

Immune Responses Induced in Mice after Intragastral Administration with Sendai Virus in Combination with Extract of *Uncaria Tomentosa*

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Summary

In the present study, the dried hydroalcoholic extract from the bark of *Uncaria tomentosa* was evaluated as a potential immunostimulator. BALP/c mice were seven times immunized intragastrically (i.g.) with a formalin-inactivated whole Sendai virus (SV) with two doses of the dry extract. The efficacy of the *U. tomentosa* was evaluated by examining specific IgA in saliva as well as IgG and haemagglutination inhibition (HI) in serum antibodies by enzyme-linked immunosorbent assay (ELISA) and HI tests. We found that the animals inoculated with 5.6 mg of the dry extract of *U. tomentosa* induced higher saliva IgA antibodies. Furthermore, the mice immunized e.g. with SV plus 0.56 mg of the extract had significantly higher IgA, IgG and HI antibody responses to SV than did those administered with the SV alone. These results suggest that dry extract from bark of *U. tomentosa* is useful as a mucosal adjuvant for mice.

Introduction

Sendai virus (SV) is a murine parainfluenza virus type 1 (MPV1) (parainfluenza family), which is formed in rodents. It causes a typical respiratory infection and has been often used as a model for respiratory virus infections (*Burek et al., 1977; Ishida & Homma, 1978; Schels & Hartl, 1991; Percy & Palmer, 1997; Liang et al., 1999; Simmons et al., 2002*). Several studies in animals have shown that vaccines consisting of inactivated parainfluenza virus, or components of the virus, may also be immunogenic when given intranasally (*Ito & Matumoto, 1986; Miyamae, 1986, 1999*).

Mucosal immunity, in which secretory IgA antibodies play the important role, is established via the mucosa-associated lymphoid tissues such as those in the respiratory tract and the gastrointestinal tract

(*McGhee et al., 1992; Amore et al., 2004*). The gut-associated lymphoid tissues (GALT), particularly the Peyer's patches, as inductive tissues, contain a large number of IgA precursor B cells, and the stimulation of them with orally administered antigens may lead to the distribution of B and T cells to mucosal effector tissues such as the *lamina propria* region of intestinal, respiratory, and genitourinary tracts and various secretory glands for subsequent antigen-specific IgA antibody responses (*Walker, 1994*).

Plants are invaluable sources of new drugs. There is an ever-growing interest in investigating different species of plants to identify their potential medicinal applications, such as immunostimulatory activity. This increasing interest is due to a tremendous historical legacy in folk medicine use of plants as medicines and their easy availability, cost effectiveness and presumed safety (*Rivera et al., 2003*).

The vine *Uncaria tomentosa* (Willd.) DC. (Rubiaceae), a Peruvian plant commonly known as 'Cat's claw' or 'uña de gato', is used for the treatment anti-leukaemic activity (*Keplinger et al., 1999*). Numerous reports have shown that extracts

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of the bark from *U. tomentosa* possess not only an anti-inflammatory activity (Aquino *et al.*, 1991; Desmarchelier *et al.*, 1997; Reinhard, 1999; Aguilar *et al.*, 2002), but also anti-viral (Aquino *et al.* 1989) and anti-mutagenic activities (Rizzi *et al.*, 1993). In addition, such extracts have been reported to enhance phagocytosis (Wagner *et al.*, 1985) and to stimulate the production of interleukin-1 and -6 in rat alveolar macrophages (Lemaire *et al.*, 1999). The aim of the present study was the assessment of the efficacy of oral immunization of mice with formalin-inactivated whole SV, with and without *U. tomentosa* as mucosal adjuvant. The results were then compared with a separate group of mice that were immunized subcutaneously without adjuvant. The efficiency of the systemic and secretory immunospecific response was evaluated by measurement of antibodies to SV, i.e. IgG, and HI in serum and IgA in saliva.

Materials and Methods

Animals

Groups of BALB/c mice, 6 inbred females in each, 7 to 8 weeks old, weighing 18 ± 2.0 g, were obtained from the breeding house-unit of the Institute of Immunology, Vilnius University (Lithuania). Animals were kept in a corridor system under conditions of semibarrier type in cages of type T-3 (Velaz, Praha, Czech Republic), with three animals per cage.

