Immunogenicity of lyophilized recombinant adenovirus-based vaccine expressing HIV-1 gagpol in mice[☆]

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Abstract

objective. To evaluate the stability and immunogenicity of lyophilized recombinant adenovirus-based vaccine expressing HIV-1 gagpol(Ad-gagpol vaccine). *Methods*. Screening the optimal novel protector excipient according to the appearance, virus titer and thermostability of the lyophilized Ad-gagpol vaccine. Western blot analysis and IFN-γ Elispot assay were used to detect the immunogenicity of lyophilized Ad-gagpol vaccine in mice. *Results*. Optimal protector excipient and buffer system of lyophilized Ad -based vaccine were identified. It was found to be fairly stable following lengthy exposure to higher temperature. The mice which administered lyophilized Ad-gagpol vaccine produced indistinguishable antibody titer and IFN-γ ELISPOT level compared with liquid Ad-gagpol vaccine group ($P > 0.05$). *Conclusion*. Lyophilized Ad-gagpol vaccine can induce high immunogenicity in mice. The protector containing human serum albumin, trehalose,mannitol, dextran and sucrose was suitable for lyophilized Ad-gagpol vaccine. [Life Science Journal. 2009; 6(1): 13 – 17] (ISSN: 1097 – 8135).

Keywords: Ad-gagpol vaccine; lyophilize; stability; immunogenicity

1 Introduction

Recombinant replication-defective adenoviral (Ad) vectors are being developed as vaccine vehicles to immunize against a number of pathogens[1]. These vectors are promising because they generate strong transgene-specific CD8+ T cell responses in both animal models and people[2]. However, recombinant adenovirus is sensitive to repeat freeze-thaw cycle and easy to lose activity. The rapid loss of vector infectivity during storage and shipment has been reported[3].

In this report, through screening (Data not shown), we identified right protectant excipient, buffer systems and stability of lyophilized recombinant adenovirus-based hiv vaccine expressing HIV-1 gagpol (Ad-gagpol vaccine) during storage at both refrigerated and clinically relevant storage temperatures. Also, evaluate the immunogenicity of lyophilized Ad-gagpol vaccine in mice. We expect that our results can contribute to open further delivery applications for vaccination strategies.

2 Materials and Methods

2.1 Cell culture

HEK293 cells were cultured in Minimum Essential Medium (MEM) supplemented with 5% (v/v) fetal bovine serum (FBS) and 2 mM glutamine. The cells were kept in a humidified atmosphere of 5% CO_2 in air at 37 ºC.

2.2 Preparation of liquid Ad-gagpol vaccine

Ad-gagpol vaccine was amplified in the HEK293 cells. The vaccine was purified from cell lysates by banding twice on CsCl gradients, followed by desalting with PD-10 Desalting columns (USA) equilibrated with sterile 10 mM Tris and 2 mM $MgCl_2$. The virus was resuspended in a PBS solution to obtain liquid Ad-gagpol vaccine.

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2.3 Measuring the crystallization and thawing characteristic of vaccine protector

Consist of 2% trehalose, 1% mannitol, 2% dextran, 0.3% albumin and 2% sucrose (w/v) in 0.01 M sodium phosphate buffer at pH 7.4 were used for this study. Crystallization was measured by registration of the resistance of the stabilizers during the freeze-thawing process. The cool-down speed was 1 ºC/minute, thawing speed was 3 ºC/minute. Change in resistance values for vaccine stabilizer is given in Figure 1.

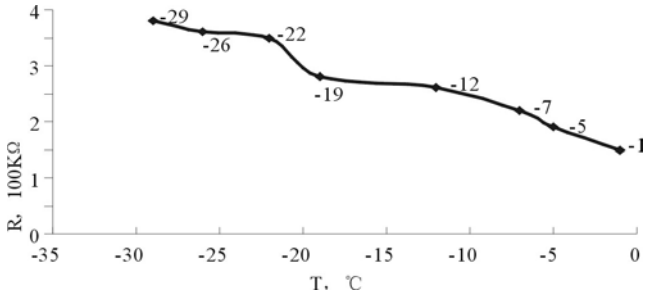

Figure 1. Measurement of the resistance of Ad-gagpol vaccine with protector during freezing and thawing treatment.

