2.3 Assessment of $CD4^+$ cells by flow cytometry

Approximately 1×10^6 cells were stained for 20 minutes with FITC-labeled anti-CD4 antibody and acquisition of data was performed on a LSR flow cytometer (BD Biosciences, San Jose, CA USA).

2.4 Cell culture for cytokine production

LP cells (5×10^5/ml), SP or PP cells (2.5×10^6/ml) were cultured in 200 μl of medium in 96-well culture plates containing 0.5 mg/ml of OVA. Supernatants were collected after 48 hours. Mitomycin C-treated BALB/c spleen cells (2×10^6/ml) were added as antigen presenting cells in LP cell cultures.

2.5 ELISA for cytokines

Cytokines in culture supernatants were measured by sandwich ELISA. Rat anti-mouse interleukin (IL)-4, IL-5, IL-6 and interferon (IFN)-γ monoclonal antibodies were used as the capture antibody, with biotinylated rat anti-mouse IL-4, IL-5, IL-6 and IFN-γ monoclonal antibodies, respectively, as the detection antibodies.

3. Results and Discussion

3.1 The number of PPs in GF TCR-Tg mice

GF TCR-Tg mice were obtained from conventional TCR-Tg mice by hysterectomy carried out under germ free conditions. The numbers of PPs from these GF TCR-Tg mice and CV TCR-Tg mice were counted and found to be significantly decreased in GF TCR-Tg mice compared with CV TCR-Tg mice (Fig. 1)

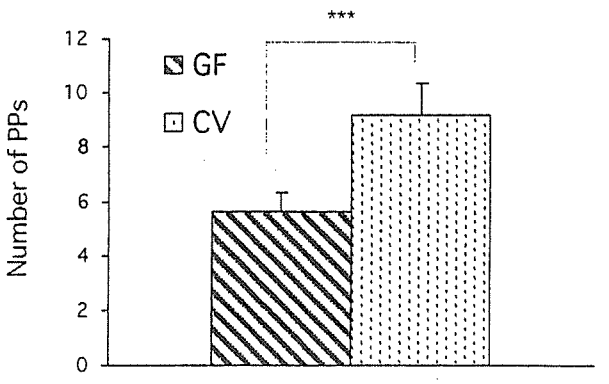

Fig. 1 The number of PPs in GF or CV TCR-Tg mice. The results are shown as mean ± SD of at least 4 experiments. ***, $p<0.001$.

3.2 Percentages of $CD4^+$ T cells in the lymphocyte fraction of SP, PP and LP of GF TCR-Tg mice

The percentages of $CD4^+$ T cells against the whole lymphocyte fraction of SP, PP and LP cells are shown in Fig. 2. The ratios of $CD4^+$ T cells were decreased in PP and LP of GF TCR-Tg mice compared with CV TCR-Tg mice. On the other hand, the ratios of $CD4^+$ T cells in SP were similar in GF and CV mice. The above results demonstrate that the GALT is poorly developed in GF TCR-Tg mice, as reported previously in other studies using germfree animals (1).

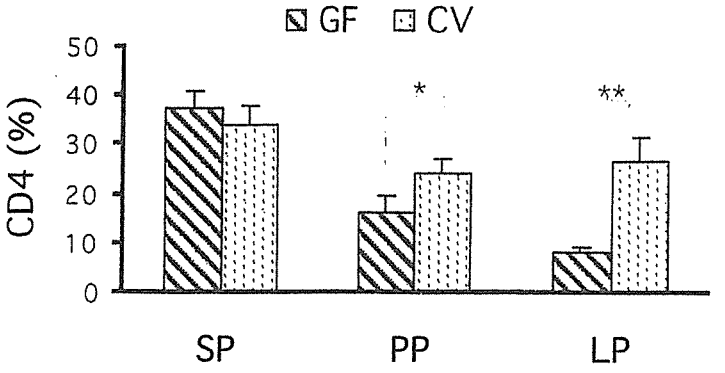

Fig. 2 Percentages of $CD4^+$ T cells in lymphocyte fraction of SP, PP and LP of GF TCR-Tg mice. The results were assessed by flow cytometry and shown as mean ± SD of 3 experiments. *, $p<0.05$; **, $p<0.005$.

3.3 Cytokine production of PP and LP cells of GF TCR-Tg mice

PP and LP cells from CV and GF mice were cultured with OVA and cytokine secretion was examined by ELISA. The results are summarized in Table 1. IL-6 and IFN-γ producing ability of PP and LP cells, and IL-5 of PP cells from GF mice, tended to be higher than CV mice. In particular, LP cells from GF mice secreted a large quantity of IL-6. These results showed that although the GALT is poorly developted in germ free conditions, intestinal T cells developing under these conditions possessed an enhanced ability to secrete cytokines in response to antigenic stimulation compared with those under conventional conditions. Therefore, continuous microbial stimulation may be involved in reducing the reactivity of intestinal T cells.

3.4 Conclusion

TCR-Tg mice are informative models for examining T cell-mediated immune responses to orally administrated protein antigens (5, 6). Our GF TCR-Tg system should be an additionally instructive resource for evaluating the effects of intestinal microflora on antigen-specific T cell responses to food proteins.

Table 1 Cytokine production of PP and LP cells in GF and CV TCR-Tg mice

		GF	CV
PP	IL-4	±	−
	IL-5	++	+
	IL-6	++	+
	IFN-γ	++	+
LP	IL-4	±	±
	IL-5	+	++
	IL-6	+++	+
	IFN- γ	++	+

Cytokines secreted in the culture supernatants were measured by ELISA. +++, ++, +, ±, − was judged based on the results of more than 3 experiments.

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Review

Modulation of Immune Functions by Foods

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Evidence is rapidly accumulating as to the beneficial effects of foods. However, it is not always clear whether the information is based on data evaluated impartially in a scientific fashion. Human research into whether foods modulate immune functions in either intervention studies or randomized controlled trials can be classified into three categories according to the physical state of subjects enrolled for investigation: (i) studies examining the effect of foods in healthy individuals; (ii) studies analyzing the effect of foods on patients with hypersensitivity; and (iii) studies checking the effect of foods on immunocompromized subjects, including patients who had undergone surgical resection of cancer and newborns. The systematization of reported studies has made it reasonable to conclude that foods are able to modulate immune functions manifesting as either innate immunity (phagocytic activity, NK cell activity) or acquired immunity (T cell response, antibody production). Moreover, improvement of immune functions by foods can normalize the physical state of allergic patients or cancer patients, and may reduce the risk of diseases in healthy individuals. Therefore, it is valuable to assess the immune-modulating abilities of foods by measuring at least one parameter of either innate or acquired immunity.

Keywords: amino acid – fatty acid – lactic acid bacteria – mineral – oligosaccharide – polyphenol – vitamin

Introduction

Foods contain various substances that can control the physiological functions of the body, and modulating immune responses is one of the most important functions of foods. Immune functions are indispensable for defending the body against attack by pathogens or cancer cells, and thus play a pivotal role in the maintenance of health. However, the immune functions are disturbed by malnutrition, aging, physical and mental stress or undesirable lifestyle. Therefore, the ingestion of foods with immune-modulating activities is considered an efficient way to prevent immune functions from declining and reduce the risk of infection or cancer.

