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The Adjuvant Effect of Sophy β-Glucan to the Antibody Response in Poultry Immunized by the Avian Influenza A H5N1 and H5N2 Vaccines

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Avian influenza virus vaccines produced in oil-emulsified inactivated form with antigen content of at least 160 hemagglutinin units (HAU) induced immunity in birds. However, in addition to enhancing the effect of the adjuvant(s), other additional supplemented biological compounds included in inactivated vaccines could produce higher levels of antibody. We examined in chickens, Vietnamese ducks, and muscovy ducks the adjuvant effect of Sophy β-glucan (SBG), a β-1,3-1,6 glucan produced by the black yeast *Aureobasidium pollulans* strain AF0-202, when administered with an avian influenza H5 subtype vaccine. In Experiment 1, 40 chickens (ISA Brown hybrid), allocated to four groups of ten each, were immunized with Oil-H5N1(VN), Oil-H5N1(CN), Oil-H5N2(CN), and saline (control group), respectively. In Experiment 2, chickens (ISA Brown hybrid), muscovy ducks (French hybrid), and Vietnamese ducks (indigenous Vietnamese) were used to further assess the effect of SBG on immunogenicity of the Oil-H5N1(VN) Vietnamese vaccine. ELISA and hemagglutination inhibition (HI) assays were used to assess the antibody response. The H5 subtype vaccines initiated significantly higher immune responses in the animals dosed with SBG, with 1.0–1.5 $\log_2$ higher HI titers and 10–20% ELISA seroconversion, compared with those not dosed with β-glucan. Notably, some of the animals dosed with SBG induced HI titers higher than 9.0 $\log_2$ following boosting immunization. Taken together, our serial studies indicated that SBG is a potential effector, such as enhancing the immune response to the H5 vaccines tested.

Keywords: Avian influenza, A/H5N1 vaccine, A/H5N2 vaccine, immunogenicity, immunomodulation, Sophy β-glucan (SBG)

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β-1,3-D Glucans are polysaccharides (complex glucose molecules) with the six-sided glucose rings connected at the 1 and 3 positions [26], and are known to possess immunomodulatory activities involving receptor recognition for enhancement of the immune response in vertebrates and invertebrates [5]. These molecules are potent reticuloendothelial-modulating agents, whose immunobiological activity is mediated by stimulating proinflammatory cytokine production [13, 16]. Many studies have shown that β-glucans potentiate the immune system, and activate B-lymphocytes and macrophages through Dectin-1, CR3, lactosylceramide, scavenger receptors, and Toll-like receptors (*i.e.*, TLR-2, TLR-6) [3, 6, 19, 20, 24, 28]. Sophy β-glucan (SBG), a product of the Sophy Company (Sophy Co., Agawa, Kochi, Japan), is a type of β-1,3-1,6-glucan produced by the black yeast *Aureobasidium pollulans* strain AF0-202, and is currently approved as a health food supplement and tested as an effective adjuvant for immunomodulation in avian influenza vaccination using a mouse model [14, 15].

Zoonotic avian influenza in poultry is a major worldwide problem. Its prevention depends on the application of poultry vaccination programs in several highly endemic countries like China, Vietnam, and Africa [18, 21]. The new genotypic avian influenza H5N1 (highly pathogenic avian influenza, HPAI) is an emerging infectious disease that caused major losses in Vietnam, Asia, and several European countries during 2003–2009 and is becoming a major global concern [9, 18]. From 2006, Vietnam has been applying a nationwide vaccination program with imported H5N1 and H5N2 vaccines. Concurrently, Vietnam has also launched a national project to implement avian flu A/H5N1 vaccine production based on the NIBRG-14 strain, the A/H5N1 master seed developed based on the reverse genetics technology by the National Institute for Biological Standards and Control (NIBSC), UK.

The ability of β-glucans to enhance the immunological response in broilers has been shown by a number of studies [4, 6, 12, 28]. These have shown that the β-glucan [(1→3)-β-glucan] may be an excellent adjuvant that improves the immune response by modulating the immune system and the effector cells to produce cytokines. To date, only one study has reported the use of extracts of mushroom mycelia as an adjuvant for A/H5N1 vaccine to enhance vaccine immunogenicity [13], whereas no experiments have investigated the effect of β-glucans on vaccine efficacy in poultry and animals.

