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SHORT COMMUNICATION

Characterization of Toll-like Receptor 7/8 Agonist Amino Acid for Antigen-Adjuvant Conjugation Co-delivery System

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Abstract

Purified or synthetic antigens including peptides require adjuvant molecules to improve vaccine immunogenicity. Toll-like receptors (TLRs) agonists are powerful vaccine adjuvant candidates. A wide variety of TLR agonists have been studied for the development of antigen-adjuvant co-delivery systems. The covalent conjugation between antigen and TLR agonist is one of promising strategies to deliver to target cells. Recently we have reported the development of novel TLR7/8 agonist amino acids. In this paper, we will describe the contribution of antigen–adjuvant conjugation using a TLR7/8 agonist amino acid to antigen-specific immune responses regarding IgG isotype distribution and cytokine production.

Keywords: Adjuvant; Co-delivery System; Peptide Vaccine; Toll-like Receptors

Introduction

Vaccine is widely considered as the most cost-effective medicine for prophylactic protection against infectious diseases, such as tuberculosis, polio, mumps, measles, and yellow fever. Vaccines are classified into different groups according to their origin or components: live-attenuated vaccines, inactivated/killed vaccines, or subunit vaccines. In comparison with live-attenuated or inactivated vaccines, peptide-based subunit vaccines can potentially induce antigen-specific immune responses and reduce the risk of adverse effects associated with microbial components. Antigenicity of peptide administered alone, however, is not strong enough to stimulate immune system appropriately and co-administration of adjuvants is necessary. Antigen and adjuvant are required to be administered at the same time and by the same route. Therefore, many research groups have been exerting to develop vaccine delivery systems [1-4]. One of the promising strategies is the co-delivery system of antigen and adjuvant to the target cells through the covalent conjugation between them, especially using Toll-like receptor (TLR) agonists as adjuvant molecules [5]. Up to now, 10 and 12 TLRs have been discovered in humans and mice, and are expressed in various immune cells including dendritic cells and macrophages [6]. Interaction between TLRs and their ligands activates and regulates the innate and adaptive immunity [7, 8]. Currently, TLR4 agonist 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL®) is approved for human use (human papilloma virus vaccine, Cervarix®, GSK and Hepatitis B vaccine, Fendrix®, GSK). Various TLR ligands have been studied for vaccine adjuvant developments, including the antigen and adjuvant co-delivery systems [9-12].

TLR7 and TLR8 recognize microbial ssRNAs as natural ligands and also synthetic compounds with small molecular weight such as guanosine analogues (Loxoribine) [13], and imidazoquinoline analogues (imiquimod and R848) [14]. TLR7 and TLR8 agonists induce Th1-type cytokine productions such as IFNs [15]. Through the structure-activity relationship study based on a TLR7 agonist imiquimod (Aldara®), we have recently developed a TLR7/8 agonist amino acid, 6-(4-amino-2-butyl-imidazoquinolyl)-norleucine **1** [16], as shown in Figure 1. As its structural features, the amino acid **1** has a TLR7/8 agonist pharmacophore on the side chain, and Boc- and Fmoc-protected analogues are capable to be used for solid phase peptide synthesis in a conventional manner. With regard to biological feature, **1** showed an adjuvant activity, in terms of antigen-specific antibody production when mixed or conjugated with peptide antigen. The balance of Th1/Th2 is influenced by vaccine components, and evaluated by determination of production of IgG isotypes, cytokines or chemokines [17, 18]. To estimate a detailed adjuvant effect of amino acid **1** and usefulness for the antigen–adjuvant co-delivery system, we examined the contribution of antigen–adjuvant conjugation to IgG isotype distribution and cytokine production in the present study (Figure 1).