In order to establish a diet capable of preserving immune functions, it is necessary to search and systematize reliable results on the immune-modulating effects of food-derived substances. To this end, we have selected reports that evaluated the immune-modulating abilities of foods in an intervention study or a randomized controlled trial. Thereafter, we classified these studies according to the physical state of their subjects into three categories: (i) studies examining immune parameters of healthy individuals whose immune functions are poorer than expected; (ii) studies analyzing immune parameters of patients with hypersensitivity; and (iii) studies checking immune parameters of immunocompromized subjects, including patients who had undergone surgical resection of cancer and newborns. We found that the measurement of at least one parameter representing either innate or acquired immunity was useful for evaluating the immune-modulating abilities of foods. This review summarizes the immune-modulating characteristics of foods that have been verified in human as well as animal studies. In addition, we briefly describe the pathways by which food-derived substances are absorbed into the body and the mechanisms through which food-derived substances exert their immune-modulating effects.

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Intestinal Transport of Foods

Molecules found in foods can be absorbed by multiple routes. Carbohydrates, proteins and lipids are broken down into monosaccharides, amino acids and fatty acids, respectively, by diverse hydrolases secreted in the gut lumen or associated with membranes of intestinal epithelial cells (IECs). These food components are actively transported via specific carrier molecules on the surface membrane of IECs and used as nutrients (Fig. 1). Vitamins and minerals in foods are also absorbed through IECs by passive diffusion or active transport using specific carrier molecules (Fig. 1). The main role of vitamins and minerals is to regulate the various physiological functions of cells.

Indigestible macromolecules such as rigid proteins are incorporated into Peyer's patches (PPs) developing throughout the intestine (1). PPs are organized lymphoid tissues that are covered by a particular epithelial layer, the follicle-associated envelope (FAE), and composed of both follicles rich in B cells and an interfollicular area filled with antigen-presenting cells and T cells. Horseradish peroxidase (HRP) given orally to mice was detectable in M cells (microfold cells) within the FAE (2). Moreover, a study using isolated intestinal loops from piglets has revealed that the absorptive rate of HRP was higher in the intestinal segments with PPs than without (3). These results demonstrate that M cells in FAE are the route for efficiently incorporating indigestible proteins (Fig. 1). Macromolecules incorporated into PPs are taken by antigen-presenting cells and induce antigen-specific immune responses. On the other hand, macromolecules with higher molecular size such as carrageenan (88 110 kDa) are hardly absorbed in the intestine.

Small molecules can be transported through IECs by endocytosis (Fig. 1). By contrast, food-derived substances are not usually transported between IECs (paracellular transport), because IECs closely connected by tight junctions do not usually allow food-derived substances to pass through. However, the barrier function of tight junctions is not stiff and breast milk-derived proteins can be transported without degradation between IECs in newborns (4) (Fig. 1). As the immune system in newborns is immature, it is reasonable that newborns incorporate breast milk-derived proteins including lactoferrin and maternal IgG to protect from infection. In contrast, dysfunction of the tight junction due to genetic defect is dangerous. Patients with food allergy have intestines with a

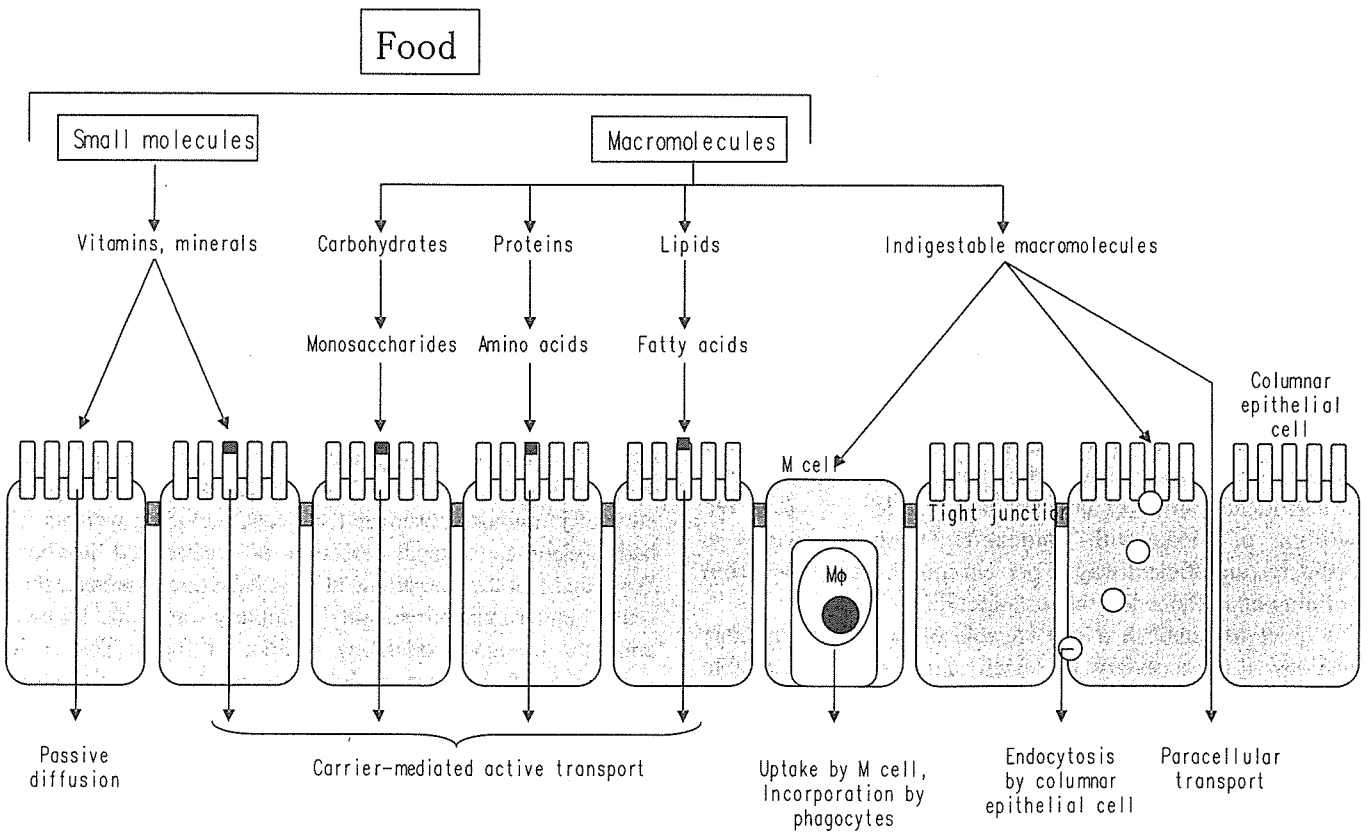

Figure 1. Mechanisms for transport of food-derived substances. Carbohydrates, proteins and lipids are digested by multiple hydrolases secreted into the gut lumen and associated with membranes of columnar epithelial cells. Specific carrier molecules transport monosaccharides, amino acids and fatty acids, and vitamins and minerals are incorporated by passive diffusion or transported by carrier proteins. In contrast, indigestable macromolecules are incorporated by M cells present in the follicle-associated envelope of Peyer's patches or through endocytosis by columnar epithelial cells. The incorporation of foods by diffusion through intercellular spaces between columnar epithelial cells does not occur except for in newborns or in subjects with a genetic deficiency in a barrier function.

reduced barrier function, and therefore an antigenic load in the gut lumen can be easily incorporated into the body (5).

The intestine and liver are important organs in terms of supply of nutrition and self-defense, and these organs are equipped with specialized immune systems. A huge number of IgA-producing cells and intraepithelial T lymphocytes (IELs) with unique physiological functions are colonized in the gut and play a pivotal role in defense against pathogens (6). Moreover, natural killer (NK) T cells are enriched in liver and highly effective in the eradication of tumor cells (7). Hazardous substances (pathogens, toxins and allergens) are recognized as antigens and activate the immune system, but most gut antigenic loads (food-derived molecules and indigenous intestinal bacteria) are harmless and the immune response to these antigens is suppressed in healthy humans (oral tolerance). In contrast, the immune system of patients with inflammatory bowel disease responds excessively to the indigenous intestinal bacteria, causing inflammation in the intestine (8).

