



Absence of protection from West Nile virus disease and adverse effects in red legged partridges after non-structural NS1 protein administration

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ABSTRACT

The red-legged partridge (*Alectoris rufa*) is a competent host for West Nile virus (WNV) replication and highly susceptible to WNV disease. With the aim to assess in this species whether the inoculation of non-structural protein NS1 from WNV elicits a protective immune response against WNV infection, groups of partridges were inoculated with recombinant NS1 (NS1 group) or an unrelated recombinant protein (mock group), and challenged with infectious WNV. A third group received no inoculation prior to challenge (challenge group). The NS1 group failed to elicit detectable antibodies to NS1 while in the mock group a specific antibody response was observed. Moreover, no protection against WNV disease was observed in the NS1 group, but rather, it showed significantly higher viral RNA load and delayed neutralizing antibody response, and suffered a more severe clinical disease, which resulted in higher mortality. This adverse effect has not been observed before and warrants further investigations.

1. Introduction

West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*) is a mosquito-borne virus with a broad vertebrate host range and different species of birds as reservoir hosts. WNV causes severe disease in horses, humans and some bird species [1]. The recent spread of this virus, remarkably in Europe and the Americas, is a matter of concern [2,3]. Effective vaccines are available for horses but not for humans or birds [4,5]. Hence, the development of new vaccines against WNV needs further efforts. For that, a better knowledge of the interaction between the host immune response and the different viral components might help identifying new targets for vaccine development.

WNV single-stranded RNA genome is translated as a single polypeptide that is cleaved to yield three structural (C, prM and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. Host adaptive immunity against WNV mostly targets structural proteins

[6]. However, NS1, which during infection is expressed as membrane and secreted forms, elicits an immune response in the host that has been involved in protection against WNV in mouse models [7]. Therefore, NS1 has been claimed as promising candidate for vaccine development [8]. The objective of this work was to assess the immune response elicited by the administration of NS1 in a natural avian host, the red-legged partridge [9] and if it conferred protection against a challenge with an infectious dose of a pathogenic WNV strain.

2. Materials and methods

2.1. Cloning and expression of recombinant proteins

Recombinant proteins used in this study were produced in the baculovirus expression system as described elsewhere [10]. Briefly, the NS1-coding region from WNV NY99 034EDV “crow” strain (obtained

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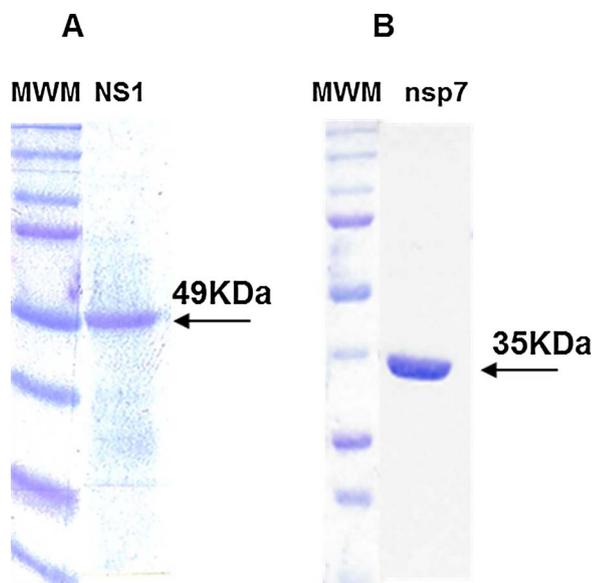


Fig. 1. Analysis by SDS-PAGE stained with Coomassie blue of the purified recombinant proteins NS1 from WNV (A) and unrelated protein (nsp7 from PRRSV), (B). MWM: molecular weight markers (from top to bottom: 250, 150, 100, 75, 50, 37.25 and 20 kDa, respectively).

through the Diagnostic Virology Laboratory, USDA, Ames, Iowa) was cloned into a baculovirus expression vector and expressed in recombinant baculovirus-infected Sf9 insect cells. Recombinant nsp7 protein derived from PRRSV (porcine respiratory and reproductive syndrome virus) strain Olot91, used as unrelated (“mock”) protein, was obtained exactly in the same way as WNV-derived NS1, during a previous work [11]. The expressed proteins were further purified from infected Sf9 cell cultures by nickel-affinity chromatography. An SDS-PAGE analysis with Coomassie blue staining was performed to assess size and purity of the obtained recombinant proteins (Fig. 1).

