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Korean Mistletoe, *Viscum album coloratum* Induces Non-Specific Immune Responses in Japanese Flounder, *Paraiichthys olivaceus*

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Effects of Korean mistletoe, *Viscum album coloratum* on the non-specific immune responses of Japanese flounder, *Paralichthys olivaceus* were examined. Flounder were inoculated with mistletoe, Freunds complete adjuvant (FCA), or phosphate-buffered saline (PBS) as a control into their peritoneal cavities. Reactive oxygen intermediate (ROI) products were more enhanced in mistletoe-injected fish kidney phagocytes than in FCA-injected ones. The level of lysozyme activity detected in the serum of fish 4 d after injection with mistletoe was also significantly higher than that found in the serum of the control fish. The appropriate concentration of mistletoe in eliciting the highest level of serum lysozyme activity was 500 μm/300 g of fish. In phagocytic activity assays, mistletoe-sensitized flounder kidney phagocytes captured more yeasts than those of the control fish. Korean mistletoe appeared to be a good activator of the non-specific immune responses of Japanese flounder.

Keywords: Mistletoe, Flounder, Lysozyme, Non-specific immunity, Kidney phagocytes

Introduction

In fish, macrophages are important cells in disease resistance. They are the main effector cells of the innate immune response (Jewhurst et al., 2004) and play a crucial role as accessory cells in both the initiation and regulation of immunity (Shen et al., 2002). Therefore, the non-specific immune responses provide immediate protection against a variety of pathogens. Phylogenetically, fish are the oldest animals showing adaptive immune responses characterized by the presence of lymphocytes able to undergo proliferation in response to specific antigens (Ono et al, 1993). Also fish possess innate immune responses based upon soluble and cellular factors of acute inflammation (Ono et al, 1993). As in mammals, adaptive and innate immunity in fish appear to be coordinated to a large degree by the cytokine network (Jorgensen et al., 2001). In fish, protective immune responses can be primed by vaccination so that the requisite defense parameters are already in place to resist infection. Fish have the aquatic environment with a large variety of diseases. While commercial vaccines are available which successfully induce protection against bacterial diseases, little is yet understood about the nature of the protective mechanisms involved. In addition, there are several other economically important diseases of fish for which no effective vaccines exist. Immunostimulants enhance the non-specific defense mechanisms, thereby preventing infectious disease. Usually, phagocytes of fish treated with immunostimulants show enhanced activity (Sakai, 2001).

Mistletoe is a semi-parasitic woody perennial commonly found growing on oak other deciduous trees. It has been shown that extracts of European mistletoe, *Viscum album* Loranthaceae, *V. album* L. possess a variety of biological activities such as induction of various cytokines (Mueller and Anderer, 1990; Mannel et al., 1991), enhancement of natural killer (NK) cell activity (Kuttan et al., 1992) and immunoadjuvant activities (Mertzer et al., 1985; Hajto, 1986). Moreover, many investigators have demonstrated that the extract of *V. album* L. augmented anti-tumor effect by enhancing the cytotoxic activity of NK cells, lymphokine-activated killer (LAK) cells and macrophages. However, even if the characteristics and biological properties of European mistletoe have been studied intensively, there is only little knowledge about the biological and physiological functions of Korean mistletoe, *Viscum album coloratum*, *V. album* c., a different subspecies of *V. album* from European mistletoe. Recently, some investigators have reported that Korean mistletoe is much more effective in inducing anti-tumor and

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immunoadjuvant activities (Lyu et al., 2000; Yoon et al., 2001). Both *in vivo* and *in vitro* research have so far mainly been focused on the effect of the mistletoe on non-specific immune responses.

In this study, we investigated the effect of Korean mistletoe on the non-specific immune responses in Japanese flounder. Chosen parameters, e.g., reactive oxygen intermediates (ROI), lysozyme activity and phagocytosis, were significantly augmented. The extract of Korean mistletoe would be utilized in elevating fish immune responses.

Materials and Methods

Fish

Japanese flounder, *Paralichthys olivaceus* weighing about 300 g were purchased from a commercial fish farm and acclimated for 2 wk to laboratory conditions in 70 L glass aquaria with recirculated and aerated water at 21-23°C, and fed daily with commercial diet during the adaptation and experimental period. They were acclimated to this environment for at least 2 wk prior to use. The health status of the animals was checked daily, and they never presented clinical symptoms and none died.

