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Comparison of the Adjuvanticity of Aluminum Salts and Their Combination in Hepatitis B Recombinant Protein Vaccine Assessed in Mice

Arash Mahboubi[1], Mohammad Reza Fazeli[2], Rasoul Dinarvand[1,3], Nasrin Samadi[2,*], Mohammad Sharifzadeh[4], Houshmand Ilka[5], Saeed Azadi[6], Roya Soleimanian[6], Hassan Kalkouei[6], Rasoul Hajikhanmirzaei[6], Mahboubeh Valadkhani[7]

[1]Department of Pharmaceutics, [2]Department of Drug and Food Control, [3]Medical Nanotechnology Research Centre, Tehran University of Medical Sciences, [4]Department of Toxicology and Pharmacology, Faculty of Pharmacy and Pharmaceuticals Quality Assurance Research Center, Medical Sciences/University of Tehran, [5]Zist Daru Danesh Ltd., [6]Biotechnology Department of Darou Pakhsh Pharmaceutical Mfg. Co., [7]Department of Biological Products, Ministry of Health, Tehran, Iran

ABSTRACT

Background: Several adjuvants have been evaluated for vaccine formulations but aluminum salts will continue to be used for many years due to their safety, low cost and adjuvanticity with different antigens. Two commonly used aluminum adjuvants, aluminum hydroxide and aluminum phosphate have different adjuvanticity properties. Commercial recombinant protein hepatitis B vaccines containing aluminum hydroxide is facing low induction of immunity in some sections of the vaccinated population. **Objective:** In this study, to follow the current global efforts in finding more potent hepatitis B vaccine formulations, adjuvanticity of aluminum phosphate, aluminum hydroxide and their combinations has been evaluated. **Methods:** The formulated vaccines were administered intra-peritoneally (i.p.) to BALB/c mice and the titer of antibody was determined after 28 days using ELISA technique. The geometric mean of antibody titer (GMT, mIU/ml), seroconversion and seroprotection rates, ED50 (ng) and relative potency (μg/dose) of different formulations were determined. **Results:** GMT of antibody titer, seroconversion and seroprotection rates showed significantly higher adjuvanticity for aluminum phosphate than other formulations. The ED50 of aluminum phosphate was approximately two fold less than other formulations. **Conclusion:** Aluminum phosphate showed more adjuvanticity than aluminum hydroxide and their combinations in hepatitis B protein vaccine. The use of aluminum phosphate as adjuvant leads to higher immunity which may result in more protective response in vaccinated groups.

Keywords: Hepatitis B vaccine, Aluminum, BALB/c mice

*Corresponding author: Dr. Nasrin Samadi, Department of Drug and Food Control, Faculty of Pharmacy and Pharmaceuticals Quality Assurance Research Center, Medical Sciences/University of Tehran, Tehran, Iran. Tel: (+) 98 21 66959090, Fax: (+) 98 21 66482608, e-mail: samadin@sina.tums.ac.ir

INTRODUCTION

The desired response to vaccines is obtained by production of antibodies with immune responses enhanced by adjuvants (1, 2, 3). The term adjuvant has been used for any material that can increase the humoral or cellular immune response to an antigen. In the conventional vaccines, adjuvants are used to induce an early, high and durable immune response while the newly developed purified subunit or synthetic vaccines which are poor immunogens, require adjuvants to increase their immune response (1-4). More than 100 compounds or formulations including organic and inorganic compounds such as aluminum salts, mineral oil, and killed mycobacteria show some degree of adjuvant properties (4, 5). The most common adjuvants approved for use in currently licensed human and veterinary vaccines are the aluminum based adjuvants (2, 3, 6, 7). Formulation of vaccines by adsorption of the antigen to a pre-formed aluminum adjuvant under controlled conditions is the most common method and the formulated vaccines are called aluminum-adsorbed or aluminum adjuvanted vaccines (6-8). Aluminum phosphate adjuvant is actually amorphous aluminum hydroxyl phosphate, $Al(OH)_m(PO_4)_n$, and aluminum hydroxide adjuvant has actually an aluminum oxyhydroxide composition, AlO(OH) (3, 9, 10).

