NOTE *Clinical Pathology*

Immunoadjuvant Activity of Crude Lectin Extracted from *Momordica Charantia* Seed

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ABSTRACT. The aim of this study is to investigate the immunoadjuvant activity of the crude *Momordica charantia* lectin (crMCL) extracted from seed using β-galactosidase (β-gal) as the model antigen. BALB/c mice were injected intramuscularly with β-gal alone or β-gal + crMCL for up to four immunizations at two-week intervals. After administration of 2 doses, the IgG-specific titer to β-gal was significantly higher in mice in the β-gal + crMCL group than in that from the animals from the β-gal alone group, while it was about the same in both groups after 1 dose. Our data suggest that crMCL may help raise antibodies under the prime and boost administration regimen and could be a potent vaccine adjuvant.

KEY WORDS: lectin, *Momordica charantia*, vaccine adjuvant.

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Vaccination is a cost-effective approach for controlling and preventing infectious diseases. Ideal vaccines should have impeccable safety records in all populations and elicit a high level of long-lived efficacy. In the practice of administering safer, inactivated vaccine, administration of antigen alone is not effective enough to achieve this goal. The use of effective vaccine adjuvants needed to enhance immune response to antigens has been investigated for the modern vaccine development [10].

Lectins are proteins or glycoproteins with carbohydrate binding specificity, which are found in both plants and animals and are involved in diverse biological functions, including immunomodulatory and immunoadjuvant properties, and some of these have been examined under clinical trial. Intranasal administration of mistletoe lectin I with ovalbumin (OVA) increased OVA-specific serum IgG and mucosal IgA [8]. Lectin isolated from Korean mistletoe (*Viscum album coloratum*) augmented keyhole limpet hemocyanine-specific IgG level [16]. However, there has been no study of the use of *Momordica charantia* seed lectin (MCL) as an immunoadjuvant.

Momordica charantia, a climber belonging to the family *Cucurbitaceae*, is commonly known as bitter gourd or bitter melon in English [4]. Fruit and seeds of bitter gourd are traditionally used as medicinal herbs and/or vegetables in Southeast Asian countries [14]. MCL, a galactose-specific glycoprotein present in the seeds of *Momordica charantia* with the $\alpha_2\beta_2$-type subunit architecture, exhibits strong type-I and weak type-2 ribosome inactivating protein activities as well as insulinomimetic activity [1, 2, 11, 12]. In this report, we aimed to elucidate the potency of the adjuvant activity of crude MCL (crMCL) on the enhancement of IgG immune response to co-administered model antigen β-galactosidase (β-gal) to provide a basis for the development of a more effective vaccine.

Extraction of crMCL was performed as described previously [15]. Briefly, *Momordica charantia* seeds were crushed into pieces, protein were subsequently extracted in phosphate-buffered saline (PBS, pH 7.4) and concentrated by ethanol precipitation, and the dried extract was re-suspended in PBS and used as crMCL. The protein concentration of crMCL was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and quantified by the Bradford protein assay kit [3] (Bio-Rad, Hercules, CA, U.S.A.), using lyophilized bovine plasma gamma globulin as a standard according to the manufacturer's instructions. The extracted crMCL showed hemagglutinating (HA) activity against red blood cells from BALB/c mice, and the HA titre corresponded with the amount of protein of crMCL. The HA titer was defined as the last dilution that showed complete HA activity, and 13 g/l of crMCL correlated to 2^{13} HA units.

Female BALB/c (*H-2d*) mice, 6–8 weeks old, from Charles River, Japan, were housed in an air-conditioned animal facility at the University of Miyazaki with a light/dark cycle of 14/10 hr and maintained on food and water *ad libitum*. Mice were always adapted for more than one week after arrival to our laboratory before use in experiments. This experiment was approved by the Animal Care and Use Committee, University of Miyazaki.

For inoculation of the mice and blood sample collection, BALB/c mice were injected intramuscularly in the right hind legs with β-gal (50 μg/mouse) (Roche Diagnostics, Indianapolis, IN, U.S.A.) with or without crMCL (130 μg/mouse = 10×2^{13} HA units) at weeks 0, 2, 4 and 6. Sera was

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collected by heart puncture under ether anesthesia two weeks after the last immunization and antibody titer was determined. Additionally, kidney, liver and spleen were collected from mice immunized with two doses for histopathological observation.

