Effects of the Subaleurone Layer of Rice on Macrophage Activation and Protection of Pollen Allergy in a Murine Model

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Abstract. *Background/Aim: Oral intake of lipopolysaccharide (LPS) has been demonstrated to be effective in the prevention of various diseases. We have found that the subaleurone layer of rice contains a large amount of LPS. The aim of this study was to evaluate the role of this layer in innate immunity. Materials and Methods: Using the Saika-style rice polishing process, a sbaleurone layer and the rice retaining a subaleurone layer and polished white rice were prepared from brown rice. Using hot-water extracts from rice, LPS content was measured by the Limulus reaction and the effect of activation of macrophages was evaluated on the basis of their phagocytic activity and nitric monoxide (NO) and tumor necrosis factor (TNF) production levels. Toll-like receptor (TLR)-2-, TLR-4- and TLR-9-transfected human embryonic kidney (HEK) cells were used to identify the activation pathway. An allergy mouse model was used to evaluate the prevention of pollen allergy. Results: When compared to polished white rice, rice retaining a subaleurone layer had a 6-fold increase in LPS and an increased macrophage activation when phagocytic activity and NO and TNF production were used as indices. TRL4 was the major pathway for such activation. Anti-allergy test by oral intake of subaleurone showed a significant preventive effect for pollen allergy. Conclusion: Compared to polished white rice, rice retaining a subaleurone layer contained a high level of LPS with higher macrophage activation. Furthermore, oral administration of the rice demonstrated a preventive effect for pollen allergy, thus indicating its utility as a functional food that has a regulatory effect on innate immunity.*

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Oral intake of lipopolysaccharide (LPS) has been demonstrated to be effective for disease prevention (1-5). Herbal medicines contain high levels of LPS and have been reported to be effective activators of host innate immunity (6). In Asian countries, rice is the most commonly consumed food, thus, we focused on LPS content in rice and found that the LPS content is higher in brown rice than in polished white rice (data not show).

Brown rice is the rice in which the thick skin (rice hull) is removed and the rice comprises of an embryo bud, endosperm and pericarp. Rice-polishing is a process to remove the bran layer (mainly the pericarp and aleurone layer) of brown rice, which covers the surface, to leave only the endosperm. Rice without the bran layer and embryo bud is called polished white rice. Rice is a staple food in Asia and consumed in large quantities compared to other foods. More often, polished white rice is chosen as the staple food over brown rice in Japan, so the effective utilization of the nutritive content of rice is important for health maintenance. Starch and proteins are the major nutrients of polished white rice; however, the removed bran layer and embryo bud -in the case of polished white rice- contains high levels of vitamin E, vitamin B1, dietary fiber and maltooligosaccharide (6). The bran layer is harder than the endosperm (starch layer) and mostly hydrophobic because of the presence of fat content; hence, when cooked in a rice cooker designed for polished white rice, it causes incomplete gelatinization resulting in poor digestion and texture (8).

In Japan, a technique was developed that enables one to leave the subaleurone layer intact (Saika-style rice polishing process). This process reportedly enables one to remove the bran layer, with poor digestion and texture, leaving the subaleurone layer intact as it is rich in nutrients. The subaleurone layer is known to contain a large amount of nutrients necessary for the germination and growth of rice, including minerals, vitamins and enzymes. Therefore, rice retaining a subaleurone layer contains more vitamins

compared to polished white rice. Plant-derived LPS originated from symbiotic Gram-negative bacteria (9), which existed highly on the tip of roots and surface of leaves and fruits. Since brown rice, a grain seed, showed a high level of LPS, whereas polished white rice had a low level of LPS, it is considered that the LPS levels in rice retaining a subaleurone layer located within the skin should be higher than polished white rice.

Phagocytes (mainly macrophages in mammals) distinguish between self and non-self, including foreign substances and apoptotic cells. The macrophages exist in the front line of the internal and external interface and play an indispensable role in maintaining health and preventing disease by eliminating non-self substances or cells (10), such as β-1,3-glucan and peptidoglycan of lactic-acid bacteria already used as ingredients of health foods. Among food derived-macrophage-activating agents, LPS is best for oral administration to evoke effective and safe way to maintain homeostasis (11). Recent information shows that humans are frequently exposed to low doses of LPS over long periods of time without harmful effect or without impairing their immunological condition (12). The LPS of Gram-negative bacteria are thought to be a useful health food additive for the prevention of allergies or infectious diseases (13).

