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Combination therapy of rabies-infected mice with inhibitors of pro-inflammatory host response, antiviral compounds and human rabies immunoglobulin

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ABSTRACT

Recent studies demonstrated that inhibitors of pro-inflammatory molecular cascades triggered by rabies infection in the central nervous system (CNS) can enhance survival in mouse model and that certain antiviral compounds interfere with rabies virus replication *in vitro*. In this study different combinations of therapeutics were tested to evaluate their effect on survival in rabies-infected mice, as well as on viral load in the CNS. C57Bl/6 mice were infected with Silver-haired bat rabies virus (SHBRV)-18 at virus dose approaching LD_{50} and LD_{100}. In one experimental group daily treatments were initiated 4 h before-, in other groups 48 or 96 h after challenge. In the first experiment therapeutic combination contained inhibitors of tumour necrosis factor-α (infliximab), caspase-1 (Ac-YVAD-cmk), and a multikinase inhibitor (sorafenib). In the treated groups there was a notable but not significant increase of survival compared to the virus infected, non-treated mice. The addition of human rabies immunoglobulins (HRIG) to the combination in the second experiment almost completely prevented mortality in the pre-exposure treatment group along with a significant reduction of viral titres in the CNS. Post-exposure treatments also greatly improved survival rates. As part of the combination with immunomodulatory compounds, HRIG had a higher impact on survival than alone. In the third experiment the combination was further supplemented with type-I interferons, ribavirin and favipiravir (T-705). As a blood-brain barrier opener, mannitol was also administered. This treatment was unable to prevent lethal consequences of SHBRV-18 infection; furthermore, it caused toxicity in treated mice, presumably due to interaction among the components. In all experiments, viral loads in the CNS were similar in mice that succumbed to rabies regardless of treatment. According to the findings, inhibitors of detrimental host response to rabies combined with antibodies can be considered among the possible therapeutic and post-exposure options in human rabies cases.

1. Introduction

Rabies encephalitis is one of the most devastating zoonotic diseases being responsible for more than 60,000 human deaths worldwide, annually [1]. Although both preventive and post-exposure vaccinations can be applied to prevent rabies-related fatalities

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and there is an increasing number of reports about survival of rabies in animals and humans [2–4], there is still no therapeutic option available to date, which could reliably prevent lethal consequences of the disease with overt clinical signs [1,5]. Our current knowledge about the pathogenesis of rabies and experiences in efforts to treat infected patients highlight that the favourable approach in treatment is combination therapy; using compounds with various mechanisms of action [1,3,6].

The immunological background of rabies virus (RABV) infection in the host is highly complex and still not yet entirely understood, involving a wide variety of cytokines and effector cells related to the innate and adaptive immune system [7]. Nevertheless, induction of pro-inflammatory signalling pathways and certain detrimental responses of the immune system triggered by RABV in the central nervous system (CNS) are known to contribute to deterioration in rabies encephalomyelitis [5,8]. Cascades induced by mitogen-activated protein (MAP-) kinases [9,10] and caspase-1-mediated pyroptosis [11,12] are key elements in RABV pathogenesis and are associated with pro-inflammatory effects in the brain during infection, as well as the overexpression of cytokines like tumour-necrosis factor-α (TNF-α) [13]. Moreover, prominent anti-rabies effects have been achieved with the inhibition of certain MAP kinases *in vitro* using sorafenib [14] and *in vivo* using U0126 [9].

We hereby report the results of *in vivo* experiments on mice infected with silver-haired bat rabies virus (SHBRV)-18 (a wild-type rabies virus strain of bat origin), and treated with different combinations of immunomodulatory compounds, human rabies immunoglobulins (HRIG) and viral replication inhibitors.

In the first experiment, inhibitors of MAP kinases (sorafenib), caspase-1 (acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethylketone [Ac-YVAD-cmk]) and TNF-α (infliximab) were included in the anti-rabies combination. In the second, this combination was supplemented with HRIG, based on reports about correlation between the quantity of neutralizing antibodies in the host and survival [2,3,15]. In the third experiment, antivirals (type-I interferons, ribavirin and favipiravir) were also administered, along with the opening of the blood-brain barrier (BBB) using mannitol-mediated osmotic disruption [16,17]. The inhibitory effect of interferons, ribavirin and favipiravir on RABV replication is well described both *in vitro* and *in vivo* [13,18–20]. Opening of the BBB helps immune effectors to invade the CNS, thus it can increase virus clearance from the brain and survival of the disease [7,21].

2. Materials and methods

2.1. Virus and compounds

Silver-haired bat rabies virus (SHBRV-18), a street rabies strain from bat origin [22] was obtained from the Thomas Jefferson University (Philadelphia, PA, USA) and propagated in mouse neuroblastoma (N2A) cells using Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Walkersville, MD, USA) supplemented with 10% foetal bovine serum (FBS, Biowest, Nuaillé, FR) and antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). Two different virus stocks were established and subsequently used in the experiments: one at a titre of $10^{5.2}$ $TCID_{50}$/ml (consistent with LD_{50} in the used mouse model) and another at $10^{6.8}$ $TCID_{50}$/ml (consistent with LD_{100}).

The tumour necrosis factor-α-inhibitor infliximab (Remicade, Janssen Biotech) was obtained from the pharmacy at the University Hospital in Leuven, Belgium; the caspase-1-inhibitor Ac-YVAD-cmk was purchased from Invivogen (San Diego, CA, USA); the multikinase inhibitor sorafenib (Nexavar tablets; 200 mg of sorafenib-tosylate) was purchased from Bayer Pharma AG (Berlin, D). Human rabies immunoglobulin (HRIG: WHO International Standard, the 2nd International Standard for ANTI-RABIES IMMUNOGLOBULIN, HUMAN) was obtained from NIBSC (London, UK). Recombinant mouse interferons (IFN-α and -β), ribavirin (Virazole) and favipiravir (T-705) were purchased from Merck (Darmstadt, D), from ICN Pharmaceuticals (Costa Mesa, CA, USA) and from BOC Sciences (New York, USA), respectively. Mannitol was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animal models and ethical statement

The inbred mouse strain C57Bl/6 was chosen for the animal experiments in accordance with numerous former studies [23–27]. Six-weeks-old female mice were ordered from Envigo Laboratories (Lake Tahoe, NV, USA). Animals were housed in BSL-3 (biosafety level 3) animal facility, in individually ventilated rodent cages. Mice had constant access to water and food, the lighting period was 12 h long daily. Experiments started after an acclimatization period of one week. All procedures during experiments were carried out according to the guidelines and regulation about animal experiments of Hungary and the Czech Republic, with the permission of the Government Office of Pest County, Food Chain Safety and Animal Health Directorate (permission number PEI/001/77-2/2014) and Institutional Expert Committee and the Ministry of Agriculture of the Czech Republic (permission number MZe 1627). To minimize suffering, mice reaching humane endpoints were euthanized by cervical dislocation under isoflurane anaesthesia. Humane endpoints were defined according to the clinical scoring system for rabies infection in mice published by Healy et al. [6] as the clinical score 3 for wild-type RABV infection (hind quarter paralysis and severe spasms).

