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Evaluation of ocean biomass products that activate cell-mediated immunity in mice cutaneously infected with herpes simplex virus type 1

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Abstract

We screened and evaluated the activation of cell-mediated immunity by ocean biomass products using a cutaneous herpes simplex virus type 1 (HSV-1) infection model. In this model, a delayed-type hypersensitivity (DTH) associated with cell-mediated immunity is an important immune defense systems. An aqueous fraction of ovary of *Seriola quinqueradiata* (A-SQ) or a lipophilic fraction of head and internal organs of *Hymenocephalus lethoemus* (L-HL) was administered orally to mice infected with HSV-1 three times daily during 8 days (days 0 to 7) after infection. They delayed the progress of herpetic skin lesions without toxicity and significantly increased the DTH reaction to inactivated HSV-1 antigen on the footpads of infected mice. Further, A-SQ and L-HL increased the production of interferon- γ from splenocytes of HSV-1-infected mice by the inactivated-HSV-1. Thus, the efficacies of A-SQ and L-HL against HSV-1 infection may be due to the augmentation of cell-mediated immunity through DTH reaction.

Keywords: Cell-mediated immunity; Herpes simplex virus; Ocean biomass; Delayed-type hypersensitivity; Immunomodulatory activity

1. Introduction

Activation of cell-mediated immunity has been shown to be valid in preventing viral infection [1-5], alleviating allergic reactions [6, 7], and suppressing the growth of tumours [8, 9]. T helper type-1 (Th1) cells and Th1 cytokines, interferon (IFN)- γ and interleukin (IL)-12, play crucial roles in cell-mediated immunity. IFN- γ and IL-12 are important immune mediators to activate the Th1 immune response leading to cell-mediated immunity. The upregulation of Th1 immunity is a valuable management to alleviate and prevent infectious diseases and maintaining quality of life (QOL).

A murine model of cutaneous herpes simplex virus type 1 (HSV-1) infection is valuable to evaluate the augmentation of cell-mediated immunity by a delayed-type hypersensitivity (DTH) reaction [4, 10-12]. DTH is an important immune defense system in intradermally HSV-1-infected mice. Using this murine model, we previously showed that Kakkon-to as a traditional herbal medicine, propolis as a dietary supplement, an aqueous extract of *Moltinga oleifera* Lam. (*M. oleifera*) as a health food, and 06CC2 strain of *Lactobacillus plantarum* as a probiotic alleviated herpetic symptoms in mice and activated DTH reaction as cell-mediated immunity associated with the increase of IFN- γ production from splenocytes [4, 5, 11, 12]. The HSV-1 infection model was verified to be able to assess the augmentation of cell-mediated immune responses through DTH reaction *in vivo* by health foods, supplements, and natural products.

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In this study, we prepared 58 fractions of ocean biomass products caught in Miyazaki Prefecture, Japan. Their activities augmenting cell-mediated immunity were assessed using a murine model of cutaneous HSV-1 infection. We found that the aqueous fraction of ovary of *Seriola quinqueradiata* (A-SQ) and the lipophilic fraction of head and internal organs of *Hymenocephalus lethocephalus* (L-HL) significantly augmented the DTH reaction associated with an increase of IFN- γ production from the splenocytes of mice infected by HSV-1.

2. Material and methods

2.1. Viruses and cells

HSV-1 7401H strain was grown in Vero E6 cell cultures and virus stock was prepared from the infected Vero cells as reported previously [13]. Splenocytes were prepared from spleens of the infected mice and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) [14].

2.2. Ocean biomass products

Most ocean biomass products were sea fishes caught in Miyazaki Prefecture, Japan. Parts of them, such as the head, skin, fin, internal organs, bone, or muscle, were freshly removed and the tissue was cut into small pieces. The pieces were lyophilized, and the lyophilized pieces were mixed with ethanol at four to five times of the weight of the lyophilisates and homogenized at 8,000 rpm for 5 min. The homogenized mixtures were filtered *in vacuo*, and the filtered ethanol extracts were evaporated *in vacuo*. The evaporated materials were used as lipophilic fractions. In addition, residues left after the filtration *in vacuo* were homogenized in distilled water at four to five times the weight of the residues at 8,000 rpm for 15 min. The homogenized mixtures were filtered *in vacuo*, and the filtered aqueous extracts were stored. The residues after filtration were again homogenized in distilled water and centrifuged at 3,000 rpm for 10 min. The filtered aqueous extracts were combined with the previously stored aqueous extracts and lyophilized. The lyophilized materials were used as aqueous fractions. We prepared 58 lipophilic and aqueous fractions deposited them at Department of Biochemistry, Graduate School of Clinical Pharmacy, Kyushu University of Health and Welfare, Nobeoka 882-8508, Japan. For *in vivo* assays, the aqueous and lipophilic fractions were dissolved and suspended, respectively, in distilled water and administered orally to mice.

