Aluminum phosphate shows more adjuvanticity than Aluminum hydroxide in recombinant hepatitis–B vaccine formulation

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Received 10 Mar 2008; Revised 20 June 2008; Accepted 30 June 2008

ABSTRACT

Background: Although a number of investigation have been carried out to find alternative adjuvants to aluminum salts in vaccine formulations, they are still extensively used due to their good track record of safety, low cost and proper adjuvanticity with a variety of antigens. Adsorption of antigens onto aluminum compounds depends heavily on electrostatic forces between adjuvant and antigen. Commercial recombinant protein hepatitis B vaccines containing aluminum hydroxide as adjuvant is facing low induction of immunity in some sections of the vaccinated population. To follow the current global efforts in finding more potent hepatitis B vaccine formulation, adjuvanticity of aluminum phosphate has been compared to aluminum hydroxide.

Materials and methods: The adjuvant properties of aluminum hydroxide and aluminum phosphate in a vaccine formulation containing a locally manufactured hepatitis B (HBs) surface antigen was evaluated in Balb/C mice. The formulations were administered intra peritoneally (i.p.) and the titers of antibody which was induced after 28 days were determined using ELISA technique. The geometric mean of antibody titer (GMT), seroconversion and seroprotection rates, ED50 and relative potency of different formulations were determined.

Results: All the adjuvanicity markers obtained in aluminum phosphate formulation were significantly higher than aluminum hydroxide. The geometric mean of antibody titer of aluminum phosphate was approximately three folds more than aluminum hydroxide.

Conclusion: Aluminum phosphate showed more adjuvanticity than aluminum hydroxide in hepatitis B vaccine. Therefore the use of aluminum phosphate as adjuvant in this vaccine may lead to higher immunity with longer duration of effects in vaccinated groups.

Keywords: Hepatitis B vaccine, Aluminum phosphate, Adjuvanticity, Relative potency, Geometric mean titer.

INTRODUCTION

An effective vaccine usually requires an adjuvant to increase the immune response. More than 100 compounds or formulations show some degree of adjuvant properties (1). At the beginning of the 20th century, researchers experimented with a wide variety of organic and inorganic compounds including aluminum salts, mineral oil, and killed mycobacteria to improve the immunogenicity of vaccines (2). The most common adjuvants approved for use in currently licensed human vaccines are the aluminum based adjuvants (3). Aluminum phosphate and aluminum hydroxide (alum) are mineral compounds which were used more than 80 years ago by Glenny et al., who discovered that a suspension of alum-precipitated diphtheria toxoid had a much higher immunogenicity than the fluid toxoid (4). Nowadays the most widely used formulation for vaccines is the antigen solution mixed with pre-formed aluminum adjuvant under controlled conditions. Such vaccines are now called aluminum-adsorbed or aluminum adjuvanted vaccines (3-5).

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Aluminum phosphate adjuvant is actually amorphous aluminum hydroxyphosphate, $Al(OH)_m(PO_4)_n$, and aluminum hydroxide adjuvant is actually an aluminum oxyhydroxide compound, AlO(OH) (6-7).

The first HBV vaccine which became available in the early 1980s (8-9) was, manufactured from HBsAg particles derived from the plasma of chronic HBV carriers,. Subsequent recombinant vaccines were expressed in yeasts such as *Saccharomyces cerevisiae* (Engerix-B, Recombivax-HB) and, more recently, in Chinese hamster ovary (CHO) cells (Hepacare), (10, 11). The response to the vaccine was determined by measurement of anti-HBs levels 1–4 months after administration of the last dose of the vaccine. The minimum protection level (seroprotection) is considered to be 10 mIU/ml while antibody responses between 1 mIU/ml to 10 mIU/ml is usually referred as seroconversion (12-13). There may be an argument for boosters where the risk of exposure is relatively high. Patients with immune deficiency, such as those with HIV infection, those receiving immunosuppressive drugs or those on dialysis programs, have a lower response and as a result boosters may also be more important in these groups (14-15). Therefore by improving the immune response using an alternative adjuvant to aluminum hydroxide, better immunity with lower antigen dose or fewer boosters might be achieved. In this study the immune response to hepatitis –B protein vaccine formulated using aluminum hydroxide (Alhydrogel) or aluminum phosphate (Adju-Phos) was evaluated by comparing the geometric mean titer (GMT,mIU/mL), the rate of seroconversion, seroprotection , ED50(ng) and relative potency in Bulb/C mice after 28 days of intra peritoneally (i.p.) injection of vaccine.

