Plasmid DNA containing multiple CpG motifs triggers a strong immune response to hepatitis B surface antigen when combined with incomplete Freund's adjuvant but not aluminum hydroxide

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Abstract. Adjuvants are important components of recombinant protein vaccines which are often poorly immunogenic. For decades, the search for new vaccine adjuvants has been predominantly empirical. In addition, combinations of more than one adjuvant plus antigen have been systematically studied. Plasmid DNA containing additional oligodeoxynucleotides with unmethylated CpG motifs (CpG ODN) entrapped in liposomes has been used as an adjuvant for DNA vaccines and has shown powerful immunostimulatory functions. In our study, the combination of plasmid DNA containing 16 additional CpG ODNs (pv-16CpG) and aluminum hydroxide (AL) or incomplete Freund's adjuvant (IFA) was used as an adjuvant for a hepatitis B surface antigen (HBsAg) vaccine to immunize C57BL/6J mice. ELISA and ELISPOT assays were used to analyze the immunological effects of the novel vaccine. A significant enhancement of the anti-HBs titer and seroconversion was observed when the CpG plasmid was combined with IFA, but not with AL. In addition, anti-HBs antibody isotype analysis revealed that the combination of CpG plasmid and IFA induced a strong HBsAg-specific IgG2a response. Moreover, the ELISPOT assays suggested that pv-16CpG suspended in IFA evoked a strong T helper 1 (Th1) immune response and high IFN-γ production. These results demonstrate that pv-16CpG suspended in IFA is able to induce cellular and humoral immune responses to HBsAg, and confirm its potential as an adjuvant for use in protein vaccines.

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Introduction

Vaccination is an effective method of decreasing the incidence of infectious diseases caused by pathogens to which individuals are susceptible in early life (1). Unlike live and attenuated organism vaccines, the inactive or dead organism vaccines and recombinant protein or subunit vaccines are generally administered with an adjuvant to aid in or enhance the immune response (2-3). Adjuvants are compounds that enhance the ability of the co-inoculated antigens to elicit an early, high and long-lasting immune response with a reduced amount of antigen (4). Owing to the highly purified recombinant proteins, which are increasingly used in the new generation vaccines but are poorly immunogenic, formulations comprising more than one adjuvant have been widely studied in various vaccines. To enhance the potency of the first generation adjuvants, including aluminum salts (also known as alums), emulsions, polymeric particles and liposomes, additional components have been added to them, generating the second generation of vaccine adjuvants. Furthermore, the second generation adjuvants have been licensed in approved vaccine products in the EU, including Pandemrix (AS03; o/w emulsion + α-tocopherol) and Fendrix or Cervarix (AS04; MPL + aluminum hydroxide) (5).

Bacterial DNA and oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN) have been studied as vaccine adjuvants for the induction of protective immunity against bacterial, viral and parasitic infections in numerous animal models (6-9). Previous studies have found that CpG ODN is able to activate a T helper 1 (Th1)-like humoral immune response with the production of IgG2a antibodies and a cell-mediated immune response with the secretion of IFN-γ cytokines (10-11). Furthermore, another study has shown that plasmid DNA directly injected into the muscle cells of mice elicited potent humoral and cellular immune responses (12). In addition, it has been shown that plasmid DNA may be useful as an adjuvant for DNA vaccines (13,14). Recently, our

laboratory revealed that plasmid DNA (pcDNA 3.1) encapsulated in cationic liposomes delivered an adjuvant effect in the cancer immunotherapy of murine colon carcinoma (15). Other studies have demonstrated that the adjuvant properties of plasmid DNA were dependent on its content of CpG motifs which interacted with TLR9 and directly induced the production of IL-12 and IFN-α by dendritic cells (16-19). As the plasmid pcDNA 3.1 contains the ampicillin resistance gene, it is commonly used for studies in mice. Considering that the plasmid vector pVAX1, which contains a kanamycin resistance gene, is permitted to be used in humans (20), and the high cost of synthesis of CpG ODN, we constructed a multiple CpG plasmid (pv-16CpG) containing 16 CpG motifs (CpG 7909) to examine its adjuvant effect in a hepatitis B surface antigen (HBsAg) vaccine when combined with aluminum hydroxide (AL) or incomplete Freund's adjuvant (IFA).