2.3. Mice

Female BALB/c (6 weeks old, 17–20 g) mice were purchased from Kyudo Animal Laboratory, Kumamoto, Japan and housed in specific pathogen-free conditions at $23 \pm 2^\circ\text{C}$ under a 12 h light/12 h dark diurnal cycle (light at 7.00 a.m.). Food (conventional solid diet CRF-1, Oriental Yeast Co., Chiba, Japan) and water were given *ad libitum* to mice. After five days acclimation, experiments were started. The experimental protocols were approved by the Animal Experiment Committee of Kyushu University of Health and Welfare, Japan, and the animal experimentation guidelines of the university were followed in the animal studies.

2.4. Murine HSV-1 infection (1st screening)

The right midflank of each mouse was shaved and scarified with 27-gauge needles. The mice were cutaneously infected with 7401 HSV-1 strain (1×10^6 plaque-forming units (PFU)/mouse) as reported previously [13, 15]. The oral administration of aqueous or lipophilic fractions of ocean biomass products (260 to 880 mg/kg) was performed once at 3 h before and twice after virus infection on day 0. From day 1 to day 7 after infection, the administration was done 3 times daily [13, 15]. Previously we showed that an aqueous extract of *M. oleifera* leaves, as a health food, was effective at the dose of 300 mg/kg against murine HSV-1 infection [12]. In this study, we used the dose of 260 to 880 mg/kg for the fractions as a similar bioactive dose of health food in the extracts. Distilled water was used for a control. Mice (seven/group) were weighed daily and the net body weights were calculated as described previously [16]. The development of skin lesions and mortality were evaluated every 8 h daily and scored as reported previously [16]. To assess the toxicity of fractions, uninfected mice (four/group) were administered the fractions at 260 to 880 mg/kg following the same schedule used for infected mice and then weighed daily as described above.

2.5. Footpad swelling due to HSV-1 antigen in mice (2nd screening)

To evaluate the effect of fractions of ocean biomass products on DTH reaction, the skin reaction of mice infected with HSV-1 was assessed to ultraviolet light (UV)-inactivated HSV-1 antigen. The mice were intradermally infected at 1×10^6 PFU/mouse of 7401H strain using a microsyringe under anesthesia [4, 11]. The fractions were administered to the infected mice for 5 days (day 0 to day 4) following the schedule as described above. The infected mice were challenged by injection of UV-inactivated virus antigen (7×10^5 PFU/10 μL) into a footpad on day 4 after infection. The swelling of footpads was measured at 24, 36, and 48 h after the challenges described previously [4].

2.6. IFN- γ production from splenocytes (3rd screening)

IFN- γ production from splenocytes of HSV-1-infected mice administered the fractions of ocean biomass products were assessed. Mice were intradermally infected with HSV-1 as described above. Spleens were removed under anesthesia on day 4 after infection, and splenocytes were prepared after erythrocytolysis [5]. The splenocytes of three infected mice were combined and suspended in RPMI-1640 medium supplemented with 10% heat-inactivated FCS. The splenocytes were added into 96-well plates at 6×10^5 cells/well, in octuplicate. The splenocytes were cultured in the presence of 0, 0.01, 0.03, 0.1, 0.3, 1, 3, or 10 $\mu\text{g/mL}$ of aqueous or lipophilic fractions and in the presence or absence of UV-inactivated HSV-1 antigen at 2×10^5 PFU/well for 24 h at 37°C. Supernatant of each well was collected after centrifugation and stored at -80°C for ELISA of IFN- γ .

2.7. ELISA

IFN- γ levels in the splenocyte culture supernatants were determined using a specific ELISA kit (eBioscience Inc., San Diego, CA) according to the manufacturer's instructions. The detection limit of the kit was more than 15 pg/mL.