Reagents

Nitroblue tetrazolium (NBT), Percoll, and Minimum essential medium (MEM) were purchased from Sigma Chemicals CO. Hanks balanced salt solution (HBSS), fetal bovine calf serum (FBS), antibiotic-antimycotic were obtained from Gibco BRL, Grand Island, NY. Sodium nitrite, sulfanylamide, phosphoric acid were purchased from ICN Biomedicals. Bakers yeast, *Saccharomyces cerevisiae* purchased from Oriental Yeast Co. Ltd., Tokyo, and thioglycollate broth was obtained from Difco Laboratories, Detroit, U.S.A.

Extraction of mistletoe

Mistletoe growing at oak in January were harvested from Kangwondo, Korea. Used mistletoe were 1 or 2 years old, and their leaves, trunks, and fruits were cut to two joint from end of a branch followed by washing with distilled water (D.W.) and drying. The vaccum wrapped mistletoe were stored at -80°C until extract. The lyophilized leaves and trunks of mistletoe were hashed up and washed in D.W. through ion exchange resin. After washing, they were pulverized at mixer for 2 min and stirred for 16 h at 4°C. Mistletoe were centrifuged at 10,000 rpm for 30 min at 4°C and the suspension was passed through and filtered with different pore sizes, 7.2, 0.45, and 0.22 μm, successively. The mistletoe extract named KM-110 was lyophilized and resuspended with D.W. in an appropriate dilution factor.

Injection of mistletoe

The flounder were divided into 5 groups of 4 or 5 fish per group. Fish in each group were intraperitoneally (I.P) injected with 200, 500, 1000 or 1500 μg/fish of mistletoe in 0.5 ml of phosphate buffered saline (PBS), respectively. The remaining group of fish was injected with an equivalent volume of sterile PBS as a control. At day 4 post-injection, blood and head kidney leucocytes were obtained from fish per group. In our preliminary study, the induction of non-specific immune response by mistletoe was the highest at day 4 post-injection.

Isolation of head kidney (HK) leucocytes

The method described by Santarem et al (1997) was used with some modification. The flounder HK previously sensitized with mistletoe was dissected out by a ventral incision, cut into small fragments and transferred to 5 ml HBSS, respectively. Cell suspensions from head kidney were obtained by teasing the HK tissues with two slide glasses in HBSS in a Petri dish (Coring). After sedimentation of tissue debris at 4°C for 1 min, the supernatants were removed. HK cell suspensions were layered over a 34-51% Percoll gradient and centrifuged at 2500 rpm for 40 min at 14°C. After centrifugation, the bands of leucocytes above the 34-51% interfaces were collected with a Pasteur pipette and washed twice at 1200 rpm for 8 min in HBSS. The viable cells concentration was determined by trypan blue exclusion.

Serum

Blood was collected from the dorsal aorta of the flounder. It was allowed to clot at 20°C for 30 min and cooled at 0°C for 1 h. Serum was obtained by centrifugation at 2500 rpm for 8 min. The sera were frozen at -20°C until used.

ROI production assay

ROI production from founder kidney cells after administration with the mistletoe was assessed by monitoring their ability to reduce nitroblue tetrazolium (Secombes, et al., 1988) The leucocytes (10^5) were washed one time with HBSS at 1000 rpm for 3 min at 4°C and incubated in 100 μl of complete media in the presence of phorbol myristate acetate (PMA, 1 μg/ml)

and NBT (mg/ml). After 1 hr at 25°C, excess amount of NBT was washed out with PBS and the leucocytes were fixed with 70% methanol. After discarding 70% methanol, the leucocytes were washed twice with PBS. The reduced formazan was solubilized with 120 μl KOH and 140 μl dimethyl sulphoxide (DMSO) and optical density values were read at 620 nm in an ELISA reader (ASYS HITECH, Austria).

Lysozyme Activity

Serum lysozyme activity was measured using a modified turbidimetric microtitre plate technique according to Ellis (1999). Briefly, a standard suspension of 0.15 mg/ml *Micrococcus lysodeikticus* (Sigma) was prepared in 66 mM phosphate buffer (pH 6.0). flounder serum (50 μl) was added to 1 ml of bacterial suspension, and the decrease in absorbance was recorded at 0.5 and 4.5 min intervals at 450 nm in a spectrophotometer (SHIMADZU UV-1600PC). One unit of lysozyme activity was defined as reduction in absorbance of 0.001/min.

