



Studies on immunomodulatory action by food-derived bacteria/fungi

Nonaka, Yuji

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Doctoral Dissertation

Studies on immunomodulatory action by food-derived bacteria/fungi
(食品由来の微生物や真菌による免疫調節作用に関する研究)

2020

Yuji Nonaka
(野中 裕司)

Contents

Abbreviations

Chapter 1.	1
------------	---

General Introduction

Chapter 2.	11
------------	----

Anti-Allergic Effects of *Lactobacillus pentosus* S-PT84 Mediated by Modulation of Th1/Th2 Immuno-Balance and Induction of IL-10 Production

Chapter 3.	25
------------	----

Protective Effect of *Lactobacillus pentosus* S-PT84 Against Restraint Stress in Mice

Chapter 4.	36
------------	----

Immunomodulating Activity of *Lactobacillus pentosus* S-PT84 on Healthy Adults:
a Pilot Trial

Chapter 5.	45
------------	----

Anti-Tumor Activities of the Antlered Form of *Ganoderma lucidum* in Allogeneic and Syngeneic Tumor-Bearing Mice

Chapter 6.	57
------------	----

Inhibitory Effects of the Antlered Form of *Ganoderma lucidum* on Immunosuppression, Tumor Growth and Metastasis in Cyclophosphamide-treated Mice

Chapter 7.	75
------------	----

General Discussion

References	81
------------	----

Acknowledgements	99
------------------	----

List of publication	100
---------------------	-----

Abbreviations

ACA, active cutaneous anaphylaxis
BRM, biological response modifier
CCR5, C-C chemokine receptor type 5
CD, cluster differentiation
CR3, complement receptor 3
CRTH2, chemoattractant receptor-homologous molecule on Th2 cells
CTL, cytotoxic T lymphocyte
CY, cyclophosphamide
Con A, concanavalin A
DEX, dexamethasone
DiO, 3,3'-dioctadecyloxycarbonylamine perchlorate
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme-linked immunosorbent assay
FAS, full analysis set
FBS, fetal bovine serum
FITC, fluorescein isothiocyanate
G. lucidum AF, antlered form of *G. lucidum*
GSH, reduced glutathione
GSSG, glutathione disulfide
IFN, interferon
IL, interleukin
LAB, lactic acid bacteria
LLC, Lewis lung carcinoma
MLR, mixed lymphocyte reaction
MRS, deMan, Rogosa and Sharpe
NK, natural killer
OVA, ovalbumin
PBMC, peripheral blood mononuclear cells
PBS, phosphate buffered saline
PD-1, programmed cell death-1
PE, phycoerythrin
PI, propidium iodide
ROS, reactive oxygen species
s.c., subcutaneously
SD, standard deviation
SE, standard error
TLR, toll like receptor
Th, T helper
i.p., intraperitoneally
p.o., post oral
QOL, quality of life

Chapter 1.

General Introduction

The immune system is the self-defense system of the organism, and this system is highly developed from the ancient in order to protect us from the risk factor of the outside of the body.

The oldest immunity record is that Edward Jenner used vaccination to prevent smallpox infection in the 1700s. Later, Louis Pasteur discovered that the cause of the disease was microorganisms, and that animals that once infected and survived acquired resistance to the microorganisms. After finding that the resistant identity was an antibody, immunology developed rapidly. In the 1960s, T cells and B cells were also found, and various lymphocytes such as NK cells were found. As the functions of these immune cells become clear, allergy is a phenomenon in which the immune function of the body reacts excessively against foreign substances, and non-self cells such as cancer proliferate and devour the host because host immune function is declined. Control of immune function is a very important function for all human beings.

In modern societies, lots of things are affected immune function by modernization. For example, the modern society is called the stress society, and it can be said that people living in the modern world are always in the environment which receives the stress such as body stress by environmental pollution, mental stress caused by the social background which repeats the sudden change, and so on. In addition, the dietary habits themselves have greatly changed due to the westernization of food and the simplification of food by the popularization of fast foods and convenience stores. Excessive stress and changes in nutritional balance have a profound effect on our immune function. In general, the immune function of an organism decreases with age, but this is not only the factor for dysfunction of the immune system. Hence, human life itself is greatly affected, including the onset and aggravation of allergies that significantly reduce the quality of life

(QOL), and the onset of cancers that threatens our life. In other words, our immune function has not been able to maintain in super-aging and ultra-modern society. There is a strong demand for functional foods that maintain them in a normal state in order to survive modern society.

Allergy and hygiene hypothesis

Half of population in Japan have some allergic symptom, and the estimated number of allergic rhinitis patients was 400,000 in 2002, but in 2014 it was increased more than 1.5 times, 650,000 patients (1). The development of antibiotics, the improvement of living standards, and the evolution of medical treatment have made it possible to control many infectious diseases. Conversely, the idea of hygiene hypothesis is proposed that the opportunity to come into contact with pathogens and the opportunity to eliminate pathogens by their own immunity are contributing to the increase in allergies (2-4). In other words, the idea is that moderate unhygiene is necessary to maintain health and the maintenance of the appropriate immune function. It is practically difficult to perform moderate unsanitary hygiene routinely, however, by using bacteria and/or fungi as functional foods, which can stimulate human immune function at a certain level, immune function can be expected to be maintained and regulated at an adequate level.

T helper (Th)1/Th2 balance

Helper T cell is one of the immunocompetent cells. The Th1 / Th2 balance hypothesis emerged in the 1980s in that there were two subtypes of Th cells with different cytokine secretion patterns and functions (5). Basically, Th1 is responsible for cellular immunity and is activated by interleukin (IL)-12 (6). By producing Th1 cytokines such as interferon (IFN)- γ , not only activate cellular immunity of the innate immune system such as macrophages and NK cells but also acquired immunity, killer T cells. These make it possible to fight with virus-infected cells and cancer cells

(6, 7). Th2 is responsible for humoral immunity, and itself is known to activate mast cells and B cells by producing Th2 cytokines such as IL-4 and IL-5 (7). Th2 cells are considered to emphasize protection against extracellular pathogens such as allergen, multicellular parasites and so on. (8). On the negative side, when the Th1 pathway is overreactive, it is connected to generate organ-specific autoimmune disease (e.g., arthritis, multiple sclerosis, type 1 diabetes) (9). Numerous studies reported that Th2 pathway is seen as underlying allergy. Therefore, the proper balance of Th1/Th2 is important, but it doesn't mean that either Th1 or Th2 immunity is strong.

Natural killer (NK) activity

NK cells are known as our bodies' frontline defense system, fighting against tumors and launching attacks against infection cells. NK cells are activated by Th1 cytokines such as IL-2 and IL-12, and themselves produce Th1 cytokines such as IFN- γ , and are the cells responsible for cellular immunity (10). NK cells develop not only the bone marrow, but also NK cells can also develop in lymph nodes and liver (11, 12). In other words, NK cells are very important for systemic defense, and maintaining NK activity at a high level is very important in preventing cancer and infectious diseases. However, NK activity is easy to change and decreases due to factors such as cancer stress (13), aging (14), cancer (15-17), and viral infection (18-20). In addition, an 11-years follow-up study has shown epidemiologically that people with low NK activity have an increased risk of carcinogenesis (17). The NK activity, which is important but easy to change, is considered to be one of the targets for maintaining and improving immune function with functional foods. Thus, NK activity was measured in various experimental models in this dissertation.

Lactic acid bacteria (LAB)

LAB are one of the most familiar fungi (bacteria) used in food. It is widely used, for

example, in the processing of yogurt and cheese, and in foods familiar to Japanese people, it has long been used, for example, in the processing of miso, soy sauce, pickles, and wine. Lots of studies have been conducted on the health effects, especially immunomodulating activity of lactobacilli. Some LABs are well known to adjust the Th1/Th2 balance toward a Th1 dominance. LABs living in the gastrointestinal tract alter the microbiota and affect bacterial immunogenicity (21-23). *Lactobacillus acidophilus* L-92 has been demonstrated to improve the symptoms of cedar pollinosis in a human study (24). Administration of the probiotic bacterium, live *L. rhamnosus* GG, significantly reduced the incidence of eczema during the first 2-4 years of life in children with a family history of atopic disease (25-27). On the other hand, several studies show that killed LAB has immunomodulatory activities (29-) including induction of IL-12 from macrophages (6, 28) and inhibition of IgE production in an ovalbumin (OVA)-induced food allergy model (29-31), and increased fecal antigen-specific IgA (32, 33) has been suggested. But, it is believed that each type and mechanism of immunomodulation can be exerted by the type of lactobacilli (34-38). For example, *L. paracasei* KW3110 inhibits Th2-dependent allergic response (31, 39), *L. paracasei* ST11 shows not only prevention of intestinal inflammation (40) but also stimulating regulatory T cells (41). *L. paracasei* subsp. *paracasei* NTU is multifunctional because it enhances dendritic cells and NK activity (42), dendritic cells-cluster differentiation 4 (CD4) T cell interaction and IgA production on Peyer's patch (43), induces proliferation of macrophages (44) and lymphocytes (42), induces IL-10 and IL-12 (44), and inhibit inflammation by downregulating toll like receptors (TLRs) expression (45). Most of reported LAB are fermented dairy- or human gut-derived. There is a debate over whether the immunostimulatory effect of LAB needs to be alive or dead.

***Lactobacillus pentosus* strain S-PT84**

The author focused on the health function of plant-derived LAB, isolated from pickles in Kyoto, and selected *L. pentosus* strain S-PT84 (Fig. 1-1) by measuring the induction activity of IL-12, one of the Th1 cytokines. It is believed that most LAB must reach the intestine alive. However, since LAB are often killed in food, we conducted various studies using heat-killed S-PT84 strains as

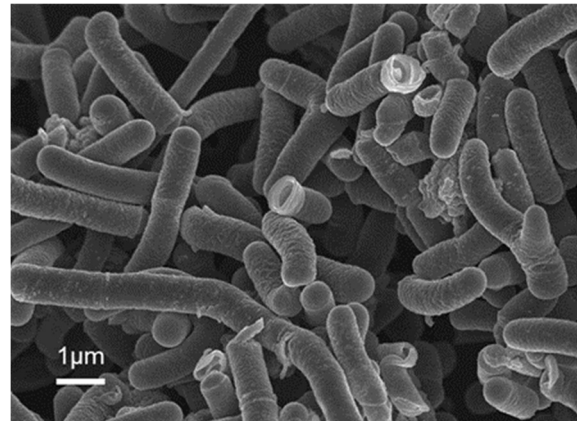


Fig.1-1 Picture of *Lactobacillus pentosus* strain S-PT84 (×10,000)

shown in Chapter 2. To begin with, in Chapter 2, experiments on immunostimulation were carried out from various angles such as effects Th1/Th2 balances and activation of NK cells in mice and effects on food allergy.

Physiologic stress can stimulate the release of epinephrine and norepinephrine through the central nervous system (46) or glucocorticoids through the hypothalamus (47, 48). In Th cell differentiation to the Th1 or Th2 phenotype, cortisol inhibits the ability of monocytes and macrophages to produce IL-12 and promotes the production of Th2 cytokines in CD4 leukocytes (49, 50). In addition, glucocorticoids are shown to increase directly the mRNA levels of Th2 cytokines in CD4⁺ T cells (49, 50). Furthermore, *in vitro* experiments have revealed that glucocorticoids directly enhance mRNA levels of Th2 cytokines in CD4⁺ T cells (51). Blood corticosterone levels have also been reported to affect NK activity (52), and a number of experimental studies have demonstrated stress and impaired immune function and exacerbation of immune-related disorders such as asthmatics (53). Since *L. pentosus* S-PT84 have immunomodulatory effects, the author examined the effects of *L. pentosus* S-PT84 strains on changes in NK activity and Th1/Th2 balance that are reduced by stress using restraint stress models

in Chapter 3.

Immunomodulatory activity of lactobacilli has been demonstrated not only at the animal level but also in humans. For example, there have been reports of a significant increase in NK activity in healthy smokers of *L. casei* strains after 3 weeks of ingestion (54), as well as reports of an increase in NK activity in a number of clinical studies in *L. casei* (54-63). In other LAB, ingestion of *L. rhamnosus* HN001 for 9 weeks increased polymorphonuclear leukocytes phagocytosis and NK activity in subjects (64). Various evidences have been clarified even when evaluated according to human life stages and health conditions. In healthy young adults, LAB intake has been reported to increase IFN production (65-67). Marcos *et. al* (67) has conducted a placebo-controlled study ingesting 200ml of yogurt containing *L. casei* DN-114001 (10^8 /ml) using university students during the academic experiment period as subjects. The subjects were in a stressed state, cortisol increased and NK activity decreased, whereas the decrease in NK activity was suppressed in subjects who took *L. casei* DN-114001. Intervention trials in elderly patients have reported that ingestion of *Bifidobacterium lactis* HN019 (10^9 - 10^{10} cfu) for 3 weeks increased the phagocytic activity of monocytes and NK activity (68, 69). Thus, after confirming the immunostimulatory activity of LAB *in vitro* or *in vivo*, it is very important to confirm the evidence in humans. In particular, a clinical study targeting humans with reduced immunity, for example, people under experimental period or elderly people as mentioned above. But first, the author challenged a pilot study targeting healthy individuals. In Chapter 4, the author prepared the *L. pentosus* S-PT84 supplement by culturing the S-PT84 in a food medium and estimate the immunomodulatory activity of *L. pentosus* S-PT84 in healthy subjects, particularly NK activity and Th1/Th2 balance by a placebo-controlled, crossover study.

Immune function and cancer

In general, cancer cells are always generated in our body, but NK cells and so on attack to them, it can be maintained a healthy state without causing cancer. Conversely, if the immune system is reduced due to factors such as stress, lack or bias of nutrition, and aging, the risk of carcinogenesis increases. Furthermore, it is well known that cancer cells themselves suppress immune functions. Therefore, anti-cancer drugs based on immunostimulation called biological response modifier (BRM), such as Krestin and Lentinan, were approved in the 1970s-80s. Later, IFN- γ and IFN- α , cytokines that activate immunocompetent cells, were approved as cancer treatments. Currently, further research on cancer and immunosuppression is ongoing. For example, it was revealed that cancer is proliferating to escape immune exclusion by skillfully using immunosuppressive factors called immune checkpoint molecules such as programmed cell death-1 PD-1 and its ligand PD-L1 by various researchers including Honjo *et al.* (70) who won the Nobel Prize in 2018. Recently, PD-1 inhibitors and PD-L1 inhibitors have been approved and are used in clinical practice. In addition, for Japanese people, infection with viruses or bacteria is the biggest risk of cancer after smoking (71). Thus, cancer and immune function are very closely linked. On the other hand, it is more important to prevent cancer by immune function than to cure cancer using immunomodulating drug for healthy people.

Tumor model suitable for evaluating immunostimulation foods

One of the purposes of maintaining immune function for us is cancer prevention. To search for a functional food material that can suppress cancer by enhancing immune function, the evaluation mode is important. Tumor bearing mice models are common and the well-known model is Sarcoma 180-tumor bearing mice. This is a model that was very used from the 1960s to the 1980s and is often transplanted to DDY mice and ICR mice. However, Sarcoma 180 is derived

from CFW mice (72) and is inoculation to DDY or ICR mice as a host. Because cancer cells are derived from different species for the host, it is not suitable for evaluating cancer suppression by immunostimulation. Therefore, an effective model is a syngeneic tumor, and the authors selected the MM46 tumor, which is a mammary carcinoma derived from C3H/He mice (73), bearing C3H/He mice as a syngeneic tumor model. According to the PubMed search, there were 71 reports using this syngeneic tumor model in 2001 when the author conducted this experiment, this model was considered to be sufficient proven. As another evaluation model, a cancer metastasis model using a syngeneic tumor Lewis lung carcinoma (LLC) in C57BL6 mice was used. LLC is lung carcinoma derived from C57BL/6 mice (74). According to PubMed research, there were 63 reports using this syngeneic tumor metastasis model until 2004 when the author conducted this experiment. There are 223 reports in October 2019.

Ganoderma lucidum

Mushrooms are one of the familiar foods of fungi and are commonly used not only as a common food but also as a supplement or as a type of Chinese medicine. Oriental mushroom, *Ganoderma lucidum* (Fr.) karst (*Reishi*) is a popular traditional Chinese herbal medicine used in China, Japan, and other Asian countries (75). It is prescribed for the prevention and treatment of various diseases such as diabetes (76) and high blood pressure(78, 79), allergy (80, 81), hepatitis (82, 83), and cancer (84, 85). Active ingredients such as β -D-glucan and triterpenoids have been isolated from *G. lucidum* and experimental data on antitumor activities *in vitro* and *in vivo* have been reported (86-93).

***Ganoderma lucidum* antlered form**

Among *G. lucidum*, antlered formed *G. lucidum* (*G. lucidum AF*, Rokkakureishi) (Fig. 1-2) is

a variant of *G. lucidum* that is rarely found in the natural world. Cultivation techniques have been developed, and *G. lucidum* AF is artificially cultivated in China, Korea, and Japan. *G. lucidum* AF



has been reported to contain large amounts of β -D-glucan (94). It has also been reported that *G. lucidum* AF triterpenoids levels were much more abundant than original *G. lucidum* (94). The anti-tumor activity of *G. lucidum* AF is expected to be stronger than that of original *G. lucidum*. In Chapter 5, the authors used a syngeneic tumor model to investigate the antitumor activity of *G. lucidum* AF.

Fig. 1-2 *Ganoderma lucidum* antlered form

Recently, anticancer drugs have advanced then human can fight many cancers. However, the side effect is inherent in the anticancer drug, and one of the side effects is the immune deficient, so immune deficiency in cancer treatment is associated with a significant risk. In Chapter 6, it was examined that whether *G. lucidum* AF can suppress the immune deficiency induced by the chemotherapeutic drugs. Furthermore, it was examined whether *G. lucidum* AF can suppress cancer metastasis when immune deficiency was occurred by chemotherapeutic drugs.

Aim of this dissertation

Since our bodies are protected by immune functions, it is no exaggeration to say that they maintain our homeostasis. However, imbalanced immune system and decreased immune function are at risk of developing allergies, which are excessive reactions of immunity, and cancers, which are the immune dysfunction will result. Therefore, keeping the immune function normal should be necessary for maintaining health. However, in recent years, studies on immunity have become

mainstream by searching for detailed types and functions of immunocompetent cells and searching for new molecular mechanisms. It must be said that there is insufficient research and discussion on how to maintain normal immune function and what is the solution. In fact, there are drugs that suppress immune function due to anti-allergy, and classic drugs that strengthen immunity to fight cancer, but there are no drugs that adjust the Th1/Th2 balance to normal or enhances NK activity. Not only that, there is no record of acceptance such health claims regarding immune functions in the Japanese “Food for specified health use” system, and “Food with functional claim” system is not accepted according to the guidelines (95). However, the author believes that food ingredients, especially those derived from microorganisms and fungi, can help us maintain normal immune functions that tend to be disturbed. From such an ambition, the author found the *L. pentosus* S-PT84 that regulates immune function and to suppress allergy (Chapter 2), as well as an inhibitory action on the decline of immune function due to restraint stress (Chapter 3). It was clarified that the immunostimulatory effect of *L. pentosus* S-PT84 is effective not only in rodents but also in humans (Chapter 4). Furthermore, the author found *G. lucidum* AF which can enhance the immune function to fight cancer and clarified its antitumor effect by immunostimulatory action in a syngeneic tumor model (Chapter 5). *G. lucidum* AF is effective in suppressing the immunosuppression induced by chemotherapeutic agents and can also be useful in suppressing metastasis and keeping QOL (Chapter 6). If the possibility of maintaining/improving immune function by food ingredients has spread scientifically even slightly through this dissertation, the author will be extremely rewarding.

Chapter 2.

Anti-Allergic Effects of *Lactobacillus pentosus* S-PT84 Mediated by Modulation of Th1/Th2 Immuno-Balance and Induction of IL-10 Production

2-1 Introduction

Allergic symptoms, such as atopic diseases and pollen allergy, are chronic diseases that are increasingly prevalent, especially in developed countries, and are a serious social problem (96).

Allergy depends on Th2 derived immune responses characterized by the production of the cytokines IL-4, 5, and 13, which regulate the production of IgE and other chemical mediators (97, 98). Th1 cytokines such as IL-2 and 12 suppress the growth of Th2 cells and the switching of B cells. IL-12 is a Th1 maturation factor that stimulates NK cells and Th1 cells to produce IFN- γ (6) and prevents the development of Th2 responses (99). Thus, modulation of Th1 cytokine production levels is expected to be beneficial not only in the prevention but also in the treatment of allergic diseases.

In Japan, many types of fermented foods such as pickles, miso, and sake have been made and eaten. These are made by fermentation by plant-derived LAB. Plant-derived LABs exhibit a wider range of sugar metabolizing activities than common LABs living in the gastrointestinal tract and used to make fermented milk products such as yogurt (100), though their physiological functions remain unclear compared to probiotics LAB. *L. pentosus* S-PT84 was selected by *in vivo* assay using IL-12 inducing activity as an index. Thereafter, its immunomodulatory activity (IL-10-inducing activity, Th1/Th2 cytokine inducing activity, and NK activity) on BALB/c mice and its inhibitory effect on OVA-induced allergy models were examined. All experiment was conducted by using heat-killed *L. pentosus* S-PT84 preparations to determine whether LAB must be alive to show immunomodulatory activity.

2-2 Materials and Methods

2-2-1 Preparation of heat-killed LAB

The bacterial strains used in this study were isolated from Kyoto pickles and kept at the Louis Pasteur Center for Medical Research. These bacterial strains were cultured in deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) at 35°C for 48 h. Cultured

bacteria were collected by centrifugation, washed twice with sterile saline, and heat-killed at 95 °C for 5 min. Heat-killed bacteria were washed with distilled water, freeze-dried, and then subjected to the following experiments. *L. pentosus* S-PT84 was deposited at the National Institute of Advanced Industrial Plant Organism Depository, Patent Microorganisms Depository (Tsukuba, Japan) as FERM ABP-1002.

2-2-2 Animals

Male BALB/c mice (7 weeks of age) were purchased from Japan SLC Inc. (Shizuoka, Japan). These animals were housed with a commercial diet (CE-2) and tap water *ad libitum* for 1 week at 25 ± 1°C and 60 ± 5% humidity under a 12 h light-dark cycle before experimentation. Experiments were performed according to “The Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals.” All experiments were conducted between 2003 and 2007.

2-2-3 IL-12-inducing activity *in vivo*

BALB/c mice were i.p. injected with 0.5 mg of each heat-killed bacterial strain in 0.2 ml sterile saline. Blood was collected 4, 6, 8, 12, and 24 h after LAB-injection and serum was prepared by centrifugation. IL-12 (p70) levels in serum were measured by enzyme-linked immunosorbent assay (ELISA) as described below.

2-2-4 IL-12- and IL-10-inducing activities *in vitro*

BALB/c mice were administered i.p. with 2 ml of 4.05% thioglycolate medium (Difco Laboratories). Peritoneal macrophages were collected 4 days after injection of the thioglycolate medium by lavage of the peritoneal cavity with 8 ml of ice-cold phosphate buffered saline (PBS). The peritoneal exudate cells were centrifuged and suspended in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS) at a density of 2×10^6 cells/ml. The cells were pre-cultured in 24-well plastic plates (BD Bioscience, Franklin Lakes, NJ) at 37°C in 5% CO₂. After 2 h, the non-adherent cells were removed, and the adherent cells were cultured in RPMI

1640 medium with 1 µg/ml of each bacterial strain for 48 h. The supernatant of the cells was collected for measurement of IL-12 and IL-10 by ELISA as described below.

2-2-5 Feeding procedure

To determine the immunomodulating effects of free feeding of *L. pentosus* S-PT84, *L. pentosus* S-PT84 (final dose: 0.075%) was added to AIN-93M diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and given to BALB/c mice. The control group was given normal AIN-93M. Mice were fed AIN-93M with or without 0.075% S-PT84 for 1 week *ad libitum*, and *ex vivo* IFN-γ- and IL-4-inducing activities, and NK activity were then measured.