In this paper, we present data obtained on immune response experiments with the newly produced avian influenza A H5 subtype vaccines and the adjuvant immunomodulatory effect of SBG tested in poultry in Vietnam. The immunomodulatory effect of β-glucans produced by the Sophy Company, Japan on avian influenza A vaccination in poultry was examined in chickens, indigenous Vietnamese ducks, and muscovy ducks.

Materials and Methods

Vaccines and Route of Administration

The three vaccines used in this study were generated from master seeds of viruses, namely, Oil-H5N1(VN) for A/H5N1 vaccine produced in Vietnam; and Oil-H5N1(CN) and Oil-H5N2(CN) for the H5 subtype poultry vaccines imported from China. The master seed strains for the Chinese and the Vietnamese vaccines were generated using reverse genetics (rg) technology [25]. The Oil-H5N1(VN) vaccine is produced in Vietnam (by the Veterinary Vaccine Company, Hanoi) from the rg-H5N1 master seed (called NIBRG-14) generated with the genes H5 and N1 from the strain A/Vietnam/1194/2004(H5N1) and the rest of the backbone segments (PB2, PB1, PA, NP, M, NS) from the A/Puerto Rico/8/34(H1N1) strain by the National Institute for Biological Standards and Control (NIBSC), UK [23]. The Oil-H5N1(CN) vaccine of China is produced from the rg-H5N1 master seed generated by the genes H5 and N1 from the strain A/Goose/Guangdong/1996(H5N1) and the rest of the backbone segments of the A/Puerto Rico/8/34(H1N1) by the Harbin Veterinary Research Institute, Heilongjiang, China. Vaccines were administered according to the manufacturer's recommendations.

SBG and Feeding Administration

The SBG product was prepared in soluble form containing the β-1,3-1,6-glucan synthesised by the yeast *Aureobasidium pullulans* (*A. pullulans*) strain AFO-202, which is currently available commercially as a health food supplement [14]. It was prepared according to the manufacturer's instructions, to a final concentration of 2% (w/v). SBG was mixed with flour and then baked at 80°C for 3 h to make a powder. This powder premix contained 40% active SBG, which was later mixed with food to 2% (w/w) concentration for feeding birds. For Experiments 1 and 2 (described below), SBG was given to the designated groups for only 2 weeks from the day of first vaccination, regardless of whether the particular groups received single or boosting vaccinations.

Experimental Design to Test Immunologic Effect of SBG

Experiment 1: Setting for study in chicken. One-day-old chickens (ISA Brown hybrid), purchased from the Company of Poultry (Hanoi, Vietnam), were raised until two weeks of age. The 2-week-old chickens were divided into 15 groups of 10 birds each (12 experimental, 3 as control) (Table 1). The experimental groups were as follows: six were fed SBG; six were not fed SBG. Within each of these six groups, three were vaccinated at day 14 only, whereas the other three were vaccinated again at day 28, each group being vaccinated with one of the three vaccines. For all birds, blood was taken on the 14th day (day 14 of age) before vaccination. Blood was collected at days 28 and 42. At day 42, all birds were sacrificed for necropsy. Evaluation was based on antibody titer by HI and ELISA, and on pathological lesions (PL). A HI titer > 3.0 $\log_2$ and an ELISA value > 0.5 are considered protective [8, 11, 18, 21].

Experiment 2: Comparison of immunization in chickens, muscovy ducks, and Vietnamese ducks. In this study, chickens (*Gallus gallus domesticus*), muscovy ducks (*Cairina moschata*), and indigenous Vietnamese ducks (member of the family Anatidae) were used.

The experimental chickens (ISA Brown hybrid), muscovy ducks (French hybrid), and indigenous Vietnamese ducks were raised until 10 days of age. Blood was collected at day 10 and the birds were divided into 10 groups of 10 birds each as follows: 2 experimental groups and one control for each of the chicken and muscovy duck groups, respectively; 3 experimental groups and one control for the Vietnamese ducks (Table 2). Additionally, from the 11th day, SBG was administered to the designated groups for 2 weeks. All experimental groups were vaccinated with a single dose of 160 HAU in 0.3 ml/dose *via* the subcutaneous route with the Vietnam-made avian influenza H5N1 vaccine designated as Oil-H5N1(VN)

Table 1. Experimental design for testing immunologic action of SBG in chickens (Experiment 1) immunized with the avian influenza A H5 subtype vaccines of China and Vietnam.