2.8. Statistical analysis

Statistical significances of differences in changes of IFN- γ levels, net increase in footpad thicknesses, and net body weights of infected mice were evaluated using Student's *t*-test. Interactions between mean scores of treated and untreated groups were analyzed using the repeated measures two-way ANOVA. A *p* value of less than 0.05 was considered to be significant statistically.

3. Results

3.1. Murine HSV-1 infection (1st screening)

We examined the anti-HSV-1 efficacy of 58 fractions prepared from ocean biomass products in a murine cutaneous HSV-1 infection model as the first screening for fractions that can activate cell-mediated immunity. Figures 1 and 2 show the representative results of active fractions (A-SQ and L-HL, respectively, as shown in Table 1). As shown in Figure 1, the oral administration of A-SQ significantly limited the development of skin lesions on days 3.6 to 4.3 after infection ($p < 0.05$ by repeated measures ANOVA). L-HL significantly delayed the development and all infected mice were dead within 14 days after infection (Figure 2). In both cases, there was no significant difference between the changes of net body weights of mock-infected mice administered water and each fraction on the 10 days after infection (data not shown). In the first screening, six fractions including A-SQ and L-HL among the 58 fractions were significantly effective in delaying herpetic skin lesions as shown in Table 1, although the six fractions did not exhibit significant direct anti-HSV-1 activity in a plaque reduction assay *in vitro* (data not shown). Thus, they were characterized as probably possessing an immunomodulatory activity contributing to the limitation of herpetic skin lesions. It was suggested that the six fractions are useful to prophylactically alleviate herpetic symptoms.

Table 1 Ocean biomass products with immunomodulatory activity

Ocean biomass product	Part	Fraction	Biological activity Screening		
			1 st	2 nd	3 rd
<i>Coelorinchus kamoharai</i> Matsubara, 1943 (CM)	Head, Shell, Internal organs	Aqueous (A-CM)	+	+	-
<i>Hoplostethus japonicus</i> Hilgendorf (HH)	Head, Internal organs	Aqueous (A-HH)	+	-	-
<i>Hymenocephalus lethoemus</i> (HL)	Head, Internal organs	Aqueous (A-HL)	+	+	-
		Lipophilic (L-HL)	+	+	+
<i>Seriola quinqueradiata</i> (SQ)	Ovary	Aqueous (A-SQ)	+	+	+
<i>Zenion japonicum</i> Kamohara (ZK)	Muscle etc.	Lipophilic (L-ZK)	+	+	-