2-2-6 Ex vivo assessment of IFN-γ and IL-4

BALB/c mice were fed AIN-93M with or without 0.075% S-PT84 for 1 week and splenocytes were prepared. The cells were suspended in RPMI 1640 with 10% FBS at a density of 5×10^6 cells/ml and were stimulated with 2.5 µg/ml concanavalin A (Con A) (Wako Pure Chemical Industries, Osaka, Japan) in 24-well plastic plates (BD Bioscience). After incubation for 24 h at 37 °C, the culture supernatant was collected and levels of IFN-γ and IL-4 were measured by ELISA as described below.

2-2-7 Measurement of NK activity (PINK method)

Yac-1 target cells were suspended in PBS at a density of 1×10^6 cells/ml and stained with 40 µg/ml of 3,3'-dioctadecyloxycarbocyanine perchlorate (DiO) (Sigma, St. Louis, MO) for 10 min at 37°C. The stained cells were washed once with PBS and resuspended in RPMI 1640 with 10% FBS at a density of 5×10^4 cells/ml. Then 100 µl of the DiO-stained target cells suspensions and 100 µl suspensions of various numbers of effector cells and 25 µg/ml of propidium iodide (PI) (Molecular Probes, Eugene, OR) were added to 96-well U-shaped microplates (BD Bioscience). The plates were centrifuged at $200 \times g$ and incubated at 37°C in 5% CO₂ for 4 h. Spontaneous cell death was determined by incubating target cells without effector cells. After incubation, DiO⁺PI⁺ cells (killed cells) were analyzed using Epics XL (Beckman Coulter, Brea, CA). NK activity

was calculated according to the formula:

$$\text{NK activity (\%)} = (\text{DiO}^+\text{PI}^+) / (\text{DiO}^+\text{PI}^- + \text{DiO}^+\text{PI}^+) \times 100 - \text{spontaneous lysis (\%)}$$

2-2-8 OVA-induced allergy model

The experimental procedure used to produce the OVA-induced allergy model is illustrated in Fig. 2-1. BALB/c mice were fed AIN-93M with or without 0.075% S-PT84 for 5 weeks, and were administered i.p. with 20 µg of OVA (Grade V, Sigma) and 2 mg of Al(OH)₃ in a total volume of 0.2 ml

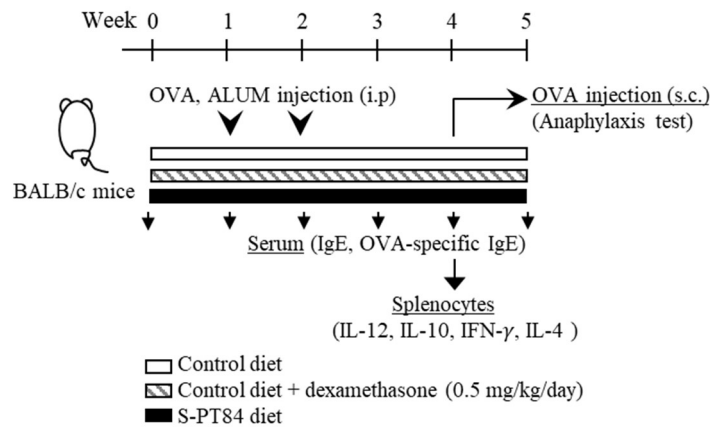


Fig. 2-1. Experimental design of OVA-induced allergy

at weeks 1 and 2. The positive control group was given drinking water containing 3.125 µg/ml dexamethasone (Nacalai Tesque) (0.5 mg/kg/day) from 1 week before the first OVA administration during the experimental period. Serum was collected from the vein of the tail every week, and total IgE and OVA-specific IgE were measured by ELISA. Splenocytes from 4 mice of each group were collected on week 4 and treated with OVA (100 µg/ml). Four days after treatment, supernatants were collected to measure produced IL-12, IL-10, IFN-γ, and IL-4 levels. The active cutaneous anaphylaxis (ACA) experiment was performed using 8-10 mice of each group at week 4 as described below.

2-2-9 Active cutaneous anaphylaxis

ACA was induced by subcutaneous challenge of OVA-sensitized BALB/c mice with OVA. An intravenous injection of 0.05 ml of 1% Evans Blue (Nacalai Tesque) was performed, and then ACA was elicited in the skin of belly after subcutaneous injection of OVA (150 µg in 0.05 ml sterile saline). Thirty minutes after OVA challenge, mice were euthanized, and a piece of skin shaved from each mice was incubated in 1 N KOH (0.7 ml) at 37°C overnight. Then, 9.3 ml acetone

containing 28% 0.6 N H₃PO₄ was added and shaken vigorously. The amount of extravasated dye was determined by measuring absorbance at 630 nm.

2-2-10 ELISA

Measurement of IL-12, IFN- γ , IL-4, IL-10, and total IgE was measured by ELISA using an OptEIA ELISA set (BD Pharmingen, San Diego, CA). Measurement of OVA-specific IgE was carried out by ELISA using OptEIA ELISA with slight modifications. Briefly, the wells of a Maxisorp immunoplate (Nunc, Roskilde, Denmark) were coated with 0.2 mg/ml OVA (Sigma) in 0.1 M carbonate buffer (pH 9.5) overnight at 4°C. After blocking the unoccupied sites on the well with assay diluent (BD Bioscience), the test samples were added. Subsequently, OVA-bound IgE was detected by biotinylated anti-mouse IgE monoclonal antibody and avidin-horseradish peroxidase conjugate (BD Bioscience). The level of OVA-specific IgE was estimated by comparison with hyperimmunized mice serum collected from BALB/c mice sensitized by i.p. administration of two doses of OVA adsorbed in Al(OH)₃. The OVA-specific IgE level of this standard serum was taken to be 1000 U/ml.

2-2-11 Statistical analysis

The significance of differences in values was tested by one-way ANOVA, followed by Fisher's PLSD as a *post-hoc* test for more than three groups. Student's *t*-test was used for two-group comparisons.

2-3 Results

2-3-1 Cytokine-inducing activities of LABs isolated from Kyoto pickles

In order to select the LAB with the best IL-12-inducing activity from 16 kinds of LAB (12 types were *L. pentosus* and 4 types were *L. plantarum*), heat-killed LAB preparations derived from Kyoto pickles, each LAB was i.p. administered to mice, and the serum IL-12 levels after 6 hours were measured (Fig. 2-2). *L. pentosus* S-PT84, DS51C, and DS25C showed strong IL-12-inducing activity *in vivo*. Especially, *L. pentosus* S-PT84 had strongest activity. In addition, when the time

course of IL-12 induction by *L. pentosus* S-PT84 was confirmed, IL-12 was most induced 6 hours after administration (Fig.2-3).

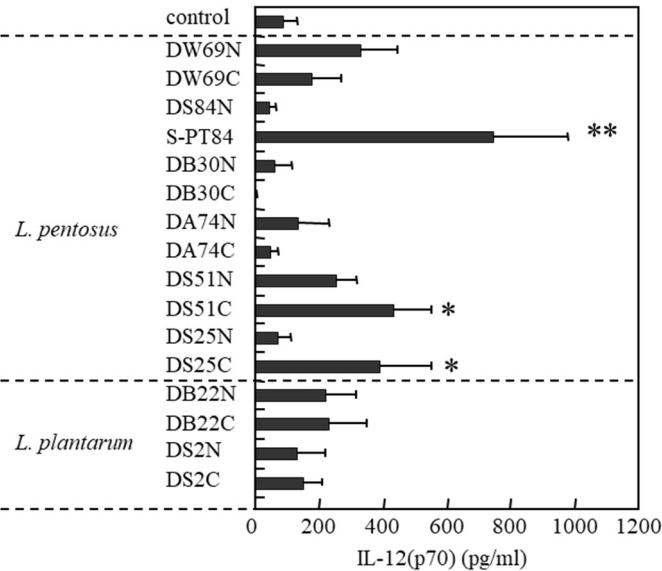


Fig. 2-2. Effects of various lactic acid bacteria strains of plant origin on IL-12 (p70) production *in vivo*. Heat-killed LAB suspended in sterile saline (0.5 mg/0.2 ml) were administered intraperitoneally to BALB/c mice, and IL-12 concentrations in serum 6 h after administration of LAB were measured by ELISA. Values are the mean $\pm$ S.E. of five mice. Asterisks indicate significant differences from the control group (*: $p<0.05$ and **: $p<0.01$).

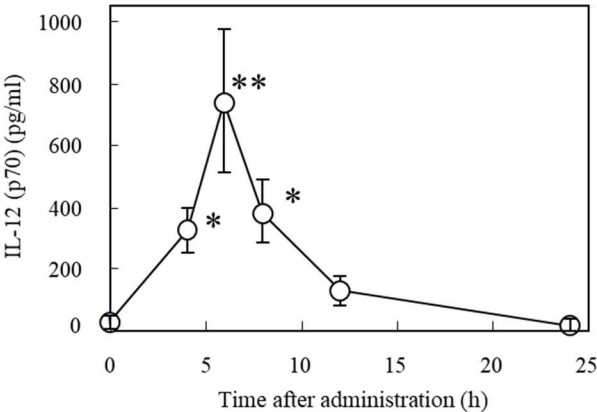


Fig. 2-3. Time course for IL-12 induction by *L. pentosus* S-PT84. *L. pentosus* S-PT84 suspended in sterile saline (0.5 mg/0.2 ml) were administered intraperitoneally to BALB/c mice, and IL-12 concentrations in serum 6 h after administration of LAB were measured by ELISA. Values are the mean $\pm$ S.E. of five mice. Asterisks indicate significant differences from 0 h group (*: $p<0.05$, **: $p<0.01$).

2-3-2 IL-12- and IL-10-inducing activities of *L. pentosus* S-PT84

In order to confirm the concentration dependence of IL-12 (p70)-inducing activity of *L. pentosus* S-PT84, *in vitro* evaluation using peritoneal macrophages was conducted. The cells were cultured with a range of doses of *L. pentosus* S-PT84 (0.1, 0.3, 1, 3, 10, 30, and 100 μ g/ml) for 48 h,

and IL-12 (p70) levels were measured (Fig. 2-4A). *L. pentosus* S-PT84 (0.3-10 µg/ml) induced IL-12 in dose-dependent manner, though in 30 or 100 µg/ml *L. pentosus* S-PT84-treated samples the IL-12 level was less than that with 10 µg/ml *L. pentosus* S-PT84. In order to confirm the reason that dose dependency of IL-12 production was not observed when *L. pentosus* S-PT84 exceeded 30 µg/ml, the concentration of IL-10, a cytokine of the immune control, in the medium was measured under the same conditions. Concentrations above 10 µg/ml of *L. pentosus* S-PT84 induced IL-10 in dose-dependent manner.

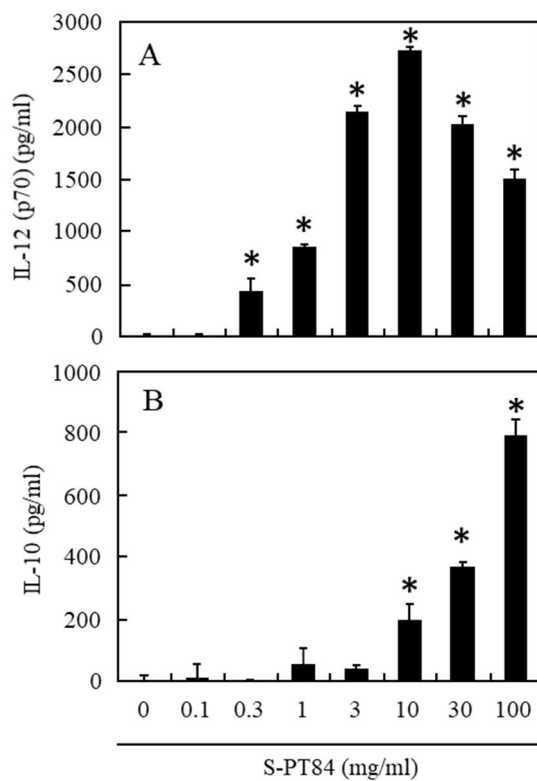


Fig. 2-4. Production of IL-12 and IL-10 by peritoneal macrophages after exposure to *L. pentosus* S-PT84.

Peritoneal macrophages were prepared from BALB/c mice. The cells were cultured in the presence of 0.1-100 µg/ml of heat-killed *L. pentosus* S-PT84 for 48 h. Culture supernatant was collected and measured for IL-12 (p70) (A) and IL-10 (B) concentration by ELISA as described in Materials and Methods. Values are the mean ± S.E. (n=3). Asterisks indicate significant differences from 0 mg/ml ($p < 0.01$).

2-3-3 Effect of oral feeding of *L. pentosus* S-PT84 on Th1 and Th2 cytokine production and NK activity

The splenocytes from the mice that received *L. pentosus* S-PT84 for 1 week were stimulated with Con A, and the cytokine-inducing activity was measured. The intake of *L. pentosus* S-PT84 did not change the IFN-γ-inducing activity, but decreased the IL-4-inducing activity, and

increased IFN- γ /IL-4 ratio significantly (Fig. 2-4 A-C). NK activity of the splenocytes was also increased by *L. pentosus* S-PT84 feeding (Fig. 2-4 D).

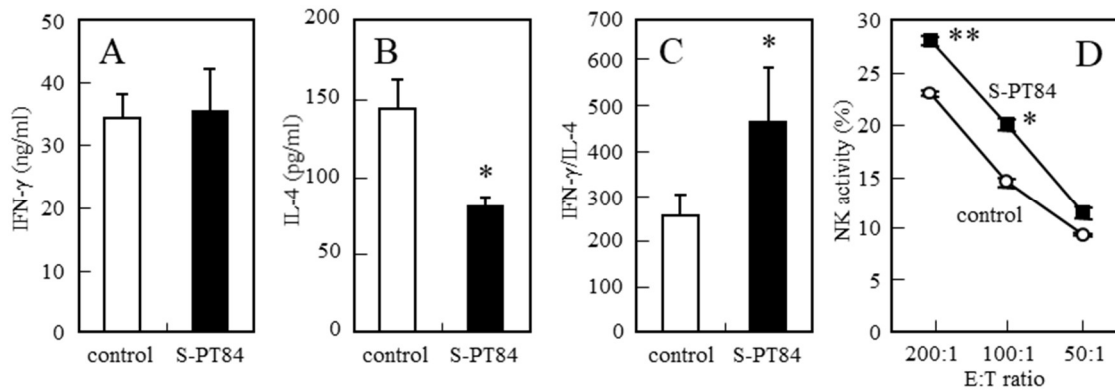


Fig. 2-5. Effect of oral feeding of *L. pentosus* S-PT84 on Th1 and Th2 cytokine production and NK activity. Mice were fed an AIN-93M diet with or without *L. pentosus* S-PT84 for 1 week, and their splenocytes were obtained. The cells were treated with Con A (2.5 μ g/ml) for 24 h, IFN- γ (A) and IL-4 (B) concentrations in culture supernatants were measured by ELISA, and the IFN- γ / IL-4 ratio was calculated (C). (D) NK activity of splenocytes from the mice. The cells were co-cultured with DiO-stained Yac-1 cells at various effector-to-target ratios for 4 h, and their cytotoxic activity was measured and calculated as described in Materials and Methods. Values are the mean $\pm$ S.E. of three mice. Asterisks indicate significant differences from the control group (*: $p < 0.05$ and **: $p < 0.01$).

2-3-4 Anti-allergic activity of *L. pentosus* S-PT84 in OVA-induced allergy model

The OVA-induced food allergy model was conducted according to Fig. 2-1. *L. pentosus* S-PT84 significantly suppressed the increase in total IgE and OVA-specific IgE produced by OVA sensitization (Fig. 2-6). The degree of inhibition of IgE produced by OVA by *L. pentosus* S-PT84 was comparable to that of dexamethasone used as a positive control for total IgE and higher for OVA-specific IgE.

In this OVA model, an ACA reaction was confirmed in order to confirm whether S-PT84 also suppressed an immunoinflammatory reaction. The ACA response induced by OVA sensitization was significantly suppressed by *L. pentosus* S-PT84, and the extent was similar to that of dexamethasone (Fig. 2-7).

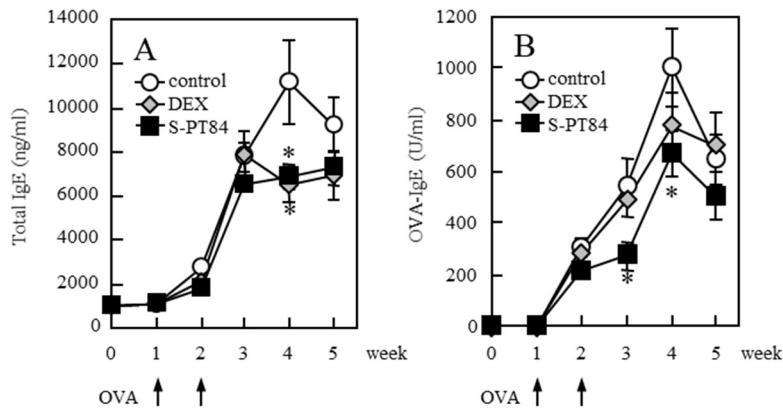


Fig. 2-6. Effects of oral feeding of *L. pentosus* S-PT84 on IgE production in ovalbumin (OVA)-sensitized mice. Mice were fed an AIN-93M diet with or without *L. pentosus* S-PT84 from week 0 for 5 weeks, and were i.p. injected with 20 μ g of OVA and 2 mg of Al(OH)₃ at weeks 1 and 2 in a total volume of 0.2 ml. The positive control group was given drinking water containing dexamethasone (DEX) (0.5 mg/kg/day) from 1 week before the first OVA administration (week 0) during the experimental period. Serum was collected from each mouse and total IgE and OVA-specific IgE were measured by ELISA as described in Materials and Methods. Values are the mean $\pm$ S.E. of 10-11 mice. Asterisks indicate significant differences from the control group (*: $p < 0.05$).

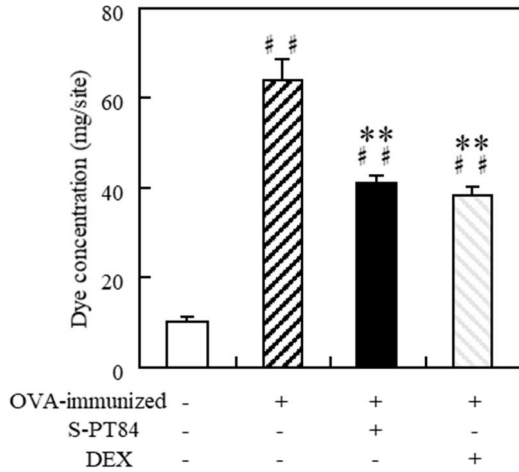


Fig. 2-7. Effects of oral feeding of *L. pentosus* S-PT84 on active cutaneous anaphylaxis (ACA) in OVA-sensitized mice. Mice were fed an AIN-93M diet with or without *L. pentosus* S-PT84 from week 0 for 4 weeks, and were i.p. injected with 20 μ g of OVA and 2 mg of Al(OH)₃ at weeks 1 and 2 in a total volume of 0.2 ml. The positive control group was given drinking water containing DEX (0.5 mg/kg/day) from 1 week before the first OVA administration (week 0) during the experimental period. On week 4, ACA was induced by subcutaneous challenge with OVA (150 μ g) as described in Materials and Methods. Values are the mean $\pm$ S.E. of 8-10 mice. Asterisks indicate significant differences from the OVA-sensitized group (the second column) ($p < 0.01$), and hash marks indicate significant differences from the control group (the first column) ($p < 0.01$).

The cytokine production activities of OVA-sensitized mouse splenocytes by Con A was measured (Fig. 2-8A-D). Th1 cytokines, IL-12 and IFN- γ levels were decreased and Th2 cytokine, IL-4 was increased significantly by OVA sensitization. IL-10 level was also increased. Th1 cytokines levels were not changed, but IL-4 level was decreased, and IL-10 level was increased by *L. pentosus* S-PT84.

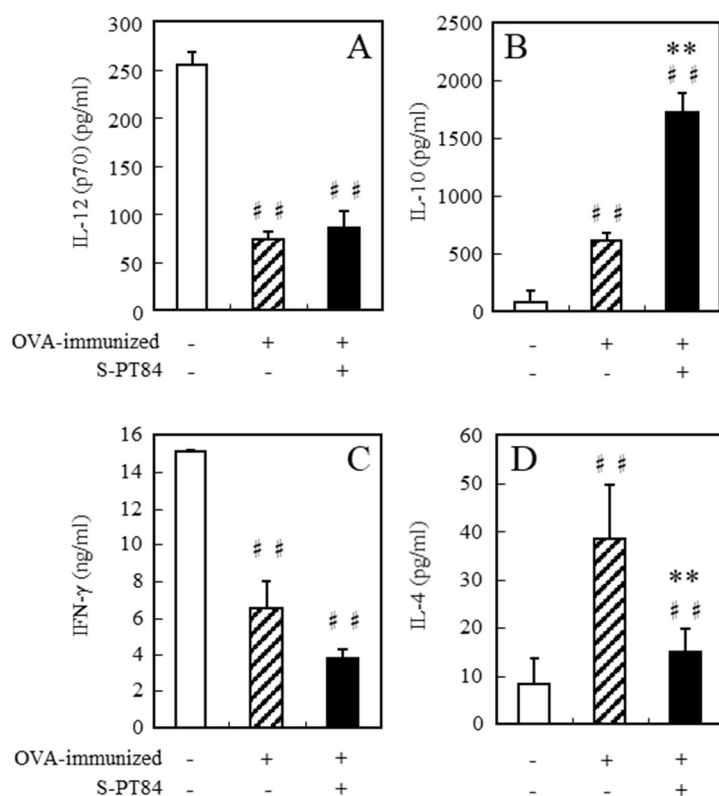


Fig. 2-8. Effects of oral feeding of *L. pentosus* S-PT84 on cytokine production in OVA-sensitized mice.

Mice were fed an AIN-93M diet with or without *L. pentosus* S-PT84 from week 0 for 4 weeks, and were i.p. injected with 20 µg of OVA and 2 mg of Al(OH)₃ at weeks 1 and 2 in a total volume of 0.2 ml. Splenocytes were derived from the mice on week 4, and were treated with OVA (100 µg/ml) for 4 days. Culture supernatants were collected and measured for IL-12 (p70) (A), IL-10 (B), IFN-γ (C), and IL-4 (D) concentrations by ELISA as described in Materials and Methods. Values are the mean ± S.E. of 4 mice. Asterisks indicate significant differences from the OVA-sensitized group (second columns) ($p < 0.01$), and hash marks significant differences from the control group (first columns) ($p < 0.01$).

2-4 Discussion

The anti-allergic effects of LAB (24-27, 29-31) have been well studied. However, most target LABs examined have been of animal origin including derived from human gut. Many types of fermented foods are consumed in Japan and these foods are fermented by LAB of plant derived (100). The purpose of this study was to discover new LAB of plant origin which show immunomodulatory and anti-allergic activity despite use in heat-killed preparations.

L. pentosus S-PT84, which has the strongest IL-12-inducing activity, was selected from 16 LABs derived from plants isolated from traditional Kyoto pickles (Fig. 2-3 and 2-4). *L. pentosus* S-PT84 modulates the Th1/Th2 balance toward a Th1-dominant state by reducing IL-4, Th2 cytokine production not by enhancing IFN-γ, Th1 cytokine (Fig. 2-5A-C), activates NK cells (Fig. 2-5D), suppresses serum IgE levels (Fig. 2-6), and the ACA reaction induced by OVA (Fig. 2-7). All of these effects were induced by heat-killed *L. pentosus* S-PT84 preparations.