It is of great interest that some substances in foods can open tight junctions between IECs. When the capsianoside contained in a sweet pepper was added to the apical side of a monolayer formed by the human IEC line Caco-2, the tight junctions transiently opened, followed by a drop of electric resistance between the apical and basal sides (9). While the tight junction basically acts as a barrier to pathogens or toxic substances in the intestine, the transient opening of tight junctions may be so important that antigens can be captured by dendritic cells in the intestinal lamina propria and immune responses to these antigens are efficiently evoked (10).

Regulation of Immune Functions by Foods

Immune-modulating abilities of foods have been investigated in a number of human studies. We tentatively classified these researches into three categories according to the state of immune system in subjects enrolled for investigation: (i) healthy individuals; (ii) patients with hypersensitivity; and (iii) subjects in immunocompromised state.

Foods Capable of Improving Immune Functions in Healthy Individuals

Immune functions are not stable and usually fluctuate within fixed limits. In addition, various endogenous and exogenous factors can influence immune functions. Corticosteroids suppress a broad range of immune functions efficiently and exhibit anti-inflammatory activity. Malnutrition, aging, stress and undesirable lifestyle are also factors lowering immune functions. The elderly exhibit higher susceptibility to infection than the young, and delayed type hypersensitivity (DTH), antigen-specific antibody production, the proliferative response of T cells and the relative proportion of T cells decline with aging (11–16). Many kinds of physical and mental stress also disturb immune functions. For instance, a surgical operation exhausts patients and is accompanied by a decline in their DTH, and caregivers of dementia patients show a decrease in NK cell activity, antigen-specific antibody production and T cell proliferation on account of depression (17,18). Moreover, NK cell activity deteriorates under not only mental stress after divorce but also physical stress of heavy exercise (19–24). It is widely known that systemic malnutrition associated with a deficiency of protein and energy causes a decline in immune functions and results in susceptibility to infection (25–28). A deficiency in vitamins and minerals induces an attenuation of immune functions including phagocytic activity, NK cell activity, DTH, antigen-specific antibody production, and the proliferative response of T cells (29,30). In addition, NK cell activity and the proliferative response of T cells decline in patients with chronic fatigue syndrome (31).

The deterioration of immune functions possibly causes loss of health. A higher risk of infection is closely linked with low NK cell activity, and increased risk of mortality in the elderly after pathogenic infection is correlated with a decline in DTH (32–35). Bodily dysfunctions in chronic fatigue syndrome patients are negatively correlated with the proliferative response of T cells (36).

These findings clearly demonstrate that immune functions in healthy individuals tend to be disturbed by various factors, and deterioration of health is closely connected with dysregulation of immune functions. On the other hand, it has been proposed that food-derived components can improve the immune functions in healthy individuals. Vitamins, minerals, and fatty acids enhance DTH (37–40), vitamins and minerals enforce antigen-specific antibody production (41–45) and vitamins, minerals and oligosaccharides increase T cells and augment their proliferative response (30,37,43,46–51). In addition, vitamins, minerals and lactic acid bacteria promote phagocytic activity and NK cell activity (30,43,52–61) (Fig. 2). The ingestion of these foods not only normalizes immune functions but also reduces the incidence of pathogenic infection (30,41,43,62–64).

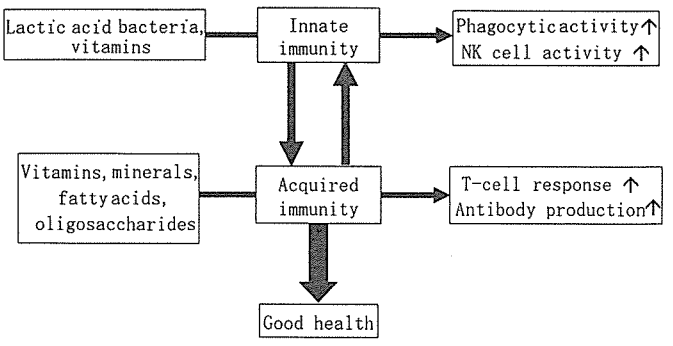

Figure 2. Modulation of immune functions by foods. The immune system is divided into innate immunity and acquired immunity, and food-derived substances can modulate either innate or acquired immunity. For example, probiotics such as lactic acid bacteria and some vitamins enhance phagocytic activity and natural killer (NK) cell activity (innate immunity), while vitamins, minerals, amino acids, fatty acids and oligosaccharides augment T cell responses and antibody production (acquired immunity). A balance of innate and acquired immunity is desirable for good health.

Table 1. Parameters available for evaluating the immune-modulating effects of foods in humans

Subjects	Beneficial changes to parameters induced by ingesting foods
Healthy individuals	Delayed type hypersensitivity ↑ (37–40), Antigen-specific antibody antibody production ↑ (41–45), Mitogen- or antigen-induced T cell proliferation and T cell number ↑ (46–51), NK cell activity and phagocytic activity ↑ (52–61)
Patients with hypersensitivity	Soluble CD4 level in serum ↓ (67), TNF-α level in feces ↓ (79), Inducible surface CD23 level ↓ (78), soluble IL-2R level in serum ↓ (67,78), Soluble VCAM level in serum ↓ (78), ECP level in feces or serum ↓ (67,86), IFN-γ production ↑ (81), TGF-β level ↓ (67), eosinophil number ↓ (81)
Subjects in immunocompromised state	Phagocytic activity ↑ (133), NK cell number ↑ (134,137), T cell number and IFN-γ level in serum ↑ (135), Delayed type hypersensitivity ↑ (133,135), NK cell activity ↑ (139), antigen-specific antibody production ↑ (140), T cell number and IgG level in serum ↑ (141)

Summarizing the results reported so far, it is reasonable to conclude that the effect of foods on immune functions can be evaluated in healthy subjects by measuring either parameters concerning innate immunity (phagocytic activity and NK cell activity) or parameters concerning acquired immunity (DTH, antigen-specific antibody production, the proliferative response of T cells and T cell number) (Table 1). Therefore, despite fears that immune functions may decline due to malnutrition, aging, stress or undesirable lifestyle, one can remain healthy and reduce the risk of infection or cancer by eating foods capable of enhancing phagocytic activity, NK cell activity, DTH, antigen-specific antibody production, the proliferative response of T cells and/or T cell numbers.

Foods Capable of Improving Clinical Symptoms in Patients With Hypersensitivity

Immune reactions are usually evoked in response to externally derived hazardous antigens. However, in patients with hypersensitivity represented by immediate type allergy, immune reaction to non-toxic antigens and sometimes to the body's own molecules is induced. The causes of hypersensitivity are mainly genetic, but environmental factors, including air pollution, dietary components and residential conditions, also play an important role. As clinical condition and immune parameters change concomitantly in allergic patients, it is possible to observe the effects of foods by measuring the immune parameters associated with allergic reactions.

Generation of pro-inflammatory cytokines and chemokines and expression of cell adhesion molecules are involved in the progression of allergic diseases including atopic dermatitis, pollinosis and allergic rhinitis. Levels of pro-inflammatory cytokines and chemokines increase and the expression of cell adhesion molecules is enhanced in allergic patients (65–70). Furthermore, eosinophils as well as mast cells secrete chemical mediators and worsen the clinical symptoms in the inflammatory areas (71,72). In order to establish an objective assessment of the clinical state of allergic patients, a skin test, the antigen-induced response and the SCORAD score have all been utilized (73,74).