In the present study, LPS content of rice retaining a subaleurone layer and the role of the subaleurone layer, in terms of the activation of innate immunity, were investigated.

Materials and Methods

Sample preparation. A subaleurone layer, rice retaining a subaleurone layer and polished white rice were prepared from Koshihikari from Nagano (brown rice), harvested in 2011, using the Saika-style rice polishing process for experiments. The subaleurone layer was provided by Toyo Rice Co., Ltd. (Wakayama-shi, Wakayama, Japan). Samples were prepared by adding 10 ml of distilled water to 1 g of each sample; after heat treatment (90˚C, 20 min), samples were sonicated for 15 min, centrifuged for 30 min at 3,500 rpm and the recovered supernatants were used (rice extracts). The displayed values are sample concentrations at the time of extraction.

Identification of a receptor for the detection of macrophage-activating components. To elucidate the Toll-like receptor (TLR) activation pathway of the test samples, human embryonic kidney (HEK) 293 cells transfected with TLR2, TRL4/MD2/CD14 or TLR9 (Invitrogen, AvenueCarlsbad, abbreviated state, USA) were used. These HEK293 cells are characterized to express interleukin (IL)-8 when activated *via* the transfected TLR pathway. Cells were adjusted to contain 4×10^5 cells/ml, then 100 μl of cells were added per well to a 96-well flat-bottom plate and cultured for 24 h at 37˚C. After culturing, cells were washed with 100 μl of fresh culture solution. Test solutions were added into each well at 100 μl and cultured for 24 h. After culturing, ELISA measurements of IL-8 (Biolegend, San Diego, abbreviated state, USA) were conducted using culture supernatants from each well.

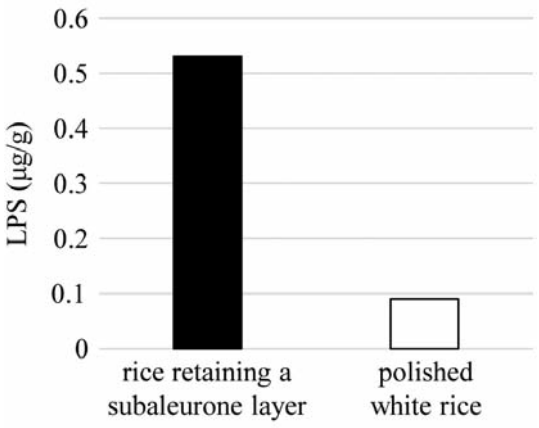

Figure 1. *Measurement of LPS content. LPS content in the prepared rice samples were measured on the basis of Limulus reaction.*

Pollen allergy relief by the subaleurone layer. As the subaleurone layer accounts for approximately 1% of rice, we used a 0.5% subaleurone layer-supplemented feed to simulate the rice containing a subaleurone layer, as well as a 5% subaleurone layer supplemented feed with a higher subaleurone layer content. Basic feed was given to a control group. BALB/c female mice (SLC, Hamamatsu-shi, Shizuoka, Japan) were intraperitoneally administered a re-suspended solution prepared by mixing 30 μg/300 μl of cedar pollen extract (Biostir, Kobe-shi, Hyogo, Japan) and Imject Alum (Thermo Scientific, Waltham, abbreviated state, USA). Three to eight days after the initial injection, the mixture was again intraperitoneally administered. PBS solution was administered to the control group (primary sensitization). Later, 0.5 μg/5 μl of cedar pollen extract antigen solution was administered into the nasal cavity of mice and nasal sensitization studies were conducted for 2 weeks. One week after the first nasal sensitization, cedar pollen extract antigen solution was injected into the nasal cavity of mice and the number of nose-scratching events within 10 min was documented.

Statistical analysis. Statistical analysis was performed using Excel 2008 ver.1.07. The Dunnett's multiple comparison test was used to compare relative gene expression levels.