It has been believed that the immunogenicity of LAB depends on survival of bacteria in

the gastrointestinal tract, resistance to gastric acid, and ability to adhere to the mucosal surface (21-23). However, some studies have suggested that non-viable LAB can also show immunomodulatory activity, and induce IL-12 production by macrophages (28, 29), inhibit IgE production in an OVA-induced food allergy model (30, 31), and increase fecal antigen-specific IgA (32, 33). These previous findings suggest that LAB do not have to be alive to exert their immunomodulatory effects such as anti-allergic activity. It is possible that killed LAB preparations exhibit such activity because the active components of LAB function as pattern ligands and trigger immunostimulation.

It has become evident that the recognition of specific microbial/pathogen-associated patterns is mediated by TLR, pattern recognition receptors that signal the presence of specific microorganisms to the host. The TLR family is the best characterized class of pattern recognition receptors in mammalian species. Some studies have reported that LAB modulate host immune reactions via TLR-2 (101,102) or TLR-9 (103) on antigen-presenting cells. TLR-2 is mainly involved in responses to cell wall components such as lipoteichoic acid from gram-positive bacteria. Grandette *et al.* (104) reported that the importance of lipoteichoic acid composition in determining the ability of *L. plantarum* which is closely related species of *L. pentosus*, to differentially impact the host immune system. TLR-2 is expressed on the surface of antigen-presenting cells such as macrophages and dendritic cells, and the number of TLR-2-positive cells in Peyer's patches was increased after LAB administration (102). It is likely that *L. pentosus* S-PT84 modulates host immunological function via TLR-2 on the surface of antigen-presenting cells. On the other hand, Forsythe *et al.* reported that *L. reuteri* decreased allergen-induced airway hyperresponsiveness in TLR-9-dependent manner (105). TLR-9 recognizes CpG oligodeoxynucleotides and was reported that AT oligodeoxynucleotides, a new group of TLR-9 ligands, were identified from LAB (103). These findings indicate that LAB show immunomodulatory activity via not only TLR-2 but also TLR-9. However, it is not clarified concerning the mechanisms of immunomodulation by LABs of plant origin, further studies of them are required for application to create functional food products.

Lots of previous studies have demonstrated that LABs exhibit anti-allergic activity by modulating Th1/Th2 balance toward a Th1 dominant state. Although these LABs show IL-12-

inducing activity *in vitro*, each patterns of Th1/Th2 cytokine production by antigen-sensitized animals differ. For example, the *L. casei* Shirota increases the production of Th1 cytokines, IL-12, and IFN- γ , and decreases that of IL-4 and IL-5 in OVA-sensitized mice (30). The *L. paracasei* KW3110 induces IL-12 production but does not affect IFN- γ and IL-4 production (31). The *L. plantarum* L137 is like *L. pentosus* S-PT84, increases IL-12, does not affect IFN- γ production, and decreases IL-4 production (29). *L. pentosus* S-PT84 does not affect Th1 related cytokines production such as IL-12 and IFN- γ , but decreases IL-4 production, and increases regulatory cytokine IL-10 production in OVA-sensitized mice (Fig. 2-9). Thus, the anti-allergic mechanisms of LAB differ depending on the strain. It is thus necessary to clarify the anti-allergic mechanisms of LAB at the strain level.

It is known that excessive Th1 causes Th1-type diseases such as rheumatic arthritis (106, 107), hepatopathy (108, 109), and type 1 diabetes (110, 111). It might have to be considered a risk of side effects of excessive LAB-mediated Th1 immunity. *L. pentosus* S-PT84 induces production of not only IL-12 but also IL-10 (Fig. 2-4). IL-12 was induced in dose-dependent manner up to 10 $\mu\text{g/ml}$, though when *L. pentosus* S-PT84 dosage reached IL-10-inducing levels, IL-12 production was suppressed. It was suggested that *L. pentosus* S-PT84 not only induces Th1, but can also be suppressed by inducing IL-10 when an excessive immune response occurs. In splenocytes isolated from OVA-sensitized mice, IL-10 was produced, although production of IL-4 was down-regulated (Fig. 2-8B and D). IL-10 is able to induce the development of regulatory T cells (112, 113) and inhibit cytokine production. These findings suggest that *L. pentosus* S-PT84 can suppress immune responses by regulatory T cells when Th1 or Th2 responses are excessive. These findings accord with previous observations that LABs induce IL-10 production in human peritoneal blood mononuclear cells (114) and cord cells (115) and induce IL-10-producing regulatory T cells (116). In this study, *L. pentosus* S-PT84 and dexamethasone at the concentration used in this study exhibited the same degrees of anti-allergic effect (Fig. 2-6 and 2-7), although it appears that the mechanism of anti-allergic effects of *L. pentosus* S-PT84 differs from those of anti-allergy drugs such as dexamethasone, which depress host immune function including production of IL-10 (117).

In conclusion, the basic immunostimulatory profile of *L. pentosus* S-PT84 has been

clarified. *L. pentosus* strain S-PT84 of plant origin, an IL-12 potentiator, can enhance NK activity, modulate Th1/Th2 balance and IL-10 production particularly in the case of excessive immunity. In the OVA-induced-allergy model, it was found that induction of IL-10 also suppressed the allergic reaction. By investigating further immune functions of *L. pentosus* S-PT84, the author believe that it will contribute to the maintenance of people's health and the prevention of immuno-related diseases.

2-5 Summary

Focusing on LAB derived from pickles in Kyoto, LAB with the strongest immunostimulatory activity were selected. The usefulness of heat-killed preparation of LAB, specifically the immunostimulatory and antiallergic activities were confirmed. Fourteen LABs derived from pickles were administered to mice, and the *Lactobacillus pentosus* S-PT84, which had the strongest activity, was selected based on the activity to induce IL-12. *L. pentosus* S-PT84 was found to have not only the activity to induce Th1 but also the activity to induce IL-10, which suppresses excessive immunity. It was indicating that it can regulate immune functions both positively and negatively. *L. pentosus* S-PT84 was found to shift the Th1 / Th2 balance to a Th1 dominance in BALB/c mice, which are Th2-type mice, based on the cytokine-producing activity of splenocytes of the mice that received *L. pentosus* S-PT84. It was also confirmed that *L. pentosus* S-PT84 increased NK activity. Furthermore, it was found that in an allergy model induced by OVA sensitization, continuous ingestion of *L. pentosus* S-PT84 suppresses IgE production in serum and allergic reaction due to OVA sensitization. These actions by *L. pentosus* S-PT84 were thought to contribute not only to regulation of Th1/2 balance and activation of immune function by IL-12, but also to immunoregulation by inducing IL-10 production.

Chapter 3.

Protective Effect of *Lactobacillus pentosus* S-PT84 Against Restraint Stress in Mice

3-1 Introduction

People in modern society can be exposed to stress throughout their lifetime. Especially, Japan is becoming a super-aged society more rapidly compared with other countries, which is forcing a lot of elderly people to work in spite of the fact that their immunological activity is compromised due to their age (118, 119). In this situation, stress is associated with the onset, course, and outcome of physical illness (120-122). In general, it is widely known that stress affects the immune system. For example, NK activity (123), phagocytosis (123), and NK T activity (124) are reported to be suppressed by restraint stress. Furthermore, shifting the Th1/Th2 balance towards Th2-dominant immunity has been reported to be induced by stress (125). These immune responses are induced by the release of corticosteroid hormones via the hypothalamic-pituitary-adrenal axis, and high levels of glucocorticoid cause apoptosis and necrosis in immature T and B cells (51, 126). Stress also induces redox imbalance associated with reactive oxygen species (ROS) production via numerous cellular cascades (127-129). Gostner *et al.* (130) reviewed the key functions that ROS and other redox-active molecules affect in immunity. Stress is now considered as an important modulator of the immune system (131-133) and a major cause of increased risk for immune-related diseases, such as infection (134), inflammatory diseases (135), and cancer (136). It is therefore important to prevent stress induced-immunodepression or disturbance.

LAB are functional foods useful for immune regulation, but it is not known whether they can maintain immune functions that are reduced by stress. As shown in Chapter 2, *L. pentosus* S-PT84 was able to regulate Th1/2 balance by inducing IL-10 when there was an excessive immune response like allergy and alleviated allergic reaction. The author next examined whether *L. pentosus* S-PT84 can suppress the immunodepression when immune function is reduced under stress. In this study, the author examined the hypothesis that *L. pentosus* S-PT84, which has potent immunomodulatory activity, might be effective for ameliorating stress-induced immune depression. The stress used in this study was restraint stress of mice as a physical stress model. Corticosterone

levels in serum were measured as a marker of stress. Splenic NK activity, IL-12 and IL-10 production from macrophages, and IFN- γ and IL-4 production from splenocytes were measured as parameters of immune activity. Reduced glutathione (GSH) and GSH/glutathione disulfide (GSSG) levels in serum and macrophages were also measured as indicators of oxidative damage. The author found that *L. pentosus* S-PT84 has a protective effect on restraint stress-induced immunodepression by immune modulating and/or antioxidative activity.

3-2 Materials and methods

3-2-1 Preparation of heat-killed LAB

The preparation method of LAB is as described in Chapter 2.

3-2-2 Animals and treatments

Male C57BL/6 mice (7 weeks of age) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Mice were reared in the manner described in Chapter 2 for one week before being used for the experiment. *L. pentosus* S-PT84 (final dose: 0.075%) was added to an AIN-93M diet (Oriental Yeast Co., Ltd.), which was then given to these mice for 1 week. The control group was given the AIN-93M diet alone. Restraint stress model was followed as the methods of Iwakabe *et al.* (124). After 1 week feeding of prepared diet, mice were restrained for 17 h with dedicated cage. The mice which were not restrained were left in a rearing cage for 17 h without food and water. Two milliliters of 4.05% thioglycollate medium (Difco Laboratories) were i.p. injected into mice at the time of peritoneal macrophage collection 4 days before *in vitro* assay. All protocols for animal procedures were approved by the Ethics Committee of Animal Experiment in accordance with the Internal Regulations on Animal Experiments at Suntory, which are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 2 Jun 2017). All animal experiments were conducted between 2003 to 2013.

3-2-3 Measurement of corticosterone

The serum corticosterone level was determined using a Corticosterone ELISA Kit for Rats

and Mice (MP Biomedicals, Santa Ana, CA, USA) .

3-2-4 Measurement of NK activity

Measurement and calculation of NK activity were in the methods described in Chapter 2.

3-2-5 *Ex vivo* assessment of cytokines

Ex vivo assessment of IL-12 (p70) and IL-10 production by macrophages and IFN- γ and IL-4 production by splenocytes were measured as described in Chapter 2.

3-2-6 Measurement of reduced GSH and GSSG level

Serum samples to be used for GSH and GSSG measurement were prepared by adding 5-sulfosalicylic acid (5%) to them and centrifuging them at $8,000 \times g$ for 10 min and their supernatants were analyzed. For measurement of the GSH level of peritoneal macrophages, 80 μ l of 10 mM HCl were added to the macrophages (1×10^6 cells), which were then frozen and defrosted three times. Subsequently, 20 μ l of 5% 5-sulfosalicylic acid were added, and the supernatants were collected after centrifugation of the samples ($8,000 \times g$ for 10 min). GSH and GSSG levels of the serum and macrophage were measured by using a total Glutathione Quantification Kit (Dojindo Laboratories, Kumamoto, Japan).

3-2-7 Statistical Analysis

The significance of differences in values was tested by one-way ANOVA, followed by Tukey's test as a *post-hoc* test.

3-3 Results

3-3-1 Suppressive effect of *L. pentosus* S-PT84 on restraint stress induced corticosterone induction

Serum corticosterone levels were measured as a marker of the stress levels of the mice induced by restraint. The serum corticosterone level was increased by restrain stress, and *L.*

pentosus S-PT84 administration significantly ameliorated the stress-induced corticosterone levels (Fig. 3-1).

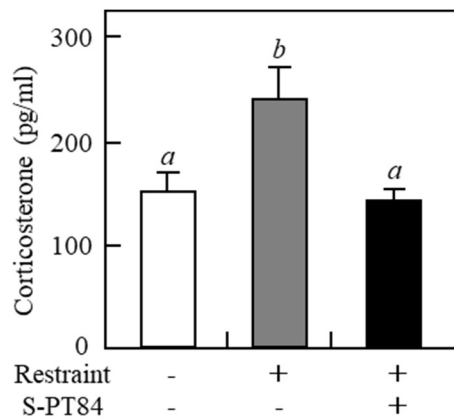


Fig. 3-1. Effect of oral administration of the *L. pentosus* S-PT84 on stress-induced changes in corticosterone in C57BL/6 mice. Corticosterone levels in the sera of mice that were fed an AIN-93M diet with or without *L. pentosus* S-PT84 for 1 week and then restrained, were measured as described in Materials and Methods. Data are presented as the mean $\pm$ S.E. of five mice. Different letters (*a* and *b*) indicate significant differences between groups ($p < 0.05$).

3-3-2 Effect of *L. pentosus* S-PT84 administration on restraint stress-induced immunodepression

The effect of *L. pentosus* S-PT84 administration on restraint stress-induced splenic immunodepression was then investigated by assay of the number of splenocytes, of splenic NK activity, and of splenocyte Con A-induced IFN- γ and IL-4 production levels. The number of splenocytes was reduced by restraint stress, but *L. pentosus* S-PT84 mitigated this reduction (Fig. 3-2A). Splenic NK activity was significantly downregulated by restraint stress, but this downregulation was ameliorated by *L. pentosus* S-PT84 feeding (Fig. 3-2B). IFN- γ production from Con A-stimulated splenocytes was reduced in stressed mice versus controls, but this reduction was not as great in stressed mice that had been fed *L. pentosus* S-PT84 (Fig. 3-2C). *L. pentosus* S-PT84 did not significantly change the stress-induced reduction in Con A-stimulated IL-4 production from splenocyte (Fig. 3-2D).

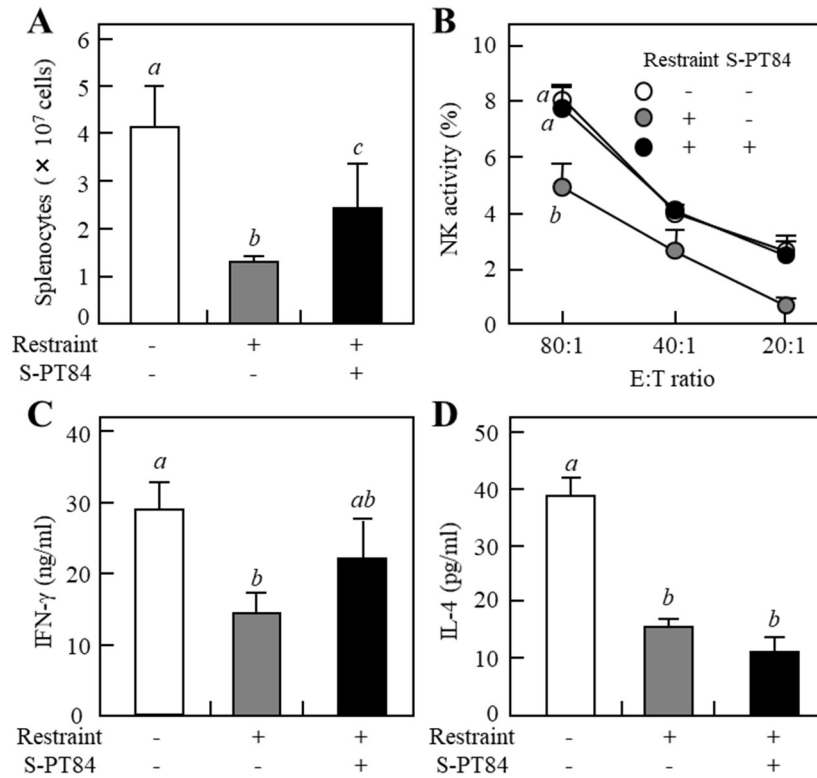


Fig. 3-2. Effect of oral administration of the *L. pentosus* S-PT84 on restraint stress-induced immunodepression of splenocytes. Splenocytes were collected from mice that were fed an AIN-93M diet with or without S-PT84 for 1 week and then restrained. The number of splenocytes (A), splenic NK activity against target YAC-1 cells (effector:target ratio = 80:1, 40:1, and 20:1) (B), and IFN- γ (C) and IL-4 (D) production levels in splenocytes cultured with Con A (2.5 μ g/ml) for 24 h were measured as described in Materials and Methods. Data are presented as the mean $\pm$ S.E. of five mice. Different letters (*a*, *b* and *c*) indicate significant differences between groups ($p < 0.05$).

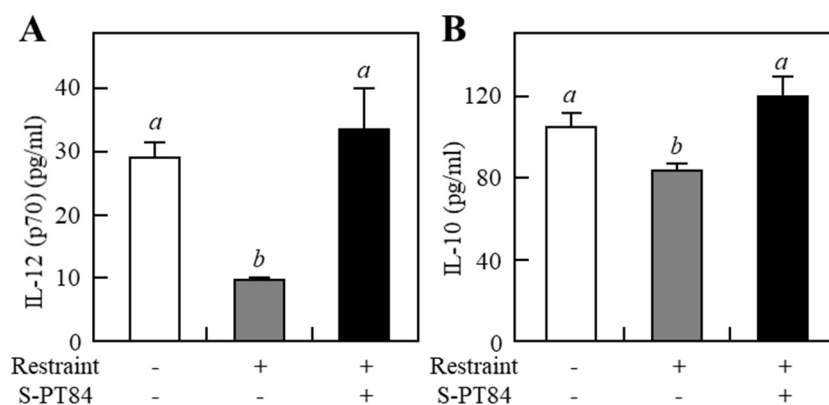


Fig. 3-3. Effect of oral administration of the *L. pentosus* S-PT84 on IL-12 and IL-10 production by macrophages in restraint stressed mice. Macrophages were collected from mice that were fed an AIN-93M diet with or without *L. pentosus* S-PT84 for 1 week and then restrained. The cells were then cultured with LPS (100 ng/ml) for 48 h. IL-12 (p70) (A) and IL-10 (B) levels in the collected culture supernatants were measured. Data are presented as the mean $\pm$ S.E. of five mice. Different letters (*a* and *b*) indicate significant differences between groups ($p < 0.05$).

To investigate the effect of *L. pentosus* S-PT84 administration on stress-induced effects on innate immune reaction, the author investigated IL-12 and IL-10 production by LPS-activated peritoneal macrophages isolated from these mice. Both IL-12 and IL-10 production by LPS-stimulated macrophages were downregulated by stress, and the decrease in IL-12 production was much greater than that in IL-10 production (Fig. 3-3A and B, respectively). In the *L. pentosus* S-PT84-fed mice, there was no stress-induced decrease in these cytokine productions by activated macrophages, and it was maintained at control levels.

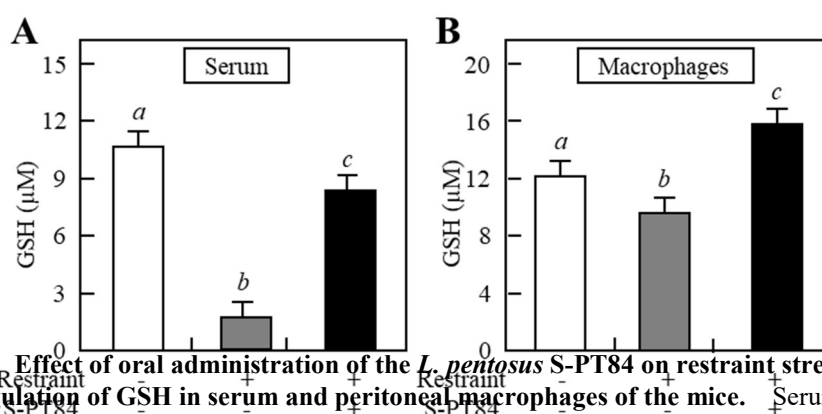


Fig. 3-4. Effect of oral administration of the *L. pentosus* S-PT84 on restraint stress-induced down regulation of GSH in serum and peritoneal macrophages of the mice. Serum and peritoneal macrophages were collected from mice that were fed an AIN-93M diet with or without *L. pentosus* S-PT84 for 1 week and then restrained. The GSH level in serum (A) and macrophages (B) was determined by HPLC. Data are expressed as the mean $\pm$ S.E. of five mice. Different letters (*a*, *b* and *c*) indicate significant differences between groups ($p < 0.05$).

3-3-3 Effect of *L. pentosus* S-PT84 feeding on restraint stress-induced changes in GSH and GSSG levels in serum and macrophages

To determine the oxidative stress induced by stress, GSH levels in serum and macrophages were measured. The serum GSH level was dramatically decreased by stress, but this decrease was suppressed by *L. pentosus* S-PT84-feeding (Fig. 3-4A). The GSH level in peritoneal macrophages was also significantly decreased by restraint stress; however, in the *L. pentosus* S-PT84-fed group, instead of decreasing with stress, the GSH level was rather higher than the control level (Fig. 3-4B).

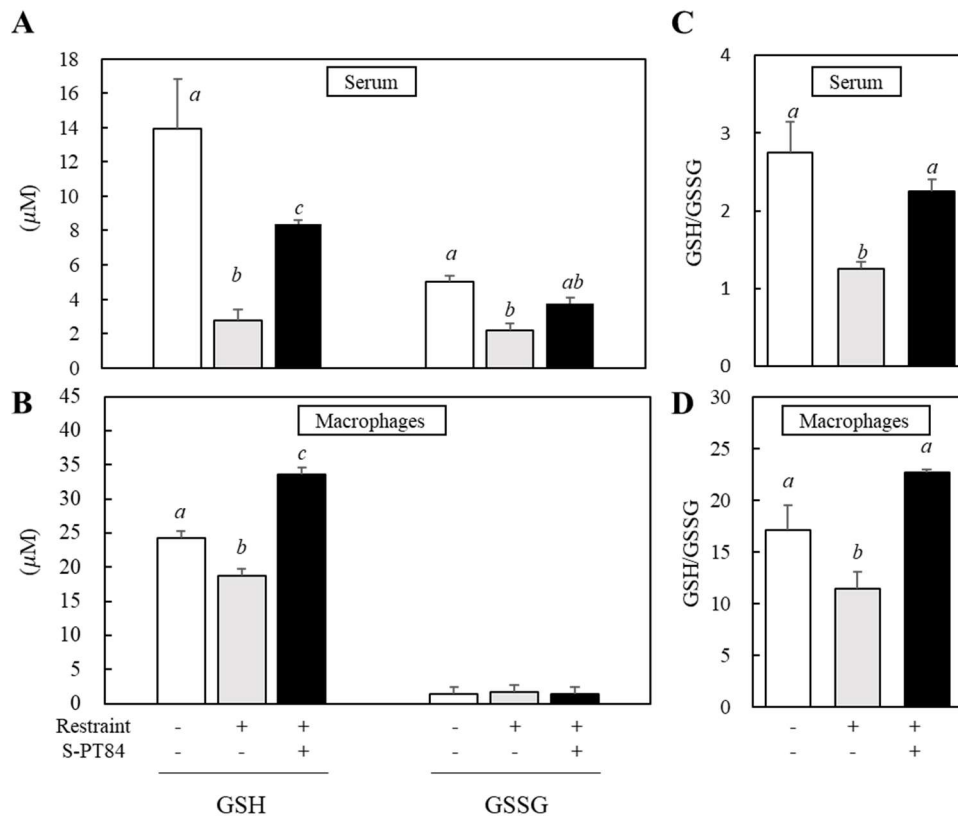


Fig. 3-5. Effect of oral administration of the *L. pentosus* S-PT84 on restraint stress-induced down regulation of GSH and GSH/GSSG in serum and peritoneal macrophages of the mice. Serum and peritoneal macrophages were collected from mice that were fed an AIN-93M diet with or without *L. pentosus* S-PT84 for 1 week and then restrained. The GSH and GSSG level in serum (A) and macrophages (B) were measured as described in Materials and Methods. Then, the GSH/GSSG serum (C) and macrophages (D) were calculated. Data are presented as the mean $\pm$ S.E. of 3 mice. Different letters (a, b and c) indicate significant differences between groups ($p < 0.05$).

