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

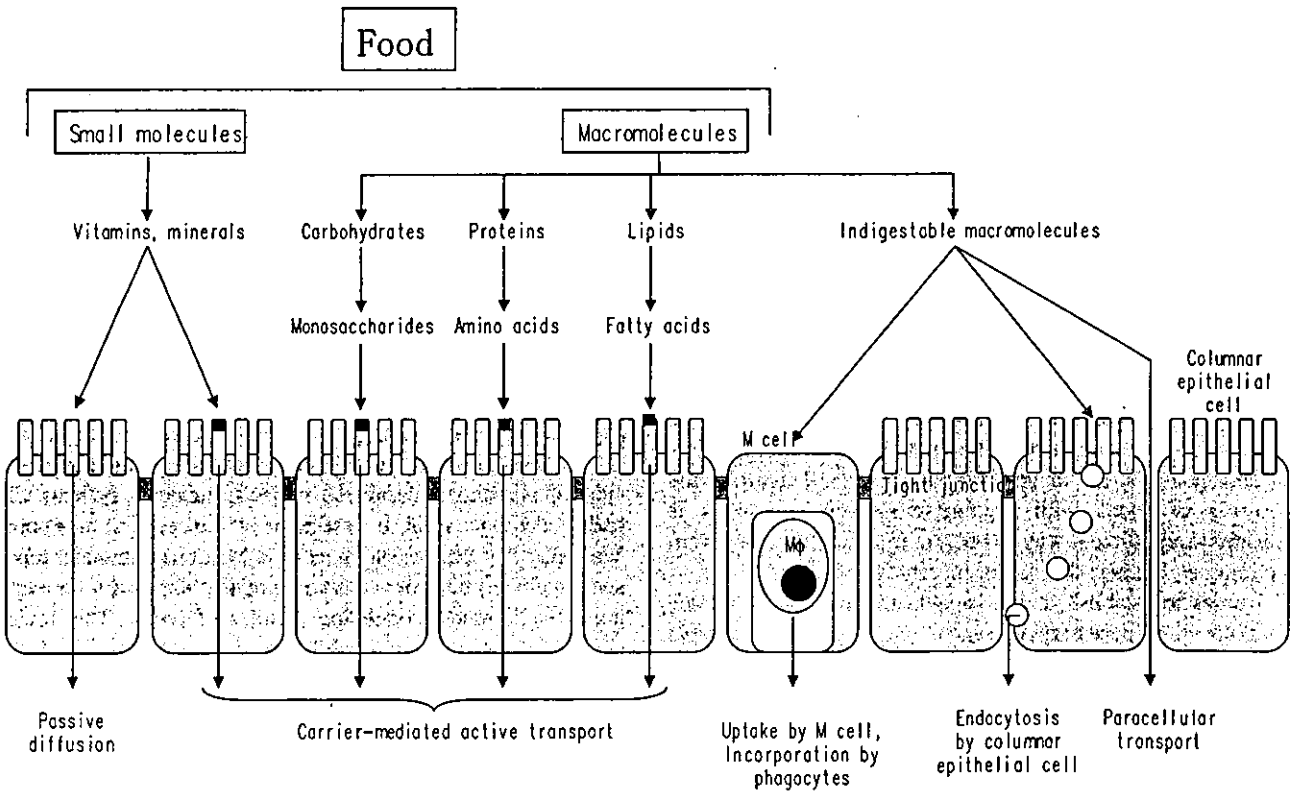

Figure 1. Mechanisms for transport of food-derived substances. Carbohydrates, proteins and lipids are digested by multiple hydrolases secreted into the gut lumen and associated with membranes of columnar epithelial cells. Specific carrier molecules transport monosaccharides, amino acids and fatty acids, 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).

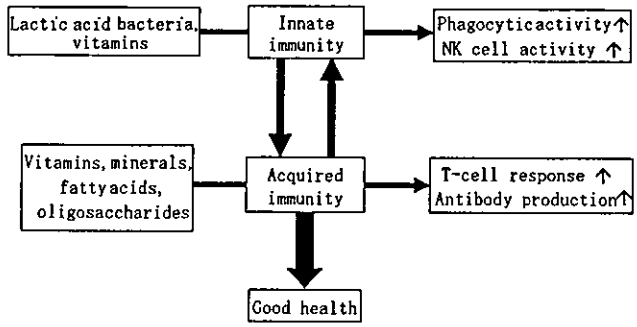

Figure 2. Modulation of immune functions by foods. The immune system is divided into innate immunity and acquired immunity, and food-derived substances can modulate either innate or acquired immunity. For example, probiotics such as lactic acid bacteria and some vitamins enhance phagocytic activity and 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

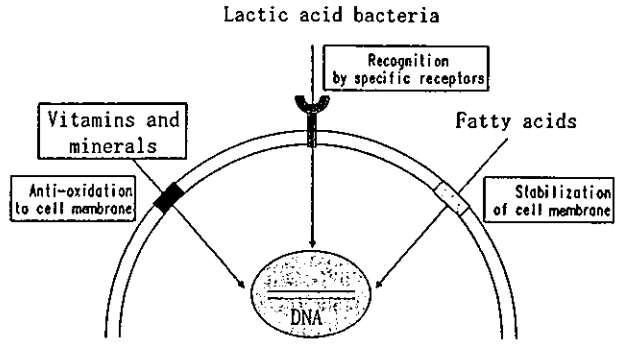

Figure 3. Mechanisms by which food-derived substances modulate the immune functions of cells. Components of probiotics are recognized by cell surface receptors such as the mannose receptor and Toll-like receptors (TLRs). It has been clarified that agonists of TLRs transmit signals to DNA by activating 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).

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.

