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Original article

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Abstract: Sophy β-glucan, a type of β-1,3-1,6 glucan produced by a black yeast *Aureobasidium pollulans* strain AF0-202, is currently approved for use as a health food supplement. The new genotypic avian influenza H5N1 is one of the major emerging infectious diseases causing concern and loss in Vietnam, Asian and several European countries during the 2003-2008 period. We examined the effect of Sophy β-glucan on the immune response against the new H5N1 vaccine made in Vietnam/China and H5N2 made in China. As a result, both H5N1 and H5N2 vaccines elicited a significantly high immune response with Sophy β-glucan supplied in drinking water in all the mice tested (2-3 log2 higher HI titer). These vaccines also elicitated ELISA positivity (ELISA (+): seroconversion) 10-20% higher than those used in groups vaccinated without Sophy β-glucan. We conclude that Sophy β-glucan showed an excellent adjuvant effect on H5N1 and H5N2 vaccinations in a mouse model.
Keywords: β-glucan, H5N1 and H5N2 vaccine, Immunostimulation, Avian influenza,

INTRODUCTION

β-glucans are deriveded from the common baker's yeast *Saccharomyces cervisiae* [1] and mushrooms such as *Basidiomycetes* spp, Reishi, Shiitake, and Maitake [2, 3]. It has been demonstrated that, specifically, β-1,3-1,6 glucan modulates systemic immunity and increases resistance to microbial challenges [4], as well as against streptococcal and staphylococcal infections [3-6]. In other words, β-glucan confers an enhanced state of host defense against bacterial infections. With regard to orally administered β-glucan, interleukin-2 was evaluated in treated animals and showed an increase in weanling pigs [5, 7]. Enhanced immunological actions have been shown with β-glucan produced by Sophy Company, Japan [8], indicating that this food supplement may be a useful new adjuvant for intradermal and oral immunizations [9].

In this paper, we discuss the enhancing effect of Sophy β-glucan on the immune response to newly produced H5N1 and H5N2 vaccines in mice in Vietnam. The experiments were conducted to examine the enhanced effect of β-glucan (Sophy Company, Japan) on immunomodulatory activity related to the two influenza virus vaccines in BALB/c mice.

MATERIALS AND METHODS

Sophyβ-glucan

β-1,3-1,6 glucan synthesized by *Aureobasidium pullulans* (*A. pullulans*) strain AFO-202 and prepared in soluble form is currently available commercially as a health food supplement [8], provided by the Sophy Co. (Kochi, Japan) in plastic sacs of 10 liters each packaged in cartons. It was used as instructed by the Sophy Co. at a final concentration of 2% in drinking water or food for animals.

BALB/c mice

Eight week old BALB/c mice of both genders (50% male and 50% female) were purchased from the Company of Experimental Animals belonging to the National Institute of Hygiene and Epidemiology (NIHE, Hanoi, Vietnam). The mice were raised in cages under standard biological conditions.

Inactivated oil-emulsified H5N1 vaccine and administration

The following avian influenza vaccines were used: 1) Vaccines imported from China (produced by the Harbin

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Veterinary Research Institute, Heilongjiang, China): inactivated oil-emulsified H5N1 vaccine made from reverse genetics (rg) based master seed (using the strain A/Goose/Guangdong/1996(H5N1)) referred to as Oil-H5N1(CN), and inactivated oil-emulsified H5N2 vaccine made from the rg master seed (using the strain A/Turkey/England/N-28/73 (H5N2)) referred to as Oil-H5N2(CN); 2) Vaccines produced in Vietnam (by the Veterinary Vaccine Company, Hanoi): inactivated oil-emulsified H5N1 vaccine made from the rg NIBRG-14 (using the strain A/Vietnam/1194/2004 (H5N1)) referred to as Oil-H5N1(VN) vaccine. The HA (hemagglutinin) antigen in the Vietnamese H5N1 vaccine was set to 160-200 HAU (HA unit) in each dose, and the vaccine was administered in 0.2 ml doses by *subcutaneous* (sc) injection at the neck of mice.