Aluminium salts, particularly phosphate or hydroxide salts, have been widely used in human and veterinary vaccines since 1930 with an excellent record of safety (21). Aluminum salts markedly induce the generation of Th2 cytokines (IL-4 and IL-5) and antibodies of the IgG1 isotype (22-23). IFA, a water-in-oil emulsion, has been widely used in veterinary vaccine products and tested in humans (24-25). The main mechanisms of action of aluminum salts and IFA are similar, they prolong the duration of antigen persistence at the injection site (5).

Since the introduction of a hepatitis B vaccine containing alum over 30 years ago, a decline in the global incidence of HBV infection has been observed (26). However, 5-10% of healthy immunocompent individuals fail to produce a protective antibody response to the HBV vaccine (27). Improving the immunogenicity of the HBV vaccine is a strategy that can be employed to overcome this problem. Therefore, new adjuvants that elicit strong immune responses to HBsAg are required. Previous studies have shown that certain adjuvant combinations induce a stronger immune response than either individual component alone (28). In this study, we used formulations of pv-16CpG with AL or IFA as adjuvants for a hepatitis B vaccine. The results showed that pv-16CpG suspended in IFA triggered a stronger immune response against HBsAg in C57BL/6J mice than pv-16CpG combined with AL. The plasmid pv-16CpG suspended in IFA induced a Th1 immune response to HBsAg, characterized by the generation of specific IgG2a antibodies and IFN-γ in antigen stimulated T cells. We suggest that pv-16CpG, which may facilitate the manufacturing process, may be used as an adjuvant for HBsAg vaccines when mixed with IFA.

Materials and methods

Plasmid pv-16CpG preparation. The plasmid pv-16CpG was constructed as follows: 16CpG motif ODN (16 x 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') fragments were synthesized and inserted into the plasmid pVAX1 (Invitrogen Life Technologies, San Diego, CA, USA) using the enzymes *Hin*dIII (5'-end of the fragment) and *Bam*HI (3'-end of the fragment) from Invitrogen. The fragment sequences were confirmed by DNA sequencing. The plasmid pv-16CpG was prepared using the Endofree Plasmid Giga kit (Qiagen, Chatsworth, CA, USA). There was no genomic DNA, small DNA fragments or RNA in the pv-16CpG DNA ($OD_{260/2280}$=1.8-2.0).

Figure 1. Schema of the immunization protocol. Mice (n=8) were immunized with HBsAg (0.1 μg) and AL (25 μg Al^{3+}), IFA (50 μl; v/v=1:1), AL + pvAX (50 μg), IFA + pvAX (50 μg), AL + pv-16CpG (50 μg) or IFA + pv-16CpG (50 μg) in weeks 0, 2 and 4. In addition, mice were immunized with the same dose of vaccine in week 31. Following the third immunization, mice (n=3) were sacrificed and an ELISPOT assay was performed. Sera were collected in weeks 1, 3, 5, 7, 9, 13, 17, 21, 25, 30, 32, 34 and 50, and the anti-HBsAg titers were measured by endpoint dilution ELISA. HBsAg, hepatitis B surface antigen; AL, aluminum hydroxide; IFA, incomplete Freund's adjuvant; pvAX, pv-16CpG, plasmid vectors; ELISA, enzyme-linked immunosorbent assay.

Reagents. AL gel adjuvant was purchased from Brenntag Biosector (Frederikssund, Denmark) and IFA was purchased from Sigma Poole (Dorset, UK). The hepatitis B surface antigen (subtype ad) protein was purchased from American Research Products, Inc. (Belmont, MA, USA).