Phagocytic Activity

Flounder head kidney leucocytes injected with mistletoe or PBS were adjusted to 1×10^6 cells/200 μl/well in 5% FBS-MEM and dispensed in 8-well slide chamber (Nunc) followed by overnight incubation at 25°C. Following incubation, 1×10^7 cells/ml of heat-treated (100°C, 1 h) Bakers' yeast were added. The mixture was incubated at 25°C for 1 h with occasional shaking and 50 μl of the mixture was smeared on a glass slide, air-dried and stained with Wrights solution. The percentage of yeast-ingested phagocytes (A) and the amount of yeast ingested per phagocyte (B) were calculated by enumerating 500 phagocytes under a microscope, and phagocytic index (PI) (Matsuyama et al. 1992). % phagocytosis = number of cells ingesting yeasts/number of cells observed×100. PI = number of cells ingesting beads/number of cells observed ×number of yeasts ingested/number of cells observed×100

Results

ROI production from flounder kidney leucocytes

The results of the ROI production are shown in Fig. 1 and 2. The level of ROI production was highly augmented in kidney cells from flounder injected with 500 or 1000 μg/fish of mistletoe compared with the control (Fig. 1). ROI product was more enhanced in kidney cells from fish injected with 500 μg of mistletoe than those injected with Freund's complete adjuvant (FCA) (Fig. 2)

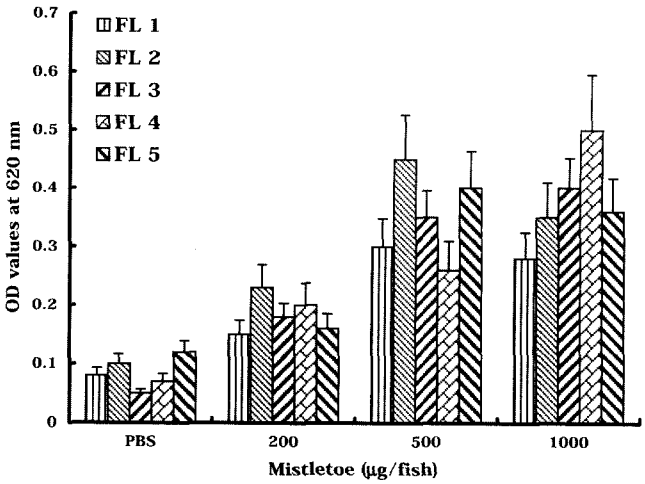

Fig. 1. NBT reduction by head kidney leucocytes from flounder after injection with mistletoe. The products of ROI were assayed by NBT reduction by kidney macrophages from 5 different fish. Results are expressed as the mean absorbance per 10^5 cells at 620 nm for triplicate samples±SD for each fish. FL, flounder. The results are representative of four experiments.

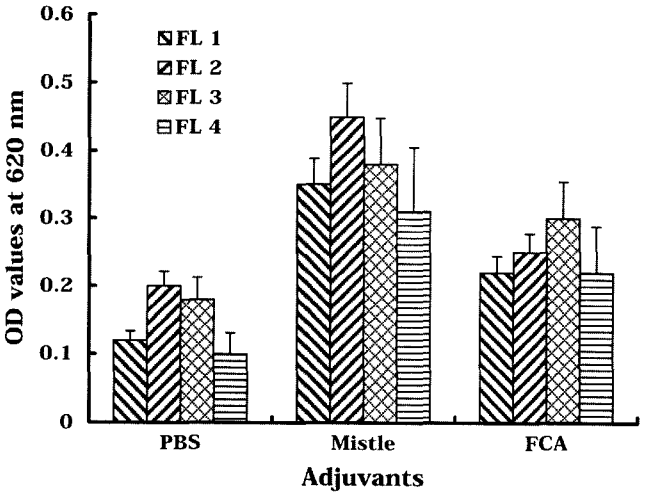

Fig. 2. NBT reduction by head kidney leucocytes from flounder after injection with mistletoe or FCA. Mistletoe (500 μg/0.5 ml of PBS/fish), 0.5 ml of PBS or 0.5 ml of 1:1 emulsified FCA were injected to 4 different fish per group, respectively. Results are expressed as the mean absorbance per 10^5 cells at 620 nm for triplicate samples±SD for each fish. FL, flounder. The results are representative of three experiments.