Vaccines	Number of chickens (age: 2 weeks old)				
	[a]Single vaccination; fed SBG	[a]Single vaccination; not fed SBG	[b]Booster vaccination; fed SBG	[b]Booster vaccination; not fed SBG	Control (no vaccination, no SBG)
Oil-H5N1(VN)	10	10	10	10	10
Oil-H5N2(CN)	10	10	10	10	10
Oil-H5N1(CN)	10	10	10	10	10

[a]Single vaccination: Vaccines were given to birds by the subcutaneous route (sc) on day 14.
[b]Booster vaccination: Vaccines were given to birds by the subcutaneous route (sc) on day 14 and day 28.
Oil-H5N1(VN): oil-emulsified inactivated H5N1 vaccine produced in Vietnam; Oil-H5N2(CN) and Oil-H5N1(CN): oil-emulsified inactivated H5N2 and H5N1 vaccines imported from China. SBG: the β-glucan product of Sophy Company (Japan).

Table 2. Experimental design for testing immunologic actions of SBG in chickens, muscovy ducks, and Vietnamese ducks (Experiment 2) immunized with the Vietnamese avian influenza H5N1 vaccine.

Days	Chickens			Muscovy ducks			Vietnamese ducks			
	Group 1 (exp)	Group 2 (exp)	Group 3 (control)	Group 4 (exp)	Group 5 (exp)	Group 6 (control)	Group 7 (exp)	Group 8 (exp)	Group 9 (exp)	Group 10 (control)
1–10	Pre-raising	Pre-raising	Pre-raising	Pre-raising	Pre-raising	Pre-raising	Pre-raising	Pre-raising	Pre-raising	Pre-raising
10	SBG	No SBG	No SBG	SBG	No SBG	No SBG	SBG	No SBG	SBG	No SBG
	0.3 ml; sc; Oil-H5N1(VN)	0.3 ml; sc; Oil-H5N1(VN)	Control (no vaccine)	0.3 ml; sc; Oil-H5N1(VN)	0.3 ml; sc; Oil-H5N1(VN)	Control (no vaccine)	0.3 ml; sc; Oil-H5N1(VN)	0.3 ml; sc; Oil-H5N1(VN)	0.3 ml; sc; Oil-H5N1(VN)	Control (no vaccine)
24									0.3 ml; sc; Oil-H5N1(VN)	
38	Blood was taken or serum collected									
42	All birds were sacrificed for inspection of pathological lesions (necropsy)									

Each group of control and experiment consisted of 10 birds.
Pre-raising: The raising period (during 10 days) with ordinary food before the start of the experiments; (exp): experiment group. SBG: Sophy β-glucan, a product of the Sophy Company (Japan); No SBG: No SBG added to the food for poultry; sc: subcutaneous (at neck) administration of the Vietnamese Oil-H5N1(VN) vaccine.

and Group 9 of the Vietnamese ducks received one more booster-dose (booster vaccination) 14 days after the first injection (Table 2). As designed in Table 2, Group 1 (chicken), Group 4 (muscovy ducks), and Groups 7 and 9 (Vietnamese ducks) were fed with SBG-supplemented food, whereas the other groups including the controls were not. Blood was collected from the single vaccinated, booster vaccinated, and control groups at days 24 and 38. At day 42, all birds were sacrificed for necropsy. Evaluation was based on an HI> 3.0 $\log_2$ being considered protective [8, 11, 21].

Immunological Assays

Preparation of blood and collection of sera. Approximately 1.0 ml of blood was collected either from the heart or the wing vein at the times indicated in Table 1 and Table 2. Blood was allowed to set at room temperature and then maintained at 4^0C for several hours. Sera were collected individually into Eppendorf tubes and maintained at –20^oC in the freezer until required.

