Effect of edible fungal polysaccharides on improving influenza vaccine protection in mice

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ABSTRACT
Fungal polysaccharides have shown broad spectrum of biological activities, including anti-inflammatory, ant-oxidative and improve immunity. However, oral administration of fungal polysaccharides for rendering the conventional vaccine against influenza virus has been reported rarely. Here, we investigated the potential of fungal polysaccharides in enhancing the influenza vaccine efficacy in a mouse model. Mice were immunized with inactivated H1N1 (A/PR8/1934) influenza vaccine combined with oral polysaccharides lentinan, tremellan, pachymaran and a mixture of the three. The results showed that mice in the polysaccharides/vaccine groups had reduced morbidity, improved viral clearance, and recovered faster than the mice receiving the conventional vaccine only after infection. This effect could be attributed to the increased levels of virus-specific serum antibody IgG and decreased levels of inflammatory cytokine IFN-γ in the lung tissue. Our finding suggests that taking fungal polysaccharides orally might be useful for improving the efficacy of conventional inactive influenza vaccines.

ARTICLE HISTORY
Received 22 April 2017
Accepted 22 April 2017

KEYWORDS
Lentinan; tremellan; pachymaran; influenza virus; immune

Introduction
Influenza viruses are a major cause of respiratory tract infections in animals (Chothe et al., 2017; Yeo et al., 2017). Vaccination is recognized as one of the most effective intervention in reducing the impact of influenza pandemics (Castilla et al., 2013; Ferguson et al., 2006; Partridge & Kieny, 2010). Although influenza vaccines have been used for decades, many immunized individuals still got infected by the virus and the potential correlates of protection induced by these vaccines are still a matter of discussion (Fichera, Felnerova, Mischler, Viret, & Glueck, 2009; Leroux-Roels et al., 2007; Lin et al., 2006; Nichol & Treanor, 2006; Trombetta & Montomoli, 2016). How to improve the efficacy of conventional inactive influenza vaccine remains a major challenge in influenza prevention.

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Supplemental data for this article can be accessed here: https://doi.org/10.1080/09540105.2017.1323326
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Over the years, fungal polysaccharides had been explored for their broad spectrum of biological activities that vary, depending on the sources from which they were isolated. Polysaccharides from *Hohenbuehelia serotina* and *Tremella aurantialba* possessed antioxidant activity (Du et al., 2015; Li & Wang, 2016). Lentinan and pachymaran have been found to have immunostimulatory activities and exert indirect inhibitory effect on cancer cells (Hamuro, Maeda, Arai, Fukuoka, & Chihara, 1971; Wasser, 2002; Wei, Hu, Chen, & Wei, 2011). *Tremella* polysaccharides were reported to attenuate sepsis through inhibiting abnormal CD4^+^CD25^+^ regulatory T cells in mice (Shi et al., 2014). Intranasally pretreatment with Ginseng polysaccharides or Lentinan could induce protective effects on influenza virus (Irinoda, Masihi, Chihara, Kaneko, & Katori, 1992; Yoo et al., 2012). However, oral administration of fungal polysaccharides for anti-influenza had rarely been reported. On the other hand, if effective, the ingestion of the polysaccharides would be more readily accepted and convenient in application than intranasal administration. To our knowledge, few studies on tremellan and pachymaran to improve the effects of influenza vaccine have been reported. In this study, we conducted studies in a mouse model to investigate the effects of lentinan, tremellan, pachymaran and a mixture of the three administrated orally on modulating the efficacy of inactivated A/PR8 (H1N1) influenza vaccine. Interestingly, these fungal polysaccharides could improve virus-specific antibody response and enhance the protective effect of the inactive influenza vaccine in their resistance to virus challenge.

**Materials and methods**

**Materials and reagents**

The laboratory-adapted A/PR8/1934 (H1N1) influenza virus was provided by the State Key Laboratory of Agricultural Microbiology of Huazhong Agricultural University. Avian Influenza Virus Enzyme-linked immunosorbent assay (ELISA) Kits were provided by Wuhan Keqian Biology Company (Wuhan, China). ELISA kits for interleukin (IL)-2 and interferon-gamma (IFN-γ) were obtained from R&D systems (Minneapolis, MN, USA). Other reagents were purchased from commercial suppliers, unless otherwise noted.

