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Communication

Dynamics of Whole Virus and Non-structural Protein 1 (NS1) IgG Response in Mice Immunised with Two Commercial Tick-borne Encephalitis Vaccines

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Abstract: The presence of a non-structural protein 1 (NS1) in tick-borne encephalitis (TBE) vaccines and the possible induction of an NS1-specific immune response in vaccinated individuals remains a somewhat controversial topic. Previously, we detected the presence of NS1 in Encepur TBE vaccine by mass spectrometry and found the induction of NS1-specific IgG antibodies in mice vaccinated with FSME-Immun TBE vaccine. Here, in this follow-up study, we examined the dynamics and extent of the NS1-specific IgG response in mice vaccinated with these two vaccines in more detail and compared it with the IgG response to the whole virus (WV). Mice were vaccinated at two-week intervals with a total of six doses of each vaccine, and levels of IgG antibodies to TBE virus WV and NS1 were measured by ELISA after each dose. Both vaccines elicited a robust anti-WV IgG response after two doses. The Encepur vaccine did not elicit NS1-specific IgG even after all six doses. In contrast, FSME-Immun vaccine triggered production of NS1-specific IgG after four doses. The results indicate that FSME-Immun is the only vaccine that elicits an NS1-specific antibody response in mice. However, compared to WV-specific IgG, the NS1-specific response is weaker, and a higher number of doses is required to induce detectable levels of NS1-specific IgG antibodies.

Keywords: tick-borne encephalitis virus; vaccine; non-structural protein 1

1. Introduction

Tick-borne encephalitis (TBE) is a tick-borne zoonosis caused by the TBE virus (TBEV), a member of the genus *Flavivirus*, family *Flaviviridae* [1]. TBEV is endemic in much of Europe and Asia [2], and recently the virus has been detected also in northern Africa [3]. TBE can manifest as a mild flu-like illness or progress to neurological disease, typically manifesting as meningitis, meningoencephalitis, or encephalomyelitis [2, 4]. TBE diagnosis relies primarily on serologic testing to detect whole virus (WV)-specific IgM and IgG antibodies [2, 5, 6]. Because the TBE vaccines currently in use are based on purified and inactivated TBEV particles [2], detection of WV-specific antibodies does not allow differentiation between antibodies elicited after vaccination and infection. Previously, it was assumed that the NS1 antigen is not present in current TBE vaccines and that those vaccinated therefore do not develop an NS1-specific antibody response. Therefore, serology based on the detection of antibodies to NS1 is believed to be a promising tool to distinguish between TBE antibodies induced by infection and those induced by vaccination [7]. However, our recent study showed that two commercial TBE vaccines do contain NS1 antigen [8]. This was demonstrated by a combination of experimental approaches that included mass spectrometry analysis of the vaccine content and analysis of NS1-specific antibody response in mice immunised with these vaccines. Mass spectrometry analysis of

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the Encepur vaccine provided clear evidence for the presence of both the envelope (E) protein (as the major surface antigen of the WV) and NS1 antigens in the vaccine preparation. Interestingly, despite the presence of detectable levels of NS1 antigen in the vaccine preparation, the Encepur vaccine did not elicit an NS1-specific serological response in mice even after six doses administered [8]. Mass spectrometric analysis of the vaccine FSME-Immun failed in part because of the high content of human serum albumin used as a stabilizer in the vaccine (sucrose is used as a stabilizer in the Encepur vaccine) [8]. Thus, only the E antigen, and not NS1, was identified in this vaccine. However, the presence of the NS1 antigen in FSME-Immun was confirmed by vaccination of naïve mice, which elicited a strong anti-NS1 antibody response in addition to anti-WV antibodies [8].

Whether immunisation with TBE vaccines elicits an NS1-specific antibody response in humans remains difficult to say. In previous studies with vaccinated individuals (in most cases with ≤ 3 doses of the vaccine), NS1-specific antibodies were not detected in the majority of cases [7, 9, 10]. Consistent with these studies, our previous work in a small cohort of vaccinated individuals found that most vaccinees who received ≤3 doses were negative for NS1-specific antibodies. In some vaccinated individuals who had received more than 3 doses of the vaccine, anti-NS1 antibodies were detected in serum by NS1-specific Western blot analysis [8]. However, these vaccinated individuals lived in the Czech Republic, a country with high TBE endemicity [11], so natural TBEV exposure in these individuals cannot be excluded.

Therefore, the possible NS1-specific antibody response in vaccinated individuals remains a controversial topic. To obtain more data and remove possible doubts about our previous work, we decided to repeat our previous experiments with vaccination of mice in this follow-up study with new lots of the two commercial TBE vaccines and to characterize in more detail the dynamics of the NS1-specific antibody response in the vaccinated animals.

2. Materials and Methods

2.1. Ethics Statement

Animal experiments were performed in accordance with Czech laws and guidelines for the use of laboratory animals. The protocol was approved by the Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Ministry of Agriculture of the Czech Republic and the Committee on the Ethics of Animal Experimentation at the Veterinary Research Institute (Approval No. 26674/2020-MZE-18134).