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

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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References

1. Weiner ML. Intestinal transport of some macromolecules in food. *Food Chem Toxic* 1988;26:867–80.
2. Owen RL. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology* 1977;72:440–51.
3. Keljo DJ, Hamilton JR. Quantitative determination of macromolecular transport rate across intestinal Peyer's patches. *Am J Physiol* 1983;244:G637–44.
4. Roberton DM, Paganelli R, Dinwiddie R, Levinsky RJ. Milk antigen absorption in the preterm and term neonate. *Arch Dis Child* 1982;57:369–72.
5. Jackson PG, Lessof MH, Baker RW, Ferrett J, MacDonald DM. Intestinal permeability in patients with eczema and food allergy. *Lancet* 1981;8233:1285–6.
6. Nanno M, Kanamori Y, Saito H, Kawaguchi-Miyashita M, Shimada S, Ishikawa H. Intestinal intraepithelial T lymphocytes. Our T cell horizons are expanding. *Immunol Res* 1998;18:41–53.
7. Smyth MJ, Crowe NY, Hayakawa Y, Takeda K, Yagita H, Godfrey DI. NKT cells-conductors of tumor immunity? *Curr Opin Immunol* 2002;14:165–71.
8. Duchmann R, Kaiser I, Harmann E, Mayet W, Ewe K, Meyer zum Buscherfelde KH. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol* 1995;102:448–55.
9. Hashimoto K, Kawagishi H, Nakayama T, Shimizu M. Effect of capsianoside, a diterpene glycoside, on tight-junctional permeability. *Biochim Biophys Acta* 1997;1323:281–90.
10. Rescigno M, Urbano M, Valzasina B, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2001;2:361–7.
11. Fagnoni FF, Vescovini R, Passeri G, et al. Shortage of circulating naive $CD8^+$ T cells provides new insights on immunodeficiency in aging. *Blood* 2000;95:2860–8.
12. Fagnoni FF, Vescovini R, Mazzola M, et al. Expansion of cytotoxic $CD8^+CD28^-$ T cells in healthy ageing people, including centenarians. *Immunology* 1996;88:501–7.
13. Stulnig T, Maczek C, Bock G, Majdic O, Wick G. Reference intervals for human peripheral blood lymphocyte subpopulations from 'healthy' young and aged subjects. *Int Arch Allergy Immunol* 1995;108:205–10.
14. Sansoni P, Cossarizza A, Brianti V, et al. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. *Blood* 1993;82:2767–73.
15. Mariani E, Ravaglia G, Forti P, et al. Vitamin D, thyroid hormones and muscle mass influence natural killer (NK) innate immunity in healthy nonagenarians and centenarians. *Clin Exp Immunol* 1999;116:19–27.
16. Ravaglia G, Forti P, Maioli F, et al. Effect of micronutrient status on natural killer cell immune function in healthy free-living subjects aged >90 y. *Am J Clin Nutr* 2000;71:590–8.
17. Vedhara K, Cox NK, Wilcock GK, et al. Chronic stress in elderly carers of dementia patients and antibody response to influenza vaccination. *Lancet* 1999;353:627–31.
18. Kiecolt-Glaser JK, Dura JR, Speicher CE, Trask OJ, Glaser R. Spousal caregivers of dementia victims: longitudinal changes in immunity and health. *Psychosom Med* 1991;53:345–62.
19. Glaser R, Kiecolt-Glaser JK. Chronic stress modulates the virus-specific immune response to latent herpes simplex virus type 1. *Ann Behav Med* 1997;19:78–82.
20. Esterling BA, Kiecolt-Glaser JK, Bodnar JC, Glaser R. Chronic stress, social support, and persistent alterations in the natural killer cell response to cytokines in older adults. *Health Psychol* 1994;13:291–8.
21. Evans DL, Leserman J, Pedersen CA, et al. Immune correlates of stress and depression. *Psychopharmacol Bull* 1989;25:319–24.
22. Kiecolt-Glaser JK, Glaser R, Strain EC, et al. Modulation of cellular immunity in medical students. *J Behav Med* 1986;9:5–21.
23. Nieman DC. Immune response to heavy exertion. *J Appl Physiol* 1997;82:1385–94.
24. Irwin M, Patterson T, Smith TL, et al. Reduction of immune function in life stress and depression. *Biol Psychiatry* 1990;27:22–30.
25. Brussow H, Sidoti J, Dirren H, Freire WB. Effect of malnutrition in Ecuadorian children on titers of serum antibodies to various microbial antigens. *Clin Diag Lab Immunol* 1995;2:62–8.
26. Ledesma F, Echevarria S, Casafont F, Lozano JL, Pons-Romero F. Natural killer cell activity in alcoholic cirrhosis: influence of nutrition. *Eur J Clin Nutr* 1990;44:733–40.
27. Vasquez-Garibay E, Campollo-Rivas O, Romero-Velarde E, et al. Effect of renutrition on natural and cell-mediated immune response in infants with severe malnutrition. *J Pediatr Gastroenterol Nutr* 2002;34:296–301.
28. Lotfy OA, Saleh WA, el-Barbari M. A study of some changes of cell-mediated immunity in protein energy malnutrition. *J Egypt Soc Parasitol* 1998;28:413–28.
29. Birmaher B, Rabin BS, Garcia MR, et al. Cellular immunity in depressed, conduct disorder, and normal adolescents: role of adverse life events. *J Am Acad Child Adolesc Psychiatry* 1994;33:671–8.
30. de la Fuente M, Ferrandez MD, Burgos MS, Soler A, Prieto A, Miquel J. Immune function in aged women is improved by ingestion of vitamins C and E. *Can J Physiol Pharmacol* 1998;76:373–80.
31. Patarca R. Cytokines and chronic fatigue syndrome. *Ann NY Acad Sci* 2001;933:185–200.
32. Marrie TJ, Johnson S, Durant H. Cell-mediated immunity of healthy adult Nova Scotians in various age groups compared with nursing home and hospitalized senior citizens. *J Allergy Clin Immunol* 1988;81:836–43.
33. Wayne SJ, Rhyne RL, Garry PJ, Goodwin JS. Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. *J Gerontol* 1990;45:M45–8.