Assays for HA(H5) antibody from immunized poultry sera by ELISA. The presence of serum anti-HA (H5) specific immunoglobulin (IgG) was determined by ELISA as previously described [8, 27] with slight modification. Briefly, the purified HA (H5) proteins at 0.5 μg/ml (H5N1, clade 1.0, purchased from the National Institute of Veterinary Research, Hanoi, Vietnam) were coated onto a 96-well microplate with 100 μl/well and incubated at 4^oC 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 the plate left at room temperature for 1 h. After washing 3 times with washing buffer, 100 μl of 1:100 serum dilution in blocking buffer was added to each well and the plate incubated at 37^oC for 1 h. Then the plate was washed 5 times and 100 μl of horseradish peroxidase-labeled goat anti-chicken IgG was added at 37^oC for 1 h. The plate was washed 5 times with washing buffer. Then 100 μl of TMB solution (3.3', 5.5'-tetramethylbenzidine and H_2O_2 in 30% concentration) was added to each well and the plate was incubated for 10 min at room temperature. The reaction was stopped by adding 50 ml of 2 M H_2SO_4. The optical density (OD) was measured at 450 nm in a microplate reader (Bio-Rad). The ELISA antibody titer was recorded as the serum dilution at which the optical density reading at 450 nm exceeded 0.5 and exceeded the reading from wells of the negative control (without antigen) by 2-fold. The mean OD of the negative group (n=30, without vaccination or β-glucan) plus three standard deviations was taken as the cut-off value. ELISA titers greater than 0.5 were considered positive [8, 27].

Assays for HA(H5) antibody from immunized poultry sera by HI. The HI test was performed as per WHO [27]. Briefly, a duplicate serial 2-fold dilution of each test serum (pretreated at 56^oC for 30 min to inactivate nonspecific inhibitors) was made. Sera in wells were then incubated with 4 HA units of the H5 antigen purchased from the National Institute of Veterinary Research, Hanoi, Vietnam (clade 1.0 of the A/H5N1 subtype; a dominant clade in Vietnam and the Southeast Asian countries), for 15 min at room temperature. Phosphate-buffered saline (1× PBS) was used instead of antigen to serum control wells. Then 0.5% (v/v) suspension of chicken red blood cells (RBC) was added to each well. The HI antibody titer was determined following the method described by Allen *et al*. [1]. Antibody titer corresponding to the reciprocal of the highest serum dilution that still inhibited hemagglutination was recorded as the realistic HI titer expressed as a $\log_2$ value. The geometric mean titer (GMT) of HI antibodies of each group was determined and compared, and titer equal to or greater than 3 $\log_2$ were considered positive [8, 17, 27].

Statistical Analysis

The 10 birds are considered technical replicates. For the studies on the immunogenicity of vaccines (with and without β-glucan supplementation),

Table 3. Summary of evaluation of the effect of SBG in chickens immunized with the avian influenza A H5 subtype vaccines of China and Vietnam by the HI and ELISA assays.

Vaccines	Tests	HI titer[a,b]				
		Single vaccination; fed SBG	Single vaccination; not fed SBG	Booster vaccination; fed SBG	Booster vaccination; not fed SBG	Control (no vaccination, no SBG)
Oil-H5N1(VN)	HI	<8.0 log_2*[c] (GMT: 6.5 log_2)	<7.0 log_2*[c] (GMT: 6.0 log_2)	<9.0 log_2*[c] (GMT: 7.5 log_2)	<8.0 log_2*[c] (GMT: 7.0 log_2)	<1.0 log_2 (not significant)
	ELISA seroconversion	75%	65%	85%	70%	10% (not significant)
Oil-H5N2(CN)	HI	<7.0 log_2*[c] (GMT: 6.1 log_2)	<6.5 log_2*[c] (GMT: 5.6 log_2)	<8.5 log_2*[c] (GMT: 7.5 log_2)	<8.0 log_2*[c] (GMT: 7.0 log_2)	<1.0 log_2 (not significant)
	ELISA seroconversion	70%	60%	75%	65%	10% (not significant)
Oil-H5N1(CN)	HI	<5.8 log_2 (GMT: 5.5 log_2)	<5.0 log_2 (GMT: 5.0 log_2)	<7.8 log_2 (GMT: 7.0 log_2)	ND	<1.0 log_2 (not significant)
	ELISA seroconversion	60%	52%	65%	ND	10% (not significant)