The first HBV vaccine was manufactured by HBsAg particles derived from the plasma of chronic HBV carriers (11, 12). To reduce the risk of infection, subunit recombinant vaccines became available in which HBsAg were expressed in yeasts such as *Saccharomyces cerevisiae* (Engerix-B, Recombivax-HB) or in Chinese hamster ovary (CHO) cells (Hepacare) (13, 14).

The response to the vaccine was determined by measuring anti-HBs antibody levels (15, 16). There may be a need for boosters where the risk of exposure to HBV is relatively high, specially among health care workers. Patients with immune deficiency diseases, such as those with HIV infection, those receiving immunosuppressive drugs or those on dialysis, have a lower response and boosters are also more important in such groups (15, 17, 18). Therefore, upon improving the immune response using aluminum phosphate or combination of aluminum phosphate and aluminum hydroxide, better immunity with lower antigen dosage or fewer boosters might be achieved. In this study the immune response to hepatitis–B protein vaccine formulated with aluminum hydroxide (Alhydrogel), aluminum phosphate (Adju-Phos) and two different combinations of them were evaluated by comparing the geometric mean of the antibody titer (GMT), the rate of seroconversion, seroprotection, ED50 and relative potency in BALB/c mice.

MATERIALS AND METHODS

HBs Antigen. The recombinant hepatitis B surface antigen used in this study was obtained from a local manufacturer (Darou Pakhsh Pharmaceutical Co., Tehran, Iran). This antigen is produced in *Pichia pastoris*, containing the gene for adw subtype of HBsAg. Properties of recombinant HBsAg was evaluated by different techniques including total protein assay of the bulk antigen using Bicinchoninic acid (BCA) method (Pierce, USA), determination of antigen content of the recombinant antigen using ELISA technique (Hepanostika HBsAg Uniform ELISA kit, Biomerieux, Netherlands), evaluation of the purity of HBsAg with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (reducing SDS-PAGE, Laemmli method) using electrophoresis

system (Mini-PROTEAN 3 Cell, BIO-RAD, USA) and staining with silver nitrate, and evaluation of the host cell nucleic acid content by PCR method using 5'-pd$(T)_{12-18}$-3'(Amersham Biosciencces, USA) as a primer in 35 cycles and detecting the repeated DNA sequences of the host (yeast). The carbohydrate content of recombinant HBsAg was measured by Anthrone method (19). Total lipids were determined by a spectrophotometric method using vanillin-orthophosphoric acid as a reagent and cholesterol as a lipid standard.

Adjuvants and Vaccine Formulation. Aluminum phosphate (Adju-Phos) and aluminum hydroxide (Alhydrogel) were purchased from Brenntag Biosector (Denmark). Adjuvant concentration was calculated on the basis of aluminum content. Vaccine formulations were prepared by mixing HBsAg (in phosphate buffer, pH 7.4) with aluminum hydroxide or aluminum phosphate and also with their combination. The mixture was shaken on a reciprocal shaker (Kűhner ISF-1-W, Switzerland) at 8°C and 200 rpm for 12 hrs. The final concentrations of HBsAg and aluminum were 20 µg/ml and 500 ppm respectively. Dilutions of 1:512 (0.03906 µg/ml), 1:64 (0.3125 µg/ml) and 1:8 (2.5µg/ml) of the vaccine were prepared by adding a buffer containing 500 ppm of the relevant aluminum adjuvant or their combinations. Engerix-B (GSK, Belgium, Lot No: AHBVB127AG) hepatitis B vaccine was used as a reference. As negative control groups, formulations containing only aluminum adjuvants or combinations of such adjuvants or HBsAg (20µg/ml) alone were prepared and administered.

Animals. Female mice (BALB/c C3H strain) obtained from Charles River Laboratories (Germany) were housed in Micro-Isolators™ at 25°C with 12 hr of light per day and 50±5% relative humidity. Food (5g) and water (6ml) were served for every mouse daily. Every dilution of the vaccine was injected to 15 mice (5-6-week old) at the start of the experiments. The injection volume was 1 ml and the route of injection was i.p. After 28 days following the injections, blood samples were collected from the heart of the anaesthetized animals for detecting HBs antibody titer. Blood samples were centrifuged at 3000 ×g for 10 min and the serum samples were stored at -20°C until analyzed.