**Preparation of polysaccharides**

The extract of polysaccharides was obtained through water extraction and alcohol precipitation, as described previously (Wang, Fang, Zhao, & Wang, 2007). Typically, raw materials were refluxed with distilled water twice, then filtrated and concentrated followed by ethanol extraction, where ethanol was added into water extraction until its concentration up to 80%. The mixture was kept at room temperature overnight and the deposition was collected by filtration. The final polysaccharides were obtained after drying up. A polysaccharide mixture was made according to the proportion (lentinan:tremellan:pachymaran = 7:2:1).

The polysaccharides including lentinan, tremellan, pachymaran and a mixture of polysaccharides were dissolved separately in physiological saline under ultrasound at 2 s interval 60 s repeated 60 times, developing into polysaccharide suspension of 20 mg/mL. Subsequently polysaccharide solutions were aliquoted and stored frozen at −20°C.
Preparation of PR8 influenza vaccine

A/Puerto Rico/8/1934 [H1N1] (PR8) virus stocks were grown in fertilized chicken eggs. Inactivation of PR8 was achieved by treating with 0.74% formaldehyde overnight at 37°C. The influenza vaccine was prepared with formaldehyde-inactivated influenza virus PR8 emulsified with MF59 adjuvant (10⁵ PFU/mL).

Animals and immunization

Female BALB/c mice (6 weeks old) were obtained from the Beijing HFK Bioscience Co. Ltd. (HFK) and maintained in State Key Laboratory of Agricultural Microbiology at Huazhong Agricultural University. The animal study was carried out in strict compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Science and Technology Department of Hubei Province. The protocol was approved by the Ethics Committee of Huazhong Agricultural University. Permit Number: 2014-0004. All efforts were made to minimize the animals’ suffering as per the previous report (Zhao, Lin, Fu, Han, & Zhang, 2006). Briefly, the mice were monitored once a day for 14 days for clinical signs and assigned clinical scores as following: 0 = normal response to stimuli; 1 = ruffled coat and slow response to stimuli; 2 = respond only to repeated stimuli; 3 = nonresponsive or walking in circles and 4 = dead. Mice exhibiting extreme lethargy or neurological signs (score = 3) were considered moribund and humanely sacrificed. At the end of the experiment, the surviving animals were sacrificed by carbon dioxide inhalation.

Scheme 1 presents the animal experimental protocol. The BALB/c mice were randomly divided into 6 groups (20 mice per group) as follows: control group, vaccine-only group, 4 polysaccharides (lentinan, tremellan, pachymaran and a mixture of the three) combined with vaccine groups. The mice were administered orally with polysaccharides (200 mg/kg) one time per day for 31 days in polysaccharides/vaccine groups, while the vaccine-only group and non-immunized control group were given...
the same volume of phosphate buffer saline (PBS). On day 10 after the administration of polysaccharides, mice were immunized with PR8/MF59 in a total volume of 100 μL via muscle injection, and boosted once 2 weeks later. On day 32, mice were intranasally challenged with $5 \times 10^3$ PFU of H1N1(A/PR8/1934) influenza virus. Mice were immediately bled after challenge for analysis of the effectiveness of polysaccharides with or without vaccine. On days 0, 3, 6 and 14 post-infection, 3 mice of each group were sacrificed for conducting lung index, antibody and cytokine analyses. The body weight and mortality of the remaining 8 mice were monitored for 14 days.

**Lung index**

On days 0, 3, 6 and 14 post influenza virus infection, 3 mice of each group were sacrificed. The lungs were obtained steriley and weighed immediately. The lung index was calculated according to the formula as follows: index (mg/g) = weight of lung/body weight.