2.4 Lyophilization

Lyophilization was carried out using shanghai LYO-0.5 freeze-drier. Equal volumes of the vaccine and protector were mixed. 0.5ml of the mixture was dispensed in sterile small vial . The vaccine vials were pre-frozen at − 40ºC for 3 hours and subjected to the first lyophilization at − 30 ºC for 10 hours and the second lyophilization at 28 ºC for 5 hours at a vacuum of 0.06 m bar. Batches of vaccine containing different stabilizer formulations were lyophilized simultaneously under identical conditions to compare the quality in terms of residual moisture and titre loss during lyophilization.

2.5 Analysis of thermostability of lyophilized vaccine

Lyophilized vaccine vials were exposed at 4 ºC and 37 ºC respectively. Samples were taken from the refrigerator at 2, 6, 10, 13, 17, 21 months; from 37 ºC at 1, 2, 3 and 4 weeks. Exposed samples were reconstituted with 0.5 ml of distilled water and titrated in HEK293 cells. For each time point, ten samples were titrated and their average log10 titer was calculated.

2.6 Measurement of titers of adenoviruses

HEK293 cells were seeded in 96-well plates at 5×10^3 cells/well. Twenty-four hours later, the medium was replaced with 50µl of medium that contained ten-fold serial dilutions of the adenovirus used, from 1×10^2 to 1×10^{-11}. The plates were centrifuged at 1000 × g for 10 min and then placed in an incubator. For determination of the CPE, the plates were incubated for a minimum of 10 days, during which time there was no evidence of a CPE in the control wells. Plates were examined daily for the CPE of the virus on the infected cells and the titer was calculated.

2.7 Mice and immunization schedule

Female BALB/c mice were divided into the liquid vaccination group, the lyophilized vaccination group, and the control group. The mice of liquid vaccination group and lyophilized vaccination group were administered a single intramuscular injection with 5×10^8 pfu Ad-gagpol vaccine. Control group mice were inoculated by PBS.

2.8 Antibody detection

Collect MoltIII B cell (stably expresses the HIV-protein cell line) supernatant, then centrifuge at 26000 rpm/minute. Resuspend the precipitation and run SDS-PAGE, transfer to cellulose membrane as antigen detecting mouse blood serum. The blocked membranes were placed in a multiscreen apparatus (Bio-Rad, USA), and approximately 100 µl of diluted serum was pipetted into individual lanes. Serum samples were diluted 1 : 50 with 3% milk-PBS. Following a 2-hour incubation at RT, the blots were removed from the apparatus and washed three times in T-PBS. The membranes were then incubated 1 hour at RT with antimouse IgG antibodies conjugated with AP and washed three times with T-PBS. The blots were visualized with NBT and BCIP in AP buffer (Sigma, USA), as recommended by the manufacturer. The blots were developed by using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

2.9 IFN-γ ELISPOT assay

An ELISPOT assay kits (USA) was used to determine vaccine elicited IFN-γ responses in BALB/c mice. Spleen lymphocytes from the immunized mice were cultured in a plate with medium. 96-well plates were coated with purified anti-mouse IFN-γ monoclonal antibodies, and incubated at 4 ºC overnight. Mice splenocytes were isolated and red blood cells were lysed by RBC lysis buffer. Cells were washed two times and re-suspended in complete culture medium. After counting, splenocytes were then adjusted to the concentration of 4×10^6 cells/ml and plated into pre-coated 96-well Elispot plate at 100 µl/well with addition of 100 µl peptide

P7G (AMQMLKETI, 1 μg/ml). The Elispot plates were incubated and developed according to the kit instruction. Finally, plates were air-dried and the resulting spots were counted with Immunospot Reader (USA). Peptide specific IFN-γ Elispot responses were considered as positive only when the responses were 4-fold above negative control with no peptide stimulation.

