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Anti-allergic effects of a mixture of *Saccharomyces cerevisiae* and its specific goat's milk immunoglobulin G rich fraction on ovalbumin sensitized BALB/c mice

By Yumi SEKEKIMURA[1], Takeshi SHIMOSATO[2] and Hajime OTANI[1]

[1]Graduate School of Agriculture, Shinshu University, Nagano-ken 399-4598, Japan. E-mail: otani84@shinshu-u.ac.jp
[2]Fiber-Nanotech Young Researcher Empowerment Center, Shinshu University, Nagano-ken 399-4598, Japan

In this study, we investigated the effects of oral ingestion of *Saccharomyces (S.) cerevisiae* and its specific immunoglobulin (Ig) G-rich fraction, prepared from goat's milk, on immune function in an allergic rhinitis mouse model, ovalbumin (OVA)-sensitized BALB/c mice. Sneezing activity in mice that were administered a feed containing a mixture of *S. cerevisiae* and its specific goat's milk IgG-rich fraction (mixture group) was significantly lower than that in mice administered the mixture-free feed (control group) at age 11 and 12 weeks, although the activity in mice that were given a feed containing *S. cerevisiae* (yeast group) was similar to that of the control group. We found that the ratio of spleen interferon-γ^+CD4$^+$ cell/interleukin (IL)-4$^+$CD4$^+$ cell was higher in the mixture and yeast groups than the control group. The number of spleen CD80$^+$CD11b$^+$ cells was significantly lower in the mixture and yeast groups than the control group. In contrast, the numbers of spleen CD122$^+$CD8α^+ and IL-10$^+$CD4$^+$ cells were significantly higher in the mixture group than the control and yeast groups while the numbers of IgE$^+$B220$^+$ and FcεRIα^+CD117$^+$ cells were significantly lower in the mixture group. These results suggested that the mixture of *S. cerevisiae* and its specific goat's milk IgG-rich fraction reduced OVA-induced allergic symptoms in BALB/c mice via the induction of suppressor T cells and the reduction of Ig E-producing cells and high affinity IgE receptor-positive mast cells rather than the adjustment of the Th1/Th2 balance.

Antiallergische Wirkungen einer Mischung von *Saccharomyces cerivisiae* und der spezifischen Immunglobulin G-reichen Fraktion von Ziegenmilch auf mit Ovalbumin sensibilisierte BALB/c-Mäuse

In der Studie wurden die Auswirkungen einer oralen Aufnahme der Hefe *Saccharomyces* (*S.*) *cerevisiae* und ihrer spezifischen Immunglobulin (Ig) G-reichen Fraktion, hergestellt aus Ziegenmilch, auf die Immunfunktion in einem Mausmodell mit allergischer Rhinitis (Ovalbumin (OVA)-sensibilisierte BALB/c-Mäuse) untersucht. Die Niesanfälle von Mäusen, die ein Futter erhielten, das eine Mischung von *S. cerevisiae* und der spezifischen IgG-reichen Fraktion aus Ziegenmilch („Mischgruppe") enthielt, waren signifikant geringer als bei Mäusen, die als Kontrollen dienten (Alter 11 und 12 Wochen). Demgegenüber waren die Niesanfälle von Mäusen, denen ein Futter lediglich mit *S. cerivisiae* gegeben wurde („Hefegruppe"), vergleichbar denen der Kontrollen. Es wurde festgestellt, dass das Verhältnis von Interferon-γ^+CD4$^+$-/Interleukin (IL)-4$^+$CD4$^+$-Milzzellen in der Misch- und der Hefegruppe im Vergleich zur Kontrollgruppe höher lag. Im Gegensatz hierzu waren die Zahlen der D122$^+$CD8α^+- und IL-10$^+$CD4$^+$-Milzzellen signifikant höher in der Mischgruppe im Vergleich zur Kontroll- und Hefegruppe, wohingegen die Anzahl der IgE$^+$S220$^+$- und FcεRIα^+CD117$^+$-Zellen in der Mischgruppe signifikant niedriger lag. Diese Ergebnisse weisen darauf hin, dass die Fütterung einer Mischung von *S. cerivisiae* und der spezifischen IgG-reichen Fraktion von Ziegenmilch die durch OVA induzierten allergischen Symptome bei BALB/c-Mäusen über die Einführung von Suppressor T-Zellen, die Reduktion von IgE-bildenden Zellen und die hohe Affinität der IgE-Rezeptor positiven Mastzellen reduziert und nicht durch die Anpassung der TH1/TH22-Balance.