34. Levy SM, Herberman RB, Lee J, et al. Persistently low natural killer cell activity, age, and environmental stress as predictors of infectious morbidity. *Nat Immun Cell Growth Regul* 1991;10:289–307.
35. Castle SC. Clinical relevance of age-related immune dysfunction. *Clin Infect Dis* 2000;3:578–85.
36. Hassan IS, Bannister BA, Akbar A, Weir W, Bofill M. A study of the immunology of the chronic fatigue syndrome: correlation of immunologic parameters to health dysfunction. *Clin Immunol Immunopathol* 1998;87:60–7.
37. Wu D, Meydani M, Leka LS, et al. Effect of dietary supplementation with black currant seed oil on the immune response of healthy elderly subjects. *Am J Clin Nutr* 1999;70:536–43.
38. Meydani SN, Barklund MP, Liu S, et al. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. *Am J Clin Nutr* 1990;52:557–63.
39. Sempertegui F, Estrella B, Correa E, et al. Effects of short-term zinc supplementation on cellular immunity, respiratory symptoms, and growth of malnourished Equadorian children. *Eur J Clin Nutr* 1996;50:42–6.
40. Han SN, Leka LS, Lichtenstein AH, Ausman LM, Schaefer EJ, Meydani SN. Effect of hydrogenated and saturated, relative to polyunsaturated, fat on immune and inflammatory responses of adults with moderate hypercholesterolemia. *J Lipid Res* 2002;43:445–52.
41. Girodon F, Galan P, Monget AL, et al. Impact of trace elements and vitamin supplementation on immunity and infections in institutionalized elderly patients: a randomized controlled trial. MIN. VIT. AOX. geriatric network. *Arch Intern Med* 1999;159:748–54.
42. Provinciali M, Montenovo A, Di Stefano G, et al. Effect of zinc or zinc plus arginine supplementation on antibody titer and lymphocyte subsets after influenza vaccination in elderly subjects: a randomized controlled trial. *Age Ageing* 1998;27:715–22.
43. Chandra RK. Effect of vitamin and trace-element supplementation on immune responses and infection in elderly subjects. *Lancet* 1992;340:1124–7.
44. Bahl R, Kumar R, Bhandari N, Kant S, Srivastava R, Bhan MK. Vitamin A administered with measles vaccine to nine-month-old infants does not reduce vaccine immunogenicity. *J Nutr* 1999;129:1569–73.
45. Wouters-Wesseling W, Rozendaal M, Snijder M, et al. Effect of a complete nutritional supplement on antibody response to influenza vaccine in elderly people. *J Gerontol A Biol Sci Med Sci* 2002; 57:M563–6.
46. Baumann W, Hanisch M, Emmrich P, Arnold W. Management of hepatic coma due to fulminant viral hepatitis B using anti-HB plasma. *Monatsschr Kinderheilkd* 1978;126:335–7 (in German).
47. Kramer TR, Udomkesmalee E, Dhanamitta S, et al. Lymphocyte responsiveness of children supplemented with vitamin A and zinc. *Am J Clin Nutr* 1993;58:566–70.
48. Fortes C, Forastiere F, Agabiti N, et al. The effect of zinc and vitamin A supplementation on immune response in an older population. *J Am Geriatr Soc* 1998;46:19–26.
49. Murosaki S, Ikematsu H, Yamamoto Y, Yukami S, Nomoto K. Effects of intake of nigerooligosaccharides-supplemented syrup on the immune function and quality of life in the healthy elderly. *Jpn Pharmacol Ther* 2001;29:815–26.
50. Murosaki S, Ikematsu H, Hirose Y, Yamamoto Y, Yukami S, Nomoto K. Effects of intake of nigerooligosaccharides-supplemented syrup on the immune function and quality of life in healthy young adult subjects. *Jpn Pharmacol Ther* 2002;30:81–90.
51. Pike J, Chandra RK. Effect of vitamin and trace element supplementation on immune indices in healthy elderly. *Int J Vitam Nutr Res* 1995;65:117–21.
52. Heuser G, Vojdani A. Enhancement of natural killer cell activity and T and B cell function by buffered vitamin C in patients exposed to toxic chemicals: the role of protein kinase-C. *Immunopharmacol Immunotoxicol* 1997;19:291–312.
53. Sheih YH, Chiang BL, Wang LH, Liao CK, Gill HS. Systemic immunity-enhancing effects in healthy subjects following dietary consumption of the lactic acid bacterium Lactobacillus rhamnosus HN001. *J Am Coll Nutr* 2001;20 (2 Suppl):149–56.
54. Nagao F, Nakayama M, Muto T, Okumura K. Effects of a fermented milk drink containing Lactobacillus casei strain Shirota on the immune system in healthy human subjects. *Biosci Biotechnol Biochem* 2000;64:2706–8.
55. Gill HS, Rutherfurd KJ, Cross ML, Gopal PK. Enhancement of immunity in the elderly by dietary supplementation with the probiotic Bifidobacterium lactis HN019. *Am J Clin Nutr* 2001;74:833–9.