2.3. Infection and treatment of animals

Mice were infected with SHBRV-18 virus strain at a dose of either $10^{5.2}$ $TCID_{50}$/ml (LD_{50}; first experiment) or $10^{6.8}$ $TCID_{50}$/ml (LD_{100}; second and third experiment) under isoflurane anaesthesia. 50 μl of undiluted virus suspension at the desired titre (i.e. $10^{3.9}$ and $10^{5.5}$ $TCID_{50}$/mouse) was inoculated to the left hind leg (intramuscularly) of the animals. Mice were assigned to different experimental groups using an online randomizer software (https://www.random.org/sequeces). Experimental groups included a virus control group, and different therapy control groups apart from the virus-infected and treated groups. The animals of the therapy control groups were inoculated with phosphate-buffered saline (PBS) instead of virus suspension. In the first and second experiment treatment was initiated either pre-exposure (4 h before infection) or (in other groups) post-exposure (48 or 96 h after infection), and then for 8 days (first experiment) or 10 days (second experiment) thereafter. In the third experiment there was only one treated group, where the start of treatment was 96 h post-infection (Table 1). In case of the virus control group no therapeutic compounds were administered, PBS was used instead.

Different combinations of the compounds were prepared for the treated groups of the experiments. The compounds were diluted in the diluent suggested by the manufacturer, or according to the literature (type-I interferons and ribavirin: PBS; infliximab and HRIG: water for injection; Ac-YVAD-cmk: dimethyl-sulfoxide (DMSO); favipiravir: 2.9% sodium bicarbonate solution (Sigma-Aldrich, St. Louis, MO, USA) [28,29]; sorafenib: DMSO in the first and second experiment, aqueous solution containing 5% cremophor (Sigma-Aldrich, St. Louis, MO, USA) and 5% ethanol (Molar, Budapest, H) in the third experiment [30–32]). The combinations used for the different treated groups in each experiment are presented in Table 1. The final volume of the therapeutic combination was

Table 1
Experimental groups and therapeutic combinations of compounds.

First experiment (n=70)			
Groups	**number of mice/group**		
start of treatment / ***group type***	-4 hours*	48 hours PI	96 hours PI
Virus control (mock-treated)	13		
Therapy control (mock-infected)	6	6	6
Infected and treated	13	13	13
Combinations			
compound	***concentration (/mouse/day)***		
TNF-α inhibitor (infliximab)	0.1 mg		
Casp-1 inhibitor (Ac-YVAD-cmk)	0.2 mg		
MAP kinase inhibitor (sorafenib)	0.6 mg		
Second experiment (n=96)			
Groups	**number of mice/group**		
start of treatment / ***group type***	-4 hours*	48 hours PI	96 hours PI
Virus control (mock-treated)	26		
Therapy control (mock-infected)	6	6	6
Infected and treated	13	13	13
HRIG control**	13		
Combinations			
compound	***concentration (/mouse/day)***		
TNF-α inhibitor (infliximab)	0.1 mg		
Casp-1 inhibitor (Ac-YVAD-cmk)	0.2 mg		
MAP kinase inhibitor (sorafenib)	0.6 mg		
HRIG	0.8 IU		
Third experiment (n=39)			
Groups	**number of mice/group**		
start of treatment / ***group type***			96 hours PI
Virus control (mock-treated)			13
Therapy control (mock-infected)			13
Infected and treated			13
Combinations			
compound	***concentration (/mouse/day)***		
TNF-α inhibitor (infliximab)	0.1 mg		
MAP kinase inhibitor (sorafenib)	0.6 mg		
HRIG	0.8 IU		
ribavirin	3 mg		
favipiravir	6 mg		
IFN-α	6000 IU		
IFN-β	6000 IU		
mannitol***	500 μl of 25% mannitol in saline		

* Before infection; ** control group with HRIG monotherapy; *** mannitol was administered 30 min after the other compounds; n: total number of mice/experiment; PI: post infection.

supplemented to 1 ml (with PBS) in all cases, and administered intraperitoneally (ip.) to the animals once daily for 8 or 10 days after the first administration. In the third experiment 500 μl of a 25% mannitol solution (dissolved in PBS) was administered (ip.) to open the blood-brain barrier 30 min after the inoculation of the therapeutic combination on every day of treatment. Body weight of mice was measured on the day of challenge and daily thereafter. The clinical status of the animals was checked twice daily (in the morning before starting the treatment and once in the late afternoon) during the entire experiments. Mice reaching clinical endpoints were euthanized and samples from the CNS and parenchymal organs were collected for virological analysis and immunohistochemistry (IHC). On the final day of experiments, all surviving mice were euthanized and samples were collected for subsequent studies.

2.4. Real-time reverse transcription PCR

The RABV RNA load in brain and spinal cord samples of experimental mice was quantified using real-time reverse transcription

PCR (qRT-PCR). After the extraction of viral RNA (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, D), SYBR Green qRT-PCR was performed using Verso 1-step RT-qPCR SYBR Green ROX Kit (Thermo Fisher Scientific, Waltham, MA, USA) with the primers previously described [33]. RNA copy numbers were determined based on a standard curve of *in vitro* transcribed SHBRV-18 RNA of known titre.

2.5. Immunohistochemistry assay

Paraffin-embedded CNS samples were cut into 4-μm thick sections and stained with hematoxylin and eosin (H&E). The same samples were also submitted for immunohistochemical investigation in order to localize and quantify the presence of RABV antigen within histological lesions. After dewaxing of sections and antigen retrieval (0.05% protease XIV solution [Sigma Aldrich, St. Louis, MO, USA] at 37 °C for 5 min) samples were incubated in 3% H_2O_2 solution for 10 min, followed by 20 min treatment with the Vectastain blocking solution (Vectastain ELITE ABC Peroxidase Kits Goat IgG 1 kit PK6105, Vector Laboratories Inc., Burlingame, CA, USA). The anti-Rabies FITC conjugated monoclonal antibody No. 5199i (Chemicon International, Temecula, CA, USA) was added to the sections at 1:400 dilution and incubated at 37 °C overnight. Antibody binding was detected by the ABC peroxidase system (Vectastain ELITE ABC Peroxidase Kits Goat IgG 1 kit PK6105, Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Slides were evaluated in a semiquantitative manner (0, +, ++, +++) based on degree of inflammation (mononuclear cell infiltration, perivascular lymphocytic cuffing) or the quantity of RABV-specific antigens found per high-power field. All samples were assessed by the same pathologist.

