NOTE *Immunology*

Starfish, *Asterias amurensis* and *Asterina pectinifera*, as Potential Sources of Th1 Immunity-Stimulating Adjuvants

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ABSTRACT. Saponin is the generic name of steroid or triterpene glycosides, and the capacities of some saponins to stimulate both Th1 immune response and production of cytotoxic T cells are useful as vaccine components against intracellular pathogens. Because saponins have been found commonly in starfish, we assessed the potential of starfish, *Asterias amurensis* and *Asterina pectinifera*, as adjuvant sources. Crude starfish saponins had hemolytic activities (EC_{50}=10 to 100 μg/m*l*) and thin layer chromatography indicated heterogeneity of their constituents. When starfish saponis were subcutaneously injected into mice with ovalbumin (OVA), OVA-specific IgG, especially IgG2a instead of IgG1 was produced in mouse blood, suggesting starfish saponins stimulated Th1 type immunity and they were potential sources of new adjuvants.

KEY WORDS: adjuvant, saponin, starfish, Th1 immunity, vaccine.

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Immunological adjuvants, originally defined as substances used in combination with a specific antigen that produce more immunity than the antigen alone, are indispensable to design effective vaccines [9]. Because adjuvants significantly affect the nature of the immune responses (e.g., Th1, Th2, antibodies, and cytotoxic T cells), adjuvants are selectively applied to elicit the appropriate type of immune response against each type of infection [4]. Although several hundred different adjuvants have been reported, the vast majority is not suitable for human use, and only aluminum-based mineral salts (alum) are the most widely used adjuvant in human vaccines [8]. MF59, consisting of emulsified squalene, is the only adjuvant licensed for human use besides alum. But, these two adjuvants were reported to favor only Th2 immune response [1, 14].

Saponins are natural glycosides of steroid or triterpene with diverse bioactivities, such as immunomodulatory, antitumor, antiinflammatory, molluscicidal, antiviral, antifungal, hypoglycemic, hypocholesterolemic [11]. Saponins are believed to be main constituents of many plant drugs and folk medicines. Especially, the capacity of saponins to activate the mammalian immunity led to significant interest in their potential as vaccine adjuvants. Quil-A and its derivatives QS-21 (*Quillaja* saponins) are derived from the bark of *Quillaja saponaria* Molina, a tree of the rose family. They have the unique capacity to stimulate both the Th1 immune response and the production of cytotoxic T cells against exogenous antigens, making them ideal for the vaccines against intracellular pathogens [12]. However, the application of *Quillaja* saponins is limited to human vaccination because of high toxicity and unstability in aqueous phase [6, 15].

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In the animal kingdom, saponins have been found exclusively in phylum Echinodermata and particularly in species of the classes Holothuroidea (sea cucumbers) and Asteroidea (starfish) [10]. Complex mixtures of saponins exist predominantly in the secondary metabolites of starfish [3]. Thus, starfish should be a potential source of various saponins. In this research, we evaluated adjuvant effect of crude saponins from two starfish species, *Asterias amurensis* and *Asterina pectinifera*.

These starfish were collected at Akkeshi, Hokkaido and Minamisanriku-cho, Miyagi Japan, and crude saponins were prepared by a classic method with minor modifications [7]. They were cut into small pieces (≈1.0 cm^3) and extracted with equal volume of ethanol overnight at 37°C. After filtration, ethanol was removed using rotary evaporator. The resulting aqueous solution was three times defatted with half volume of diethylether, followed by three time extraction with equal volume of n-butanol. The combined n-butanol layers were dried under reduced pressure and we call them crude saponins.

First, compositions of crude saponins were analyzed by thin layer chromatography (TLC), in which samples were developed on a commercial silica gel 60 plate (Merck, Darmstadt, Germany) with chloroform: methanol: H_2O (60:40:10, v/v/v) and detected with H_2SO_4: ethanol (1:1), heating at 100°C. TLC analysis clearly showed the heterogeneity of each preparation and commercial Quil-A, and the difference between these mixtures (Fig. 1a). Components of crude starfish saponins were more hydrophobic (Rf of *A. amurensis* >0.34, Rf of *A. pectinifera* >0.29) than those of commercial Quil-A (Rf=0.20–0.35). It is known that Quil-A is a heterogenous mixture of saponins and even QS-21, which is a relatively hydrophobic in *Quillaja* saponins, carries many sugars [12]. The number of saccharides might cause the difference in hydrophobicity between *Quillaja* saponins and starfish saponins.