Preparation of blood and collection of sera for testing

Blood samples of about 0.4 ml were collected directly from tail vein of mice before and about 21 days post-vaccination. Blood was left to set at room temperature and kept at a cold temperature (in the second compartment of a refrigerator) for several hours. Sera were collected in Eppendorf tubes and kept at -20°C in a freezer until used. In several cases, sera were also prepared from blood samples obtained directly from the heart or from mice by sacrifice.

Assays for HA(H5) antibody from immunized mice by ELISA

The presence of serum anti-HA(H5) specific immunoglobulin (IgG) was determined by enzyme linked immunosorbent assay (ELISA). Briefly, the purified HA(H5) proteins at a concentration of 0.5 µg/ml (H5N1 clade 1.0, purchased from the National Institute of Veterinary Research, Hanoi, Vietnam) were coated on 96-wells of a microplate at 100 µl/well and incubated at 4°C overnight. The plate was washed three times with washing buffer (0.05% Tween 20 in PBS buffer-PBST), and then 150 µl of blocking buffer (5% skim milk in PBST) was added and left at room temperature for 1 hour. After washing 3 times, the other 100 µl of 1: 100 mice sera diluted in blocking buffer was added on the plate and incubated at 37°C for 1 hour. Then, the plate was washed 5 times, and 100 µl of horseradish peroxidase-labeled goat anti-mouse Ig(G+M+A) was added and incubated at 37°C for 1 hour. The plate was washed 5 times. 100 µl of TMB solution (3, 3, 5, 5-tetramethylbenzidine and H_2O_2 in 30% concentration) was added to each well and incubated for 10 min at room temperature, the incubation being stopped by adding 50 µl of 2 M H_2SO_4. The optical density (OD) was measured at 450 nm in a microplate reader (Bio-Rad). The mean optical density (OD) of the negative group (n = 30, without vaccination or β-glucan) plus three standard deviations was taken as the cut-off value.

Assays for HA(H5) antibody from immunized mice sera by HI

The hemagglutination inhibition (HI) test was performed as instructed [10]. Briefly, sera were twofold serially diluted and incubated with 8 HA units of the H5 antigen (H5N1 clade 1.0 purchased from the National Institute of Veterinary Research, Hanoi, Vietnam) and then with a 0.5% (vol/vol) suspension of chicken red blood cells (RBC) per well. Antibody titers corresponding to the reciprocal of the highest serum dilution that inhibited hemagglutination were expressed as HI titers, and the geometric mean titers (GMT) were calculated from the average of HI titers in the samples.

Experiment design

Eight week old BALB/c mice of (50% male and 50% female) were purchased from the Company of Experimental Animals (Hanoi, Vietnam) and divided into 8 groups of four for each for the experiment and 2 groups of two for each for the control as shown in Table 1, then administrated the food supplied by the same company. Sophy β-glucan was mixed in drinking water for the vaccinated mice groups. Eight groups of mice were provided with water supplemented with Sophy β-glucan at a 2% concentration for 7 days before and during the period of the immune response (one dose vaccination of 21 days and two-dose vaccination of 42 days); 4 other two-dose vaccinated groups of mice, without Sophy β-glucan; and the control groups (neither vaccination nor Sophy β-glucan) (Table. 1). A summary of the experi-

Table 1. Kinds of vaccine and number of mice used for vaccination.

Vaccine	Animals	Numbers of animals used			
		One dose vaccination with β-glucan	Two dose vaccination with β-glucan	Two dose vaccination without β-glucan	Control: Neither vaccination nor β-glucan
Oil-H5N2 (China)	mice (BALB/c)	2 males, 2 females	2 males, 2 females	2 male, 2 female	1 male, 1 female
Oil-H5N1 (China)	mice (BALB/c)	2 males, 2 females	2 males, 2 females	0	0
Oil-H5N1 (Vietnam)	mice (BALB/c)	2 males, 2 females	2 males, 2 females	2 male, 2 female	1 male, 1 female