MATERIALS AND METHODS

HBs antigen

The recombinant hepatitis B surface antigen used in this study was produced in *Pichia pastoris*, a histidine requiring strain, GS115 (his4) containing the gene for the *adw* subtype of HBsAg and was obtained from a local manufacturer (Darou Pakhsh Pharma. Co., Tehran, Iran). Purity and quantity of the HBsAg was assessed by different techniques. Antigen content of bulk was estimated using ELISA technique (Hepanostika HBsAg Uniform ELISA kit, Biomerieux, Netherlands) while purity of HBsAg was assessed by conducting reducing SDS-PAGE, Laemmli method using electrophoresis system (Mini-PROTEAN® 3 Cell, BIO-RAD, USA) according to the manufacture protocol. Total protein content of the bulk antigen was estimated using bicinchoninic acid (BCA) method (Pierce, USA) according to the manufacture protocol. The content of host cell nucleic acid in the bulk purified antigen was determined by PCR technique using 5'-pd$(T)_{12-18}$-3'(Amersham Biosciencces,USA) as a primer and 35 thermo cycles. The content of carbohydrates in bulk preparation was assessed using Anthrone method. Total lipids were determined by the spectro-photometric method using vanillin-ortho-phosphoric acid as reagent and cholesterol as lipid standard.

Vaccine formulations

Aluminum phosphate (Adju-Phos®) and aluminum hydroxide (Alhydrogel ®) were purchased from Brenntag Biosector (Denmark). Concentrations of the adjuvants were calculated on the basis of their aluminum contents. Vaccine was formulated by mixing the HBsAg bulk in phosphate buffer (pH 7.4) containing dibasic sodium phosphate anhydrous (1.12 g/l) and monobasic sodium phosphate monohydrate (1.1 g/l) with aluminum hydroxide or aluminum phosphate in 0.9% NaCl and 0.05 mg/ml thiomersal. The mixture was shaked on a reciprocal shaker (Kühner ISF-1-w) at 25°C and 140 rpm for 6 hrs and then at 4°C for 18hrs. The final concentration of HBsAg and aluminum was 20µg/ml and 500 ppm respectively. Dilutions of 1:512 (0.03906µg/ml), 1:64 (0.3125µg/ml) and 1:8 (2.5µg/ml) of vaccine were prepared by addition of phosphate buffer, pH 7.4 containing 500 ppm of the relevant aluminum adjuvant. Engerix-B® (GSK, Belgium, Lot No: AHBVB127AG) hepatitis B vaccine which included aluminum hydroxide as adjuvant was used as a control vaccine. Formulations containing only aluminum adjuvants were served as negative control.

Animals

Mice of the female Balb/C C3H strains of 5-6 weeks old were obtained from Charles River Laboratories (Germany) and housed in Micro-Isolator™ at 25°C, 12hrs day and night cycle with 50±5% of relative humidity. Food (5g) and water (6ml) were served, for each mouse daily. All studies were performed in accordance with the procedures issued by the Institutional Animal Care and Use Committee. Each dilution of vaccine at volume of 1mL was injected i.p.. to 15 mice. After 28 days the blood samples were collected from the heart of anaesthetized animal to determine HBs antibody titer. The serum of the blood samples were separated by centrifuging at 3000×g for 10 min and stored at -20°C.

Determination of anti HBs titers

Anti HBs antibody was determined by ELISA technique using Diasorin, ETT-AB-AUK-3 anti-HBs antibody ELISA kit (Italy) according to the manufacture protocol. The seroprotection level was achieved when the antibody titer was at least 10mIU/ml and antibody response between 1mIU/ml to 10mIU/ml was considered as seroconversion.

Statistics

ED50 for each formulation was evaluated by SPSS VER.12 using Probit method while the relative potencies of formulations was evaluated by quantal responses method (16). . Geometric mean anti-HBs Ag titers (GMTs) were calculated by taking the anti-log of the mean of the log titer transformations. Antibody titers below the cut-off the assay were given an arbitrary value of half the cut-off for the purpose of GMT calculation. GMT and other data processing were taken with Microsoft Excel 2007.