When the immune parameters representing clinical symptoms characteristic of atopic dermatitis, pollinosis and allergic rhinitis normalize, the patients recover from allergic diseases (67,75). Therefore, normalization of these immune parameters by foods is helpful in that allergic patients recover their health and persons with a predisposition to allergies may avoid falling ill. Parietaria extract (76,77), herbal extract (78) and lactic acid bacteria (67,79–89) have been found to suppress allergic diseases in human subjects as well as animal models.

Based on findings reported to date, we conclude that the following immune parameters can be used to evaluate the effects of foods on the clinical symptoms of allergic patients: (i) parameters to directly assess clinical symptoms in allergic patients: skin test (75,82), skin-induced response (76), SCORAD score (74,78,90); (ii) parameters that vary in association with the clinical symptoms of allergic patients: TNF-α level (65,66,79,91), soluble CD4 level (67), soluble CD23 level or inducible surface CD23 level (68,78,92), soluble IL-2R level (67,68,93,94), soluble VCAM level (70,78), amount of granular protein in eosinophils (ECP, EPX) (67,86,95); (iii) parameters possibly involved in the clinical symptoms of allergic patients: IgG_4 level (75), IL-4/IFN-γ production (81,96), TGF-β level (67,97,98), eosinophil number (81,99) (Table 1).

An allergic reaction is a sequential immune response involving the processing and presentation of the allergen, activation of allergen-specific T and B cells, production of IgE against the allergen, and activation of mast cells and eosinophils triggered by the allergen. Therefore, food-derived materials could prevent allergy by counteracting at least one step in the cascade of allergic reactions. It has been reported that a variety of foods contain substances able to prevent an allergic reaction (100–102).

Foods Capable of Improving Immune Functions in Subjects in an Immunocompromised State

Cancer patients are usually immunosuppressed and at high risk of infection due to a reduction of immune functions. Therefore, foods capable of enhancing the immune responses of cancer patients with disturbed immune functions are valuable.

Invading pathogenic bacteria or viruses are captured and killed by phagocytes such as neutrophils and macrophages, and NK cells recognize and lyse infected cells. Activated NK cells and T cells produce huge amounts of IFN-γ, which further augments the anti-bacterial activity of macrophages (103–106).

Pathogens that have escaped capture by phagocytes or NK cells are incorporated and processed by professional antigen-presenting cells, which stimulate T cell clones expressing

antigen receptors specific for pathogens. Activated antigen-specific T cells secrete various arrays of cytokines necessary for antibody production, and pathogen-specific antibodies play an important role in the exclusion of pathogens invading the airway, intestine and urinary tract (107–109). IgA secreted in the intestinal mucosa can neutralize toxins produced by pathogens and prevents diarrhea (110), and IgG circulating in sera is principally for defense against infection in the upper respiratory tract (107).

The incidence of infection increases and the aggravation of infectious diseases occurs when innate and acquired immune functions decline or are insufficient. Patients with undetectable levels of NK cell activity suffer frequent viral infections and the transfer of NK cells into suckling mice can render the recipient mice resistant to infection for murine cytomegalovirus (111,112). Patients with Gaucher disease, who were highly susceptible to serious bacterial infections, had macrophages with impaired anti-bacterial activity and the rate of infection among marrow transplant recipients 100–365 days after transplantation was negatively correlated with the total number of B cells and monocytes (113,114). On the other hand, several reports have shown that the improvement of depressed immune functions by ingesting foods reduced infection rates and mitigated the severity of infectious disease (43,115–118). When assessing the anti-infectious capabilities of foods, phagocytic activity, NK cell activity, T cell number, production of antigen-specific antibodies and total IgG level can be regarded as useful parameters.

NK cells exhibit cytotoxic activity against not only infected cells but also cancer cells (119–121). IFN-γ produced by activated NK cells suppresses the proliferation of cancer cells and activates cytotoxic T cells and macrophages (122,123). While NK cells kill cancer cells in an antigen non-specific manner, cytotoxic T cells recognize specific antigens of cancer cells for killing. Moreover, macrophages secrete molecules toxic to cancer cells and induce the apoptosis of cancer cells (124–126).

The proliferation and metastasis of cancer cells accelerate when immune functions are disturbed. It has been found that cancer patients have lower NK cell activity than healthy controls and persons with lower NK cell activity are subject to higher rates of cancer incidence, metastasis and aggravation of cancer (127–131). The macrophages infiltrating solid tumor have less phagocytic activity (132). On the other hand, when cancer patients ingest foods capable of improving immune functions, the prognosis becomes much better (133–137). Based on the reports of clinical trials with cancer patients, phagocytic activity, NK cell number, T cell number, DTH and IFN-γ production are all useful immune parameters for assessing the effect of foods on prognosis after surgical operation for cancer (Table 1). Moreover, it has been reported that NK cell activity deteriorates in AIDS patients (138), and branched chain amino acids, probiotics and vitamin A improves virus-triggered diseases (139–141).

Newborns exhibit immature immune functions and are vulnerable to pathogenic infection. Supplementation of vitamins in malnourished children and ingestion of probiotics in newborns enhance immune functions and prevent viral infection (118,142).

Mechanisms by which Foods Influence Immune Functions

Food-derived substances incorporated into the body via various routes modulate immune functions. Taking into consideration that malnutrition or calorie restriction cause reduced activity in immune functions, nutritional condition is indispensable for the development of the immune system (143). Moreover, food-derived substances exhibit a special role in influencing immune functions.

The way that food-derived substances modulate immune functions is either indirect or direct. Comparative analyses of conventional and germ-free animals revealed that indigenous intestinal microflora play a pivotal role in the development of host immune systems. Ingestion of probiotics stabilizes the intestinal microflora, and normalization of the intestinal microflora by probiotics could lead to modulation of the host immune system (144). In addition, probiotics such as lactic acid bacteria are recognized by specific receptors on the surface of phagocytic cells. Additionally, vitamins, minerals or fatty acids affect cellular functions by preserving the cell membrane or regulating gene expression after being incorporated into lymphocytes (Fig. 3). One group of foods represented by lactic acid bacteria stimulates innate immunity (phagocytic activity, NK cell activity), while other foods, including vitamins and minerals, activate acquired immunity (T cell response, antibody production). However, as innate immunity and acquired immunity are closely linked, both groups of foods may regulate both immune systems. It has been reported that various nutrients found in foods exhibit anti-infectious functions (145). We would like to briefly

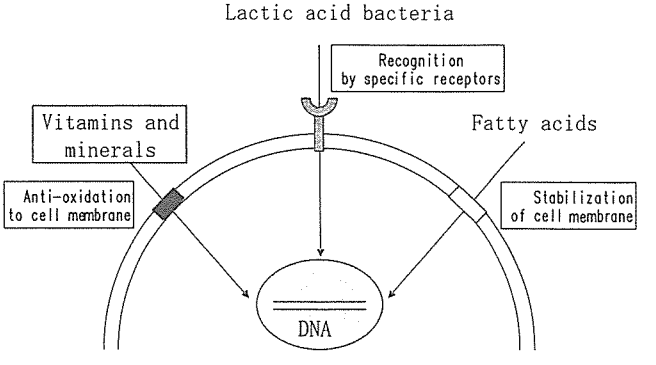

Figure 3. Mechanisms by which food-derived substances modulate the immune functions of cells. Components of probiotics are recognized by cell surface receptors such as the mannose receptor and Toll-like receptors (TLRs). It has been clarified that agonists of TLRs transmit signals to DNA by activating intracellular adaptor molecules such as MyD88. In contrast, vitamins and minerals prevent the oxidation of cell membranes and affect gene expression. Furthermore, fatty acids modulate immune functions by stabilizing the cell membrane and regulate the transcription of genes.

describe the pathways by which food-derived substances exert their immune-modulating abilities.

