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Differences in Biological Response Modifier-like Activities According to the Strain and Maturity of Bananas

Haruyo IWASAWA* and Masatoshi YAMAZAKI

Department of Medical Life Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-machi, Sagamihara-city, Kanagawa 229-0195, Japan

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We have studied the neutrophil-increasing effects of fruits and vegetables and their priming effects on cytokine induction. Among fruits, bananas exhibited the most marked priming effects. Therefore, we evaluated possible differences in the biological response modifier (BRM)-like activities of bananas (such as the effects on neutrophil accumulation and macrophage morphology, and the priming effects on cytokine induction), according to their strain and maturity, using a conventional strain and a strain for highland cultivation. As a result, the total intraperitoneal leukocyte count and % neutrophils increased in parallel with the concentration and maturity of banana extracts. These effects were more marked in the highland strain. After the addition of banana extracts, marked macrophage spreading was observed, and morphological changes differed according to the strain and maturity of bananas. The priming effects on TNF-α or IL-12 induction also differed according to the maturity and strain of bananas, and could also be confirmed after oral administration. These results suggest that banana intake is associated with various BRM-like activities, and these effects differ according to the maturity level of the bananas.

Keywords: banana, BRM, macrophage, cytokine, strain, maturity

Introduction

In conventional dietetics, much importance has been attached to nutrients and only minor attention has been focused on other components. However, foods contain not only nutrients but also large amounts of compounds called phytochemicals. About 10,000 types of phytochemicals are considered to be present in nature. In our department, the various effects of foods on immune function have been studied (Yamazaki, 1992; Yamazaki and Ueda, 2000). The immune system is an important biological defense mechanism and its balance is maintained by various factors. The immune system is affected by the nutritional state of the body and is impaired in states such as malnutrition (Sakamoto, 1992). Therefore, to maintain an effective immune system, optimal nutrient intake is necessary. However, for further improvement and enhancement of immune capacity, the effects of phytochemicals are also marked. Phytochemicals have been shown to play various important roles in protecting the body from disease, and we have reported on their anti-oxidative, anti-inflammatory, anti-allergic, and anti-cancer effects (Yamazaki and Ueda, 1997; Ueda and Yamazaki, 1997; Ueda and Yamazaki *et al.*, 1999; Ueda and Yamazaki, 1997).

To evaluate immune function, cytokines, which are physiologically active substances produced by leukocytes, are often measured due to their quantifiability, sensitivity, reproducibility, and significance. For example, phagocytes, such as macrophages, produce cytokines, e.g., interleukin-1 (IL-1), IL-6, IL-8, IL-12, tumor necrosis factor (TNF), and interferon, when stimulated or activated (Yamazaki and Iwasawa, 2004). In addition to cytokines, many compounds such as bacteria, their cell components, and neutral polysaccharides stimulate immune function (Ohashi and Ueda *et al.*, 1992). Some of these compounds are used as biological response modifiers (BRMs) (immunostimulants). Therefore, the measurement of induced cytokines facilitates estimation of the leukocyte activation state, i.e., immunological state. For the production of cytokines, such as TNF, by macrophages, 2-stage stimulation is necessary, i.e., priming stimulation for macrophage activation and triggering stimulation for cyto-

*To whom correspondence should be addressed.
E-mail: ricky@pharm.teikyo-u.ac.jp

kine release from activated macrophages. Priming stimulation is essential for macrophage activation and is known to be induced by BRMs. Therefore, evaluation of the priming effects is appropriate for measurement of the cytokine-induction ability of food components.

We have evaluated the neutrophil-increasing effect of common fruits and vegetables, and their priming effect on TNF-α induction (Yamazaki, 1992; Yamazaki and Ueda, 2000; Yamazaki and Ueda, 1997; Maeda and Ueda *et al.*, 1997; Yamazaki and Iwasawa, 2005). Among vegetables, garlic, ginger, cabbage, eggplant, and Japanese radish, which have not been considered important in terms of nutrition, showed marked activity levels comparable to those of immunostimulants. Among fruits, banana, kiwi, watermelon, and grape revealed marked activities, and the activity of banana was comparable to that of lentinan, a chemical immunostimulant. There are about 300 wild and 20 edible strains of banana. Some studies have compared the amounts of components in bananas among strains and at varying maturity levels (Kanazawa and Sakakibara, 2000; Suzuno and Ishida, 2005; Garcia-Moreno and Nogales-Alarcon *et al.*, 1980), but no studies have compared BRM-like activities between strains and levels of maturity. Therefore, using a conventional strain and that for highland cultivation, we evaluated possible differences in BRM-like activities (effects on neutrophil accumulation and macrophage morphology, and priming effects for cytokine induction) between the two strains and among maturity levels.