Results

Measurement of the level of LPS in rice retaining a subaleurone layer. Prepared rice sample extracts were used to measure LPS levels (Figure 1). Rice retaining a subaleurone layer contained 0.53 μg of LPS/g. Polished white rice contained 0.09 μg of LPS/g. The results indicate that rice retaining a subaleurone layer contained a 5.9-fold increase in LPS compared to the LPS content in polished white rice.

Activation of macrophage phagocytosis by rice retaining a subaleurone layer. Macrophages are the fundamental cell population for removing foreign substances and exhaust

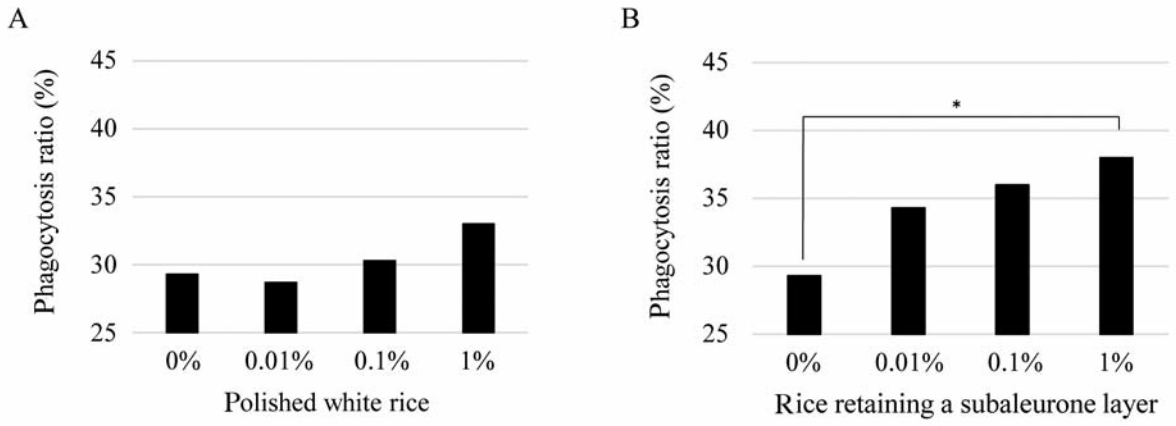

Figure 2. *Measurement of phagocytosis ratio. The mouse macrophage-like cell line J774 was used. Graphs show a comparison of the phagocytic activity of extracts from polished white rice (A) and rice retaining a subaleurone layer (B) using latex beads. The displayed values on the horizontal axis are sample concentrations at the time of extraction. Significant differences (*p<0.05) were obtained between the group of sample and control.*

products (14) and, because of this function, they play a key role in innate immunity. Therefore, we first measured the phagocytic activity of macrophages (uptake of foreign particles within the cell) to evaluate the effect of rice retaining a subaleurone layer. The same samples used for LPS level measurements were used in this experiment. The phagocytic activity of the macrophage-like mouse cell line (J774.1) was evaluated using latex beads (Figure 2). The extract from rice retaining a subaleurone layer facilitated the phagocytic activity at 0.1 mg/ml, which was statistically significant. No such effect was seen in the extract from polished white rice.

Measurements of nitric monoxide (NO) and tumor necrosis factor (TNF) production by rice retaining a subaleurone layer. We compared the activation of innate immunity by rice retaining a subaleurone layer with that caused by polished white rice using the production of NO and TNF. RAW246.7 cells were used as macrophages. As shown in Figure 3, no production of NO was observed when extracts from polished white rice were added at a concentration of 10 mg/ml; however, when 10 mg/ml extract from rice retaining a subaleurone layer were added, NO production was increased. No increase in the production of TNF was observed when extracts from polished white rice were added at 10 mg/ml; however, when 0.1-1.0 mg/ml extracts from rice retaining a subaleurone layer were added, an increase in its production was observed. Rice retaining a subaleurone layer was shown to have higher biological activity compared to polished white rice.