14 Allergy (mouse model, antiallergic effects, *Saccharomyces cerevisiae*/goat's milk)

14 Allergie (Mausmodell, antiallergische Effekte, *Saccharomyces cerevisiae*/Ziegenmilch-IgG)

1. Introduction

The number of patients diagnosed with allergic diseases such as atopic dermatitis, allergic asthma and allergic rhinitis has increased significantly in many industrial countries. These type I allergic diseases are generally considered to be attributable to a skewed T helper type 1 (Th1)/T helper type 2 (Th2) balance (1). However, the allergic diseases may be halted through the suppression of high affinity immunoglobulin (Ig) E receptor-positive mast cell and antigen-presenting cell functions, and/or the enhancement of suppressor cell functions (2).

In a previous study (3), we demonstrated, via microarray analysis of mRNA extracted from normal mouse Peyer's patch cells, that the expression profile of genes related to immunoglobulin production and the development of immune diseases was reduced in mice administered a mixture of *Escherichia coli* and its

specific cow's milk IgG in their feed as compared to those not fed this mixture. In contrast, the gene expression of marker proteins on Th1, T helper type 3 and negative regulatory T cells was found to increase significantly in the mice administered the mixture. Moreover, MIZUTANI et al. (4) reported that cow's milk IgG stimulated immunoglobulin formation in mouse cell cultures. These findings suggest that the oral ingestion of a mixture of edible microorganisms and their specific milk IgG fraction reduces development of type I allergic symptoms.

In this study, we prepared an anti-*Saccharomyces* (*S.*) *cerevisiae* IgG-rich fraction from milk produced by Shiba goats, which had been immunized with a commercial *S. cerevisiae* powder for bread-making, and investigated immune functions in an allergic rhinitis mouse model, ovalbumin (OVA)-sensitized BALB/c mice orally administered with *S. cerevisiae* and its specific goat's milk IgG-rich fraction.

2. Materials and methods

2.1 Materials

Phycoerythrin (PE)-labeled anti-mouse interleukin (IL)-4 monoclonal antibodies (mAb, clone 11B11), PE-labeled anti-mouse interferon (IFN)-γ mAb (clone XMG1.2), PE-labeled anti-mouse IL-10 mAb (clone JES5-16E3), PE-labeled anti-mouse CD80 mAb (clone 16-10A1), PE-labeled anti-mouse CD8α mAb (clone 5H10-1), biotin-labeled anti-mouse CD4 mAb (clone RM4-5), biotin-labeled anti-mouse CD45R/B220 (B220) mAb (clone RA3-6B2), biotin-labeled anti-mouse CD117 mAb (clone 2B8), biotin-labeled anti-mouse CD11b mAb (clone M1/70) and phycoerythrin/cyanine 5 (PE/Cy5)-labeled streptavidin were purchased from BioLegend (San Diego, CA, USA). Anti-mouse FcεRIα mAb (clone G-14) and PE-labeled anti-goat IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). OVA, brefeldin A (BFA), ionomycin, streptomycin and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep was obtained from Beckman Coulter (Marseille, France). Defined fetal bovine serum (FBS) was obtained from HyClone Laboratories (Road Logan, UT, USA). Penicillin was purchased from MP Biomedicals (Costa Mesa, CA). RPMI-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan). 3,3',5,5'-tetramethyl benzidine (TMB) was purchased from KPL (Gaithersburg, MD). Bovine serum albumin (BSA) and aluminum hydroxide gel were obtained from Sigma-Aldrich (St. Louis, MO, USA). Complete Freund's adjuvant was purchased from Nacalai Tesque (Kyoto, Japan). All chemicals used in this study were of the highest analytical grade commercially available.

2.2 Yeast and its specific goat milk IgG-rich fraction

Saccharomyces cerevisiae JCM7255 was obtained from the Japan Collection of Microorganisms (JCM, Saitama, Japan). The yeast was inoculated in YM broth containing 1% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract (pH 6.2), cultivated for 72 h at 25°C, collected by centrifugation, washed three times with sterile saline and lyophilized.

