Comparative Immunology, Microbiology and Infectious Diseases 56 (2018) 30–33

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

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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ARTICLE INFO

Keywords:
Flavivirus
West Nile virus
NS1
In-vivo
Adverse effects
Immunization
Red-legged partridge
Avian

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 polyprotein 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 through the Diagnostic Virology Laboratory, USDA, Ames, Iowa) was cloned into a baculovirus expression vector and expressed in

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https://doi.org/10.1016/j.cimid.2017.12.006
Received 16 November 2017; Received in revised form 29 December 2017; Accepted 29 December 2017

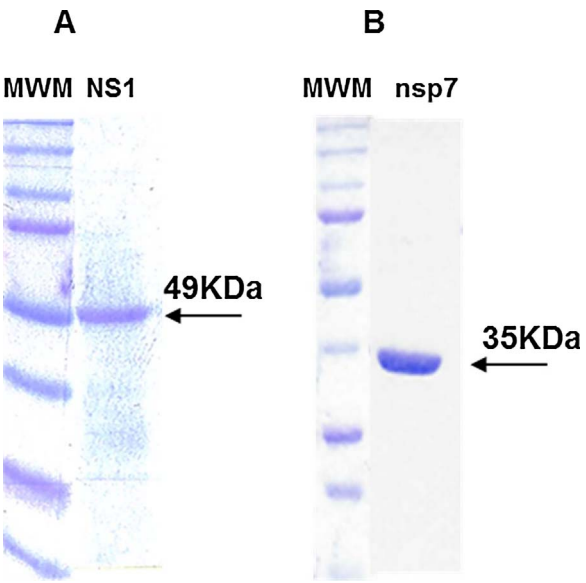

Fig. 1. Analysis by SDS-PAGE stained with Coomassie blue of the purified recombinant proteins NS1 from WNV (A) and unrelated protein (nsp7 from PRRSV), (B). MWM: molecular weight markers (from top to bottom: 250, 150, 100, 75, 50, 37 25 and 20 kDa, respectively).

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 (10^4 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 $TCID_{50}$ of WNV strain Eg101 for 1 h at 37 °C and 5% CO_2. This was followed by the addition of 50 μl of a suspension (2×10^5 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% CO_2 atmosphere until cytopathic effect (cpe) were observed in control wells containing 10 $TCID_{50}$ 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 Log_{10} 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 the NS1 group as compared to the other groups (Fig. 2D, Table 1).