2.6. Fluorescent antibody virus neutralization test

Serum samples were collected from all surviving mice in virus-infected groups of the first experiment. Sera were heat inactivated at 56 °C for 30 min, then fluorescent antibody virus neutralization (FAVN) test was performed to determine the anti-rabies antibody contents, following the description of the chapter about rabies diagnostic methods in OIE (World Organisation for Animal Health) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [34]. Antibody titres were calculated based on the titration results of each serum sample and the OIE reference serum (0.5 IU/ml).

2.7. Data analysis

Kaplan-Meier survival curves of mice in different experimental groups were compared with the Mantel-Cox log-rank test. RNA load data resulted by qRT-PCR were compared between groups using the Kruskal-Wallis test followed by Dunn's multiple comparison test and 2-way ANOVA with Tukey test (dead versus survived). RABV-specific antigen levels in CNS samples determined by IHC were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Anti-RABV neutralizing antibody levels determined with FAVN were analysed with Student's *t*-test. All statistical tests were performed using the GraphPad Prism 5.04 software. Significance level was $P < 0.05$ in all cases.

3. Results

3.1. Clinical course and body weight changes

Mice were infected with the wild-type RABV strain SHBRV-18 and treated with different combinations of antiviral and immunomodulatory compounds in three separate experiments (see experimental setup in Table 1). The clinical course was similar in all animals in which the clinical signs of rabies appeared. Usually the disease started with ruffled hair and hunched back, but these were not always evident as in some mice the first signs affected the inoculated (left hind) leg: twitching and paralysis were observed, which was present in every case (clinical score (CS-) 1 [6]). This first stage of the clinical course was followed by recurrent and progressive spasms (CS-2). Once spasms became particularly severe (affecting the whole body with high intensity) the condition was considered to be consistent with CS-3. By this stage hind quarter paralysis also developed in most of the animals. The consecutive clinical scores followed each other in short time span, often overlapping, therefore the boundaries were not always obvious between them. Mice reaching CS-3 (humane endpoint) were euthanized. In most cases, the whole clinical course starting from the onset of CS-1 signs until euthanasia lasted 12–36 h. In the first experiment, when mice where infected with LD_{50} dose of virus, the first clinical signs appeared 6 days post infection (DPI), and the first mortality occurred 7 DPI. In the second and third experiments (challenge with LD_{100} virus dose) the first clinical signs appeared 5 DPI, whereas the first mouse reached human endpoints 6 DPI.

One mouse in the first experiment (infected and treated group, first administration of therapeutics 48 h post infection) reached CS-1 16 DPI (the latest among all mice in the experiment), its left hind leg was paralysed, but the progress of the disease uniquely stopped at this point, the mouse remained in this condition, and survived until the end of the experiment (28 DPI).

The body weight of mice was measured daily, starting with the day of challenge (Fig. 1A–D). The mean (±SD) body weight at the start of experiments was 17.58 ± 0.80 g in the first, 18.19 ± 1.04 g in the second, and 17.12 ± 0.85 g in the third experiment. In all three experiments, mice showing clinical signs of rabies started to consistently lose weight after the onset of clinical disease (due to the lack of food intake and spasms). The average weight loss of rabid mice until euthanasia was 17.39% of the initial body weight. Mice that remained asymptomatic (in therapy control groups or surviving mice in infected groups) did not lose weight during the experiment.

In the third experiment mice of the therapy control group suffered severe loss of body weight from the start of treatment (4 DPI) despite gaining weight before that point (Fig. 1D). These animals showed clinical signs from 7 DPI onwards, which included apathy, permanent recumbency, disinclination to move, eventually leading to death in approximately 50% of the group. The signs were different from the clinical course caused by rabies infection (and this group was not inoculated with virus), therefore this effect can be attributed to toxicity. Additionally, in this experiment there were mice showing not rabies-specific clinical signs described above in the virus-infected and treated group as well (while other animals in the same group died of typical rabies infection), further strengthening the conclusion about toxicity.

3.2. Survival of SHBRV-18-infected mice

The challenge virus dose in the first experiment was LD_{50} ($10^{3.9}$ $TCID_{50}$/mouse), thus the expected mortality in the untreated virus control group was 50%. 6 mice survived out of 13 virus control animals until the end of experiment (28 DPI), which is 46.2%. In the infected and treated groups, higher survival rates were observed: there were 8 survivals (61.5%) in the pre-exposure treatment group (first administration of therapeutics was 4 h prior to challenge), 9 survivals (69.2%) in the 48 h treatment group (first administration was 2 DPI) and 10 survivals (76.9%) in the 96 h treatment group (first administration was 4 DPI) out of 13 animals per group. The survival curves of groups with different timing of treatment did not differ significantly, neither did the treated groups compared

to the virus control (Fig. 2A). The uninfected therapy control animals remained healthy throughout the experiment (100% survival).

In the second experiment virus dose was raised to LD_{100} ($10^{5.5}$ $TCID_{50}$/mouse), and HRIG was included in the therapeutic combination. Although expecting a 100% mortality in the virus control group, 3 mice out of 26 (11.5%) survived until 28 DPI, the final day of experiment. Survival rates in the treated groups were notably higher: 7 mice survived (53.8%) in the 48 h treatment group, 6 (46.2%) in the 96 h treatment group from 13 infected animals. In the pre-exposure (−4 h) treatment group only one mouse showed clinical signs and reached humane endpoints; in effect, 12 of 13 (92.3%) mice survived challenge with SHBRV-18 virus. According to Mantel-Cox log-rank test, the survival curve of the 96 h group is not significantly different from the virus control ($P = 0.0610$), but the 48 h group and the −4 h group differ significantly, with $P = 0.0167$ and $P < 0.0001$ compared to the virus control, respectively (Fig. 2B). To elucidate whether HRIG is solely responsible for the beneficial effect, an extra group was included in the