In order to confirm whether the reduction of the GSH levels due to restraint stress reduces the total GSH or the ratio of GSH/GSSG, small-scale experiments were conducted to examine changes in the levels of GSH and GSSH (Fig. 4-5). The GSSG levels in serum were decreased by restraint stress (Fig. 3-5A), but these did not change in macrophages (Fig. 3-5B). GSH/GSSG ratio was decreased by restraint stress, but S-PT84 feeding suppressed the decreases in both serum and macrophages (Fig. 3-5C and D). In other words, both the absolute amount of glutathione and the GSH/GSSG ratio decreased due to restraint stress but S-PT84 feeding was found to suppress both decreases.

3-4 Discussion

Stress is a modern social problem for all people, and it is therefore important to control both the mind and the body in order to overcome stress. One of the problems caused by stress is immunodepression (131-133). In the present study, the author examined if oral administration of *L. pentosus* S-PT84, which has been shown to have immune modulating activity, particularly activity that enhances Th1 immunity (137-140), could ameliorate restraint stress-induced immunodepression. Administration of *L. pentosus* S-PT84 to mice suppressed restraint stress-induced corticosterone elevation (Fig. 3-1). The author consider that this effect of *L. pentosus* S-PT84 is due to immune modulating (Fig. 3-2 and 3-3) and antioxidative (Fig. 3-4 and 3-5) activities. Specifically, oral administration of *L. pentosus* S-PT84 suppressed the decrease in NK activity (Fig. 3-2B) and IFN- γ (Fig. 3-2C) production induced by stress.

The stress utilized in this research was applied according to the design described in a previous report by Iwakabe *et al.* (125). That previous report indicated that application of restraint stress to mice significantly decreased Th1 immunity but did not change Th2 immunity. Our results differed from those of this previous research in that the author found that such stress decreased both Th1 (IFN- γ) and Th2 (IL-4) cytokine production by Con A-stimulated splenocytes by more than 50% (Fig. 3-2C and 3-2D). However, although the reasons for the different results between this study and that previous study are unclear, the author consider that the author's results suggest that the restraint stress model in this study is a working model that can be used for examination of restraint stress-induced immunodepression. In the present study, *L. pentosus* S-PT84 feeding ameliorated all parameters of restraint stress-induced immunodepression that were measured in this stress model, except for the restraint stress-induced decrease in production of IL-4, a Th2 cytokine. *L. pentosus* S-PT84 is a LAB that was identified in a screening in which the intensity of IL-12 induction in macrophages and confirmed some immunomodulating effects *in vivo* (137). Therefore, especially when the immune function is down regulated by restraint stress and so on, IL-12-induced by *L. pentosus* S-PT84 is expected to enhance Th1 cytokine production and NK activity.

Lots of studies have demonstrated that the Th1/Th2 balance is closely related to the reduced GSH level within antigen-presenting cells such as macrophages and that a GSH regulates

IL-12 production (141-144). Stress generally induces oxidative stress and decreases the GSH level in some organisms and cells, which causes damage to leukocytes (145) and hepatocytes (146) and so on. Therefore, the much larger decrease in the production of IL-12 induced by stress was confirmed (Fig. 3-3) as compared with that in IL-10 in LPS-activated macrophages and it might be caused by a restraint stress-induced GSH decrease. Maintenance of the IL-12-producing activity of macrophages in stressed mice by feeding them *L. pentosus* S-PT84 was partially mediated by *L. pentosus* S-PT84-induced negation of the restraint stress-induced GSH (GSH/GSSG ratio) decrease (Fig. 3-4 and 3-5). It is reported that orally administered *L. rhamnosus* GG increased the levels of endogenous antioxidants like GSH against oxidative stress caused by *Giardia intestinalis* in the intestine (147). *L. salivarius* increased the GSH level in the liver of LPS-treated mice (148). In addition, liver damage induced in a chronic alcohol-fed mouse was prevented by *L. rhamnosus* through an effect on endogenous antioxidants such as GSH and superoxide dismutase (149). These reports support the notion that *L. pentosus* S-PT84 canceled the stress-induced GSH decrease; however, the exact mechanism by which LAB increase GSH remains to be elucidated. As far as the author know, there has been only one report regarding this mechanism, in which *L. plantarum*, a member of the same genus as *L. pentosus*, was reported to increase plasma GSH levels by activating the protein kinase C pathway (150). However, it is still unclear how the oxidative state in serum and immunocompetent cells is regulated after oral ingesting of LAB. These mechanisms as well as absorption and/or distribution of LAB should be further investigated to elucidate the immunomodulatory effects of LAB.

The stress response is very complicated. In general, stress enhances sympathetic nerve activity and induces glucocorticoid production. Increased sympathetic nerve activity decreases NK activity (150, 151). In another research, the authors college demonstrated that oral administration of *L. pentosus* S-PT84 suppresses sympathetic nerve activity in the mouse spleen (152). It is considered that one of the mechanisms behind the immunomodulatory effects of *L. pentosus* S-PT84, such as enhancement of NK activity, involves sympathetic nerve activity. On the other hand, glucocorticoids also reduce immune functions (51, 52, 126). Several reports concluded that restraint stress-induced changes in immune functions were caused by an increased glucocorticoid

(125, 154). A recent study showed that induction of corticosterone production under stress is due to c-FOS induction (155), which is followed by oxidative damage as shown by the presence of reactive oxygen species, lipid peroxidation, and a decrease in the GSH/GSSG ratio (156). It is also reported that exposure to far-infrared rays protects against a stress-induced increase in c-FOS induction, oxidative burdens, and serum corticosterone level via induction of glutathione peroxidase (156). However, in the present study, despite the fact that the corticosterone level was suppressed (Fig. 3-1) and NK activity (Fig. 3-2B) and IFN- γ inducible activity (Fig. 3-2C) were kept at control levels by *L. pentosus* S-PT84 feeding, the number of splenocytes was increased and was not kept at the control level (Fig. 3-2A). Thus, although it is considered that one mechanism of *L. pentosus* S-PT84 amelioration of stress-induced immunodepression might be by *L. pentosus* S-PT84 induction of the cellular redox system, the existence of another mechanism is implied by the author's data. To the best of the author's knowledge, no report has suggested that LAB directly regulate glucocorticoid production. In the present study, *L. pentosus* S-PT84 ameliorated restraint stress-induced corticosterone elevation and immunodepression, supporting the above potential mechanism of *L. pentosus* S-PT84 effects.

The authors examined whether *L. pentosus* S-PT84 normalizes or recovers immune function in immunosuppression mice using a restraint stress model. In conclusion, *L. pentosus* S-PT84 ameliorated restraint stress-induced depression of the immune function. One of the mechanisms of amelioration involved may be associated with *L. pentosus* S-PT84 induction of GSH in serum and macrophages. The detailed mechanisms by which LAB affect cellular redox or autonomic nerves and by which LAB compromise the immune function need to be examined in future research. It is considered that *L. pentosus* S-PT84 can be expected to be adequate immune function even in an excessive immune state such as allergy or in an immunodepression state during stress.

3-5 Summary

Immune function decreases during stress. It was confirmed whether *Lactobacillus pentosus* S-PT84 could alleviate the immune function reduced by restraint stress. By applying restraint stress to the mice, corticosterone was elevated, and consequently the number of splenocytes was reduced. In splenocytes, decreased Th1 cytokine and IL-10 production and decreased NK activity were observed. In addition, GSH levels and GSH/GSSG ratio in serum and liver were decreased. All of these phenotypes caused by restraint stress were significantly suppressed by allowing mice to take *L. pentosus* S-PT84 one week before restraint stress. These results cannot be explained solely by the mechanism by which *L. pentosus* S-PT84 attenuates immunodepression through immunocompetent cells activation, but it is possible that *L. pentosus* S-PT84 regulates immunomodulation via the hypothalamus-pituitary-adrenal nervous system. It was also suggested that immune regulation might be performed through oxidation control *in vivo*. In conclusion, *L. pentosus* S-PT84 was shown to be useful for immunodepression due to restraint stress.

Chapter 4.

Immunomodulating Activity of *Lactobacillus pentosus* S-PT84 on Healthy Adults: a Pilot Trial

4-1 Introduction

Maintaining an immune function in a proper state in humans is very important for maintaining a healthy state, particularly in an aging society and a stressed society like the modern society. Various papers have reported that ingesting several LAB for a certain period of time not only helps to regulate the intestine, but also helps maintain and improve immune function (21-45, 54-69). The authors have found a plant-origin LAB, *L. pentosus* S-PT84, which exhibits an immunomodulatory activity *in vivo* with the ability to induce IL-12 production. *L. pentosus* S-PT84 suppresses excessive immune reactions, such as allergies, by induction of IL-10 and suppression of Th2 (Chapter 2). It also ameliorates immunodepression induced by stress, shifting to Th1-dominant by inducing IL-12 production, maintaining NK activity (Chapter 3). However, their usefulness has only been shown only *in vivo*, and it is not yet known whether it really helps to regulate our immune function. Therefore, the author decided to examine how the human immune function changes when *L. pentosus* S-PT84 is ingested for 4 weeks in a crossover study. The immune functions evaluated were NK activity, which tends to change due to daily stress (13), and Th1/Th2 balance, and various antibodies.

4-2 Materials and Methods

4-2-1 Preparation of S-PT84 supplement

L. pentosus S-PT84 is a plant origin LAB, the fermentation medium was prepared based on plant materials. *L. pentosus* S-PT84 was inoculated into a medium in which sesame, defatted soybean, and kelp were mixed at a ratio of 1: 3: 1, and cultured at 37 ° C for 18 hours and sterilize. Fermentation medium (1,575 mg) was taken as one day's intake, and honey and dextrin were mixed as excipients to form a supplement. Finally, the intake volume of supplements is 3.7 g/day, and this includes about 11×10^8 *L. pentosus* S-PT84 (about 26 mg in dry weight). The placebo

supplement was prepared in the same manner as the *L. pentosus* S-PT84 supplement, except for the inoculation and culture of *L. pentosus* S-PT84.

4-2-2 Study protocol and participants

Thirty-three healthy subjects working at Suntory Research Center (Shimamoto, Osaka), were measured their NK activity, and divided into 2 groups to be equal sex, age, and NK activity. One group was 16 (male/female = 8/8) and the other group was 17 (male/female = 10/7). One group intake S-PT84 supplement, the other group intake placebo supplement for 4 weeks, followed by NK activity, IFN-γ and IL-4 production after phytohemagglutinin stimulation and CD4⁺CCR5⁺/CD4⁺CRTH2⁺ ratio of PBMC and various immunoglobulin titers in plasma were measured. After a 6-week washout period, the supplements to be taken were replaced for 4 weeks, and the same contents were measured. Excluded were those who had a cold during the period, and those who were judged to be unable to continue the test for unavoidable reasons. The subjects who were able to complete the test and used for analysis [Full analysis set (FAS)] were as shown in Table 4-1. In conducting this study, subjects were given sufficient explanations regarding the research content and methods, and written informed consent was obtained. This study was conducted with the approval of the ethical committee for healthy individuals at Suntory Limited.

Table 4-1 Average age and Number of subjects (FAS).

	Average age		Number of subjects (male/female)	
	placebo	S-PT84	placebo	S-PT84
Term 1	34±3.0	35±7.5	12 (7/5)	15 (6/8)
Term 2	37±7.8	37±10.4	15 (8/7)	17 (10/7)

Subjects who had a cold or retired by unavoidable reasons were excluded during the intake period.

4-2-3 Separation and culture of PBMC

PBMCs were collected by using Lymphoprep™ (Alere Technologies AS, Oslo, Norway). The collected PBMC were suspended in RPMI 1640 medium (Nacalai Tesque) with 10% FBS at a density of 2 × 10⁶ cells/ml and culture to measure NK activity and cytokine producing activity.

4-2-4 Assessment of NK activity

NK activity was measured by the PINK method described in Chapter 2 at an effector/target ratio = 20/1 using PBMC as an effector cell and K562 as a target cell.

4-2-5 Ex vivo assessment of IFN- γ and IL-4

IFN- γ and IL-4 produced from PBMC were measured by the method described in Chapter 2. Cytokine production using the culture supernatant 22 hours after phytohemagglutinin (25 μ g/ml) stimulation instead of 24 hours culture with Con A. ELISA kit for them were Human IFN- γ , and IL-4 ELISA Ready- SET Go! (eBioscience, San Diego, CA).

4-2-6 Assessment of CD4⁺CCR5⁺/ CD4⁺CRTH2⁺ ratio on PBMC

CD4⁺, CCR5⁺, and CRTH2⁺ cells were stained for 45 min using CY-CHROME™ conjugated anti-human CD4 monoclonal antibody (BD Pharmingen), phycoerythrin (PE)-Cy™5 Mouse Anti-Human CD195 (CCR5) antibody (BD Pharmingen), and PE Rat Anti-Human CD294 (CRTH2) respectively. The cells were washed with PBS, and CD4, CCR5, and CRTH2 expression in PBMC was analyzed using Epics XL (Beckman Coulter). The analysis was based on 15,000 cells gated as PBMC and calculated using EXPO32 software.

4-2-7 Assessment of various immunoglobulin titers

Immunoglobulin titer in serum was commissioned to FALCO bio systems Ltd. (Kyoto Japan) for analysis.

4-2-6 Statistical analysis

The significance of differences in values comparison before and after the intake of S-PT84 supplement was tested using Paired-*t* test. The Student's-*t* test was used to compare the S-PT84 supplement intake group and the placebo supplement intake group.

4-3 Result

4-3-1 Effect of *L. pentosus* S-PT84 on human NK activity

In the group that took the placebo supplement, NK activity did not change before and after the intake period, but in the group that took the *L. pentosus* S-PT84 supplement, NK activity increased significantly after the intake period compared to before the intake period (Fig. 4-1 A). When compared with the rate of change in NK activity, it was significantly greater in the S-PT84 supplement intake group than in the placebo intake group (Fig. 4-1 B).

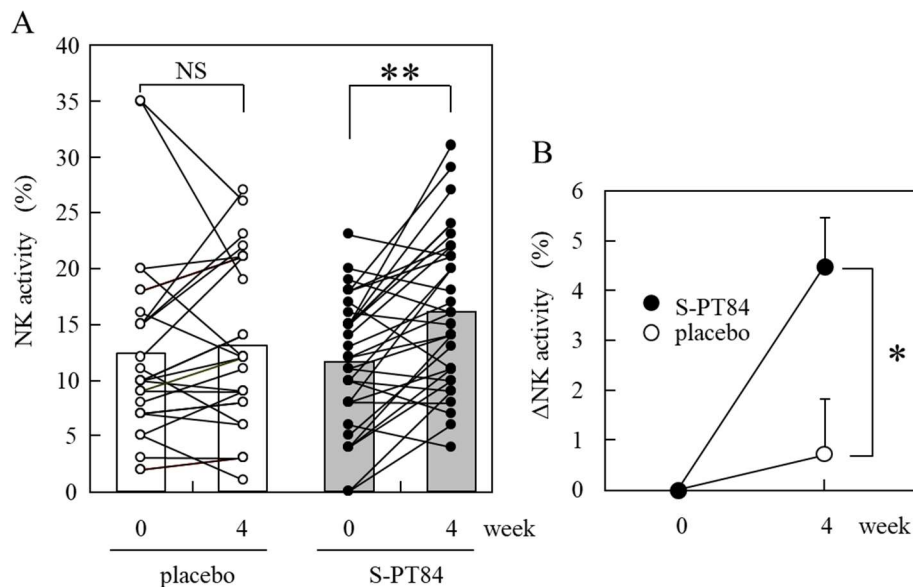


Fig. 4-1. Effect of *L. pentosus* S-PT84 supplementation for 4 weeks on NK activity of PBMC. PBMC were collected from healthy subjects who intake *L. pentosus* S-PT84 or placebo supplement for 4 weeks. The cells were co-cultured with DiO-stained K562 cells at effector/target ratio = 20/1 for 4 h, and their cytotoxic activity was determined by the PINK method as described in Materials and Methods. Values are the mean $\pm$ S.E. Asterisks indicate significant differences from the control group (*: $p < 0.05$ and **: $p < 0.01$).

4-3-2 Effect of *L. pentosus* S-PT84 on Th1/Th2 cytokine producing activity and Th1/Th2 phenotype on PBMC

The difference in cytokine-producing activity by stimulating PBMC with phytohemagglutinin were examined. Ingestion of *L. pentosus* S-PT84 supplement tended to increase IFN- γ production, but it was not a significant difference (Fig. 4-2 A). IL-4 producing activity did not change before and after the intake period and supplements (Fig. 4-2 B). The IFN- γ /IL-4 ratio did not either

change significantly before and after the intake period (Fig. 4-2 C). On the other hand, when compared with the change in the IFN- γ /IL-4 ratio, the IFN- γ /IL-4 ratio was significantly higher in the *L. pentosus* S-PT84 supplement intake group than in the placebo intake group (Fig. 4-2 D).

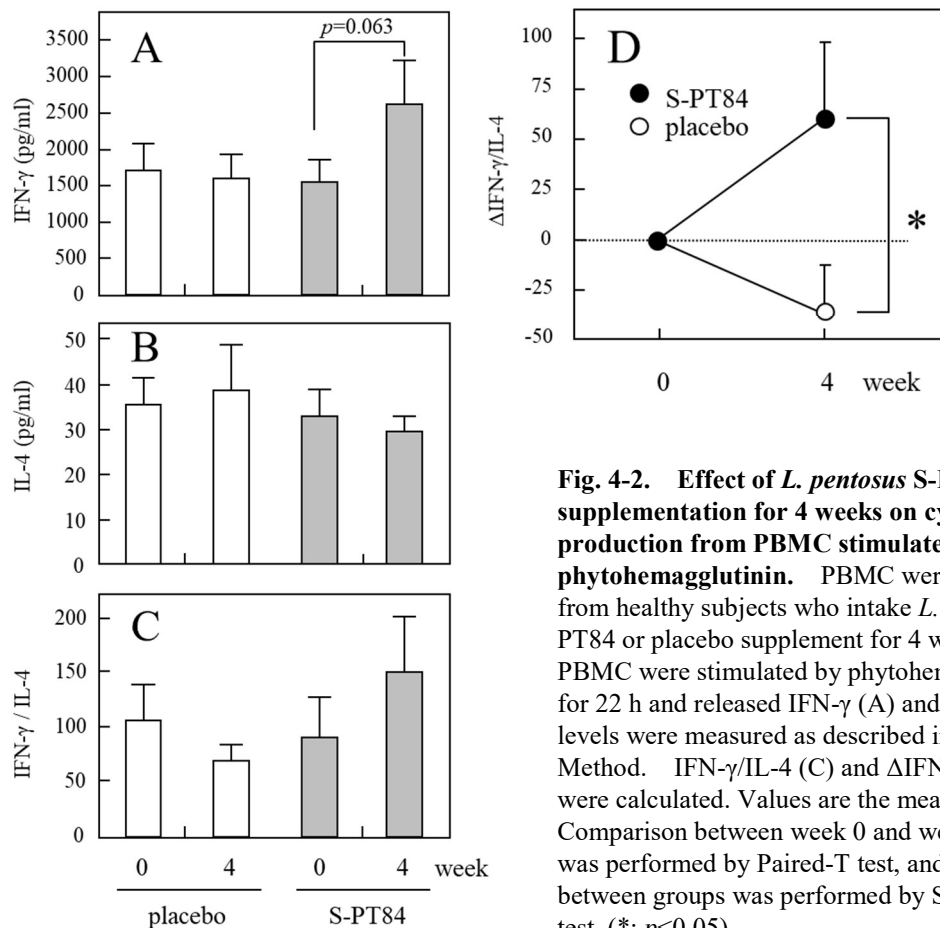


Fig. 4-2. Effect of *L. pentosus* S-PT84 supplementation for 4 weeks on cytokine production from PBMC stimulated by phytohemagglutinin. PBMC were collected from healthy subjects who intake *L. pentosus* S-PT84 or placebo supplement for 4 weeks. PBMC were stimulated by phytohemagglutinin for 22 h and released IFN- γ (A) and IL-4 (B) levels were measured as described in Material and Method. IFN- γ /IL-4 (C) and Δ IFN- γ /IL-4 (D) were calculated. Values are the mean $\pm$ S.E. Comparison between week 0 and week 4 (A-C) was performed by Paired-T test, and comparison between groups was performed by Student's *t* test. (*: $p < 0.05$)

In order to determine whether the helper T in PBMC is Th1-type or Th2-type, the ratio of CD3⁺CCR5⁺ (Th1 cells) PBMC to CD3⁺CRTH2⁺ (Th2 cells) cells was measured by flow cytometry (Table 4-2). These did not change with or without *L. pentosus* S-PT84 supplementation.

4-3-3 Effect of *L. pentosus* S-PT84 on Immunoglobins titers in PBMC

Various immunoglobulin antibody titers were measured before and after the supplement intake period (Table 4-2). There was no significant change in all antibody titers before and after the intake period, and *L. pentosus* S-PT84 supplement group and placebo group.

In addition, the blood parameters before and after each intake period were confirmed, but there were no safety problems due to the intake of these supplements.

Table 4-2 Effect of S-PT84 supplement on CD4⁺CCR5⁺/CD4⁺CRTH2⁺ ratio and immunoglobulin titers.

supplement		placebo		S-PT84	
week		0	4	0	4
CD4 ⁺ CCR5 ⁺ /CD4 ⁺ CRTH2 ⁺		6.93 ± 1.14	5.62 ± 1.01	6.67 ± 0.94	6.47 ± 0.92
IgE	IU/ml	172.2 ± 35.7	168.5 ± 34.6	198.2 ± 40.7	195.2 ± 40.6
IgG	mg/dl	1204 ± 49.3	1150 ± 48.9	1180 ± 34.3	1149 ± 37.3
IgA	mg/dl	212.7 ± 14.3	204.7 ± 14.2	215.6 ± 14.1	211.9 ± 13.8
IgM	mg/dl	111.0 ± 6.0	103.1 ± 5.4	111.5 ± 5.0	108.6 ± 5.2

CD4⁺CCR5⁺/CD4⁺CRTH2⁺ of PBMC and various immunoglobulin in serum from subjects were measured as described in Materials and Methods. Values are the mean ± S.E.

4-4 Discussion

The exploratory human trial conducted this time showed that NK activity increased not only *in vivo* but also in humans by ingesting *L. pentosus* S-PT84 for 4 weeks (Fig. 4-1). Although there was no significant change in the production activity of Th1 and Th2 cytokines, the production ratio of Th1/Th2 cytokines was significantly increased by taking *L. pentosus* S-PT84 (Fig. 4-2). The ratio of Th1/Th2 cells did not change at all (Table 4-2), suggesting that S-PT84 *L. pentosus* activates Th1 cells, dose not increase Th cells. *L. pentosus* S-PT84 had no effect on Th2 cells in this study, in addition to having no effect on antibody production in serum (Table 4-2). This trial was conducted as an exploratory and was conducted as a pilot in-house trial, however the increase in NK activity even under such limited conditions was sufficient to confirm the immunomodulatory potential of *L. pentosus* S-PT84. Some subjects had decreased NK activity compared to before taking *L. pentosus* S-PT84, but NK activity of most of the subjects had increased by ingesting *L. pentosus* S-PT84 (Fig. 4-1 A). Regarding Th1/Th2 balance, the potential to shift to Th1 predominance by *L. pentosus* S-PT84 intake was confirmed (Fig. 4-2 D), even though Th2 cytokine production did not decreased and Th1 cytokine production did not increased significantly (Fig. 4-2 A and B).

