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SHORT COMMUNICATION

Beneficial Effects of the Mixed Adjuvant of CpG plus Monophosphoryl Lipid A in Immunization with a Recombinant Protein Vaccine for Hepatitis A

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Abstract In an effort to develop a new vaccine for hepatitis A, which is mainly transmitted via contaminated foods and water, recombinant virus protein 1 (VP1) of hepatitis A virus was used as an antigen. Several adjuvants in a single or a mixed form, i.e., alum, CpG oligodeoxynucleotide, monophosphoryl lipid A (MPL), alum plus MPL, and CpG plus MPL, were also tested for their immunological properties. When their effects on the production of VP1-specific IgG relative to that of total IgG and the levels of and balance between Th1- and Th2-type cytokine productions were compared, CpG plus MPL was found to have highly beneficial effects, providing a new insight in selection of adjuvant for development of a new vaccine.

Keywords adjuvant · cytokine · hepatitis A · immunoglobulin · recombinant vaccine

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Vaccines using live (attenuated) or killed viruses have protected human beings from a number of diseases for many decades. However, due to safety issues arising from the use of whole virus particles and the difficulty of manufacturing a large quantity of certain viruses, searches for safer and cheaper vaccines are ongoing (Levine, 2011).

Advances in molecular biology and technology for the production of recombinant proteins made it feasible to use a single viral antigen (or a combination of antigens) as a vaccine (Cox, 2012). While such recombinant protein vaccines have advantages in the safety and the cost of production, it is also known that purified recombinant viral antigens generally hold a weaker immuno-genicity compared to whole virus particles. Thus, it is often imperative to administer such an antigen with an adjuvant to attain a sufficient level of immune response against antigen (Aguilar and Rodriguez, 2007; Tagliabue and Rappuoli, 2008; Yang and Yang, 2009).

Alum, hydrated aluminum hydroxide, is currently the most widely used and clinically approved adjuvant (Mbow et al., 2010). Lipopolysaccharide (LPS), a component of cell wall of Gram-negative bacteria, has long been studied as an adjuvant in both preclinical and clinical settings (McAleer and Vella, 2008). Monophosphoryl lipid A (MPL), a derivative of LPS with less toxicity and pyrogenicity, was also developed as a safer alternative of LPS (Cluff, 2009). CpG ODN, a class of single stranded oligodeoxynucleotides with a sequence of cytosine followed by guanine, is also known to possess a strong adjuvant activity (Seo et al., 2009; Vollmer and Krieg, 2009). Although working mechanisms of these adjuvants are not yet fully understood, it is known that alum mainly hoists antibody (Ab)-mediated immune responses (Marrack et al., 2009; Mbow et al., 2010), whereas MPL and CpG promote cell-mediated immune responses as well as Ab-mediated immune responses via Toll-like receptors (TRLs) (van Duin et al., 2006).

