EXPERIMENTAL PAPER

Development of novel plant-based adjuvant formulation against rubella and hepatitis B vaccine antigen

AMIT GUPTA*, SUSHAMA R. CHAPHALKAR

Department of Immunology and Virology
Vidya Pratishthan's School of Biotechnology
Baramati-413133
Maharashtra, India

*corresponding author: e-mail: amitvsbt@gmail.com; amitgupta@vsbt.res.in

Summary

Introduction: Numerous metabolites present in the aqueous extract from plants are responsible for inducing adjuvant activity against rubella and hepatitis B vaccine antigen (HBsAg). One of the medicinal plants, *Adhatoda vasica* has been pointed out with great potential of vaccine adjuvant property. **Objective:** The objective of our study is to evaluate the adjuvant potential of aqueous leaves extract of *Adhatoda vasica* against rubella and hepatitis B vaccine antigen (HBsAg). **Methods:** For these studies, our group evaluated the antibody (IgG) titre of HBsAg and rubella vaccine antigen using variable doses (0.625–5 mg) of aqueous leaves extract of *Adhatoda vasica* and also determined the lymphocyte (splenocyte) proliferation assay (0.625–5 mg; 50 μ l) in mice model studies *ex vivo* (i.e. immunized with HBsAg subcutaneously). **Results:** The results showed that aqueous leaves extract showed anti-HBsAg and anti-rubella titre and also enhanced the lymphocyte proliferation assay at higher doses (5 mg) as compared to control. **Conclusion:** Aqueous leaves extract of *Adhatoda vasica* showed adjuvant activity against HBsAg and rubella vaccine antigen.

Key words: *Adhatoda vasica*; hepatitis B vaccine; rubella vaccine; adjuvant

INTRODUCTION

Vaccination is considered to be one of the greatest medical achievements and showed its significant reduction of mortality rate related to various infectious diseases [1, 2]. However, the existence of various diseases such as malaria,

tuberculosis and cancer poses a great threat to human beings and these challenges has driven the emergence of novel plant based adjuvant pertaining to immunogenicity for vaccine design approach [3, 4]. In this regard, lot of efforts have been made and put into the development of new safe adjuvant candidate for vaccine design approach. Indeed, development of vaccines containing proposed adjuvant candidate has proven difficulty, especially against chronic infections or cancer [5, 6]. To overcome these obstacles, large research efforts, especially immunological investigations, are put to better understanding of the key elements required to generate protective immunity. The majority of available vaccines enhanced the generation of antibodies (IgM and IgG, neutralize targeted pathogens) through cellular immune response which is required to protect against various infections [7, 8]. As per the literature, balanced and complete immune response involving both humoral immunity (high-affinity neutralizing antibodies limiting pathogen transmission) and cellular immunity (cytotoxic CD8+ T cells killing infected cells) is required to control malaria, hepatitis C, HIV/AIDS etc., which highlights the need for the development of new vaccine design approaches containing adjuvant [9]. Moreover, epitopes of various pathogenic micro-organisms targeted by protective immune responses have been identified and led to the design of safer and more specific recombinant vaccines, although they are often less immunogenic (pertaining to antibody titre) than more traditional vaccines based on live attenuated pathogens [10]. In this regard, selection or addition of proposed adjuvant candidate, especially from medicinal plant (*Adhatoda vasica*) will likely be required to achieve protective immunity with these vaccine antigens, i.e. HBsAg and rubella vaccine. Scientific researchers suggest that the best way to generate the adjuvant for vaccine antigen that is capable for inducing broad immune response with high levels of antibodies and cytotoxic T lymphocytes is to mimic a pathogenic infection [11] while remaining as safe as possible. In this regard, plant-based candidates have been explored and increasingly studied for last few years with the aim of developing new effective vaccines. Of particular interest, the utilization of plant based candidates especially *Adhatoda vasica* has gained increasing significance as an effective tool in the development of novel vaccine adjuvant against infectious diseases and cancer. In the present study, the recent advances in the use of aqueous leaves extract as adjuvants and understand their mechanism of action was described. In order to determine its immunogenic properties the design of novel, safe, and efficacious prophylactic and therapeutic vaccines against disease will be enabled.

