealing (95 °C, 15 s) and amplification (60 °C, 1 min) using ABIPrism 7000 thermocycler. Absolute quantification was performed using a MV-NP cDNA standard and data analysis was performed using ABI Prism® 7000 Sequence Detection System (Applied Biosystems).

2.10. Internal standard using house-keeping gene

The presence of murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined in all RNA samples. The RT was performed for 60 min at 37 °C and 10 min at 98 °C with 10 μl of RNA in a total volume of 25 μl using the reaction mix described above and 0.8 μM of reverse primer (5′-CGT GGT TCA CAC CCA TCA CAA A-3′). Specific cDNA was amplified using PCR Syber Green Master Mix 2× (Applied Biosystems, Courtaboeuf, France) including 0.2 μM of forward primer (5′-AAT TCA ACG GCA CAG TCA AGG C-3′), 0.2 μM of reverse primer. 10 μl of cDNA sample was added for a total volume of 50 μl. The PCR was performed on an ABI Prism 7000 (Applied Biosystems) using the conditions described above.

2.11. Preparation of the measles cDNA standard

The product of a conventional RT-PCR carried out on the N gene of the MV CAM/RB genomic RNA was subcloned into the vector pCR® 4-TOPO® using TOPO TA Cloning® Kit for sequencing (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions. Plasmid was linearized, purified and transcribed into RNA. The cDNA was obtained after RT step with reverse primer as described above. After quantification, serial dilution of measles cDNA standard was made in water.

2.12. Statistics

Mean virus titers of the treated and untreated groups were compared using Mann–Whitney nonparametric statistical test and survival-rate curve by nonparametric Kaplan Meier test.

3. Results

3.1. In vivo toxicity

The LD50s of α-CD, RBV and the RBV/α-CD (1:3) complex were determined after intraperitoneal injection in CBA/ca mice. RBV was devoid of any lethal effect even at the maximum dose (480 mg/kg). The LD50 of α-CD alone was 850 mg/kg. The LD50 of the RBV/α-CD (1:3) complex was 55 mg/kg of RBV and 657 mg/kg of α-CD. These findings enable mice to be treated with 40 mg/kg of free or complexed RBV.

3.2. Intracerebral infection with measles virus

Mice were challenged with 1000 PFU of MV CAM/RB, corresponding to about 5 times the LD50 (213 PFU per mouse) on day 0. Three groups of 15 mice treated during ten days (day-1 to day 8) with either distillated water, RBV and RBV/α-CD were used. From day 4 on, animals started to show encephalitis signs, including limb weakness, tremors, arched vertebral column and hyperactivity to different degrees. Mock-treated mice developed more overt symptoms and in a greater proportion (data not shown).

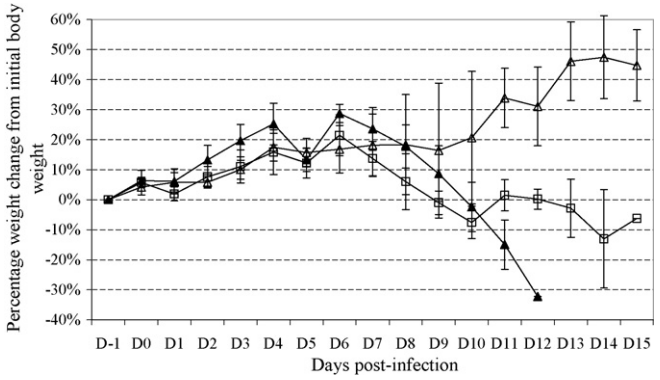

Fig. 1. Percentage weight change in CBA/ca mice intracranially injected with measles virus CAM/RB strain according to treatment with distillated water (▲), ribavirin (40 mg/kg) (□) and ribavirin/alpha-cyclodextrin complex (molar ratio 1:3; 40 mg/kg of ribavirin) (△).

Daily body-weight variation was determined as a marker of morbidity after virus-injection. For the first 7 days (day-1 to day 5), mice of all groups continued to thrive irrespective of the treatment (0.5 g/day on average), except after intracranial injection (day 1, day 2) where weight did not increase. From day 6 on, the weight of mock-treated mice continually decreased until day 12, whereas the weight of RBV-treated mice stayed unchanged until day 14, even for recovering animals. Only RBV/α-CD-treated surviving mice continued to gain weight (Fig. 1).

3.3. Effect of RBV and RBV/α-CD treatment

On day 21 mortality rates of 100, 80 and 40% were determined for the groups treated with distilled water, RBV and RBV/α-CD, respectively. Infected mice treated with distilled water died on days 8–12 post-infection. RBV-treated mice (40 mg/kg) have a mean death-time of 11.5 days (range 8–15 days) after virus injection (Fig. 2). The survival rate of RBV/α-CD-treated mice was significantly higher ($p < 0.05$) compared to mock-treated mice. Although more mice survived in the RBV/α-CD-treated group compared to RBV-treated group, the difference was not statistically significant ($p = 0.06$). Similarly, the difference in survival rates between RBV- and mock-treated mice was not significant ($p = 0.2$).