LAB may not enhance the immune function of humans with normal immune function. For example, *L. plantarum* L-137 is very similar to *L. pentosus* S-PT84 in that it has a strong IL-12 production ability like *L. pentosus* S-PT84 and suppresses casein-induced allergic reactions *in vivo* (29). Immunoregulatory effects on healthy individuals have also been investigated (157), and it was a test of ingesting 10 mg of heat-killed *L. plantarum* L-137 per day for 12 weeks. However, the Th1 cytokine production ability was not change. At the 4th week after ingestion, the Th1/Th2 cytokine production balance was significantly shifted toward the Th1 type, but despite continuing ingestion, it went down gradually. *L. casei* Shirota increased mouse IL-12 production and IFN- γ production (28), and when cocultured *in vitro* with human PBMCs, Th1 cytokine production increased, and NK activation is observed (158). However, according to Harbige *et al.* (159), even though 1.3×10^9 *L. casei* Shirota were ingested by healthy individuals for 4 or 14 weeks, there was no change in T cell activation, IFN- γ or IL-12 production activity, and only the proportion of NKT cells increase was observed. Therefore, a few studies have evaluated the immune function of healthy individuals by combining multiple LABs instead of single use of LAB. According to Lee *et al.*, when multiple LAB strain (*L. paracasei* ssp. *Paracasei*, *B. animalis* ssp. *Lactis*, and *L. plantarum*) taken in healthy people but over 60 years old, IL-12 and IFN- γ production activity and NK activity of PBMC were increased (160). Furthermore, Olivares *et al.* (161) reported that even in healthy people, continuous intake of fermented product made with *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 increased NK activity and promoted IgA production. In this way, it is difficult to evaluate the immunomodulatory effect of a single LAB in healthy individuals, but this time we are able to demonstrate a certain potential for *L. pentosus* S-PT84 in human trials targeting healthy individuals.

Examination of the immunomodulatory effect of LAB on humans is often confirmed by LAB's immunoregulatory function in subjects who are not healthy but whose immune function is not normal. For example, smokers decreased NK activity by smoking, but NK activity increased by taking *L. casei* Shirota for 3 weeks (55). NK activity was also reduced during the study period, but students who took 200 ml of *L. casei* DN-114001 (10^8 /ml) every day had reduced NK activity (67). In addition, there are reports on the immunoregulatory effects of LAB on elderly (68, 69, 162),

allergic rhinitis patients (163, 164), and atopic patients (165, 166). All of these have increased production of Th1 cytokines or improved allergic symptoms by LAB. It is thought that those who are strongly required to improve their immune function are those who are strongly stressed with abnormal immune function, humans who have illness, and those who have allergies. The author would like to investigate in the future whether *L. pentosus* S-PT84 is effective in regulating the immune function of these people.

The limitation in this study was that the dropout rate was high for reasons such as catching colds. Since a cold is generally a virus or bacterial infection, immune condition changes greatly. It was excluded because it was not possible to continue such a test in ethics or data. However, this trial was the first exploration test, and even if it dropped out in the first period of crossover, it was possible to participate if there was no problem in the other period, and it was also included in the analysis as FAS. The trial started in early November, the second in mid-January, and was completed by mid-February. In Japan, the hay fever season begins around the end of February, and this is thought to have a significant impact on immune function. However, since the number of test subjects who catch a cold increases in November, it is important to secure a large number of subjects in consideration of the season and cold preparations when conducting an immune-related human trial. Based on this study, the author's colleagues have confirmed the immune function enhancing effect of the *L. pentosus* S-PT84 strain in healthy subjects again. At that time, intake of $1.5\text{--}4.5 \times 10^9$ cells *L. pentosus* S-PT84 for 2 to 4 weeks has been confirmed to increase NK activity and IFN- α production against viruses (167). Hence, the authors believe that *L. pentosus* S-PT84 will definitely increase human NK activity.

In conclusion, this pilot study suggested that *L. pentosus* S-PT84 may activate NK cells even in humans and shift the Th1/Th2 balance to Th1 dominant. In the future, it will be necessary to obtain further evidence by conducting large-scale tests outside the company, as well as in human tests not only for healthy individuals but also for subjects with impaired immune function.

4-5 Summary

Lactobacillus pentosus S-PT84 increase immune function by NK activation and IL-12 induction and shifting Th1/Th2 to Th1 dominant, and show anti-allergic effect, ameliorate immunodepression induced by stress *in vivo*. In the next step, the author tried exploratory study of *L. pentosus* S-PT84 in humans. The healthy volunteers were divided into two arms, one taking *L. pentosus* S-PT84 [about 11×10^8 cells (about 26 mg dry weight)] supplements and the other group taking placebo supplements for 4 weeks. After a 6-week washout period, the taking supplement was replaced and ingested for another 4 weeks. Before and after each intake period, NK activity and IFN- γ and IL-4 producing activity, the Th1/Th2 cells ratio of PBMC, and various immunoglobulin antibody titers in the serum were measured. Intake of *L. pentosus* S-PT84 supplement increased NK activity. In addition, no significant changes in IFN- γ and IL-4 production were observed, but the IFN- γ /IL-4 ratio was significantly increased by taking *L. pentosus* S-PT84 supplement. Other parameters were unchanged. These results suggest that *L. pentosus* S-PT84 has a potential to activate NK cells and to shift the Th1/Th2 balance to Th1 dominant in humans.

Chapter 5.

Anti-Tumor Activities of the Antlered Form of *Ganoderma lucidum* in Allogeneic and Syngeneic Tumor-Bearing Mice

5-1 Introduction

Our bodies are at risk of cancer every day, but because of our immune function, cancer cells do not grow and do not become cancer easily. If the immune function is superior to the proliferation ability of cancer cells, it will not become cancer, but cancer cells generally have a function of suppressing immune function (168). Therefore, in order to avoid cancer with our own immunity, we have to maintain immune function normally. Even if it becomes cancer, it is necessary to maintain immune function even a little to suppress cancer. The relationship between cancer and immunity have been studied for more than 40 years, and mushroom-derived β -glucan preparations such as Lentinan, Schizophyllan and Krestin have been developed as pharmaceuticals based on the concept of BRM (169). In addition, cytokine preparations such as IFN- γ and IFN- α have been developed to stimulate the immunity necessary for anti-tumor immunity (170). However, since these are pharmaceuticals and are not expected to be effective by oral administration, they cannot be taken in daily life. In order to maintain immunity that does not cause cancer in daily life, foods that can be eaten instead of drugs are one of the most familiar and easy to continue.

Mushrooms are known that can be expected to have an anticancer effect through immune enhancement. For example, anticancer effect of *Agaricus blazei* Murrill (171, 172), *Meshimakobu* (*Phellinus igniarius*) (173, 174), *G. lucidum* (84, 85) are well known. Among them, *G. lucidum* has lots of physiological activity that has been used as a traditional Chinese medicine for a long period. *G. lucidum* AF is a type of *G. lucidum*. Although many physiological activities have been reported for *G. lucidum* (76-93), there are not many reports on *G. lucidum* AF since the cultivation method has just been established in the last 20-30 years. It has been reported that *G. lucidum* AF can activate macrophages *in vivo* and induce Th1 immunity (94). However, there is no report on the antitumor effect and immunological mechanism of *G. lucidum* AF in a syngeneic tumor mouse model. Analysis of immunity against tumors is possible if it is effective in syngeneic tumor model.

In addition, if *G. lucidum* AF is effective in this model, it can be expected to maintain and enhance immune functions to avoid cancer.

In this chapter, the anti-tumor effects of oral administration (p.o. administration and free-feeding) of *G. lucidum* AF were investigated by not only in allogeneic Sarcoma 180 tumor-bearing ddY mice but also in syngeneic MM 46 mammary carcinoma-bearing C3H/He mice. In addition, as an action mechanism of the anti-tumor effect, immunomodulatory activity of *G. lucidum* AF was evaluated using splenocytes and lymphocytes from regional lymph nodes of syngeneic MM46-bearing mice.

5-2 Materials and Methods

5-2-1 Materials

G. lucidum AF in this study, were grown commercially in China, Korea, and Japan. All *G. lucidum* AF samples (Chinese 4, Japanese 3, and Korean 1) were purchased from Shinwa Bussan (Osaka, Japan). They were crushed to a 200-mesh path equivalent. A *G. lucidum* AF-containing diet was prepared from AIN-93M chow (Oriental Yeast). Sarcoma 180 cells were kindly donated by Dr. M. Hayashi (Kitasato Institute for Life Sciences and School of Pharmaceutical Sciences, Kitasato University, Tokyo). MM 46 cells were from the collection of the Institute of Medical Mycology, Teikyo University.

5-2-2 Animals

Male ddY and C3H/He mice (5-6 weeks old) were purchased from Japan SLC and Charles River Japan (Yokohama, Japan) respectively. Mice were reared in the manner described in Chapter 2 for one week before being used for the experiment. Experiments were performed according to the “Guidelines for the Care and Use of Experimental Animals” of the Japanese Association for Laboratory Animals. This animal experiment was conducted in 2001-2004.

5-2-3 Anti-tumor tests

Allogeneic tumor model: Sarcoma180 cells (1×10^6 cells) were inoculated i.p. into ddY mice

for passage culture. Tumor cells were collected from the peritoneal cavity 7 days after inoculation and then suspended in MEM medium (Nacalai Tesque). The cells were subcutaneously (s.c.) inoculated into the left chest of ddY mice (1×10^6 cells/0.1 ml/mouse). *G. lucidum* AF (0.005, 0.05, or 0.5 g/kg/day) was suspended in 0.6 ml of distilled water and p.o. administered to mice for 19 consecutive days starting from the day before inoculation. The same volume of water was administered to the control mice. The tumor diameter was monitored and the tumor weight was measured 18 days after inoculation. An AIN-93M diet with or without 2.5% *G. lucidum* AF was also administered to the mice by free feeding from the day before until 100 days after inoculation. The tumor diameter and the survival rate were monitored during the experiment.

Syngeneic tumor model (175): MM 46 mammary carcinoma (1×10^6 cells) was inoculated i.p. into C3H/He mice. Tumor cells for the experiment were prepared similarly to Sarcoma 180 cells. The cells were inoculated s.c. into the inguinal region of C3H/He mice (2×10^6 cells/0.2 ml/mouse). The mice were equivalently divided into two groups according to tumor size 7 day after inoculation. Then each group of mice was administered an AIN-93M diet with or without 2.5% *G. lucidum* AF, and the survival rate was monitored until 100 days after inoculation. Half of the mice in each group were euthanized at 28 days after inoculation, and the tumor weight was determined.

The average diameter was calculated using a slide caliper as follows:

$$\text{Tumor diameter (mm)} = \sqrt{\text{the largest diameter (mm)} \times \text{the smallest diameter (mm)}}$$

The anti-tumor test was performed at least twice for reproducibility check and representative results are shown.

5-2-4 Preparation of splenocytes and lymphocytes

Splenocytes and lymphocytes were prepared from spleens and regional lymph nodes from MM 46 tumor-bearing mice euthanized 28 days after tumor inoculation as described above. After removing the spleen or the regional lymph node from a mouse, each single-cell suspension of splenocytes or lymphocytes was prepared by passing it through a 70 μm nylon mesh. The cells were washed with a hemolytic buffer (155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM ethylenediaminetetraacetic acid (EDTA) and PBS in that order. The cells were suspended in RPMI

1640 medium containing 10% FBS and a 1% antibiotic-antimycotic mix cocktail (Nacalai Tesque).

5-2-5 Flow cytometric analysis

CD4⁺ and CD8⁺ cells were stained for 45 min using CY-CHROME™ conjugated anti-mouse CD4 monoclonal antibody (BD Pharmingen) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 monoclonal antibody (Immunotech, Marseille, France) respectively. The cells were washed with PBS, and CD4 and CD8 expression in lymphocytes was analyzed using Epics XL (Beckman Coulter) as described in Chapter 4.

5-2-6 Assay for cytokine production

Splenocytes and lymphocytes (5×10^6 cells/ml each) were stimulated with 2.5 µg/ml of Con A and measured IFN-γ and IL-4 levels described in Chapter.2.

5-2-7 Statistical analysis

The statistically significant differences for each value were tested by one-way ANOVA, followed by Fisher's PLSD as a *post-hoc* test. Cumulative survival curves were constructed using Kaplan-Meier methods, and significant differences were tested by the log-rank test.

5-3 Results

5-3-1 Selection of highly bioactive *G. Lucidum* AF using allogeneic tumor model

The antitumor activity of *G. lucidum* AF from eight different localities (one in Korea, four in China, and three in Japan) was compared using Sarcoma 180 tumor-bearing mice. In this experiment, total β-D-glucan content was over 40% in all eight different localities, but there was no correlation between antitumor activity and total β-glucan content of *G. lucidum* AF (Fig. 5-1). *G. lucidum* AF China1 (from *Anhui*) and China3 (from *Shangtong*) exhibited the significant activity, inhibiting tumor growth by about 48% 18 days after inoculation. Especially for China3, the reproducibility was also good, it was used in all the experiments described below. Daily p.o. administration of *G. Lucidum* AF (0.5 g/kg/day) significantly inhibited the growth of Sarcoma 180

tumors (Fig. 5-2A), and significantly reduced tumor weight on day 18 at doses of 0.05 and 0.5 g/kg/day (Fig. 5-2B).

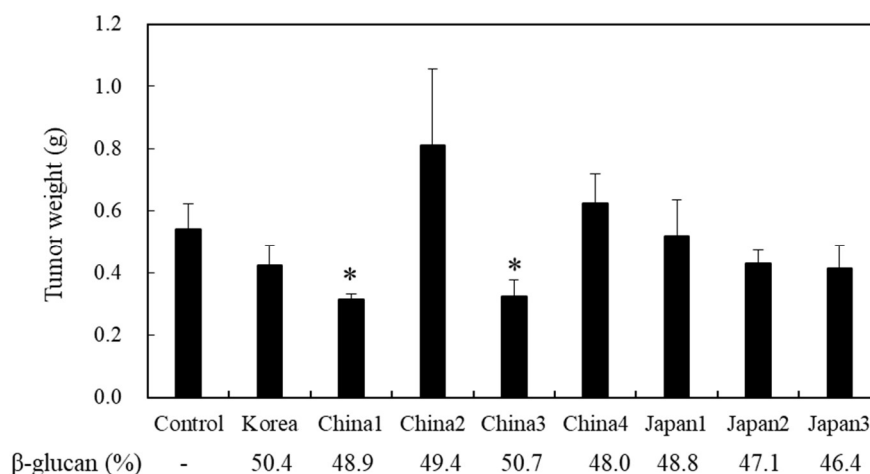


Fig. 5-1. Anti-tumor effects of various *G. lucidum* AF in Sarcoma 180 tumor-bearing mice. Sarcoma 180 (1×10^6 cells) was inoculated s.c. into ddY mice. *G. lucidum* AF was p.o. administered to mice for 19 consecutive days starting from 1 day before inoculation. The tumor weight was determined 18 days after inoculation. Determination of the tumor diameter is described in Materials and Methods. Data are the means $\pm$ S.E. (n = 8-9). Asterisks indicate significant differences from the control group (*, $p < 0.05$; one-way ANOVA followed by Fisher's PLSD). β -glucan contents (%) were measured by consigning to Japan Food Research Laboratories (Tokyo, Japan).

5-3-2 Anti-tumor effects of *G. lucidum* AF in allogeneic tumor model

Since p.o. administration may induce experimental stress in mice, the anti-tumor effect of *G. lucidum* AF by free-feeding in mice with Sarcoma 180 tumors was investigated. The differences in the anti-tumor effects of the control (AIN-93M) and the *G. lucidum* AF (0.156, 0.625, or 2.5% containing AIN-93M) were compared. In the group receiving 2.5% *G. lucidum* AF, tumor growth was significantly suppressed from day 15 after tumor inoculation. The group with a low amount of *G. lucidum* AF also showed a tendency to suppress tumor growth compared to the control group (Fig. 5-3A). The body weight of the *G. lucidum* AF-fed group was slightly lower than that of the control group but was not significantly different. There was no difference between groups in food intake.

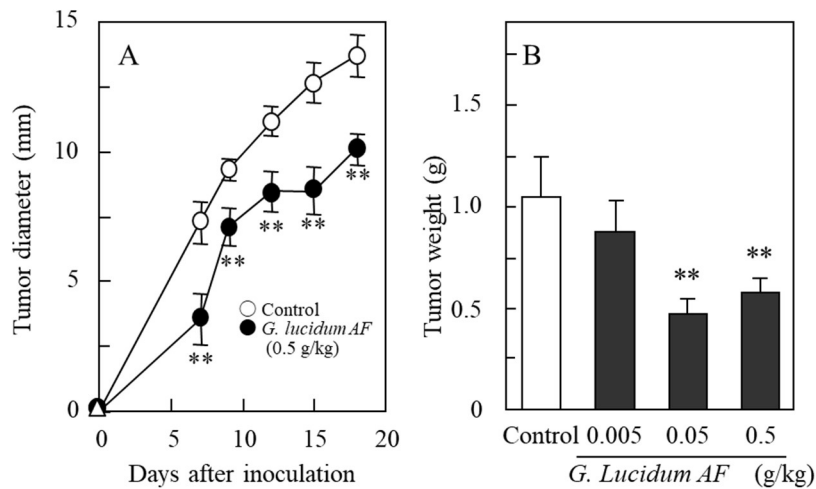


Fig. 5-2. Anti-tumor effects of *G. lucidum* AF in Sarcoma 180 tumor-bearing mice. Sarcoma 180 (1×10^6 cells) was inoculated s.c. into ddY mice. *G. lucidum* AF was p.o. administered to mice for 19 consecutive days starting from 1 day before inoculation. The tumor diameter (A) was monitored, and tumor weight (B) was determined 18 days after inoculation. Determination of the tumor diameter is described in Materials and Methods. Data are the means $\pm$ S.E. ($n = 13-15$). Asterisks indicate significant differences from the control group (**, $p < 0.01$; one-way ANOVA followed by Fisher's PLSD).

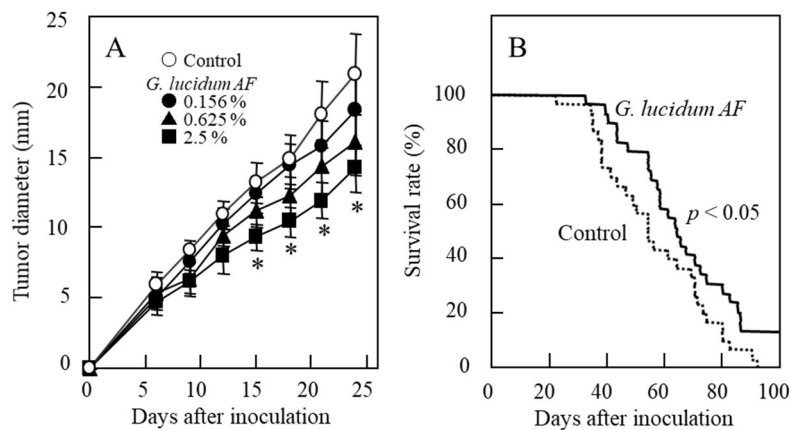


Fig. 5-3. Anti-tumor and life span-elongation effects of *G. lucidum* AF in Sarcoma 180 tumor-bearing mice. Sarcoma 180 (1×10^6 cells) was inoculated s.c. into ddY mice. The mice were fed an AIN-93M diet or *G. lucidum* AF (0.156, 0.625, and 2.5%) from 1 day up to before tumor inoculation. A, The tumor diameter was determined as described in Materials and Methods. The data are the means $\pm$ S.E. ($n = 10$). Asterisks indicate significant differences from the control group (*, $p < 0.05$; one-way ANOVA followed by Fisher's PLSD). B, Survival rate was monitored ($n = 29-30$). Cumulative survival curves were constructed using the Kaplan-Meier methods, and significant differences were tested with the log-rank test. The life span of mice fed the *G. lucidum* AF diet was significantly prolonged.

compared to the control group (Fig. 5-3B). Survival periods giving 50% lethality were 54 and 64 days in control and *G. lucidum* AF-fed group, respectively.

5-3-3 Anti-tumor effect of *G. lucidum* AF in MM 46 tumor-bearing mice; syngeneic tumor model

G. lucidum AF (2.5% diet) also showed anti-tumor activity against syngeneic MM46 tumors in C3H/He mice, significantly reduced tumor weight 28 days after tumor inoculation, despite ingestion starting 7 days after tumor inoculation. A lower concentration of *G. lucidum* AF diet also tended to suppress tumor growth (Fig. 5-4A).

Survivals of MM 46 tumor-bearing mice were observed up to 100 days after tumor

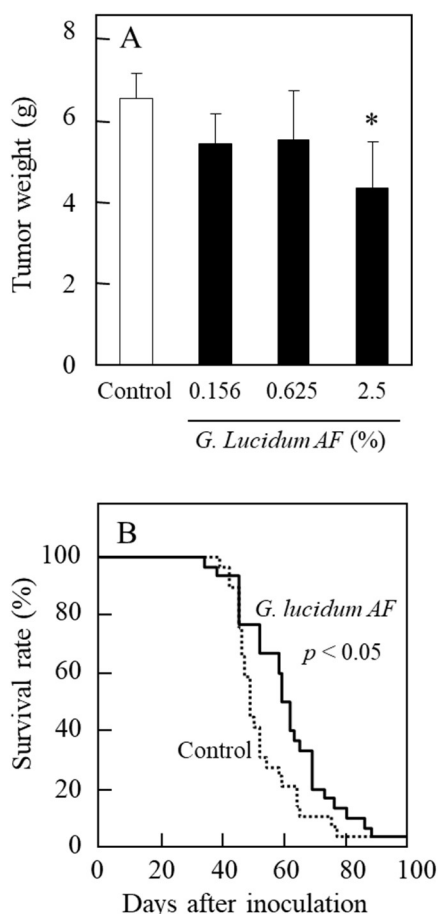


Fig. 5-4. Anti-Tumor and Life Span-Elongation Effects of *G. lucidum* AF in MM 46 Tumor-Bearing Mice. MM 46 mammary carcinoma was inoculated s.c. into the inguinal region of C3H/He mice (2×10^6 cells/0.2 ml/mouse). The mice were divided equivalently into two groups according to the tumor size 7 days after inoculation. Then each group of mice was administered an AIN-93M diet or *G. lucidum* AF (0.156, 0.625, 2.5%). A, Ten arbitrarily-chosen mice in each group were euthanized 28 days after inoculation, and the tumor weight was determined. The data are the means $\pm$ S.E. Asterisks indicate significant differences from the control group (*, $p < 0.05$; one-way ANOVA followed by Fisher's PLSD). B, The survival rate was monitored ($n = 29-30$). Determination of the survival rate is described in Materials and Methods. Cumulative survival curves were constructed using Kaplan-Meier methods, and significant differences were tested with the log-rank test.

inoculation. The longevity of mice fed the *G. lucidum* AF 2.5% diet was significantly prolonged compared to the control group (Fig. 5-4B). Survival periods giving 50% lethality were 49 and 62 days for control mice and 2.5% *G. lucidum* AF-fed mice, respectively. In both groups, a decrease in

food intake was observed around day 42 after tumor inoculation, but body weight was not significantly different between the two groups.

5-3-4 Immunostimulatory effect of *G. lucidum* AF in MM46 tumor-bearing mice

Splenocytes and lymphocytes were prepared from spleens and regional lymph nodes of MM46 tumor-bearing mice euthanized 28 days after tumor inoculation, and the immunostimulatory effect of *G. lucidum* AF was examined. Analysis of the population of CD4⁺ and CD8⁺ cells of splenocytes showed that the population of CD4⁺ and CD8⁺ cells was reduced by MM46 inoculation (Fig. 5-5). *G. lucidum* AF significantly restored the loss of CD8⁺ cells in splenocytes. There were no changes in cell populations in lymphocytes from any of the groups of regional lymph nodes.