+, active fraction; -, inactive fraction

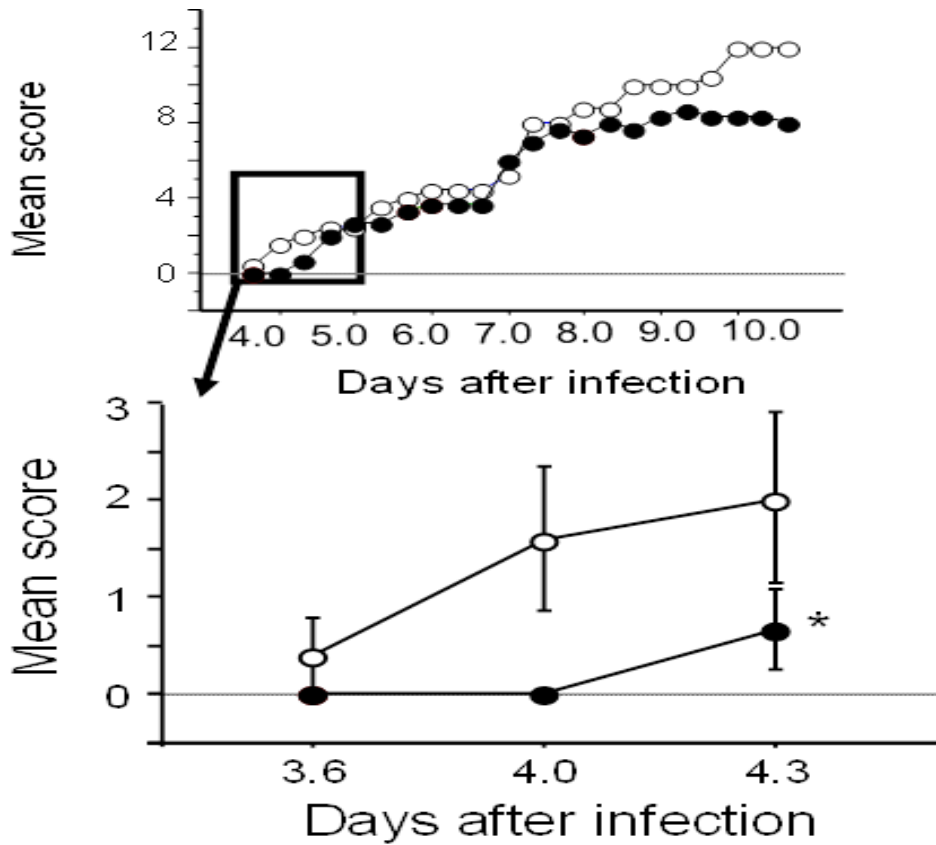


Figure 1 Effect of A-SQ on the development of herpetic skin lesions of mice infected cutaneously with HSV-1

Lower graph is an enlargement of the upper graph from 3.6 to 4.3 days. Seven mice in each group were infected with HSV-1 at 1×10^6 PFU/mouse and administered A-SQ (●) or distilled water as control (○) as described in text. * $p < 0.05$ versus control by repeated measures ANOVA. Bars indicate standard errors.

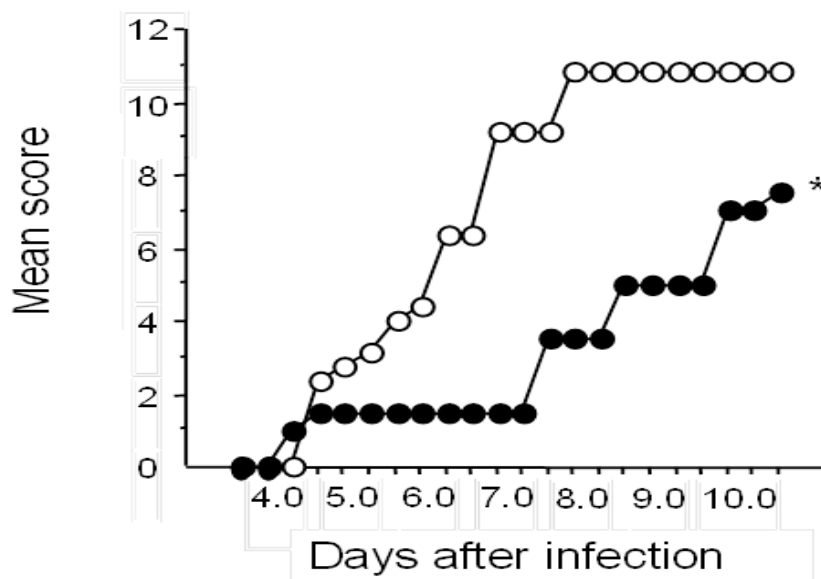


Figure 2 Effect of L-HL on the development of herpetic skin lesions of mice infected cutaneously with HSV-1

Seven mice in each group were infected with HSV-1 at 1×10^6 PFU/mouse and administered L-HL (●) or distilled water as control (○) as described in text.* $p < 0.05$ versus control by repeated measures ANOVA. Bars indicate standard errors.

3.2. Footpad swelling due to HSV-1 antigen in mice (2nd screening)

It has been reported that DTH skin reaction is a major defense system in the clearance of HSV-1 in mice infected intradermally with HSV-1 [4, 10, 11]. Effects of the six fractions selected in the first screening on the DTH reaction were examined in HSV-1-infected mice as the second screening. As shown in Figure 3, the oral administration of A-SQ or L-HL was significantly effective in increasing the thickness of footpads at 24, 36, and/or 48 hours after the inactivated HSV-1 antigen challenge. The DTH reaction has been reported to reach maximal levels between 24 and 48 hours after a challenge in intradermally HSV-1-infected mice [4, 11]. In our study, the maximum level of swelling due to A-SQ or L-HL was also observed at 24 to 48 hours after the challenge (Figure 3). The swelling times were consistent with previous reports. Thus, A-SQ and L-HL augmented the DTH reaction in HSV-1-infected mice and were suggested to have some components that act as immunomodulators for the DTH reaction in the HSV-1-infected mice. Among the six fractions, five containing A-SQ and L-HL significantly augmented the DTH reaction except the aqueous fraction of head and internal organs of *Hoplostethus japonicus* Hilgendorf (A-HH) as shown in Table 1 and were suggested to be immunomodulators contributing to the activation of cell-mediated immunity.