RESULTS

Specifications of bulk antigen

The concentration of bulk antigen which was estimated by the Hepanostika HBsAg uniform ELISA kit was 39.98 µg/ml. Total proteins content of the bulk antigen which was determined by BCA method was 29.18 µg/ml. The ratio of total antigen/total protein was 1.3 which was within the upper acceptable range of 0.8-1.4. The result on the purity of HBsAg assessed with SDS-PAGE is depicted in Figure 1. Single bands of 24 kDa are shown in lanes 1 and 3 which corresponds to monomer of HBsAg indicating high purity of the antigen which was used (16).

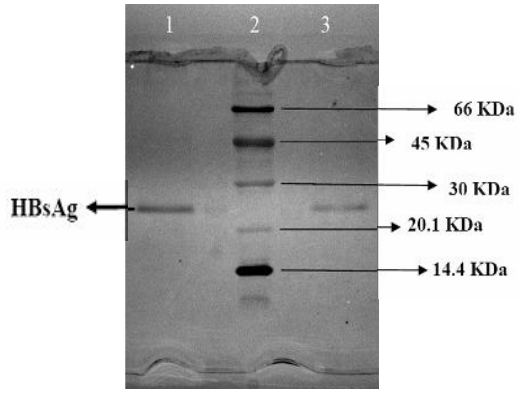

Figure 1. SDS-PAGE analysis of purified HBsAg . The gel was stained with AgNO3. Lane 1. Undiluted purified HBsAg bulk, Lane 2. Low molecular weight calibration kit (14.4-97KDa , GE Healthcare), Lane 3. Diluted (1:2) purified HBsAg bulk.

Determination of nucleic acid contamination by PCR showed less than 10 pg of DNA/dose (Fig. 2). The lipid content of HBsAg was 0.34mg/mg protein while carbohydrate content was 168 µg/mg protein which are indicative of acceptable quality of the used antigen (16).

Figure 2. Agarose gel electrophoresis of the PCR analysis for nucleic acid contamination.
Lanes1, 3, 4, 5 and 6 are the positive controls containing 10, 100, 50, 25 and 5 pgs of the host DNA respectively while lane 2 is the sample and lane 7 is the negative control lacking either the host DNA or the sample.

Seroconversion of the formulated vaccines

The results of 28 days seroconversion rates of various dilution of HBs vaccine formulations containing aluminum phosphate or aluminum hydroxide and also those of the control vaccine is shown in Figure 3. All formulations showed 100% of seroconversion at concentrations of 2.5 µg/ml and higher while at lower HBsAg concentrations (0.3125 µg/ml, 0.039 µg/ml), aluminum phosphate-containing formulations showed higher seroconversion rate (93%, 20%) compared to aluminum hydroxide based formulation (13%, 0%) as well as the control vaccine (73%, 0%).

ED50

ED50 (The dose which could induce seroconversion in 50% of the vaccinated population) for individual formulations were calculated with statistical software package SPSS VER.12 using Probit method with 95% confidence limit and the results are shown in Figure 4. The lowest ED50 was of the aluminum phosphate formulated vaccine which corresponds to better immunogenicity.

Seroprotection of the formulated vaccines

Seroprotection rates of various dilution of HBs vaccine formulations containing aluminum

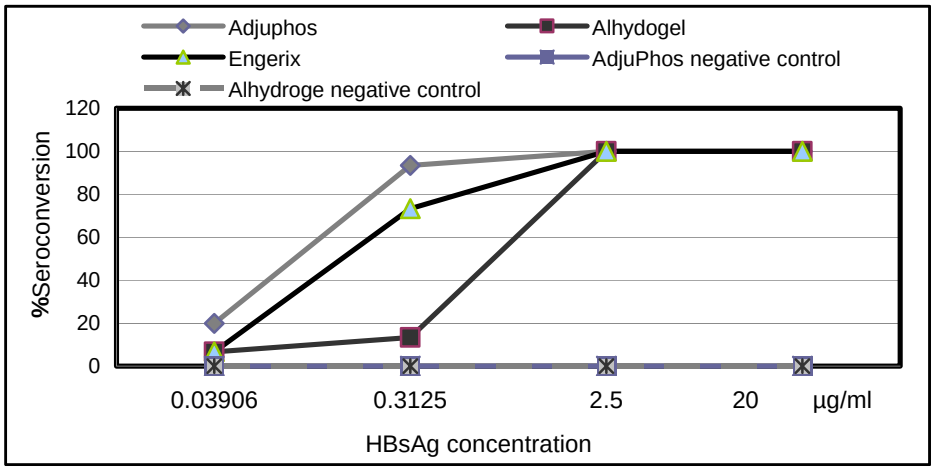

Figure 3. Seroconversion effect of different formulations containing Adju-Phos (aluminum phosphate), Alhydrogel (Aluminum hydroxide), AdjuPhos (aluminum phosphate) negative control, Alhydrogel (Aluminum hydroxide) negative control and Engerix after 28 days of i.p. injection in Balb/C mice.