**Viral titer in lung**

Lung tissue samples were homogenized in sterile condition in ice-cold PBS. The samples were centrifuged at 10,000 rpm at 4°C for 5 min. The supernatants were collected for virus measurement. The number of viral particles was quantified by a commercial available ELISA kit, based on the detection of nucleoprotein (NP) (Zhang et al., 2006).

**Serum PR8-specific Immunoglobulin G (IgG)**

On days 0, 3, 6 and 14 post influenza virus infection, serum samples were collected, aliquoted and stored frozen at $-20^\circ$C. PR8-specific IgG in sera were measured by ELISA as reported previously (Yam et al., 2015). Briefly, ELISA plates were coated with purified inactivated PR8 virus at $10^5$ PFU/mL overnight. After blocking, 1:400 diluted sera were added and incubated for 1 h at 37°C. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000) was added. The enzyme reaction was stopped quickly by pipetting Stop Solution into each well, and absorbance value was detected by measuring the optical density (O.D.) at 630 nm. The antibody response was evaluated with the O.D. values, which were corrected by subtracting the baseline value, that is, the control without the serum.

**Analysis of cytokine production in the lung**

Tissue samples were homogenized and then centrifuged at 10,000 rpm at 4°C for 5 min. The supernatants were collected for the measurement of the cytokines. Analysis of IL-2, IL-4, IL-5 and IFN-γ in the lung tissue was performed by ELISA, following the manufacture’s instruction (R&D Systems, Minneapolis, MN, USA).
**Statistical analysis**

Results were expressed as the mean ± standard error of the mean (SEM). P-values were calculated using a two-tailed unpaired t-test. Significance difference was set at a P-value of .05. All graphing and statistical analyses were performed using the GraphPad Prism 5.0 graphing program (GraphPad Software, San Diego, CA, USA).

**Results**

**The role of polysaccharides on enhancement of protective efficacy of PR8-vaccine**

Mice pretreated with polysaccharides followed by PR8-vaccination and those receiving the flu vaccine only were challenged with influenza virus. On day 7 post-infection, the mice (n = 8) with mock immunization began to die, and five of the eight mice died within 2 weeks (Figure 1(a)). With PR8-vaccination, one of the eight mice died on day 10 post-infection. However, all polysaccharides pretreated groups, either individual polysaccharides or mixture of the three polysaccharides, followed by the vaccination survived throughout our analysis.

Mouse body weight is another important parameter to assess the general health status upon virus infection. The body weight of mice in the control group dropped significantly after infection (Figure 1(b)). The vaccine-only group showed slight weight loss. Nevertheless, the mice pretreated with polysaccharides in combination with the vaccine were found to retain their body weight throughout the study, significantly better than the one observed in the vaccination-only group.

The body weight of mice in the mixed polysaccharides/vaccine group was actually increased during our investigation time (Figure 1(b)). Therefore, the mice receiving both fungal polysaccharides and vaccination presented less severity of the disease condition, which highlights the synergistic action of these polysaccharides in boosting the protective effect of the flu vaccine against influenza infection.

The weight of the lung, reflecting the extent of lung damage caused by the viral infection, was measured after virus infection. As shown in Figure 1(c), the lung index in the control group started to increase on day 3 after infection and reached 15 (mg/g) on day 6 and remained high afterwards. The vaccinated group showed a significant increase in the lung index on day 6, but then a reduction on day 14. However, there were no increases in the lung index when the mice were combined pretreated with polysaccharides and vaccine. This finding indicates that the tested polysaccharides offer better lung protection when used in combination with the vaccines, presumably by reducing the virus load or rendering the host resistant to virus-induced pulmonary lesion.

**Polysaccharides combined with vaccine reduced viral titer in the lung tissue**

After virus challenge, the viral titer in the lung was analyzed. In the control group, the viral titer was very high on days 3 and 6 after infection, and then declined on day 14 (Figure 2). Although the vaccinated group presented low titers in general compared to the non-immunized group, it did show a slight increase in the viral titers. In contrast, the titers
in several combination treatment groups show significantly further reduction in virus titers on days 6 and 14. The reduced titers found in these groups were correlated with their better clinical condition and less damage in the lung (Figure 1), further validating our assertion that these polysaccharides could synergize the vaccination to reduce virus production or accelerate virus clearance.