Animals and immunizations. Six to eight-week-old female C57BL/6J mice (n=8 mice per group) were purchased from the Experimental Animal Center of Sichuan University and were housed in our animal research facility. The mice were immunized intramuscularly (hind limbs) three times at 2-week intervals (in weeks 0, 2 and 4) with 0.1 μg HBsAg and adjuvant in a total volume of 100 μl (Fig. 1). The adjuvant amounts used for each immunization were: 25 μg Al^{3+}, 50 μg pvAX, 50 μg pv-16CpG or IFA 50 μl (v/v 1:1), mixed by syringe. The mice were administered a booster immunization with the same dose in week 31. Blood samples were collected from the animals in weeks 0, 1, 3, 5, 7, 9, 11, 13, 17, 21, 25, 30, 32, 34 and 50 following the immunization. The serum was divided into aliquots and was maintained at -70˚C.

Enzyme-linked immunosorbent assay (ELISA). To measure the HBsAg-specific antibody response, a 96-well plate was coated overnight at 4˚C with 100 μl HBsAg solution (1 μg/ml) in sodium carbonate buffer. The plate was washed three times with 0.1% Tween in PBS (PBST), and then blocked with 5% skimmed milk in PBST for 1 h at 37˚C. After being washed 5 times with PBST, the plate was incubated with serially diluted mice sera in 5% skimmed milk for 1 h at 37˚C, followed by further washes. Bound antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 (Southern Biotechnologies, Birmingham, AL, USA) diluted at 1:400 for 1 h at 37˚C. The plate was washed again and incubated with 100 μl substrate-chromogen solution (SureBlue™ TMB Microwell Peroxidase substrate, KPL, Inc., Gaithersburg, MD, USA) for 20 min at room temperature. A volume of 0.5 M H_2SO_4 (100 μl) was added to terminate the reaction and the absorbance was read at 450 nm using a Bio-Rad (Hercules, CA, USA) microtiter plate reader. ELISA titers were expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above that of non-immunized serum with a cut-off of 0.2.

IFN-γ and IL-4 ELISPOT assays. To measure the numbers of IFN-γ and IL-4 secreting cells, the dual-color ELISPOT Mouse IFN-γ/IL-4 kit (R&D systems, Inc., Minneapolis, MN, USA) was used. Following the third immunization, the mice were sacrificed and their splenocytes were separated as described in the manual of the EZ-sep™ mouse 1X. (Dakewe Biotech Co., Ltd., Shenzhen, China). The splenocytes were added to triplicate wells at a concentration of $5x10^5$ cells/well and cultured in the presence of HBsAg (5 μg/ml) or ConA (1 μg/ml) for 48 h. Stained spots were counted using a computer-assisted ELISPOT image analyzer (Immunospot, Cellular Technology Ltd., Cleveland, OH, USA).

Electrophoretic mobility shift assay (EMSA) and scanning electron microscopy (SEM) experiments. Binding assays were performed by incubating pv-16CpG (10 μg) with various amounts of AL in a final volume of 100 μl of 10 mM Tris-HCl and 270 mM sorbitol (pH 7.4). After incubating at 37˚C for 10 min, the complexes were centrifuged at 3000 rpm for 5 min. The deposits were subjected to electrophoresis through a 0.8% agarose gel at 120 V for 15 min. The DNA was visualized by ethidium bromide staining. The SEM experiments were performed using a JSM-5900LV SEM instrument to inspect the sizes of the particles. The samples were prepared according to the prescription of administration for mice and were then gold-coated and observed under an accelerating voltage of 20 kV.

Statistical analysis. The studies were performed on groups of 3-5 individual mice. Statistical comparisons were carried out using one-way analysis of variance (ANOVA) followed by Tukey's test. Results were considered statistically significant at $p<0.05$.