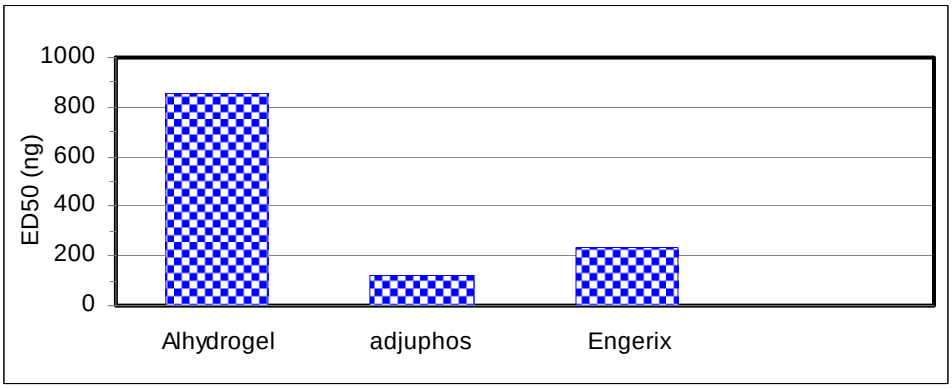

Figure 4. ED50 of different formulations containing AdjuPhos (aluminum phosphate) or Alhydrogel (Aluminum hydroxide) and Engerix after 28 days of i.p. injection in Balb/C mice($p<0.05$).

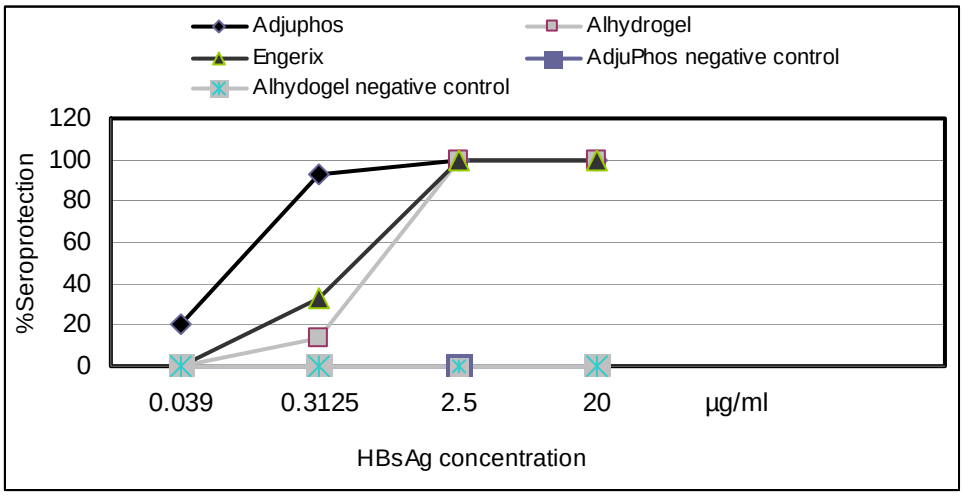

Figure 5. Seroprotection effect of different formulations containing Adju-Phos (aluminum phosphate), Alhydrogel (Aluminum hydroxide), AdjuPhos (aluminum phosphate) negative control, Alhydrogel (Aluminum hydroxide) negative control and Engerix after 28 days of i.p. injection in Balb/C mice.

Table 1. Geometric mean antibody titers (GMT) of different formulations containing Adju-Phos(aluminum phosphate) or Alhydrogel(Aluminum hydroxide) and Engerix after 28 days of i.p. injection in Balb/C mice.