Materials and Methods

Banana (Musa species) Common edible Cavendish (regular banana), a commercially available strain, and Sweetio, a highland cultivar (grown at more than 500 m above sea level), were kindly donated by Dole Japan, Ltd. immediately after ethylene gas spraying for ripening. The day of ethylene spraying was designated as day 0. Maturity levels on days 1, 3, 5, 7, and 10 were evaluated. In the color chart, day 1 corresponds to 2, day 3 to 5-6, and day 5 to 7. Brown spots (sugar spots) on the peel increased after day 7, and the entire banana peel turned brown on day 10. The flesh was weighed, cut into appropriate sizes, and mixed with a 3-fold weight of deionized water using a mixer for 30 seconds. The homogenate was centrifuged at 3,000 rpm for 5 minutes, the supernatant was obtained and passed through a filter, and the suspended substances were removed to obtain the sample solution. The osmotic pressure and pH of the sample were adjusted for intraperitoneal (*i.p.*) administration.

Mice Male ICR mice (5 or 6 weeks old) were purchased from Sankyo Labo. Service Corporation, INC. (Tokyo, Japan). C3H/HeNCrl mice (6 weeks old) were purchased from Oriental Yeast Co., Ltd. The animals were given a standard laboratory diet and water *ad libitum*. Experiments were conducted following the Guidelines for Animal Experimentation (No. 105) and Notification (No. 6) implemented by the government.

Media RPMI1640 was purchased from Nissui Seiyaku Co. and used for the incubation of peritoneal cells.

Measurement of accumulated neutrophils Male ICR mice were injected *i.p.* with 0.5 or 0.05 mL of banana juice. Peritoneal exudate cells (PEC) were collected in saline after 6 hours and added to a 5% volume of FCS. Some of the PEC were stained with Türk reagent and counted under a light microscope. A portion of the remaining cells was placed on a glass slide, using an auto cell smear, to assess the percentage of neutrophils in peritoneal cells by direct counting under a light microscope.

In vitro observation of differences in morphological changes of macrophages Peritoneal cells were collected in saline from male C3H/HeNCrl mice. Peritoneal cells were cultured in 24-well plates at 3.0×10^4 cells / mL of 5%FCS-RPMI / well at 37°C in 5% CO_2 for 2 hours. The wells were washed twice with warm phosphate-buffered saline (PBS) (-) before the addition of 1 mL of 5%FCS-RPMI containing banana juice. The wells were observed under a light microscope from days 1 to 7.

Measurement of priming activity for cytokine production after i.p. administration Male ICR mice were injected *i.p.* with 0.5 mL of banana juice. PEC were obtained after 4 days. These cells were cultured in 96-well plates at 1.0×10^6 cells in 200 μL of 5%FCS-RPMI / well at 37°C in 5% CO_2 for 2 hours. One hundred microliters of the supernatant was discarded and replaced with 100 μL of 5%FCS-RPMI containing 10 μg of *Enterococcus faecalis*. Two hours later, the TNF-α concentration of the culture supernatant was evaluated by ELISA (Endogen, Pierce Biotechnology Inc., U.S.A.). Twenty hours later, the IL-12 concentration of the culture supernatant was evaluated by ELISA.

Measurement of priming activity for cytokine production after per oral (p.o.) administration Male ICR mice were *p.o.* administrated 5% banana juice for 2 weeks *ad libitum*, excluding weekends. PEC were obtained and cultured in 96-well plates at 1.0×10^6 cells in 200 μL of 5%FCS-RPMI / well at 37°C in 5% CO_2 for 2 hours. One hundred microliters of the supernatant was discarded and replaced with 100 μL of 5%FCS-RPMI containing 10 μg of *Enterococcus faecalis*. Two hours later, the TNF-α concentration of the culture supernatant was evaluated by ELISA (Endogen, Pierce Biotechnology Inc., U.S.A.). Twenty hours later, the IL-12 concentration of the culture supernatant was evaluated by ELISA.

Measurement of dopamine and serotonin contents of bananas For dopamine, 4 g of banana peel or pulp was added to 2 mL of methanol (Wako Pure Chemical Industries, Osaka, Japan) and mashed well. For serotonin, 4 g of banana peel or pulp was mashed well with DIW. Homogenized substances were extracted at 3000 rpm for 5 minutes. The resulting supernatants were subjected to thin layer chromatography (TLC) and sprayed with ninhydrin reagent. Chromatographs were warmed at 90°C for 10 minutes. Dopamine and serotonin levels were determined colorimetrically. Dopamine and serotonin standards were purchased from Wako Pure Chemical Industries (Osaka, Japan) and ICN Biomedicals INC. (Hyland Ave., CA, U.S.A.), respectively.

Results

Differences in neutrophil accumulation activity according to bananas strain and maturity level Using bananas on day 3, which had turned yellow and were ready to eat, the dose-response in terms of neutrophil accumulation was evaluated. In general, the intraperitoneal leukocyte count in mice is about 5×10^6 cells and the percentage of neutrophils is only about 5%. However, after inoculation with an immunostimulant, neutrophils increase to 60-90%. The leukocyte (PEC) count in the group injected with 0.05 mL of banana extract was similar to that in the control mouse group. However, the leukocyte counts following administration of 0.5 mL of the regular and highland banana extracts were 4 and 5 times higher, respectively, than the count in the control group (Fig. 1A). Neutrophils increased to 20% and 30% even with the administration of 0.05 mL of the regular and highland banana extracts, respectively, and to 60% and 90% after 0.5 mL administration (Fig. 1B). These results reveal a dose-dependent increase in the neutrophil-accumulation effect of bananas. Even using bananas on day 10, which showed a large amount of sugar spots on the peel, dose-dependent neutrophil-accumulation was observed (data not shown).