Results

Seroconversion induced by IFA + pv-16CpG. In week 1 following the immunization, 20% of mice in the AL + pvAX, IFA + pvAX and IFA + pv-16CpG groups had produced anti-HBs (titer ≥100) (Fig. 2). In week 3 post-immunization, the anti-HBs seroconversion rate of the AL and AL + pvAX groups had also reached 20%. In addition, at this time, the rates in the IFA + pvAX and IFA + pv-16CpG groups were 40 and 80%, respectively. However, no seroconversion was identified in the AL + pv-16CpG and IFA groups. The seroconversion rate in the IFA + pv-16CpG group was clearly improved compared with the other 5 groups. However, 5 weeks after immunization, the seroconversion rates in the AL + pv-16CpG and IFA groups were 40 and 20%, respectively. Moreover, while the seroconversion rate of the AL + pvAX group reached 60%, the rates in the other 3 groups were 100%.

Enhancement of anti-HBs titer by IFA + pv-16CpG. The kinetics of the HBsAg-specific humoral immune responses were monitored over the 50 weeks following immunization. C57BL/6J mice were immunized by intramuscular (i.m.) injection of 0.1 μg HBsAg with various adjuvants at different time points (Fig. 2). When the double adjuvant combinations were used, the anti-HBs titer of the IFA + pv-16CpG group in week 13 (first peak) was 50-fold higher than that of the IFA group and 320-fold higher than that of the AL group (Fig. 3). However, there was no increase in the anti-HBs titers when the combination of AL and pv-16CpG was used as the adjuvant. The IFA + pvAX group underwent a significant increase compared with the remaining groups, with the exception of the IFA + pv-16CpG group. Throughout the study, the titers of the IFA + pv-16CpG group were 5-8-fold higher than that of the IFA + pvAX group at 50 weeks. The antibody titer of the IFA group underwent a sustained growth following the first immunization, and this increasing trend stopped in week 17, while in the other groups, it stopped in week 13. Twenty-one weeks after immunization, the titers of the 6 groups exhibited a sustained decrease. To detect the memory response evoked by the vaccine, the mice were boosted with the same dose of vaccine in week 31. A 2- to 20-fold increase in anti-HBs titers was observed in all the groups. However, the anti-HBs titer of the IFA + pv-16CpG group in week 32 was

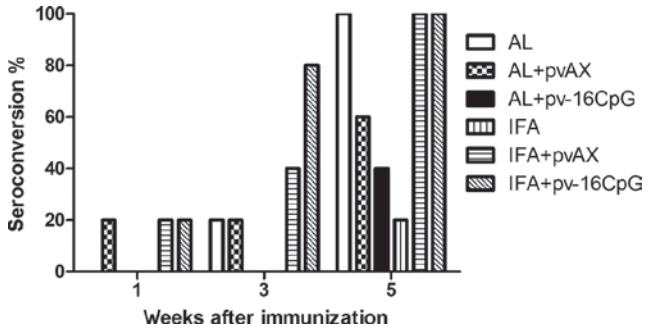

Figure 2. Percentage of seroconversion for C57BL/6J mice immunized in weeks 0, 2 and 4 using HBsAg with various adjuvants. C57BL/6J mice (n=5) were immunized 3 times by intramuscular (i.m.) injection with 0.1 μg HBsAg formulated with 25 μg AL, IFA (50 μl; v/v=1:1), AL + pvAX (50 μg), IFA + pvAX (50 μg), 25 μg AL + 50 μg pv-16CpG, or 50 μg IFA + pv-16CpG (50 μl). Sera obtained in weeks 1, 3 and 5 were assayed for anti-HBsAg by endpoint-dilution ELISA assay. Seroconversion was defined as a dilution titer ≥100 with a cut-off value of 0.2. HBsAg, hepatitis B surface antigen; AL, aluminum hydroxide; IFA, incomplete Freund's adjuvant; pvAX, pv-16CpG, plasmid vectors; ELISA, enzyme-linked immunosorbent assay.