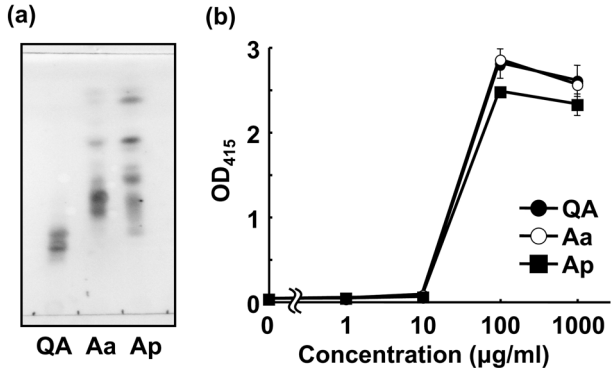

Fig. 1. Hemolytic activities and heterogeneity of starfish saponins. (a) Quil-A (QA), crude saponins from *A. amurensis* (Aa) and *A. pectinifera* (Ap) were developed on TLC plate with chloroform: methanol: H_2O (60:40:10, v/v/v) and detected with H_2SO_4: ethanol (1:1), heating at 100°C. (b) Quil-A (QA) and crude saponins (Aa and Ap) were respectively added into diluted dog blood at indicated concentrations, and hemolytic activities were evaluated after 30 min incubation at 37°C. OD_{415} values of supernatants represented the amount of hemoglobin, released by hemolysis.

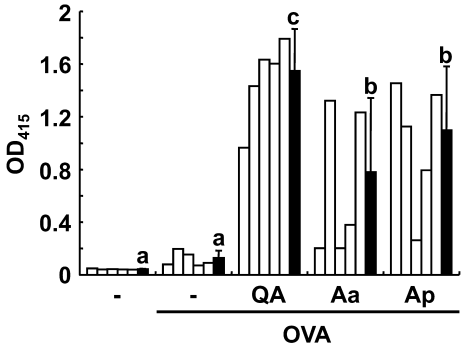

Fig. 2. Adjuvant effect of starfish saponins. Mice were immunized with 100 μl PBS containing 25 μg OVA alone, 25 μg OVA + 50 μg Quil-A (QA), 25 μg OVA + 1 mg *A. amurensis* saponins (Aa), or 25 μg OVA + 1 mg *A. pectinifera* saponins (Ap). OVA-specific IgG was detected by ELISA. White bars represented the OD_{415} value of individual mouse and the data of mouse no. 1 to 5 were arranged from left side in each group, while black bars represented the average ± SD of each group. According to Scott-Knott cluster analysis method, these groups were significantly discriminated as "a", "b", and "c".

Next, hemolytic activity is one of the common properties of saponins and maybe related to toxicity of saponins [11]. To characterize our preparations, crude saponins from two starfish species and commercial Quil-A were tested for hemolytic assay, following a reported method with minor modifications [13]. They were dissolved in phosphate buffered saline (PBS), and added into beagle blood, which was 50-fold diluted with PBS. After 1 hr incubation at 37°C, supernatants of each mixture were collected by centrifugation (100 × *g*, 10 min) and light absorbances at 415 nm (OD_{415}) were measured to detect the amount of hemoglobin, released by hemolysis. Similar hemolytic activities were detected in saponins from two starfish species and Quil-A, and the EC_{50} values were 10 to 100 μg/m*l*, suggesting starfish saponins were as toxic as Quil-A (Fig. 1b). Actually, skin necrosis occurred in the mice, into which starfish saponins were subcutaneously injected (data not shown). Further separation of individual saponins may yield a great variety of pure saponins, and give lower toxic, but active, compounds, because it has reported that the hemolytic and adjuvant activities of saponins are related to their chemical structures. Although both QS-18 and QS-21 were the saponin components of Quil-A and were equally active, the former was much more lethal in mice [2]. The structure modification of *Quillaja* saponins resulted in the altereation of immunomodulating and toxicological properties [5, 6].