[a]Vaccines were given to birds by subcutaneous route with 0.3 ml/dose containing 160 hemagglutinin units of antigen on day 14 (single vaccination) or on days 14 and 28 (booster vaccination) (see Table 1).
[b]Mean log_2 titer (GMT) for the 10 birds in each group.
*[c]Comparison of the day 42 HI value for the booster vaccination group and the single vaccination group, fed with SBG and not fed with SBG, analyzed by Student's t-test. P-values < 0.05 were considered to be statistically significant.
Oil-H5N1(VN): oil-emulsified inactivated H5N1 vaccine of Vietnam; Oil-H5N2(CN) and Oil-H5N1(CN): oil-emulsified inactivated H5N2 and H5N1 vaccines of China. SBG: Sophy β-glucan, a β-glucan product of the Sophy Company (Japan); HI: Hemagglutination Inhibition Test; < log_2: Individual chicken among the experimental birds tested showing the highest HI titer; GMT: Geometric mean HI titer; ELISA: enzyme-linked immunosorbent assay (%: positive seroconversion); ND: Not done.

the titer in each chick was considered an independent experimental unit for analysis. Data are presented as Mean ± S. D. Comparisons between experimental groups were analyzed by Student's t test. A p-value of 0.05 was used to determine statistical significance in all analyses. P values < 0.05 were considered to be statistically significant.

Results

Effect of SBG on Antibody Response to Different Avian Influenza A Vaccines in Chickens

The efficacy of SBG on the immunological response and antibody production to H5 vaccines was assessed by determination of HI and ELISA titers.

Groups dosed with SBG had significantly higher (P<0.05) GMT HI titers compared with those of the nonsupplemented groups for both single and booster vaccinated groups (Table 3). A similar trend of increased positive seroconversion (P<0.05) measured by ELISA in SBG-supplemented groups compared with the control groups was also noted after single as well as booster vaccinations (Table 3). In the control group, no significant HI and ELISA titers were noted (Table 3). Additionally, the addition of SBG significantly increased (P<0.05) antibody values in single and booster vaccinated birds. The GMT HI titer was 1.0–1.5 log_2 higher and the ELISA value 10–20% higher (P<0.05) in the vaccinated groups adjuvanted with SBG, compared with groups without adjuvant (Table 3).

Immunologic Effect of SBG on Antibody Response to the Vietnamese H5N1 Avian Influenza Vaccine in the Domestic Chicken, Muscovy Ducks, and Vietnamese Ducks

Results from Experiment 1 in chickens showed that the level of antibody induced by the H5 vaccines was greater (P<0.05) in animals fed with SBG. We decided to assess the immunogenicity of SBG in commonly raised poultry such as muscovy ducks, Vietnamese ducks, and domestic chickens. The vaccine used in Experiment 2 was the NIBRG-14 based inactivated-oil H5N1 vaccine produced in Vietnam, designated Oil-H5N1(VN). Antibody production in immunized birds, either fed with SBG for 2 weeks or not fed SBG, was measured by the HI test (see Table 2). The HI titers in response to the addition of SBG are summarized in Table 5. Groups 1, 2, 4, 5, 7, 8, and 9 were vaccinated at day 10. A booster dose was given on day 24 only to Group 9 Vietnamese ducks (see Table 4). As shown in Table 4, after a single dose of vaccine, all birds had a protective serum antibody response as measured by HI assay. Many birds had antibody levels between 5.0 and 7.0 log_2. A value lower than 3.0 log_2 was obtained in one bird

Table 4. Antibody responses in immunized chickens, Muscovy ducks, and Vietnamese ducks after immunization with the Vietnamese H5N1 avian influenza vaccine[a].

		Chickens				Muscovy ducks				Vietnamese ducks						
	Group 1–10 (all birds)	Group 1 (single dose; fed SBG)		Group 2 (single dose; not fed SBG)		Group 4 (single dose; fed SBG)		Group 5 (single dose; not fed SBG)		Group 7 (single dose; fed SBG)		Group 8 single dose; not fed SBG)		Group 9 (booster dose; fed SBG)		Control (Groups 3, 6, 10) (no vaccine; no SBG)
HI titer (log_2)	Day 10	Day 24	Day 38	Day 24	Day 38	Day 24	Day 38	Day 24	Day 38	Day 24	Day 38	Day 24	Day 38	Day 24	Day 38	Day 38
	Number of birds having the corresponding HI titer															
<1	10															10
<3					1											
4				1	2			1	2			1	1	1		
5		1	2	2	3	2	2	4	4	1	1	2	3	1	1	
6		1	1	3	2	3	4	2	2	2	3	3	3	4	1	
7		4	3	3	2	2	2	2	2	3	3	3	2	2	3	
8		2	3	1		3	2	1		4	3	1	1	2	2	
9		2	1												2	
10															1	