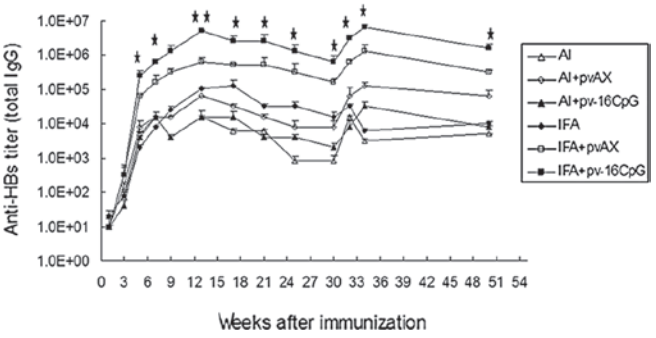

Figure 3. IgG antibody titer kinetics against HBsAg in C57BL/6J mice immunized with AL, IFA, AL + pvAX, IFA + pvAX, AL + pv-16CpG or IFA + pv-16CpG. Mice were immunized as described above. ELISA titers are defined as the reciprocal of the highest dilution resulting in a reading of two standard deviations above that of non-immunized mice with a cut-off value of 0.2. Sera obtained from each mouse were used to detect the titer by ELISA in each group. Data are presented as the group mean for total IgG titer to HBsAg. Vertical lines are the SEM. Two other experiments produced similar results; *$p<0.05$; **$p<0.001$. HBsAg, hepatitis B surface antigen; AL, aluminum hydroxide; IFA, incomplete Freund's adjuvant; pvAX, pv-16CpG, plasmid vectors; ELISA, enzyme-linked immunosorbent assay.

