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Assessment of structurally modified plant virus as a novel adjuvant in toxicity studies

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ABSTRACT

Spherical particles (SPs) generated by thermally denatured tobacco mosaic virus (TMV) coat protein can act as an adjuvant, as they are able to enhance the magnitude and longevity of immune responses to different antigens.

Here, the toxicity of TMV SPs was assessed prior to it being offered as a universal safe adjuvant for the development of vaccine candidates. The evaluation included nonclinical studies of a local tolerance following the single administration of TMV SPs, and of the local and systemic effects following repeated administrations of TMV SPs. These were conducted in mice, rats and rabbits. General health status, haematology and blood chemistry parameters were monitored on a regular basis. Also, reproductive and development toxicity were studied. No significant signs of toxicity were detected following single or repeated administrations of the adjuvant (TMV SPs).

The absence of toxicological effects following the injection of TMV SPs is promising for the further development of recombinant vaccine candidates with TMV SPs as an adjuvant.

1. Introduction

Interest in vaccine adjuvants is growing rapidly. The reason for this is the desire to develop novel, safe and highly effective subunit vaccines. A large number of different materials are known to have adjuvant activity, (mineral salts, oils, polymers, peptides, nucleic acids etc.). Many of them are currently tested as part of modern vaccine candidates in preclinical and clinical trials.

In recent years, plant viruses have become increasingly used in the development of new biotechnologies in medicine, including vaccinology, and in the delivery of drugs and diagnostics (Acosta-Ramírez et al., 2008; Denis et al., 2008; Savard et al., 2011, 2012; Babin et al., 2013; Karpova et al., 2012; Trifonova et al., 2014; Nikitin et al., 2014). The main advantages of plant virus-based vaccines and adjuvants are biosafety (the absence of common pathogens in plants and mammals), and optimal size for absorption by antigen-presenting cells (McCormick and Palmer, 2008; Nikitin et al., 2016).

Recently, we studied the structure and properties of the spherical particles (SPs) generated by thermal remodelling of the tobacco mosaic virus (TMV) (Atabekov et al., 2011). Molecular structure and features of rod-shaped TMV particles are well-known. Virions have the form of rigid cylinders 300 nm in length and 18 nm in diameter, and consist of 2130 coat protein subunits packaged in a helix around the genomic (+) RNA (Klug, 1999). The phenomenon of TMV remodelling to SPs is associated with changes in the secondary structure of TMV coat protein on heating up to 94 °C (Dobrov et al., 2014). Varying the initial virus concentration makes it possible to control SP size (Atabekov et al., 2015). SPs are very stable to external factors, and biodegradable (Nikitin et al., 2011). The unique feature of SPs is their high immunopotentiating properties. SPs act as an effective adjuvant and stimulate an immune response to different antigens (Trifonova et al., 2014, 2017; Karpova et al., 2012). It is important to note, that TMV SPs efficacy is ten times higher than aluminium-based adjuvant (Trifonova et al., 2017).

In this work, we have studied local and systemic effects following single and repeat dose injections in model animals, including physiological, histological and hematological changes, as well as conducting an analysis of reproductive and developmental toxicity studies.

Abbreviations: spherical particles, SPs; tobacco mosaic virus, TMV; intramuscularly, IM; intravenously, IV; intraperitoneally, IP; plaque-forming cell, PFC; sheep red blood cells, SRBC; delayed-type hypersensitivity, DTH; trinitrobenzenesulfonate, TNBS; standard deviation, SD; white blood cells, WBC; virus-like particles, VLPs; magnetic resonance imaging, MRI

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2. Materials and methods

2.1. Animals

Animal studies were performed under protocols approved by the Federal Research Centre of Biotechnology of the Russian Academy of Sciences Animal Ethics Committee (Ethics Committee Session № 170511), in accordance with national law and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Young adult laboratory animals were used: Wistar outbred rats (140–160 g, 7–8 weeks old), Standard Chinchilla rabbits (3.2–3.5 kg, 5–6 months old) and BALB/c line mice (20–22 g, 8–9 weeks). Animals were kept and handled according to the "European Convention for the protection of Vertebrate animals used for experimental and other scientific purposes (ETS No123)" and federal standards "Laboratory animals keeping and care guidance. Requirements for facilities and procedures" (Russian Federal Standard 33215-2014) and "Laboratory animals keeping and care guidance. Specifications for laboratory rodents' and rabbits' keeping and care" (Russian Federal Standard 33216-2014). Rodents were marked with safety dye, sorted by sex, experimental groups and placed into grid cages with the minimum space for one animal: 100 cm^2 for mice, 500 cm^2 for rats. Breeding for the reproductive toxicity study was performed in individual cages for each pair of animals. Rabbits were housed separately, in grid cages with 5000 cm^2 of individual space. Hay and tree branches were used for environmental enrichment in all cases. Animal facilities were sufficiently ventilated (15 vol per hour), with a comfortable humidity (50 ± 10%) and temperature (18 ± 2 °C for rabbits and 22 ± 2 °C for rats), and a light/dark (12/12) cycle was maintained. "Full ration pellets for mice, rats and hamsters" (MEST LLC, Russia) was given to rats and mice; "Full ration pellets for rabbits" (MEST LLC, Russia) was given to rabbits. The food and drinking water were available without restriction. Intramuscular injections of sterile TMV SPs solution in PBS, or sterile apyrogenic PBS as a control, were performed into the quadriceps muscle group (Nebendahl, 2000). Intravenous injections of SPs solution, of a maximum dose, had duration of at least 10 min. The blood samples were collected from rabbits' marginal ear vein, under local anesthesia, and from rodents' lateral tail vein.