[a]Vaccine was given to birds by subcutaneous route with 0.3 ml/dose containing 160 hemagglutinin units (HAU) of antigen on day 10 (single vaccination) and on day 24 (booster vaccination) (see Table 2).
The Vietnamese avian influenza vaccine, designated as Oil-H5N1(VN), is an NIBRG-14 based oil-emulsified inactivated H5N1 vaccine produced in Vietnam; HI: Hemagglutination Inhibition Test; < log_2: Birds showing the lowest HI titer; SBG: Sophy β-glucan product.

on day 38 (Table 4). Two chickens in Group 1 fed with SBG had a HI titer of 9.0 log_2, one persisting to day 38. Three Vietnamese ducks in Group 9 (booster immunized) and fed with SBG had an HI titer higher than 9.0 log_2 on day 38. Tables 4 and 5 and Fig. 1 show that animals fed SBG had significantly higher ($P<0.05$) HI values compared with nonsupplemented and control groups (Table 5).

DISCUSSION

Vaccination is an extremely effective strategy for protection of birds against avian influenza, but protection depends on the nature of the vaccine itself and the method of the application [21]. HI is the preferred test to evaluate antibody levels in birds immunized with influenza vaccines [10]. As

Table 5. Summary of evaluation of the effect of SBG in poultry (chickens, muscovy ducks, Vietnames ducks) immunized with the Vietnamese H5N1 avian influenza vaccine by HI test.

Days	Chickens HI titer[a,b] (log_2)			Muscovy ducks HI titer[a,b] (log_2)			Vietnamese ducks HI titer[a,b] (log_2)			
	Group 1 (single dose; fed SBG)	Group 2 (single dose; not fed SBG)	Group 3 (control)	Group 4 (single dose; fed SBG)	Group 5 (single dose; not fed SBG)	Group 6 (control)	Group 7 (single dose; fed SBG)	Group 8 (single dose; not fed SBG)	Group 9 (booster dose; fed SBG)	Group 10 (control)
Highest HI titer	9.0	8.0	<1.0	8.0	8.0	<1.0	8.0	8.0	10.0	<1.0
GMT (HI) 2 weeks after injection	7.3	6.1	<1.0 (not significant)	6.6	5.8	<1.0 (not significant)	7.0*[c]	6.1*[c]	7.6*[c]	<1.0 (not significant)

[a]Birds were immunized by subcutaneous route with 0.3 ml/dose containing 160–200 hemagglutinin units of antigen on day 10 (single vaccination) and on days 10 and 24 (booster vaccination) (see Table 2).
[b]Mean log_2 titer (GMT) for 10 birds in each group.
*[c]Comparison of the day 42 HI value for the booster vaccination group and the single vaccination group, fed with SBG and not fed with SBG, using the unpaired Student's t-test assuming equal variance: $P<0.01$.
The Vietnamese avian influenza vaccine, designated as Oil-H5N1(VN), is an oil-emulsified inactivated H5N1 vaccine produced in Vietnam;
HI: Hemagglutination Inhibition Test; < log_2: Individual chicken among the experimental birds tested showing the lowest HI titer; GMT: Geometric mean HI titer.