2.2. Vaccination of Mice

BALB/c mice (females, 6 weeks old, Envigo) were used for the vaccination experiment. Mice in the first group (N=6) were vaccinated with Encepur (GSK Vaccines, Brentford, UK; Lot No.: AEA33A1C). The second group of mice (N=6) were vaccinated with FSME-Immun (Pfizer, New York, NY; Lot No.: EK3932). The mice in the third group (N=6) received only the adjuvant and served as controls. A single vaccine dose consisted of a mixture of vaccine antigen (0.25 μg), 10% Alhydrogel adjuvant (InvivoGen, San Diego, CA), and PBS (Serana Europe GmbH, Pessin, Germany). The total volume of the vaccine dose was 0.15 ml. Vaccine doses were prepared immediately before use. Mice were vaccinated subcutaneously dorsally in the neck region. A total of six vaccine doses were administered on two-weeks intervals. Blood samples were collected from the tail vein of the mice 7 days after each vaccination. The concentration of specific anti-NS1 TBEV antibodies and anti-whole virus TBEV antibodies was measured by ELISA.

2.3. Detection of Antibodies in Mouse Sera

Detection of anti-NS1 specific IgG antibodies in the sera tested was performed using the Mouse Anti-Tick-Borne Encephalitis virus NS1 IgG Elisa Kit (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer's instructions. The assay used evaluates the concentration of specific anti-NS1 antibodies in arbitrary units (U/ml). Anti-TBEV whole virus IgG antibodies in the tested sera were measured using the

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IMMUNOZYM FSME IgG All-Species Kit (Progen GmbH, Heidelberg, Germany) following the manufacturer's instructions. The assay evaluates the concentration of specific IgG antibodies to TBEV in Vienna units (VIEU/ml).

2.4. Statistical analysis

Differences in antibody levels between the tested groups were analysed using Mann–Whitney *U* test. The analyses were performed by GraphPad Prism 7 for Windows (version 7.04). A p-value of < 0.05 was considered significant.

3. Results and Discussion

We previously investigated whether immunisation with Encepur or FSME-Immun vaccines elicits an NS1-specific immune response in mice [8]. In total, the mice received six doses of the vaccine with a 2-week interval between doses. Negative controls received six doses of the adjuvant. After the third and sixth doses of vaccine, serum samples were collected and the concentrations of anti-WV and anti-NS1 antibodies were measured by ELISA. After 3 doses of vaccine, a robust antibody response against WV was detected, but all mice were negative for NS1-specific antibodies. After 6 doses, all vaccinated mice had high levels of WV-specific antibodies, and mice that had received FSME-Immun vaccine were also positive for NS1-specific antibodies. Mice vaccinated with the Encepur vaccine were negative for anti-NS1 antibodies. Neither WV- nor NS1-specific antibodies were detected in control mice that received only the adjuvant [8]. Results suggested that FSME-Immun, but not Encepur vaccine, can induce NS1-specific immune responses in vaccinated mice [8]. However, the NS1-specific antibody response after vaccination with the commercial vaccines has been controversial because other studies have not found NS1-specific antibodies in serum samples from human vaccinees with completed vaccination regimens [7, 9, 10, 12]. One recent study compared a dynamics and extend of NS1-antibody responses in TBE vaccination breakthroughs and unvaccinated TBE patients, and found that neither the dynamics nor the extent of NS1-antibody formation differed significantly between these two groups, arguing against substantial NS1-specific priming and an anamnestic NS1-antibody response in vaccination breakthroughs [13]. Based on these conflicting observations, we decided to repeat our previous experiments (i) with a different vaccine batch to see if the NS1-specific antibody response was dependent on a particular vaccine batch, (ii) with more mice per group to increase the statistical power of the analysis, and (iii) with analysis of WV and NS1-specific IgG levels after each vaccine administration to characterise the detailed dynamics and extent of the immune response. The present study not only confirmed the previous results but also provided a more detailed insight into the kinetics and intensity of the NS1-specific IgG antibody response in the vaccinated mice. Similar to the previous study [8], mice received six doses of the vaccine 2 weeks apart. Negative controls received six doses of the adjuvant. However, unlike the previous study, blood samples were collected seven days after each dose and the concentrations of WV- and NS1-specific IgG were measured (Figure 1A). The kinetics of the WN-specific IgG antibody response were similar for both vaccines. Significantly increased ($p < 0.01$) concentrations of WN-specific IgG were detected after two doses of either vaccine, and then the concentrations increased with the number of vaccine doses administered (Figure 1B). At the end of the experiment, i.e. after six doses, significantly higher levels of WV-specific IgG ($p < 0.01$) were detected in mice immunised with FSME-Immun vaccine than in mice receiving Encepur vaccine (Figure 1C). Consistent with the results of the previous study [8], all blood samples from mice immunised with Encepur vaccine were negative for the presence of NS1-specific IgG (Figure 1D). In contrast, mice immunised with FSME-Immun developed detectable levels ($p < 0.01$) of NS1-specific IgG after 4 doses, and the levels of these antibodies then increased after each subsequent dose until the end of the experiment (Figure 1D). Unfortunately, comparison of NS1-specific IgG levels obtained in our previous and current study was not possible because different standards and arbitrary units were used in the previous and current ELISA assays, albeit from the same manufacturer.