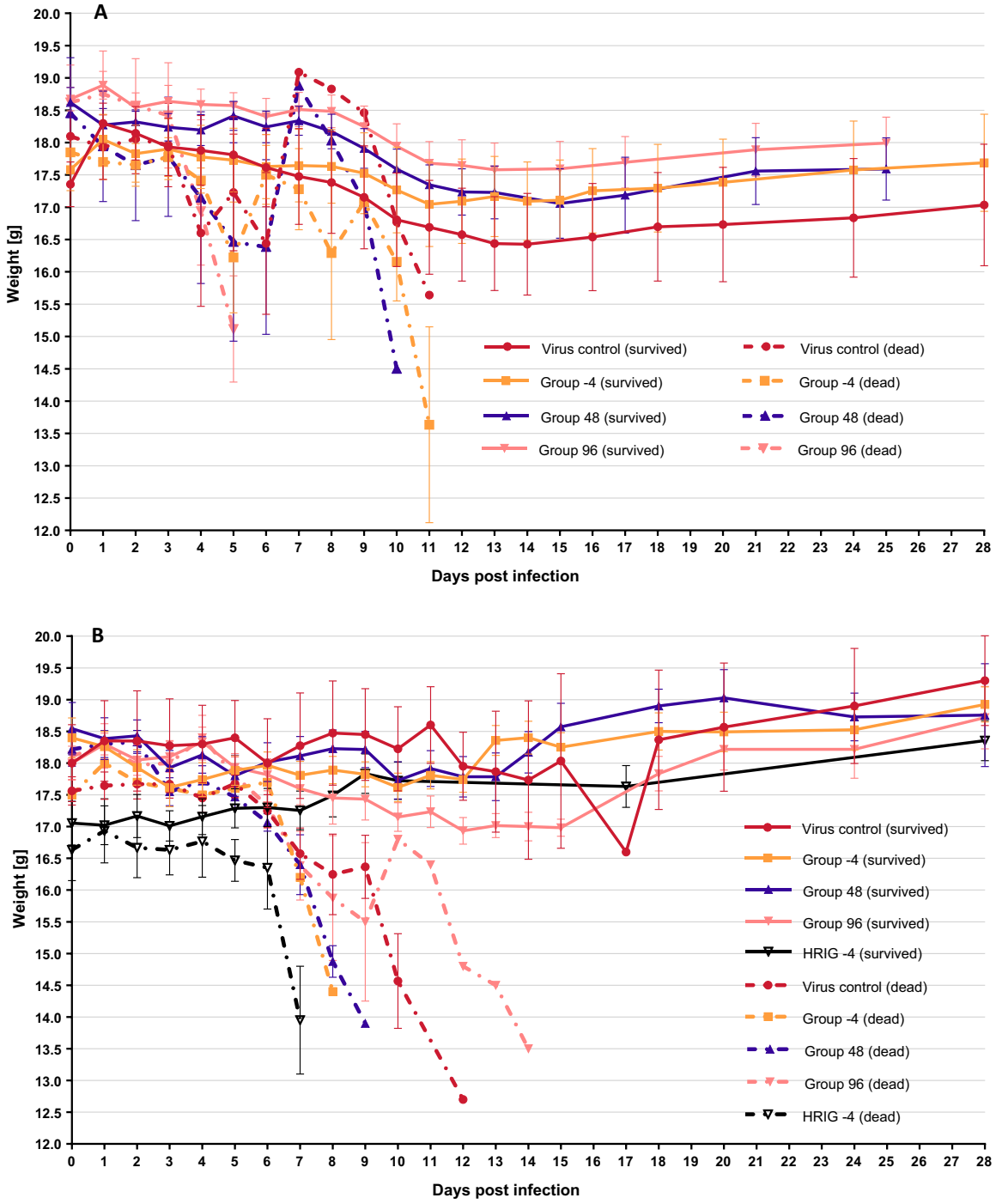

Fig. 1. Body weight of mice in different experimental groups. Mice were infected with SHBRV-18 by intramuscular route in the left hind leg and treated with the therapeutic combination starting 4 h before infection (group −4), 48 h post-infection (group 48), or 96 h post infection (group 96). HRIG −4: control group in the second experiment treated with HRIG only. Values are shown as mean ± SEM. (A): first experiment, (B): second experiment, (C): therapy control groups of first and second experiments, (D): third experiment.