Preparation of goat's milk IgG-rich fraction specific to *S. cerevisiae* was carried out as described previously (5). In brief, Shiba goats (*Capra hircus*) were immunized with an intradermal injection of a commercial *S. cerevisiae* powder for bread making (Hoshino Natural Leaven, Tokyo, Japan: 10 mg) suspended in a mixture of 5 ml sterile saline solution and 5 ml complete Freund's adjuvant. The immunization was carried out a total of 10 times at 7-day intervals approximately 3 months prior to delivery. Milk was then collected every day, from delivery to 60 days postpartum. The collected milk was centrifuged at 1,200 × g for 30 min at 4°C to obtain skim milk. The skim milk was adjusted to pH 4.6 with 1 M HCl. The whey was collected by centrifugation at 1,200 × g for 20 min at 4°C, and 40%-saturated with ammonium sulfate. The precipitate was collected by centrifugation, dissolved in a small amount of distilled water, dialyzed against tap water, lyophilized and used as *S. cerevisiae*-specific goat's milk IgG-rich fraction.

2.3 Feeding procedure

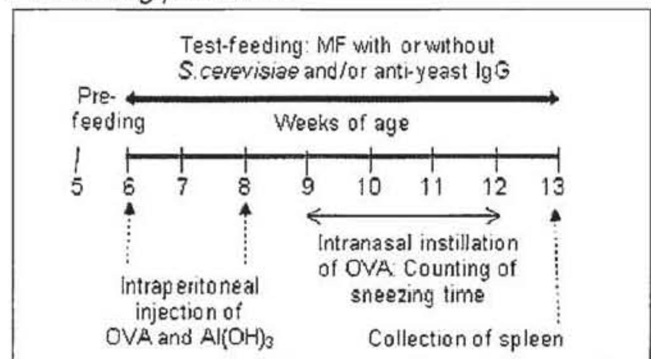

Fig. 1: Schedule for the test-feeding of mice. Six-week-old BALB/c mice were given either commercial feed, MF (control group), MF containing S. *cerevisiae* (yeast group) or MF containing a mixture of S. *cerevisiae* and its specific goat's milk IgG-rich fraction (mixture group) for 7 weeks. The mice were intraperitoneally injected with an antigen solution containing OVA and aluminum hydroxide at 6 and 8 weeks of age. Mice were instilled with OVA dissolved in distilled water via the intranasal cavity between 9 and 12 weeks of age. The spleens of mice were collected at 13 weeks of age.

Five-week-old male BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). As shown in Fig. 1, the mice were first assigned to three test regimens, administered a commercial mouse powder feed (MF, Oriental Yeast Company, Tokyo, Japan) for a week, and then given either MF (control group), MF containing 0.005% *S. cerevisiae* JCM7255 (yeast group) or MF containing a mixture of 0.0025% *S. cerevisiae* JCM7255 and 0.0025% its specific goat's milk IgG-rich fraction (mixture group) between 6 and 13 weeks of age. The mice were then intraperitoneally injected with 200 µl saline containing 20 µg OVA and 2 mg aluminum hydroxide gel at 6 and 8 weeks of age. The mice were intranasally challenged by instillation with 10 µl distilled water containing 100 µg OVA three times a week between 9 and 12 weeks of age. The total number of sneezing events was then counted for 10 min, 1 min from the intranasal instillation. Feed and water were supplied *ad libitum* throughout the course of the experiment. The mice were housed at 23 ± 2°C under

a standard 12-h light-dark cycle. Spleen samples were collected immediately following a lethal dose of ether at 13 weeks of age. All animal experimentation undertaken during this study was conducted in accordance with the guidelines for the Regulation of Animal Experimentation at Shinshu University and according to Law No. 105 and Notification No. 6 of the Japanese government.

2.4 Cell suspensions and functional analysis

Spleen samples were homogenized in RPMI-1640 medium containing 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspensions were then washed three times in this medium and adjusted to 1×10^6 viable cells/ml. The cell surface markers for CD11b, CD117 and B220 were then observed using a specific biotin-conjugated anti-mouse mAb for 15 min at 4°C, followed by incubation with PE/Cy5-labeled streptavidin for 15 min at 4°C. $CD80^+CD11b^+$ cells were incubated with PE-labeled anti-mouse mAb specific for CD80 for 15 min at 4°C. For $Fc\varepsilon RI\alpha^+CD117^+$ and IgE^+B220^+ cells, the cell surface antigens for FcεRIα or IgE were labeled using the specific anti-mouse antibodies for 15 min at 4°C, followed by incubation with PE-labeled anti-goat IgG for 15 min at 4°C. The cell numbers were then determined using a Guava personal cell functional analyzer (Guava PCA: Guava Technologies, Hayward, CA, USA).

