

# Nanoparticles as Adjuvant in Development of Vaccine Formulations

Vahid Asgary\*, Reza Ahangari Cohan, Omid Kord Mafi, Mohammad Sadeq Khosravy, Rouzbeh Bashar, and Alireza Janani

**Abstract**—Scientists have been considered nanoparticles as promising tools because of high applicability. Recently, nanoparticles are evaluated for their capability to increase immune responses as an adjuvant. On the other hand, neutralizing antibodies were approved as the main response to vaccination in many disease. Therefore, we employed inactivated rabies virus to evaluate AgNPs adjuvanticity. Different concentration of AgNPs (5, 10, 15 and 20 mg/ml) were added to inactivated rabies virus. The mice were immunized by two i.p. injection of each concentration on first and 7th day. The inactivated virus and Alum were used as negative and positive controls, respectively. The bloods was collected from healthy and immunized mice one week later. The serum was isolated from each sample and the amount of neutralizing antibodies was determined by RFFIT. The results showed that 10, 15 and 20 mg/kg of AgNPs had significant difference compared to control but 5 mg/kg of AgNP did not show significant effect. Virus loaded AgNPs can raise neutralizing antibodies against rabies virus at least 10 mg/kg in mice. Herewith with showed that AgNPs have adjuvant effect to produce immune responses against rabies virus which is important in clinical treatment of rabies disease.

**Keywords**—Adjuvant, Silver Nanoparticle, Neutralizing Antibody

## I. INTRODUCTION

VACCINATION is one of the most effective approaches in managing healthcare cost in all countries. Unfortunately, the vaccines do not usually show good immunogenic properties similar to native microorganisms because of either chemical modifications or usage of none whole live microorganism [1]. In many cases, adjuvants are employed to evoke more powerful immune responses. The optimally formulated adjuvant must be safe, stable before administration, readily biodegraded and eliminated, able to promote an antigen-specific immune response, inexpensive to produce, and easy to use [2]. Until recently, however, only one type of adjuvant-aluminum salts, have been widely used

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within licensed human vaccines [3], even though a variety of novel adjuvants have been evaluated in the past few decades such as oligonucleotides, emulsions(w/o or o/w) with the most famous member named Freund's adjuvant, ISCOMATRIX and etc [4]. The adjuvants enhance immunogenicity of through sustained release of antigen in injection site and stimulation of innate immunity [5, 6, and 7]. At present, the choice of adjuvants for human vaccination reflects a compromise between a requirement for adjuvanticity and an acceptable low level of side effects. Other problems with the development of adjuvants include restricted adjuvanticity of certain formulations to a few antigens, use of aluminum adjuvants as reference adjuvant preparations under suboptimal conditions, non-availability of reliable animal models, use of non-standard assays and biological differences between animal models and humans leading to the failure of promising formulations to show adjuvanticity in clinical trials [8]. Some of the side effects can be ascribed to an unintentional stimulation of different mechanisms of the immune system whereas others may reflect general adverse pharmacological reactions which are less expected. Due to the limited adjuvanticity effect of aluminum salts, constant mutation of existing microbes, and ever identification of new disease-causing microbes, extensive search of more effective adjuvants has been the focus of many scientists for many years [4]. In this study, the ability of silver nanoparticles (AgNPs) to evoke immune response against rabies virus was investigated and the results were compared to, commercially available adjuvant, Alum.

## II. MATERIAL AND METHODS

### A. Loading of inactivated rabies virus on AgNPs

0.5 ml of inactivated virus (Institute of Pasteur of Iran, Lot Number: 92-2) was added to AgNPs (Sigma-Aldrich, USA, size < 100 nm) at different concentrations (5, 10, 15 and 20 mg/Kg) and incubated for an overnight at 4 °C with gentle stirring. Four groups with six female NMRI mice (average weight ~20 g, Institute of Pasteur of Iran) were chosen for in vivo test. In addition, Rabies Vaccine (containing Alum, Institute Pasteur, Iran) and inactivated virus (without Alum, Institute Pasteur, Iran) were used as positive and negative controls, respectively. The mice were injected i.p. two times with 0.5 ml of each vaccine at 1th and 7th day and the blood samples were collected one week later (14th day). The Serum was subsequently isolated by centrifugation of each sample at 5000 rpm / 10 min after 1 hr. incubation at RT and stored at -20 °C for further analysis.

### B. Determination of neutralizing antibody titers by RFFIT

The isolated serums were inactivated by incubation at 56 °C for a half hour. Then, three-fold serial dilutions of reference (WHO Reference) and sample serums were prepared out to 12th well of a 96-well plate using MEM medium as triplicate. Subsequently, 50 µl of live rabies virus (CVS-11, Institute of Pasteur of Iran, Iran) with sufficient quantity to infect 80 % of cells in each well was added to each well and incubated at 37 °C for 1 hr in CO<sub>2</sub> incubator. MEM instead of CVS and PBS instead of serum were used as negative and positive controls, respectively. 50 µl of BSR cell suspension with MEM supplemented with FBS 10 % (5×10<sup>4</sup> cells / well) was added to each well and incubated at 37 °C for 24 hr in CO<sub>2</sub> incubator. The plate were rinsed by PBS three times and fixed using 80 % cold acetone for 30 min at 4 °C. Finally, the plate was stained by 50 µl FITC-conjugated anti nucleocapside polyclonal antibody (Bio-Rad, USA) and the percentage of infection was determined by fluorescent microscope in each well. The neutralizing antibody titers were calculated using Reed & Muench method.