Adhatoda vasica is a medicinal plant commonly grown in different habitat conditions including different types of soil throughout India, Sri Lanka and Malaysia. As per the literature, leaves of this medicinal plant have been used to relieve asthma, bronchitis and cough [12-14]. In addition, leaves (pasted) are applied on the abdomen for relieving urinary infections and also relieve from piles, peptic ulcers etc.

One of the contagious viral diseases, rubella, is reported mostly in children and pregnant women. This disease showed some symptoms like fever, rashes etc. and can be prevented through vaccination already available in the market, i.e. rubella [15] vaccine (live attenuated virus propagated on human diploid cells). This rubella vaccine (using single dose) is almost effective (more than 95%) against this disease but when in combination with measles and mumps vaccine known as MMR vaccine. Similarly, hepatitis B, viral disease that directly attacks our digestive system, especially liver, showed various symptoms such as cirrhosis (scarring) of the liver, including cancer [16]. Hepatitis may show short- (acute hepatitis, first six months) and long-term (chronic hepatitis, virus persists within the body) illness. For the elimination of this viral disease, HBsAg vaccine was produced (Serum Institute of India) containing HBsAg (20 μ g) adsorbed on aluminium hydroxide (0.5 to 0.8 mg). This immunological research will add to the understanding how to formulate proposed adjuvant candidate extracted from *Adhatoda vasica* in vaccines that are targeted specifically against viral infections.

MATERIAL AND METHODS

Plant material

Fresh mature plant leaves of *Adhatoda vasica* were gathered in January 2016 from the Vidya Pratishthan's nakshatra udyan which mentioned the importance and medicinal use of this tree in Marathi book language, *Nakshatra vriksha*.

Preparation of plant material

The collected plant leaves of *A. vasica* were properly cleaned and washed with distilled water in order to remove the dust particles. The fresh healthy plant leaves were selected and macerated in liquid nitrogen to form powder. This powder is usually macerated in phosphate-buffered saline and filtered using Whatman filter paper. The supernatant was collected after filtration and quantified the secondary metabolites in the aqueous extract using HPTLC.

HPTLC study

This study has been used for quantitative analysis of secondary metabolites in the aqueous leaves extract of *A. vasica*. As per the HPTLC results, it revealed the presence of secondary metabolites, i.e. saponin, terpenoids, flavonoids and

alkaloids in the phytochemical profile of aqueous extract of leaf of *A. vasica*. These HPTLC results almost showed the excellent reliability, reproducibility, accuracy and precision of the method. The retardation factor (Rf) value of secondary metabolites, i.e. terpenoid (0.97), alkaloids (0.21 and 0.96; peak area 390 nm) and saponin (0.35; peak area 290). In addition, traces of metal are reported in 1 g of leaves i.e. Cu (13 ppm); Fe (154 ppm); Mn (35 ppm); Mg (236 ppm); Ca (3512 ppm) and Zn (5 ppm).

Elisa

Indirect Elisa was performed using standard HBsAg and rubella vaccine (1:1000 dilution) as coating antigen. Variable doses (0.625–5 mg) of aqueous leaves extract of *A. vasica* were used as anti-HBsAg as well as rubella serum as a standard for the estimation of IgG antibody titre. Horse anti-serum was used as secondary antibody and optical density was measured at 450 nm [9].

Lymphocyte proliferation assay

All these studies were conducted under ethical guidelines of animal ethics committee with registration No. 1814/PO/ERE/S/15/CPCSEA.