Anti-HBs EIA (Total Antibody). Anti HBs antibody was determined by ELISA technique using Diasorin, ETT-AB-AUK-3 anti-HBs antibody ELISA kit (Italy). Seroprotection level was achieved with an antibody titer of at least 10 mIU/ml and an antibody response of 1-10 mIU/ml was considered as seroconversion (15, 16).

Statistics. ED50(ng) for each formulation was evaluated by SPSS VER.12 using Probit method with $p<0.05$ while the relative potencies of formulations were evaluated using quantal responses method (19). Geometric mean of anti-HBs Ag titers (GMTs) were calculated by taking the anti-log of the mean of the log of titer transformations. Antibody titers below the assay cut-off were given an arbitrary value of half the cut-off for the purpose of GMT calculation. GMT (mIU/ml) calculations, SD and other data processing were performed with Microsoft Excel 2007.

RESULTS

Quality of HBsAg. Antigen content of the bulk sample as assayed by Hepanostika HBsAg Uniform ELISA kit was 37.68 µg/ml. Total protein content of the bulk antigen determined by BCA method was 29.38 µg/ml.

Purity of HBsAg was evaluated with SDS-PAGE technique. The single 24 KDa monomer of HBsAg was observed on the gel (Figure 1), indicating high purity of the antigen.

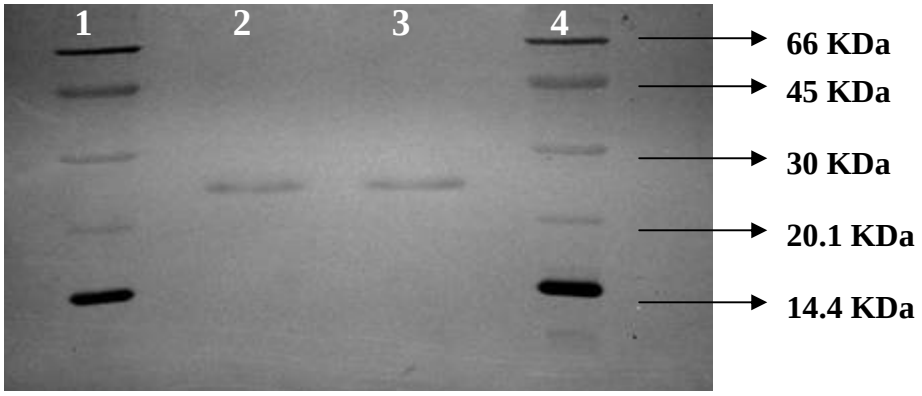

Figure 1. SDS-PAGE analysis of purified HBsAg . The gel was stained with AgNO3. Lane 1. Low molecular weight calibration kit (14.4-97KDa , GE Healthcare, Amersham Biosciences). Lane 2. Purified HBsAg. Lane 3. Purified HBsAg. Lane 4. Low molecular weight calibration kit (14.4-97KDa , GE Healthcare, Amersham Biosciences).

Determination of nucleic acid contamination by PCR showed less than 10 pg of DNA/dose (data not shown). The lipid content of HBsAg was 0.36 mg/mg protein and the carbohydrate content was 165 µg/mg protein indicative of the good quality of HBsAg.

Seroconversion of Formulated Vaccines. The percent of seroconversion versus HBsAg dose in mice after 28 days of vaccination is shown in Figure 2. At lower HBsAg concentrations, aluminum phosphate-containing formulations showed higher immunogenicity than formulations with either aluminum hydroxide or their combination. Seroconversion was reduced upon increasing the amount of aluminum hydroxide in the formulations.