The neutrophil-accumulation activity was more marked for the highland than the regular banana variety, showing a difference between the two strains. Therefore, in addition to strain, the influence of maturity was evaluated after 0.5 mL administration. As a result, both the intraperitoneal leukocyte (PEC) count and neutrophils increased in parallel with maturity (Figs. 2A, 2B). This maturity-dependent activity was also more marked for the highland than the regular banana variety. These results suggest that banana juice contains an immunostimulatory component that increases leukocyte levels, and either this component or its activity increases with the maturity level. The content of this component may be greater in the highland variety.

Differences in macrophage morphological changes in vitro according to banana strain and maturity level Since banana extracts exhibited leukocyte-increasing activity, the morphological changes of leukocytes were evaluated. Macrophages recognize foreign matter, migrate to their sites, and engulf them by extending pseudopodia. Activated macrophages are known to show morphological changes, such as the extending of pseudopodia and an increase in size. Mouse intraperitoneal macrophages were incubated in medium con-

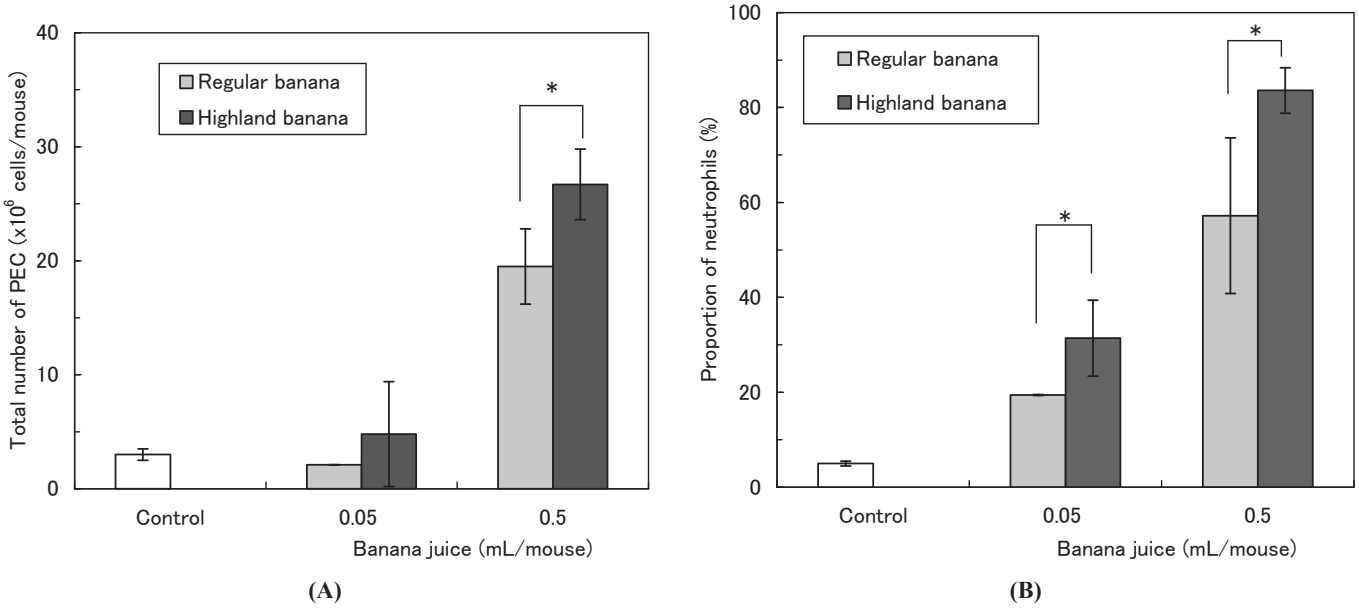

Fig. 1. Dose-dependent neutrophil accumulation by intraperitoneal injection of regular or highland banana juice.
The data is represented as mean ± S.D. (n=3). A : Total cell number of peritoneal exudate cells (PEC). B : Proportion of neutrophils in PEC. Statistical differences were analyzed by Student's t-test. Significant differences were between regular and highland banana. Fig. A: *p = 0.07, Fig. B: *p < 0.05.