Briefly, mice were immunized on day 0 with HBsAg (100 μ l) subcutaneously and on day 4, splenocytes were excised removed aseptically from the abdominal cavity of mouse [17]. Splenocytes (10^6 cells/ml; 100 μ l) were taken in 96 well plate in presence of HBsAg (20 μ g/ml; 10 μ l) and rubella (≥ 1000 CCID₅₀; 10 μ l) vaccine for 48 h incubation in carbon dioxide incubator along with variable concentration of aqueous leaves extract of *A. vasica* in a final volume of 0.2 ml. These studies were conducted and analyzed at the VSBT, Baramati, Maharashtra, India, from January to February 2016. After incubation, MTT (2.5 mg/ml; 10 μ l) solution was added in 96 well plates. Again the plate was incubated for next 3–4 h; then centrifuged, the supernatant was discarded and formazon crystals were settled at the bottom. These crystals were dissolved in dimethyl sulphoxide and the optical density was measured at 570 nm.

Statistical analysis

The difference between control and treated groups of *A. vasica* were determined by one way ANOVA test (Bonferroni multiple comparison test).

RESULTS

Elisa

Elisa results confirmed that aqueous leaves extract of *A. vasica* showed anti-HBsAg and anti-rubella vaccine titre at higher doses, as shown in figure 1. Anti-HBsAg and anti-rubella cell culture supernatant was used as standard for this studies and results showed the enhancement of anti-HBsAg and anti-rubella titre, as compared to control.

Figure 1. ELISA assay. Indirect Elisa was performed using standard HBsAg and rabies vaccine (1:1000 dilution) as coating antigen. Aqueous leaves extract of *Adhatoda vasica*, anti-HBsAg and anti-rubella samples were used for the estimation of anti-HBsAg and anti-rubella antibody titre. Horse anti-serum used as secondary antibody and optical density measured at 450 nm. The difference between the control and standard is determined by one way ANOVA test

Lymphocyte proliferation assay

The effect of aqueous leaves extract of *A. vasica* on lymphocyte proliferation assay as shown in figure 2. The results showed that aqueous leaves extract showed anti-HBsAg and anti-rubella proliferation at a dose range of 5 mg. Anti-HBsAg and anti-rubella cell culture supernatant was used as standard for these studies and results showed enhancement of proliferation as compared to control.

Figure 2.

Lymphocyte (splenocyte) proliferation assay. Spleen cells (10^6 cells/ml) were treated with variable doses of aqueous leaves extract in presence HBsAg and rubella vaccine antigen (as already described in materials and methods section). Values are expressed in mean $\pm$ SE. The difference between the control and standard is determined by one way ANOVA test

DISCUSSION

Most of the vaccines administered currently (immunization, challenging as well as booster) in animals and humans enhance the humoral- (antibody) and cell-mediated immune response. During vaccination, it protects individuals or animals against prophylactic as well as therapeutic infections [1, 2]. Recently, researchers focused on various medicinal plants tried to use as an adjuvant for vaccine antigen pertaining to enhancement of antibodies and cell mediated immune response. Moreover, these vaccines are available commercially and often administered in conjunction with less toxic adjuvants, especially alum (approved for human use) compared to Freund's Complete Adjuvant; QS-21 and QuilA, saponin-based or RIBI (not approved for human use) or even without the use of any adjuvant. In present study, we discuss the immunogenicity or adjuvant activity of aqueous leaves extract of *A. vasica* against rubella and HBsAg.

Medicinal plants were considered to be alternative sources of vaccine adjuvants against specific vaccine (protein) antigens [18-20]. However, administration (subcutaneously/intraperitoneally) of these medicinal plants in the form of primary and secondary metabolites were used as an adjuvant for vaccine antigen and showed some benefits to our immune system [18-20]. Despite of

this, number of attempts were taken, and no real anti-HBsAg and anti-rubella plant-based vaccine containing adjuvant has been developed. For its isolation, extraction as well as purification of adjuvants from various medicinal plants which were considered to be safe and totally free from animal or human pathogens and also showed some beneficial effects in terms of cost, distribution and production [19, 20].