Results and Discussion

In this study, M2e peptide (SLLTEVETPIRNEWG-CRCNDSSD), the ectodomain of influenza A virus matrix

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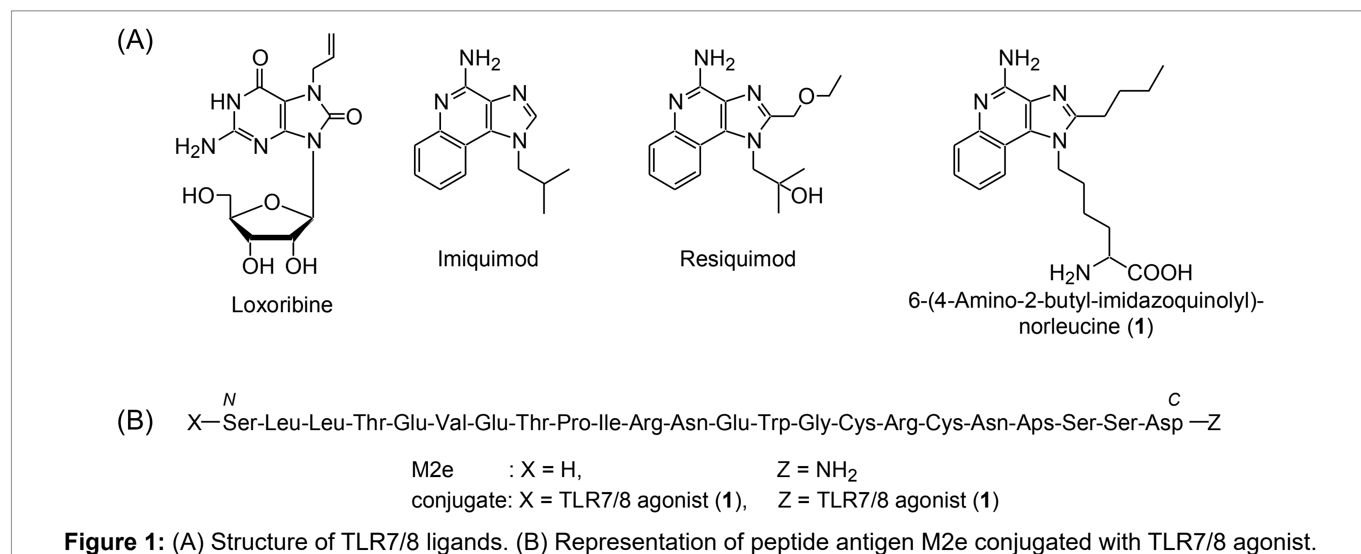

Figure 1: (A) Structure of TLR7/8 ligands. (B) Representation of peptide antigen M2e conjugated with TLR7/8 agonist.

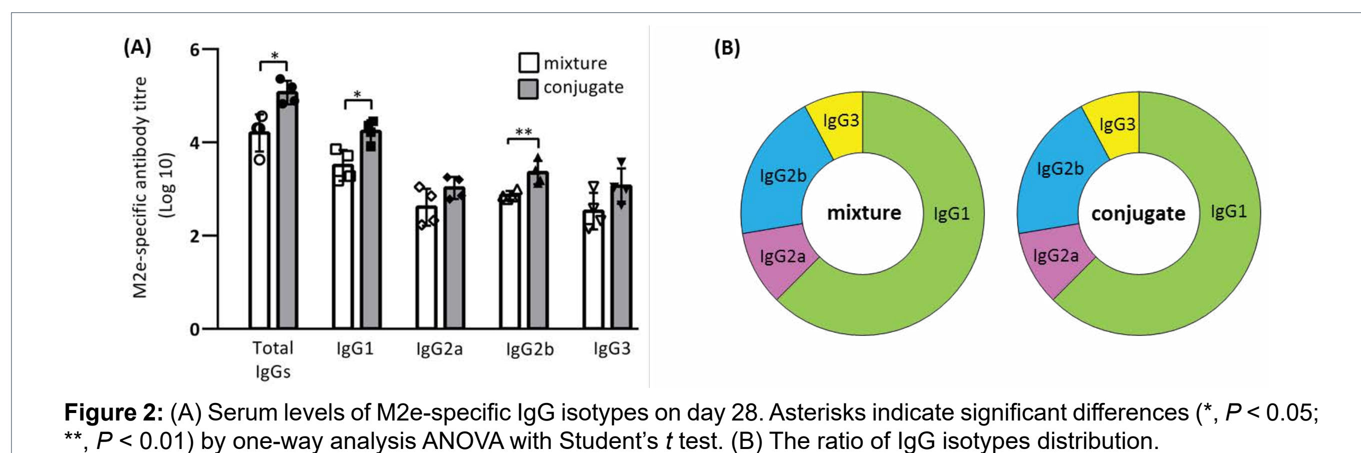

Figure 2: (A) Serum levels of M2e-specific IgG isotypes on day 28. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) by one-way analysis ANOVA with Student's *t* test. (B) The ratio of IgG isotypes distribution.