56. Chiang BL, Sheih YH, Wang LH, Liao CK, Gill HS. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (Bifidobacterium lactis HN019): optimization and definition of cellular immune responses. *Eur J Clin Nutr* 2000;54:849–55.
57. Santos MS, Gaziano JM, Leka LS, Beharka AA, Hennekens CH, Meydani SN. Beta-carotene-induced enhancement of natural killer cell activity in elderly men: an investigation of the role of cytokines. *Am J Clin Nutr* 1998;68:164–70.
58. Kiremidjian-Schumacher L, Roy M, Wishe HI, Cohen MW, Stotzky G. Supplementation with selenium and human immune cell functions. II. Effect on cytotoxic lymphocytes and natural killer cells. *Biol Trace Elem Res* 1994;41:115–27. [Erratum in: *Biol Trace Elem Res* 1994;46:183.]
59. Schiffrin EJ, Brassart D, Servin AL, Rochat F, Donnet-Hughes A. Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. *Am J Clin Nutr* 1997;66:515S–20S.
60. Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *J Dairy Sci* 1995;78:491–7.
61. Arunachalam K, Gill HS, Chandra RK. Enhancement of natural immune function by dietary consumption of Bifidobacterium lactis (HN019). *Eur J Clin Nutr* 2000;54:263–7.
62. Sempertegui F, Estrella B, Correa E, et al. Effects of short-term zinc supplementation on cellular immunity, respiratory symptoms, and growth of malnourished Equadorian children. *Eur J Clin Nutr* 1996;50:42–6.
63. Field CJ. Use of T cell function to determine the effect of physiologically active food components. *Am J Clin Nutr* 2000;71:1720S–7S.
64. Junghans V, Gutgesell C, Jung T, Neumann C. Epidermal cytokines IL-1beta, TNF-alpha, and IL-12 in patients with atopic dermatitis: response to application of house dust mite antigens. *J Invest Dermatol* 1998;111:1184–8.
65. Turchet P, Laurenzato M, Auboiron S, Antonie JM. Effect of fermented milk containing the probiotic *Lactobacillus casei* DN-114 001 on winter infections in free-living elderly subjects: A randomized, controlled pilot study. *J Nutr Health Aging* 2003;7:75–7.
66. Sakurai T, Inagaki N, Nagai H. The effect of anti-tumor necrosis factor (TNF)-alpha monoclonal antibody on allergic cutaneous late phase reaction in mice. *Life Sci* 1994;54:PL291–5.
67. Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. *Clin Exp Allergy* 2000;30:1604–10.
68. Ito H, Nakamura Y, Takagi S, Sakai K. Effects of azelastine on the level of serum interleukin-4 and soluble CD23 antigen in the treatment of nasal allergy. *Arzneimittelforschung* 1998;48:1143–7.
69. Colver GB, Symons JA, Duff GW. Soluble interleukin 2 receptor in atopic eczema. *Br Med J* 1989;298:1426–8.
70. Schleimer RP, Sterbinsky SA, Kaiser J, et al. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. *J Immunol* 1992;148:1086–92.
71. Koller DY, Halmerbauer G, Frischer T, Roithner B. Assessment of eosinophil granule proteins in various body fluids: is there a relation to clinical variables in childhood asthma? *Clin Exp Allergy* 1999;29:786–93.
72. Bousquet J, Chanez P, Lacoste JY, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033–9.
73. Leonardi A, Battista MC, Gismondi M, Fregona IA, Secchi AG. Antigen sensitivity evaluated by tear-specific and serum-specific IgE, skin tests, and conjunctival and nasal provocation tests in patients with ocular allergic disease. *Eye* 1993;7:461–4.
74. [No authors listed] Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. *Dermatology* 1993;186:23–31.
75. Thien FC, Atkinson BA, Khan A, Mencia-Huerta JM, Lee TH. Effect of dietary fish oil supplementation on the antigen-induced late-phase response in the skin. *J Allergy Clin Immunol* 1992;89:829–35.
76. La Rosa M, Ranno C, Andre C, Carat F, Tosca MA, Canonica GW. Double-blind placebo-controlled evaluation of sublingual-swallow immunotherapy with standardized Parietaria judaica extract in children with allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 1999;104:425–32.
77. Purello-D'Ambrosio F, Gangemi S, et al. Sublingual immunotherapy: a double-blind, placebo-controlled trial with Parietaria judaica extract standardized in mass units in patients with rhinoconjunctivitis, asthma, or both. *Allergy* 1999;54:968–73.
78. Latchman Y, Banerjee P, Poulter LW, Rustin M, Brostoff J. Association of immunological changes with clinical efficacy in atopic eczema patients treated with traditional Chinese herbal therapy (Zemaphyte). *Int Arch Allergy Immunol* 1996;109:243–9.