ED50 (ng) which is the induced dose of seroconversion in 50% of vaccinated population for individual formulations were calculated with statistical software package SPSS VER.12 using probit method and the results are shown in Figure 3. The lowest ED50 was obtained for aluminum phosphate formulated vaccine corresponding to a better immunogenicity. ED50 was raised upon increasing the amount of aluminum hydroxide in the formulations. The percent of seroprotection versus HBsAg dose in mice after 28 days of vaccination was determined and is shown in Figure 4. At lower HBsAg concentration, aluminum phosphate-containing formulations showed higher immunogenicity than formulations with aluminum hydroxide or their combinations. The rate of seroprotection decreased with an increase in the amount of aluminum hydroxide. GMT titers of different formulations of hepatitis B vaccine are depicted in Table 1. Using 20 µg/ml antigen concentration, the titer of GMT obtained with aluminum phosphate (3883.484 mIU/ml) was significantly higher (3-fold) compared to those of aluminum hydroxide based formulations and also that of the reference vaccine (1030.67 mIU/ml). The amount of GMT in all dilutions was lower when the amount of aluminum hydroxide was increased in the formulations. Relative potency (µg/dose) which shows the potency of the formulation in comparison with the reference vaccine was assessed according to the seroconversion of the formulation using quantal responses method.

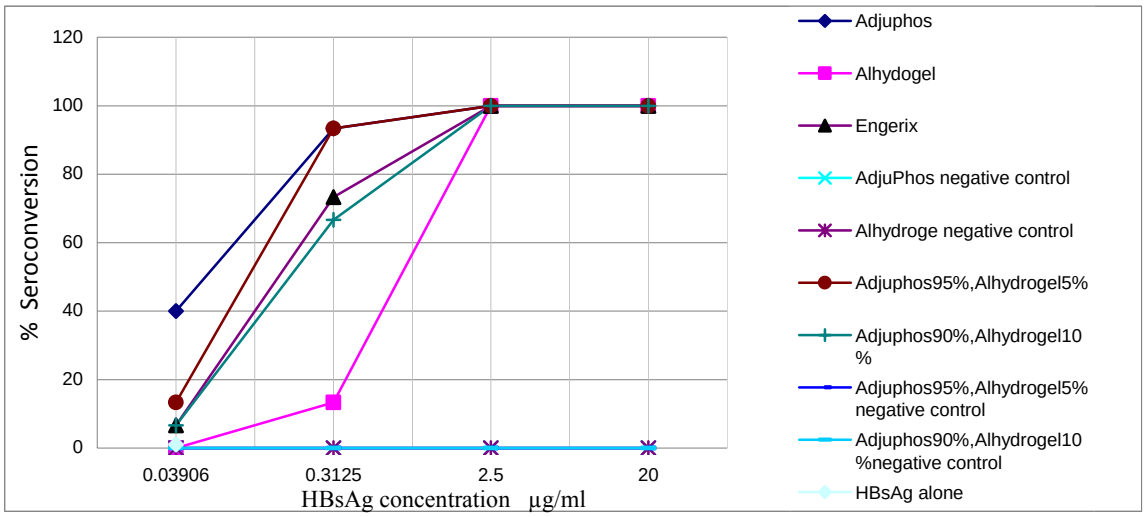

Figure 2. Seroconversion effect of different formulations containing Adju-Phos (aluminum phosphate); Alhydrogel (aluminum hydroxide); Adju-Phos 90%, Alhydrogel 10%; Adju-Phos 95%, Alhydrogel 5%; Adju-Phos, negative control; Alhydrogel, negative control; Adju-Phos 90%, Alhydrogel 10%, negative control; Adju-Phos 95%, Alhydrogel 5%, negative control; HBsAg alone; and Engerix-B after 28 days of i.p. injection in BALB/c mice. The colourful version is available at: www.iji.ir