3.4. Measles viral load in the brain of infected mice

Virus infection was assessed by quantifying a significant increase in MV RNA copy numbers (range 6.8–8.8 $\log_{10}$ Eq. copies/ml) between days 2 and 5 in all treatment groups (Table 1). Between days 5 and 6, the viral load further increased in the mock- and RBV-treated groups, and slightly decreased in the RBV/α-CD-treated group. Thus,

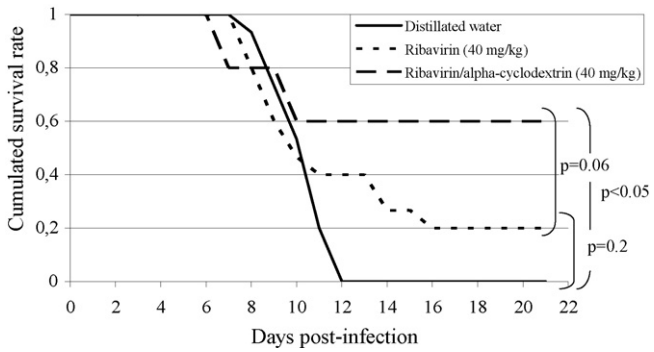

Fig. 2. Survival rates after intracranial challenge of CBA/ca mice with measles virus CAM/RB according to treatment with distilled water, ribavirin (40 mg/kg), ribavirin/alpha-cyclodextrin complex (molar ratio 1:3; 40 mg/kg ribavirin). 15 animals per group.

on day 6, the viral load was significantly lower (minus 0.60 $\log_{10}$ Eq. copies/ml) in the RBV/α-CD-treated group compared to RBV- and mock-treated groups ($p = 0.02$ and 0.04).

4. Discussion

RBV has an antiviral activity against MV replication both *in vitro* (Huffman et al., 1973) and *in vivo* (Forni et al., 1994). Although conflicting results about its clinical efficiency have been reported: in measles pneumonitis and encephalitis in children treated for malignant disease, RBV treatment was not clearly effective (Kernahan et al., 1987). It is thought that RBV does not cross the blood–brain barrier (Morrey et al., 2002; Sidwell et al., 1973) most probably because of its low lipid solubility, which led to CD evaluation as a drug carrier towards the blood–brain barrier.

Determination of the complex antiviral activity necessitated *in vivo* toxicity evaluation on mice. The LD50 of α-cyclodextrin in CBA/ca mice after intraperitoneal injection is closely related to those obtained after intravenous injection in mice (LD50 of 842–1070 mg/kg) and in rats (LD50 of 788 mg/kg) (Frank et al., 1976; Uekama and Otagiri, 1987). The complex ribavirin/α-cyclodextrin at molar ratio 1:3 is more toxic than both molecules alone at the same dose and than the complex ribavirin/β-cyclodextrin at molar ratio 1:1 (Jeulin et al., 2006). Behaviour of cyclodextrins on an *in vitro* model of blood–brain barrier has been described and the threshold of toxicity was 1 mM for native α-cyclodextrin, explained by efflux of phosphatidylcholine, sphyngomyelin and cholesterol (Monnaert et al., 2004). *In vivo* α-cyclodextrin concentration in the blood–brain barrier could not be evaluated in our model, but the slight removal of cholesterol may result in an increase in membrane fluidity and explain both toxicity and antiviral activity improvement. Alpha-cyclodextrin toxicity on the blood–brain barrier could be reduced by using α-cyclodextrin derivatives (Monnaert et al., 2004). For example, sulfated amphiphilic α-, β- and γ-cyclodetxrin are in the course of development for complexation with another antiviral molecule, acyclovir (Dubes et al., 2003). Data of antiviral activity has not yet been published.

We have previously demonstrated that the complex RBV/α-CD enhances the antiviral activity of RBV on MV *in vitro* (Grancher et al., 2004). Here, for the *in vivo* antiviral activity evaluation, we have chosen weaning CBA/ca mice (3–4-week-old) susceptible to rodent brain adapted and neurovirulent measles CAM/RB strain (Niewiesk et al., 1993). In this model, the susceptibility of MV is age related (Griffin et al., 1974). Inoculation of 3–4-week-old mice with CAM/RB strain requires a significantly higher LD50 (212.7 PFU versus 27 PFU) than for 1–2-day-old CBA mice using Edmonston strain (Neighbour et al., 1978). A higher virus dose injection on older mice was preferred because of frequent cannibalism of 1–2-day-old MV infected mice by their mother, observed in pilot experiments. To avoid any influence of age on the results, mice from the same litter were randomly distributed between the different time and treatment points. Increasing measles viral load from day 2 to day 6 indicated an active infection in the brain. Clinical signs of infection were first apparent 4 days after inoculation and infected mice exhibited some growth retardation when compared with control mice as previously described by Neighbour et al. (1978). After day 6 the mortality of mock- and RBV-treated mice did not allow us any comparison between the 3 groups of animals.