79. Majamaa H, Isolauri E. Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol* 1997;99:179–85.
80. Pessi T, Sutas Y, Hurme M, Isolauri E. Interleukin-10 generation in atopic children following oral Lactobacillus rhamnosus GG. *Clin Exp Allergy* 2000;30:1804–8.
81. Wheeler JG, Shema SJ, Bogle ML, et al. Immune and clinical impact of Lactobacillus acidophilus on asthma. *Ann Allergy Asthma Immunol* 1997;79:229–33.
82. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 2001;357:1076–9.
83. Kalliomaki M, Salminen S, Poussa T, Arvilommi H, Isolauri E. Probiotics and prevention of atopic disease: 4-year follow-up of a randomized placebo-controlled trial. *Lancet* 2003;361:1869–71.
84. Rautava S, Kalliomaki M, Isolauri E. Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. *J Allergy Clin Immunol* 2002;109:119–21.
85. Kirjavainen PV, Arvola T, Salminen SJ, Isolauri E. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? *Gut* 2002;51:51–5.
86. Rosenfeldt V, Benfeldt E, Nielsen SD, et al. Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *J Allergy Clin Immunol* 2003;111:389–95.
87. Hattori K, Yamamoto A, Sasai M, et al. Effects of administration of bifidobacteria on fecal microflora and clinical symptoms in infants with atopic dermatitis. *Jpn J Allergol* 2003;52:20–30 (in Japanese).
88. Shida K, Makino K, Morishita A, et al. *Lactobacillus casei* inhibits antigen-induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. *Int Arch Allergy Immunol* 1998; 115:278–87.
89. Ishida Y, Bandou I, Kanzato H, Yamamoto N. Decrease in ovalbumin specific IgE of mice serum after oral uptake of lactic acid bacteria. *Biosci Biotechnol Biochem* 2003;67:951–7.
90. Portales P, Aries MF, Licu D, et al. Immunomodulation induced by Avene spring water on Th1- and Th2-dependent cytokine production in healthy subjects and atopic dermatitis patients. *Skin Pharmacol. Appl Skin Physiol* 2001;14:234–42.
91. Benlounes N, Dupont C, Candalh C, Blaton MA, Bloom M, Heyman M. Effect of terfenadine on TNF alpha release from peripheral blood mononuclear cells during cow's milk allergy. *Clin Exp Allergy* 1997;27:942–8.
92. Kim K.M, Nanbu M, Iwai Y, et al. Soluble low affinity Fc receptors for IgE in the serum of allergic and nonallergic children. *Pediatr Res* 1989;26:49–53.
93. Kagi MK, Joller-Jemelka H, Wuthrich B. Correlation of eosinophils, eosinophil cationic protein and soluble interleukin-2 receptor with the clinical activity of atopic dermatitis. *Dermatology* 1992;185:88–92.
94. Sutas Y, Hurme M, Isolauri E. Down-regulation of anti-CD3 antibody-induced IL-4 production by bovine caseins hydrolysed with Lactobacillus GG-derived enzymes. *Scand J Immunol* 1996;43:687–9.
95. Nishioka K, Saito C, Nagano T, Okano M, Masuda Y, Kuriyama T. Eosinophil cationic protein in the nasal secretions of patients with mite allergic rhinitis. *Larynagoscope* 1993;103:189–92.
96. Kaburagi Y, Shimada Y, Nagaoka T, Hasegawa M, Takehara K, Sato S. Enhanced production of CC-chemokines (RANTES, MCP-1, MIP-1alpha, MIP-1beta, and eotaxin) in patients with atopic dermatitis. *Arch Dermatol Res* 2001;293:350–5.
97. Wuthrich B, Joller-Jemelka H, Kagi MK. Levels of soluble ICAM-1 in atopic dermatitis. A new marker for monitoring the clinical activity? *Allergy* 1995;50:88–9.
98. Hansen G, McIntire JJ, Yeung VP, et al. $CD4^+$ T helper cells engineered to produce latent TGF-β1 reverse allergen-induced airway hyperreactivity and inflammation. *J Clin Invest* 2000;105:61–70.
99. Wilson N, Pedersen S. Inflammatory markers in clinical practice. *Am J Respir Crit Care Med* 2000;162:S48–51.
100. Nagura T, Hachimura S, Hashiguchi M, et al. Suppressive effect of dietary raffinose on T-helper 2 cell-mediated immunity. *Br J Nutr* 2002;88:421–6.
101. Takano H, Osakabe N, Sanbongi C, et al. Extract of *Perilla frutescens* enriched for rosmarinic acid, a polyphenolic phytochemical, inhibits seasonal allergic rhinoconjunctivitis in humans. *Exp Biol Med (Maywood)* 2004;229:247–54.
102. Nagafuchi S, Hachimura S, Totsuka M, et al. Dietary nucleotides can up-regulate antigen-specific Th1 immune responses and suppress antigen-specific IgE responses in mice. *Int Arch Allergy Immunol* 2000;122:33–41.
103. Hunter CA. How are NK cell responses regulated during infection? *Exp Parasitol* 1996;84:444–8.
104. Tay CH, Szomolanyi-Tsuda E, Welsh RM. Control of infections by NK cells. *Curr Top Microbiol Immunol* 1998;230:193–220.
105. Fleming SD, Campbell PA, Some macrophages kill Listeria monocytogenes while others do not. *Immunol Rev* 1997;158:69–77.
106. Vazquez-Torres A, Fang FC, Oxygen-dependent anti-Salmonella activity of macrophages. *Trends Microbiol* 2001;9:29–33.
107. Daele J, Zicot AF. Humoral immunodeficiency in recurrent upper respiratory tract infections. Some basic, clinical and therapeutic features. *Acta Otorhinolaryngol Belg* 2000;54:373–90.
108. Bloom PD, Boedeker EC. Mucosal immune responses to intestinal bacterial pathogens. *Semin Gastrointest Dis* 1996;7:151–66.
109. Uehling DT, Johnson DB, Hopkins WJ. The urinary tract response to entry of pathogens. *World J Urol* 1999;17:351–8.
110. Marteau P, Vaerman JP, Dehennin JP, et al. Effects of intrajejunal perfusion and chronic ingestion of Lactobacillus johnsonii strain La1 on serum concentrations and jejunal secretions of immunoglobulins and serum proteins in healthy humans. *Gastroenterol Clin Biol* 1997;21:293–8.
111. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* 1989;320:1731–5.
112. Bukowski JF, Warner JF, Dennert G, Welsh RM. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. *J Exp Med* 1985;161:40–52.
113. Marodi L, Kaposzta R, Toth J, Laszlo A. Impaired microbicidal capacity of mononuclear phagocytes from patients with type I Gaucher disease: partial correction by enzyme replacement therapy. *Blood* 1995;86:4645–9.
114. Storek J, Espino G, Dawson MA, Storer B, Flowers ME, Maloney DG. Low B-cell and monocyte counts on day 80 are associated with high infection rates between days 100 and 365 after allogeneic marrow transplantation. *Blood* 2000;96:3290–3.
115. Braga M, Vignali A, Gianotti L, Cestari A, Profili M, Carlo VD. Immune and nutritional effects of early enteral nutrition after major abdominal operations. *Eur J Surg* 1996;162:105–12.
116. Scaglione F, Cattaneo G, Alessandria M, Cogo R. Efficacy and safety of the standardised Ginseng extract G115 for potentiating vaccination against the influenza syndrome and protection against the common cold [corrected]. *Drugs Exp Clin. Res* 1996;22:65–72.
117. Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S, Arvilommi H. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain. *Pediatr Res* 1992;32:141–4.
118. Coutsoudis A, Kiepiela P, Coovadia HM, Broughton M. Vitamin A supplementation enhances specific IgG antibody levels and total lymphocyte numbers while improving morbidity in measles. *Pediatr Infect Dis J* 1992;11:203–9.
119. Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989;47:187–376.
120. Hercend T, Schmidt RE. Characteristics and uses of natural killer cells. *Immunol Today* 1988;9:291–3.
121. Ravetch JV, Lanier LL. Immune inhibitory receptors. *Science* 2000;290:84–9.
122. Tannenbaum CS, Hamilton TA. Immune-inflammatory mechanisms in IFNgamma-mediated anti-tumor activity. *Semin Cancer Biol* 2000;10:113–23.
123. Ikeda H, Old LJ, Schreiber RD. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev* 2002;13:95–109.
124. Albina JE, Reichner JS. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. *Cancer Metastasis Rev* 1998;17:39–53.
125. Zavadova E, Loercher A, Verstovsek S, Verschraegen CF, Micksche M, Freedman RS. The role of macrophages in antitumor defense of patients with ovarian cancer. *Hematol Oncol Clin North Am* 1999;13:135–44.
126. te Velde AA, Figdor CG. Monocyte mediated cytotoxic activity against melanoma. *Melanoma Res* 1992;1:303–9.
127. Balch CM, Tilden AB, Dougherty PA, Cloud GA. Depressed levels of granular lymphocytes with natural killer (NK) cell function in 247 cancer patients. *Ann Surg* 1983;198:192–9.
128. Brenner BG, Friedman G, Margolese RG. The relationship of clinical status and therapeutic modality to natural killer cell activity in human breast cancer. *Cancer* 1985;56:1543–8.
129. Strayer DR, Carter WA, Mayberry SD, Pequignot E, Brodsky I. Low natural cytotoxicity of peripheral blood mononuclear cells in individuals with high familial incidences of cancer. *Cancer Res* 1984;44:370–4.