**Figure 1.** Polysaccharide/vaccine pretreatment improved the protective effects against influenza virus. The mouse survival rate after infection ($n=8$). (a) The change of mouse body weight after infection ($n=8$). (b) The changes of lung index after infection ($n=3$). (c) *$P<.05$, **$P<.01$, compared with the vaccine-only group. The results represent means ± SEM.
Polysaccharides combined with inactivated influenza vaccine elicited stronger specific antibody responses than the one induced by the vaccination only

Serum samples were collected on days 0, 3, 6 and 14 after virus infection. Serum IgG antibodies specific to influenza virus in various treatment groups are shown in Figure 3. While vaccination of inactive virus elicited specific antibody compared to the control group, the combination of polysaccharides and vaccination induced higher level of serum antibodies on days 3 and 6. In particular, the antibody titers in the groups of pachymaran/vaccine and mixture/vaccine demonstrated statistically significant increase over the vaccination-only group on day 3. The group pretreated with mixture of polysaccharides retained higher antibody levels even on day 14, although the observed increase

Figure 2. The viral titer of lung tissue homogenate after virus infection. When mice pretreated with polysaccharides/vaccine, lung tissue were collected on days 3, 6 and 14 after virus infection. The viral titer decreased in polysaccharides/vaccine groups. *P < .05, **P < .01, compared with the vaccine-only group. The results represent means ± SEM (n = 3).

Figure 3. The change of PR8-specific IgG in mouse sera after virus infection. When mice pretreated with polysaccharides/vaccine, sera were collected on days 0, 3, 6 and 14 after virus infection. IgG level in sera increased in polysaccharides/vaccine groups. *P < .05, **P < .01, compared with the vaccine-only group. The results represent means ± SEM (n = 3).
compared to the vaccination-only group was not statistically significant. These findings suggest that the elevated antibody levels may account for the better protection elicited by the combination treatment against influenza infection.

**Cytokine analysis in lung tissue after infection**

The level of IL-2 in lung tissue was increased in a time-dependent manner in different groups after infection (Figure 4(a)). Significantly increase was observed in the tremel-lan/vaccine group on day 6, while in the phachymaran/vaccine group and mixture/vaccine group on day 14. The level of lung IFN-γ in the control group remained high, whereas it was at a lower level in other groups from day 0 to 14 (Figure 4(b)). It suggests that polysaccharides in combination with vaccine increase the level of cytokine IL-2 and reduce the level of cytokine IFN-γ induced by influenza virus. Figures S1 and S2 show the levels of IL-4 and IL-5 in lung tissue. Except for the lentinan/vaccine group, lung IL-5 level was increased significantly in polysaccharides/vaccine groups on day 3 compared to that of vaccine-only group, whereas IL-5 retained high level among all groups on days 6 and 14 post-infection.

![Figure 4](image-url)  
*Figure 4. Changes of cytokines after influenza virus infection. The change of IL-2 level in mouse lung tissue after infection. (a) The change of IFN-γ level in mouse lung tissue after infection. (b) **P < .01, compared with the vaccine-only group. The results represent means ± SEM (n = 3).