2.2. Experimental inoculations in red-legged partridges

Animal care, handling and experimental procedures were authorized by the INIA Committee of Ethics and Animal Experimentation (Reference: 2013-012) according to Council Directive 2010/63/EU (Spanish Royal Decree 53/2013).

Three-week-old red-legged partridge chicks were injected intramuscularly with purified recombinant WNV NS1 (15 µg/chick) adjuvanted in Montanide ISA-70® (“NS1 group”, n = 10) or the same amount of unrelated mock protein in the same adjuvant (“mock group”, n = 7). Two additional groups (“challenge”, n = 8, and “control”, n = 7) received buffered saline solution (BSS). Two weeks later, all chicks received a “boost” injection consisting of the same substance and dose as previously administered. Thirty days after the first injection, an infectious dose (10⁴ pfu/chick) of WNV pathogenic strain Morocco-2003 [9] was administered subcutaneously to all chicks, except the control group.

2.3. Clinical follow-up and analysis of samples

Clinical parameters were monitored daily during 2 weeks. Severely affected birds, showing irreversible signs, including: severe neurological signs such as paralysis, unresponsiveness, ataxia and prostration, or severe weakness, apathy, anorexia or weight loss over 40%, were humanely euthanized for welfare reasons, by intravenous injection of embutramide (T61®, Intervet – Schering-Plough, Madrid, Spain). Blood samples were collected prior to each administration or challenge and at 1, 3, 5, 7, 9, 11 and 14 days post-challenge. Viral load in blood was assessed by real-time reverse-transcription polymerase chain reaction

(RRT-PCR), using an already described method [12]. Antibody production against E, NS1 and mock proteins was determined by different types of ELISA methods: anti-E antibodies were measured using a commercial epitope-blocking ELISA (INgezim® West Nile Compac, INGENASA, Spain) [13], whereas antibodies to NS1 and nsp7 (mock) proteins were measured using “in-house” methods. Briefly, serum samples (1:50 dilutions) were incubated in recombinant-protein-coated microplate wells for 1 h, then washed and incubated with peroxidase-conjugated 1A3 anti-avian immunoglobulins monoclonal antibody (MAb) [14,15]. This MAb recognizes an epitope in the light chains of bird immunoglobulins, common to all immunoglobulin isotypes. Colour was developed following standard procedures. Signals above 2× background were considered positive. Neutralizing antibodies were assessed by a micro-virus-neutralization test (VNT) in 96-well plates, as described [13]. Briefly, serum samples were inactivated at 56 °C for 30 min prior to testing. Two-fold dilutions of test sera (25 µl) in Eagle’s Minimal Essential Medium (EMEM) supplemented with L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, were incubated with the same volume (25 µl) of 100 TCID₅₀ of WNV strain Eg101 for 1 h at 37 °C and 5% CO₂. This was followed by the addition of 50 µl of a suspension (2 × 10⁵ cells/ml) of Vero E6 cells in the same medium, plus foetal calf serum to reach a final concentration of 5%. The plates were further incubated for 6–7 days at 37 °C and 5% CO₂ atmosphere until cytopathic effect (cpe) were observed in control wells containing 10 TCID₅₀ of virus. Virusneutralization positive samples were those inhibiting cpe at 1:5 or higher dilutions. Neutralizing serum titre was regarded as the highest value of the reciprocal serum dilution giving a complete absence of cpe. For graphical representation, geometric mean titres were calculated for each group of birds at each time point and expressed in a Log₁₀ scale.