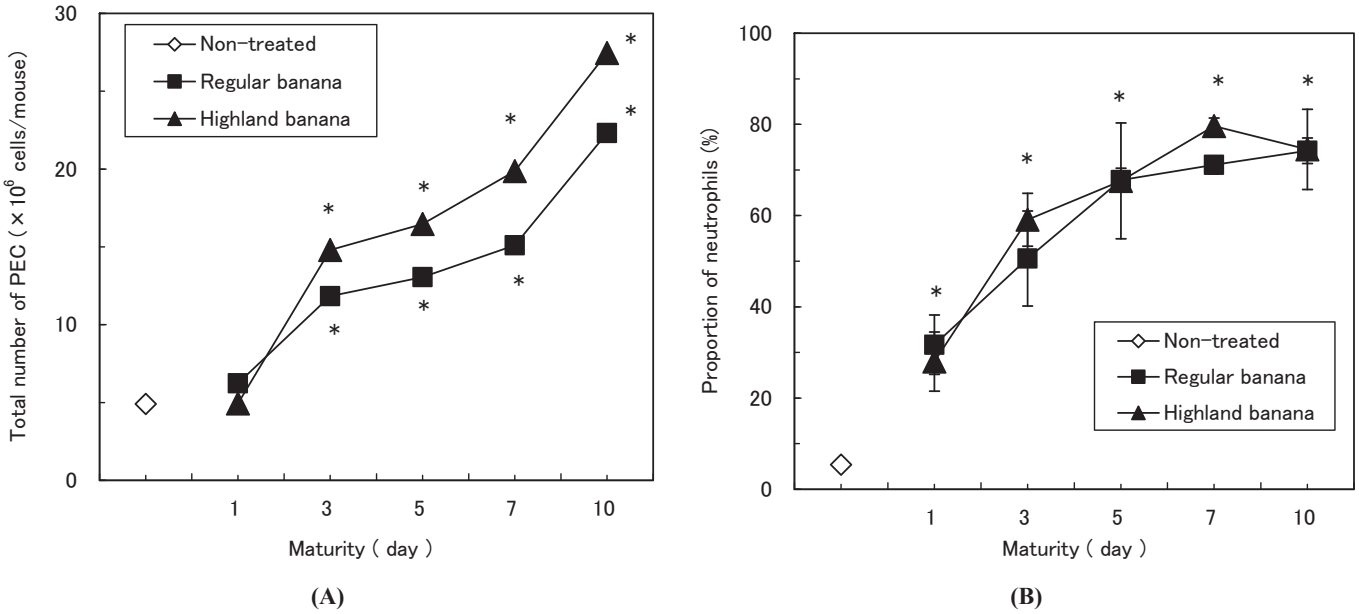

Fig. 2. Differences in neutrophil accumulation by intraperitoneal injection according to banana strain and maturity level.
The data is represented as mean ± S.D. (n=3). A : Total cell number of peritoneal exudate cells (PEC). B : Proportion of neutrophils in PEC. The X-axis represents the maturity of bananas. Statistical differences were analyzed by Student's t-test. Significant differences were from the non-treated group. Figs A and B: *$p < 0.05$.

taining 10% banana extract. No morphological changes were observed immediately after the addition of macrophages. After 2 days, many spread macrophages were observed in the banana-administered group, though a few spread macrophages were also present in the control group (Fig. 3). This macrophage-spreading effect was the most marked 2-3 days after addition of banana extracts. After 1 week, most cells were dead in the control group, whereas in the banana-administered group, most cells remained alive and spread cells were observed. This spreading effect was marked when bananas on day 3 (maturity level) or more were used, and particularly marked when regular bananas on day 3 (after ripening) were used. The concentration of banana extracts in this study was 10%, but even extracts at a low concentration (0.03%) exhibited macrophage-spreading effects.

Macrophage morphological changes differed among banana maturity levels. Elongated macrophages were frequently observed on days 1-5 after ripening, and multi-directional macrophage spreading was frequently observed on days 7 and 10. These results show that banana juice contains a component that induces the morphological change and activation of macrophages.

Differences in the priming effect of i.p. administration on cytokine production according to banana strain and maturity level Since banana extracts increased the leukocyte count and morphologically activated macrophages, their qualitative activation of macrophages was evaluated. The priming effects of *i.p.* sample administration on cytokine production were evaluated first. The priming effects of regular and highland bananas were similar to those of the control on day 1 after ripening, but the levels of TNF-α and IL-12 induction markedly increased on day 3 or more in the banana-administered groups (Fig. 4 A and B). These results suggest that banana juice has marked priming effects on TNF-α and IL-12 induction. The priming effects of banana on TNF-α induction did not markedly change between day 3 and 10, but tended to increase with the maturity level. The priming effects of banana on IL-12 induction increased with the maturity level of each strain, being most marked on day 7, and greater for the highland banana.

Differences in the priming effects of p.o. administration on cytokine production according to banana strain and maturity level Since the priming effects of banana could be confirmed by *i.p.* administration, the priming effects of *p.o.* banana administration were evaluated. In this study, a regular and a highland banana on days 3 and 7, which have been shown to exhibit high-level activity and were ready to eat, were evaluated. As a result, the levels of TNF-α and IL-12 induction increased in all samples, indicating *p.o.* priming effects just like *i.p.* priming (Fig. 5 A and B). Priming effects on both TNF-α and IL-12 induction were more marked on day 7 for the regular banana and on day 3 for the highland banana. At the same maturity levels, priming effects on day 3 were more marked for the highland banana, and those on day 7 were more marked for the regular banana. These results suggest that *p.o.* banana intake also qualitatively increases immune capacity though priming effects, depending on the strain and degree of maturation.