**Discussion**

Due to their potential ability to activate innate immune response, polysaccharides may also contribute to enhanced adaptive immunity (Chen & Seviour, 2007; Li, Wang, & Wang, 2013; Roupas, Keogh, Noakes, Margetts, & Taylor, 2010; Sun & Zhou, 2014). Some studies reported that polysaccharide alone has protective effects against flu (Roxas & Jurenka, 2007). Chinese herb isatis has been used for centuries in traditional medicine for the treatment of influenza due to immunostimulatory functions of isatis polysaccharide (Roxas & Jurenka, 2007). Plant polysaccharides (mannan, delta inulin, β-glucan, starch, dextran and pectin) were also used as vaccine adjuvants and delivery vehicles (Rosales-Mendoza, Salazar-González, Decker, & Reski, 2016). A single dose vaccine can achieve immune-protection against influenza when delta inulin was used as the adjuvant (Honda-Okubo, Ong, & Petrovsky, 2015). Mannan conjugated to the whole inactive H1N1 influenza viruses and administered intranasally in mice elicited higher serum IgG than immunization with the virus alone (Proudfoot et al., 2015). Mouse body weight loss was delayed and lung virus replication was inhibited when mice were immunized together with influenza virus-like particles intramuscularly one time and Ginseng polysaccharide intranasally (Yoo et al., 2012). The adjuvant effect of mushroom mycelia extracts was reported by intranasal co-administration of the extracts and inactivated A/PR8 (H1N1) influenza virus hemagglutinin vaccine in BALB/c mice (Ichinohe et al., 2010). Many antidote findings implicate the function of polysaccharides for protection against flu. However, few studies were conducted in an unbiased way to directly investigate the role of fungal polysaccharides in such protection. To address this issue, we focused our study on investigating the effect of three types of fungal polysaccharides and their mixture in modulating the efficacy of PR8-vaccines.

To test the potential of fungal polysaccharides enhancing the influenza vaccine efficacy, inactive virus vaccine (10^5 PFU/mL) was used. Our results demonstrated that the oral fungal polysaccharides combined with the inactive influenza vaccine could protect BALB/c mice from a lethal challenge of influenza virus. The polysaccharides/vaccine suppressed lung inflammatory response and improved survival rate in the mouse model after influenza virus infection. The polysaccharides combined with conventional vaccine elicited stronger specific antibody response against virus. Higher antibody titers are beneficial for complete protection (Potter & Oxford, 1979). Gao et al. also reported that increased levels of protection from virus were closely correlated with serum antibody titers (2006). In this study, there is no observed difference among groups after immunization. Polysaccharides/vaccine-induced serum IgG titers have been shown to be protective against influenza infection. As a whole, mixed polysaccharides combined with vaccine elicited the highest antibody response among all groups on days 6 and 14, suggesting that polysaccharide mixture has the synergistic effect against influenza virus.

To further explain the enhanced protection and increased viral clearance in mice vaccinated with polysaccharides/vaccine, cytokines were examined. IL-2 is capable of facilitating the secretion of immunoglobulins produced by B cells and inducing the differentiation and proliferation of natural killer cells (Fernández-Ruiz et al., 2015). In this study, IL-2 increase in a time-dependent manner may be related to the secretion of specific immunoglobulins against virus.
IFN-γ plays an important role in host immunoregulatory response (Saurwein-Teissl et al., 2002). Virus infection induces the rapid production of type I interferon (IFN) in the control group, which is consistent with the report (Dutta et al., 2013). IFN-γ is one of the major participants in cytokine storm (Walsh et al., 2014). An excessive and aberrant inflammatory response will damage host tissues, participating in the enhanced morbidity and mortality. There is no significant difference of IFN-γ among groups after immunization. However, increased level of IFN-γ was presented in the control group. Polysaccharides combined with vaccine blunted the production of IFN-γ, and lessened pulmonary injury in this study.

**Conclusions**

Our findings indicated that mice vaccinated with conventional vaccine combined with oral polysaccharides had stronger protective effects than mice receiving vaccine only. Polysaccharides/vaccine could enhance the survival rate of infected mice, and decrease pulmonary infection. Therefore, the conventional inactive vaccine combined with oral polysaccharide may be helpful to prevent influenza virus infection.

**Acknowledgements**

Yun Chang, Xiangliang Yang, Minghua Hu and Yanhong Zhu designed the research; Qian Zhang and Lu Xu conducted the research; Xiangliang Yang and Minghua Hu reviewed all experiment. Yanhong Zhu and Yun Chang wrote the paper. All authors read and approved the final manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work is supported by the grant of Natural Science Foundation of Hubei Province (2015CFA041).

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