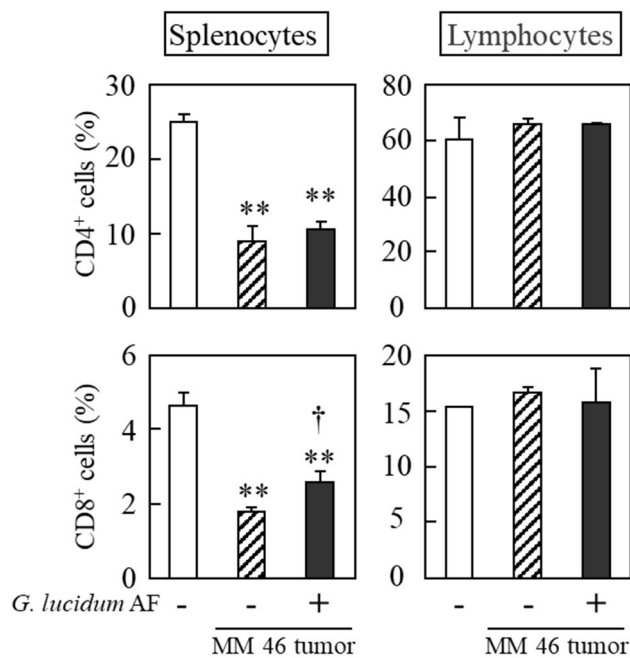


Fig. 5-5. Changes in CD4⁺ and CD8⁺ cell populations in spleens and regional lymph nodes from MM 46-tumor bearing mice. Splenocytes and lymphocytes were prepared as described in Materials and Methods. CD8⁺ and CD4⁺ cells were analyzed using Epics XL. Open bars indicate normal mice (without tumor inoculation), hatched bars indicate tumor bearing mice fed a control diet, and closed bars indicate tumor bearing mice fed the *G. lucidum* AF-containing diet. The data are the means $\pm$ S.E. (n = 10). **, $p < 0.01$ vs. open bar; †, $p < 0.05$ vs. hatched bar; one-way ANOVA followed by Fisher's PLSD.

IFN- γ and IL-4 production of splenocytes or lymphocytes after Con A stimulation was also measured. Inoculation with MM46 significantly reduced both IFN- γ production and IL-4 in splenocytes and lymphocytes in regional lymph nodes, but *G. lucidum* AF significantly suppressed the decrease, respectively (Fig. 5-6).

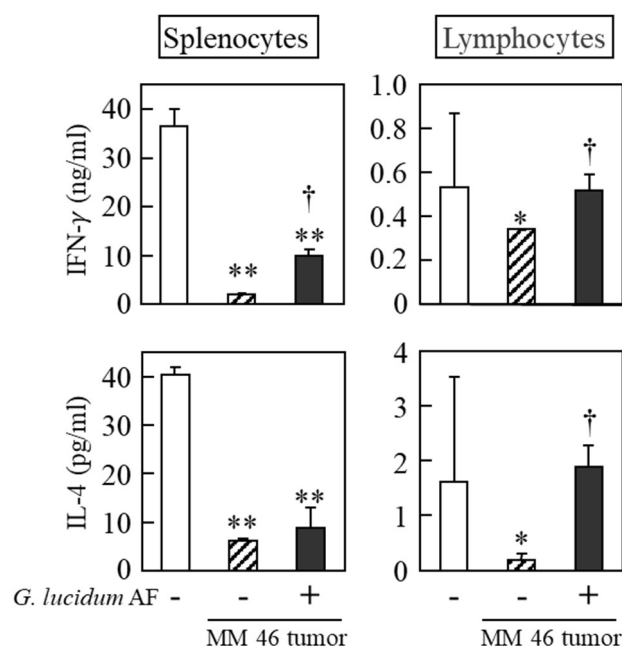


Fig. 5-6. Cytokine production from spleens and regional lymph nodes from MM 46-tumor bearing mice.

Splenocytes and lymphocytes were prepared as described in Materials and Methods. The cells were stimulated with 2.5 $\mu\text{g/ml}$ of Con A for 24 h. The levels of IFN- γ and IL-4 in the culture supernatant were measured as described in Materials and Methods. Open bars indicate normal mice (without tumor inoculation), hatched bars indicate tumor bearing mice fed a control diet, and closed bars indicate tumor bearing mice fed the *G. lucidum* AF-containing diet. *, $p < 0.05$ vs. open bar; **, $p < 0.01$ vs. open bar; †, $p < 0.05$ vs. hatched bar; one-way ANOVA followed by Fisher's PLSD.

5-4 Discussion

The experiments demonstrated the anti-tumor activity of oral taking of *G. lucidum* AF, variant of *G. lucidum*, not only in allogeneic Sarcoma180 tumor-bearing mice (Fig. 5-1, 5-2, 5-3) but also in syngeneic MM46 mammary carcinoma-bearing mice (Fig. 5-4). *G. lucidum* AF not only inhibited the increase in tumor weight, but also extended the lifespan of tumor-bearing mice when given orally as a free-feed (Fig. 5-3 and 5-4). Furthermore, it was also found that *G. lucidum* AF protected tumor-bearing mice from immunosuppression due to tumor growth (Fig. 5-5, 5-6).

It has been expected that *G. lucidum* AF has strong anti-tumor activity because of the high content of β -D-glucan in its fruiting body (Fig. 5-1). β -D-glucan, a major component of *G. lucidum* AF, shows immuno-stimulating activities *in vivo* and *in vitro* (84-90). β -D-glucan content of *G. lucidum* AF is about 40%, and the total ratio of β -D-(1,3)-glucan in β -D-glucan is reported to be about 85% (94). The report shows that oral administration of *G. lucidum* AF produces Th1-related immunopotentiating activity in BALB/c mice, but in this study, there was no correlation between the anti-tumor activity and β -D-glucan content of *G. lucidum* AF (Fig. 5-1), although all the total β -D-glucan contents were more than 40%. The author should take notice that the anti-tumor

activity of *G. lucidum* AF could not be predicted by the total β -D-glucan content. *G. lucidum* AF from Shandong, China was used in all experiments, which exhibited the highest activity and reproducibility of eight samples.

Recently, various structures and bioactivities of β -D-glucans derived from *G. lucidum* have been reported, *e.g.*, α -D-(1 $\rightarrow$ 3)-glucan (86, 176), protein-bound β -glucan (177), β -D-(1 $\rightarrow$ 6)-glucan with β -(1 $\rightarrow$ 3) or β -(1 $\rightarrow$ 4) branches (178), and β -D-(1 $\rightarrow$ 3)-glucan with β -(1 $\rightarrow$ 6) branches (87, 89). The cytotoxic actions of triterpenes, such as ganoderiol, lucidenic acid, and ganoderic acid, in *G. lucidum* AF have been also studied strenuously (92, 93). To better understand the mechanism of anti-tumor activity of *G. Lucidum* AF by oral administration, the active ingredients need to be searched and determined.

The authors considered that it was better to test the anti-tumor activity of *G. lucidum* AF by oral administration with meals to avoid experimental stress due to gastric gavage. In Sarcoma 180 tumor-bearing mice, feed containing *G. lucidum* AF was given *ad libitum* one day before tumor inoculation. And despite the start of feeding from 7 days after tumor inoculation, it showed anti-tumor effect and prolonged the lifespan in syngeneic MM46 tumors-bearing C3H/He mice. These results suggest that taking *G. lucidum* AF as a food not only helps prevent, but also suppresses cancer progression.

The mechanism of the anti-tumor effect of *G. lucidum* AF is not yet clarified. At this point, we consider as follows: The immunological mechanism may be related to inhibition of MM46-tumor growth, because MM 46 carcinoma in C3H/He mice is moderately antigenic (179), and its growth can be inhibited by immuno-stimulating agents, including mushroom β -D-glucans such as lentinan (179, 180) and PSK (181-183). In this study, *G. lucidum* AF significantly countered the depression of splenic CD8⁺ cells and protected against the decrease in IFN- γ and IL-4 production from lymphocytes in regional lymph nodes in MM 46 tumor-bearing mice. In addition, *in vivo* depletion of IFN- γ abrogated the anti-tumor vaccination effect (184), indicating that IFN- γ might play an important role in immunomodulating anti-tumor action. These findings suggest that one of the anti-tumor mechanisms of *G. lucidum* AF might be due to its IFN- γ -inducible activity and killer T cell activation under the cancer condition.

In summary, this study confirmed the anti-tumor activity of *G. lucidum* AF against not only allogeneic but also syngeneic tumors. In addition, it was suggested that one of the mechanisms of the anti-tumor effect of *G. lucidum* AF is alleviation of tumor-mediated immunosuppression. Mushroom-derived BRM is commonly co-treated with chemotherapeutics to combat side effects such as myelosuppression and leukopenia (185, 186). *G. lucidum* AF is clinically expected to reduce chemotherapeutic immunosuppression. The anti-tumor activity of *G. lucidum* AF in combination with chemotherapeutic agents is the subject of next research. Ultimately, further studies of *G. lucidum* AF will prove useful as an adjunct in the prevention of cancer recurrence and in the medical treatment of cancer, and at least improve the quality of life of people suffering from cancer.

5-5 Summary

The anti-tumor activity of *Ganoderma lucidum* AF cultivated from Japan and other countries were compared, and *G. lucidum* AF from Shandong, China with high bioactivity and high reproducibility was selected. The anti-tumor effect of *G. lucidum* AF is evaluated by not only in the allogeneic tumor model using Sarcoma180-bearing ddY, but also in the syngeneic tumor model using MM46-C3H/He mice. In both model, the effect of extending the survival rate of tumor-bearing mice by *G. lucidum* AF feeding was also confirmed. In addition, this anti-tumor effect was confirmed not by oral administration of *G. lucidum* AF, but by free feeding mixed with experimental diet. Analysis of immune function in a syngeneic tumor model of MM46-tumor-bearing mice revealed that intake of *G. lucidum* AF suppressed the tumor-induced reduction of the proportion of killer T cells in splenocytes and recovered IFN- γ production from splenocytes and lymphocytes. These results suggest that *G. lucidum* AF maintains the immune function of the host by suppressing the decrease in immune function caused by tumor and exerts an anti-tumor effect.

Chapter 6.

Inhibitory Effects of the Antlered Form of *Ganoderma lucidum* on Immunosuppression, Tumor Growth and Metastasis in Cyclophosphamide-treated Mice

6-1 introduction

Chemotherapeutic drugs are most often used to treat cancer, but show mainly side effects, including weight loss, neutropenia, and immunosuppression. While having pharmacological benefits, it may increase the risk of cancer metastasis and bacterial infection, which in turn leads to poor patient QOL. Cyclophosphamide (CY) is one of the most commonly used chemotherapeutics for many years (187). Synergistic anti-tumor effects have been reported in a model with MCA207 sarcoma-bearing mice combined with CY and IL-12 (188, 189). Anti-tumor treatment with CY and IL-15 was found to activate natural killer (NK) cells and tumor-specific $\alpha\beta$ and $\gamma\delta$ T cells (190). In addition, IL-2 is expected to enhance the anti-tumor effect of CY (191).

β -glucan, a BRM, has been reported to reduce immunosuppression by chemotherapeutic agents and enhance anti-tumor activity. For example, a combination of lentinan from *Lentinus edodes* and CY completely suppressed the growth of Rous sarcoma and D2 fibrosarcoma (192). SCG from *Sparassis crispa* also increased cytokine production from splenocytes and suppressed CY-induced leukopenia in mice (193, 194). Therefore, β -glucan having a strong immunostimulatory effect can be expected to reduce its side effects and exert synergistic anti-tumor activity when combined with a chemotherapeutic agent such as CY.

As shown in Chapter 5, the antitumor effect of *G. lucidum* AF on syngeneic tumors has been clarified. *G. lucidum* AF significantly suppressed the depletion of the number of splenic CD8⁺ T cells and protected against reduced IFN- γ production in splenocytes and lymph node lymphocytes in MM46 tumor-bearing mice. In addition, it has been reported that *G. lucidum* AF has high contents of β -(1 $\rightarrow$ 3)-D-glucan and promotes phagocytic activation of macrophages and induction of Th1 cytokine production. Therefore, *G. lucidum* AF is expected to act as a good BRM in combination with chemotherapeutics for cancer treatment.

In this chapter, the effect of *G. lucidum* AF on reducing CY-induced immunosuppression was assessed by measuring mouse body weight, splenic NK activity, mixed lymphocyte reaction (MLR), CTL activity, and cytokine production. Then, a syngeneic MM46-tumor bearing C3H/HeN mice model was used to confirm the combination of the anti-tumor effects of CY and *G. lucidum* AF. Furthermore, using C57BL/6 mice injected with LLC into the tail vein after CY administration, the inhibitory effect of *G. lucidum* AF on metastasis worsened by CY was examined.

6-2 Materials and Methods

6-2-1 *G. lucidum* AF

G. lucidum AF was purchased and prepared that containing diet as described in Chapter 5.

6-2-2 Animals

C3H/HeN, C57BL/6, and Balb/c mice (6 weeks old, male) were purchased from Charles River Laboratories Japan. Mice were reared in the manner described in Chapter 2 for one week before being used for the experiment. The animals were subsequently fed specific diets in accordance with the protocol described next. All experiments were performed in accordance with the “Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals.” This animal experiment was conducted in 2003-2006.

6-2-3 CY-induced immunosuppression

C57BL/6 mice (n = 12 per group) were fed on the AIN-93M diet or 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was i.p. injected into each group of mice. The control mice (n = 12 per group) were fed on the AIN-93M diet or 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week without this CY treatment. Body weights were monitored daily for 8 days. Splenocytes and lung lymphocytes were prepared 3, 5 and 7 days after CY administration. The spleen was passed through a 70- μ m nylon mesh, and then washed with a hemolysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) and PBS and counted the whole numbers of splenocytes by cell counter. The lung was passed through a 70- μ m nylon mesh,

and non-parenchymal cells were fractionated by using Lympholyte M (Cedarlane Laboratories). The prepared splenocytes and lung lymphocytes were suspended in RPMI1640 (Nacalai Tesque) containing a 10% FBS and 1% antibiotic-antimycotic cocktail, and then assayed as described next.

6-2-4 White blood cell population analysis

Smear preparations were prepared from blood sample collected from mice 4 to 8 days after CY administration. Thereafter, the number of white blood cells, neutrophil cells, and lymphocyte cells were counted by microscopic examination, and the number of blood cells in 1 μ l was calculated.

6-2-5 NK activity (PINK method)

Measurement and calculation of NK activity were in the methods described in Chapter 2.

6-2-6 MLR and CTL activity

Splenocytes (1×10^7 cells/ml) from BALB/c mice were incubated in an RPMI1640 medium containing 50 μ g/ml of mitomycin C (Kyowa Hakko Industry, Tokyo, Japan) at 37°C for 30 min. The medium was removed by centrifugation, and the cells were washed 4 times with the RPMI1640 medium. One ml of the mitomycin C-treated splenocytes (2.5×10^6 cells/ml) and 1 ml of splenocytes (5×10^6 cells/ml) from CY-treated C57BL/6 mice were mixed in a 12-well multiplate (BD Bioscience) and cultured at 37°C in 5% CO₂ for 4 days. The non-adhering viable cells were fractionated by using Lympholyte M and then counted. CTL activity was measured by a modified PINK method, using P-815 cells as the target cells.

6-2-7 Flow cytometric analysis

CD4⁺, CD8⁺, CD3⁺, NK1.1⁺, and CD3⁺NK1.1⁺ cells were respectively stained for 45 min at a density of 1×10^6 cells/ml with the PE-Cy5-conjugated anti-mouse CD4 monoclonal antibody (BD Pharmingen), FITC-conjugated anti-mouse CD8 monoclonal antibody (Immunotech), PE-conjugated anti-mouse CD3 monoclonal antibody (Immunotech), and FITC-conjugated anti-mouse

NK1.1 monoclonal antibody (Immunotech). Cells labeled with each antibody were analyzed using Epics XL (Beckman Coulter) as described in Chapter 4.

6-2-8 Th1 and Th2 cytokine measurements

IFN- γ and IL-4 from Con A- stimulated splenocytes were assessed as described in Chapter 2.

6-2-9 Anti-tumor model

MM 46 mammary carcinoma was inoculated into the inguinal region of C3H/HeN mice as described in Chapter 5. The mice were divided into four equal groups (10 animals/group) according to the tumor size 7 days after inoculation. Three groups were then administered with AIN-93M or a 2.5% *G. lucidum* AF-containing AIN-93M diet, and then 50 mg/kg or 150mg/kg of CY (Endoxan®, Shionogi Pharmaceutical Co., Osaka, Japan) diluted with saline was i.p. injected 14 days after inoculation. The body weight was measured 7, 14, 21, and 28 days after inoculation, and the tumor weight was determined 28 days after inoculation. To the other group was administered AIN-93M alone without CY as the control. The average tumor diameter was calculated as described in Chapter 5.

6-2-10 Anti-metastatic mode

The LLC metastasis experiment on CY-treated C57BL/6 mice was performed as shown in Fig. 6-1. LLC cells were s.c. injected into C57BL/6 mice. The solid tumor was excised 10 days later, and then treated with a 0.25% trypsin-Hanks solution at 37°C. The viability of the cells was confirmed to be greater than 80%. The LLC cells were adjusted to 1×10^6 cells/ml with a Hanks solution, and then 0.2 ml of the tumor cell solution was injected into the tail vein of C57BL/6 mice at 0.2 ml/head 3, 5 and 7 days after the CY administration (150 mg/kg). The AIN-93M diet or a 2.5% *G. lucidum* AF-containing AIN-93M diet was fed from 7 days before CY administration. The control mice were fed with the AIN-93M diet for 1 week without CY administration. The lungs were excised 14 days after LLC inoculation and fixed with Bouin's solution (a saturated picric acid

aqueous solution: formalin: glacial acetic acid = 15: 5: 1). Metastases in the lung were determined by measuring the number of nodules in the fixed tissue.

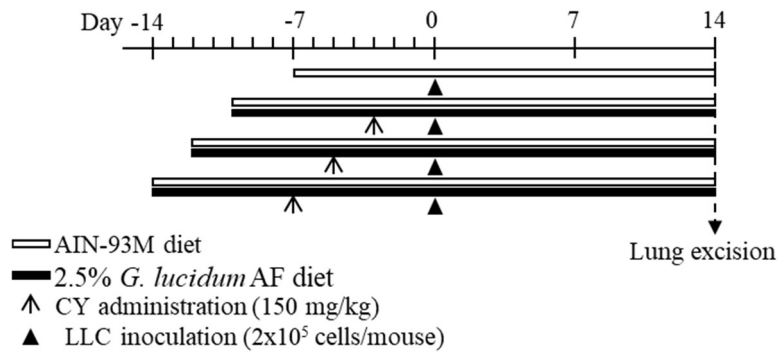


Fig. 6-1. Design of the LLC metastasis model.

6-2-11 Statistical analysis

One-way ANOVA was performed, followed by Fisher's PLSD as a *post-hoc* test for 3-group comparisons. Student's *t*-test was used for 2-group comparisons.

6-3 Results

6-3-1 Inhibitory effect of *G. lucidum* AF on changes in body weight and immunocompetent cell profiles caused by CY.

The change in body weight after CY administration was examined (Fig. 6-2). The weight of the mice was reduced by CY administration, and recovery started 4 days later. When *G. lucidum* AF was given to mice as a 2.5% diet 7 days before CY administration, CY-induced body

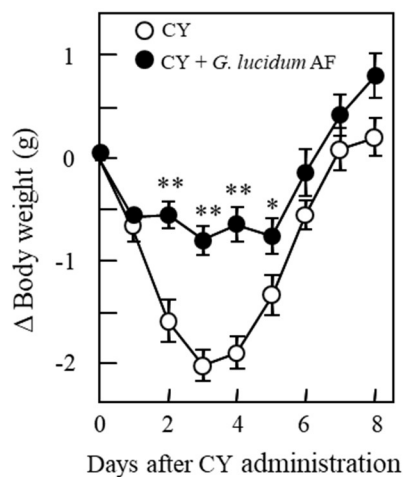


Fig.6- 2. Effects of *G. lucidum* AF on CY-induced body weight loss. C57BL/6 mice were fed on an AIN-93M diet or a 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was i.p. injected into each group of mice. Body weights were monitored daily for 8 days. Each value is the mean $\pm$ S.E. (n=12). Asterisks indicate significant differences from the CY-treated group (*, $p < 0.05$ and **, $p < 0.01$; Student's *t*-test).

weight loss was significantly suppressed.

Four to eight days after CY administration, the number of leukocytes, lymphocytes and neutrophils in the blood was measured (Fig. 6-3). CY administration significantly decreased white blood cells, lymphocytes, and neutrophils, and then gradually recovered. On day 7 of CY administration, leukocytes increased abnormally, more than twice the normal level. This increase after day 7 of administration was also remarkable in neutrophils. The group taking *G. lucidum* AF from 1 week before CY administration, abnormal increase of leukocytes and neutrophils on day 7 was significantly suppressed. The recovery of lymphocytes was not significant, but there was a tendency for the recovery to be accelerated by taking *G. lucidum* AF.

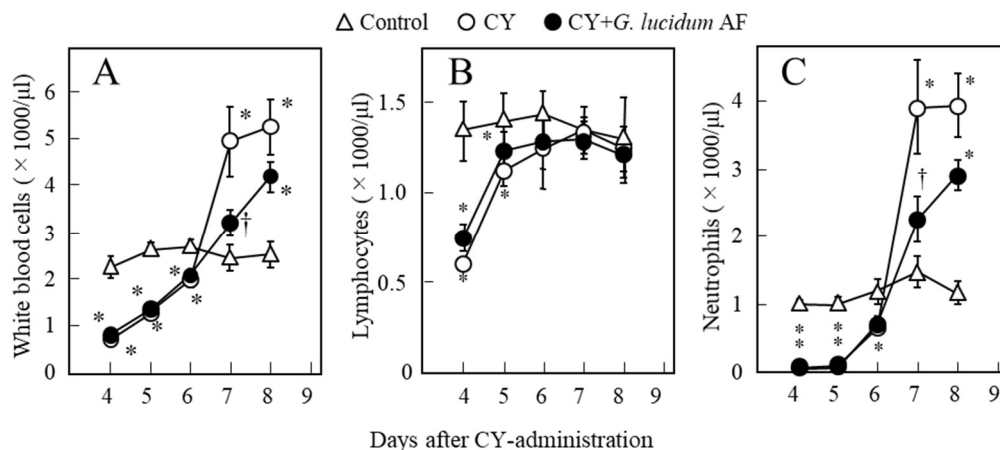


Fig. 6-3. Changes of number of white blood cells, lymphocytes, and neutrophils in CY-treated C57BL/6 mice. C57BL/6 mice were fed on an AIN-93M diet or a 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was i.p. injected into each group of mice. Blood samples were collected 4-9 days after the CY administration. Number of the cells were counted as described in Materials and Methods. Each value is the mean $\pm$ S.E. (n= 6-8). *, **: significant difference from control, (*, $p<0.05$ and **, $p<0.01$; one-way ANOVA followed by Fisher's PLSD as a *post-hoc* test) †: significant difference from CY ($p<0.05$; one-way ANOVA followed by Fisher's PLSD as a *post-hoc* test).

The numbers of CD4⁺ (helper T) cells, CD8⁺ (killer T) cells, CD3⁺ (T) cells, NK1.1⁺ (NK) cells, NK1.1⁺CD3⁺ (NKT) cells of splenocytes isolated 5 and 7 days after CY administration were measured (Table 6-1). The number of splenocytes and all kinds of immunocompetent cells were decreased 5 days after CY administration, but the decrease was significantly suppressed in mice that had taken *G. lucidum* AF from 7 days before CY administration. Furthermore, the number of

splenocytes, CD8⁺ cells, and NK1.1⁺ cells in mice that had taken *G. lucidum* AF recovered to normal levels 7 days after CY administration. However, in mice that did not take *G. lucidum* AF, the number of immunocompetent cells was significantly lower, even though the number of splenocytes were more than double that of the normal state.

Table 6-1. Changes of number of splenocytes and immunocompetent cells of splenocytes in CY-treated C57BL/6 mice. Number of the cells were counted as described in Materials and Methods. Each value is the mean $\pm$ S.E. (n=3). *a, b, c*: significant difference between groups with different letters ($p < 0.05$; one-way ANOVA followed by Fisher's PLSD as a *post-hoc* test).