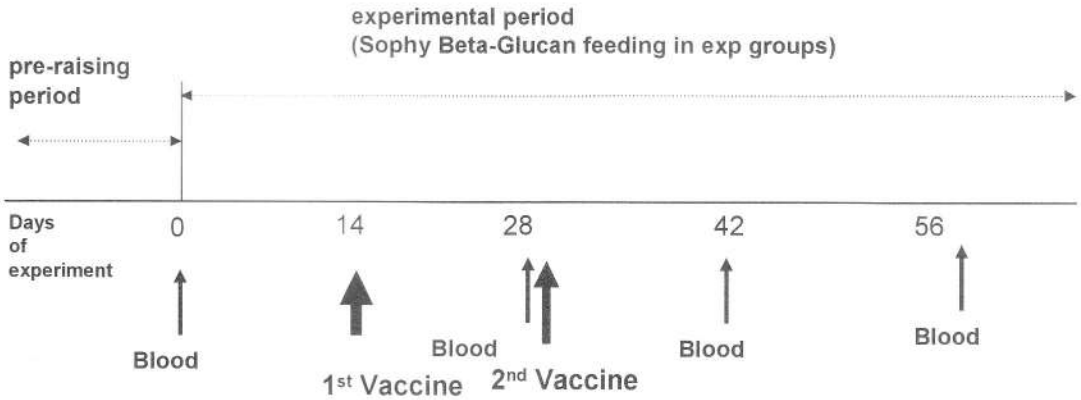

Fig. 1. Experiment design of the immunological action of Sophy β-glucan in BALB/c mice immunized with H5N1 and H5N2 vaccines from China and Vietnam.

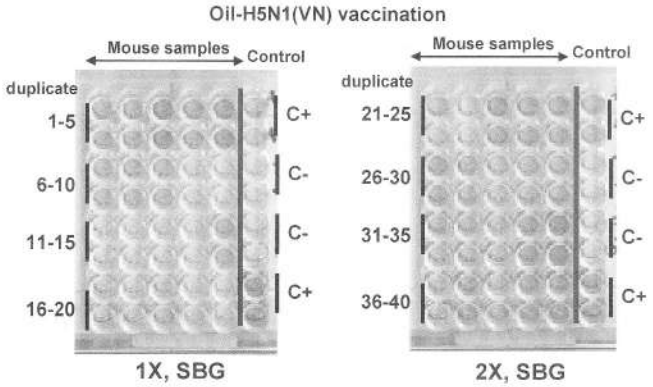

Fig. 2. ELISA plates (as a example) demonstrating color changes resulting from the reaction between H5 antigen and sera obtained from mice immunized in one and two shots with Oil-H5N1(VN) vaccine.

Note: Samples were tested in duplicate (indicated by the short vertical bar); quantity of samples in one line of wells shown by numbers; 1X: one shot vaccination; 2X: two shot vaccination; SBG: Sophy Beta Glucan; Control: C+: known antigen (H5) with positive antibody (H5-antibody); C-: known antigen (H5) with negative sera; all were also carried out in duplicate.

ment design is presented in Figure 1. At the end, blood was taken for serum collection and all animals were sacrificed for pathological examination. Evaluation was based on the antibody titer by HI (hemagglutination inhibition test) and ELISA (Enzyme linked immunosorbent assay). HI>3.0 log2 (>1:8) is considered to be protective and ELISA>0.5 is positive (protective) [11, 12].

Results and Discussion

We assessed the effect of Sophy β-glucan on immunological action and antibody production to H5N1 and H5N2 vaccines (from strains derived from Chinese and Vietnamese origin) in BALB/c mice fed with Sophy β-glucan.

As shown in Table 2, the immune response in Sophy β-glucan-fed mice immunized with H5N1 and H5N2 vaccines (Chinese and Vietnamese) was more enhanced than that in groups without Sophy β-glucan. The ELISA titer also showed that the titer reached 80% positivity in the twice vaccinated mice. The difference in antibody induction in the presence and absence of Sophy β-glucan was significant ($P<0.01$) (Table 2). Sera from the groups vaccinated with oil-inactivated vaccines in addition to Sophy β-glucan have a value of HI that is ~1.5 log2 higher (GMT ~1.0 log2 higher) and ELISA positivity (ELISA (+): seroconversion) that is ~10-20% higher than those from the groups vaccinated without Sophy β-glucan.