2.4. Statistical analysis

Statistical analyses were performed using the Student’s *t*-test, except for comparison of mortalities in the different groups of partridges, which was analyzed using the Gehan-Breslow-Wilcoxon test.

3. Results

Cloning, expression of recombinant NS1 protein from WNV in a baculovirus-insect cell system and subsequent purification, resulted in a single polypeptide migrating as a unique band of the expected 49 kDa size in a SDS-PAGE gel stained with Coomassie blue (Fig. 1A). Unrelated (“mock”) protein nsp7 from PRRSV, obtained from a previous work using the same baculovirus expression system and purification scheme, resulted in a single 35 kDa polypeptide by SDS-PAGE analysis, as expected (Fig. 1B).

Overall, the group of NS1-inoculated chicks did not show any protection from WNV challenge. By opposite, after WNV challenge, the observed clinical course of the disease was more severe in this group than in any of the other groups. Mortality in the NS1 group was higher (6 out of 10) and occurred faster (5–8 dpi) than in the other groups (Fig. 2A). Challenge group had two fatalities (7 and 8 dpi), mock group had one (8 dpi) while all control chicks survived. Statistically significant differences (*p* < 0.05) were found in mortality curves between NS1-inoculated and control or mock groups, respectively (Fig. 2A). Differences between NS1-inoculated and challenge groups were marginally significant (*p* = 0.06). Weight gain at 5–7 dpi was lower in the NS1 group than in the other groups (Fig. 2B). Viral RNA load at 3 dpi was significantly higher in the NS1 group than in the other groups (Fig. 2C). No antibodies were detected against NS1 in any of the chicks before challenge, while anti-NS1 antibodies were evident in all groups after challenge (Table 1). However, antibodies against mock (nsp7) protein were detectable in 3 out of 7 individuals in the mock group before challenge, and in all of them after challenge (Table 1). Interestingly, anti-E and neutralizing antibodies developed slightly later in