		$\times 10^7$ cells	$\times 10^6$ cells				
		splenocytes	CD4 ⁺	CD8 ⁺	CD3 ⁺	NK1.1 ⁺	CD3 ⁺ NK1.1 ⁺
Day 5	control	6.55 $\pm$ 2.21 ^a	8.01 $\pm$ 2.65 ^a	2.81 $\pm$ 0.65 ^a	11.10 $\pm$ 3.30 ^a	3.38 $\pm$ 0.98 ^a	0.35 $\pm$ 0.09 ^a
	CY	0.59 $\pm$ 0.22 ^b	0.76 $\pm$ 0.32 ^b	1.25 $\pm$ 0.48 ^b	1.89 $\pm$ 0.74 ^b	0.38 $\pm$ 0.18 ^b	0.06 $\pm$ 0.03 ^b
	CY+ <i>G. lucidum</i> AF	1.16 $\pm$ 0.37 ^c	1.38 $\pm$ 0.48 ^c	2.24 $\pm$ 0.77 ^c	3.31 $\pm$ 0.80 ^c	0.85 $\pm$ 0.67 ^c	0.12 $\pm$ 0.04 ^c
Day 7	CY	13.45 $\pm$ 5.36 ^b	3.35 $\pm$ 0.61 ^b	1.91 $\pm$ 0.41 ^b	5.05 $\pm$ 0.98 ^b	3.31 $\pm$ 1.64 ^a	0.16 $\pm$ 0.07 ^b
	CY+ <i>G. lucidum</i> AF	6.27 $\pm$ 2.29 ^a	3.70 $\pm$ 1.11 ^b	2.76 $\pm$ 0.84 ^a	5.90 $\pm$ 1.58 ^b	2.64 $\pm$ 1.06 ^a	0.18 $\pm$ 0.07 ^b

6-3-2 Inhibitory effect of *G. lucidum* AF on CY-induced immunosuppression

Three days after CY administration, CY reduced splenic NK activity compared to that of control group, but the decrease was suppressed by *G. lucidum* AF intake (Fig. 6-4). The order of the intensity of NK activity 5 days after CY administration was as follows: *G. lucidum* AF intake group, CY administration group, and control group. And a significant difference was observed among these three groups, respectively (Fig. 6-4B). However, the population of NK1.1⁺ cells in splenocytes in these groups was similar and not significantly different (6.4%, 7.3%, 5.2%, respectively). Seven days after CY administration, all groups were not significantly different. NK activities in lung lymphocytes were also measured at 3, 5, and 7 days after CY administration. There was no significant difference in lung NK activity 3 days after CY administration (Fig. 6-5A), but CY significantly reduced NK activity 5 days after CY administration. *G. lucidum* AF alleviated the decrease in lung NK activity after 5 days (Fig. 6-5B), and the activity of *G. lucidum* AF administration group 7 days after CY administration was higher than that of the control group (Fig. 6-5C).

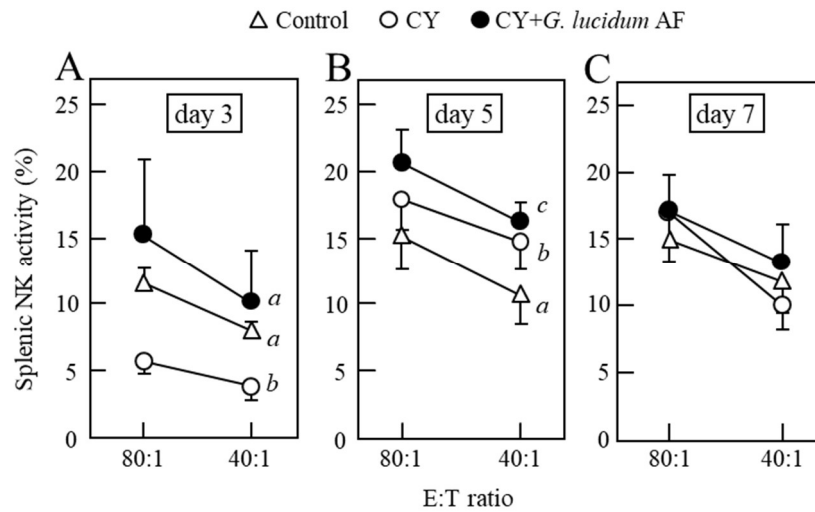


Fig. 6-4. Changes of splenic NK activities in CY-treated C57BL/6 mice. C57BL/6 mice were fed on an AIN-93M diet or a 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was i.p. injected into each group of mice. Splenocytes were prepared 3, 5 and 7 days after the CY administration. NK activities were determined as described in Materials and Methods section at different effector: target cell ratios (40: 1 and 80: 1). A, B and C show splenic NK activities 3, 5 and 7 days after CY administration, respectively. Each value is the mean $\pm$ S.D. (n= 4). a, b and c: significant difference between groups with different letters ($p < 0.05$; one-way ANOVA followed by Fisher's PLSD as a *post-hoc* test).

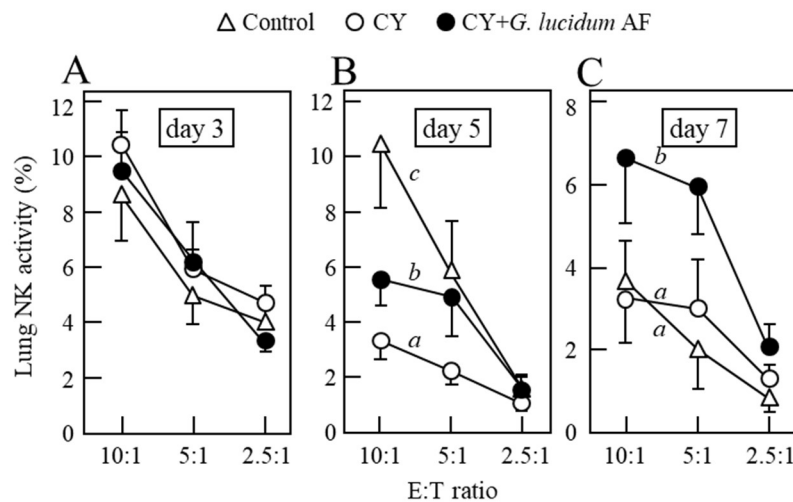


Fig. 6-5. Changes of lung NK activities in CY-treated C57BL/6 mice. C57BL/6 mice were fed on an AIN-93M diet or a 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was i.p.-injected into each group of mice. Lung lymphocytes from 12 mice were prepared and mixed 3, 5 and 7 day after CY administration. NK activity was measured as described in the Materials and Methods section at various effector:target cell ratios (2.5:1, 5:1, and 10:1). A, B and C show lung NK activities 3, 5 and 7 days after CY administration, respectively. Each value is the mean $\pm$ S.D. a, b and c: significant difference between groups with different letters ($p < 0.05$; one-way ANOVA followed by Fisher's PLSD as a *post-hoc* test).

MLR was performed as described in the Materials and Methods section. The numbers of non-adherent cells 5 and 7 days after CY administration were significantly lower than in the control mice. However, the cell numbers recovered to the control level by 7 days after CY administration in the *G. lucidum* AF-fed groups (Fig. 6-6A).

After testing MLR, the numbers of non-adherent cells after MLR 5 and 7 days after CY administration were significantly lower than that of control mice. However, in the *G. lucidum* AF group, no recovery was observed 5 days after CY administration, but the number of non-adherent cells recovered to control levels after 7 days (Fig. 6-6A).

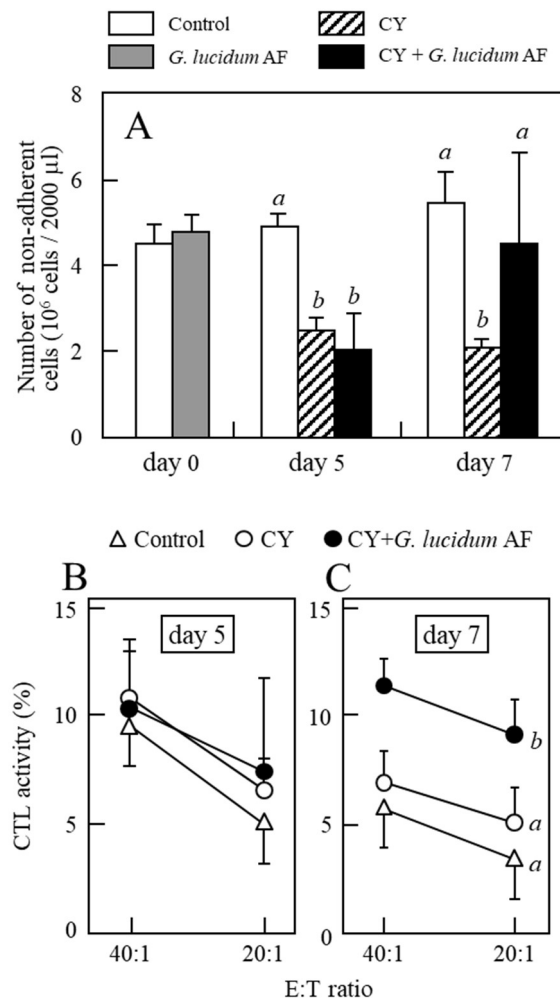


Fig. 6-6. Changes in MLR of splenocytes from CY-treated C57BL/6 mice. C57BL/6 mice were fed on an AIN-93M diet or a 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was i.p.-injected into each group of mice. Splenocytes were prepared 5 and 7 days after CY administration. MLR with mitomycin C-treated splenocytes from BALB/c mice was performed as described in the Materials and Methods section. Non-adherent cells were counted after MLR (A). B and C present CTL activity against P-815 cells at different effector:target cell ratios (40:1 and 80:1). Each value is the mean $\pm$ S.D. (n= 4). a, b: significant difference between groups with different letters ($p < 0.05$; one-way ANOVA followed by Fisher's PLSD as a *post-hoc* test).

Using non-adherent cells after MLR, CTL activity was measured by adjusting the E:T ratio. Five days after CY administration, there was no significant difference among the three groups (Fig. 6-6B), which was maintained at the control level despite CY treatment. Seven days after CY administration, the *G. lucidum* AF administration group had significantly higher CTL activity than the other two groups (Fig. 6-6C).

The change in Th1 and Th2 cytokine production ability of Con A-stimulated splenocytes was examined. For the Th1 cytokine IFN- γ (Fig. 6-7A), *G. lucidum* AF promoted IFN- γ production in splenocytes. On day 5 of CY administration, there was no change in Th1 cytokine-producing ability, but the enhancing effect of *G. lucidum* AF was canceled. On the 7th day, CY decreased IFN- γ production ability, but *G. lucidum* AF suppressed it. The Th2 cytokine IL-4 was greatly enhanced on day 5 of CY administration but was partially suppressed by *G. lucidum* AF (Fig. 6-7B). Conversely, on the seventh day, the IL-4 production ability decreased, and the decrease was completely suppressed by *G. lucidum* AF.

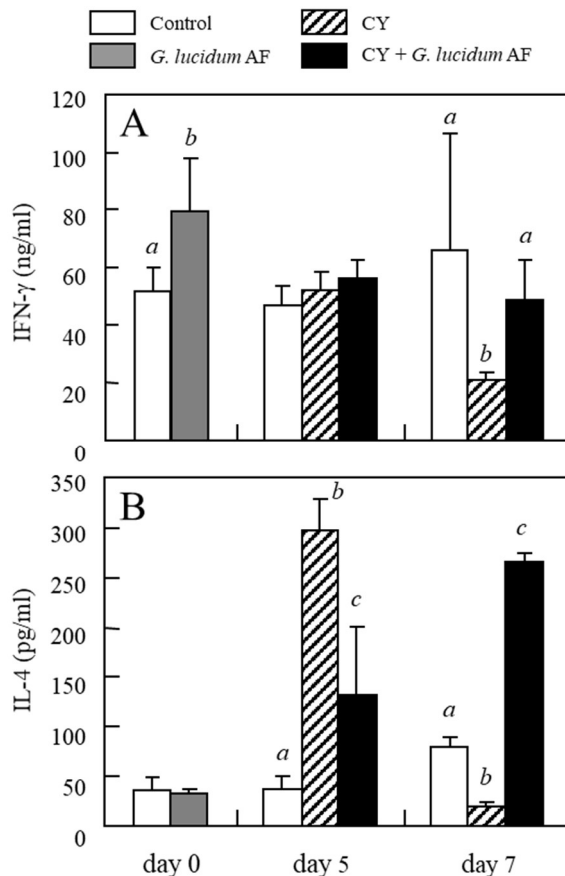


Fig. 6-7. Changes of cytokine production from splenocytes in CY-treated C57BL/6 mice. C57BL/6 mice were fed on an AIN-93M diet or a 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week, and then 150 mg/kg of CY was i.p.-injected into each group of mice. Splenocytes were prepared 5 and 7 days after CY administration. The control mice were fed on the AIN-93M diet or 2.5% *G. lucidum* AF-containing AIN-93M diet for 1 week without the CY treatment, and then splenocytes were similarly prepared. The IFN- γ (A) and IL-4 (B) production was measured as described in the Materials and Methods section. Each value is the mean $\pm$ S.D. (n=4). a, b and c: significant difference between groups with different letters [p <0.05, Student's t -test (day 0) or one-way ANOVA followed by Fisher's PLSD as a *post-hoc* test (5 and 7 days after CY administration)].

6-3-2 Effect of combined use of *G. lucidum* AF and CY on MM46 tumor-bearing mice

In preliminary experiments, the anti-tumor effect of CY was not significant at doses below 50 mg/kg (data not shown). Therefore, the combined effects of 50 mg/kg CY and *G. lucidum* AF were compared to 50 and 150 mg/kg CY. As shown in Fig. 6-7A, following administration of CY at a dose of 150 mg/kg, mice lost weight as a side effect, but no weight loss was seen in the other groups. In the CY (50 mg/kg) plus 2.5% *G. lucidum* AF and CY (150 mg/kg) groups, tumor growth was significantly suppressed from day 17 and day 21 after MM46 tumor inoculation, respectively (Fig. 6-7B). The final tumor weight was also significantly smaller than the control (Fig. 6-7C). In other words, it was found that the combination of CY (50 mg/kg) and 2.5% *G. lucidum* AF was more effective than CY alone at the same dosage.

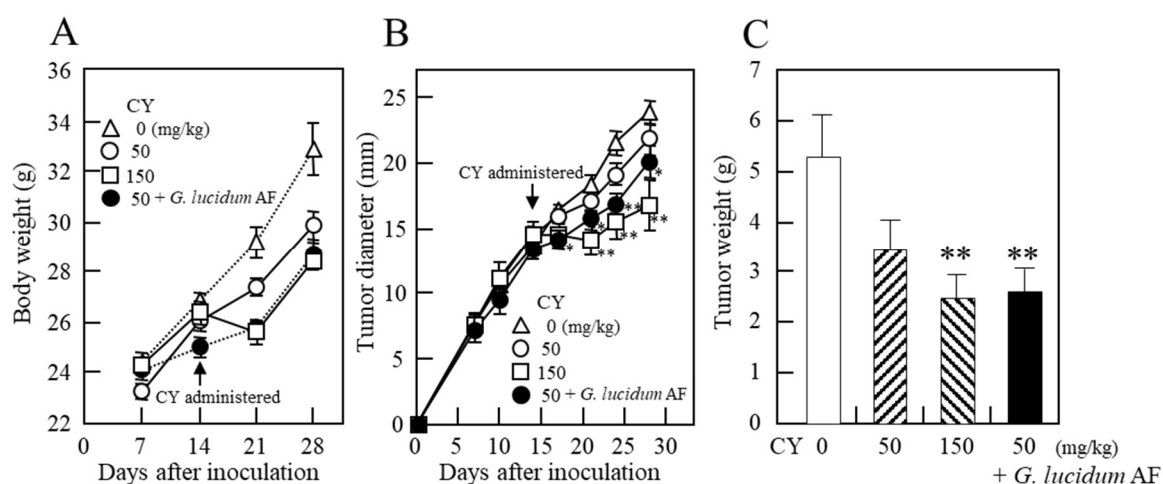


Fig. 6-8. Anti-tumor effects of *G. lucidum* AF and CY in syngeneic MM 46 tumor-bearing mice. C3H/HeN mice were s.c. inoculated with MM 46 mammary carcinoma (2×10^6 cells/0.2 ml/mouse) into the inguinal region. The mice were divided into 4 equal groups according to tumor size 7 days after inoculation. The *G. lucidum* AF (●) groups were then administered with the 2.5% *G. lucidum* AF-containing AIN-93M diet. CY was i.p. injected 14 days after the inoculation. Changes in the mean body weight (A), tumor diameter (B), and tumor weight 28 days after tumor inoculation (C) were measured. Each value is the mean $\pm$ S.E. ($n = 10$). Asterisks indicate significant difference from the control group (*, $p < 0.05$ and **, $p < 0.01$; one-way ANOVA, followed by Fisher's PLSD as a *post-hoc* test).

6-3-3 Effect of *G. Lucidum* AF on lung metastasis in CY-treated mice

Lung metastasis experiments were performed using CY-treated mice as shown in Fig. 6-1. When LLC was injected into the tail vein of C57BL/6 mice 3 or 5 days after CY administration, the

number of LLC lung metastases clearly increased (Fig. 6-8A). When LLC was injected 7 days after CY administration, the number of metastases was at the control level. When *G. lucidum* AF was ingested since one week before CY administration, CY metastasis of LLC was suppressed on any day. In particular, LLC metastasis 7 days after CY administration was significantly suppressed by *G. lucidum* AF. Fig. 6-8B shows typical findings of lung metastasis without or on day 7 of CY administration and *G. lucidum* ingestion.

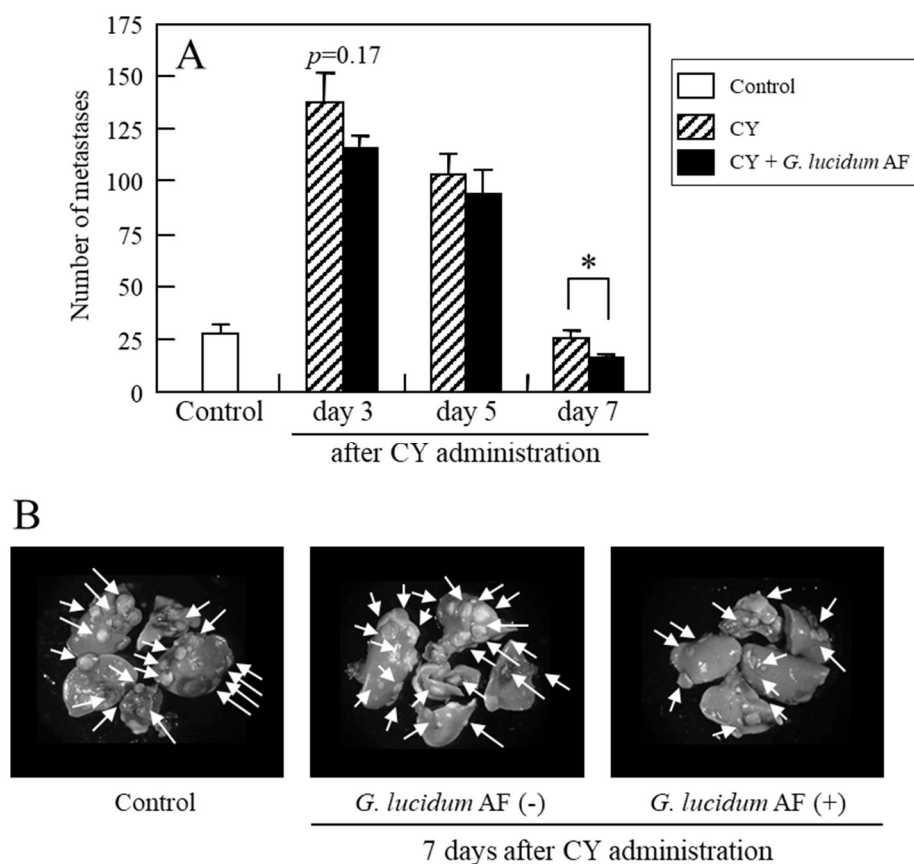


Fig. 6-9. Effects of *G. lucidum* AF on Lung Metastasis in CY-treated Mice. The LLC metastasis experiment was performed as described in the Materials and Methods section and Fig. 6-1. A, Number of lung metastases. Each value is the mean $\pm$ S.D. ($n = 10$). Asterisks indicate significant difference (*, $p < 0.05$; Student's *t*-test). B, Typical views of lung metastases in LLC-inoculated mice without CY administration (as the control) and LLC-inoculated mice 7 days after CY administration with or without *G. lucidum* AF-feeding. Arrows indicate metastatic nodules.

6-4 Discussion

G. lucidum AF is a medicinal mushroom used as a Chinese medicine in Korea, China and Japan. *G. lucidum* AF showed immunostimulatory antitumor activity when administered 2.5% *G. lucidum* AF diet to syngeneic MM46 tumor-bearing C3H/HeN mice (Chapter 5). In this chapter, the author examined whether *G. lucidum* can maintain the immune function decreased by the chemotherapeutic agent, CY using CY-treated C57BL/6 mice. In addition, the anti-tumor effect and anti-metastatic effect when CY and *G. lucidum* AF were used in combination as phenotypes for maintaining immune function were confirmed using C3H/HeN mice and C57BL/6 mice, respectively.

In a preliminary study, 150 mg/kg CY inhibited MM46 growth under the same experimental conditions as in this experiment (Fig. 6-8), but 2.5% *G. lucidum* AF did not enhance the anti-tumor effect (Data not shown). This might be due to the high anti-tumor activity of 150 mg/kg CY itself. Therefore, in this study, the authors evaluated the efficacy of a combined *G. lucidum* AF and ineffective dose of CY (50 mg/kg), and confirmed that it was nearly as effective as 150 mg/kg CY (Fig. 6-8). These results suggest that the combination of *G. lucidum* AF and a chemotherapeutic agent can be expected to be more effective than each single use.

MM 46 carcinoma is moderately antigenic (179) and has been reported to be attacked by NK cells (195). Therefore, analyzing cellular immunity, including NK and CTL activity, is very important in elucidating the anti-tumor effects through immune function on MM46 tumor growth. The number of NK cells and killer T cells were down regulated 5 and 7 days after CY treatment (Table 6-1), and *G. lucidum* AF suppressed those downregulations except for NK cells on 7 days. On day 5 and 7, NK activity (Fig. 6-4) and the number of NK cells seem to correlate to some extent. Since NK activity of *G. lucidum* AF-treated mice is high for the number of NK cells, it is considered that not only the number of cells but also the cell killing activity of each cell was enhanced by *G. lucidum* AF ingestion.

The mitogenic activity of T cells after MLR of CY-treated mice was down-regulated on days 5 and 7 (Fig. 6-6A). On the other hand, the MLR-induced CTL activity was at the same level as that of control mice (Fig. 6-6B and C). When T cell population was compared (Table 6-1), CY

administration reduced Th cells (CD4⁺) to less than 10% of normal levels and total T cells (CD3⁺) to about 20%. However, killer T cells (CD8⁺) are only reduced by about 45%. These results suggest that the total number of T cells and the function for antigens were reduced, but the number of CTLs was maintained more than other T cells, and the activity was maintained at a normal level at this time. By the ingestion of *G. lucidum* AF, the number of splenocytes and T cells on day 5 were both lower than those of the group administered with CY alone (Table 6-1), but the function were decreased (Fig. 6-6A). However, on day 7, killer T has completely recovered to normal levels but the number of splenocytes have not yet recovered by *G. lucidum* AF ingestion. It is considered that the apparent CTL activity was increased due to the preparation based on the number of splenocytes (Fig. 6-6C). However, it seems possible to say that *G. lucidum* AF intake accelerates the recovery of CTL important for tumor immunity. The increase in the number and activity of CTLs by *G. lucidum* AF is consistent with the results in Chapter 5 using MM46 tumor-bearing C3H/HeN mice. These findings suggest that one of the characteristic immunological effects of *G. lucidum* AF is the rapid recovery of CD8⁺ population and activation of CTL activity under tumor or chemotherapeutic-induced immunosuppressive conditions.