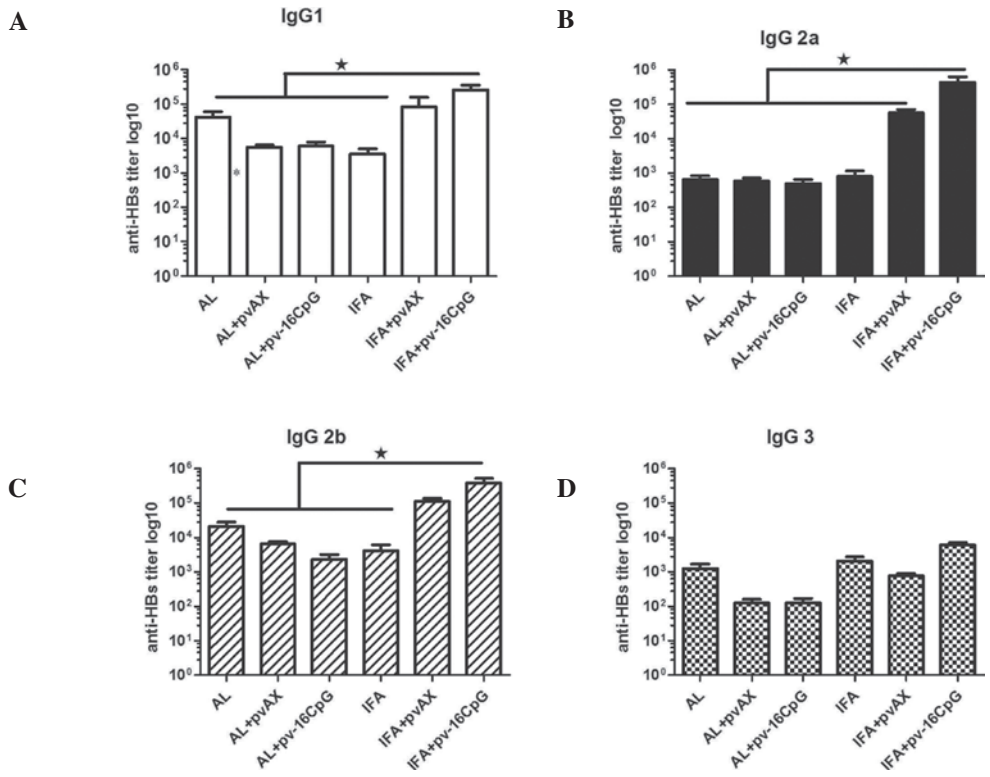

Figure 4. Antibody isotypes in C57BL/6J mice 7 weeks after immunization with various vaccines. Anti-HBs serum IgG isotype titers were determined by end-point dilution ELISA assay. Data are presented as the mean ± SEM of ELISA titers obtained from the sera of 5 animals. Each graph is representative of at least three experiments. ELISA, enzyme-linked immunosorbent assay; AL, aluminum hydroxide; pvAX, pV-16CpG, plasmid vectors; IFA, incomplete Freund's adjuvant.

40- or 300-fold higher than that in the AL and IFA groups, respectively. Furthermore, the anti-HBs antibody titer in the IFA + pv-16CpG group in week 32 was 800-fold higher than that in the AL + pv-16CpG group (Fig. 3).

Antibody isotype profiles evoked by IFA + pv-16CpG. The ratio of IgG2a to IgG1 antibody levels is often used to evaluate the type of Th response, with a value >1 indicating a predominantly Th1 response and <1 indicating a predominantly Th2 response. Immunization with AL or IFA alone generated a Th2 response, with higher levels of anti-HBs antibodies of the IgG1 isotype than those of the IgG2a isotype (Fig. 4A and B). The values of IgG2a/IgG1 were 0.021 and 0.25 in the AL and IFA groups, respectively, 7 weeks after immunization. When the plasmid pv-16CpG combined with AL was added as the adjuvant, the anti-HBs titers of the IgG1 and IgG2a isotypes were lower than those in the AL group. In addition, the IgG2a/IgG1 ratio was 0.063, 7 weeks after immunization. When the plasmid pvAX was combined with AL or IFA, the values of IgG2a/IgG1 in week 7 were 0.078 and 0.5, respectively. This finding suggested that the combination of plasmid and AL or IFA induced a Th2 immune response. However, with an increased effect on IgG1 and IgG 2a antibody titers, the combination of plasmid pv-16CpG and IFA evoked a Th1 response (Fig. 4A and B) with a value of IgG2a/IgG1 >1 (2.0). Moreover, the IgG2b and IgG3 antibody titers in the IFA + pv-16CpG group increased significantly compared with those in the IFA group, but decreased in the AL + pv-16CpG group when compared with the AL group (Fig. 4C and D). These findings suggest that the plasmid pv-16CpG has great potential for improving the immune response when combined with IFA but not with AL.

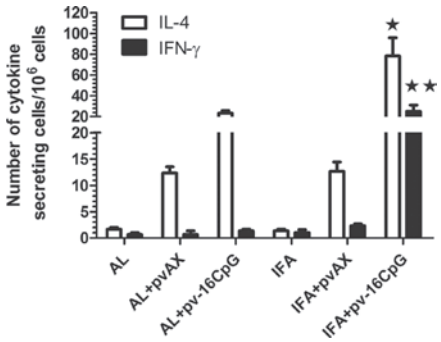

Figure 5. Frequency of HBsAg-specific IFN-γ- and IL-4-secreting cells in splenocytes of mice 5 weeks after immunization. C57BL/6J mice (n=3) were immunized with 0.1 μg HBsAg combined with AL, IFA, AL + pv-16CpG, IFA + pv-16CpG, AL + pvAX or IFA + pvAX in weeks 0, 2 and 4. The number of cytokine-secreting cells were determined by a computer-assisted ELISPOT image analyzer. Bars show the mean ± SEM number of cytokine-specific cells in splenocytes as determined by the ELISPOT assay. *p<0.05; **p<0.001. HBsAg, hepatitis B surface antigen; AL, aluminum hydroxide; IFA, incomplete Freund's adjuvant; pv-16CpG, pvAX, plasmid DNA.