Lysozyme activity

To study whether mistletoe has an impact on increasing lysozyme activity, sera were harvested from flounder treated with 500, 1000 or 1500 μg/fish of mistletoe. Fig. 3 shows lysozyme activity in serum of flounder sensitized with mistletoe. Mistletoe-injected flounder shows high level of lysozyme activity compared to the control fish. The appropriate concentration of mistletoe in eliciting the highest level of serum lysozyme activity was revealed to 500 or 1000 μg/fish (Fig. 3).

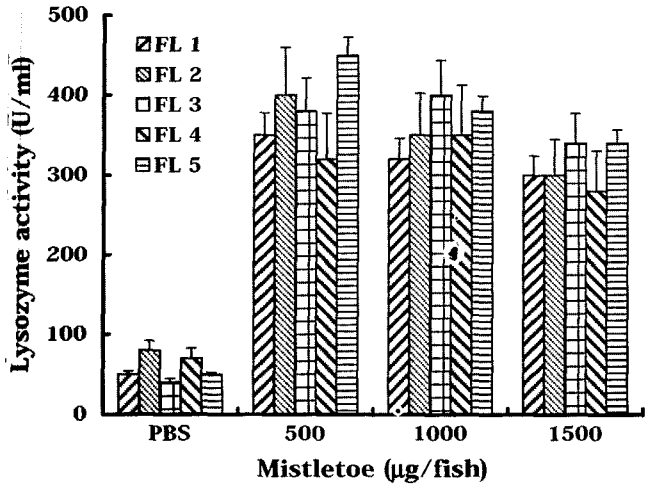

Fig. 3. Serum lysozyme activity of flounder injected with mistletoe. Results are expressed as the mean units/ml of triplicate serum samples±SD for each fish. One unit of lysozyme activity was defined as reduction in absorbance of 0.001/min. The results are representative of four experiments.

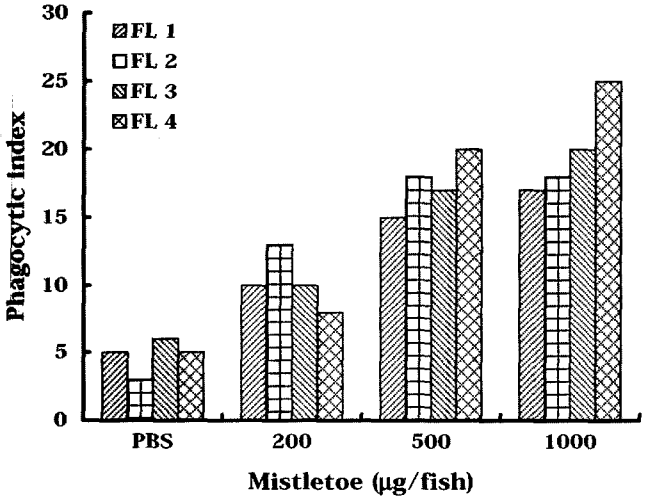

Fig. 4. Phagocytic activity of flounder kidney leucocytes injected with mistletoe. Fish were injected intraperitoneally with 0, 200, 500 or 1000 μg/ fish of mistletoe. Phagocytic activity was measured on day 4. The results are representative of three experiments.

Phagocytic activity

As shown in Fig. 4, Phagocytes from mistletoe-injected (500 or 1000 μg/fish) flounder were observed to engulf more yeasts than those from the control fish. Considering an induction of phagocytic activity by mistletoe treatment, no significant phagocytic difference was observed between 500 μg and 1000 μg-injected flounder (Fig. 4).