Identification of a receptor for macrophage activating components in rice retaining a subaleurone layer. We hypothesized that LPS, peptidoglycan, β-glucan and bacteria-derived nucleic acid were macrophage activation substances contained in rice retaining a subaleurone layer. To examine their contributions, hTLR2-transfected HEK cells, hTLR4-transfected HEK cells and hTLR9-transfected HEK cells were used and evaluated on the basis of the amount of IL-8 secretion (Figure 4).

HEK cells transfected with TLR2 or TRL4/MD2/CD12 demonstrated a sample dose-dependent increase in the concentration of IL-8. TRL2-transfected HEK cells showed a 1.5-fold increase in IL-8 compared to control in the presence of extracts from rice retaining a subaleurone layer, whereas HEK cells transfected with TRL4/MD2/CD12 demonstrated a 4.1-fold increase compared to control; this indicated the generation of a stronger signal *via* TLR4 than *via* TRL2. Although no increase in IL-8 concentration was observed in HEK cells transfected with TLR9 when milled rice was used, a dose-dependent increase in IL-8 concentration was observed when rice retaining a subaleurone layer was used. Based on the abovementioned observations, macrophage activation by rice retaining a subaleurone layer goes through TLR2, TLR4 and TLR9, with TRL4 being the major pathway. The activating component in rice retaining a subaleurone layer was suggested to be mainly derived from the subaleurone layer.

Pollen allergy relief by intake of subaleurone layer-supplemented feed. Using a BALB/c murine model for pollen allergy, immunized with intraperitoneal or nasal administration of cedar pollen extracts, the degree of symptoms was evaluated on the basis of the number of nose-scratches.

As shown in Figure 5, comparison of mice fed with basic feed, the mice fed with 0.5% subaleurone layer-supplemented feed or 5% subaleurone layer-supplemented feed showed that the number of nose-scratches decreases by 40.5% and 57.8%, respectively. These results suggest that the subaleurone layer does have a relief effect on pollen allergy.

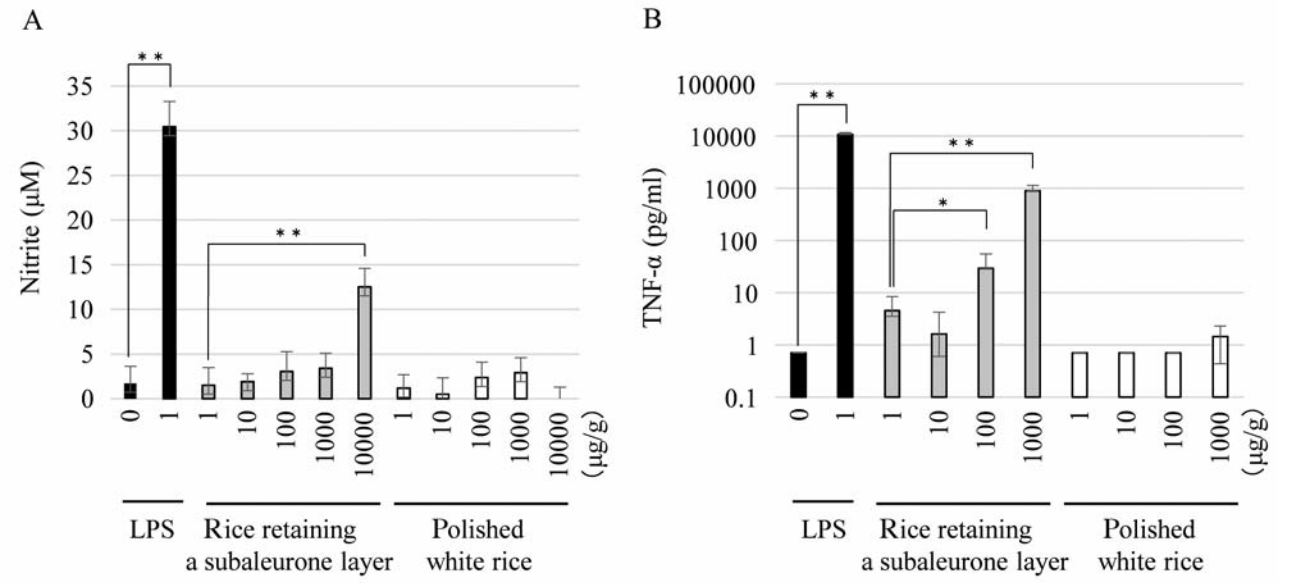

Figure 3. *Measurement of the production of NO and TNF. Bars in black, gray and white indicate the level of NO (A) and TNF (B) production after the addition of purified LPS (positive control), extracts from rice retaining a subaleurone layer or extracts from polished white rice, respectively. The displayed values on the horizontal axis are sample concentrations at the time of extraction. Data are shown as means±SD. Significant differences (**p<0.01, *p<0.05) were obtained between the group of sample and control.*

Discussion

It is reported that LPS is contained in various edible plants and herbal medicines (9). The LPS contained in these plants is mainly derived from symbiotic Gram-negative bacteria. Some of the Gram-negative bacteria are called endophytes, which are known to enter, survive and facilitate the prevention of infection in the plan (symbiotic bacteria) (15). As Gram-negative symbiotic bacteria are known to be present in rice and wheat, we are likely to consume them on a daily basis. Therefore, higher LPS content in rice is possibly due to symbiotic bacteria. We speculate that the amount of LPS in brown rice and in rice retaining a subaleurone layer is increased when compared to that in polished white rice because symbiotic bacteria are deemed to exist mainly in the rice skin.

In the present study, rice retaining a subaleurone layer had an increased LPS content and macrophage activation when compared to polished white rice. According to previous studies, the effective dose of LPS for humans is approximately 10 μg/kg of body weight/day (2-3). Therefore, assuming a person consumes 300 g of rice retaining a subaleurone layer every day, the total amount of LPS is calculated to 159 μg in a day. As the recommended daily intake of LPS is approximately 500 μg, the LPS content in rice retaining a subaleurone layer covers approximately 30% of the recommended amount (2-3).

Oral administration of a subaleurone layer facilitates the prevention of pollen allergy. In addition, as oral administration of LPS has an anti-allergy effect (5), this effect -of the subaleurone layer- likely originates from LPS with the effect mainly caused *via* TLR4; however, TRL2 and TLR9 are also involved and may function synergistically with TLR4.

We have previously found that oral administration of *Pantoea agglomeans*-LPS was useful for preventing hyperlipidemia (rabbit), diabetes mellitus (mouse and human), various infectious diseases (mouse, shrimp), analgesia (mouse, rat and human) *etc*. (16-26) without toxicity (27). *Pantoea agglomerans* can fix nitrogen and phosphorus levels and it adheres to various kinds of plants, such as rice, sweet potato, apple and pear (28-32). These facts suggest that plant symbiotic Gram-negative bacteria have a long history of being consumed in foods (rice, wheat, *etc*.) with apparent safety. LPS in subaleurone layer of rice is considered as the same LPS found in wheat.

Based on previous research, if macrophage activation is successfully induced, factors for various diseases, such as oxidized lipid, denatured protein and glycosylated end product, are actively removed. These are foreign substances generated within the body that have high cytotoxicity and can induce chronic inflammation. Therefore, these factors are reported to trigger lifestyle diseases, including diabetes, arteriosclerosis, atopic dermatitis, pollen allergy, Alzheimer's disease and depression. In the future, using rice with high-LPS content may help us prevent preventive against modern diseases.

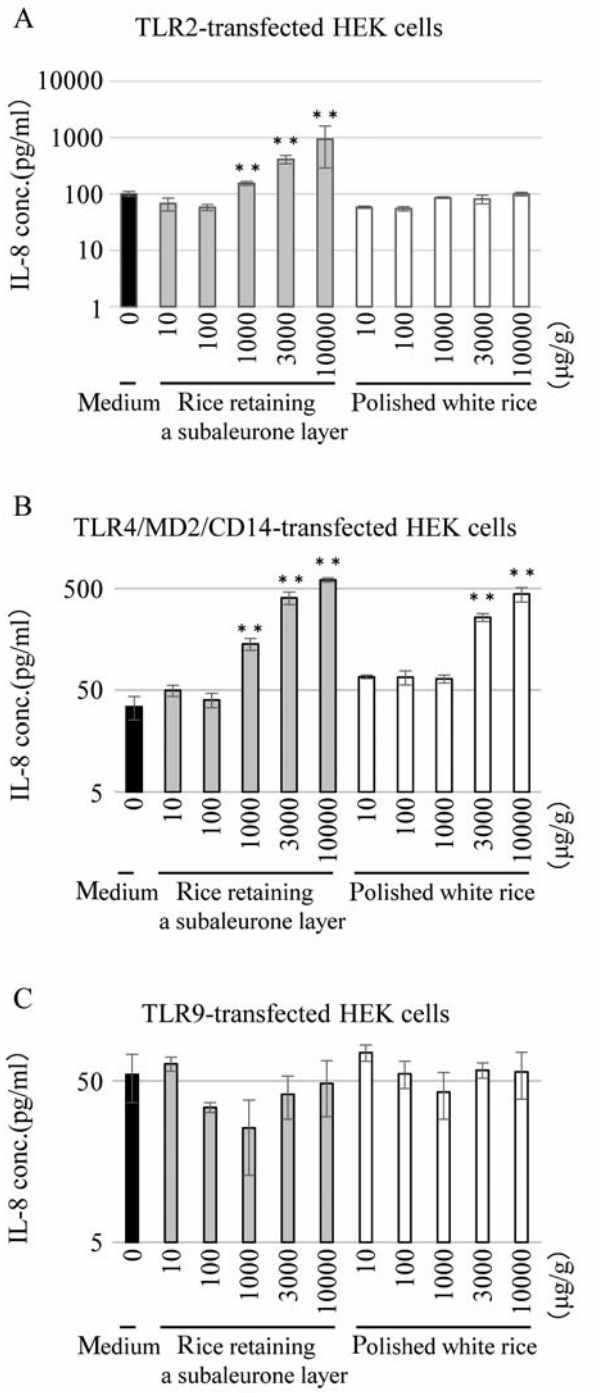

Figure 4. *Measurement of IL-8 production. Extracts from polished white rice and from rice retaining a subaleurone layer were added to each HEK cell line and the concentration of the generated IL-8 was measured using the ELISA method. A: TLR2-transfected HEK cells, B: TLR4/MD2/CD14-transfected HEK cells, C: TLR9-transfected HEK cells (black, medium; gray, extracts from rice retaining a subaleurone layer; white, extracts from polished white rice). The displayed values on the horizontal axis are sample concentrations at the time of extraction. Data are shown as means±SD. Significant differences (**p<0.01) were obtained between the group of sample and medium.*

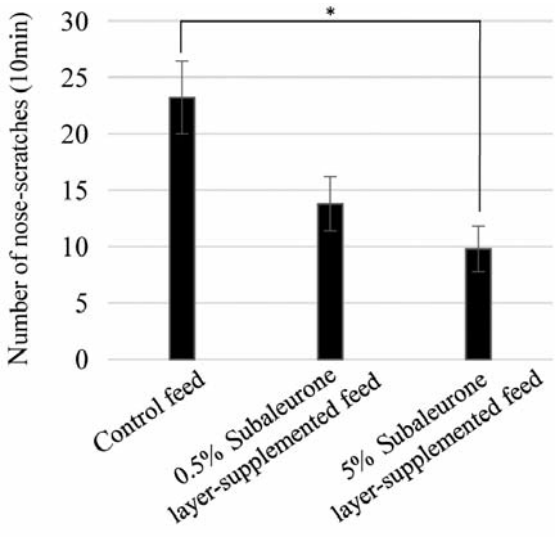

Figure 5. *The number of nose-scratches by pollen allergy murine-models. The left, middle and right columns show the number of nose scratches by mice after being sensitized with pollen that fed with control feed, 0.5% subaleurone layer-supplemented feed or 5% subaleurone layer-supplemented feed, respectively. Data are shown as means±SD. Significant differences (*p<0.05) were obtained between the group of sample and control.*

Conflicts of Interest

The Authors have no financial conflicts of interest.

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