Chips of deciduous trees sterilized for 20 min. at 120°C were used for bedding. The bedding was changed twice weekly. Temperature was maintained at $20 \pm 2^\circ$ daylight lamps realized C. Illumination during 12 h/day. Ventilation was carried out by circulation of unfiltered air ($0.5 \text{ m}^3/\text{sec}$). Relative humidity and noise were maintained at $55 \pm 5\%$, and 50 dB, respectively.

Food granules were produced by of Kėdainiai (Lithuania) and consisted of gross energy 13.6 MJ/kg, crude protein (20%), crude oil (6.2%), and crude fibre (3.9%). The feed was sterilized for 20 min. at 120°C and balanced as to amino acids and vitamins. Water was provided *ad libitum*.

Virus

SV strain Fushimi used for immunization of mice was obtained from the Institute of Virology (Moscow, Russia) and was grown, purified by centrifugation and inactivated as described previously (Bizhanov & Vyshniauskis, 2000). For the preparation of the mixture for immunization of animals the viral suspension was used at 500 µg in 200 µl of phosphate-buffer saline (PBS) pH 7.2.

Adjuvant

The stem bark of *U. tomentosa* was obtained from Pharmaceutical Company "Evalar" (Biisk, Russia). 200 g of bark was extracted for 20 h at room temperature (RT 20°C) with 30% (v/v) EtOH (drug/solvent ration 1:10). The extract was filtered with a stainless-steel mesh. The filtrate was clarified at 2000 g and RT for 20 min and then vacuum dried. The yield of the extract was about 10.0% (w/v). The formalin-inactivated whole Sendai virus and dry extract of the *U. tomentosa* were mixed immediately before immunization.

Immunization protocol

The study consisted of four treatment groups with six mice in each group. The treatment groups were: Group A: SV alone. The viral suspension containing 500 µg of SV (measured as total protein) in 200 µl of PBS per mouse. This mixture was used in B and C Groups.

Group B: SV+0.56 mg *U. tomentosa*. The viral suspension was mixed with 0.56 mg of the dry extract *U. tomentosa*.

Group C: SV+5.6 mg *U. tomentosa*. The viral suspension mixed with 5.6 mg of the dry extract *U. tomentosa*.

Group D: The viral suspension, 5.0 µg of SV (measured as total protein) in 50 µl of PBS per mouse, was administered subcutaneously (s.c.) into the posterior thigh muscle.

All the groups of the mice were immunized intragastrically (i.g.), except Group D, seven times at two-day intervals (at days 0,2,4,6,8,10 and 12). The immunogen mixture was administered i.g. into the

stomach using a 20-gauge stainless steel feeding needle attached to a 1-ml syringe, 200 μ l/mouse. Group D was immunized twice at days 0 and 12. The animals were not anesthetized during immunizations.

Collection of samples

Saliva and serum were collected for antibody measurements at days 2, 7 and 14 after the last immunization. Saliva samples were collected with absorbent wicks consisting of synthetic fibres and cellulose (Polyfiltronics Group Inc., Rockland, MA, USA) after a single intraperitoneal injection of 0.1 mg pilocarpine-HCl (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 200 μ l of PBS, and the net weight recorded. Two wicks saturated with saliva were obtained from each mouse, frozen at -20°C in 1.5-ml microcentrifuge tubes, and subsequently extracted with 400 μ l of PBS with 0.05% Tween 20, as described earlier (Haneberg *et al.*, 1994). Blood samples were collected from the lateral femoral vein in heparinized capillary tubes (Vitrex, Herlev, Denmark) and were separated and stored at -20°C until they were analysed (Aaberge *et al.*, 1992). The final blood samples were taken by cardiac puncture during CO₂-anaesthesia.

Sera pretreatment

Sera samples were heat inactivated at 56°C for 30 min and then, to avoid a false positive reaction in haemagglutination inhibition (HI) test sera, were pretreated by kaolin and chicken erythrocytes. For this pretreatment, 30.0 μ l of 25% acid-washed kaolin (Sigma, St. Louis, MI, USA) was added to 10.0 μ l of serum, mixed briefly using a vortex mixer, and incubated for 2 h for 4°C. Following centrifugation at 2000 g and 4°C for 10 min, the supernatant was removed to a clean centrifuge tube, 10.0 μ l of packed chicken erythrocytes was added, and the suspension was gently mixed. After an overnight incubation at 4°C, the erythrocytes were pelleted by centrifugation at 200 g and 4°C for 10 min, and the supernatant was removed for using in the HI test.