In conclusion, Sophy β-glucan exerted an enhancing effect on immune response in mice vaccinated with H5N1 and H5N2 oil-vaccines. This indicates that Sophy β-glucan is an excellent adjuvant to potentially enhance the immune responses of living organisms. The immune system is known to work in a complicated manner with multifunctional interactions between immune cells. The immune response starts with the antigen uptake by macrophage and dendritic cells. Concurrently, β-glucan is known to be taken up by pattern recognition receptors (PRR), ie TLR-2 and DECTIN-1, and through MyD88, a range of cytokines and interleukins produced to regulate differentiation of the effector cells like B and T lymphocytes [13]. Sophy β-glucan did induce DNA synthesis in all lymphoid and myeloid cells and played an important role in the regulation of various immune responses, such as cell proliferation/differentiation and adhesion, and immunoregulatory cytokine production [8]. Unlike the attenuated vaccine, where antigen genes might be activated to go through multiplication, the inactivated oil-vaccine of H5N1 given in a fixed dose had a constant antigen amount (in this particular case, it was set to 160 HAU), so the level of antibody induced was dependent

Table 2. Immunological effects of Sophy β-glucan on influenza vaccination as an adjuvant using HI and ELISA tests.

Vaccines	Test	BALB/c mice HI titer			
		1×vaccination with SBG	2×vaccination with SBG	2×vaccination without SBG	Control: No vaccination, No SBG
Oil-H5N2 (China made)	HI*	<3.5 $\log_2$ (GMT: 3.1 $\log_2$)	<4.5 $\log_2$ (GMT: 4.0 $\log^2$)	<4.5 $\log_2$ (GMT: 3.6 $\log_2$)	<1.5 $\log_2$ (not significant)
	ELISA**	40%	70%	60%	ND
Oil-H5N1 (China made)	HI*	<3.8 $\log_2$ (GMT: 3.0 $\log_2$)	<5.5 $\log_2$ (GMT: 4.8 $\log_2$)	ND	<1.0 $\log_2$ (not significant)
	ELISA**	30%	80%	ND	ND
Oil-H5N1 (Vietnam made)	HI*	<3.5 $\log_2$ (GMT: 3.0 $\log_2$)	<6.0 $\log_2$ (GMT: 5.5 $\log_2$)	<5.0 $\log_2$ (GMT: 4.2 $\log_2$)	<1.0 $\log_2$ (not significant)
	ELISA**	30%	80%	65%	ND

Note: 1×vaccination: one dose vaccination; 2×vaccination: two dose vaccination.; SBG: Sophy β-glucan; HI: hemagglutination inhibition protective titer is 3.0 log2 or higher.; ELISA: enzyme linked immunosorbent assay (seroconversion in %).; GMT: Geometric mean titer.; ND: not done.

*: Mean $\log_2$ titers for the four animals were estimated in each group. Statistical tests of differences of HI value between one-dose vaccination group (1×vaccination) and two-dose vaccination group (2×vaccination) with and without SBG were performed using the unpaired Student t test assuming equal variance: There were significant differences in HI value between one-dose vaccination group with SBG and two-dose vaccination group with SBG ($P<0.01$) in oil-H5N2 (China: CN) as well as in oil-H5N1 (Vietnam: VN). In oil-H5N1 (CN), statistically significant difference in HI value was detected between one-dose vaccination group and two-dose vaccination group with SBG. There were also statistically significant differences in HI value between two-dose vaccination groups with and without SBG ($P< 0.01$) in oil-H5N2(CN) as well as in oil-H5N1(VN). All of statistical comparisons between experimental groups and control group (no vaccination/no SBG) revealed significant differences ($P<0.01$).

**: In ELISA tests, there are differences among the three experimental groups (one-dose vaccination group with SBG, two-dose vaccination group with SBG and two-dose vaccination group without SBG), and the highest value was obtained from two-dose vaccination group with SBG in all of three vaccines (Oil-H5N2 from China, Oil-H5N1 from China, and Oil-H5N1 from Vietnam).

to a large extent on the effective response in the immune system of the body. As seen from the outcome of the experiments, the immune system in the immunized animals fed with Sophy β-glucan was highly modulated.

Finally, interactive modulation between biologically active compounds and functioning cells in the context of vaccine efficacy is an important area of research with broad medical implications. In particular, one of the biopolymers - newly commercially available Sophy β-glucan - offers significant potential immunomodulatory and biologically active compounds applicable to a wide range of disease systems. This family of biopolymers also has significant potential in vaccine delivery based on interactive activity as an excellent adjuvant.

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