Furthermore, the adjuvant activities of crude saponins from *A. amurensis* and *A. pectinifera* were tested by an animal experiment using mice. The use of these animals and the procedures performed on them were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine (ID No.: 21–18). On days 1 and 14, ICR mice (six week old, female, CLEA Japan, Tokyo, Japan) were subcutaneously immunized with 100 μl PBS containing 25 μg ovalbumin (OVA, Sigma, St. Louis, MO, U.S.A.) alone, 25 μg OVA + 50 μg Quil-A (Sigma), or 25 μg OVA + 1 mg each crude saponins. On day 27, mouse sera were collected and enzyme-linked immunosorbent assay (ELISA) was performed to detect OVA-specific IgG in the sera, as described before [16]. It revealed that IgG production was facilitated by the crude saponins, although the degree of response varied in individual mice (Fig. 2). Compared with Quil-A, which was effective equally for all five mice, the effects of starfish saponins were unstable, suggesting the activities were weaker than Quil-A. Furthermore, the irregular severeness of skin necrosis, which was often affected by mouse scratching or biting itself, probably caused the variation of each mouse condition. Although it was not significantly different, the adjuvant activity of *A. pectinifera* seemed to be higher than that of *A. amurensis*, implying that saponin variation between these species affected the difference of activities. Starfish saponins were still crude and further purification of individual saponins should make the relative activities higher and reveal the correlation of structure and activity.

Finally, to analyze the Th1/Th2 balance, elicited by the immunization, amount of two IgG isotypes in the sera, such as IgG1 and IgG2a, were measured by ELISA (Fig. 3). Both IgG1 and IgG2a were exceptionally produced in a mouse treated with OVA alone (mouse no. 3) and a mouse treated with OVA + Quil-A (mouse no. 5). However, almost all the mice, treated with OVA + saponins, produced only IgG2a, while almost all the mice, treated with PBS and OVA respectively, produced neither IgG1 and IgG2a. In fact, several reports have shown that Th1 type immunity was pre-

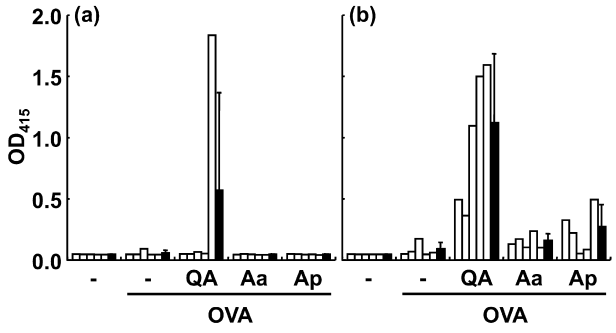

Fig. 3. Stimulation of Th1 antibody production by starfish saponins. Mice were immunized with 100 μl PBS containing 25 μg OVA alone, 25 μg OVA + 50 μg Quil-A (QA), 25 μg OVA + 1 mg *A. amurensis* saponins (Aa), or 25 μg OVA + 1 mg *A. pectinifera* saponins (Ap). OVA-specific IgG1 (a) and IgG2a (b) were detected by ELISA. White bars represented the OD_{415} value of individual mouse and the data of mouse no. 1 to 5 were arranged from left side in each group, while black bars represented the average ± SD of each group.

dominantly or partially stimulated by plant saponins, e.g., *Quillaja* saponins, ginseng saponins, *Panax notoginseng* saponins, *Platycodon grandiflorum* saponins, and *Polygala* saponins [12]. These reports supported our idea that Th1 immune response was induced by starfish saponins.

Taking all results into consideration, we suggested that starfish saponins were important candidates for Th1 immunity-stimulative adjuvants. This study is an important step leading to the development of novel vaccine adjuvants and effective utilization of marine resources.

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