protein 2 (M2) [19, 20], was used as a model antigen. Two peptide, M2e and M2e conjugated with TLR7/8 agonist amino acid **1** at its both *N*- and *C*-termini, were prepared by Fmoc-solid phase peptide synthesis as previously reported [16], with a minor modification. To avoid a side reaction during repetitive coupling steps, the aromatic amino group of amino acid **1** was protected by Trt group. Briefly, Fmoc-6-(4-amino-2-butyl-imidazoquinolyl)-norleucine and Trt-Cl were reacted in dioxane in the presence of trimethylamine [21].

Female BALB/c mice (n = 4/group) were subcutaneously injected with 50 µg of immunogens (M2e mixed with 10 equimolar of TLR7/8 agonist **1**, or conjugated with 2 equimolar of TLR7/8 agonist **1**) in sterile-filtered PBS (total volume of 50 µL) on days 0, 7, 14, and 21. To evaluate the ratio of IgG isotype distribution, the amount of Th2-associated isotype (IgG1) and Th1-associated isotypes (IgG2a, IgG2b, and IgG3) in the collected serum on day 28 were determined by ElISA (Figure 2 (A)). Each IgG isotype controls were purchased from Medical & Biological Laboratories Co., LTD and the control IgG and corresponding HRP-conjugated goat anti-mouse IgG isotype secondary antibodies were purchased from Abcam [22]. It was observed that the amounts of all IgG antibody isotype productions were increased by the covalent conjugation of TLR 7/8 agonist **1** to peptide antigen in comparison with the case of mixture. In regard with Th1/Th2 bias evaluation, the ratios of IgG isotypes distribution were shown in Figure 2 (B). The ratio of Th1- and Th2-associated isotype distribution was not significantly changed by the conjugation. The amounts of Th1-associated isotypes (IgG2a, IgG2b, and IgG3) were smaller than that of Th2-associated isotype (IgG1) in both cases (Figure 2).

To assess the antigen-specific cytokine production, splenocytes (1×10^6 cell/mL) from the immunized mice were treated with M2e (10 µg/mL). The levels of IFN-γ (Th1-type cytokine) and IL-4 (Th2-type cytokine) productions in cell culture supernatant after 24-hr and 48-hr antigen restimulations were determined using ELISA kits [23]. As shown in Figure 3, there was no detectable or low level of cytokine productions in the case of immunization with the mixture (M2e and 10 equimolar of TLR7/8 agonist amino acid **1**). Meanwhile, the conjugation of peptide antigen and TLR7/8 agonist **1** remarkably increased IFN-γ and IL-4 productions. The splenocytes from immunized mice with the conjugate vaccine could induce a sufficient immune response to peptide antigen alone. Moreover, a production of IFN-γ, but not IL-4, was amplified by prolongation of restimulation time (48 hr). TLR7 and 8 agonists are generally characterized as a Th1-type adjuvant [14, 15]. It was observed that prolonged antigen restimulation