Vaccine	Dose (µg/ml)	GMT (mIU/ml)	Standard Deviation (mIU/ml)
Engerix-B	20	691.77	360.65
	2.5	339.04	182.22
	0.3125	4.52	2.67
	0.03906	1.08	2.35
Adju-phos	20	3685.484	1987.52
	2.5	998.59	523.62
	0.3125	77.16	37.22
	0.03906	2.13	2.42
Alhydrogel	20	689.24	352.56
	2.5	320.62	181.76
	0.3125	1.38	2.44
	0.03906	1.26	2.88

Table 2. Relative potency and its lower limit and upper limit of different formulations containing Adju-Phos (aluminum phosphate) or Alhydrogel (Aluminum hydroxide) and Engerix-B after 28 days of i.p. injection in Balb/C mice.

Formulation	Lower limit	Relative potency	Upper limit
Adju-phos	29.7	33.33	37.58
Alhydrogel	6.3	7.74	9.42
Engerix-B	-	20	-

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

phosphate or aluminum hydroxide and also those of the control vaccine is depicted in Figure 5. All formulations showed 100% of seroprotection at concentrations of 2.5 µg/ml and higher while at lower HBsAg concentrations (0.3125 µg/ml, 0.039 µg/ml), aluminum phosphate-containing formulations showed higher seroprotection rate (93%, 20%) compared to aluminum hydroxide based formulation (13%, 0%) and also the control vaccine (33%, 0%).

Geometric mean antibody titers (GMT)

GMT titers of different formulations of hepatitis B vaccine are depicted in Table 1. Using a concentration of 20 µg/ml antigen the titer of GMT obtained with aluminum phosphate (3685 mIU/ml) was significantly higher (four folds) compared to those of aluminum hydroxide based formulation (689 mIU/ml) and also that of control vaccine (692 mIU/ml). Three fold differences in the GMT titers were observed at lower concentrations of antigen.

Relative potency

According to the seroconversion rates the relative potencies of the formulations were estimated using the quantal responses method (16). Engerix –B vaccine was used as the reference vaccine. Relative potency, lower limit and upper limit of relative potency are shown in Table 2. The relative potency of aluminum phosphate formulated vaccine was significantly higher (33.33) than the aluminum hydroxide based formulation (7.74).

DISCUSSION

Immunogenicity of two aluminum salts formulated recombinant hepatitis B vaccine were assessed in Balb/C mice. These adjuvants can induce rapid secretion of antibody and increase the antibody titer, which may lead to decrease of booster doses of vaccines. A potent adjuvant needs less antigen to achieve the desired immune response, therefore the cost of production will be reduced (1,2,5). Administration of aluminum salts with HBsAg causes much more immunity than HBsAg alone. Using the Engerix-B and Recombivax vaccines the seroprotection levels (above 10 mIU/ml) reported in the United States in different vaccinated groups were 83–100% and 69–99% respectively (12). Similar results have been reported with recombinant vaccines throughout the world targeting different risk groups (12). Therefore almost 17% or 31% of vaccinated groups, may remain non-protected (12-13). 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 (12-14). Also long-term follow-up studies have shown that the duration of vaccine-related immunity declines after several years. A number of such studies, where monitoring continued up to 12 years after vaccination, showed that anti-HBs levels declined over time and that half of the vaccinated persons had levels below10 mIU/ml (17-19). In this study the results of GMT for aluminum phosphate adjuvant were about three folds higher than

aluminum hydroxide and ED50 of aluminum phosphate was about 2 fold less than aluminum hydroxide formulations. Similar results were obtained for seoconversion and seroprotection. Relative potency of aluminum phosphate also indicated that aluminum phosphate adjuvanticity is more than aluminum hydroxide. Better adjuvanticity of aluminum phosphate may be due to ligand exchange between phosphate group of antigen and hydroxyl group of adjuvant, mechanism of the presentation of antigen to immune competent cells and the production of various lymphokines such as interleukins and tumor necrosis factor (3-7). It may also be due to the better effect of aluminum phosphate on the increase of Gr1 in spleen and higher secretion of IL-4 necessary for the priming of the B cells (6, 7). In conclusion the use of aluminum phosphate as adjuvant in hepatitis B vaccine may leads to higher immunity with more durable effect in vaccinated groups in comparison to aluminum hydroxide.

ACKNOWLEDGEMENTS

We wish to thank Biotechnology Department of Darou Pakhsh Pharmaceutical Manufacturing Company for their co-operation and providing the HBs antigen. We would like also to thank the Deputy for Research of Tehran University of Medical Sciences for funding the work.

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