Assay of HBsAg-specific T cell immune response by ELISPOT. To characterize the immune responses induced by the various formulations, the production of IL-4 and IFN-γ by HBsAg-exposed splenocytes from immunized mice were examined by re-stimulating the splenocytes with HBsAg. The number of IL-4-secreting T cells in the mice immunized with

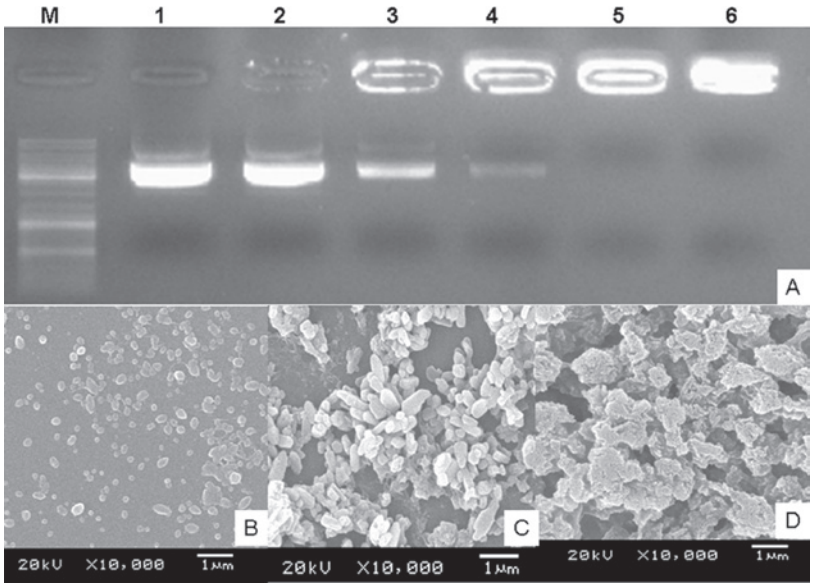

Figure 6. Electrophoretic mobility shift assay (EMSA) and scanning electron microscopy (SEM) of AL and pvAX or pv-16CpG complexes. (A) EMSA of AL and pv-16CpG complex: M, marker; lane 1, pv-16CpG (10 μg); lane 2, pv-16CpG (10 μg) + AL (5 μg); lane 3, pv-16CpG (10 μg) + AL (10 μg); lane 4, pv-16CpG (10 μg) + AL (20 μg); lane 5, pv-16CpG (10 μg) + AL (40 μg); lane 6, pv-16CpG (10 μg) + AL (80 μg). (B) SEM micrograph of AL (25 μg) diluted and vortexed in a total volume of 100 μl. (C) SEM micrograph of AL and pvAX complex: AL (25 μg) and pvAX (50 μg) diluted and vortexed in a total volume of 100 μl. (D) SEM micrograph of AL and pv-16CpG complex: AL (25 μg) and pv-16CpG (50 μg) diluted and vortexed in a total volume of 100 μl. Each graph is representative of at least three experiments. AL, aluminum hydroxide; pvAX, pv-16CpG, plasmid vectors.

AL + pv-16CpG or IFA + pv-16CpG were significantly higher than that in the AL, AL + pvAX, IFA or IFA + pvAX groups (Fig. 5). The number of IL-4-secreting splenocytes in the mice immunized with AL + pv-16CpG or IFA + pv-16CpG were approximately 15- and 30-fold higher than in the mice immunized with AL or IFA alone, respectively (Fig. 5). When the plasmid pvAX was added to AL or IFA, the number of IL-4-secreting splenocytes was also lower than that in the IFA + pv-16CpG group ($p<0.05$). Furthermore, the highest number of IFN-γ-secreting T cells was found in the mice receiving IFA + pv-16CpG. The number of IFN-γ-secreting T cells in the mice receiving IFA + pv-16CpG was almost 30-fold higher than that in the mice from the other groups ($p<0.001$). Moreover, the higher IFN-γ secretion indicated a potent type 1 cell-mediated immune response.