Discussion

We have studied whether non-specific immune response of flounder kidney leucocytes was affected by the injection of mistletoe. Mistletoe, *V. album* is native to both Europe and Asia. *V. album* grows in Europe, northwest Africa, and southwest and central Asia and Japan: the Asian plant is a special variety. It is propagated by birds that eat the berriers and then excrete by wiping the sticky pulp off their beaks. Mistletoe contain lectins, protein toxins, and polysaccharides. Also, mistletoe's lectins are cytotoxic glycoproteins of approximately 10,000 molecular weight and activates macrophages and lymphocytes, leading to secretion of various kinds of cytokines. Lectins are believed to mediate pathogen recognition, which can lead to neutralization of the invading organism (Vasta et al., 1994; Weis et al., 1998) during the early stages of an infection (Holmskov et al., 1994; Ni and Tizard, 1996; Lu, 1997). Mistletoe, therefore, has been widely used for therapeutic purpose in Europe and in Korea as well. Furthermore, mistletoe preparations had increase in natural killer cells, T-helper cells, cytokine release, and peripheral blood mononuclear cells and lymphocytes. In mammalians, Korean mistletoe has shown 10 to 1000 times higher anti-tumor effect than Europe mistletoe. Administration with mistletoe showed an increased non-specific immune response. In our preliminary study, eel kidney leucocytes were not able to induce *in vivo* or *in vitro* nitric oxide (NO) production when administrated with mistletoe (data not shown). In gilthead seabream head-kidney leucocytes, however, macrophage activating factor (MAF) could induce NO production by macrophages when used in conjunction with bacterial lipopolysaccharide (LPS) (Mulero and Meseguer, 1998). Also, concerning turbot macrophage NO production induced by LPS (Tafalla and Novoa, 2001), NO was produced from coelomocytes of the echinoderm starfish, *Asterias forbesi* upon stimulation with several different microbial products (Gregory et al., 2001). But there is increasing evidence that fish are able to generate reactive nitrogen species such as NO (Cameron, et al., 2003; Singru et. al., 2003). Therefore, it is necessary to further study whether flounder kidney phagocytes do not possess the mechanism to synthesize NO against foreign stimulators or mistletoe itself is not able to induce NO synthesis from kidney phagocytes. On the other hand, ROI production from flounder kidney leucocytes was readily elicited by the mistletoe administration, suggesting that ROI might be better activation indicator than NO in flounder kidney leucocytes. In fact, ROIs such as the superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen, play an important role in the antimicrobial activity of phagocytic cells. In *in vivo* experiments, the level of ROI production was highly augmented in kidney leu-

cocytes from flounder injected with 500 or 1000 μg/fish of mistletoe. At higher concentration of mistletoe, ROI product was much more expressed. However, mistletoe with more than concentration of 1000 μg/fish (300 g) failed to up-regulate the further induction of ROI production. Usually, immunostimulants do not show a linear reaction between doses and effect but a maximum effect at intermediate doses and no effect and even toxicity at high doses (Bliznakov and Adler, 1972; Gialdroni-Grassi and Grassi, 1985). The fact has been established in fish through *in vivo* (Kenyon et al., 1985; Anderson and Jeney, 1992) and *in vitro* studies (Siwicki et al., 1990). In *in vitro* experiment, 10 μg/ml of mistletoe was revealed to an optimal concentration to induce ROI production without any cellular cytotoxicity. In rechallenging test, mistletoe failed to reactivate flounder leucocytes with an elevated ROI production (data not shown), indicating that non-specific fish immune response is similar to a mammalian system regarding to a cellular memory process.

According to Fulton (1957) and Dianoux and Jolles (1969), 'true' lysozymes have to satisfy the following criteria: (1) the enzyme lyses *Micrococcus lysodeikticus* cells; (2) is readily adsorbed by chitin-coated cellulose; (3) is a low molecular weight protein; and (4) is stable at acidic pH at higher temperatures, but is inactivated under alkaline conditions. In the assay of lysozyme activity in serum from the mistletoe-injected fish, it was found that mistletoe plays a critical role in evoking lysozyme activity from flounder kidney phagocytes. In the injection of 1500 μg/fish of mistletoe, lysozyme activiy was down-regulated relatively to a low concentration of mistletoe.

It is necessary to ascertain that phagocytes are working on phagocytosis in the presence of foreign pathogens. In our study, instead of some pathogens, yeasts were treated to either phagocytes from mistletoe-injected flounder or those from mistletoe non-treated flounder. Expectedly, phagocytes from mistletoe-treated flounder were aggregated together and bunch of yeasts were engulfed by the phagocytes, indicating that mistletoe play an excellent role in activating the non-specific immune response in flounder.

In conclusion, considering that most of all indicators for non-specific immune mechanism were revealed with positive results, Korean mistletoe could be a promising immunoadjuvant inducing the non-specific immune response in fish. Based on our preliminary results, the specific immune response by mistletoe needs to be further studied and further fish immunoadjuvant for a diet should be developed in future.

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