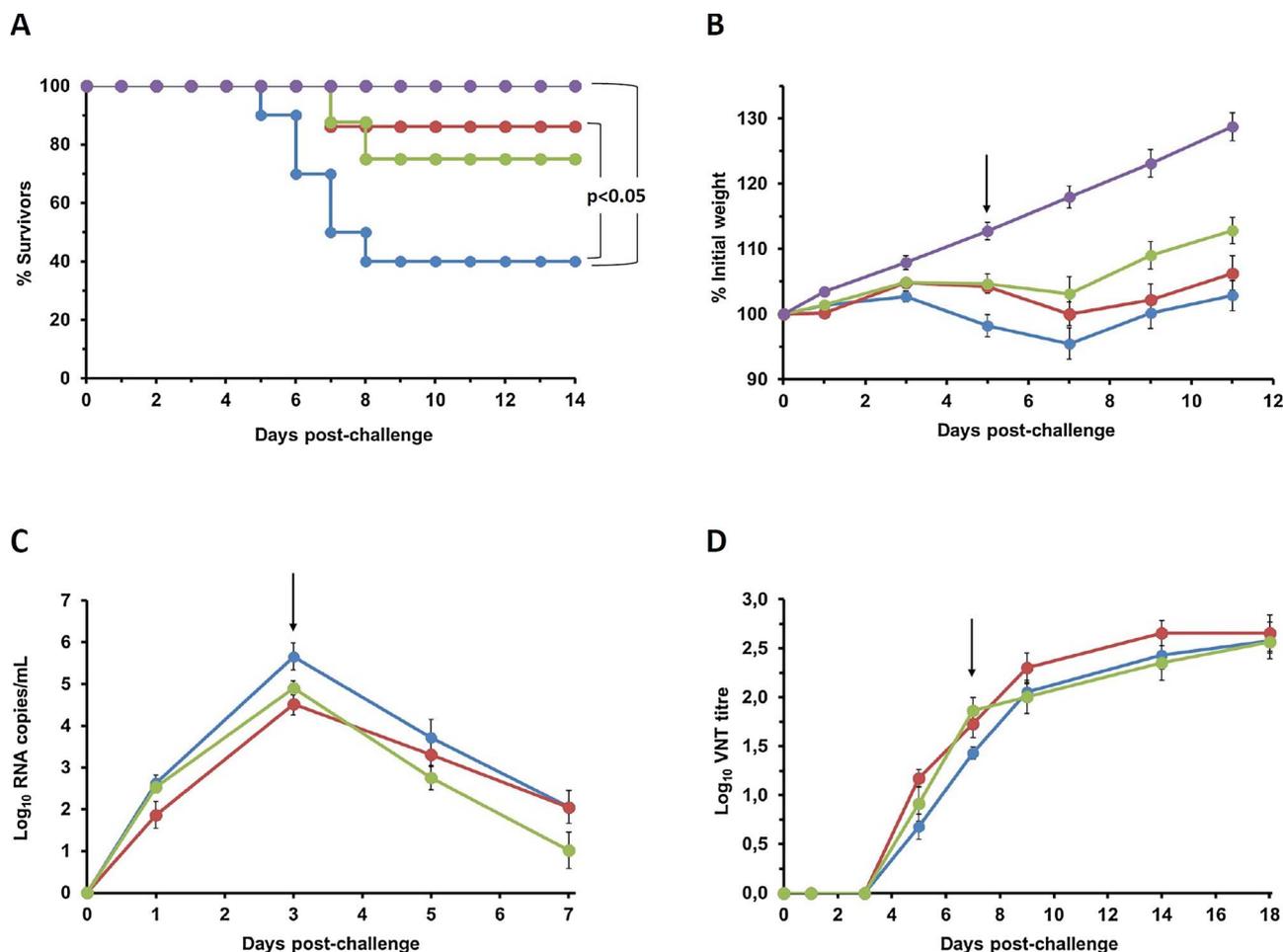


Fig. 2. Course of A) survival; B) weight gain, C) viral load in blood and D) neutralizing antibody titres, in the different groups of red-legged partridge chicks (*Alectoris rufa*) after challenge with WNV. Blue: group inoculated with NS1 and subsequently challenged with WNV (NS1 group); red: group inoculated with unrelated protein nsp7 from PRSSV and subsequently challenged with WNV (mock group); green: group receiving WNV challenge only (challenge group); purple: group receiving neither injection nor challenge (control group). Statistically significant differences ($p < 0.05$) among groups analysed by the Gehan-Breslow-Wilcoxon test are indicated in A), whereas in B), C) and D), arrows point to values where significant differences (Student's *t*-test) are found between NS1 group and the other groups. Error bars represent the standard error of the data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the NS1 group as compared to the other groups (Fig. 2D, Table 1).

4. Discussion

Administration of recombinant NS1 from WNV in a natural avian host, the red-legged partridge, followed by inoculation of a pathogenic dose of WNV, lead to a more severe course of the disease caused by WNV infection, as shown by the differences in mortality curves observed. Statistical significance was reached when comparing NS1-

inoculated and two of the other groups (mock-inoculated and control), whereas comparison between NS1-inoculated and challenge groups was marginally significant ($p = 0.06$). Probably, a higher number of individuals in each group would have allowed for a substantial increase in statistical significance when comparing survival curves. Nevertheless, other clinical signs such as mean weight gain, as well as analytical parameters such as the viral load in blood, support the finding that a more severe course of the infection and subsequent disease occurred in the chicks primed with NS1.

Table 1
Summary of the results obtained in the ELISA tests.