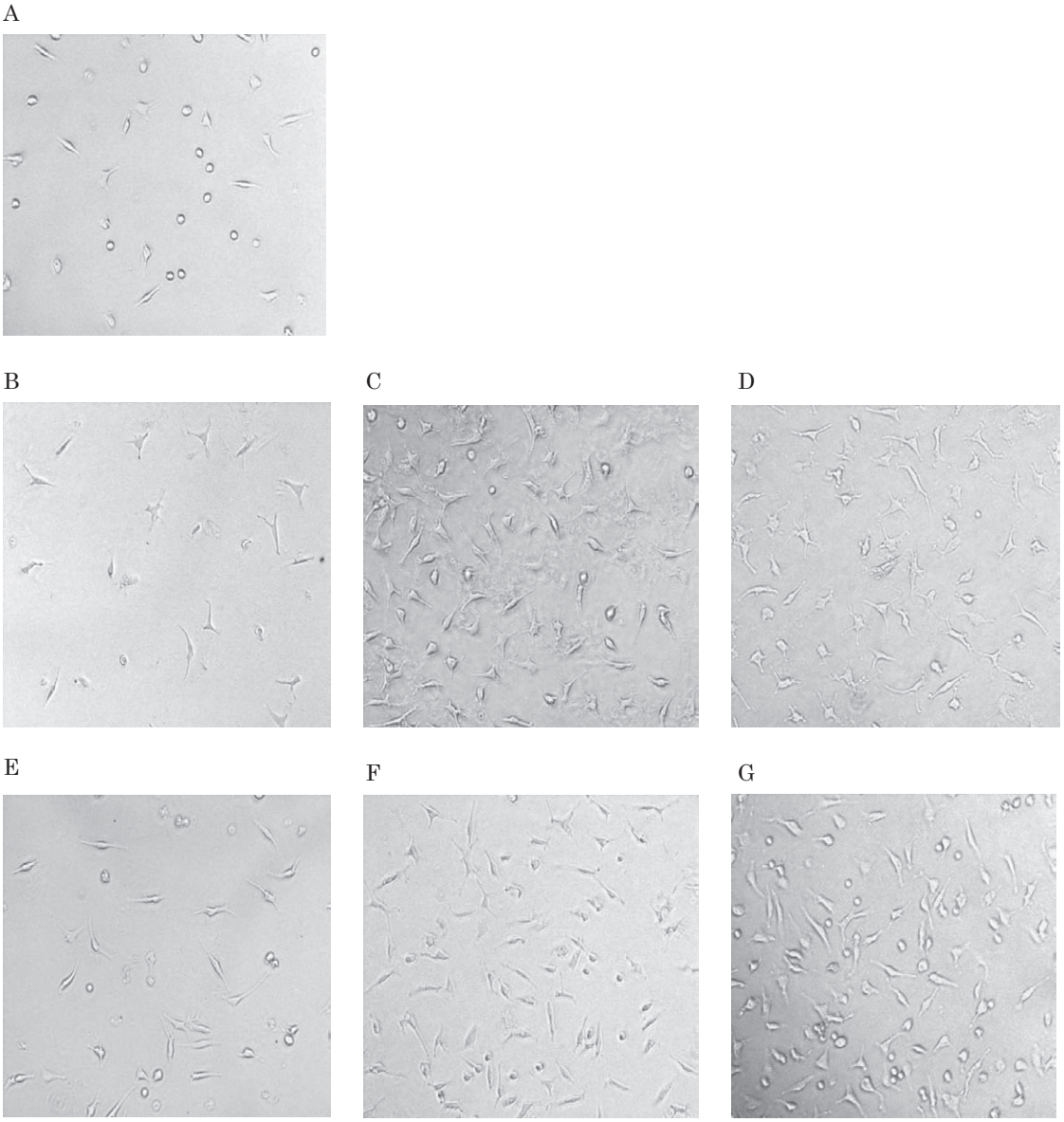

Fig. 3. Difference of *in vitro* morphological changes of macrophages according to banana strain and maturity level.
The cells were observed under a microscope for 1 week. Photos were taken after 3 days. A) Saline, B) Regular banana, day 1, C) Regular banana, day 3, D) Regular banana, day 10, E) Highland banana, day 1, F) Highland banana, day 3, G) Highland banana, day 10.

The priming effect of cytokine production was observed both in mice subjected to intraperitoneal injection and those subjected to oral administration. Although lipopolysaccharide (LPS) shows both priming and triggering effects, banana juice did not demonstrate a triggering effect for cytokine production. The cytokine-priming substances in banana juice are different from LPS, since the juice did not show the triggering effect for cytokine production. In fact, the contamination of LPS to banana juice was negligibly lower than 20 pg/mL. The time interval of cytokine-priming of the juice was similar to that of immunopotentiators such as muramyl dipeptide. These data suggest that banana juice contains cytokine-priming substances similar to immunopotentiators.