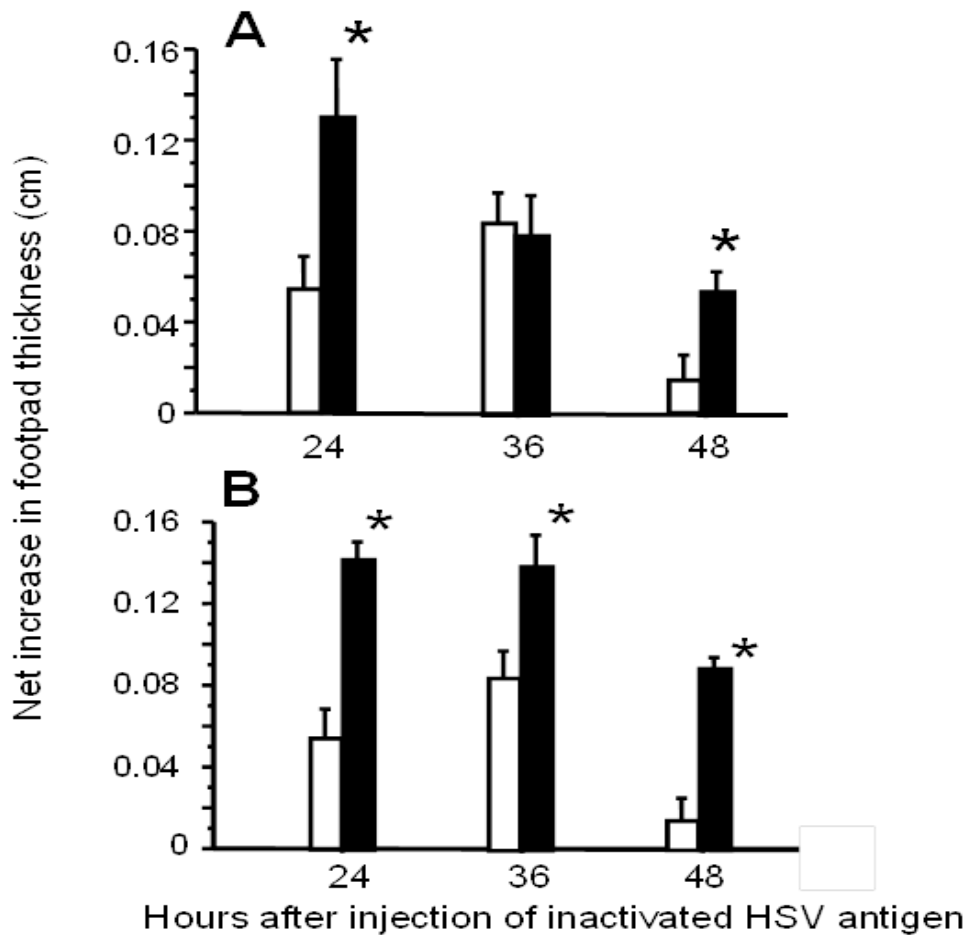


Figure 3 Time course of skin reactions to HSV-1 antigen in mice infected intradermally with HSV-1

Five HSV-1-infected mice were administered A-SQ (A) or L-HL (B) (closed columns) or distilled water (open columns), and the mean percent swelling was determined at 24, 36, and 48 h after injection of HSV antigen into footpads as described in text. * $p < 0.05$ versus control by Student's t -test. Bars indicate standard errors.

3.3. IFN- γ production from splenocytes (3rd screening)

IFN- γ is a critical cytokine for innate and adaptive immunity against viral infections. It is an important activator of macrophages and Th1 immunity. As a Th1 cytokine, IFN- γ has been reported to be a potent stimulator of lymphocyte

migration into skin and a major mediator of lymphocyte recruitment in DTH [17]. We prepared splenocytes from mice infected intradermally with HSV-1 and examined IFN- γ production from the splenocytes in the presence of various concentrations of the fractions with or without inactivated HSV-1 antigen *in vitro*. As shown in Figure 4, A-SQ (Figure 4A) and L-HL (Figure 4B) were significantly effective in augmenting IFN- γ production by inactivated HSV-1 antigen from splenocytes ($p < 0.05$ by Student's *t*-test). Both fractions exhibited the bell-shaped concentration dependency that is characteristic of a cytokine reaction. However, the other three fractions among the five failed to increase significantly the production of IFN- γ in the presence of the antigen (Table 1). Thus, A-SQ and L-HL were significantly effective in increasing IFN- γ production from splenocytes of HSV-1 infected mice.