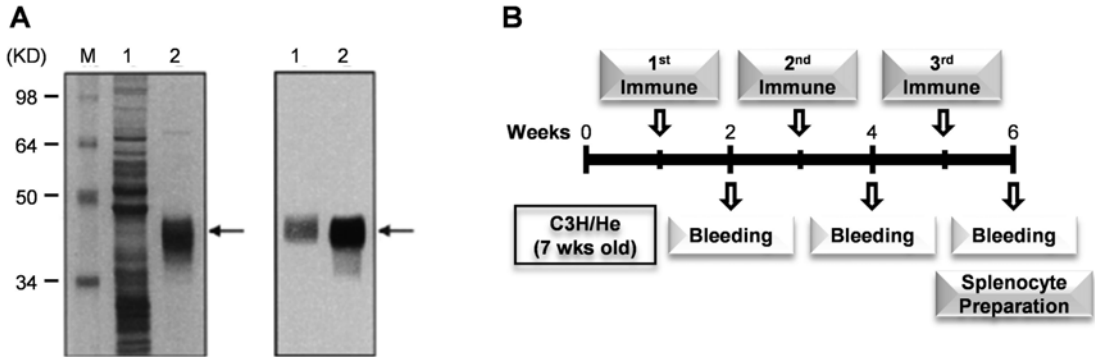

Fig. 1 Preparation of recombinant VP1 fusion protein and mouse immunization schedule. (A) HisX6-VP1 protein stably expressed in Drosophila S2 cell line was affinity-purified from the culture supernatant as described (Lee et al., 2009). Proteins, before (Lane 1) and after (Lane 2) purification, were analyzed by SDS-PAGE (Left) and Western blotting using mAb for V5 epitope in the fusion protein as a part of HisX6 motif (Right). (B) Ten micrograms of purified recombinant VP1 protein was injected intraperitoneally (i.p.) into groups (5 mice per group) of C3H/He (Orient Bio, Korea) mice with PBS, alum (1 mg/head), CpG (50 mg/head), MPL (50 mg/head), alum plus CpG, and CpG plus MPL, respectively, three times every two weeks. CpG (TCCATGACGTTCCTGACGTT) was synthesized in St Pharm Co. (Korea), and alum and MPL were purchased from Thermo Science (USA) and Sigma (USA), respectively. One week after each injection, blood was drawn for analysis of total and VP1-specific IgG levels in the serum, respectively (Fig. 2). One week after the final injection, splenocytes were made to examine ex vivo patterns of cytokine productions (Fig. 3).

Hepatitis A (Hep A) is an acute infectious disease of liver caused by Hepatitis A virus (HAV), which is a single-stranded RNA virus belonging to Picornaviridae (Martin and Lemon, 2006). HAV is transmitted via contaminated foods, water or direct contact with an infected individual (Koopmans and Duizer, 2004). Even though HAV infection is more widespread in developing countries where the standard-level of quality control of food and water processing is often overlooked, it also becomes increasingly problematic in developed countries as more people travel around the globe to contract the virus from indigenous people (Connor, 2005). HAV infection resolves mostly without showing much clinical symptoms when occurring in adolescents, but it often causes serious illnesses (e.g., acute liver damage) when it occurs in adults (Jeong and Lee, 2010).

Killed HAV has been used as a vaccine for Hep A, effectively protecting people from HAV infection. However, the use of whole virus particles poses safety issues and, therefore, demands for a safer HAV vaccine remain high (Nothdurft, 2008). In the present study, we used a recombinant HAV envelop protein (VP1) as an immunogen to address the possibility of its use as an alternative vaccine for Hep A (D'Hondt, 1992). In addition, we examined efficacies of various adjuvants, in single or mixed forms, in the promotion of host immune responses against VP1.

The recombinant HisX6-VP1 fusion protein expressed in S2 Drosophila cells was affinity-purified and used in the experiments (Fig. 1A) (Lehr et al., 2000; Lee et al., 2009). C3H/He mice (5 mice per group) were immunized with the antigen mixed with an adjuvant every two weeks, for three times (Fig. 1B). One week after each immunization, blood was drawn to examine the levels of total immunoglobulin G (IgG) and VP1-specific IgG in the serum by enzyme-linked immunosorbance assay (ELISA). Differences in the level of total IgG became highly apparent among groups of animals immunized with VP1 after the third immunization. MPL and alum plus MPL showed the strongest effects on the production of total IgG followed by alum, CpG, and CpG plus MPL (Fig. 2A).

Effects of the adjuvants on the production of VP1-specific IgG began to be noticeable after the second immunization, and more apparent increase was observed after the third immunization. Data showed that CpG, alum plus MPL and CpG plus MPL had better effects in the production of VP1-specific IgG; thus, levels of VP1-specific IgG in groups of animals immunized with CpG, alum plus CpG and CpG plus MPL, were significantly higher than those in groups of animals immunized with alum and MPL (Fig. 2B). When effects of respective adjuvants on the production of VP1-specific IgG relative to those on the production of total IgG were compared, it seemed apparent that CpG and CpG plus MPL had a preferential effect on the production of VP1-specific IgG; thus they revealed a superior effect on the production of VP1-specific IgG even though their effects on the production of total IgG were moderate compared with those of others (Fig. 2).