Differences in dopamine and serotonin contents of bananas according to banana strain and maturity level Bananas are known to contain serotonin. In recent years, bananas have also been shown to contain dopamine. We examined differences in these components between the two strains and changes due to ripening by TLC. As a result, the dopamine content in the banana peel decreased after ripening in each strain. The dopamine content was greater in the highland banana until day 3, but the two strains became more similar on day 5 or more, when sugar spots appeared (Fig. 6A). The dopamine content in the flesh only slightly changed after ripening, but markedly differed between the strains; the dopamine content in the highland banana was about 3 times that in the

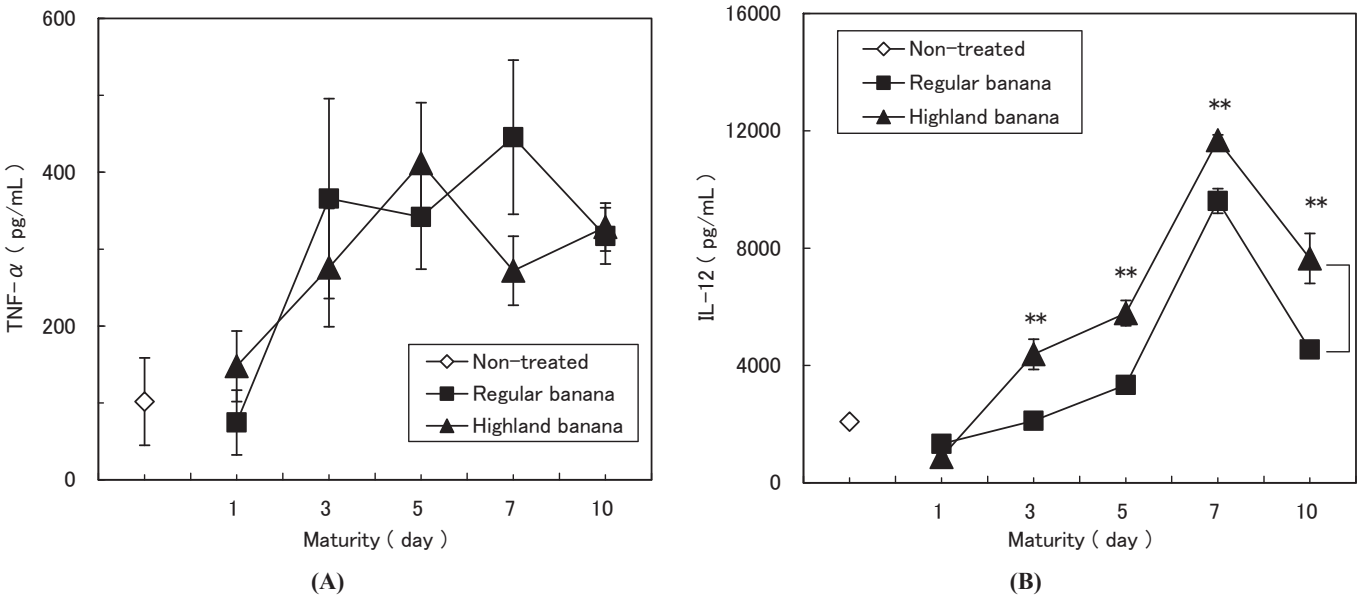

Fig. 4. Difference in the priming activity of intraperitoneal injection on cytokine production according to banana strain and maturity level. The data is represented as mean ± S.D. (n=3). A : Tumor necrosis factor (TNF)-α concentration in the culture supernatant. B : Interleukin (IL) -12 concentration in the culture supernatant. The X-axis represents the maturity of the bananas. Statistical differences were analyzed by Student's t-test. Significant differences were between regular and highland banana. Fig. B: **p < 0.01.