3 Results

3.1 Physical characters

Appearance of the lyophilized Ad-gagpol vaccine still keep good, white and loose at 37 ºC for 2 months. Furthermore, it can be reconstituted rapidly with distilled water.

3.2 Crystallization and thawing temperatures of stabilized Ad-gagpol vaccines

Freeze and thawing of the Ad-gagpol vaccine and protectors clearly showed that only at low temperatures the products are completely frozen (Figure 2). Parts of the products crystallized between 0 ºC and − 19 ºC, whereas the main part crystallized up to a temperature of − 19 ºC. The Eutectic point temperature with a minimum range in the resistance value is reached below − 22 °C. The thawing curve mirrors in general the crystallization curve of the products.

Figure 2. Shape of lyophilized Ad-gagpol vaccine stored at the 60st day at 37 ºC.

3.3 Thermostability of lyophilized Ad-gagpol vaccine

Virus titration was carried out to determine the infectivity of the freeze-dried vaccine after exposure at 4 ºC and 37 ºC for different time intervals. The infectivity titres thus obtained were subjected to regression analysis. Table 1 and Table 2 summarize the results of regression analysis at different temperatures. It could be possible to calculate half-life (time required for loss of half the original titre, i.e. 0.30 $\log_{10}$ PFU based on the degradation constant) by assaying more number of samples over a long period beyond.

The titer of Ad-gagpol vaccine before and after lyophilized decreased by 0.17 $\log_{10}$ (Table 3). As for lyophilized vaccine, 0.50 $\log_{10}$ and 0.60 $\log_{10}$ titer reduced after heating at 37 ºC for 14 days and 21 days, respectively. Its half-life is 11.07 days at 37 ºC. The loss was very small.While as for liquid vaccine, the half-life is 1.03 days. 2.50 $\log_{10}$ titer reduced after heating at 37 ºC for 7 days (Table 1, Figure 3). We also assess long-term stability of lyophilized Ad-gagpol vaccine stored at 4 ºC, a drop in titer of approximately 0.6Log_{10}PFU/ml for 17 months, but the titer of the liquid vaccine drop apparently 1.6Log_{10}PFU/ml after stored for 2 months (Table 2, Figure 4). These data showed lyophilized vaccine viruses were found to be fairly stable following lengthy exposure to higher temperature, compared with the current liquid vaccine preparations.

Table 1. Comparison of degradation values of lyophilized and liquid Ad-gagpol vaccine at 37 ºC

Temperature (ºC)	Stabilizer	Initial titer	Regression equation	Half-life (days)
37	lyophilized	7.6	y = – 0.0271x + 7.54	11.07
37	liquid	8.2	y = – 0.2914x + 8.0467	1.03

Table 2. Comparison of degradation values of lyophilized and liquid Ad-gagpol vaccine at 4ºC

Temperature (ºC)	Stabilizer	Initial titer	Regression equation	Half-life (months)
4	lyophilized	7.6	y = – 0.0281x + 7.4683	10.68
4	liquid	7.8	y = – 0.3181x + 7.3157	0.94

Table 3. Virus titers of Ad-gagpol vaccine before and after lyophilization

Group	Titer (Log_{10} PFU/ml)		
	Before lyophilization	After lyophilization	Loss
lyophilized	7.43	7.26	0.17
Liquid	8.30	6.80	1.50

3.4 Humoral immune responses

The mouse blood serum according to 1 : 50 dilution, anti-P24 antibody was used to detect humoral immune