130. Purtilo DT, Strobach RS, Okano M, Davis JR. Epstein-Barr virus-associated lymphoproliferative disorders. *Lab Invest* 1992;67:5–23.
131. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: An 11-year follow-up study of a general population. *Lancet* 2000;356:1795–9.
132. Hermann M, Niemitz C, Marafioti T, Schriever F. Reduced phagocytosis of apoptotic cells in malignant lymphoma. *Int J Cancer* 1998;75:675–9.
133. Braga M, Vignali A, Gianotti L, Cestari A, Profili M, Carlo VD. Immune and nutritional effects of early enteral nutrition after major abdominal operations. *Eur J Surg* 1996;162:105–12.
134. Isenberg J, Stoffel B, Wolters U, et al. Immunostimulation by propionibacteria–effects on immune status and antineoplastic treatment. *Anticancer Res* 1995;15:2363–8.
135. Kemen M, Senkal M, Homann HH, et al. Early postoperative enteral nutrition with arginine-omega-3 fatty acids and ribonucleic acid-supplemented diet versus placebo in cancer patients: an immunologic evaluation of Impact. *Crit Care Med* 1995;23:652–9.
136. Song JX, Qing SH, Huang XC, Qi DL. Effect of parenteral nutrition with L-arginine supplementation on postoperative immune function in patients with colorectal cancer. *Di Yi Jun Yi Da Xue Xue Bao* 2002;22:545–7.
137. Braga M, Gianotti L, Vignali A, Carlo VD. Preoperative oral arginine and n-3 fatty acid supplementation improves the immunometabolic host response and outcome after colorectal resection for cancer. *Surgery* 2002;132:805–14.
138. Plaeger-Marshall S, Spina CA, et al. Alterations in cytotoxic and phenotypic subsets of natural killer cells in acquired immune deficiency syndrome (AIDS). *J Clin Immunol* 1987;7:16–23.
139. Takegoshi K, Nanasawa H, Itoh H, Yasuyama T, Ohmoto Y, Sugiyama K. Effects of branched-chain amino acid-enriched nutrient mixture on natural killer cell activity in viral cirrhosis. *Arzneimittelforschung* 1998;48:701–6.
140. Araki K, Shinozaki T, Irie Y, Miyazawa Y. Trial of oral administration of Bifidobacterium breve for the prevention of rotavirus infections. *Kansenshogaku Zasshi* 1999;73:305–10 (in Japanese).
141. Coutsoudis A, Kiepiela P, Coovadia HM, Broughton M. Vitamin A supplementation enhances specific IgG antibody levels and total lymphocyte numbers while improving morbidity in measles. *Pediatr Infect Dis J* 1992;11:203–9.
142. Hatakka K, Savilahti E, Pönkä A, et al. Effect of long term consumption of probiotic milk on infections in children attending day care centers: Double blind, randomized trial. *Br Med J* 2001;322:1–5.
143. Amati L, Cirimele D, Pugliese V, Covelli V, Resta F, Jirillo E. Nutrition and immunity: laboratory and clinical aspects. *Curr Pharm Des* 2003;9:1924–31.
144. Fuller R. Probiotics in human medicine. *Gut* 1991;32:439–42.
145. Field CJ, Johnson IR, Schley PD. Nutrients and their role in host resistance to infection. *J Leukocyte Biol* 2002;71:16–32.
146. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003;21:335–76.
147. Agrawal S, Agrawal A, Doughty B, et al. Different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-fos. *J Immunol* 2003;171:4984–9.
148. Houdjik APJ, Rijnsburger ER, Jansen J, et al. Randomized trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma. *Lancet* 1998;352:772–6.
149. Morlion BJ, Stehle P, Wachtler P, et al. Total parenteral nutrition with glutamine dipeptide after major surgery. A double blind controlled study. *Ann Surg* 1998;227:302–8.
150. Gionotti L, Braga M, Fortis C, et al. A prospective randomized clinical trial on perioperative feeding with arginine, omega 3 fatty acid, and RNA-enriched enteral diet. Effect on host response and nutrient status. *J Parent Ent Nutr* 1999;23:314–20.
151. Nagafuchi S, Totsuka M, Hachimura S, et al. Dietary nucleotides increase the proportion of a TCR $\gamma\delta^+$ subset of intraepithelial lymphocytes (IEL) and IL-7 production by intestinal epithelial cells (IEC); implications for modification of cellular and molecular cross-talk between IEL and IEC by dietary nucleotides. *Biosci Biotechnol Biochem* 2000;64:1459–65.
152. Carver JD. Dietary nucleotides: cellular immune, intestinal and hepatic system effects. *J Nutr* 1994;124:144S–8S.
153. Cantorna MT, Nashold FE, Hayes CE. Vitamin A deficiency results in a priming environment conductive for Th1 cell development. *Eur J Immunol* 1995;25:1673–9.
154. Schwager J, Schulze J. Modulation of interleukin production by ascorbic acid. *Vet Immunol Immunopathol* 1998;64:45–57.
155. Moriguchi S, Itoh T. Vitamin E enhances T cell differentiation through increased epithelial cell function in rat thymus. *Nutr Res* 1997;17:873–83.
156. McKenzie RC, Rafferty TS, Beckett GJ. Selenium: an essential element for immune function. *Immunol Today* 1998;19:342–5.
157. Prasad AS. Effects of zinc deficiency on Th1 and Th2 cytokine shifts. *J Infect Dis* 2000;182:S62–8.
158. Fujikawa M, Yamashita N, Yamazaki K, Sugiyama E, Suzuki H, Hamazaki T. Eicosapentanoic acid inhibits antigen-presenting cell function of murine splenocytes. *Immunology* 1992;75:330–5.
159. Hughes DA, Pinder AC. N-3 Polyunsaturated fatty acids inhibit the antigen-presenting function of human monocytes. *Am J Clin Nutr* 2000;71:357S–60S.