Probiotics ingested may be partially digested in the gut and incorporated into M cells present in FAE, and then captured by dendritic cells or macrophages in the interfollicular area of PPs. These professional phagocytic cells hold various receptors on their surface capable of binding common structures of microbes, the pathogen-associated molecular patterns (PAMPs). Among the receptors for PAMPs, molecular structure and functions of TLRs (Toll-like receptors) have been recently unveiled. Ten TLR families (TLR1–TLR10) have been identified and ligands recognized by some TLRs have been determined. TLR2 recognizes peptidoglycans and lipopeptides as TLR4 does lipoteichoic acids and lipopolysaccharides. Moreover, the CpG oligonucleotides universally detected in bacterial DNA are recognized by TLR9. The signaling response to stimuli recognized by TLRs is mainly mediated by an intracellular adaptor molecule, MyD88 (myeloid differentiation factor 88). Thereafter, the nuclear transport of NF-κB (nuclear factor-κB) is stimulated and *de novo* synthesis of cytokines is induced (146). It has been proposed that stimuli through TLR2 activate both JNK (c-Jun N-terminal kinase) and ERK (extracellular signal regulated kinase) and induce production of IL-10, while stimuli through TLR4 activate JNK and induce production of IL-12 (147).

Immune-modulating effects of amino acids such as glutamine and arginine have been evaluated. Ingestion of glutamine improved nitrogen retention and lowered incidence of bacteremia in patients with trauma, and enteral supplementation of glutamine-enriched diet enhanced the recovery of immune functions and reduced the length of hospital stay after surgical operation in cancer patients (148,149). Glutamine is a nutrient for immune cells and acts as precursor for glutathione, which circumvents oxidant stress and improves cell-mediated immunity. Arginine is a substrate for synthesis of nitric oxide and improves helper T-cell numbers. Peri-operative feeding of arginine and n-3 polyunsaturated fatty acids (PUFAs) restored DTH and decreased infection rates in colorectal cancer patients (150).

Nucleotides are rich in foods containing nucleic acid/nucleoprotein and supplementation of nucleotides is important for growth of infants. Addition of nucleotides increased the proportion of TCRγδ-bearing IELs through stimulating IL-7 production by IECs in mice (151), and ingestion of formula supplemented with nucleotides augmented NK cell activity and IL-2 production in human infants (152). It remains to be elucidated whether immune cells may utilize ingested nucleotides as substrates for synthesis of nucleic acids.

Vitamins and minerals exhibit important immune-modulating functions by entering cells and regulating gene expression. Vitamin A affects the differentiation of epithelial cells and inhibits IFN-γ production by T cells at the transcriptional level, which results in stimulation of antibody-mediated immune responses (153). Vitamin C prevents the production of reactive oxygen intermediates and reduces DNA damage in immune cells. Moreover, vitamin C inhibits the transcription of NF-κB, and down-regulates the production of pro-inflammatory cytokines (154). Vitamin E is also an anti-oxidant and exerts an anti-inflammatory effect. Vitamin E stabilizes the membrane of immune cells and enhances the binding of antigen-presenting cells and T cells (155).

Table 2. Major food-derived substances that modulate immune functions

Nutrients/nutricines	Immune-modulating functions
Nutrients/calorie	Indispensable for normal development of immune system
Amino acids	
Glutamine	Trophic for immune cells, circumvention of oxidant stress
Arginine	Substrate for synthesis of nitric oxide, enhancement of Th cells
Fatty acids	
n-3 PUFAs	Anti-inflammatory
Vitamins	
Vitamin A	Regulation of Th1/Th2 balance
Vitamin C	Circumvention of oxidant stress
Vitamin E	Circumvention of oxidant stress, anti-inflammatory
Minerals	
Selenium	Stimulation of cell-mediated immune response
Zinc	Stimulation of cell-mediated immune response
Nucleotides	Stimulation of cell-mediated immune response
Probiotics	
Peptidoglycan, lipoteichoic acids	Stimulation of IL-12/IL-10 production
CpG oligonucleotides	Anti-inflammatory

Minerals prevent the oxidation of lipids in the cell membrane, which can reduce oxidative stress affecting immune cells. For instance, selenium is indispensable to the function of reducing enzymes such as glutathione peroxidase and thioredoxin reductase, and is needed to stimulate cell-mediated immune functions (156). Furthermore, zinc may be required for the translocation and binding of NF-κB to DNA (157).

Long-chain PUFAs in foods can modulate immune functions. Dietary n-3 PUFAs alter the lipid composition of the cell membrane and regulate the function of immune cells. Antigen-presenting cells from mice and humans fed n-3 PUFAs exhibited the capacity to suppress excessive activation of T cells (158,159). As a result, n-3 PUFAs can act as anti-inflammatory agents.

Major food-derived substances and their immune-modulating functions are summarized in Table 2.

CONCLUDING REMARKS

We have reviewed and systematized studies reporting the effects of food-derived materials on immune functions in intervention studies or randomized controlled trials in order to clarify whether the immune-modulating activities of foods have been evaluated in a scientific manner. This search has revealed the following points: (i) many foods or food-derived materials improve or enhance immune functions in a wide range of human subjects; and (ii) foods with immune-modulating activities affect either innate or acquired immunity. Phagocytic activity

and NK cell activity are representative parameters of innate immunity, and phagocytes and NK cells rapidly kill pathogenic bacteria, viruses and cancer cells in an antigen-independent manner. In contrast, DTH, antigen-specific antibody production and the proliferative response of T cells are major parameters reflecting acquired immunity, which is responsible for the antigen-specific exclusion of pathogenic bacteria, viruses and cancer cells. Many kinds of foods can improve parameters exhibiting either innate or acquired immunity.

Ingestion of foods does not always change many immune parameters. Therefore, it is useful to define immune parameters affected by foods. Vitamins, minerals, amino acids, proteins, carbohydrates or lipids, for example, enhance parameters of acquired immunity. In contrast, probiotics, including lactic acid bacteria, mainly augment parameters of innate immunity. These findings support that food-derived materials act on different immune cells or distinct molecules of the cells and improve at least one parameter of either innate or acquired immunity. In other words, these results mean that one can evaluate the immune-modulating abilities of foods by analyzing parameters of either innate or acquired immunity.

The components in foods that improve immune functions and the mechanisms by which foods exert immune-modulating effects are still far from fully understood. To confirm the scientific basis of the immune-modulating activities of foods, there is a need to keep on systematizing newly obtained scientific data on foods.