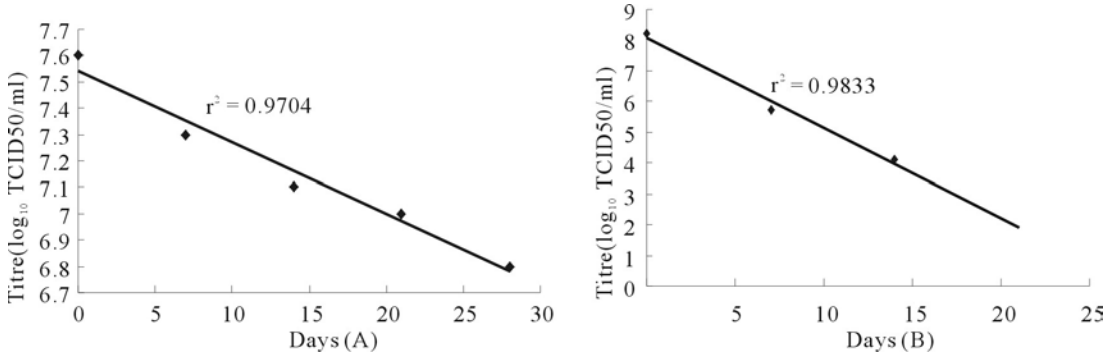

Figure 3. Degradation curves (37 ºC) for lyophilized and liquid Ad-gagpol vaccine. A: ophilized Ad-gagpol vaccine B: liquid Ad-gagpol vaccine.

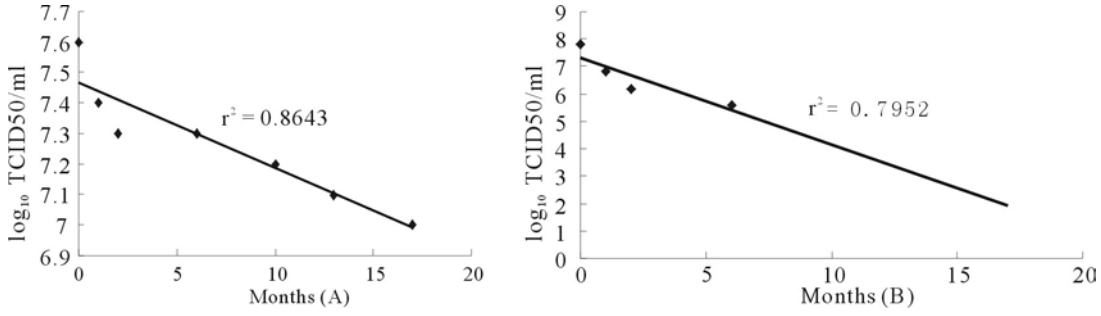

Figure 4. Degradation curves (4º C) for lyophilized and liquid Ad-gagpol vaccine. A: lyophilized Ad-gagpol vaccine B: liquid Ad-gagpol vaccine.

response in blood serum with Western blot law. Figure 5 demonstrates that two vaccines groups produce the same antibody level.

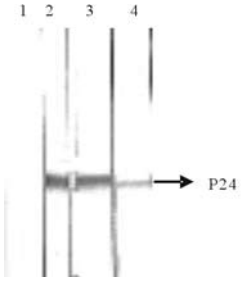

Figure 5. Western blot analysis the antibody level of lyophilized and liquid Ad-gagpol vaccine immunized mice. 1: PBS negative control, 2: lyophilized Ad-gagpol vaccine, 3: liquid Ad-gagpol vaccine, 4: mice P24 Ab positive control.

3.5 IFN-γ ELISPOT

Ag-specific CD8 T cells were analyzed by IFN-γ ELISPOT. The evaluated results for IFN-γ production are expressed as the mean numbers of IFN-γ secreting cells (spots) per 10^5 splenocytes. The number of IFN-γ-secreting lymphocytes elicited by lyophilized vaccine and liquid vaccine in mice was approximated (275 versus 238, $P > 0.05$, Figure 6). These results illustrate that the mice which administered lyophilized Ad-gagpol vaccine produced indistinguishable IFN-γ ELISPOT level compared with liquid Ad-gagpol vaccine group.