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Effects of oral administration of extracts of *Hypericum perforatum* (St John's wort) on brain serotonin transporter, serotonin uptake and behaviour in mice

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Abstract

The pharmacological effects of extracts of *Hypericum perforatum* (St John's wort) were characterized in-vitro and ex-vivo, in relation to its behavioural effects. In in-vitro experiments, St John's wort inhibited brain synaptosomal [^{3}H]serotonin uptake in mice with little effect on specific [^{3}H]paroxetine binding. For selective serotonin-reuptake inhibitors (SSRIs), the IC50 value for [^{3}H]serotonin uptake (molar concentration of unlabelled drug necessary to displace 50% of specific uptake) correlated well with the inhibition constant K_i value for [^{3}H]paroxetine binding in mouse brain. Oral administration of St John's wort (900 mg kg^{-1}), paroxetine (1 mg kg^{-1}) and sertraline (10 mg kg^{-1}) brought about significant increases in the K_m value for [^{3}H]serotonin uptake into brain synaptosomes 4 h later, and only SSRIs suppressed specific [^{3}H]paroxetine binding in mouse brain. St John's wort and SSRIs significantly inhibited marble-burying behaviour in mice and the time-course of attenuation of this behaviour by St John's wort was similar to that of [^{3}H]serotonin uptake inhibition. In the forced swimming test, St John's wort, but not SSRIs, suppressed the immobility time of mice after oral administration. These results provide the first in-vivo evidence to suggest that the mode of antidepressant action of St John's wort differs from that of SSRIs. Thus, this study may have a significant impact on phytotherapy with St John's wort.

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Introduction

Extracts of *Hypericum perforatum* (St John's wort) are frequently prescribed in Germany and other European countries in the treatment of mild to moderate depression, anxiety and sleep disorders. Several recent reviews of controlled clinical studies with St John's wort have come to the conclusion that it represents an effective antidepressive principle, superior to placebo (Linde et al 1996; Volz 1997; Wheatley 1997; Wong et al 1998) and having a similar effect to some standard antidepressant drugs (Philipp et al 1999; Brenner et al 2000; Schrader 2000; Woelk 2000). Furthermore, this extract has proved to be free of cardiac and anticholinergic side effects, which are typical for the tricyclic antidepressants (Ernst et al 1998). In accordance with the clinical studies, many recent pharmacological studies with St John's wort also support its antidepressive activity, and attention has increasingly focused on hyperforin (a phloroglucinol derivative) as an active ingredient. St John's wort and hyperforin (80–200 nM) inhibit synaptosomal monoamine uptake in-vitro (Chatterjee et al 1998), and these hyperforin concentrations are close to its plasma C_{max} value in human subjects given daily St John's wort (300 mg of the extract containing 5% hyperforin) (Biber et al 1998). In in-vivo experiments, acute systemic treatment with St John's wort exerts significant antidepressant activity in some behavioural tasks (forced swimming test, learned helplessness and tail suspension test) (Chatterjee et al 1998; Butterweck et al 2003) and St John's wort (500 mg kg^{-1}, p.o.) or hyperforin alone (10 mg kg^{-1}, i.p.) significantly enhances levels of brain serotonin, noradrenaline (norepinephrine) and dopamine in rats (Calapai et al 1999; Kaehler et al 1999).