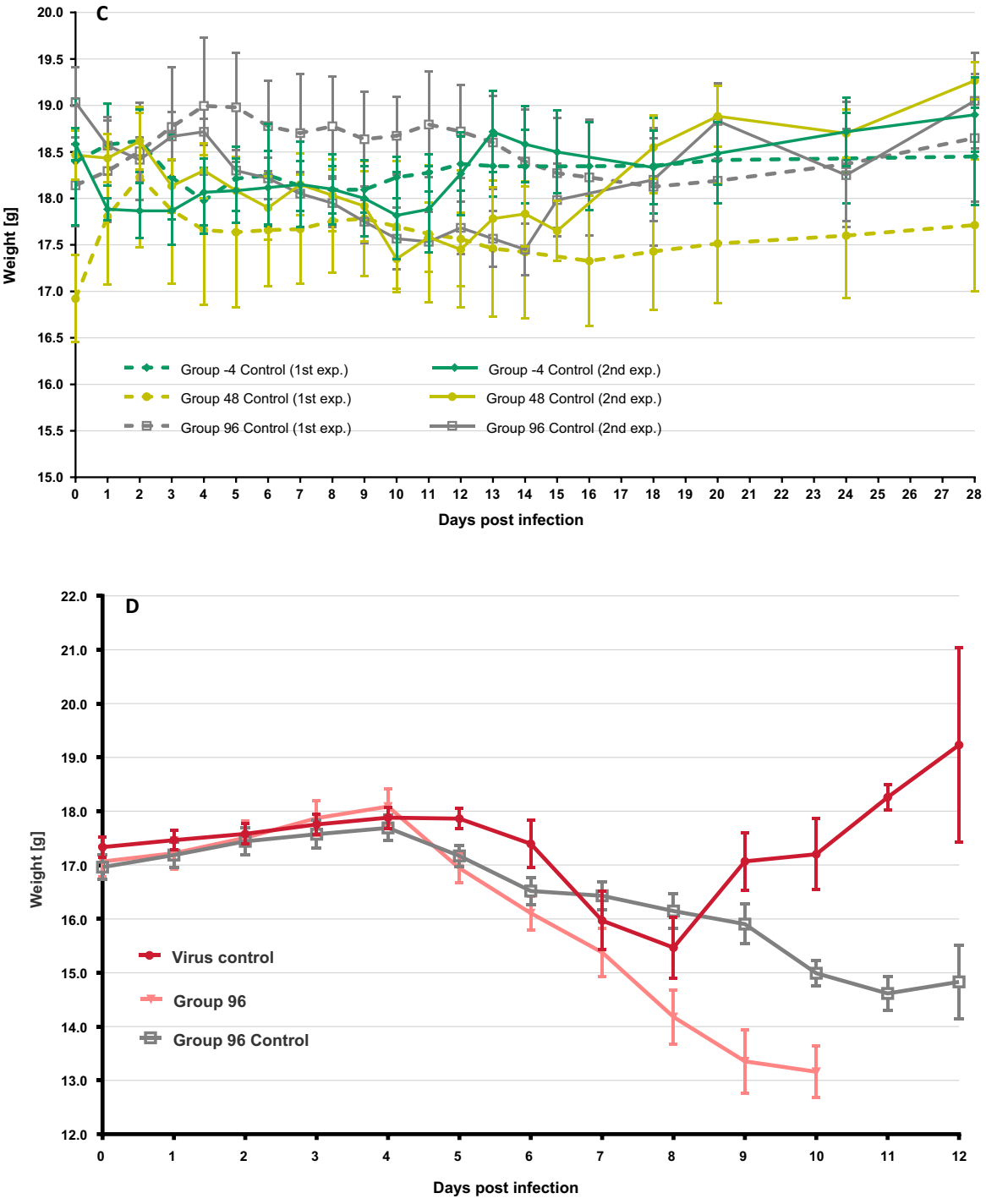

Fig. 1 (*continued*)

experiment (n = 13), in which treatment was carried out with HRIG only, following the administration schedule of the −4 h (pre-exposure) treatment group. In this group, 10 mice survived infection (76.9%); less than in the −4 h combination treatment group. There was significant difference in survival between the HRIG monotherapy group and the virus control group (P = 0.0032). All animals in the therapy control groups survived in good health.

In the third experiment, therapeutic combination was supplemented with antiviral compounds ribavirin, favipiravir and recombinant murine type-I interferons. There was only one infected and treated group, with initiation of treatment 96 h post infection. Despite using LD_{100} virus dose 2 mice out of 13 survived infection in the virus control group (15.4%). In the infected and treated group there was no surviving mouse; by 10 DPI all animals were terminated. It is noteworthy that 2 mice in the group died without showing rabies-specific clinical manifestations, they showed signs similar to the therapy control group that was described earlier (signs of toxicity). Mice in the therapy control group were clearly affected with toxicity, resulting in a 53.8% mortality (Fig. 2C).

3.3. SHBRV-18 RNA load in CNS samples

Brain and spinal cord samples were collected from mice reaching humane endpoints of rabies and from all surviving mice on the

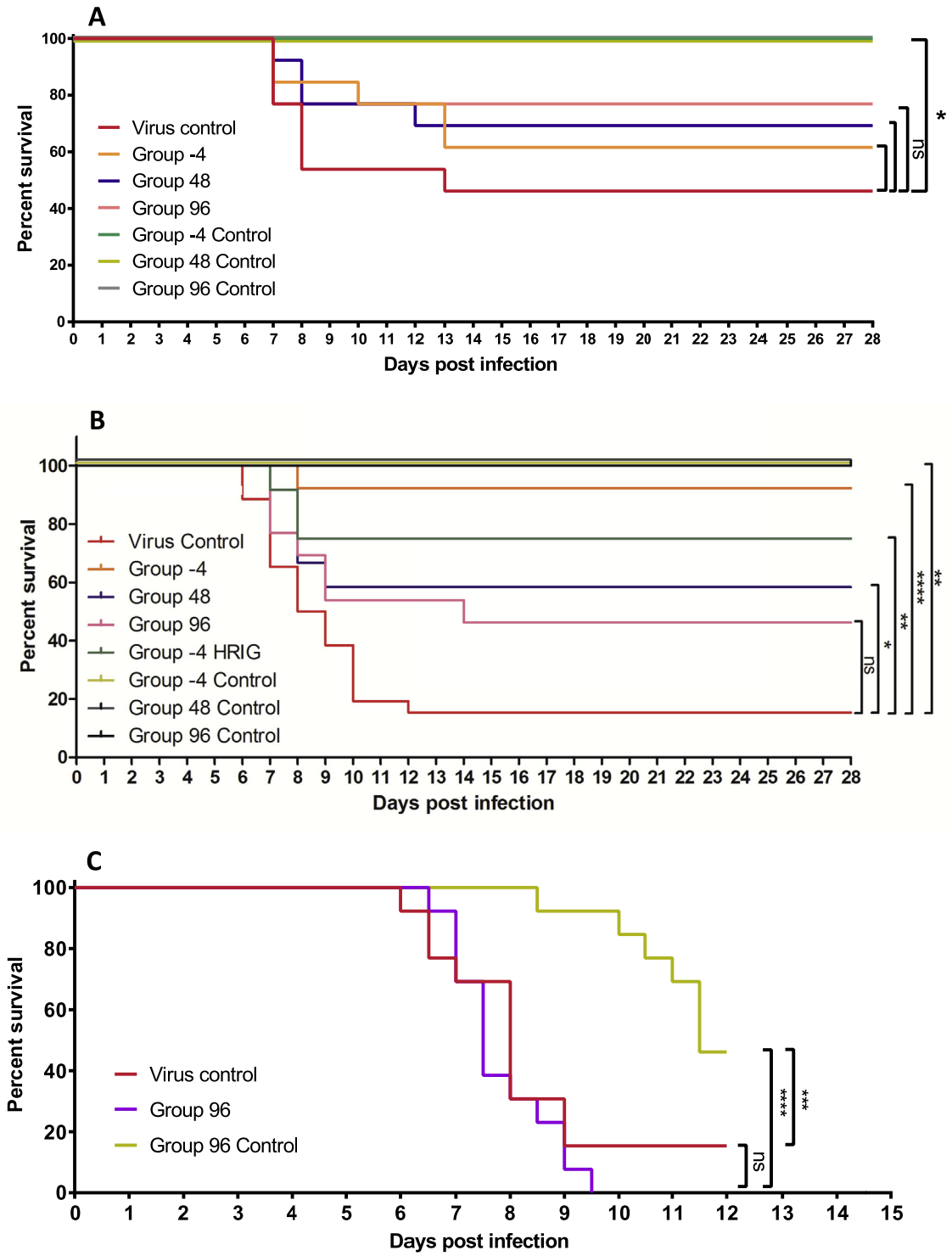

Fig. 2. Kaplan-Meier survival curves of mice in different experimental groups. Mice were infected with SHBRV-18 by intramuscular route in the left hind leg and treated with the therapeutic combination starting 4 h before infection (group −4), 48 h post-infection (group 48), or 96 h post infection (group 96). HRIG −4: control group in the second experiment treated with HRIG only. Statistical analysis: log-rank (Mantel-Cox) test. *P < 0.05; **P < 0.01; ****P < 0.0001. ns: not significant. (A): first experiment, (B): second experiment, (C): third experiment.