β-gal-specific antibody titers were determined by ELISA, as described previously [5]. Briefly, 96-well Nunc-immuno MaxiSorp assay plates were coated with 50 μl β-gal (5 μg/ml) in coating buffer (0.1 M Na_2HPO_4, pH 9.0) per well at 4°C overnight. After blocking the wells with 10% fetal calf serum in PBS, serial two-fold dilutions of sera were incubated for 2 hr at 37°C. Biotinylated γ-chain-specific goat anti-mouse IgG (Sigma-Aldrich, St.Louis, MO, U.S.A.), or, to determine antigen-specific IgG isotypes, mouse IgG1 antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), mouse IgG2a antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), mouse IgG2b antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), mouse IgG2c antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.) or mouse IgG3 antibody (polyclone, BETHYL, Montgomery, TX, U.S.A.), was added as secondary antibodies. Color reactions were developed with streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Buckinghamshire, U.K.) and ABTS Peroxidase Substrate Solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.). ABTS Peroxidase Stop Solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) was added to stop the color development and the OD value of each well was determined using a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Hercules, CA, U.S.A.) at 405 nm. Endpoint titers were expressed as the reciprocal log2 of the last dilution which produced an optical density at 405 nm of 0.1 unit above the values of the negative control, which was prepared from animals injected with PBS.

To determine the immunoadjuvant effect of crMCL, antigen-specific antibodies were examined in sera of the mice immunized with β-gal alone or β-gal + crMCL. After a single injection, β-gal-specific IgG antibody was detected in all immunized mice (Table 1) while no antibody titer for β-gal was detected in animals injected with crMCL only (data not shown). With two doses, anti-β-gal IgG antibody titer in the β-gal + crMCL group was significantly higher than that in the β-gal alone group ($P<0.05$). However, production of anti-β-gal IgG reached a plateau after the third inoculation, which was about the same after the fourth immunization. These results suggest that crMCL could significantly improve antigen-specific total IgG antibody response in the regimen of priming with the booster vaccination (2 doses).

To obtain preliminary insights into the nature of the immune responses after 2 doses, the IgG subclass was analyzed by ELISA. Significantly larger amounts of IgG1, IgG2b, IgG3 ($P<0.01$) and IgG2c ($P<0.05$) were seen in β-gal + crMCL-injected mice, compared with β-gal-injected mice, however, the IgG2a antibody level did not reach the detection limit (<3 log2) (Fig. 1). IgG1 was the predominant IgG subclass, followed by IgG2b in both β-gal alone and β-gal + crMCL-injected mice.

Table 1. β-gal-specific total IgG response (log_2) in sera from immunized mice

Immunization		β-gal alone	β-gal + crMCL
Priming	(1 dose)	12.5 ± 1.29	13.3 ± 0.96
Priming + boost	(2 doses)	14.5 ± 1.29	16.5 ± 0.58*
Priming + 2 boosts	(3 doses)	17.0 ± 0.00	17.0 ± 1.41
Priming + 3 boosts	(4 doses)	17.5 ± 0.58	17.3 ± 0.50

* Indicates a significant difference observed in the β-gal + crMCL group after two doses, compared with the β-gal group ($P<0.05$) by Student's *t*-test. Data are represented as mean ± S.D. (n=4).