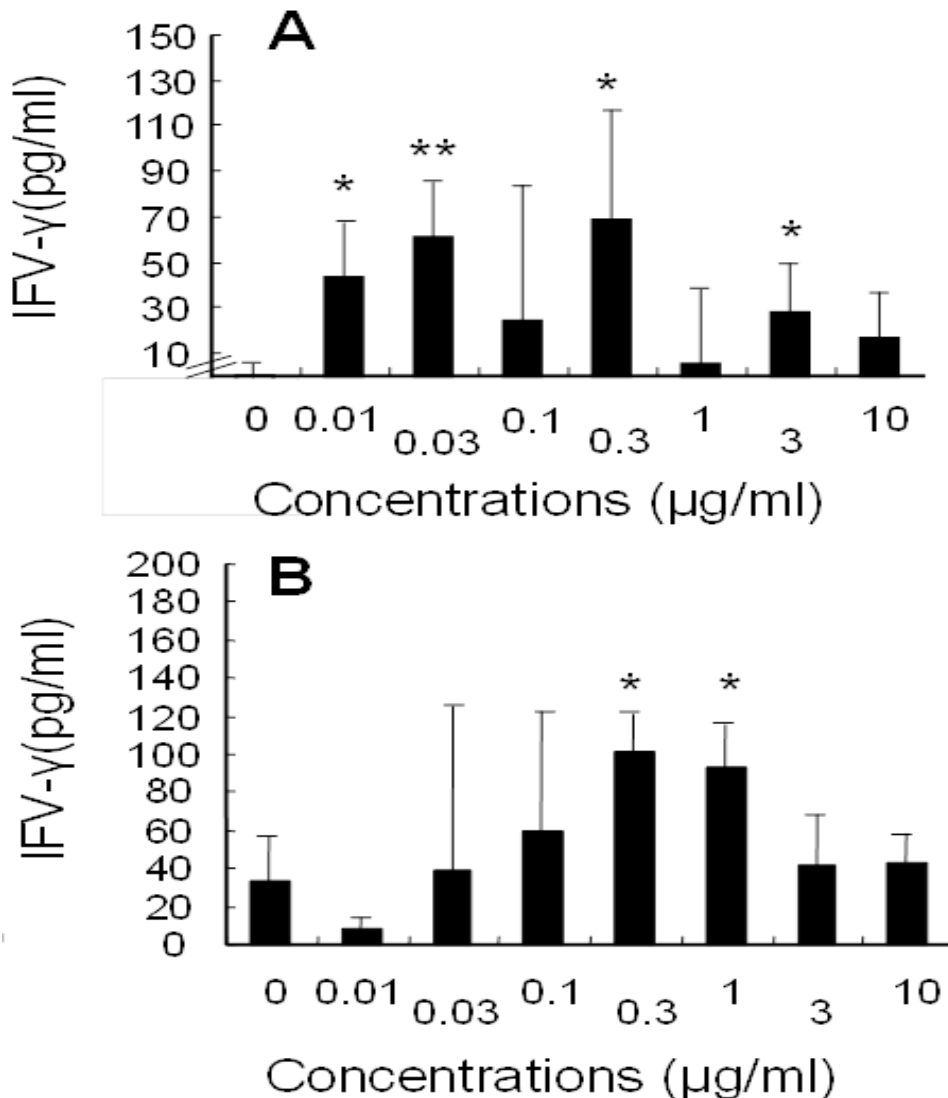


Figure 4 Effect of A-SQ or L-HL on IFN- γ production from splenocytes by inactivated HSV-1 antigen

Splenocytes were prepared from mice intradermally infected with HSV-1 on day 4 and incubated in the presence of various concentrations (0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 $\mu\text{g/mL}$) of A-SQ (A) or L-HL (B) and in the presence or absence of inactivated HSV-1 antigen for 24 h as described in text. At each concentration of A-SQ (A) or L-HL (B), IFN- γ levels in the absence of inactivated HSV-1 antigen were subtracted from that in the presence. Bars show mean \pm standard errors of the subtracted values ($n=8$). * $p < 0.05$ and ** $p < 0.01$ versus values at 0 $\mu\text{g/mL}$ by Student's *t*-test.

