CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Clin Exp Vaccine Res 2021;10:259-263
https://doi.org/10.7774/cevr.2021.10.3.259
pISSN 2287-3651 • eISSN 2287-366X

The effect of apolipoprotein A-I and perfluorocarbon emulsion on the production of specific antibodies in mice

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Received: July 15, 2021
Accepted: September 15, 2021

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No potential conflict of interest relevant to this article was reported.

Adjuvants are often required to improve the potentially low immunogenicity of vaccines. In this study, it is proposed to use an emulsion based on fluorocarbons as an adjuvant. Since this emulsion adsorbs only a small range of proteins, apolipoprotein A-I (ApoAI) was used as an anchor. Antigen and ApoAI were combined by creating a fusion construct. Results showed that the combined use of a perfluorocarbon emulsion and ApoAI during immunization significantly increases the specific antibody titer in mice and in its effectiveness this system is close to the incomplete Freund's adjuvant.

Keywords: Adjuvants, Perfluorocarbons, Apolipoprotein A-I, Adsorption, Synthetic biology

Immunization is considered to be one of the most effective pathways to fight infectious diseases. Adjuvants play an important role in vaccination. Although adjuvants are used to create most of the inactivated vaccines, the process of developing new adjuvants is lengthy and slow. So far, only several adjuvants have been approved for medical application, while some of them are a combination of several already known adjuvants: Alum, MF59, AS03, virosomes, AS04, QS-21, and AS01 [1,2]. Thus, there is necessity for creation of new types of adjuvants.

An perfluorocarbons emulsion (PFCE) can be considered as a new adjuvant. These emulsions were previously applied as plasma substitutes, ultrasound contrast agents, and others for medical applications [3,4]. In PFCE, the perfluorocarbon (PFC) drops are coated with a layer of triblock copolymer (130PEG-38PPG-130PEG). Our early studies showed that PFCE can adsorb significant amount of proteins which mainly consists of immunoglobulins and various lipoproteins [5,6]. It is impossible to use this emulsion as an adjuvant directly. But protein can be bound to a particle by creation of genetically engineered fusion proteins, consisting of two parts. The first part should be responsible for ability of entire fusion protein to be adsorbed on the surface of PFC. The second part should be target antigen. We believe that a plausible candidate for the first part is apolipoprotein A-I (ApoAI) which as shown earlier is strongly adsorbed on the surface of the emulsion [5,6].

Thus, this study's objective was to assess the potential of using PFCs as an adjuvant for recombinant proteins linked into a single polypeptide chain with ApoAI. The dC fragment (amino acids 553 to 723) of human heat shock protein 90 β (HSP90AB1) was used as a model antigen to create a fusion protein.

The study showed that the specific antibody titer to the dC fragment was significantly higher when the mice were immunized with the ApoAI_dC-HSP90β than when the pure dC fragment was used. Using ApoAI_dC-HSP90β with PFCE as an adjuvant induced an immune response with the production of antibodies to the dC fragment. This immune response was comparable to the ApoAI_dC-HSP90β emulsified in incomplete Freund's adjuvant (IFA).

The emulsion was obtained in high-pressure homogenizer. The average diameter of particle was 100 nm. PFCs composition: 10 volume% of 2:1 mix of perfluorodecalin (Kirovo-Chepetsk Chemical Plant Ltd., Kirovo-Chepetsk, Russia) and perfluorocyclohexylpiperidine (Russian Research Center of Applied Chemistry branch, Perm, Russia), 4% proxanol 268 (Scientific Research Institute of Organic Intermediates and Dyes, Moscow, Russia), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 8 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 10 mM glucose (Sigma-Aldrich, St. Louis, MO, USA).

Complementary DNA (cDNA) encoding ApoAI and dC fragment of HSP90AB1 were synthesized by polymerase chain reaction using cDNA from human liver. For fusion protein, pET23b-dC-HSP90β and pET23b-ApoAI_dC-HSP90β gene constructs were created and transformed into BL21 (DE3). Protein synthesis was induced by 1 mM IPTG (isopropyl-β-d-thiogalactopyranoside; Anatrace, Maumee, OH, USA). Protein purity was assessed by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) electrophoresis.

The amount of adsorbed proteins was measured as described previously [5].