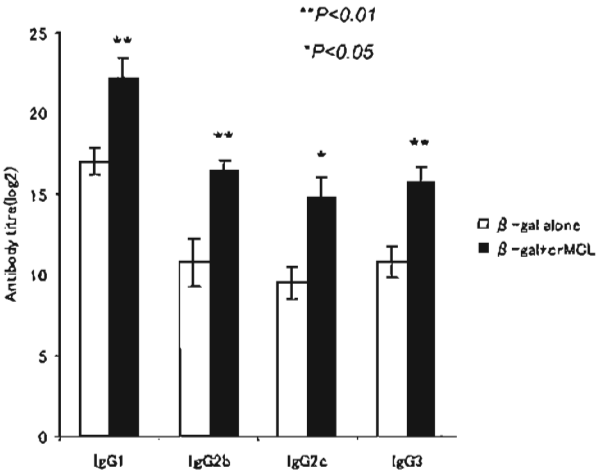

Fig. 1. Determination of IgG subclass response to β-gal in sera from mice immunized i.m. with two doses of β-gal (50 μg/mouse) alone or β-gal (50 μg/mouse) together with crMCL (130 μg/mouse). Sera were collected at two weeks after the second immunization for IgG subclasses by ELISA. After two doses, a significant difference was observed between the β-gal + crMCL group compared to β-gal alone by Student's *t*-test for each of the β-gal-specific IgG subclasses: IgG1, IgG2b, IgG3 ($P<0.01$) and IgG2c ($P<0.05$). IgG2a level did not reach the detection limit (<3 log_2) in any of the groups. The predominant IgG subclass was IgG1, followed by IgG2b. Data are represented as mean ± S.D. from four mice.

To examine the pathologic effects of crMCL, organs including the liver, spleen and kidney were examined histologically at 2 weeks after the second inoculation. No significant histopathological lesions were observed in any of the groups (data not shown). These findings indicate that crMCL has no obvious side effects on the organs of immunized mice for the doses of crMCL used in this study.

Vaccination is the first line of defense to reduce morbidity and mortality during a pandemic, such as influenza. However, commercial vaccines would be unsuitable in the current situation. To enhance the immunogenicity of a vaccine, one of strategies is to use an adjuvant with dose-sparing potential [13]. The data obtained in this study indicated that crMCL is a potent immunoadjuvant when admixed with model antigen β-gal via intramuscular administration. In this study, we used 50 μg of β-gal as a model antigen based on previous experiments [5]. Even with administration of

antigen alone, 3 doses were thought to be sufficient to achieve a maximum antigen-specific IgG level. Further investigation is required to determine whether the adjuvant activity of crMCL is more effective for lower amounts of antigen.

Serum IgG level to β-gal was significantly different in sera collected from mice following two immunizations; moreover, antigen-specific IgG subclass antibody response was effectively enhanced, as for IgG1 and IgG2b. This suggests that crMCL could actively induce a strong humoral immune response when followed by a booster immunization. This would be advantageous for the development of effective vaccination programs, as an unprimed population would likely require at least two doses to induce immunity [13]. Furthermore, Hehme *et al.* [6] reported that a two-dose regimen and an adjuvant system were required for a pandemic vaccine to elicit a satisfactory immune response. Therefore, this crMCL would be a promising adjuvant for vaccinations.

Here, we focused on antigen-specific IgG production enhanced by crMCL because we speculate that its effects are mainly on IgG. Induction of IgA may vary with lectin identity. Lavelle *et al.* [9] reported that several lectins poorly stimulated local specific IgA secretion as intranasal adjuvants because of insufficient binding to mucosa. Yoon *et al.* [16] indicated that Korean mistletoe lectin had no apparent effect on antigen-specific IgM production after subcutaneous injection. The effect of MCL on IgA and IgM induction remain to be determined.

The source of crMCL is seeds of bitter gourd, which are part of the normal diet. However, in studies on immune response by bitter gourd extract, Ike *et al.* [7] found that mice died after the intraperitoneal inoculation of whole or pulp juices of bitter gourd. Different procedures for extraction and route of administration may be responsible for this discrepancy, and these alternatives need to be considered from a safety prospective. In the present study, during the period of four immunizations, all animals at least survived without signs of severe illness prior to the sampling time point. Furthermore, histopathological analysis showed that no significant lesions after two doses in either group on various organs, including the liver, spleen and kidney from animals. These results suggest that crMCL used in this study does not cause severe side effects, although further analysis is required to ensure its safety.

In conclusion, our results show that antigen-specific IgG antibody is significantly enhanced by crMCL via intramuscular administration with the prime and boost regimen. This suggests that crMCL may be promising for use as a vaccine adjuvant. As lectin tested in this study was in the crude extract form, further study is needed to focus on purified MCL and to determine the specific effects, as well as its mechanisms, to stimulate immune responses.

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