The balance between Th1- and Th2-type cytokine productions in the body after immunization is important for preventing potential adversary effects of vaccination such as allergy against environmental antigens (Nguyen and Casale, 2011). Indeed, alum, known to favor the production of Th2-type cytokines, tends to increase the occurrence of allergy, suggesting that the biased production of Th2-type cytokines results in the hyper-production of immunoglobulin E (IgE) for such antigens (Baylor et al., 2002; Marrack et al., 2009).

We investigated the effect of each adjuvant on the cytokine production by culturing splenocytes from immunized mice in the presence or absence of VP1 and measuring levels of representative Th1- and Th2-type cytokines, i.e., interleukin-10 (IL-10) and IL-12 (O'Garra and Murphy, 2009), respectively, in the culture supernatants (Fig. 3). In IL-10 production (Fig. 3A), all adjuvants

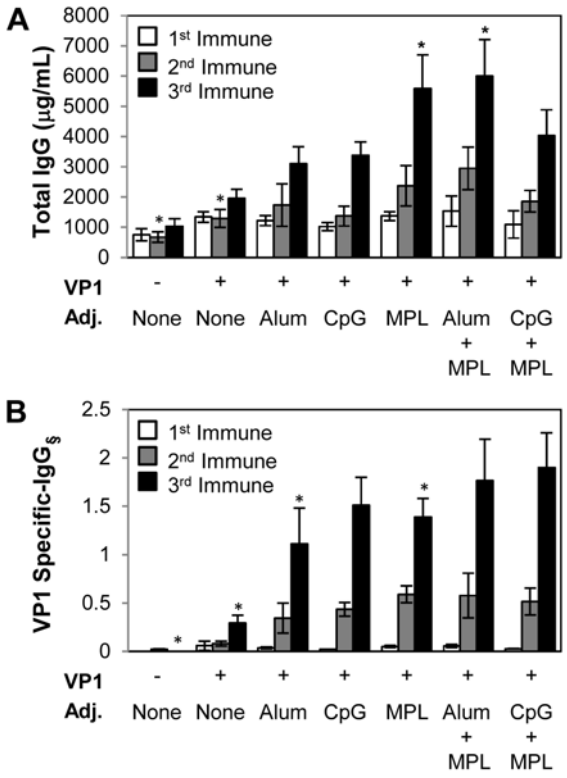

Fig. 2 Effects of adjuvants on the production of total and VP1-specific IgG. Experiments were performed to compare the efficacies of adjuvants in the production of total IgG and VP1-specific IgG (Fig 1B). Amounts of total and VP1-specific IgG in the serum prepared 1 week after each immunization (1st, white; 2nd, grey; 3rd, black) were measured using ELISA. Briefly, for the measurement of total and VP-1-specific IgG in the serum, plates were coated with goat anti-mouse IgG antibodies (Abs) and VP-1 antigen used for immunization of mice, respectively. The protein-coated plates were then treated with a diluted serum followed by treatment with goat anti-mouse IgG Abs labeled with hose-radish peroxidase, which was used for colorimetric measurement. *T*-tests were performed to determine the statistical significance between the value of CpG plus MPL group and that of other group. * indicates $p < 0.05$. (§: Note that amounts of VP1-specific IgG were denoted as optical density values directly from ELISA.)