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特集II 粘膜免疫をめぐる新たな進歩

特異抗原経口投与時における小腸上皮内リンパ球の遺伝子発現変化*

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Key Words：intestinal intraepithelial lymphocyte, interleukin-10, ovalbumin, TCR transgenic mouse

はじめに

腸管粘膜は常在細菌や食物由来抗原に常に曝されている．これら管腔内抗原との相互作用の最前線にある腸管上皮細胞の間に存在するT細胞が，腸管上皮内リンパ球(intestinal intraepithelial lymphocyte；IEL)である[1)2)]．IELはその数においてIEL以外の通常のリンパ組織に存在するT細胞に匹敵するが，これらとは性状が異なる独自の細胞群である．IELはT細胞レセプター(TCRαβ, TCRγδ)および補助レセプター分子(CD4, CD8αα, CD8αβ)の発現により異なるサブセットに分類され，それぞれ異なる生理機能を果たすものと考えられる．IELの生理機能としては，上皮層の恒常性維持や感染防御，免疫応答の制御などが報告されているが，いまだ十分には明らかにされていない．

CD8αα$^+$ TCRαβ$^+$IEL(αβ-IEL)とTCRγδ$^+$IEL(γδ-IEL)については，胸腺外分化IELであり，粘膜固有層に存在するクリプトパッチに由来するものであることが報告された[3)4)]が，CD8αα$^+$ αβ-IELは胸腺のCD4$^+$CD8$^+$細胞に由来するという説[5)6)]もあり，いまだ結論は得られていない．これらのIELは自己抗原や非古典的MHCクラスIbなどを認識するものと考えられるが，その詳細は不明である．

一方，通常の末梢リンパ組織にも存在するCD4$^+$あるいはCD8αβ$^+$の表現型をもつαβ-IELとCD4$^+$ CD8αα$^+$ αβ-IEL[7)]は，おそらく末梢で活性化されたT細胞が上皮内にホーミングしてIELとなったものであり，管腔内の抗原に対する特異的な反応を担っているものと考えられる．しかしながら，その生理機能，とくに経口摂取された食物由来抗原に対するIELの応答や生理的意義についてはいまだ不明な点が多い．

われわれは，IELが食物抗原の認識にかかわる可能性およびそこでIELが果たす役割について検討するため，MHCクラスII分子拘束的に卵白アルブミン(OVA)を認識するTCRトランスジェニックマウスを用い，OVAを経口的に摂取させた場合にIELに生じる変化を，とくに遺伝子発現変化に注目して解析した．

特異抗原の経口摂取によりIELに生じる変化

OVA-TCRトランスジェニックマウス(DO11.10マウス)にOVAを含む食餌(卵白食)あるいは，カゼインを含む食餌(カゼイン食：対照食)を自由摂取させ，特異抗原の経口摂取によりIELに生じる変化について検討を行った．フローサイトメ

* Altered gene expression in intestinal intraepithelial lymphocytes derived from mice fed a specific antigen.

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トリーによりIELサブセット構成を解析した結果，3日間の卵白食摂取によって$CD4^+CD8\alpha\alpha^-\alpha\beta$-IELが増加することが示された．

また，3日間の卵白食摂取により，DO11.10マウスTCRのクロノタイプ抗体である$KJ1.26^+$のIELにおいてCD69の発現上昇，CD45RBの発現低下，$CD62L^+$細胞の増加が明らかとなった．CD69の発現上昇は$CD4^+$IELおよび$CD8\alpha^+$IELで，$CD62L^+$細胞の増加は$CD8\alpha^+$IELで認められた．一般に末梢T細胞においてCD69は初期活性化マーカーであり，一方CD62Lは活性化に伴い発現低下することが知られている分子であり，この結果が意味するところを明らかにするには，さらに詳細な解析が必要であろう．しかしながら，これらの結果から，特異抗原を経口摂取することにより，IELにおいて少なくともなんらかの変化が生じることが明らかとなった．この結果の解釈にあたっては，①IELが抗原提示を受け活性化した，あるいは②ほかのリンパ球が抗原を認識しIELに作用を及ぼした，という両者が考えられる．

$CD4^+$ IELが管腔内抗原に対して反応することに関しては，これまでにも報告がある[8)9)]．一方，食物抗原の摂取に対してIELが反応する例としては，セリアック病患者において，グルテンの経口摂取がIELを増加させることが知られている[10)]．またわれわれのグループは，DO11.10マウスのTCRと同じ抗原を認識するOVA-TCRトランスジェニック(OVA23-3マウス)を用い，同様にOVAを経口摂取させることにより，$CD4^+CD8\alpha\alpha^-\alpha\beta$-IELが増加し，*in vitro*抗原刺激に対する増殖応答性が高まることを報告している[11)]．これらの事実から，IELが食物抗原に対してもなんらかの反応を示すことは確かであると考えてよいであろう．

特異抗原の経口摂取によるIELの遺伝子発現変化

次に特異抗原の経口摂取でIELに生じる変化を分子レベルで明らかにするため，DNAマイクロアレイ法により遺伝子発現変化を解析した．卵白食あるいは対照食を3日間自由摂取させた群から，それぞれIELを調製し，セルソーターによりTCRαβあるいはTCRγδを発現するIEL(whole IELとする)を精製した．GeneChip®システム(Affymetrix社)を用いて両群のwhole IELの遺伝子発現の違いを検討した結果，2回の実験でともに卵白食摂取によりIELにおいて有意に発現が上昇した遺伝子98個，発現が低下した遺伝子24個を同定した(表1)．発現が上昇した機能分類別の遺伝子数としては，半数近くが細胞周期・細胞増殖に関する遺伝子であり，次いで，免疫・炎症関連，細胞骨格関連，転写調節関連の遺伝子が多く認められた．2回の実験でともに3倍以上の発現増加が認められたものを表2に示した．また，CD4遺伝子の発現上昇が検出されており，これは$CD4^+$ $CD8\alpha\alpha^-$ $\alpha\beta$-IELが増加したことと符合する結果である．

表1　特異抗原の経口摂取によりwhole IELにおいて発現の変化した遺伝子の機能別分類

機能別分類	発現上昇（遺伝子数）	発現低下（遺伝子数）
細胞周期・細胞増殖	44	1
免疫関連	14	2
細胞骨格	12	0
転写因子・転写調節	8	1
代謝・ハウスキーピング	8	6
シグナル伝達	3	1
アポトーシス	3	0
そのほか・機能未知	2	6
ESTs	4	7
合計	98	24

DO11.10マウスに卵白食あるいはカゼイン食(対照食)を3日間自由摂取させた後，IELを調製し，$TCR\alpha\beta^+$あるいは$TCR\gamma\delta^+$IEL(whole IEL)をセルソーターにより精製した．whole IELからRNAを抽出し，Affymetrix社のGeneChip®システム(Murine Genome U74Av.2 Array)により遺伝子発現の解析を行った．2回の実験でともに，対照食群と比較して卵白食群で発現が上昇あるいは低下した遺伝子数を示した．

細胞周期・細胞増殖に関する遺伝子の発現増大が顕著に認められたことから，IEL中に食物抗原を認識して増殖した細胞が含まれていることが推測された．一般に，IELは*in vitro*で抗原刺激をした場合，その増殖性は低いことが知られている．IELの中で増殖関連遺伝子の発現が上昇する細胞は，おそらくOVAを認識する$KJ1.26^+$細胞であることが予想され，ここでも次の2つの可能性が考えられる．すなわち，①IELが上皮内で抗原を認識し増殖した，②パイエル板，腸間膜

表2　特異抗原の経口摂取によりwhole IELにおいて発現が上昇した遺伝子

Accession No.	遺伝子名	発現倍率（卵白食群/対照食群）	
		実験1	実験2
細胞周期・細胞増殖			
AF002823	budding uninhibited by benzimidazoles 1 homolog (Bub1)	16.0	26.0
AF016583	checkpoint kinase 1 homolog (Chek1)	11.3	3.5
AW213883	RIKEN cDNA A730011O11 gene (A730011OllRik)	9.9	4.9
X60980	thymidine kinase 1 (Tk1)	8.0	4.9
AW209238	transforming, acidic coiled-coil constaning protein 3 (Tacc3)	5.7	19.7
AB025409	CDC28 protein kinase 1 (Cks1)	4.9	26.0
M14223	ribonucleotide reductase M2 (Rrm2)	4.9	12.1
U01915	topoisomerase (DNA) II alpha (Top2a)	3.7	42.2
X66449	S100 calcium binding protein A6 (S100a6,calcyclin)	3.7	4.0
AJ223087	cell division cycle 6 homolog (Cdc6)	3.5	7.0
AI317217	cyclin-dependent kinase inhibitor 3 (Cdkn3)	3.5	9.8
X82786	antigen identified by monoclonal antibody Ki 67 (Mki67)	3.3	9.8
免疫関連			
M37897	interleukin 10 (IL-10)	9.2	22.6
X15986	lectin, galactose binding, soluble (Lgals1, galectin1)	3.3	8.0
細胞骨格			
AJ223293	kinesin family member 11 (Kif11)	4.9	13.0
AI591702	kinesin family member 23 (Kif23)	3.5	4.3
転写因子・転写調節			
M90397	B-cell leukemia/lymphoma 3 (Bcl3)	9.2	4.0
代謝・ハウスキーピング			
D55720	karyopherin (importinn) alpha 2 (Kpna2)	-3.3	3.7
シグナル伝達			
AI838080	stathmin 1 (Stmn1)	4.6	21.1
EST			
AI122538	RIKEN cDNA 2810417H13 gene (2810417H13Rik)	4.9	21.1