### C. Statistical Analysis

All results are presented as mean ± SD. The significance of differences between groups was determined by student's t test. The SPSS software (version 16) was used for all computer analyses. The differences were significant when  $\alpha < 0.01$ .

## III. RESULTS

Statistical analysis showed that 10, 15 and 20 mg/kg of AgNPs had significant difference compared to inactivated virus (without adjuvant) group but 5 mg/kg of AgNP did not show significance value. In AgNPs received groups, no significant difference between 15 and 20 mg/kg of AgNPs was found. However, a significant difference was observed with respect to 10 mg/kg AgNP group. Although the positive control group (Alum) had most significant neutralizing antibody level (Figure 1), statistical analysis did not show any significant differences with regard to 15 and 20 mg/kg groups.

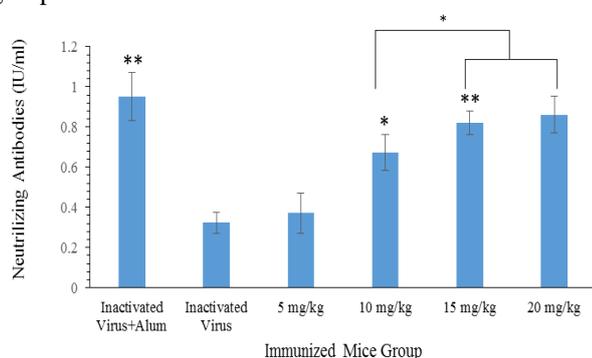


Fig. 1 Determination of neutralizing antibodies in immunized mice raised by different amount of AgNPs. Alum and inactivated virus were used as positive and negative controls, respectively. All data were duplicated and presented as Mean ± SD and significant level were assumed as follow; \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

## IV. DISCUSSION

In present investigation, we assessed adjuvant effect of AgNPs on rabies vaccine and the results were compared to commercially available rabies vaccine. The identity of AgNPs was previously shown by Xu et al which constituted only of silver and oxygen atoms that confirmed adjuvant observed effect was only due to by AgNPs [9]. The mean size of AgNPs in water was reported 141 nm and a negative charge of -30.6 mv as determined by Dynamic Light Scattering (DLS) method. Although the in vitro and in vivo toxicity of Ag ions has been proved but no toxicity was observed because it was shown that the release of Ag ions from AgNPs was negligible [9]. Unfortunately, Many adjuvant candidates which have potent action on immune system, have remarkable poisonous effects on peripheral microenvironments lead to fail in achievement of an efficient and safe immunopotentiator [10].

In current study we also elucidate the adjuvanticity effect of AgNPs as previously shown by Xu et al. The adjuvant effect on rabies vaccine was observed above 10 mg/kg as intraperitoneal (i.p.) injected to mice. In similar study, carried out by Xu, the effect was shown at 0.4 mg/kg for two protein model, Ovalbumin (OVA) and Bovine serum Albumin (BSA) when compared to PBS received group [9]. It must be noted that subcutaneous (s.c.) immunization had better effect because many studies illustrated that route of administration plays important role in immune system stimulation [11].

With increasing of adjuvant concentration, the adjuvanticity effect was also increased reach to a plateau qua no significant discrepancy was observed between 15 mg/kg and 20 mg/kg concentrations. In addition, adjuvanticity effect is comparable with Alum at 15 mg/kg and 20 mg/kg concentrations whereas in Xu study this phenomenon was observed at lower concentrations for both protein models (2 mg/kg and 10 mg/kg for BSA and OVA, respectively). This observation may be due to used antigen type as our antigen is a complex antigen. Although it was reported that the adjuvanticity effect of AgNPs is not depended on kind of antigen but it must noted that they used similar antigens and this may explain the same obtained results [9]. Another reasons may be related to protocol of immunization and type of employed mice. Furthermore, it was reported balb/c mice had more potent immune humoral responses [12]. It is noteworthy that our antigen is lonely more immunogenic rather than BSA or OVA and increase of immune responses by adjuvant is more difficult.

The mechanisms of adjuvant effect of AgNPs is not clearly known. But investigations suggested that AgNPs act via cytokines release, recruitment of leukocytes and up-regulation of Major Histocompatibility (MHC) class II expression of peritoneal macrophages. In addition it reported that AgNPs like Alum stimulate the T helper II depended response leads to neutralizing antibodies. Although it was not argued but it was reported that AgNPs in aqua tend to be aggregated and it may be the presumptive mechanism of in addition mentioned reasons [9].

## V. CONCLUSION

We showed herewith the adjuvanticity effect of AgNPs on raising of rabies neutralizing antibodies. The AgNPs can be

more evaluated on various antigens and assessment of involved mechanisms in and overall we confirmed the indubitable effect of nanotechnology in developing of new biomaterials which can be used in new application areas.

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