Haemagglutination inhibition

Anti-SV virus antibody titres were measured by HI. Serial 2-fold dilution in 50 μ l of PBS of serum was prepared in duplicate in 96-well plates (U-shaped bottom, Nunc A/S Roskilde, Denmark). Four haemagglutinin units of SV in 50 μ l were added to each well and the plates were incubated at room temperature (RT) for 1 h; afterwards 50 μ l of 1% chicken erythrocytes was added. The results were read after incubation for 2 h at 4°C. The antibody titres were expressed as the reciprocal of the highest dilution of serum at which complete inhibition of haemagglutination was seen and the titres were converted to a base-2 logarithmic scale.

Quantitation of antibodies

SV-specific IgA in saliva and SV-specific IgG antibodies in serum were analyzed by enzyme-linked immunosorbent assay (ELISA) using Nunc Immuno Plates (MaxiSorp F96; Nunc A/S Roskilde, Denmark). The plates were coated at least overnight at 4°C with the formalin-inactivated SV at a concentration of 7.0 μ g/ml in 0.01 M PBS. Non-specific protein-binding sites were blocked with PBS containing 5% skimmed milk (Oxoid Ltd., Hampshire, UK), and after 1 h incubation at 37°C, followed by 30 min at RT, the plates were washed with PBS containing 0.05% Tween 20. Serum samples and extracts of saliva were applied to the ELISA plates (100 μ l per well), serially diluted two-fold in the blocking solution, and incubated at 4°C overnight. The plates were then washed with PBS containing 0.05% Tween 20, before being incubated for 1 h at RT with peroxidase-conjugated goat antibodies directed against mouse IgA or IgG (Sigma, USA) both diluted 1:1000 in blocking buffer (100 μ l per well). After washing, bound antibodies were detected with *o*-phenylenediamine (Sigma, USA) in 0.05 M phosphate-citrate buffer, pH 5.0 containing 0.012% H₂O₂. Colour was developed for 30 min at room temperature and afterwards the reaction was stopped by addition of 50 μ l per well 1.25 M H₂SO₄. The optical densities were read at 492 nm in Titertek Multiscan Plus MK

II (Labsystems, Helsinki, Finland). The antibody titres were expressed, as the reciprocal of the highest dilution of serum optical density, which was twofold higher than that of the negative samples. The titres were converted to a base-2 logarithmic scale. The unknown samples were corrected for the weights of the original samples and for dilutions made during the extraction from wicks and preparation for ELISA.

Total protein estimation

The protein content of inactivated SV was calculated as described earlier (Lowry et al., 1951).

Ethical committee approval

We have performed the experiment on mice after having received permission No 0086 of the Ethics Committee on the Use of Laboratory Animals, at the State Food and Veterinary Service (Vilnius, Lithuania).

Statistical analysis

The mean of the IgA and IgG, and antibody titres in HI were compared using unpaired two-tailed Student's *t*-test. All values were expressed as geometric mean titres \pm standard error and were considered to be statistically significant at $P < 0.05$.

Results

The IgA antibody response after immunization with SV in combination with U. tomentosa.

Figure 1 shows that the IgA titres in Group C were significantly higher than those in Groups A, B and D at days 2, 7 and 14 after the last immunization and also that there was significant difference in IgA titres among Groups A, B and D. Notably, IgA titres were significantly increased within 2 to 14 d after the last immunization only in Group C.

The IgG antibody response after immunization with SV in combination with U. tomentosa.

There was a significant difference in IgG titres among Groups A, B, C and D at days 7 and 14 after the last immunization (Figure 2). In Groups A and

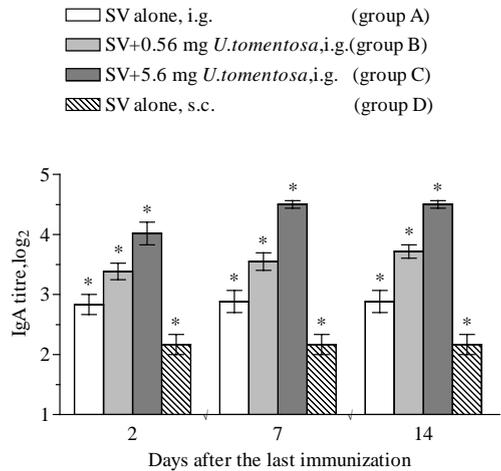


Figure 1. Saliva IgA antibody response in mice after i.g. and s.c. immunizations with SV alone or admixed with the extract *U. tomentosa*. Asterisks indicate groups the values of which differ significantly from those of others on the same day.