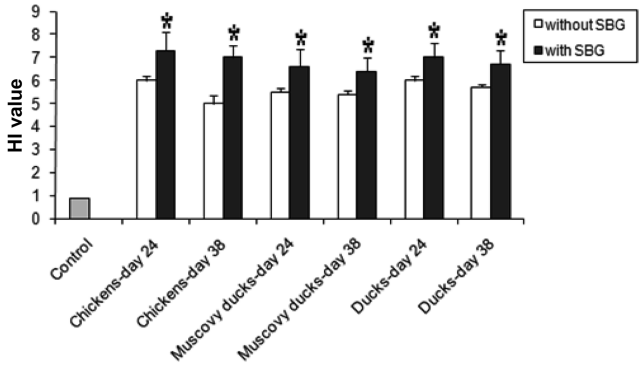

Fig. 1. Effect of supplementation of SBG on immunity in Chickens, Muscovy duck, and Ducks (Vietnamese ducks) vaccinated with the Vietnamese H5N1 avian influenza vaccine.
Single-dose vaccine was given to birds by the subcutaneous route with 0.3 ml/dose containing 160 hemagglutinin units (HAU) of antigen. Sera were collected on days 24 and 38, and subjected to HI test. Comparison of the HI value of the vaccination groups, fed with SBG and not fed with SBG, was analyzed by the Student's t-test. The HI values were statistically compared with those of non-adjuvanted chickens and with the controls (no vaccine, no SBG) (*$p<0.05$).

shown in Tables 3–5, prior to vaccination, all birds had no avian influenza A antibody. Two weeks after the primary immunization, they developed an increased HI antibody ($P<0.05$) titer, especially after the secondary dose, compared with the control groups (Tables 3–4). The mean GMT HI titer and seroconversion as measured by ELISA in Experiment 1 for chickens and Experiment 2 for additional waterfowl indicated that the level of antibody was significantly higher ($P<0.05$) in vaccinated groups fed SBG compared with groups not fed SBG (Tables 4–5 and Fig. 1). A HI value > 3.0 $\log_2$ in vaccinated birds and a seroconversion of over 70% are considered protective against avian influenza of the same hemagglutinin subtype [10, 21]. The immunogenicity of inactivated vaccines appeared to be improved by using an immunomodulatory adjuvant such as SBG.

The immune responses of the chickens fed SBG and immunized was significantly better than in those groups not fed SBG (Tables 3 and 5; and Fig. 1). In the SBG-fed birds, the HI titers (as expressed in GMT value) of the booster vaccinated birds were greater ($P<0.05$) than those that received a single vaccination, and significantly greater ($P<0.01$) when compared with nonsupplemented birds. The ELISA titer was higher for booster vaccinated birds and reached 80% positivity (Table 3). The difference in antibody induction between SBG-supplemented and nonsupplemented groups was significant ($P<0.05$) (Tables 3 and 5; and Fig. 1) following vaccination. Although we have not done the HI and ELISA assays in animals without SBG and with booster vaccination for Oil-H5N1(CN), the HI and ELISA assays in animals vaccinated with Oil-H5N1(VN) and Oil-H5N2(CN) with single and booster vaccinations showed clearly that the SBG-supplemented chickens had significantly higher immune responses, including higher HI titers and seroconversion rates, as compared with the nonsupplemented groups (Table 3). In other words, the use of SBG as an adjuvant appeared to enhance immune responses in all animals and with different H5 subtype vaccines tested in this study.

The immune response starts with antigen uptake by the macrophage and the dendritic cells and concurrently glucan by a number of pattern recognition receptors (*i.e.*, TLR-1/2, TLR-6, and DECTIN-1) [7]. Through MyD88 and NFκβ, a range of cytokines and interleukins are produced to regulate the differentiation of the effector B and T lymphocytes [5, 16, 28]. Ikewaki *et al.* [14] showed that the SBG induced DNA synthesis in 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. Unlike attenuated vaccines, where expression of antigen persists to maintain the antigenic polypeptide source for antibody response, inactivated oil-emulsified vaccines contain a fixed amount of antigen (in our study, the H5 antigen was set to 160 HAU, equal to 10 to 33 μg of hemagglutinin protein per dose) [21, 22]. Our results demonstrated that the antibody titer was higher ($P<0.05$) in those fed SBG than in those not fed SBG.

The addition of SBG to poultry offers potential benefits on the immune response to newly developed avian influenza H5 vaccines. SBG is accepted as a health food supplement, currently available commercially in a soluble form to humans [14]. The immunomodulatory effect of β-glucan in general, and SBG in particular, indicates that the glucan adjuvants may be a good source for a multitude of purposes including the enhancing effect of the immune system in animals and humans [2, 19].

In conclusion, from our studies, and previously in a mouse model [15], SBG is a potential immune initiator for H5 vaccines. SBG offers a way of modulating immune cells in their response to avian influenza vaccines. This study demonstrates an effective interaction between β-glucans and inactivated avian influenza A vaccines in enhancing the immune response.

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