4 Discussion

Adenovirus is significantly inactivated at pH values below 6, and undergoes capsid degradation during repeated freeze-thaw cycles[4]. Scientists generally place their novel vectors in buffer, add glycerol and store the preparation at − 80 ºC. Vectors stored under these conditions must be quickly shipped to remote sites on dry ice,which is somewhat costly[5].This formulation also requires extensive dilution before administration to reduce the toxicity of glycerol.

Although freeze-dry is a common method in preserving live biological samples, it usually leads to protein denaturation and drop in cell viability. Thus, selecting the optimal protector for the lyophilization process of the vaccine is a critical step in vaccine

production. This is directly related to the infective titer and stability of the vaccine. Surface adsorption, freeze-thaw and free-radical oxidation are the major inactivation pathways for Ad during storage[6]. The noncovalent interactions of viral structures are sensitive to variations in pH, temperature, and composition of the surrounding environment, particularly to osmotic stress[7].

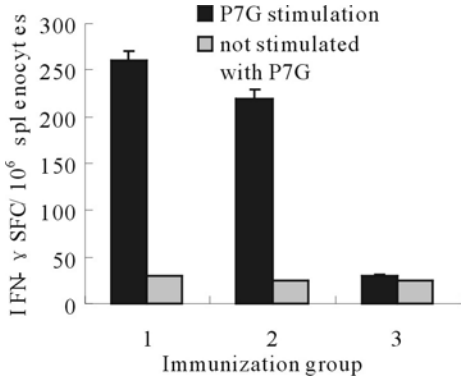

Figure 6. Cell-mediated immune response by ELISPOT. 1: lyophilized Ad-gagpol vaccine; 2: liquid Ad-gagpol vaccine; 3: PBS negative control.

Through screening, the protector containing human serum albumin, trehalose, mannitol, dextran and sucrose showed good protective effect on lyophilized adenovirus-based live vaccine.

Carbohydrate and polyalcohol enhance the hydrophobic interaction with protein through influencing the water molecule conformation, thus preventing heat denaturation and enhanced stability for proteins in solution. When water is removed during drying, the protectors can hydrogen bond with the protein as water does, thereby preserving the native structure during processing by thermodynamic stabilization of the native conformation. Stabilization during storage is then a result of preservation of the native structure in the solid state , regardless of the mechanism responsible for such preservation[8]. Furthermore, trehalose and dextran inhibit potential for virus adsorption and aggregation. The free-radical oxidation inhibitor mannitol was determined to be effective stabilizer of virus. The addition of 1% albumin prevented viral aggregation and allowed the purified virus to retain its activity after filter sterilization. Furthermore, viral activity was retained within the 1% albumin solution for at least 1 week at 37 ºC and for 2 weeks at 4 ºC, whereas viral activity within the albumin-free solution was quickly lost[9].

According to the results on the appearance, infective titer, accelerated thermal stability and long term stability tests, combination of trehalose, mannitol, dextran, albumin and sucrose makes a suitable protector compound. At the same time, it is pharmaceutical acceptable and convenient for large-scale production of the vaccine.

Excipients employed in vector formulations must be suitable for use *in vivo*. To determine the potentiality of trehalose, mannitol, dextran, albumin and sucrose cause a loss of immunogenicity for lyophilized Ad-gagpol vaccine, The mice of liquid vaccination group and lyophilized vaccination group were administered a single intramuscular injection with 5×10^8 pfu Ad-gagpol vaccine.The IFN-γ ELISPOT assay and immunoblot assay were used to monitor the cellular and humoral immune responses. The results (Figure5, Figure6) indicated that the immunogenicity of lyophilized Ad-gagpol vaccine and liquid Ad-gagpol vaccine was indisti nguishable,showing that trehalose, mannitol, dextran and inositol did not significantly alter the immunogenicity of the vaccine.

These results demonstrate that lyophilized Ad-gagpol vaccine is as effective to induce immune response in mice as liquid vaccine. In addition,it can be shipped and stored at the room temperature,supporting its further evaluation and application in clinical studies.

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