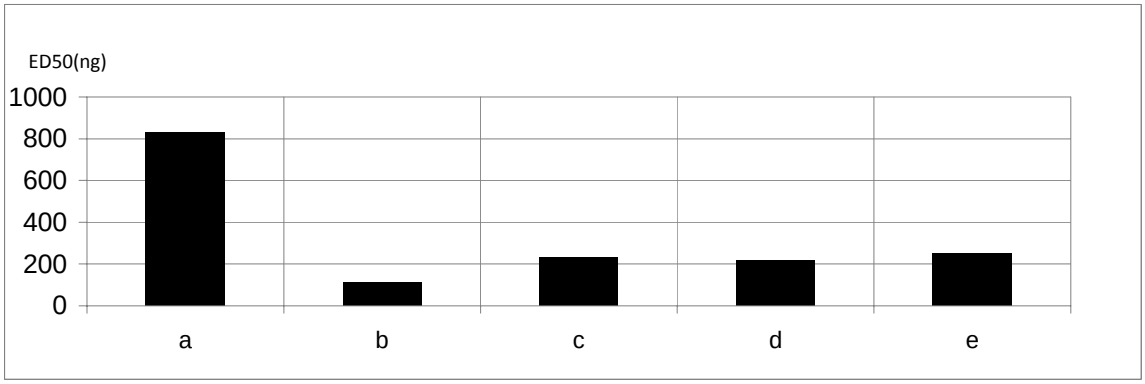

Figure 3. ED50 of different formulations containing a) Alhydrogel (aluminum hydroxide) or b) Adju-Phos (aluminum phosphate); c) Engerix; d) Adju-Phos 95%, Alhydrogel 5%; and e) Adju-Phos 90%, Alhydrogel 10% after 28 days of i.p. injection to BALB/c mice, $p < 0.05$.

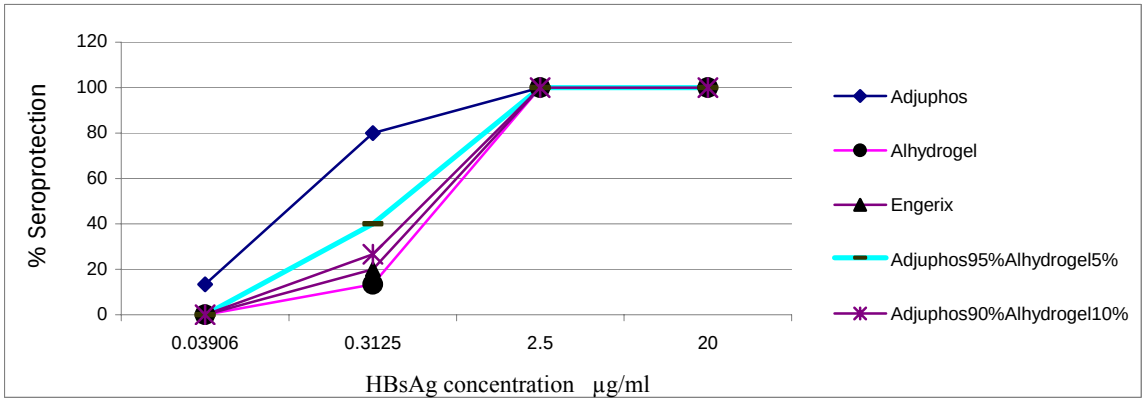

Figure 4. Seroprotection effect of different formulations containing Adju-Phos (aluminum phosphate); Alhydrogel (aluminum hydroxide); Adju-Phos 90%, Alhydrogel 10%; Adju-Phos 95%, Alhydrogel 5%; and Engerix-B after 28 days of i.p. injection in BALB/c mice (all controls had no seroprotection). The colourful version is available at: www.iji.ir

Table1. GMT of different formulations containing Adju-Phos (aluminum phosphate) or Alhydrogel (Aluminum hydroxide), Adju-Phos, 90%, Alhydrogel, 10%; Adju-Phos, 95% Alhydrogel, 5%; and Engerix after 28 days of i.p. injection in BALB/c mice. Data are presented as mean ± SD.

HBsAg Conc. / Formulation	GMT (mIU/ml)			
	20 µg/ml	2.5 µg/ml	0.3125 µg/ml	0.0390 µg/ml
Adju-Phos 100%	3883.484 ± 1246.43	415.744 ± 186.54	35.03 ± 20.43	2.59 ± 1.62
Alhydrogel 100%	1011.535 ± 324.36	259.96 ± 186.94	1.476 ± 1.02	0
Adju-Phos 90% Alhydrogel 10%,	2747.09 ± 1592.35	408.21 ± 221.43	4.87 ± 3.22	1.11 ± 0.96
Adju-Phos 95% Alhydrogel 5%	3177.047± 1343.65	175.29 ± 53.26	9.51 ± 6.22	1.992 ± 1.23
Engerix	1030.67 ± 456.32	339.04 ± 176.53	4.52 ± 2.78	0.98 ± 0.68
HBsAg alone	0	0	0	0

Engerix –B vaccine was used as the reference vaccine. Relative potency, and the lower and the upper limits of relative potency are shown in Table 2. As seen, aluminum phosphate alone shows the highest relative potency due to its better adjuvanticity. The relative potency was reduced upon increasing the amount of aluminum hydroxide in other formulations.