4. Discussion

We characterized the basis of the biological efficacy of A-SQ and L-HL in the alleviation of HSV symptoms in mice and found that oral administration of A-SQ and L-HL were effective in activating the DTH reaction based on cell-mediated immunity [4], which is a major host defense immunity against intradermal HSV infection, and in elevating IFN- γ

production from splenocytes of HSV-1-infected mice. In the cutaneous HSV-1 infection model in mice, we administered 260 to 880 mg/kg of A-SQ and L-HL orally to mice. The doses for mice correspond to the bioactive doses of health foods in humans as described in Materials and Methods. It suggested the possibility that their oral administrations could be helpful to maintain and improve QOL in humans.

A-SQ and L-HL were effective in limiting the development of HSV-1 skin lesions (Figures 1 and 2), but they had no direct anti-HSV-1 activity *in vitro* (data not shown). It is probable that A-SQ and L-HL possess immunomodulatory activities contributing to the limitation of development of HSV-1 skin lesions. HSV-1 causes epithelial lesions upon establishment of productive infection and spreads to the brain, resulting in potentially fatal herpes encephalitis [18]. In our study, L-HL was more effective in delaying the development of HSV-1 skin lesions than A-SQ (Figures 1 and 2). The larger delay due to L-HL may be due to a strong limitation of the productive HSV-1 infection in skin and spread to the brain based on the immunomodulatory activity stimulated by L-HL administration.

A-SQ and L-HL augmented footpad swelling in HSV-1-infected mice (Figure 3). This was probably due to augmentation of the DTH reaction. The DTH reaction is caused by activation of cell-mediated immunity through Th1 immune response [4, 12]. A-SQ and L-HL probably augmented the cell-mediated immunity based on Th1 immunity in the HSV-1-infected mice, resulting in a delay of herpetic symptoms. A-SQ and L-HL are foods and not anti-HSV-1 medicines. They may be helpful as an immunomodulatory adjuvant for the management of HSV-1 symptoms. Their daily intake may contribute to promotion and maintenance of health.

The production of IFN- γ was augmented by HSV-1 antigen in the splenocytes of HSV-1-infected mice administered A-SQ and L-HL (Figure 4). Previously we showed that Brazilian propolis AF-08, probiotic *Lactobacillus plantarum* strain 06CC2, and aqueous extracts of *M. oleifera* leaves augmented DTH reaction and alleviated the symptoms of herpetic skin infection in mice [4, 5, 12]. Also, they have been confirmed to augment IFN- γ production from splenocytes of HSV-1 infected mice in the presence of inactivated HSV-1 antigen [4, 5, 12]. However, in the case of *Lactobacillus plantarum* strain 06CC2, the gene expression of IL-12 was not augmented in Peyer's patches of HSV-1 infected mice, although the gene expression of IFN- γ was significantly enhanced [5]. IFN- γ and IL-12 as Th1 cytokines have been demonstrated to regulate Th1 development independently and it was shown that autocrine mechanism of IFN- γ largely contribute to the overall strength of T cell stimulation [19]. Thus, the augmentation of IFN- γ level is probably necessary for the establishment of Th1 immunity more than IL-12. The augmentation of IFN- γ correlated with augmentation of the DTH reaction and was suggested to protect against HSV-1 infection [14]. A-SQ and L-HL possibly have active compounds that act as biological response modifiers. The analysis of their components may be interesting.

5. Conclusion

We evaluated the mode of anti-herpetic action of A-SQ and L-HL using an HSV-1 infection model in mice. A-SQ and L-HL were demonstrated to have immunomodulatory activities against intradermal HSV-1 infection in mice. Especially, the immunological activity associated with IFN- γ production leading to Th1 immunity in mice may contribute to the elucidation of various pharmacological actions of A-SQ and L-HL in health and disease. Such augmented immunomodulatory activity associated with IFN- γ production demonstrated a beneficial effect of A-SQ and L-HL for alleviating infectious diseases and maintaining host health.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest in manuscript submitted.

Statement of ethical approval

The experimental protocols were approved by the Animal Experiment Committee of Kyushu University of Health and Welfare, Japan, and the animal experimentation guidelines of the university were followed in the animal studies.

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