final day of experiments. According to the results of SYBR Green qRT-PCR viral RNA loads were similar in all mice that succumbed to rabies within one experiment, regardless of the therapy received. The majority of mice that survived challenge were free from detectable amount of RABV, indicating that the virus was cleared from the CNS by the end of the experiment. However, in some surviving mice PCR provided positive results for the presence of viral RNA both in the spinal cord and the brain. The titres in surviving animals were significantly lower than those reaching humane endpoints, but their samples were taken at the end of experiment (28 DPI) which can mean that the clearance of the virus from the CNS was already in progress at the given time point.

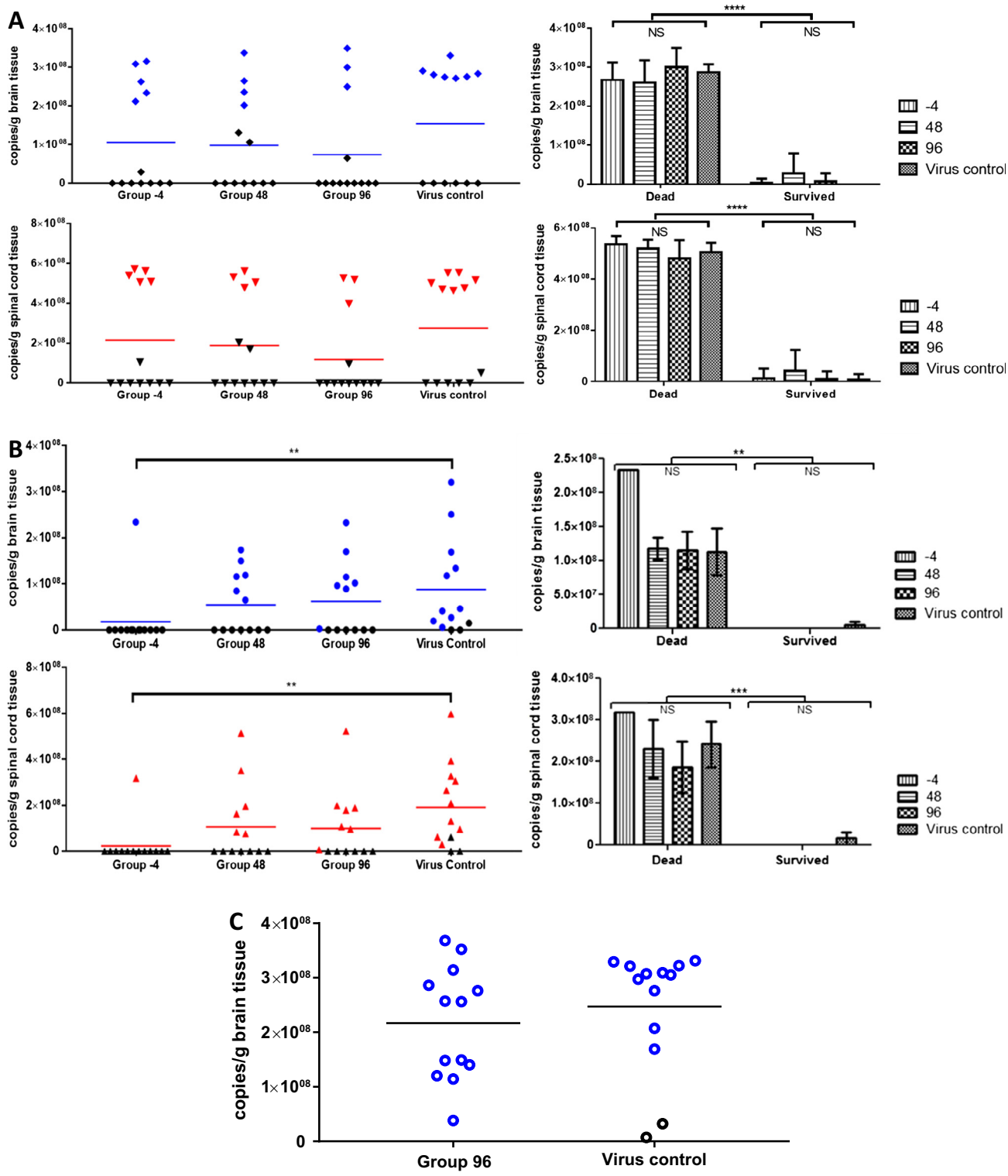

Fig. 3. SHBRV-18 viral RNA load in brain and spinal cord samples of mice in different experimental groups. Samples were collected from mice (n = 13 per group) exhibiting disease consistent with clinical score >3 and from surviving mice at the end of experiments. Values were determined using SybrGreen qRT-PCR. Statistical analysis: Kruskal-Wallis test with Dunn's multiple comparison test and 2-way ANOVA (dead vs. survived). **P < 0.01; ***P < 0.001; ****P < 0.0001 NS: not significant. At the left side charts: black indicators show survived mice; blue or red indicators show dead mice. (A): first experiment, (B) second experiment, (C): third experiment.

Viral RNA was also present in the brain and spinal cord of the surviving mouse in the 48 h treatment group of the first experiment with the continual CS-1-consistent status described earlier (see 3.1).