表1と同じ実験において，2回の実験でともに3倍以上，対照食群と比較して卵白食群で発現が上昇した遺伝子を示した．

リンパ節などで抗原を認識し増殖過程にあるT細胞が上皮内に移動した，という可能性である．

IELにおけるIL-10遺伝子発現およびIL-10産生の上昇

免疫関連遺伝子のうち，特異抗原の経口摂取でもっとも顕著な発現上昇が認められたのはIL-10遺伝子であった．定量的リアルタイムRT-PCR法により，IL-10遺伝子の発現を確認したところ，卵白食摂取期間が3日，7日，14日のいずれにおいても，whole IELにおいて，対照食群と比較してIL-10遺伝子の発現上昇が認められた．また，両群から分離したwhole IELを抗CD3抗体および抗CD28抗体を用いて*in vitro*で刺激した場合の培養上清中へのIL-10分泌を測定したところ，蛋白質レベルでも卵白食摂取群のIELでより強いIL-10発現が確認された(図1)．一方，ほかのサイトカイン遺伝子については，DNAマイクロアレイ解析からは卵白食摂取で増加するものは認められなかった．IL-4およびIFN-γについては，RT-PCRおよび*in vitro*抗原刺激によるサイトカイン産生試験でも検討したが，ともに両群で差が認められなかった(図1)．

IL-10遺伝子を発現するIELサブセット

次にwhole IELのうち，どの画分がIL-10遺伝子を発現しているのかを検討した．BALB/cマウスより調製したIELを$CD4^+$IELと$CD4^-$IELに分画し，IL-10遺伝子発現を調べたところ，$CD4^+$IELにおいて顕著な遺伝子発現が検出された(図2)．また，ほかのリンパ組織についても検討したところ，パイエル板$CD4^+$T細胞で発現が認められ

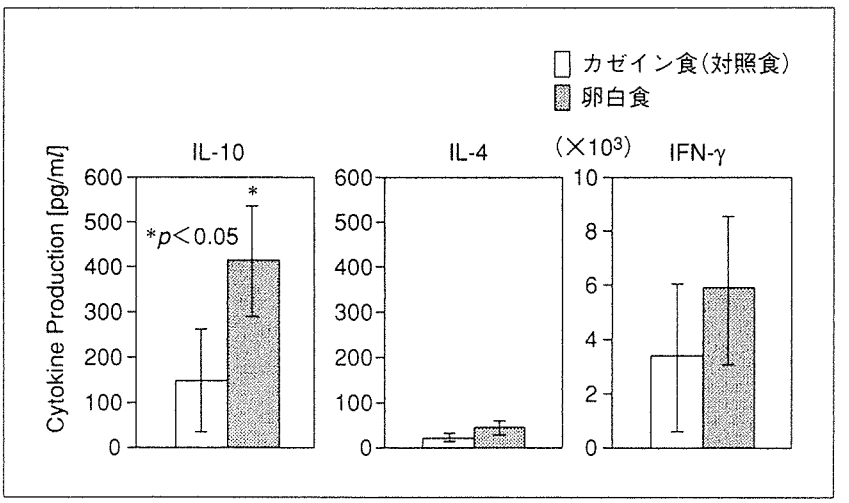

図1　特異抗原の経口摂取によるwhole IELのサイトカイン産生の変化
DO11.10マウスに卵白食あるいはカゼイン食(対照食)を3日間自由摂取させた後，IELを調製し，TCRαβ⁺あるいはTCRγδ⁺IELをセルソーターにより精製した．これを抗CD3抗体および抗CD28抗体で刺激した後，培養上清中のサイトカイン産生量をELISA法で測定した．

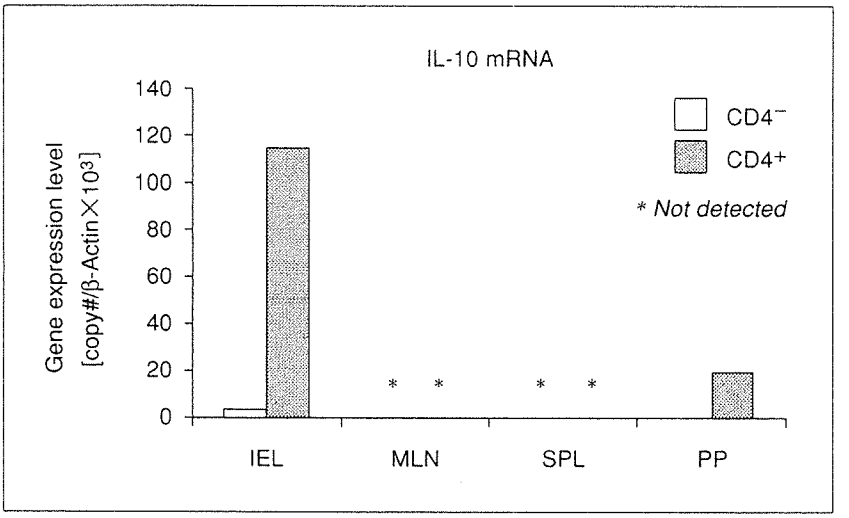

図2　IEL，腸間膜リンパ節(MLN)，脾臓(SPL)およびパイエル板(PP)由来CD4⁺あるいはCD4⁻T細胞におけるIL-10遺伝子発現の比較
BALB/cマウスの各リンパ組織よりT細胞を調製し，CD4⁺およびCD4⁻画分に分画した．各細胞よりRNAを抽出し，リアルタイムPCRを用いた定量的RT-PCR法によりIL-10遺伝子の発現を測定した．

たが，CD4⁺IELと比べるとその発現は低かった．腸間膜リンパ節，脾臓においては，CD4⁺T細胞およびCD4⁻T細胞ともにIL-10遺伝子の発現は認められなかった．この実験は，*ex vivo*で生体から取り出したままの細胞からRNAを抽出しIL-10 mRNAの存在量を解析したものであり，*in vitro*でのTCR刺激を加えていない状態の結果であることに注意が必要である．また，この結果は通常のマウスを用いて得られたものであり，IELに対して*in vivo*においても人為的な抗原刺激は行っていないことも重要なポイントである．したがって，CD4⁺IELは①管腔内抗原の刺激により，あるいは②上皮細胞間という環境下にあることにより，恒常的にIL-10遺伝子を発現していることが示された．どちらの影響によるものであるかについては今後の検討が必要である．