When observing intracellular cytokines in $CD4^+$ cells, spleen cells were incubated at 37°C in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml BFA, 2 μg/ml ionomycin and 20 ng/ml PMA for 4 h. The cell surface marker antigens for CD4 were then labeled using a specific biotin-conjugated anti-mouse mAb for 15 min at 4°C, followed by incubation with PE/Cy5-labeled streptavidin for 15 min at 4°C. Intracellular cytokines were measured following permeabilization and labeling with a PE-labeled anti-mouse cytokine mAb specific for IL-4, IFN-γ or IL-10. In order to achieve this, cells were fixed with IntraPrep reagent 1 for 15 min, washed and then permeabilized with IntraPrep reagent 2. The cells were then incubated with the appropriate antibodies and intracellular cytokine levels were determined using a Guava PCA.

2.5 Statistical analysis

Data is presented as the mean ± standard deviation (SD). Statistical analyses were performed using the Dunnett's multiple comparison test for one-way analysis of variance or Student's *t*-test. Differences were considered significant when *P* values were less than 0.05.

3. Results and discussion

BALB/c mice were fed either a control feed, MF (control group), MF containing *S. cerevisiae* (yeast group) or MF containing a mixture of *S. cerevisiae* and its specific goat's milk IgG-rich fraction (mixture group) between 6 and 13 weeks of age. Under these conditions, we observed no significant differences in the body weight and average intake of feed among the control, yeast and mixture groups (data not shown). These results indicate that there is little difference in both the nutritional value and the stress levels among the control, yeast and mixture groups. As mice housed under stressful conditions generally gain less weight than their control counterparts, the differences among the effects of the control, yeast and mixture groups in this study are considered to reflect the physiological functions of the yeast and mixture.

Allergic rhinitis was induced by the continuous nasal administration of OVA after intraperitoneal injection of OVA in BALB/c mice (6). We have induced acquired allergic symptoms by the continuous nasal administration of OVA in BALB/c mice. As shown in Fig. 2, the sneezing activity at age 11 weeks was significantly lower in the mixture group than the control and yeast groups, and that at 12 weeks was significantly lower in the mixture group than the control group. This result indicates that the mixture of *S. cerevisiae* and its specific goat's milk IgG-rich fraction may suppress allergic rhinitis.

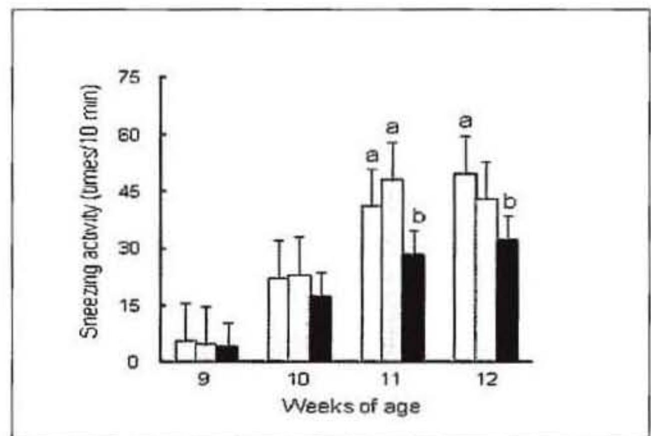

Fig. 2: Sneezing activity of the control (□), yeast ( ), or mixture group (■). Results are expressed as mean ± standard deviation (n=6). Items indicated with different letters (i.e. a, b) were significantly different (*P<0.05).

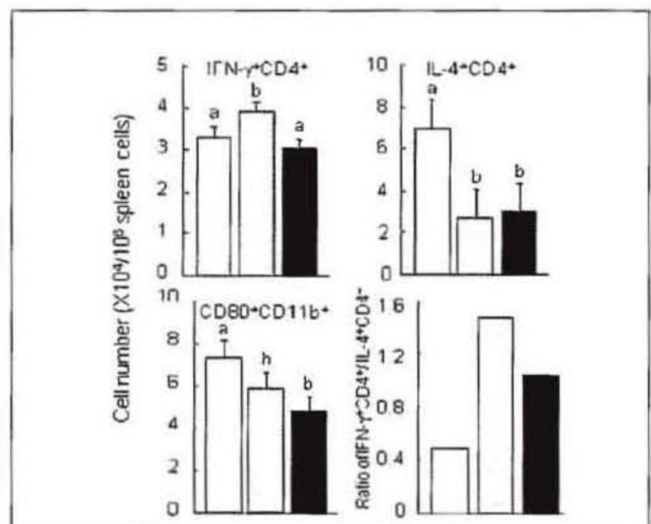

Fig. 3: $IFN\text{-}\gamma^+CD4^+$, $IL\text{-}4^+CD4^+$ and $CD80^+CD11^+$ cell numbers, and the ratio of $IFN\text{-}\gamma^+CD4^+$ cell/$L\text{-}4^+CD4^+$ cell in spleens of the control (□), yeast ( ), or mixture group (■). Results represent mean ± standard deviation (n=6). Items indicated with different letters (i.e. a, b) were significantly different (*P<0.05).