In the first experiment, there was no significant difference in RNA loads among groups, while the viral titres between diseased and surviving mice significantly differed (P < 0.0001) in case of brain and spinal cord samples as well (Fig. 3A). In the second experiment, there was significant difference between the virus control and the pre-exposure (−4 h) treatment group (brain: P = 0.0039; spinal cord: P = 0.0011), but the results of other groups did not differ significantly (Fig. 3B). In the third experiment viral

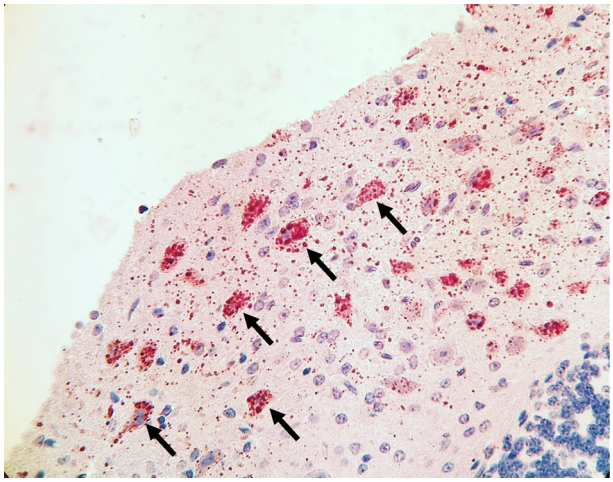

Fig. 4. Rabies-specific antigens in the cerebrum of a mouse (third experiment, virus control group) infected with SHBRV-18. Immunohistochemistry image, 40× objective. Antigens were stained with anti-rabies FITC conjugated monoclonal antibody No. 5199i. Some of the RABV+ neurons are indicated with black arrows.

RNA was detectable from both surviving mice in the virus control group, and also from those two mice in the infected and treated group that died due to toxicity before the clinical signs of rabies could appear. In the brain of animals with rabies-related signs in the virus control or the treated group there was high amounts of SHBRV-18 RNA detected, and the difference between the two groups is not significant (Fig. 3C).

3.4. Histopathology and immunohistochemistry of CNS samples

The right hemisphere of the brain of mice and a part of the spinal cord from the thoracolumbar region were analysed for the degree of inflammation (histopathology) and the quantity of viral antigens (IHC). High amount of RABV-specific antigens were detected in all mice that showed clinical signs of rabies infection, especially in the spinal cord, brainstem, cerebellum and cerebrum. However, antigens were virtually missing from the hippocampus (there was only one mouse of which hippocampus was found to be mildly positive), which is in accordance with the findings of Healy et al. [6]. Antigens were mainly localized in the soma of neurons, but they could also be detected in axons (Fig. 4). Extensive presence of rabies-specific antigens was accompanied by substantial inflammation (mononuclear cell infiltration, perivascular lymphocytic cuffing) in the CNS. Viral antigens were also found in the spinal cord of those clinically healthy surviving animals that were positive for viral RNA with qRT-PCR, but not in the brain. The mouse that survived despite showing CS-1 signs of rabies was the only survival of infection in which antigens were also detected in the brain (cerebrum and brainstem). Comparing mice positive for RABV in their CNS, viral antigen loads did not differ significantly between groups with different treatment or compared to the virus control group except for the 96 h treatment group of the first experiment, in which the quantity of antigens both in the brain and spinal cord is significantly lower than that in the virus control (Fig. 5). Additionally, taken all treated groups together, antigen levels were also significantly lower compared to the virus control group.

3.5. Detection of anti-RABV antibodies in surviving mice

In surviving animals of the virus-infected groups in the first experiment, the neutralizing antibody-level was determined from serum samples using FAVN test. The results show that all mice that survived RABV challenge had a detectable amount of antibodies in the blood, showing that their infection was successful, leading to seroconversion. Antibody titres varied between 0.29 and 1.50 IU/ml, with an average of 0.61 ± 0.45 (mean ± SD). There was no correlation between treatment regimen and antibody titres. The average antibody level in sera of mice with positive PCR results for RABV in the CNS was significantly higher: 1.09 ± 0.30 ($P = 0.0420$). The mouse that survived despite showing CS-1 signs of rabies in the 48 h treatment group had an antibody titre of 0.87 IU/ml.

4. Discussion

There is a considerable demand on the development of novel treatment strategies against rabies encephalitis to overcome the current disappointing success rate in attempts to treat human rabies [35]. After former empirical approaches by clinicians facing rabies cases now there is increasing knowledge available about rabies pathogenesis and factors related to survival [7,8,11,26], allowing faster progress in this field in recent years. This study reports another promising result with partial success in treatment of mice with different combinations of therapeutics after wild-type RABV challenge.

In our first experiment, mortality of rabies-infected mice treated with the combination of sorafenib, infliximab and caspase-1 inhibitor was reduced by approximately 30% compared to the mock-treated group, though the difference between survival curves was not significant. Surprisingly, higher survival rates were

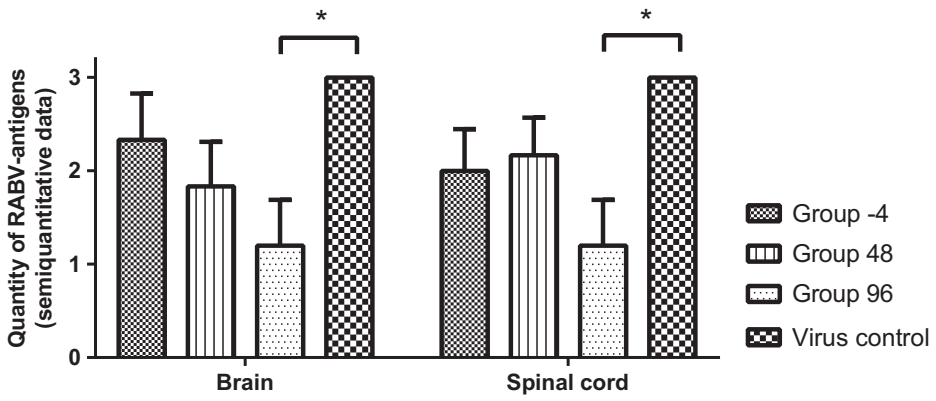

Fig. 5. Quantity of rabies-specific antigens detected by immunohistochemistry (semi-quantitative analysis: 0, +, ++, +++) in the brain and spinal cord samples of mice in different experimental groups; first experiment. Data is shown as mean ± SEM. Statistical analysis: Kruskal-Wallis test with Dunn's multiple comparison test, *$P < 0.05$.