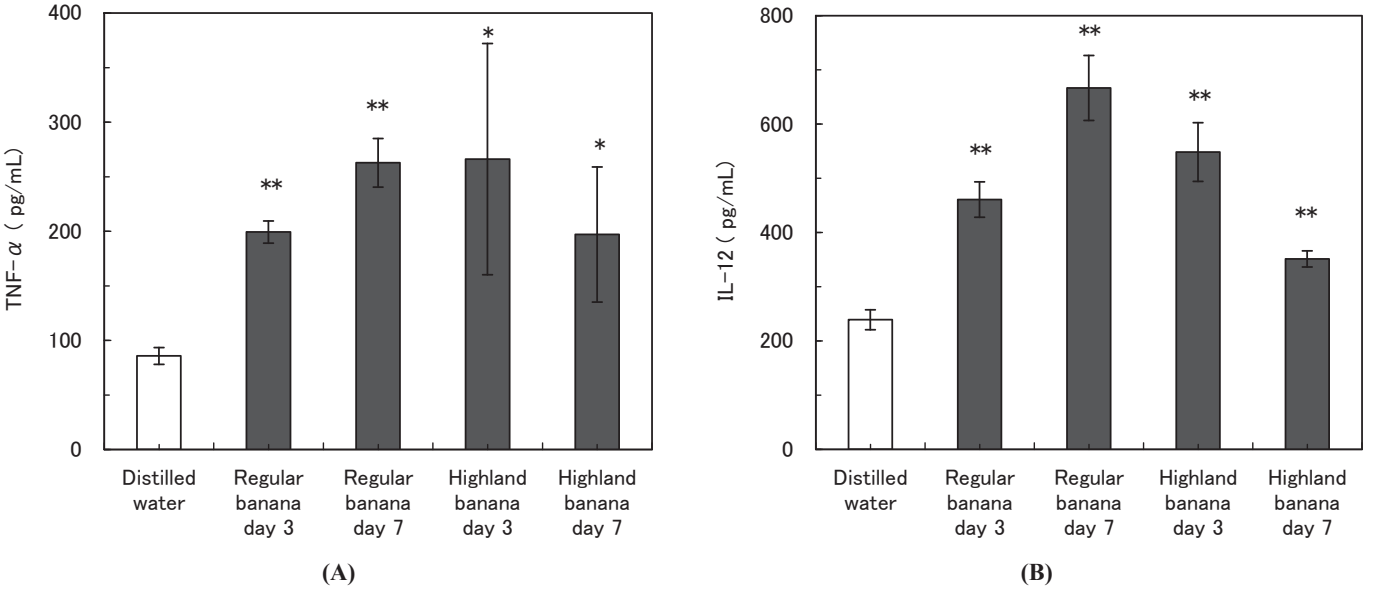

Fig. 5. Difference in the priming activity of per oral (*p.o.*) administration on cytokine production according to banana strain and maturity level. The data is represented as mean ± S.D. (n=3). A : Tumor necrosis factor (TNF)-α concentration in the culture supernatant. B : Interleukin (IL) -12 concentration in the culture supernatant. Statistical differences were analyzed by Student's t-test. Significant differences were from the non-treated group. Figs A and B: *p < 0.05, **p < 0.01.

regular banana (Fig. 6B). Dopamine was detected in papayas as well as bananas, but was negligibly present in other fruits such as oranges and apples (date not shown), showing that dopamine is a characteristic component. Differences in the serotonin content between the strains and among maturity levels were also evaluated. The serotonin content in the peel decreased after ripening (Fig. 7A). Serotonin content in the pulp changed after ripening, was maximal on day 1 and minimal on day 3, and increased thereafter (Fig. 7B). The serotonin content in each part of the banana did not differ between the two strains. These results confirmed that bananas contain dopamine and serotonin as characteristic neurotransmitters at high concentrations, and that their contents differ among strains and maturity levels.

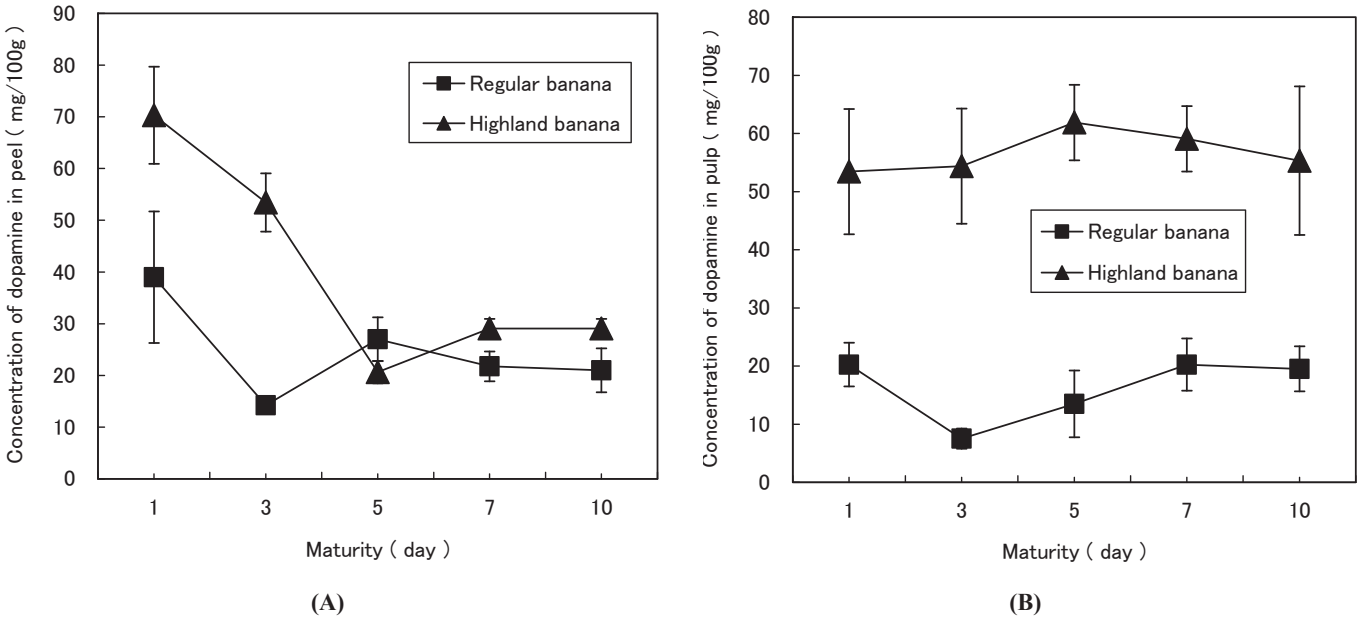

Fig. 6. Differences in dopamine content of bananas according to banana strain and maturity level.
The data is represented as mean. A : Concentration of dopamine in peel. B : Concentration of dopamine in pulp.