As shown in Fig. 3, the number of spleen IFN-γ^+CD4$^+$ cells was higher in the yeast group than the control and mixture groups, while the numbers of spleen IL-4$^+$CD4$^+$ and CD80$^+$CD11b$^+$ cells in the yeast and mixture groups were significantly lower when compared to the control group. In addition, the ratio of IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$ was obviously higher in the yeast and mixture groups than the control group. The spleen plays an important role in the systemic immune system. It is well known that IL-4, IL-5 and IL-13, which are produced by Th2 cells, stimulate IgE production (7). Allergic rhinitis is generally caused by degranulation that occurs following antigen cross-linking of IgE molecules present on mast cells (7). On the other hand, Th1 cells mainly secrete IFN-γ, which inhibits IL-4 production by the Th2 cells (8). Hence, the Th1/Th2 balance in type-I allergic disease patients is in a Th2-dominant state (1). Moreover, it is well known that the activation of Th cells requires the interaction of costimulatory molecules, as well as T cell receptor and major histocompatibility complex molecules, delivered by antigen-presenting cells. CD80 and CD86, as the most important costimulatory molecules, are involved in the allergic immune responses (9,10). The upregulation of CD80 on dendritic (CD11b$^+$) cells of OVA-immunized BALB/c mice plays a pivotal role in allergic immune responses, namely Th2 immune responses (11). These facts suggest that the anti-allergic effect of the mixture is not completely attributable to the adjustment of the Th1/Th2 balance.

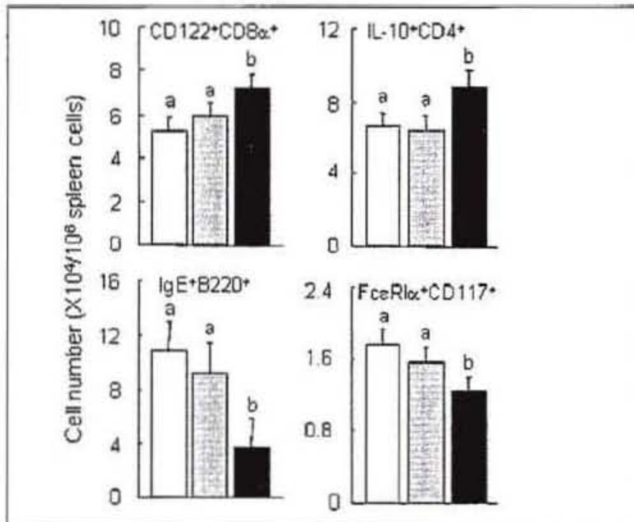

Fig. 4: CD122+CD8α+, IL-10+CD4+, IgE+ B220+ and FcεRI α +CD117+ cell numbers in the control (□), yeast ( )), or mixture group(■). Results represent mean ± standard deviation (n=6). Items indicated with different letters (i.e. a, b) were significantly different (*P<0.05).

As shown in Fig. 4, on the other hand, the numbers of spleen CD122$^+$CD8α^+ and IL-10$^+$CD4$^+$ cells were significantly higher in the mixture group than the control and yeast groups, while those of spleen IgE$^+$B220$^+$ and FcεRIα^+CD117$^+$ cells were significantly lower in the mixture group than the control and yeast groups. CD8$^+$CD122$^+$ cells are known as naturally occurring regulatory T cells (12). Regulatory T cells producing IL-10 suppresses inflammatory responses via inhibition of the synthesis of various cytokines and IgE production (13). IgE$^+$B220$^+$ cells are IgE-producing B cells (14). Moreover, mast cells are normally defined by the cell surface expression of FcεRIα and CD117$^+$. FcεRIα is an essential molecule in the development of type I allergic diseases (15). These facts suggest that the reduction of allergic symptoms by the mixture of *S. cerevisiae* and its specific goat's milk IgG-rich fraction is attributable to the increase of IL-10-producing regulatory T cells and decrease of IgE-producing B cells and high affinity IgE receptor-positive mast cells.

In conclusion, we propose that the mixture of *S. cerevisiae* and its specific goat's milk IgG-rich fraction reduced OVA-induced allergic symptoms in BALB/c mice via the induction of suppressor T cells and the reduction of IgE-producing cells and high affinity IgE receptor-positive mast cells rather than the adjustment of the Th1/Th2 balance.

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