observed in groups with later initiation of treatment. The quantities of RABV antigens in the CNS follow a similar scheme: within the treated animals the lowest antigen level was found in the group with the latest start of treatment (96 h group), while the highest was observed in the −4 h treatment group, showing that the viral loads were reduced in the later treated groups even if mice eventually succumbed to rabies. However, differences in antigen levels may not be representative as only few slices of the brain and spinal cord of each mouse were evaluated. It is possible that the slightly higher survival rate and lower rabies-specific antigen levels in the groups with delayed start of treatment were due to the fact that in these groups drug administration finished later and could presumably prevent late mortalities around 12–14 DPI (Fig. 2A). Considering viral RNA levels, there were no significant differences observed among experimental groups with qRT-PCR. It is noteworthy that four surviving mice in the treated groups were PCR-positive for SHBRV-18 RNA in the brain and spinal cord and also positive with IHC for rabies antigens in the spinal cord. Only one of these four animals had viral antigens in the brain and showed rabies-related signs throughout the experiment. In that mouse (48 h treatment group) signs appeared relatively late (16 DPI) and its clinical status was stabilized at CS-1 (paralysed left hind leg). Wild-type RABV is known to substantially delay immune response by various immune evasion mechanisms [7]. Therefore, we hypothesise that the immunomodulatory therapeutic combination used in our experiment delayed the escalation of encephalitis and neuronal damage long enough for the recruitment of immune effector cells and their infiltration into the CNS. In IHC-/PCR-positive surviving animals that phenomenon could prevent the development of a clinical disease or at least stop the clinical course at early stages. Virus neutralizing antibodies were demonstrated in the serum samples of these mice (using fluorescent antibody virus neutralization test), which is also considered as a crucial factor in survival after rabies infection [2,36].

For the better utilization of anti-rabies antibodies, the therapeutic combination was supplemented with HRIG in the second experiment. Based on experience gained during the first experiment, the challenge virus dose was raised to LD_{100} and the treatment period was prolonged to 10 days following the first administration of therapeutics in all treated groups. The combination fulfilled the expectations in further enhancement of survival: mortality was significantly reduced in the −4 h and 48 h treatment groups compared to the virus control. The effect in case of the 96 h group was still notable though falling just below the significance level ($P = 0.0610$). Pre-exposure treatment (−4 h group) almost completely prevented the development of clinical disease and hence mortality: only one animal reached humane endpoint with this treatment regimen. RNA loads in the CNS were also significantly reduced in the −4 h group compared to the virus control group, but in the other two groups RNA levels were not significantly lower. Regarding the difference between survival of −4 h, 48 h and 96 h treatment groups, trends are opposite to the findings of the first experiment: earlier initiation of therapy increased survival rates. This difference can be attributed to the use of HRIG in the combination: the early presence of anti-rabies antibodies might lead to an increased initial immune response against the virus before an overwhelming RABV multiplication in the CNS [37]. However, in the additional −4 h treatment group with HRIG monotherapy survival rate was lower than in case of the combination therapy with the same timing of treatment. This finding supports the conclusion that the positive effect on survival function is not caused by HRIG alone and that the use of immunomodulatory compounds in combination with antibodies is favourable over the sole administration of antibodies.

In the third experiment with the addition of ribavirin, favipiravir and type-I interferons to the combination there was only one infected and treated group in which therapeutics were first administered 96 h post infection. To enhance the effect of the combination, mannitol was added as BBB opener daily 30 min after each treatment [38]. We expected significantly higher survival among the treated animals than the virus control, despite the late start of therapy. This experiment was unsuccessful due to the high toxicity caused by the combination. There was a drastic decrease in body weight of the therapy control mice starting from the day after first treatment (Fig. 1D) followed by inactivity, apathy and in some animals, death. Until 12 DPI (when the experiment was terminated due to the loss of all mice in the infected and treated group) there was a 53.8% mortality in the therapy control group. The group of infected and treated mice also suffered from toxicity: two mice died after showing clinical signs not specific for rabies but similar to those observed in the therapy control group. In other animals it was difficult to reveal the actual impact of toxicity because rabies is also associated with weight loss and the clinical signs of rabies emerged in the majority of mice in this group around 6–7 DPI (before the appearance of toxicity-related signs). The mortality in the treated group was 100% in contrast to the virus control group with 84.6%. It is likely that toxicity impaired the immune functions of treated mice leading to a lower resistance to virus infection. The cause of toxicity cannot be undoubtedly determined. As the individual ingredients of the combination in the used dose are non-toxic (Ac-YVAD-cmk, infliximab and sorafenib: first and second experiment of current study; interferons [39,40]; ribavirin [41]; favipiravir [28]), some type of interaction can be present between certain components. A plausible explanation is the use of cremophor EL as vehicle for poorly water-soluble sorafenib in the third experiment. In the first and the second experiments, DMSO was used, based on the recommendation by the research group of the National Veterinary Research Institute in Puławy, Poland. The study protocol was changed for the third experiment, because cremophor EL is suggested by the literature for ip. administration of sorafenib in mouse model [30–32]. In result, we observed high toxicity, while the group from Puławy investigated a similar therapeutic combination with DMSO as solvent of sorafenib without any toxic effect [42]. It is thus recommended to avoid the use of cremophor EL as vehicle of sorafenib in combination treatments in the future.

Based on the findings of this study we conclude that the combination of inhibitors of certain pro-inflammatory cytokines (TNF-α) and molecular pathways (MAPK, Casp-1 mediated cascades) improve survival chances of mice infected with neurovirulent, wild-type rabies virus. The effect of these immunomodulators on survival is more pronounced if therapy lasts longer, preferably until at least 12–14 DPI. With the addition of anti-rabies antibodies (HRIG) to the combination, survival rates are highly enhanced, noting that earlier start of treatment leads to a more significant increase in survival. We expected that the addition of virus replication inhibitors with established anti-rabies effect like type-I interferons, ribavirin and favipiravir, as well as the use of BBB openers could provide further protection against the development of clinical disease and death. However, this could not be demonstrated due to toxicity that prevented the observation of any possible anti-rabies effect. Our results suggest that inhibitors of detrimental host responses to rabies, preferably in combination with antibodies should be considered among the potential therapeutic or post-exposure options against rabies encephalitis. Nonetheless, the interpretation of the results achieved using a mouse model for a human rabies situation should be cautious, since the relevant immunological processes following a RABV infection and subsequently the clinical manifestations of the disease highly vary among different host species. In dogs, in has been demonstrated that in early stages of rabies the transcription of inflammatory cytokines was moderate; with notable differences between furious

and paralytic clinical forms [43], questioning the hypothesis about immune-mediated neurological damage [5]. However, recent transcriptomic studies in mice show a pronounced up-regulation of several chemokines and cytokines in association with rabies infection, leading to the activation of various cell death pathways related to the innate immune system (including CASP-1 and TNF-α mediated cascades) [44]. Taken all these data together with our findings it is clear that more research is needed to elucidate the exact mechanisms of action of immunomodulatory compounds during RABV infection in different species, as well as to reveal more targets for therapeutic intervention in rabies.

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Conflict of interest

The authors declare that no conflicts of interest exist.

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