The mitogenic activity after MLR was also down-regulated 7 days after the CY treatment (Fig. 6-6A). However, the MLR-induced CTL activity of CY-treated mice was equal to the control level (Fig. 6-6C). These findings suggest that, although the T cell function was reduced, the CTL activity was maintained at the control level. On the other hand, despite the number of CD3⁺ T cells being down-regulated (Table 6-1), the functions of T cells such as the CD8⁺ number (Table 6-1), mitogenic activity (Fig. 6-6A), and IFN- γ induced activity (Fig. 6-7A) were recovered or maintained at the control levels by *G. lucidum* AF. These findings suggest that the CD8⁺ T cells were highly activated, and maintained the general T cell functions and enhanced the CTL activity (Fig. 6-6C) in the *G. lucidum* AF-fed mice. The finding of up-regulation of the CTL activity by *G. lucidum* AF agrees with that of a previous study that used *G. lucidum* AF-fed MM 46-bearing C3H/HeN mice (Chapter 5). These findings suggest that one characteristic immunological effect of *G. lucidum* AF is speedy recovery of the CD8⁺ population and activation of CTL activity under the immunosuppressive condition induced by a tumor or chemotherapy.

β -glucans act as pathogen-associated molecular patterns and are recognized by appropriate cell surface receptors which initiate immune responses. In particular, opsonic receptor complement receptor 3 (CR3, CD11b/CD18)(196, 197) and dectin-1(198, 199) are well known receptors for β -glucan. Harada *et al.* (193, 194) have reported that the SCG (6-branched 1, 3- β -D-glucan)-induced cytokine levels were increased concomitant with the up-regulation of CR3 and dectin-1 levels by CY. Fungal cells and crude β -glucans like zymosan bind to TLR 2 and 4 and activate innate immune responses (200). It is reported that OM-174, a chemically defined TLR 2 and 4 agonist, reduced tumor growth and prolonged survival in CY-treated B16 melanoma-bearing mice (201). The structure of the specific β -glucan in *G. lucidum* AF that has immunomodulating activity has yet to be determined. However, the β -glucan content in *G. lucidum* AF is more than 40% and is comparatively high in medical mushrooms. The author therefore speculates that one of the active components of *G. lucidum* AF is the specific structure of β -glucan. It also appears that an innate immune response is generated *via* the activation of CR3, dectin-1, and TLR2/4 by the β -glucans of *G. lucidum* AF. Further study is needed to determine the specific structure of the β -glucans of *G. lucidum* AF which activate these receptors.

CY down-regulated not only IFN- γ but also IL-4 production, while *G. lucidum* AF reduced both of these down-regulations by day 7 (Fig. 6-7), showing that Th2-type responses were also enhanced by *G. lucidum* AF. The authors found that ingestion of *G. lucidum* AF increased not only IFN- γ but also IL-4 production in MM46 tumor-bearing mice (Chapter 5). Oral administration of β -glucan to C57BL/6 and CR3-deficient mice has been reported to stimulate CR3 of granulocytes, increase cytotoxicity against tumor cells, and act as an adjuvant for anti-tumor antibodies (202, 203). Furthermore, it has been reported that orally administered β -(1-3)-D-glucan enhances not only cellular immunity but also antibody production in CY-treated mice (204). These findings suggest that, as already explained, the anti-tumor effect of the combination of *G. lucidum* AF and CY in MM46-bearing mice depends on the regulatory effect of *G. lucidum* AF on both cellular and humoral immunity (Fig. 6-8). This report indicates that the anti-tumor activity of lentinan, a polysaccharide containing β -(1-3)-D-glucan, against MM46 carcinoma was correlated with increased cellular and humoral immune responses to MM46 antigen (179).

Various immune mechanisms are known to inhibit LLC metastasis. For example, IL-2-activated NK cells and MHC-restricted CTL inhibited LLC metastasis (191, 199). Macrophage-activated β -glucan, such as schizophyllan, inhibited metastasis by infiltrating CTLs and macrophages into tumor and metastatic areas (205). In this study, the authors found that *G. lucidum* AF tended to inhibit the increase in LLC metastasis accelerated by CY administration (Fig. 6-9). One possible mechanism for suppression of LLC metastasis by *G. lucidum* AF, on day 7, the lung NK activity of the *G. Lucidum* AF-fed group was significantly higher than the lung NK activity of the CY-administered group, there is a possibility of activating the lung NK activity (Fig. 6-4C). However, no correlation was found between anti-metastatic activity and lung NK activity by day 3 or 5 after CY administration. It has been reported that The triterpenoid fraction of *G. lucidum* inhibited angiogenesis and metastasis in LLC-grafted C57BL/6 mice (206), and the lanostane triterpenoid ganodate phosphate Me purified from *G. lucidum* mycelium showed an inhibitory effect on lung metastasis through IL-2 and IFN- γ -upregulation and activated NK activity (207). In addition, the amount of triterpenoids in *G. lucidum* AF is higher than that of normal types of *G. lucidum* (90), so it is expected that the triterpenoids in *G. lucidum* AF will also exhibit anti-metastatic activity. Therefore, it is considered that a combination of specific structure of β -glucan and triterpenoids in *G. lucidum* AF exhibited an anti-metastatic effect in CY-treated mice.

Lin (208) has reviewed the broad immunomodulatory functions of *G. lucidum*, including promotion of the function of antigen-presenting cells, the mononuclear phagocyte system, humoral immunity, and cellular immunity. *G. lucidum* also inhibits the formation of oxidative stress-induced invasive cancers by inhibiting the transcription of AP-1 and NF- κ B mediated by the phosphorylation of extracellular signal-regulated protein kinase (209). Combinations of *G. lucidum* AF and various chemotherapeutic drugs can thus be expected to reduce adverse effects and to enhance anti-tumor activity, suggesting that immunotherapy with *G. lucidum* AF would be superior to anti-cancer therapy with a single recombinant cytokine.

In conclusion, ingestion of *G. lucidum* AF before CY administration suppressed the reduction of NK activity by CY, accelerated the recovery of reduced T cell function, and enhanced the anti-tumor and anti-metastatic effects. Although beneficial in treating cancer, chemotherapeutic

drugs can cause serious and difficult side effects for patients, impair QOL, and increase the risk of infection. Although it is difficult to completely cure cancer by ingesting functional foods such as *G. lucidum* AF, the combination of *G. lucidum* AF and chemotherapeutic drugs may lead to more effective cancer treatment and improved QOL. The author eagerly hopes that the evidence will become clearer through clinical trial studies and it will help to maintain the health and improve QOL of many people.

6-5 Summary

It is known that chemotherapeutic agents not only kill cancer cells but also damage immune cells, causing a decrease in immune function. Therefore, it was examined whether the immunosuppression caused by cyclophosphamide (CY), a typical chemotherapeutic agent, could be suppressed by taking *Ganoderma lucidum* AF. Although the weight of C57/BL6 mice was reduced by the administration of CY, the weight loss was significantly suppressed by ingesting *G. lucidum* AF in advance for one week. Furthermore, *G. lucidum* AF was found to significantly suppress abnormal rebound of white blood cells, especially neutrophil count, and reduction of killer T cells and NK cells after CY administration. *G. lucidum* AF was also found to maintain not only the number of immunocompetent cells but also NK activity, which is temporarily reduced by CY, and accelerate the number and function recovery of T cells, especially killer T cells. In the MM46-C3H syngeneic tumor model used in Chapter 5, CY exerts an antitumor effect at a concentration of 150 mg/kg and does not work at 50 mg/kg, but 50 mg/kg when used in combination with *G. lucidum* AF. However, a significant anti-tumor effect was observed. The inhibitory effect of *G. lucidum* AF on lung metastasis was confirmed using a model in which Lewis lung carcinoma (LLC), which is also syngeneic, was metastasized to the lungs of C57BL/6 mice. Metastasis of LLC increased by CY administration, but metastasis tended to be suppressed by *G. lucidum* AF, and was significantly suppressed on day 7 of CY administration. In *G. lucidum* AF mice, the decrease in NK activity in the lung was suppressed, which is thought to be the mechanism of metastasis suppression. These results revealed that *G. lucidum* AF suppressed CY-induced side effects, particularly immune decline, or accelerated recovery.

Chapter 7.

General Discussion

The immune function of people is a defense system for maintaining life for us, but it has the aspect that it changes greatly with aging, daily living habits, stress, and diseases. Especially in modern society where the sanitary environment is well established, it seems that people's immune functions tend to be disturbed because of less immune stimulation (2-4) or more daily stress (131-133) than before. One result is an increase in the allergic population in Japan (1). Diseases resulting from the breakdown of immune function include allergies, cancer, type I diabetes, and autoimmune diseases (9). Although there may be an idea that these illnesses should be treated after getting ill, the author wanted to protect their health by keeping their immune functions normal in their daily lives. It is also preferable if the maintenance of immune function can be achieved by familiar and convenient approach such as eating functional food. Therefore, the author used the hygiene hypothesis against itself and conducted research on the theme of whether these would help maintain immune function using microorganisms and fungi that familiar to Japan and the over world. As a microorganism, LAB derived from Japanese pickles, *L. pentosus* S-PT84 strain, was selected. As the fungus, *G. lucidum* AF, which succeeded in artificial cultivation was used.

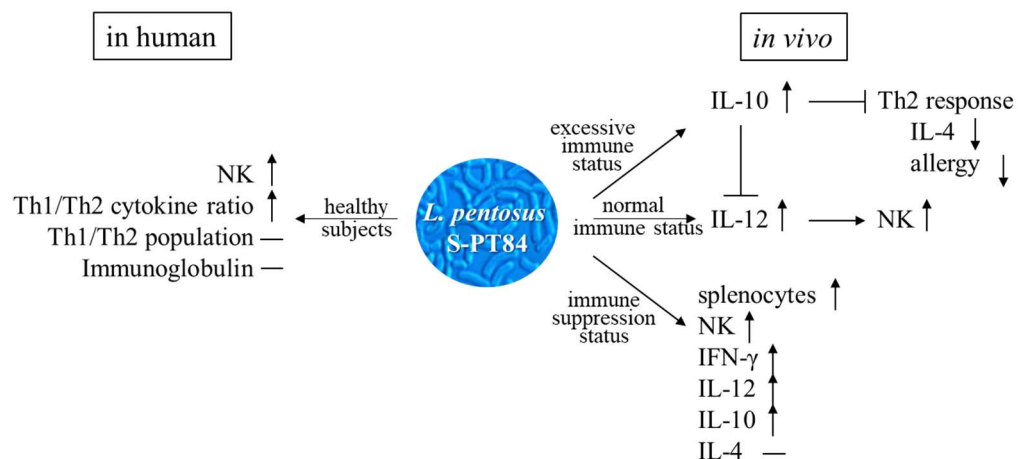


Fig. 7-1. The overview of immunomodulating activity of *L. pentosus* S-PT84.

From the results about the immunomodulatory action of *L. pentosus* S-PT84 in mice (shown in Chapter 2 and Chapter 3) and humans (shown in Chapter 4), it can be explained by the action mechanism shown in Fig. 7-1. Since this strain was screened by IL-12-inducing activity as an index (Fig. 2-2), it was predicted that IFN- γ induction would be increased, but IL-4-inducing ability was lowered instead (Fig. 2-5). *L. pentosus* S-PT84 is unique in that it induces not only IL-12, but also IL-10 which works for immunosuppression. From the experiments in Chapter 2 and Chapter 3, *L. pentosus* S-PT84 probably suppresses excessive immunity via IL-10 when immune function is excessive, and induces IL-12 when Th1 function is reduced. It is thought that it is effective in enhancing, that is, maintaining the homeostasis of immune function. Since Balb/c mice is considered to be a Th2-type (210), if the mice were sensitized with OVA, it could be immune overly reaction. Therefore, as shown in Chapter 2, *L. pentosus* S-PT84 increased not IL-12 but IL-10, and suppressed IL-4 and IgE production (Fig. 2-8) in the OVA model, because the action of IL-10 is thought to suppress IL-4 reaction or allergic reactions in an excessive immune state. Many LAB have been reported to adjust the Th1/Th2 balance (31, 103) and suppress allergic reactions (24, 29-31, 39), but the author was able to find another activity of LAB, relieved against immunosuppression induced by restraint stress as shown in Chapter 3.

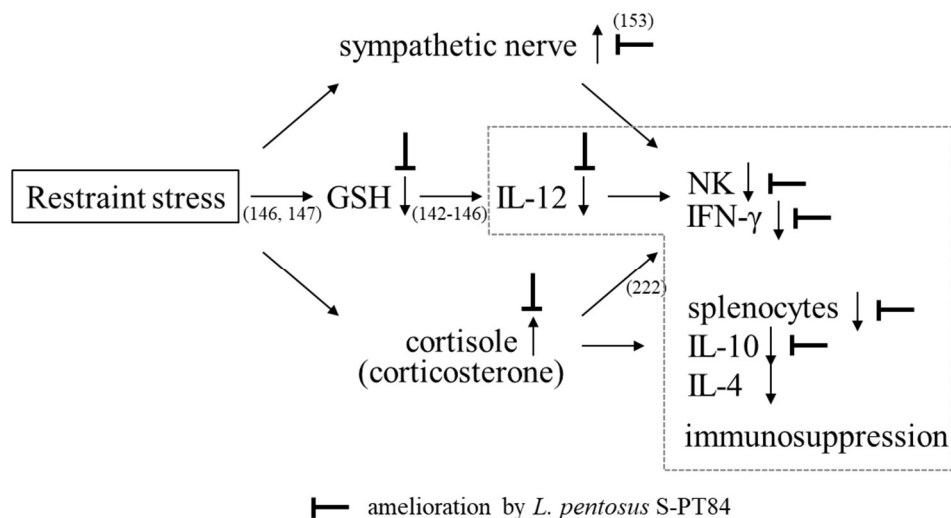


Fig. 7-2. Predicted mechanism of *L. pentosus* S-PT84 on restraint stress-induced immunosuppression.

Although the response of stress is so complex that there are some unclear points, the relationship among stress and immunosuppression and effect of *L. pentosus* S-PT84 on restraint stress-induced immunosuppression is considered as shown in Fig. 7-2. LAB is thought to induce immune responses through receptors such as TLR-2 (101, 102) and TLR-9 (103). The suppression of immune depression by stress caused by *L. pentosus* S-PT84 and the possibility of being mediated by these receptors are considered to be sufficient, but suppression of corticosterone elevation and GSH reduction are not be able to be explained by currently known TLR signaling. However, *L. plantarum* CMU995 (211) and *L. plantarum* KSFY02 (212) are also reported as LAB that increase GSH, and several anti-stress LAB that suppress cortisol increase due to stress have been reported (213-215). Those reports suggest that some LAB have the same anti-stress effect as *L. pentosus* S-PT84. In particular, Takada *et al.* (214) consider that the mechanism is due to gut-brain interaction, but it is even more interesting when the function of LAB is discovered in addition to immunity. The results of a human test that *Lactobacillus casei* DN-114001 suppressed NK activity, which decreases at the period of the examination under stress have been reported (67), but this may be the same mechanism. In any case, *L. pentosus* S-PT84 suppresses an excessive immune response in the case of excessive immunity such as allergy (Chapter 2), and enhances immune function in an immunosuppressed state such as when stress is applied (Chapter 3). In terms of these points, it is very useful for maintaining human health.

G. lucidum AF was also confirmed to have an effect of suppressing immune decline caused by cancer (Fig. 5-5, 5-6) as shown in Chapter 5 and chemotherapeutic agents (Fig. 6-3, 6-4, 6-5, 6-6) as shown in Chapter 6. Fig. 7-3 summarizes the effects of *G. lucidum* AF that have been revealed.

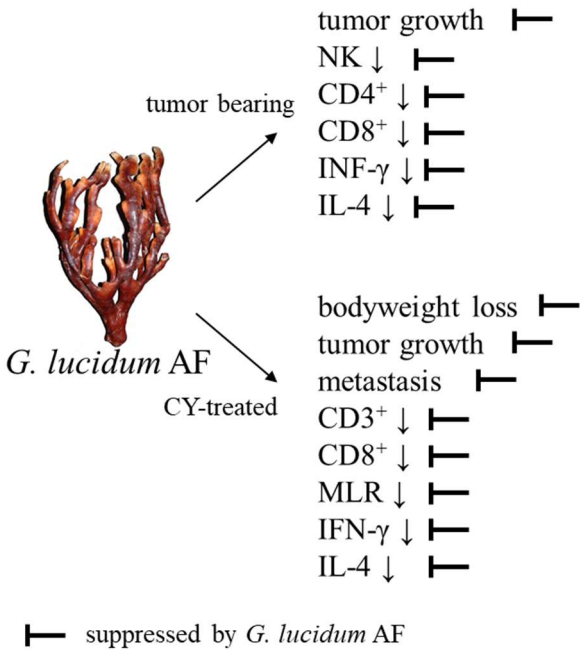


Fig. 7-3. The overview of immunomodulative activity of *G. lucidum* AF.

One of the most frightening things about cancer is that although cancer cells are non-self, the immune system is compromised, and the immune defense system fails. Chemotherapeutic drugs to fight cancer can also cause immune depression. Hence when human get cancer, they lose the weapon to fight cancer with our own immunity. Of course, it is impossible to cure cancer with functional food alone. However, it has been suggested that polysaccharides of *G. lucidum* AF activate lymphocytes in patients with lung cancer (216), and there is also a clinical report intake of *G. lucidum* AF for 6 months increases the antioxidation and hepatoprotective activity in healthy individuals (217). From these research results, it is considered that the prevention of cancer can be expected by daily intake of functional foods like *G. lucidum* AF that enhance our immune functions.

What can be said from the results of this dissertation is that the immunomodulatory effects of these food ingredients have proved by oral administration except for some *in vitro* and i.p. administration experiments. In both ingredients, indigestible components are the most components, and it is unlikely that they are absorbed as they are. However, the fact that the intravital immune function is affected by the oral intake of these food ingredients may indicate that the gut immunity has somehow reacted. Dendritic cell is said to be able to contact the luminal environment and present antigens in the body (218, 219). Both *L. pentosus* S-PT84 and *G. lucidum* AF might be recognized by such antigen-presenting cells in the gut lumen. As mentioned in each chapter, research on the components and mechanisms of action of these food ingredients that are not single component is still necessary.

Both LAB and *G. lucidum* AF used in this research showed a unique physiological activity, but it doesn't seem to be anything as long as it is LAB or *G. lucidum* AF. This is because when the author started the research, the physiological activities of several plant-origin LAB (Chapter 2, Fig. 2-2) and *G. lucidum* AF (Chapter 5, Fig. 5-1) were evaluated and selected the ones that showed the highest activity. And the author was able to discover various physiological activities and usefulness. Regarding *L. pentosus* S-PT84, the research by the author's colleagues has progressed further and 15 papers have been published as at December 2019. Table 7-1 summarizes the physiological activities related to immunity of *L. pentosus* S-PT84. Since microorganisms are not a single compound, they might have various active components and action mechanism. And the

most important thing is whether to show efficacy in humans. One of the biggest achievements of the author's research was that the potential of *L. pentosus* S-PT84 was confirmed in humans (Chapter 4). This is an in-house pilot study, but its potential has been re-examined by the authors colleague (167). In the future, not only *L. pentosus* S-PT84 but also *G. lucidum* AF should be able to raise its level of evidence by conducting more accurate verification tests. The author believes that the level of evidence can be increased by conducting high verification tests, for example, the randomized control study such as *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 has been verified to prevent influenza infection (220), and the symptoms of hay fever can be alleviated as *L. acidophilus* L-92 (24) and *L. rhamnosus* GG (221). It can be expected that it will help improve the QOL of many people as functional foods by regulating immune function when it could be obtained a high level of verification testing and results. Furthermore, if this kind of research results can be recognized as “foods with functional claims”, it will be possible to further contribute to maintaining the health of people. Unfortunately, however, the current Japanese regulation for foods with functional claims does not accept health claims regarding enhancement of immune function or prevention/improvement of allergies and infectious diseases (95). However, maintaining the immune function is maintaining the homeostasis and protecting the body from various health problems. The author hopes that such health claims will be recognized and the importance of maintaining immune function will become more widely known. Through this dissertation, the author studied the immunoregulatory effects of food components, especially LAB and fungi, and wanted to contribute to improving the QOL of people all over the world. Especially in today's aging and stressful environment, people's immune function tends to be disturbed, and people are exposed to dangers such as allergies, infections and cancer every day. Fortunately, the author was able to find *L. pentosus* S-PT84 that modulate immune function to adequate condition even when the immune function is reduced or excessive. In addition, *G. lucidum* AF was also found to recover the immune function that is reduced by cancer and chemotherapeutic agents. The author believes these research results are connected to Suntory's products and contribute to improving the QOL of many people, but more specifically, building evidence for humans and helping to maintain the happy and health of many more people.

Table 7-1. Various immunomodulating activity of *L. pentosus* S-PT84

Efficacy	species / cells	reference
anti-allergy		
NK activation	BALB/c	137
IL-10, 12 induction		
Shift to Th1 dominant		
activation of Th1	dendritic cell, NK1.1	138
anti influenza virus	BALB/c	139
IFN- α , γ , IL-12 induction		
IFN- α induction	C57BL/6, BALB/c, DBA/2	140
IgA induction	BALB/c	
Anti Salmonella infection		
NK activation via modulation of sympathetic nerve activity	BALB/c	153
enhancing anti-influenza specific IgA production	BALB/c	223
NK activation	Human	224
(combination with vitamin B)		
anti candida infection	ICR	225
anti stress-induced immunosuppression	C57BL/6	226
anti LPS-induced chronic inflammation	C57BL/6	227

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A. Original papers

1. Ashida H, Kihara K, **Nonaka Y**, Fukuda I, Shiotani B, Hashimoto T. The heterocyclic amine, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole induces apoptosis in cocultures of rat parenchymal and nonparenchymal liver cells. *Toxicol Appl Pharmacol* 2001, **177**, 59-67
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15. 立石 法史, 中村 淳一, **野中 裕司**, 体脂肪低減効果を有するケルセチン配糖体配合飲料の研究開発 特定保健用食品「伊右衛門 特茶」の開発 化学と生物 2018, **56**, 408-413
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B. Miscellaneous Papers

バイオテクノロジーシリーズ このこの生理活性と機能 シーエムシー出版

C. Patent

1. 特許 6396970 集中力向上剤
2. 特許 6463312 ケルセチン配糖体配合容器詰め飲料
3. 特許 6050862 集中力向上剤
4. 特開 2015-65943 色調安定性を有するケルセチン配糖体飲料
5. 特開 2012-183063 ケルセチン配糖体配合容器詰め飲料
6. 特開 2013-121332 アスパルテーム含有酸性飲料
7. 特開 2012-90526 生姜エキスの製造方法
8. 特許 5721352 ビタミン類含有飲料
9. 特許 5789089 集中力向上剤
10. 特許 5351032 甘味料の呈味が改善された飲食品
11. 特許 5498046 マンガン含有物及びその製造法
12. 特許 5496535 茶殻抽出物を用いた乳酸菌の培養方法

13. 特再公表 2008-90631 フコイダン由来オリゴ糖
14. 特許 5676078 風味増強剤及び香料組成物
15. 特許 5175167 高甘味度甘味料を含有する炭酸飲料
16. 特再公表 2007-13613 フコイダン又はフコイダン加水分解生成物と免疫賦活素材とを含む組成物
17. 特許 4917025 ラクトバチルス・ペントーサスを含む、免疫調節作用を有する組成物
18. 特再公表 2006-93267 免疫調節作用を有する発酵組成物
19. 特開 2009-65877 重合カテキン入り容器詰飲料
20. 特許 4981593 精製茶抽出物の製造方法
21. 特許 5117148 アスパルテームを含有する酸性飲料
22. 特開 2009-65872 重合カテキン含有飲料
23. 特開 2009-65871 茶抽出物及びその製造方法
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26. 特許 451515 免疫調節作用を有する乳酸菌
27. WO10-074258 風味増強剤及び香料組成物
28. WO09-035048 アスパルテームを含有する酸性飲料
29. WO07-013609 フコイダン由来オリゴ糖