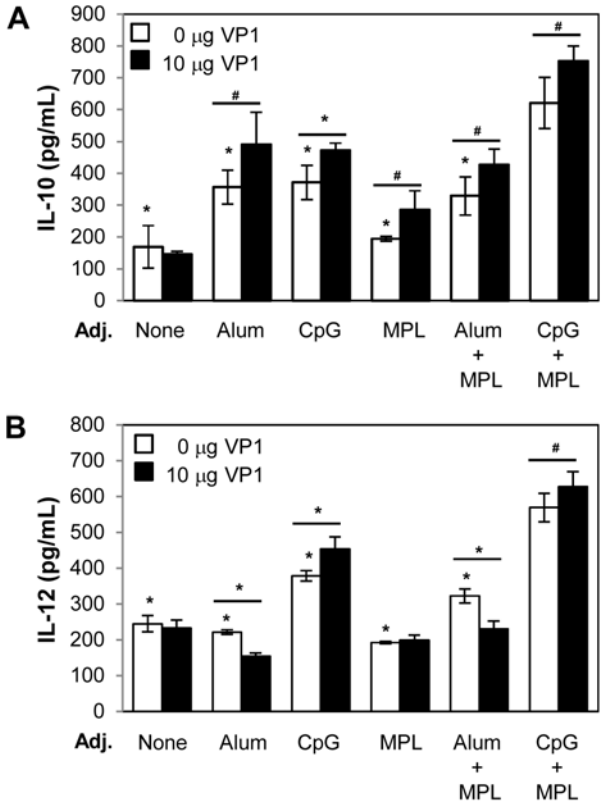

Fig. 3 Effects of adjuvants on the production of IL-10 and IL-12 by splenocytes ex vivo. Splenocytes, prepared from a group of mice 1week after the final immunization, were combined together and cultured in the presence or absence of VP1 protein for 3 days. IL-10 and IL-12 in the supernatants were measured using ELISA kits (BD Bioscience, USA). *T*-tests were performed to determine the statistical significance between the value (open bars) of CpG plus MPL group and that of other group (open bar). The values obtained when splenocytes were cultured in the presence or absence of VP1 antigen were also compared. * and # indicate $p < 0.05$ and $0.05 < p < 0.1$, respectively.

showed positive effects to varying degrees; note that CpG plus MPL showed a particularly strong effect compared with others. The splenocytes produced IL-10 even when they were cultured in the absence of VP1, suggesting that IL-10 in the culture supernatants was produced independently of antigen stimulation and at least in part by non-T cells, e.g., macrophages, monocytes and/or dendritic cells (Ogawa et al., 2008). However, the addition of VP-1 to the culture significantly increased the level of IL-10 production, indicating that VP1-specific T cells had been programmed to produce IL-10 during immune activation following the immunization.

Either alum or MPL had little effect on the production of IL-12, whereas CpG, alum plus MPL, and CpG plus MPL showed positive effects; note that as in the IL-10 production, CpG plus MPL showed the strongest effect on the IL-12 production (Fig. 3B). In addition, effects of supplementation of VP1 varied; thus, splenocytes from mice immunized with either CpG or CpG plus MPL increased the level of IL-12 production when cultured with the protein, whereas splenocytes from mice immunized with alum and alum plus MPL decreased the level of IL-12 production. Taken together, alum and alum plus MPL favored the production of Th2-type cytokines, whereas CpG and CpG plu MPL favored the production of both Th1- and Th2-type cytokines.

It must be ideal if an adjuvant promotes the production of antibodies only specific to the vaccinated antigen without changing the levels of other antibodies. It is also desirable if it promotes the production of cytokines, not biased to either Th1- or Th2-type and therefore is able to induce not only humoral but also cellular immune responses (MacLeod et al., 2011). Taking into consideration the production of VP1-specific IgG relative to that of total IgG and the pattern of Th1- and Th2-type cytokine productions, we conclude that a mixed adjuvant of CpG plus MPL has a unique and preferable effect as an adjuvant of hep A vaccine using recombinant VP1 protein. We anticipate that this study may

lead to development of a new vaccine for the disease caused by a food (water)-borne virus increasingly problematic not only in developing but also in developed countries.

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