The measurement of protein fluorescence was performed on fluorescence spectrophotometer (Varian Cary-Eclipse; Varian Inc., Palo Alto, CA, USA) at excitation wavelength 297.5 nm. PFCE (0.5 mL), 1.5 mL of 1× phosphate-buffered saline (PBS) and 150 μL of protein (225 μg) solution were added to quartz cuvette. Fluorescence was measured at 340 nm for ApoAI_dC-HSP90β and 343 nm for dC fragment.

Equimolar rates of 1 nM protein per animal and equal vaccine volume (100 μL) were used for all immunizations. To prepare a suspension based on ALU (AlumVax Hydroxide; OZ Biosciences, San Diego, CA, USA), the protein solution was mixed with adjuvant in 1:1 ratio. To prepare emulsions based on IFA, equal volumes of adjuvant and protein solution were emulsified using syringe. The PFCE were centrifuged at 18,000 g for 10 minutes at rate of 200 μL per animal to prepare PFCE-based vaccine for immunization. Then protein solution in 1× PBS was added.

Experiments were carried out on male 9 weeks old ICR (CD-1) mice (n=10). Mice were immunized intraperitoneally

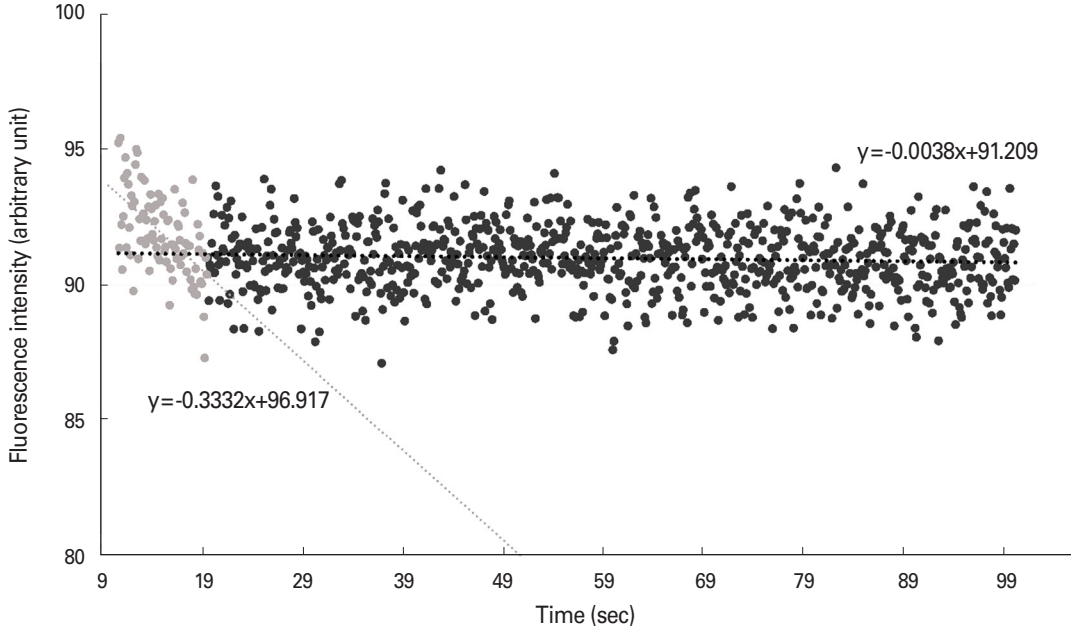

Fig. 1. Change of fluorescence of ApoAI_dC-HSP90β after adding of PFCE (emulsion based on perfluorocarbons) to the cuvette. The area where the fluorescence is decreasing is marked in light gray (the grey line is the result of regression analysis for this area). The dark grey color shows the area where the fluorescence does not change (the black line is the result of regression analysis for this area, which shows no change in fluorescence). The formula is given for each line.

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twice, at 1st and 3rd weeks. Blood was collected from tail vein every week for 5 weeks.

The study protocol was approved by the institutional ethics committee of Institute of Cell Biophysics, Russian Academy of Sciences. All experiments were carried out according to international regulations listed in the EU Directive 2010/63/EU for animal experiments and Institute of Cell Biophysics, Russian Academy of Sciences manual for working with laboratory animals (no. 57; December 30, 2011; permission: no. 2 dated June 12, 2020).

In order to determine titers of C-specific antibodies as well as to analyze the distribution of immunoglobulin G (IgG) by subclass, enzyme-linked immunosorbent assay method was used. The total content of mouse antibodies was detected using peroxidase-antiperoxidase method [7]. For the analysis of IgG subclasses, horseradish peroxidase-linked goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 were used (Bio-Rad, Hercules, CA, USA). Titer was calculated as dilution that resulted in OD495 ≥0.3.