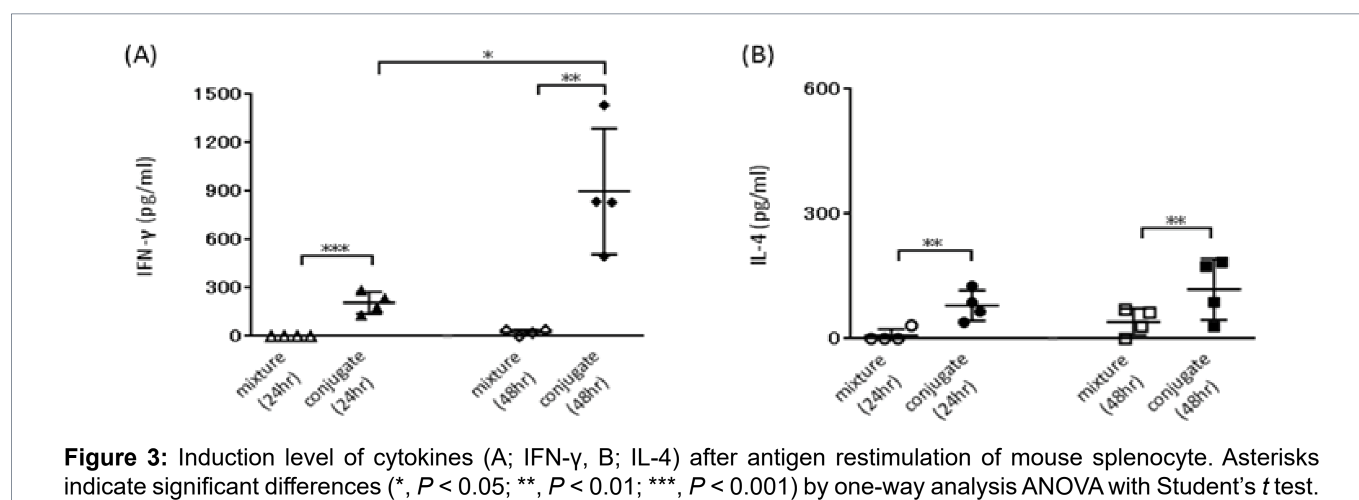

Figure 3: Induction level of cytokines (A; IFN-γ, B; IL-4) after antigen restimulation of mouse splenocyte. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) by one-way analysis ANOVA with Student's *t* test.

showed induction of a Th1-type cytokine response with high levels of IFN-γ and low levels of IL-4 (Figure 3).

In summary, we evaluated the antigen–adjuvant co-delivery system using TLR7/8 agonist amino acid **1**. The covalent conjugation induced a quantitative change of antigen-specific responses. This system, in which antigen and adjuvant efficiently activate immune responses by targeting the same immune cells, can reduce the amount of adjuvant dose and would contribute to the development of future vaccine delivery systems with less risk of side effects. As the design of adjuvants composed of different ligands in single molecules (TLR2–TLR9 ligands heterodimer [24], TLR4–TLR7–TLR9 ligands heterotrimer [25], and TLR2–TLR7 ligands heterodimer [26, 27] as well as TLR2–NOD2 (nucleotide-binding oligomerization domain-containing protein 2) ligands heterodimer [28] were reported recently, similar attempts using multiple different adjuvant molecules would be applied to the antigen–adjuvant conjugation. Therefore, searching optimized or suitable combinations from a wide variety of different adjuvant molecules including TLR7/8 agonist amino acid **1** to conjugate will be beneficial researches for the development of the next generation of antigen-adjuvant co-delivery system.

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21. R_f = 0.50 (CH_3Cl/MeOH/AcOH = 8:3:1), ESI-MS: 834.3941 g/mol, $[M+H]^+$ m/z 834.3907 (calcd., 834.4019), [M+Na]+ 856.3695 (calcd.,856.3839). ^{1}H-NMR (400 MHz; C*D*Cl_3): 0.83 (3H, t, *J* = 7.2 Hz), 1.22-1.96 (10H, m), 2.68-2.90 (2H, br), 4.17-4.41 (6H, m) 5.77 (1H, br), 7.12-7.82 (28H, m); ^{13}C-NMR (100 MHz;, $CDCl_3$): 14.7, 23.4, 27.6, 30.7, 33.0, 46.7, 48.2, 55.2, 67.6, 120.6, 120.1, 126.1, 127.7, 128.0, 128.6, 130.2, 142.2, 144.8, 145.0, 146.1, 148.7, 154.5, 156.8.

22. The following control antibodies were used: IgG (ab37355), IgG1 (M075-3), IgG2a (M076-3), IgG2b (M077-3) and IgG3 (M078-3). The following secondary antibodies were used: anti-IgG (ab97265), anti-IgG1 (ab97240), anti-IgG2a (ab97245), anti-IgG2b (ab97250) and anti-IgG3 (ab97260).

23. Mouse IL-4 Quantikine® ELISA Kit, and mouse IFN-*γ* Quantikine® ELISA Kit (R & D Systems, USA) were used.

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