Survival rates, used to evaluate the therapeutic effect of the treatment, showed a significant improvement ($p < 0.05$) for treatment by the complex at a dose of 40 mg/kg per day (60% survival of infected mice 21 days after MV virus injection). Thus the same dose of RBV had an effect only if complexed with α-CD. This is in agreement with Honda's results showing no efficacy

Table 1
Comparison of the effects of intraperitoneal treatment with distilled water, ribavirin (RBV) and ribavirin/α-cyclodextrin (RBV/α-CD) at molar ratio 1:3 on a measles virus encephalitis model in mice

Treatment	Mean of virus titer ($\log_{10}$ Eq. copies/ml) ± standard deviation and GADPH load ($\log_{10}$ Eq. copies/ml)				
	Day 2	Day 3	Day 4	Day 5	Day 6
Distilled Water (10 μL)	6.80 ± 0.2 ($n = 3$)	7.44 ± 0.14 ($n = 3$)	8.10 ± 1.00 ($n = 9$)	8.30 ± 0.20 ($n = 6$)	9.10 ± 0.12 ($n = 8$)
GADPH	5.86	5.92	5.78	5.86	5.80
Ribavirin (40 mg/kg)	6.80 ± 0.33 ($n = 3$)	7.00 ± 0.19 ($n = 3$)	7.66 ± 0.13 ($n = 9$)	8.80 ± 1.00 ($n = 6$)	9.10 ± 0.80 ($n = 10$)
GADPH	5.99	5.94	5.92	5.77	5.76
RBV/α-CD (1:3) (40 mg/kg of RBV)	7.10 ± 0.11 ($n = 3$)	6.80 ± 0.03 ($n = 3$)	7.99 ± 0.17 ($n = 8$)	8.54 ± 0.4 ($n = 6$)	8.48 ± 0.20 ($n = 9$)
GADPH	5.84	5.72	5.83	5.92	5.75

n: number of mice in each group. GADPH load was used as an internal standard.

of free RBV in measles infected hamsters, when administered intraperitoneally at the dose of 50 mg/kg/day (Honda et al., 1994).

Our results thus indicate that the *in vivo* antiviral effect of RBV relies on its combination with α-CD. A previous study using the complex ribavirin/β-cyclodextrin (1:1) indicated no significant improvement of antiviral activity *in vivo* (Jeulin et al., 2006), in accordance with the *in vitro* results (Grancher et al., 2004), whereas β-cyclodextrin showed the best interaction with ribavirin regarding its higher stability constant (Grancher et al., 2005).

Other carriers of RBV have already been studied *in vivo*, for instance the lipophilic dihydropyridine (DHP)-hydrophilic pyridinium in a murine model with Japanese encephalitis virus (Prokai et al., 2000) with slight efficacy (40–50% survival). Liposomal encapsulated RBV was more efficiency than free RBV in treatment of mice infected with Rift Valley fever, influenza or herpes simplex viruses (Gangemi et al., 1987; Kende et al., 1985). Moreover positive results were obtained after intrathecal injection of RBV which avoids problems of brain accessibility but is obviously not routinely practical (Honda et al., 1994; Hosoya et al., 2004). The improved efficiency of RBV/α-CD complex *in vivo* could be explained by an enhancement of the passage through the blood–brain barrier. Thus it remains to be determined if the penetration of RBV is enhanced because of an alteration of the blood–brain barrier or to a facilitated transport. One other possibility is that the RBV/α-CD complex is significantly more stable than RBV alone and accumulates in the brain over time because of its slow clearance (Gilbert and Wyde, 1988). This needs to be examined more closely by monitoring RBV concentration into the brain by high performance liquid chromatography, as has been described in mice and hamsters (Gilbert et al., 1991; Ishii et al., 1996).

In conclusion, the antiviral activity of RBV was only effective when administered after complexation with α-CD. The lower viral load in the RBV/α-CD-treated group compared to the RBV alone treated group further indicates that the complexation of RBV with α-CD increases its bioavailability, and thus its antiviral effect in the brain. No measles viral load was found in recovered animals (day 21).

The CBA/ca mice model described herein is the first study of vectorisation of RBV on the measles infection and the first *in vivo* evaluation of an antiviral activity of RBV/α-CD complex. It will provide opportunities to test other compounds for treatment of measles or other viral encephalitis.

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