The data were analyzed using the nonparametric Mann-Whitney U criterion. Values are presented as mean±standard deviation ($p<0.05$). The significance of regression was assessed by analyzing the significance of difference between theoretical regression points and experimental points.

The purity of synthesized dC and ApoAI_dC-HSP90β was above 90%. Before immunization experiments, the ability of dC and ApoAI_dC-HSP90β to adsorb on the surface of PFCs was tested. It was found that dC fragment is almost not detected on the emulsion after three washes. Under the same conditions, 12.4%±0.79% of initially added 4 nM ApoAI_dC-HSP90β remained on the surface of emulsion particles. Adsorption process occurs quickly and is fully completed in less than 10 seconds. It is known that upon contact of proteins with fluorocarbon compounds, significant (up to complete) fluorescence quenching occurs [6,8]. Thus, quenching of intrinsic fluorescence during adsorption indicates the presence of contact between ApoAI_dC-HSP90β and fluorocarbon core of emulsion (Fig. 1).

To determine how the addition of ApoAI to dC fragment affected antibody responses, mice were immunized with 1 nM dC and ApoAI_dC-HSP90β fragments in the presence or absence of emulsion mix that included PBS solution, IFA, colloidal ALU, and PFCE. Adding compounds such as IFA and ALU makes it possible to compare the effectiveness of the activation of antibody responses when using a fluorocarbon emulsion in comparison with traditional adjuvants.

As our data shows (Fig. 2), in all formulations throughout the entire duration of experiment, groups of animals immunized with ApoAI_dC-HSP90β had several times (approximately 5–15 times) higher titers of specific antibodies than

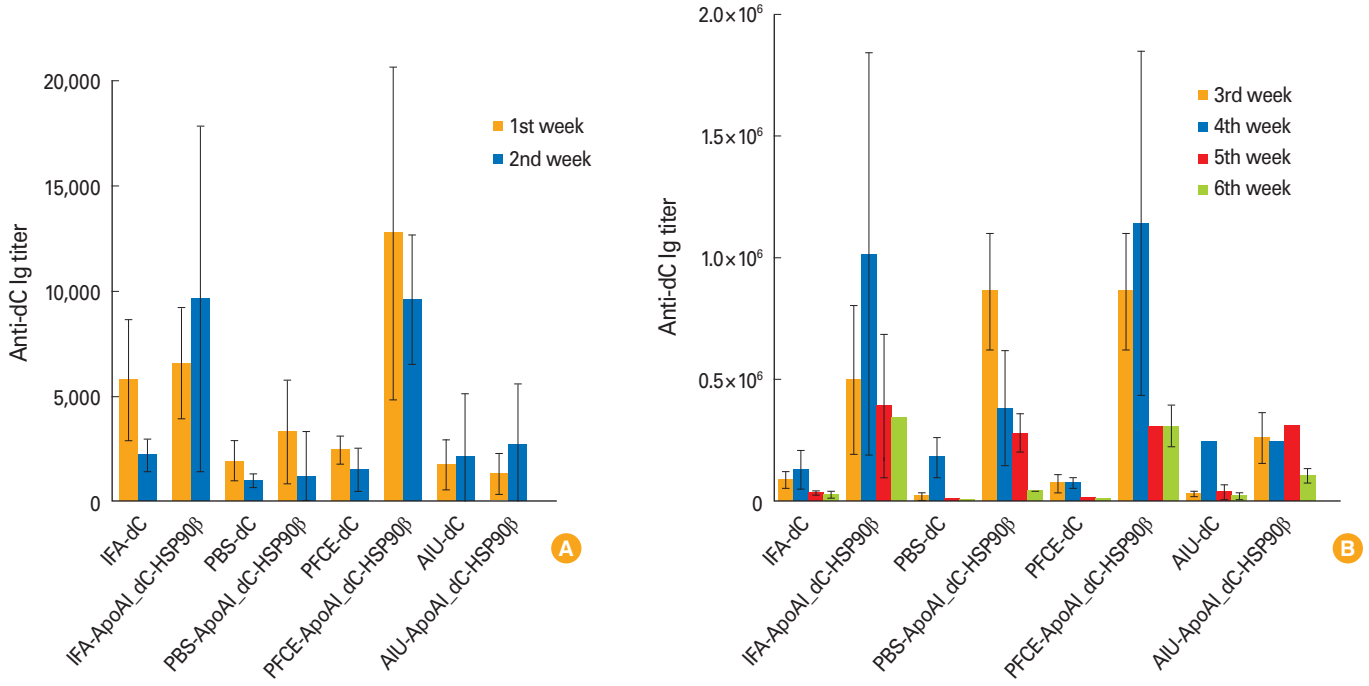

Fig. 2. Antibody titer values to the dC fragment from 1st to 6th week after immunization. For clarity, the data are divided into two diagrams: (A) on the left—the primary immune response from 1st to 2nd week and (B) on the right—the secondary immune response from 3rd to 6th week.