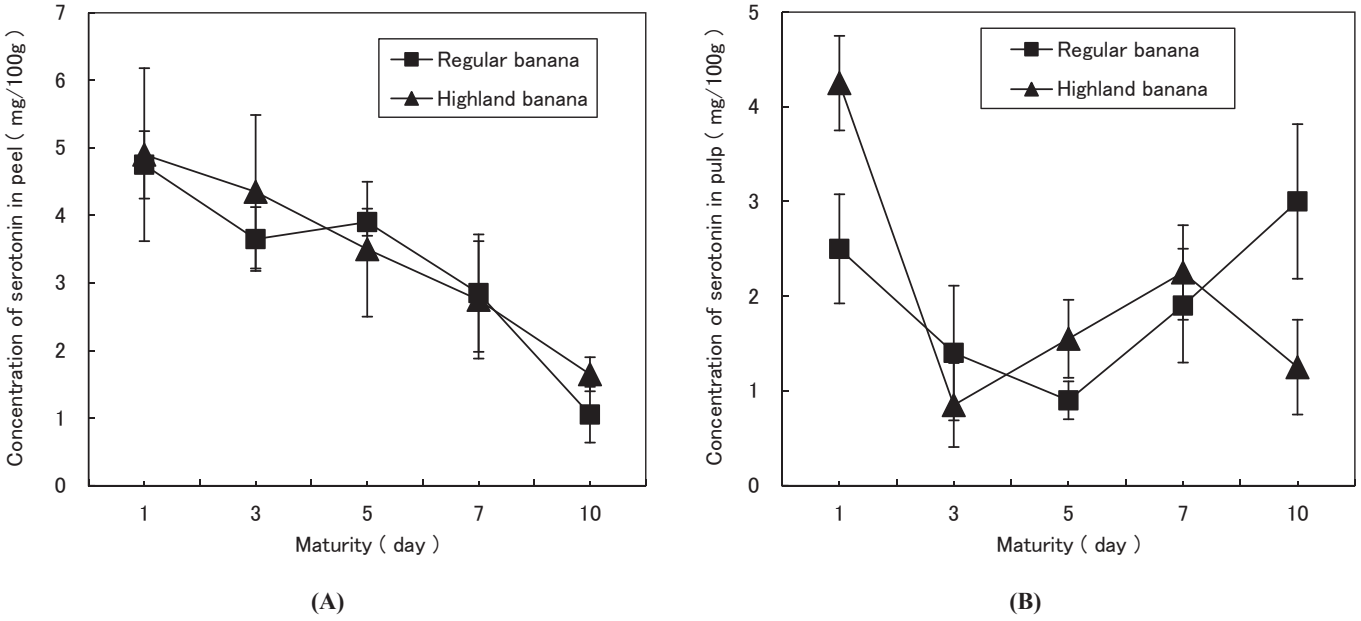

Fig. 7. Differences in serotonin content of bananas according to banana strain and maturity level.
The data is represented as mean. A: Concentration of dopamine in peel. B: Concentration of dopamine in pulp.

Discussion

We evaluated possible differences in the BRM-like activities of bananas between strains and among maturity levels. Banana extract administration resulted in a dose-dependent accumulation of neutrophils and macrophage activation. In the *i.p.* experiment, the priming effects on cytokine induction increased with maturity and were slightly more marked for the highland banana. In the *p.o.* administration experiment, the activity exhibited by the regular banana increased with maturity, while that of the highland banana was highest when it was ready to eat. Comparison between the two strains revealed a higher activity level in the highland banana on day 3, and in the regular banana on day 7. In this study, oral banana administration promoted immune capacity both quantitatively and qualitatively, even after the digestion/absorption process. The degree of this activity differed among strains and maturity levels. Previous experiments have shown that the active component responsible is heat-resistant and water-

soluble. The isolation and identification of this component are future tasks.

Bananas contain dopamine and serotonin. Their contents differed between the two strains and among maturity levels. The dopamine content of the peel decreased after ripening in both strains. Since dopamine turns brown with time, sugar spots on the peel of banana may be associated with the action of dopamine. The dopamine content of the flesh was greater in the highland banana. Since the highland variety is cultivated for a longer period and under a more severe environment, the content of dopamine, with its anti-oxidative effects, may be a useful self-defense mechanism. The more marked BRM-like activities of the highland banana may also be due to environment-associated differences in the contents of components. Dopamine is secreted during eating and pleasurable activities, and markedly affects motor function. Dopamine is administered clinically for its diuretic effects, its action of vasoconstriction as well as its effects on acute circulatory failure, such as cardiogenic shock. Serotonin is associated with mental stabilization such as sleep, has anti-depression and anti-anxiety effects, and promotes gastrointestinal movement. These monoamines in banana may enhance the effects of monoamine oxidase inhibitor drugs (Walker and Shulman *et al.*, 1996).

Bananas are widely eaten as an energy source before exercise or during fatigue. Whether dopamine and serotonin are transferred to the brain after oral intake is unclear, but these components may be associated with the effects of oral intake of bananas. In recent years, dopamine has been reported to play an important role in immune balance (Nakano and Matsushita, 2007). The possible involvement of dopamine in the immunostimulatory effects of bananas requires further evaluation. Bananas contain many anti-oxidative substances. We also intend to evaluate differences in the anti-oxidative activity among strains and maturity levels. Due to the association between immunostimulatory and anti-oxidative effects, oral banana intake has the potential to help prevent lifestyle-related diseases and carcinogenesis.

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