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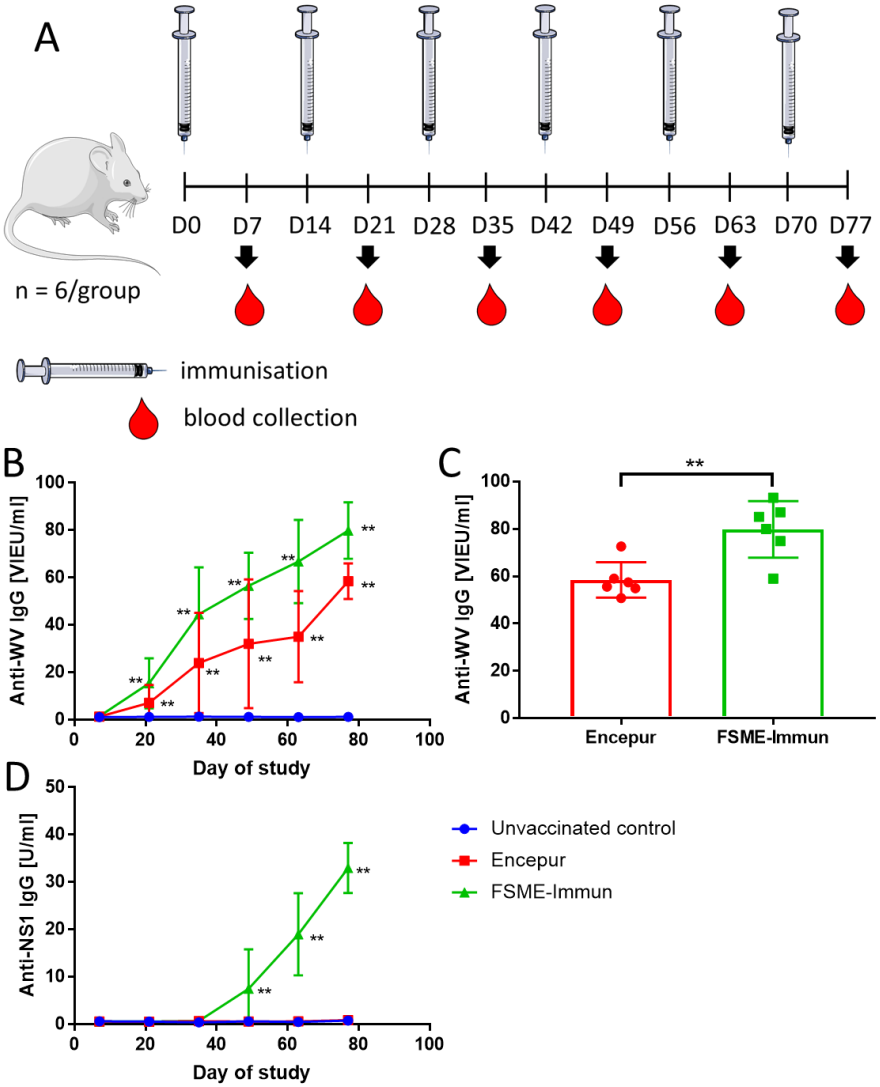

Figure 1. (A) Experimental protocol. Mice were immunised with either Encepur or FSME-Immun vaccines. Control mice received only the adjuvant. A total of six doses of vaccine were administered two week apart. Blood samples were collected from the tail vein of the mice 7 days after each vaccination. (Figure created using Servier Medical Art, available at www.servier.com). **(B)** Dynamics of whole virus (WV)-specific IgG in mice vaccinated with either Encepur or FSME-Immun. Mice receiving only the adjuvant were used as controls. **(C)** Comparison of WV-specific IgG levels in mice vaccinated with six doses of Encepur or FSME-Immun vaccine. **(D)** Dynamics of NS1-specific IgG in mice immunised with either Encepur or FSME-Immun vaccines. Mice receiving adjuvant only were used as controls. **, $p < 0.01$.

The results show that FSME-Immun is the only vaccine that elicits an NS1-specific antibody response in mice. Why the Encepur vaccine does not elicit an NS1-specific antibody response, despite containing detectable levels of NS1 antigen [8], remains enigmatic. After FSME-Immun vaccination, the NS1-specific response is much weaker compared with WV-specific IgG, and a higher number of doses is required to induce detectable amounts of NS1-specific IgG antibody. Our mouse experiments have shown that 3 doses representing the complete vaccination scheme are insufficient to elicit detectable levels of NS1-specific antibodies, and this could explain that there was no detection of NS1 antibodies in studies involving participants who received only the basic vaccination scheme consisting of three doses of the vaccine [7, 9, 10, 12, 13]. Whether humans who received higher numbers of doses (more than 3) of FSME-Immun develop detectable NS1-specific antibodies remains unknown and will be the subject of our next study. In any case, this and our previous study [8] suggest that any attempt to distinguish between vaccination

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and infection based solely on the detection of NS1 antibodies should be taken with caution.

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