In this study, HBsAg and rubella vaccine antigen were tested with variable doses of aqueous leaves extract of *A. vasica* using lymphocyte proliferation assay in animal model studies *ex vivo* using Swiss mice. When these vaccines i.e. HBsAg and rubella were exposed to splenic lymphocytes in presence of aqueous leaves extract, there is dose dependent change in the proliferation as compared to standard vaccine and control. In addition, aqueous leaves extract tested against these vaccine antigens i.e. HBsAg and rubella vaccine for the estimation of IgG antibody titre. These antigens were recognized strongly by aqueous leaves extract of *A. vasica* containing primary as well as secondary metabolites and antibody-secreting cells from vaccines. Additionally, there was good correlation between the aqueous leaves extract of medicinal plant (*A.vasica*)-made vaccine adjuvant candidate and the conventional vaccine antigen both by Elisa assay. The conclusion was that the aqueous leaves extract of *A. vasica* had good vaccine adjuvant potential, but needed good preservatives for its long-term use in a clinical trial. In short, identifying an adjuvant activity that can accommodate the aqueous extract of *A. vasica*, increase the immune response and have a low toxicity level will increase the possibility of formulating a vaccine that will aid in the prevention and control of viral disease. Studying this interaction will allow us to further elucidate the effectiveness of antigen adjuvant combinations.

CONCLUSION

The proposed adjuvant candidate (*A. vasica*) was selected due to their promising results in previous studies as well as their integral role in cellular and humoral immunity. These findings about plant-based adjuvants can be used to formulate other vaccine antigens.

Conflict of interest: Authors declare no conflict of interest.

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OPRACOWANIE NOWEGO ADIUWANTU ROŚLINNEGO PRZECIWKO ANTYPENOM SZCZEPIONKI PRZECIW RÓŻYCZCE I WIRUSOWEMU ZAPALENIU WĄTROBY TYPU B

AMIT GUPTA*, SUSHAMA R. CHAPHALKAR

Department of Immunology and Virology
Vidya Pratishthan's School of Biotechnology
Baramati-413133
Maharashtra, India

*autor, do którego należy kierować korespondencję: e-mail: amitvsbt@gmail.com; amitgupta@vsbt.res.in

Streszczenie

Wstęp: Wiele metabolitów obecnych w wyciągach wodnych z roślin jest odpowiedzialnych za indukcję adiuwantowej aktywności przeciwko antypenom szczepionek przeciw różyczce i wirusowemu zapaleniu wątroby typu B (HBsAg). Jedną z roślin leczniczych, *Adhatoda vasica* ma duży potencjał adiuwacyjny w szczepionce. **Cel:** Celem naszej pracy była ocena możliwości adiuwacyjnych wyciągu wodnego z liści *Adhatoda vasica* przeciwko antypenowi szczepionki przeciwko różyczce i wirusowemu zapaleniu wątroby typu B (HBsAg). **Metody:** Na użytek badań nasza grupa oceniła miano przeciwciała (IgG) HBsAg i antypenu szczepionki przeciwko różyczce przy pomocy różnych dawek (0,625–5 mg) wyciągu wodnego z liści *Adhatoda vasica*, a także określiła proliferację limfocytów (splenocytów) (0,625–5 mg; 50 µl) w modelu mysim *ex vivo* (tj. immunizowanych przez podawanie podskórne HBsAg). **Wyniki:** Na podstawie otrzymanych wyników wykazano, że wyciąg wodny z liści wpływał na miano przeciw HBsAg i przeciwróżyczkowe, a także w wyższych dawkach (5 mg) wzmacniał proliferację limfocytów w porównaniu do grupy kontrolnej. **Wniosek:** Wyciąg wodny z liści *Adhatoda vasica* wykazał działanie adiuwacyjne przeciwko antypenowi HBsAg i antypenowi szczepionki przeciwko różyczce.

Słowa kluczowe: *Adhatoda vasica*; szczepionka przeciwko wirusowemu zapaleniu wątroby typu B; szczepionka przeciwko różyczce; adiuwant