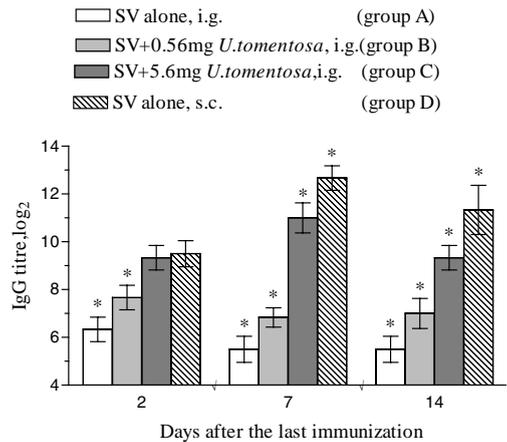


Figure 2. Serum IgG antibody response in mice after i.g. and s.c. immunizations with SV alone or admixed with the extract *U. tomentosa*. Asterisks indicate groups the values of which differ significantly from those of others on the same day.

B, IgG titres were significantly reduced within 2 to 7 d after the last immunization and in Group A remained low whereas, in the Group B responses increased slightly again at final day of observation. In contrast, IgG titres were observed to peak at day 7 after the last immunization in C and D Groups and then were significantly reduced by day 14 after the last immunization.

The serum HI antibody response after immunization with SV in combination with U. tomentosa.

There was significant difference in the HI antibody titres among all Groups at days 7 and 14 after the last immunization (Figure 3). In all days of the observation the HI antibody titres remained at a similar low level in Group A. On the other hand, the responses were significantly increased within 2 to 14 d after the last immunization in other groups and in Group D were higher at all days of the observation.

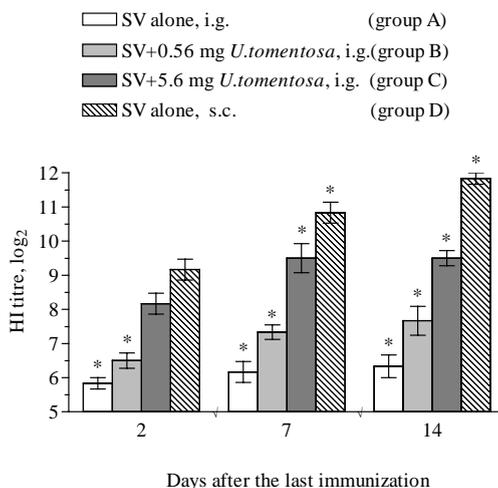


Figure 3. Serum HI antibody response in mice after i.g. and s.c. immunizations with SV alone or admixed with the extract *U. tomentosa*. Asterisks indicate groups the values of which differ significantly from those of others on the same day.

Discussion

In this study, we have demonstrated that i.g. immunization with SV in combination with dry extract of the *U. tomentosa* could also prime the immune system for both secretory and systemic booster antibody responses to later repeated i.g. immunizations. Furthermore, addition of a dry extract of the *U. tomentosa* to the immunization mixture had an effect on the enhancement of the animals' immune response over a long time.

It is important, that the high titres to SV in saliva indicate that the induction of antibody responses had taken place in the mucosa of the upper respiratory tract. In addition, the animals immunized either i.g. or s.c. with SV alone showed high serum IgG and HI antibody titres. Induction of both systemic and secretory antibody responses is a desirable characteristic of mucosal administered vaccines (Kunisawa *et al.*, 2001). These findings suggest that repeated immunization with the whole virus in combination with dry extract of the *U. tomentosa* as mucosal adjuvants may allow immune memory to develop as well as to form the protection mechanism against viral infection (Lycke & Holmgren, 1987).

Our results suggest that non-proliferating vaccines based on extracts containing biological active components from plants may be effective when given i.g. and that they may be used in conjunction with similar vaccines.

This work is a necessary step prior to the use of the vaccine formulations. The intragastral mode of immunization has the prospect to increase the efficacy and safety of vaccines and also to develop an active intragastral parainfluenza vaccine.

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