Discussion

Vaccines are widely used in the protection and eradication of a wide range of diseases, including smallpox, polio, measles, whooping cough and hepatitis. Sub-unit vaccines, including HBsAg-based vaccines, have a good record of safety but low efficacy in eliciting an immune response (29). Therefore, adjuvants which enhance the immunogenicity of these vaccines are urgently required.

Aluminum salts (particularly aluminum hydroxide or phosphate), which have been licensed as adjuvants for human use, have been widely and successfully used in a great number of licensed vaccines although they induce a poor cytotoxic T cell and Th1 immune response (30-31). As shown in Figs. 4 and 5, AL induces a Th2 immune response, with predominantly IgG1 antibody and IL-4 secretion from immunized splenocytes. When the plasmid pv-16CpG was combined with AL, it induced a weaker immune response than AL alone.

IFA, a water-in-oil emulsion adjuvant, which produces a high and sustained antibody response, has been used in experimental vaccines in mice, rabbits, guinea pigs and rhesus macaques (24). Furthermore, IFA induces a Th2 type response, which is not effective for a cellular immune response and limits the adjuvant activity of IFA. Our results showed that when IFA was used alone, it induced predominantly IgG1 antibody (Fig. 4A and B) and IL-4 production, as detected by the IL-4/IFN-γ ELISPOT assay (Fig. 5). However, mixing or using IFA in conjunction with other adjuvant-active compounds has been successful in animal testing and preclinical trials (24). Furthermore, findings of another study have shown that insect DNA and CpG suspended in IFA was a more powerful adjuvant in mice than CFA, which is considered as the 'gold standard' for adjuvant function (32). In our study, the use of a combination of IFA and pv-16CpG as the adjuvant for a HBsAg vaccine, resulted in higher anti-HBs antibody titers than the other adjuvants tested (at least 10-fold higher than the other groups throughout the experiment; Fig. 3) and significantly increased IFN-γ production, even compared with the AL + pv-16CpG group ($p<0.001$). The booster immunization in week 31 resulted in a profoundly increased and sustained memory response in the IFA + pv-16CpG group. The data suggest that pv-16CpG suspended in IFA evokes cellular and humoral immune responses.

The data demonstrated that pv-16CpG exhibited a strong adjuvant activity when combined with IFA but not with AL. The reasons which may explain this result include the fact that the mechanism of action of adjuvant aluminum salts involves the formation of an antigen depot at the injection site (33), the electrostatic attractive forces between the aluminum salt (positive charge) and antigen (negative charge) (34). As pv-16CpG itself may be considered as a polyelectrolyte with a high negative charge (35), there is likely to be an electrostatic interaction

between AL and pv-16CpG. As shown in Fig. 6, pv-16CpG was completely adsorbed by AL when the ratio was 1:10 by weight. This interaction may reduce the ability of the aluminum salt to form an antigen depot from which the antigen is slowly released. However, there may be not an electrostatic interaction between pv-16CpG and IFA. Moreover, in the microdroplets of oil formed by mixing, the electrostatic attractive force between pv-16CpG and the HBsAg protein may contribute to the slow release of antigen. Due to the limitations of the technology, EMSA could not be performed for IFA and plasmid DNA.

Another explanation for our results is that the size of the antigen or the antigen formulated with an adjuvant is a significant factor for its efficient uptake by antigen-presenting cells (APCs). The range of particle sizes which allows the most efficient uptake by APCs is 10-3,000 nm. In addition, the average particle sizes of emulsions (such as IFA) and mineral salts (such as AL) are 200-8,000 and 800-12,000 nm, respectively (36). The particle size of AL is smaller than 1 μm, but becomes larger than 1.5 μm when pv-16CpG is combined with it (Fig. 6B and C), and this change may affect the take up of the antigen by APCs. The mechanism of adjuvant activity in the vaccine comprising pv-16CpG suspended in IFA requires further study.

In conclusion, the results of our studies have shown that pv-16CpG may be used as an adjuvant for HBsAg vaccines when combined with IFA but not with AL, and evoked cellular and humoral immune responses against HBsAg.

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