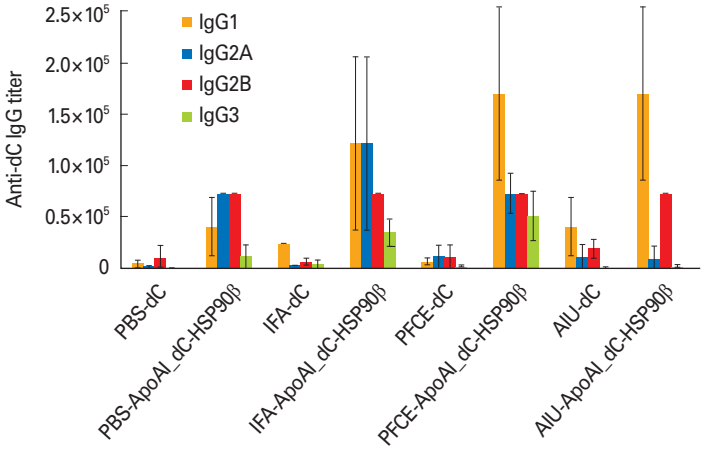

Fig. 3. The level of the immune response in immunoglobulin G (IgG) subclasses. Samples were collected on the third week after the first immunization.

animals immunized with pure dC.

Overall, for all formulations the adding of ApoAI to dC fragment significantly (by orders of magnitude) increased immunogenicity of dC fragment most likely because of physiological role of ApoAI. It plays a key structural role in high-density lipoprotein (HDL) particles, which contain 2–5 ApoAI molecules per particle [9]. Once released into plasma, ApoAI binds to lipids to form lipoprotein particles in a process dependent on transporter-dependent ATP-binding cassette transporter, subfamily A, member 1 (ABCA1). Equally important, lipid-free ApoAI mediates cholesterol efflux through ABCA1, thereby promoting the reverse cholesterol transport pathway [10]. Thus, it can be assumed the following: when ApoAI_dC-HSP90β enters the bloodstream, it begins to function as a lipid-free form of ApoAI. Namely, it begins to participate in formation of HDL through ABCA1. These HDLs carry antigen on their surface in the form of dC fragment. Because lipid-free ApoAI and HDL have affinity for scavenger receptor class B-1 and ABCA1 [11], available on cell membranes of the phage system (neutrophils, macrophages), then phage system cells can capture antigen more efficiently due to interaction with these receptors. However, more precise mechanisms of this activity are yet to be examined by future research.

After administration of different formulations, immune response was analyzed and IgG subclasses were determined. It was shown when using ApoAI_dC-HSP90β, spectrum of IgG response is broader than using pure dC. This effect was observed regardless of used adjuvant (Fig. 3). It indicates that ApoAI_dC-HSP90β had a significant activating effect on mice immune system.

When comparing the groups with the addition of ApoAI-HSP90β for each IgG subclass, it can be noted that IgG1 was the most common subclass for almost all preparations.

Groups PFCE-ApoAI_dC-HSP90β and IFA-ApoAI_dC-HSP90β showed the broadest IgG spectrum. It is known that different IgG subclasses are responsible for different functions of immune system. Only in tandem it is possible to achieve the most complete protection effect [12]. Thus, it can be assumed that vaccine based on PFCE-ApoAI will have the most pronounced protective properties.

The observed effect of enhanced antibody responses in variant with adding of ApoAI to another protein has a potential for the development of vaccines of a fundamentally new type. However, additional studies are required for a more detailed immunity induction assessment using similar fusion constructs and PFCE, as well as assessments of cellular and protective immunity. Also, it is assumed that use of a linker other than ApoAI (or modification of this protein—chemical or genetic) may significantly increase the percentage of protein adsorbed on PFCE. As a result, it will have a positive effect on the final level and dynamics of the specific antibody response. Our laboratory continues the research in this field.

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