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

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Citation
The Journal of Veterinary Medical Science, 73(2): 227-229

Issue Date
2011

URL
http://ir.obihiro.ac.jp/dspace/handle/10322/3587

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Japanese Society of Veterinary Science
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 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 (EC50=10 to 100 µg/ml) and thin layer chromatography indicated heterogeneity of their constituents. When starfish saponins 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.

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 cm3) 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: H2O (60:40:10, v/v/v) and detected with H2SO4: 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.
and the EC50 values were 10 to 100 detected in saponins from two starfish species and Quil-A, released by hemolysis. Similar hemolytic activities were detected in starfish species and commercial Quil-A were tested for hemolytic assay, following a reported method with minor modification [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 (OD415) were measured to detect the amount of hemoglobin, released by hemolysis.

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 (OD415) 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 EC50 values were 10 to 100 μg/ml, 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 sapogenins of Quillaja, implying that saponin variation resulted in the alteration 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-
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.

ACKNOWLEDGMENTS. We greatly thank staff of Shizugawa Nature Center and Akkeshi Marine Station, Aquatic Research Station, Field Science Center for Northern Biosphere, Hokkaido University for their helping us to collect starfish. This work was partly supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES