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Potency of Water Extracts of *Rhizopus oryzae* on the *Salmonella* Infection Rat

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Rhizopus oryzae **U-1 water extract (ROU-we) was administered orally to rats at 10 mg/kg body weight for 9 days.** ***Salmonella enteritidis*** **was inoculated at a dose of 10^9 CFU/animal. The following protective effects of ROU-we against infection were examined: cell counts of** ***Salmonella*** **in organs of infected rats (liver, spleen, and mesenteric lymph nodes); phagocytic capacity of collected peripheral monocytes and peritoneal macrophages; cell counts of respective helper T-cell subclasses (Th0, Th1, and Th2); and the leukocyte percentage in peripheral blood. The** ***Salmonella*** **cell count in the liver of the ROU-we group decreased significantly compared to that of the control group and the peripheral monocytes' phagocytic capacity increased 4.5-fold. Moreover, the ROU-we group's Th1 response was higher than the infected control group. However, the healthy control group and the ROU-we group showed similar Th1/Th2 balance and cell count tendencies. These results suggest that ROU-we activated peripheral monocytes and improved Th1/Th2 balance, thereby strengthening immunity against** ***Salmonella*** **infection.**

Keywords: *Rhizopus oryzae,* immunostimulative effect, infection, *Salmonella enteritidis,* Th1/Th2

Introduction

Fungi of the *Rhizopus* genus have traditionally been used in China for fermentation to produce alcohol, and to produce tempeh and fermented soy foods in Southeast Asia. However, one of the *Rhizopus* species, *Rhizopus delemar,* also shows physiologic action to biologic objects. It has been reported that *R. delemar* water extract administration engenders improvement of reproductive functions, such as improving the fertilization rate, fecundity, and hatchability in quails and chickens (Ushikoshi, 1963), extending the mating season in rats (Ushikoshi, 1963), synthetically advancing ovarian steroid hormones in rats and rabbits (Seto *et al.,* 1970; Higuchi *et al.,* 1979; Saito *et al.,* 1980; Horiuchi *et al.,* 1985), and improving the rate of pregnancy in cows (Umezu *et al.,* 1973; Sato, 1976).

In addition to the above characteristics, other effects of *R. delemar* water extract have been reported in recent years. It was clinically confirmed that it improves allergic dermatitis in dogs and cats, and that it is unreactive to steroids as its common curative drug (Abe *et al.,* 2004). Another effect is stepped-up production of superoxides to human postmitotic neutrophils *in vitro* (Zhang *et al.,* 1999). However, the mechanism of *R. delemar* water extract remains unclear.

These recent reports show modulating effects on immune cells as a new function of *R. delemar* water extracts. Superoxide production by neutrophils is essential for protection against infection by microorganisms (Stossel, 1974). Although englobement by neutrophils is essential for protection against infection, *R. delemar* water extract could possibly activate macrophages that facilitate antigen presentation as well as englobement action. However, macrophage and dendritic cells not only have antigen presentation to the adaptive immune system; they also serve an important role in differentiation of naïve T cells into Th1 cells and Th2 cells, and exert an influence on the directional character of proper immunity (Ohteki *et al.,* 1999; Fukao *et al.,* 2000a; Fukao *et al.,* 2000b). Thus, *R. delemar* water extract is expected to excite natural immune systems to intensify proper immunity.

For the assessment procedure in this study, we used model rats infected with *Salmonella* whose Th1-predominant immune state engenders protection against infection, anticipating protection effects on bacterial infection of *R. delemar* water extract. We examined whether oral administration of *R. delemar* water extract to infected model rats can function effectively for protection against bacterial infection through increased macrophage englobement or improved balance between Th1 and Th2 to improve or mitigate the infection state.

Rhizopus delemar is hereafter referred to as *Rhizopus oryzae* because it has been reclassified as *R. oryzae* (Kirk *et al.,* 2001).

Materials and Methods

Fungal strain and ROU-water extract The *R. oryzae* used in this study, U-1, is a fungus that we isolated from fermentation using ground barley and bran as the culture medium. Before incubation at 24 for 4 days, *R. oryzae* U-1 was inoculated to malt extract medium (malt extract, 20 g; polypeptone, 1 g; and glucose, 20 g/l; pH 7). After in-

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cubation, the fungus bodies were collected, suspended in water (10 g/100 ml) at 45 and then stirred for 30 min. This solution was centrifuged at 3000×*g* for 20 min. Then the supernatant was concentrated *in vacuo* to produce a dried powder. The powder was dissolved in distilled water (1 g/10 ml) and filtered using a 0.20-μm filter. This extract solution was used as *R. oryzae* U-1 water extract (ROU-we) in this study.

Bacterial strains and growth conditions *Salmonella enteritidis* JCM 1652, which was isolated from human feces, was obtained from the Japan Collection of Microorganisms. The strain was incubated using nutrient broth (Eiken Chemical Co. Ltd., Tokyo, Japan) at 37 for 18 h.

Animals and Experimental protocols The infection conditions for *S. enteritidis* to rats were referred from precedent reports of the literature (Naughton *et al.,* 1996; Islam *et al.,* 2000; Havelaar *et al.,* 2001). The animals used were 8-week-old specific pathogen-free (SPF) male Wistar rats (Japan SLC Inc., Shizuoka, Japan) with body weights of 190±10 g. The ROU-we solution described above was administered orally at a dose of 10 mg/ROU-we powder per kg of body weight using a probe once a day for 9 days. Subsequently, the rats were fasted overnight, and inoculated orally with 1 ml of nutrient broth containing 1×10^9 CFU/ml *Salmonella enteritidis* JCM 1652 using a probe. In a preliminary experiment undertaken for this study, cell counts detected in organs were unstable for 1×10^8 CFU/rat of *S. enteritidis* administration; for that reason, 1×10^9 CFU/rat was administered. After administration of *Salmonella,* the rats were kept for 5 days under normal conditions. They were then sacrificed to collect blood, mesenteric lymph nodes (MLNs), liver, spleen, and peritoneal exudate cells (PECs). The PECs were collected via peritoneal lavage using 40 ml of Hanks solution (Invitrogen Corp., Carlsbad, CA, USA). Each unit of material was cooled to 4 immediately after collection and used at once for the experiments described below. As control groups, a "healthy control group" without *Salmonella* infection and an "infected control group" with *Salmonella* infection were designated, both without the administration of ROU-we. For both groups, distilled water was administered instead of the ROU-we solution. Furthermore, nutrient broth instead of the *Salmonella* culture fluid was administered to the group without *Salmonella* infection.

Bacterial counts and identification *Salmonella* cell counts per gram of organ tissue (wet weight) were obtained by plate culture. Collected liver, spleen, and MLN tissues were homogenized, serially diluted with saline, then smeared onto DHL agar medium (Eiken Chemical Co. Ltd.). The DHL agar medium was incubated at 37 for 18 h; the black colonies were then counted.

Measurement of phagocytic capacity Peritoneal lavage (Hanks solution) was removed from PECs by centrifugation. The PEC was adjusted to a cell count of 1×10^5 cells/ml using RPMI1640 medium and was incubated for 2 h in 96-well microplates at 37 with 5% CO_2. The buffy coat that was separated from the collected blood was adjusted to 1×10^6 cells/ml using RPMI1640 medium (Invitrogen Corp.) containing 10% fetal calf serum (Nippon Biotest Laboratories Inc., Tokyo, Japan), and incubated for 2 h in 96-well microplates at 37 with 5% CO_2. After incubation, the plates were washed three times with PBS, and cells that adhered to the bottom surface of the plates were determined as peritoneal macrophages and peripheral blood monocytes. The RPMI1640 medium containing 0.5% FluoSpheres (1.0μm, yellow-green fluorescent) (Invitrogen Corp.) was added, incubated for 1 h, then washed three times with PBS. The fluorescence intensity for each well was then measured using a microplate fluorometer (Fluoromark; Bio-Rad Laboratories Inc., Hercules, CA, USA).

Isolation of lymphocytes from spleen Spleen tissue was cut into pieces using a surgical knife in RPMI1640 medium; the cells were filtered with No. 200 nylon mesh and underwent Percoll (Amersham Biosciences, Piscataway, NJ, USA) density gradient separation to separate only the lymphocytes (Hewitt *et al.,* 1980). The lymphocytes were incubated at 37, 5% CO_2, for 10 h; then phytohemagglutinin (PHA) (Remel Inc., Lenexa, KS, USA) was added to the final concentration of 90μg/ml, which was incubated for an additional 8 h.

Flow cytometric analysis of helper T lymphocytes The lymphocytes described above were dyed using a kit (Cytofix/Cytoperm Plus with GolgiPlug; BD Biosciences, San Jose, CA, USA): Cy5-labeled antirat CD4 antibody, FITC-labeled antirat IFN-γ antibody, and PE-labeled antirat IL-4 antibody (all from BD Biosciences). As for IFN-γ and IL-4, cytokines accumulated in cells were dyed directly (Andolph *et al.,* 1999; Cousins *et al.,* 2002). Measurements were carried out using EPICS XL (Beckman Coulter Inc., Fullerton, CA, USA); all cell counts per 5,000 lymphocytes were obtained, respectively, for CD4+/IFN-γ+ as Th1 cells, CD4+/IL-4+ as Th2 cells, and CD4+/IFN-γ+/IL-4+ as Th0 cells.

Measurement of leukocyte count For blood collected from rats, EDTA was used as an anticoagulant. The white blood cell count was measured using an automatic blood cell counter. The percentage was calculated from blood smear specimens for rod neutrophils, filamented neutrophils, eosinophils, lymphocytes, and monocytes.

Statistics The respective measured values were analyzed using Student's *t*-test. Values of $p<0.05$ considered statistically significant.

Results

Figure 1 shows the body weight gain for the 9 days preceding and the 5 days following *S. enteritidis* inoculation. No significant difference was found among groups before *S. enteritidis* inoculation. In terms of the body weight gain for 5 days after inoculation, although the values of healthy controls and the infected controls were 13±2.4 and 17±5.1, respectively, the ROU-we administration group showed a significant increase of 27±5.1 ($P<0.05$, $n=5$).

Figure 2 shows viable cell counts of *S. enteritidis* separated from the MLNs, liver, and spleen. In the MLNs and spleen, cell counts for the ROU-we administration group did not differ greatly from those of the infected

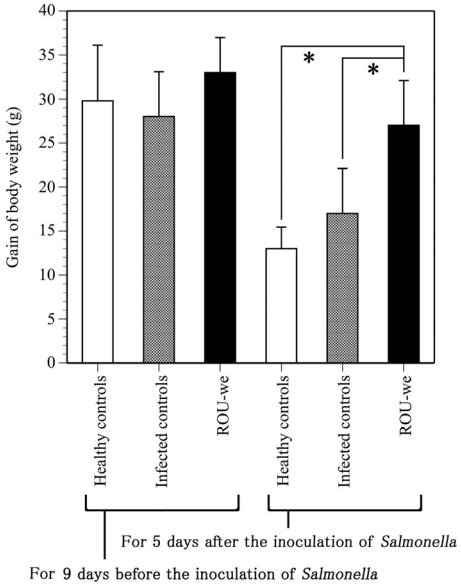

Fig. 1. Body weights during over 9 days prior to and 5 days after *S. enteritidis* inoculation. Distilled water was administered orally to healthy controls (□) and infected controls (▩) every day for 9 days before *Salmonella* inoculation. The solution of ROU-we (10 mg/kg B.W.) was administered to the ROU-we administration group (■) every day for 9 days before *Salmonella* inoculation. The values are increased body weight of each group in both periods (for 9 days and 5 days). The bar above the column shows the standard deviation of values of 5 animals for each group (*: $p<0.05$, $n=5$).

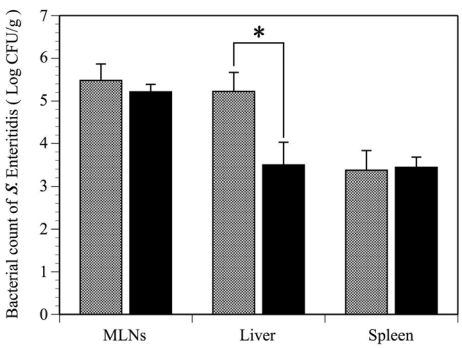

Fig. 2. Viable cell counts of *S. enteritidis* that entered respectively into MLN (mesenteric lymph nodes), liver, and spleen. Infected controls (▩) received distilled water for 9 days, followed by *Salmonella* inoculation; after 5 days, *Salmonella* was detected from organs by plate culture. The ROU-we administration group (■) similarly received the solution of ROU-we (10 mg/kg body weight) instead of distilled water for 9 days, followed by *Salmonella* inoculation. The values represent the logarithm of cell counts per gram of organ tissue. The bar above the column shows the standard deviation of values of 5 animals for each group (*: $p<0.05$, $n=5$).

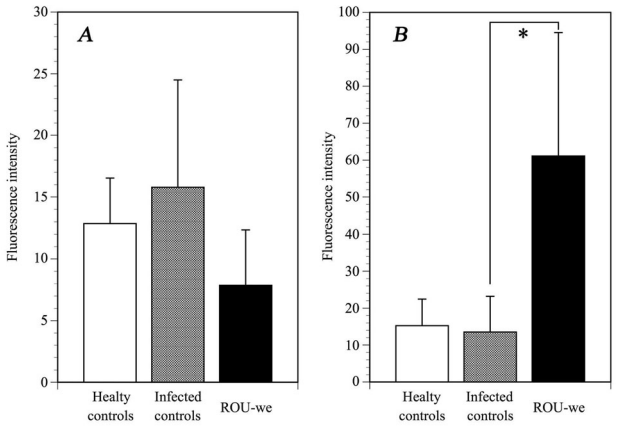

Fig. 3. Measurements of phagocytic capacity of peritoneal macrophages (A) and peripheral blood monocytes (B). □, healthy controls; ▩, infected controls; ■, ROU-we-administered animals. The bar above the column shows the standard deviation of 5 animals for each group (*: $p<0.05$, $n=5$).

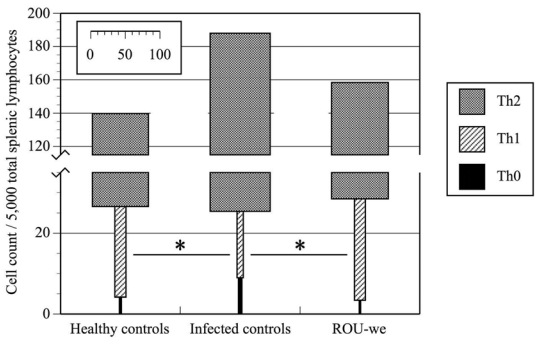

Fig. 4. The number of helper T cells and ratios of each subclass. The values are per 5,000 lymphocytes. The bar width indicates the ratio of each Th-cell to total helper T-cell count as 100% (scale at upper left) (*: $p<0.05$, $n=5$).

control group, but the *Salmonella* viable cell count in the liver decreased significantly (3.5±0.5 log CFU/g vs. 5.2±0.4 log CFU/g by infected controls, $p<0.05$, $n=5$).

Figure 3-A shows the phagocytic capacity of peritoneal macrophages. Each value indicates the fluorescence intensity of fluorescent beads per well, which were phagocytized by cells. For the ROU-we administration group, the value was 7.9±4.5, which was not significantly different from that of the healthy controls (12.9±3.7) or the infected controls (15.8±8). However, the phagocytic capacity of peripheral blood monocytes was 15.3±7.2 for the healthy control group, 13.5±9.7 for the infected control group, and 61.1±33.4 for the ROU-we group, showing 4-fold and 4.5-fold increases, respectively, in the ROU-we group compared to the control groups. This increase was significant for the infected control group ($p<0.05$, $n=5$, Fig. 3-B).

Figure 4 shows the helper T-cell count and the ratio of each subclass to total helper T cells (Th0+Th1+Th2) for respective groups. For the healthy control group, the Th

Table 1. Cell count of leukocytes and differential counts*.

	Total leukocytes (cells/μl)	Differential counts (%)				
		Neutrophils		Lymphocytes	Monocytes	Eosinophils
		Rod neutrophils	Filamented neutrophils			
Healthy controls	7,867	2.0	42.2	49.2	4.3	2.3
Infected controls	10,367	3.4	64.1	27.6	3.9	0.7
ROU-we**	7,367	3.1	61.7	31.5	3.2	0

* It showed mean value of each groups ($n=3$).

** The ROU-we group received ROU-we solution (10 mg/kg body weight) for 9 days, followed by *Salmonella* inoculation.

1 cell count was 22.4±3.9 and the Th2 cell count was 113.2 ±46.6; for the infected control group, the Th1 cell count was 16.4±4.3 with a significant decrease ($p<0.05$, $n=5$). The Th2 cell count of the infected control group showed no statistically significant difference in the 5% risk rate, although an upward trend was observed (162.5±17.9; $p=0.069$, $n=5$). For the ROU-we group, the Th1 cell count was 25.1±2.1 and the Th2 cell count was 129.8±28.8, showing no significant difference regarding the healthy control group; however, a significant increase in the Th1 cell count was observed ($p<0.05$, $n=5$) in the infected control group. The Th2 cell count showed no statistically significant difference at the 5% level, but a downward trend (129.8±28.8; $p=0.076$, $n=5$) was observed in the infected control group. The Th0 cell count was not significantly different among the groups.

Table 1 shows the percentage of leukocytes for the respective groups. In the infected control group, increases were noted in all white blood cell counts because of the remarkable increase in neutrophil count. A decrease in the lymphocyte count was observed, exhibiting a typical white blood cell picture at an early stage of bacterial infection. The total white blood cell count for the ROU-we group was closer to that of the healthy control group than the infected control group. However, the neutrophil/lymphocyte ratio showed a marked increase and rod neutrophils showed a slight increase; consequently, the increase of neutrophils in response to *Salmonella* inoculation was confirmed.

Discussion

This study elucidated the effects of ROU-we on each stage of innate immunity and adaptive immunity using a *S. enteritidis* rat infection model as the model of cytozoic bacterial infection. In the model, *S. enteritidis* reaches the small intestine via oral inoculation. It enters the body by a type III secretion system via M cells, migrates within the living body both hematogenously and lymphogenously, and enters the respective organs (Grutzkau *et al.,* 1990; Kubori *et al.,* 1998). The increase or decrease in the viable cell count of *Salmonella* in respective organs is the clearest and most direct indicator of protection by the test substance against infection in this infection model. The results of this study indicate that ROU-we administration significantly reduced the *Salmonella* viable cell count in the liver, suggesting that ROU-we has some protective effects against *Salmonella* infection. Consequently, the phagocytic capacity of peripheral blood monocytes was measured as a parameter of innate immunity and showed a marked 4.5-fold increase for the ROU-we administration group compared to that of the infected control group, indicating a highly activated status (Fig. 3). Inherent tissue macrophages are present in intestinal mucosa in preparation for invasion of foreign bodies. However, it is important for monocytes in peripheral blood to transmigrate and differentiate rapidly into macrophages to reach the infected site to assist phagocytosis by neutrophils at the early stage of infection, as well as trigger an adaptive immunity that is appropriate for the infecting microorganism. In fact, macrophages might quickly recognize cytozoic *Salmonella* and produce IL-12 and IFN-γ so that Th0 cells differentiate into Th1 cells (Ohteki *et al.,* 1999; Fukao *et al.,* 2000a; Fukao *et al.,* 2000b).

Helper T cells, which play a central role in adaptive immunity, are differentiated from the precursor Th0 cells by various factors including cytokines, mainly to either Th1 or Th2 cells (O'Garra *et al.,* 1998). The Th1 cells produce cytokines such as IL-2, which control the proliferation of T cells; they also produce IFN-γ and TNF-β, which control inflammatory reactions, enhance the proliferation of cytotoxic T cells (CTL) and the arming of macrophages. Th1 cells also participate in protection via cellular immunity against infection by cytozoic microorganisms (such as viruses, *Salmonella, Listeria, Leishmania,* acid-fast bacteria, and *Salmonella typhi*) (Abbas *et al.,* 1996; Mosmann *et al.,* 1996). Th2 cells are involved in protection against infection by extracellular parasitic microorganisms, and the Th2 reaction is expected to be superior under stress or in allergic reactions (Chen *et al.,* 2003). In the present study we expected that when the helper T cell count was measured in the *Salmonella* infection model rats, the infection would be prevented by the increase of Th1 cells in the infected control group. However, an increase of Th2 cells contrary to protection against infection was observed because of the load (stress) of the invasion induced by the large amount of Salmonella (1×10^9 CFU) (Fig. 4). In the ROU-we group, the Th1 cells increased significantly compared to those of the infected control group, but the Th2 cell count revealed a trend toward diminishment, showing a status preferred for protection against infection by cytozoic bacteria. The increase of Th1 cells was from 8.7% to 15.8%, or about twice that by the ratio to total Th cells. Furthermore, the enhancement of cellular immunity from this increase is inferred to strongly bolster protection against infection. Moreover, a slight decrease of Th0 cells was noted in the ROU-we administration group, but a rapid induction of differentiation is also possible considering the increase of Th1 cells. The capability of ROU-we to induce Th0 cell differentiation must be tested *in vitro* in future studies.

The number of neutrophils among white blood cells is reported to increase considerably on day 5 after inoculation of *Salmonella,* reverting to an almost normal value on day 11 in *Salmonella* infection model rats (Havelaar *et al.,* 2001). In the present study, blood collection and cell

counting were also carried out on day 5 after infection, and similar remarkable increases of neutrophils and in the neutrophil/lymphocyte ratio were observed in the infected control group. In the ROU-we administration group, despite inoculation with *Salmonella,* a decreased total white blood cell count and neutrophil counts were evident, at almost the same level as that of the healthy control group. The decrease in neutrophil counts was examined after evaluating the experimental results described above, and we inferred that a recovery from infection status was more rapid than in the infected control group. Resistance against *Salmonella* was also implied because the ROU-we administration group showed a significant body weight gain compared to the healthy and infected control groups after *Salmonella* inoculation (Fig. 1).

In summary, the effects of ROU-we on the *Salmonella* infection model are a specific acceleration of protection against infection in the innate immune system through enhancement of the phagocytic capacity of peripheral blood monocytes, with subsequent facilitation of transition to a superior Th1 reaction of adaptive immunity (increased Th1 cells/decreased Th2 cells). The results of this study further demonstrated the link between the alleviation of infection and immune cell behavior.

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