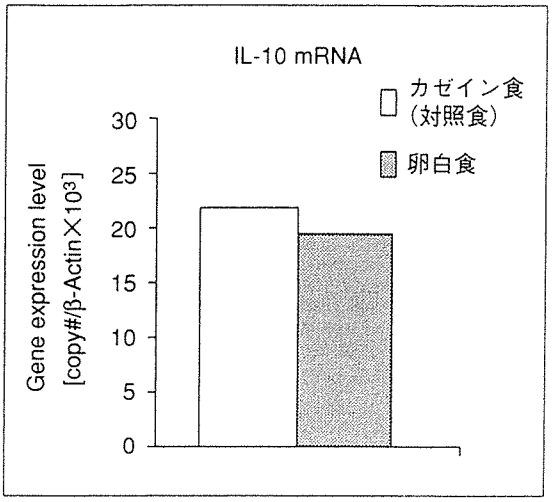

図3　卵白食あるいは対照食を摂取したDO11.10マウス由来のCD4⁺IELにおけるIL-10遺伝子の発現
DO11.10マウスに卵白食あるいはカゼイン食(対照食)を3日間自由摂取させた後，IELを調製し，CD4⁺IELをセルソーターにより精製した．細胞からRNAを抽出し，リアルタイムPCRを用いた定量的RT-PCR法によりIL-10遺伝子の発現を測定した．

一方，卵白食あるいは対照食を摂取させたDO11.10マウスからCD4⁺IELを精製してIL-10遺伝子の発現を調べたところ，両者の間には顕著な差が認められなかった(図3)．すなわち，特異抗原の経口摂取によるIELのIL-10遺伝子発現増強に対して，CD4⁺IEL1細胞あたりのIL-10遺伝子発現量の上昇が寄与するところは少なく，主にIL-10遺伝子を発現するCD4⁺IELの数の増加によって生じたものであることが示唆された．また，腸間膜リンパ節CD4⁺T細胞を*in vitro*抗原刺激した場合，対照食群ではIL-10産生は認められなかったが，卵白食摂取でその産生が認められた．しかしながら，1細胞あたりのIL-10産生量は，腸間膜リンパ節CD4⁺T細胞よりも，CD4⁺IELの方が高いことが示された．

ある種の制御性T細胞(Tr1)はIL-10を高産生し，その分化発達にはIL-10が必要であることから[12]，CD4⁺IELが食物抗原に対する免疫応答や炎症反応の抑制的制御に関与していることが推察された．SCIDマウスに脾臓CD4⁺CD45RB^{hi}T細胞を移入して起こる腸炎症は，CD8αα⁺ αβ-IELを移入することにより抑制されることが報告されている[13]．この抑制はIL-10$^{-/-}$マウス由来の同細胞ではみられないことから，この反応がIL-10依存的であることは明らかにされているが，この細胞を抗CD3抗体および抗CD28抗体で刺激してもIL-10を産生しない[13]．また，αβ-IELにおけるIL-10遺伝子発現は低いという結果も得られているが[14]，αβ-IEL中のCD4⁺IELの存在比が小さいことを考えると理解できる結果である．われわれの結果を合わせて考えると，CD4⁺IELがIELにおけるIL-10の主な供給源となっていることが示唆される．DasらはCD4⁺CD8αα⁺ αβ-IELがIL-10を産生し，CD4⁺CD45RB^{hi}T細胞の移入で起こる腸炎症をIL-10依存的に抑制することを報告している[7]．われわれの結果では，特異抗原の経口摂取によりCD4⁺CD8αα⁻ αβ-IELが増加したことから，このIELサブセットがIL-10遺伝子発現増大に寄与しているものと考えられる．したがって，IL-10遺伝子を高発現するCD4⁺CD8αα⁻ αβ-IELもCD4⁺CD8αα⁺ αβ-IELと同様に制御性T細胞としての機能を有している可能性が考えられる．また，食物抗原の摂取によりこのような制御性T細胞がIEL中に誘導されてくる可能性が示唆された．

おわりに

本研究ではDNAマイクロアレイ解析により，特異抗原の経口摂取によって，IELに増殖中の細胞が増加すること，小腸上皮内にCD4⁺CD8αα⁻ IELが増加すること，CD4⁺IELはIL-10遺伝子を恒常的に発現していることが示された．これらの結果から示唆された事象については，今後実験的な裏づけをしていく必要がある．これまでに主にγδ-IELとαβ-IELの異同に着目し，網羅的な遺伝子発現解析により特徴的な発現を示す分子からIELの特性・機能を推定する研究が報告されている[14)~16]．各IELサブセットでの発現が明らかにされた分子に注目して研究を進めることで，IELの分化経路や生理機能などに新しい知見が得られることが期待される．

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Serum vitamin C–periodontal relationship in community-dwelling elderly Japanese

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Abstract

Objective: To determine the relationship between serum vitamin C and periodontitis as estimated by clinical attachment loss (CAL) in community-dwelling elderly Japanese.

Material and Methods: This analysis was confined to 413 Niigata citizens aged 70 years in whom the data for serum vitamin C and CAL were available. High-pressure liquid chromatography method was used to ascertain the serum vitamin C levels while CAL was assessed on six sites of all teeth present including third molars by means of pressure-sensitive probes. Other variables included gender, smoking, toothbrushing frequency, number of teeth present and random blood sugar levels.

Results: Serum vitamin C concentration was inversely related to CAL ($r = -0.23$, $p < 0.00005$) at bivariate level. Multiple linear regression analysis showed that CAL was 4% greater in subjects with lower serum vitamin C levels than in subjects with higher serum vitamin C levels notwithstanding smoking, diabetes, oral hygiene, gender or number of teeth present.

Conclusion: The findings suggested that serum vitamin C might have relatively weak but a statistically significant relationship with periodontitis in this elderly population.

Key words: elderly; periodontitis; serum vitamin C

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Investigations into vitamin C (ascorbate/ascorbic acid)–periodontal relationship go as far back as the 18th century when a British naval physician revealed that scurvy, which was accompanied by putrid gums could be successfully treated with oranges and lemons (Rubinoff et al. 1989). Since then numerous experimental as well as epidemiological studies in both humans and animals have attempted to address this issue but the findings have been rather incoherent: some have failed to suggest any significant relationship between vitamin C and periodontal disease (Waerhaug 1958, Barros & Witkop 1963, Russel et al. 1965, Enwonwu & Edozien 1970, Woolfe et al. 1980, 1984) while others reported that the deficiency of this vitamin could not be correlated with severe periodontitis but with gingival inflammation or acute necrotizing ulcerative gingivitis (Enwonwu 1972, Shannon 1973, Leggott et al. 1986, Melnick et al. 1988). Still others have observed a weak association between vitamin C and periodontitis (Ismail et al. 1983, Nishida et al. 2000). However, the majority of workers who have looked into ascorbic acid–periodontal relationships in humans estimated the dietary intake of vitamin C (Ismail et al. 1983, Nishida et al. 2000) in comparison to the few who have assessed serum or plasma ascorbic acid levels (Leggott et al. 1986, Melnick et al. 1988, Pussinen et al. 2003), which might provide much reliable information than the former method (Simon & Hudes 2001). On the other hand, little or virtually nothing has been reported in the literature on the association between vitamin C and periodontal status of the elderly who may be at a higher risk of developing vitamin C deficiency compared with other age groups (Rubinoff et al. 1989, Pussinen et al. 2003). In view of these facts, it is important to explore the link between serum ascorbic acid levels and periodontitis in an elderly population. Accordingly, the main purpose of the present investigation was to ascertain the relationship between serum vitamin C levels and periodontitis as measured by clinical attachment loss (CAL) among senior citizens in Japan.

Material and Methods

This was a part of the ongoing oral and general health survey, which has been carried out in senior citizens of Niigata city, Japan since 1998. The study methodology has been described in