2.2. TMV SPs production (test substances)

TMV SPs were generated by TMV heating, according to (Trifonova et al., 2015). The size and morphology of TMV SPs were controlled by transmission electron microscopy and nanoparticle tracking analysis, as described previously (Nikitin et al., 2013, 2015). TMV SPs were used in apyrogenic PBS sterile solution at concentration of 1 mg/ml.

2.3. Study design

2.3.1. Single dose toxicity studies

Single dose reaction was evaluated on rats, rabbits and BALB/c mice. Five groups of rats, each consisting of six males and six females, received, in a single injection, 50 or 400 μg of SPs intramuscularly (IM), 100 μg intravenously (IV), 3000 μg intraperitoneally (IP) (the IP route proved to be the only feasible way to administer 3 ml per rat safely) and 400 μl PBS IM as a control. Five groups of mice, each consisting of six males and six females, received a single administration of SPs – 10 or 100 μg IM, 10 or 500 μg IV and 100 μl PBS IM as a control. Two groups of rabbits, each consisting of three males and three females, received 30 mg SPs or 30 ml PBS in slow IV injections. All animals were observed daily, and their weight, respiratory rate, heart rate and temperature were recorded. Blood samples were collected the day before SPs were administered, and two days after. Animals were euthanized (three rodents and one or two rabbits per group, on the first, fourth, seventh and fourteenth days) and an autopsy was undertaken. Macroscopic and histologic examinations of the main organs and the site of administration were carried out. The weight of organs was rounded to the nearest mg, and organ/body weight ratio was calculated.

Table 1
Repeated dose toxicity study groups, treatment and dosage.

Group	Treatment	Dosage per animal
Rats		
1 (5 males + 5 females)	PBS	200 μl
2 (5 males + 5 females)	SPs	20 μg *(low dose)*
3 (5 males + 5 females)	SPs	200 μg *(high dose)*
Rabbits		
4 (3 males + 3 females)	PBS	1000 μl
5 (3 males + 3 females)	SPs	100 μg *(low dose)*
6 (3 males + 3 females)	SPs	1000 μg *(high dose)*

2.3.2. Repeated dose toxicity studies

To evaluate potential local and systemic reactions following single injections or three consecutive intramuscular injections, at two-week intervals, SPs in low and high doses were administered on rats and rabbits. Phosphate buffer saline was used as a control (Table 1). Each group of rodents contained five males and five females, while each group of rabbits consisted of three males and three females. The groups were numbered sequentially.

All animals were observed daily, and weight, respiratory rate, heart rate and temperature were recorded. Animals were euthanized on the 42nd day, and an autopsy was undertaken. Macroscopic and histologic examinations of the main organs and site of administration were carried out. Blood samples were collected just before the first administration, and at the days 18 and 41. ECG evaluation was performed at days: one day before the first administration, 17th and 40th (Fig. 1). ECG was measured on a II limb lead with PowerLab 4/26 (ADInstruments, Australia) station, and automatically processed with LabChart (ADInstruments, Australia) software. PR and QT segments, QRS complex duration and P, R, S, T waves' amplitudes for rabbits were evaluated.

2.3.3. Reproductive and developmental toxicity

Fertility and reproductive toxicity, and prenatal developmental toxicity, studies were performed as follows. Thirty male and 30 female young adult outbred Wistar rats were used. Animals were divided into three groups, each consisting of ten males and ten females. The first study group was exposed to 20 μg, and the second to 200 μg, of SPs in PBS IM. The control group was injected with 200 μl of sterile apyrogenic PBS. Males were treated 14 days and one day before mating, and females were treated 14 days before mating, and after one and 14 days of gestation. The mating ratio was 1:1, and a positive vaginal smear was considered to represent 'day zero' of gestation. Males were euthanized on the first day of females' gestation, and females were euthanized on 'day 20' of gestation. Animals were observed daily for clinical signs of altered health status. The following parameters were monitored: number of required mating cycles, implantation sites, live and dead fetuses, dams' weight gain, preimplantation loss, male/female fetus ratio. The fetuses were weighed and examined for visual abnormalities, then one half of the fetuses were subjected to visceral examination, and the other half subjected to skeletal examination using common protocols (Narotsky and Kavlock, 2003).

The whole set of studies was performed in compliance with federal regulatory requirements for recombinant active pharmaceutical ingredients.

2.4. Statistical analysis

Data were analyzed using IBM SPSS Statistics (23.0.0.0 64 bit for Windows). The accepted significance level was $p = 0.05$. The Levene test was used for variance homogeneity significance, and Brown and