Table 2. Relative and lower and upper limits of potency for different formulations containing Adju-Phos (aluminum phosphate); Alhydrogel (aluminum hydroxide); Adju-Phos 90%, Alhydrogel 10%; Adju-Phos 95%, Alhydrogel 5%; and Engerix-B after 28 days of i.p. injection in BALB/c mice.

Formulation	Lower limit	Relative potency	Upper limit
Adju-phos	26.57	29.61	33.07
Alhydrogel	5.06	6.32	7.81
Adjuphos 95%, Alhydrogel 5%	23.44	28.27	34.28
Adjuphos 90%, Alhydrogel 10%	14.83	19.06	24.44
Engerix-B	-	20	-

The upper confidence limit (p=0.95) of the estimated relative potency is not less than1.0.

DISCUSSION

In this study the immunogenicity of two aluminum salts and their combinations, formulated with recombinant hepatitis B vaccine were assessed in BALB/c mice. These adjuvants can induce rapid secretion of antibody and increase the antibody titer, leading to decreased booster doses of the vaccine. The use of adjuvants enables the application of fewer antigens in achieving the desired immune response, thus reducing vaccine production costs (1, 3, 4, 5, 8). Administration of aluminum salts with HBsAg causes much more immunity than HBsAg used alone. Using the Engerix-B and Recombivax vaccines which are aluminum hydroxide adjuvanted, the seroprotection levels (above10 mIU/ml), reported in different vaccinated groups, were 83–100% and 69–99%, respectively (15). Similar results have been reported with recombinant vaccines throughout the world targeting different risk groups (15). Therefore, depending on the vaccinated groups, from 17% to 31% of vaccinated groups may remain non-protected (15, 16). On the other hand for high-risk groups such as healthcare workers, it is suggested that one should aim for levels above 100 mIU/ml (15-17). Also long-term follow-up studies have shown that immunity declines after several years. A number of such studies, where monitoring continued up to 12 years after vaccination, showed that anti-HBs antibody levels declined over time and that half of the vaccinated persons had levels below 10 mIU/ml (20-22).

Other studies have shown that aluminum phosphate stimulated a high increase in anti-HBs antibody titers and a switch from a TH2 to a TH1 response profile as evidenced by an increase in IgG2a subclass anti-HBs antibodies (23-25). This shift effect may lead to a better balance between TH1 and TH2 that is better cellular and humoral immunity with longer durability after the vaccination (25-27).

In this study the results of GMT for aluminum phosphate were higher than aluminum hydroxide or their combination and there was an inverse relation between GMT and the amount of aluminum hydroxide used. ED50 of aluminum phosphate was about 7-fold less than that of aluminum hydroxide formulation and 2 fold less than that of their combination. Similar results were obtained for seroconversion and seroprotection. Relative potency of aluminum phosphate also indicated that aluminum phosphate adjuvanticity is more than aluminum hydroxide or their combinations.

The aluminum adjuvants allow the slow release of antigens, prolonging the time for interaction between the antigen and the antigen-presenting cells and lymphocytes. This property may differ between aluminum phosphate and aluminum hydroxide. Better adjuvanticity of aluminum phosphate may be due to less ligand exchange between phosphate group of the antigen and the hydroxyl group of aluminum phosphate ($Al(OH)_m(PO_4)_n$,) in comparison to aluminum hydroxide (AlO(OH)), leading to a less stable binding of the adjuvant to antigen with higher elution rate. Different mechanisms of antigen presentation to immune competent cells and the production of different lymphokines such as interleukins and tumor necrosis factor (3-10) may be some of the other factors responsible in raising antibody titer.

In conclusion aluminum phosphate is a much more potent adjuvant than aluminum hydroxide or a combination of both in hepatitis B vaccine production. Decrease in immunity upon increasing the amount of aluminum hydroxide in the combinations indicates that the formulation of hepatitis B vaccine with aluminum phosphate could lead to a better protection in vaccinated groups.

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