Sample collection time (dpi)*	Anti-E Abs (ELISA, % competition + s.d)				Anti NS1 Abs (n° positives/total)				Anti-mock Abs (n° positives/total)
	NS1	Mock	Challenge	Control	NS1	Mock	Challenge	Control	Mock
-28	neg	neg	neg	neg	0/10	0/7	0/8	0/7	0/7
-14	"	"	"	"	"	"	"	"	"
-1	"	"	"	"	"	"	"	"	3/7
1	"	"	"	"	"	"	"	"	"
3	"	"	"	"	0/10	0/7	1/8	"	"
5	"	43.8 ± 10.2	44.6 ± 20.8	"	3/9	3/7	"	"	7/7
7	59.7 ± 13.8	58.3 ± 23.6	60.9 ± 25.4	"	5/7	4/7	7/8	"	"
9	72.4 ± 8.3	71.0 ± 12.1	75.6 ± 11.4	"	4/4	6/6	6/6	"	6/6
14	75.6 ± 8.1	71.9 ± 12.1	74.5 ± 11.2	"	4/4	"	"	"	"

*Abbreviations: Abs: antibodies; dpi: days post infection; neg: negative.

Furthermore, since NS1-specific antibodies were undetectable before challenge, consequently the involvement of antibodies in the adverse effect observed seem to be unlikely. However a very low level of NS1-specific antibodies, not detectable by the ELISA employed, and playing some role in this phenomenon, could not be discarded.

The severity of WNV disease observed after NS1 administration reminds that observed in severe cases of dengue haemorrhagic fever [16]. The mechanism behind this phenomenon is known as antibody-dependent enhancement of infection (ADE), but should be discarded here since the NS1 antigen is not incorporated into the virion. Hence, other as yet unknown mechanisms might be mediating this effect, including cellular and/or innate immunity. Unfortunately, the methodology available to analyse the immune response in the red-legged partridge is limited, precluding further studies.

The same immunization procedure raised antibodies against the mock protein, but failed to raise antibodies to NS1 above detectable levels (Table 1). Thus, the absence of detectable antibodies to NS1 before challenge was not due to impaired immunocompetence of the chicks, since they responded normally to the mock protein. Alternatively, a lack of immunogenicity of the recombinant NS1 could be argued to explain this finding. However, this same NS1 preparation otherwise induced specific antibodies to high titres when administered to mice, from which we successfully obtained monoclonal antibodies to NS1. Therefore, the low immune responsiveness found against the NS1 used in this study could be due to a lack of immunogenicity of this molecule in the red-legged partridges, but does not necessarily affect other species, such as mice.

Remarkably, NS1-primed chicks responded later to WNV challenge, with a delayed neutralizing antibody response, one of the primary host's responses leading to protection and survival. WNV NS1 exhibits a range of activities against both natural and adaptive immune responses. Notably, it inhibits complement activation by withdrawing factor H, binding of C1s, C4 and the plasma regulator C4b-binding protein. All these activities may help WNV to evade complement-driven antiviral responses such as, for instance, pathogen opsonization and/or lysis, priming of B cells and enhancement of T cell killing of infected target cells [6]. Bearing this in mind, it is possible that NS1 administered to chicks may have affected complement-mediated antiviral mechanisms. For instance, inhibition of B-cell priming through binding of activated mediators such as C3b, C3dg and C3d to complement receptor type 2 (CR2), may interfere in B-cell activation and maturation [17], which may explain the less efficient antibody response observed. Alternatively, NS1 may trigger other pro-inflammatory mechanisms, which alone or in combination with complement, may lead to WNV disease exacerbation. This adverse effect has not been observed in rodent models [8].

5. Conclusions

This work revealed an adverse effect elicited by priming partridges with WNV NS1 prior to challenge with infectious WNV. The mechanisms behind it are not understood, though they appear different from ADE observed during certain flaviviral infections. Whether this effect is mediated by complement abrogation or by interference with other antiviral immune responses warrants further investigation.

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Declarations of interest

None.

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