Although a wide variety of bioactive compounds, such as phenylpropanes, flavonol derivatives, biflavones, proanthocyanidines, xanthones, phloroglucinols, some amino

acids, naphthodianthrones and essential oil constituents, have been identified in the extracts (Nahrstedt & Butterweck 1997), it is still not clear which components could account, wholly or partly, for the antidepressant activity of St John's wort. Because elimination of hyperforin from St John's wort does not result in a loss of behavioural pharmacological activity (Butterweck et al 2003) and the brain-to-plasma ratio of hyperforin is only 4%, the corresponding brain concentration of hyperforin is probably far from the levels required to affect the neurotransmitter mechanisms (Cervo et al 2002). Thus, the exact mechanism by which St John's wort exerts its antidepressant effect still remains to be resolved. Most previous studies on St John's wort and its ingredients have involved in-vitro experiments and behavioural effects but little in-vivo study has been undertaken to establish the pharmacological relevance of in-vitro findings. Therefore, it would be of general importance to elucidate the in-vivo mode of action of St John's wort under the influence of pharmacokinetic and pharmacodynamic factors. In this study, the binding characteristics of St John's wort to serotonin transporters and its effects on serotonin uptake in mouse brain were examined in-vitro and ex-vivo, in relation to its behavioural effects. Mice received St John's wort (100–900 mg kg^{-1}), paroxetine (1 mg kg^{-1}) and sertraline (10 mg kg^{-1}) orally at doses chosen to significantly increase the extracellular serotonin in rat brain (Calapai et al 1999; Malagié et al 2000; Bymaster et al 2002).

Materials and Methods

Chemicals

[^{3}H]Paroxetine (806.6 GBq mmol^{-1}) and [^{3}H]serotonin (1.0 TBq mmol^{-1}) were purchased from Dupont-NEN Co. Ltd (Boston, MA). The *Hypericum perforatum* dry extract (St John's wort) was kindly supplied by Indena (Milan, Italy). The content of hypericin (0.3%) and hyperforin (3.2%) was quantified by Indena. Fluvoxamine maleate was purchased from Tocris (UK). Fluoxetine hydrochloride, paroxetine hydrochloride and sertraline hydrochloride were kindly donated by Eli Lilly pharmaceuticals (Greenfield, IN), GlaxoSmithKline pharmaceuticals (West Sussex, UK) and Pfizer Inc. (Groton, CT), respectively. All other drugs and materials were obtained from commercial sources.

Drug administration

Male ICR strain mice, 6–8 weeks old (Japan SLC Inc., Shizuoka, Japan), were housed five per cage in the laboratory with free access to food and water, and were maintained on a 12-h dark–light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%). Mice were fasted for 16 h before the administration of drugs. St John's wort (100, 300 and 900 mg kg^{-1}), paroxetine (1 mg kg^{-1}) or sertraline (10 mg kg^{-1}) was administered orally, and control mice received vehicle. St John's wort was suspended in distilled water and sonicated for 10 min before oral administration; SSRIs were dissolved in distilled water. At 1–12 h after the administration, mice were exsanguinated by taking the blood from the descending aorta under light anaesthesia with diethyl ether and the brain was perfused with 0.9% NaCl from the aorta. Then, the whole brain was removed and used for [^{3}H]paroxetine binding or [^{3}H]serotonin uptake experiments. All the procedures used in this study were conducted according to guidelines approved by the Experimental Animal Ethical Committee of the University of Shizuoka.

[^{3}H]Paroxetine binding assay

The whole brain tissue was homogenized in 19 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCl with a Polytron homogenizer and the homogenate was centrifuged at 40 000 *g* for 15 min. The pellet was resuspended in 24 volumes of the buffer. All steps for the tissue preparation were performed at 4°C. The binding assay for serotonin transporters in brain homogenates from mice was performed by using [^{3}H]paroxetine, as previously described (Habert et al 1985). Briefly, the brain homogenates (approximately 400 μg of protein) were incubated with different concentrations of [^{3}H]paroxetine (0.1, 0.3, 0.5, 1.0 and 2.0 nM) for 2 h at 20°C in the buffer. The reaction was terminated by rapid filtration (Cell Harvester; Brandel Co., Gaithersburg, MD) through Whatman GF/B glass fibre filters, and filters were rinsed three times with 2 mL of ice-cold buffer. Tissue-bound radioactivity was extracted from filters overnight in scintillation fluid (2 L of toluene, 1 L of Triton X-100, 15 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene) and it was determined in a liquid scintillation counter. Specific binding of [^{3}H]paroxetine was determined experimentally from the difference between counts in the absence and presence of 10 μM fluoxetine. All assays were conducted in duplicate. Every binding experiment was performed using fresh tissues. Protein concentration was measured according to the method of Lowry et al (1951) using bovine serum albumin as standard.

Synaptosomal uptake of [^{3}H]serotonin

Synaptosomal preparations from the whole brain of mice were used for serotonin uptake as previously described (Chatterjee et al 1998; Singer et al 1999). The tissue was homogenized in 0.32 M sucrose solution with a Teflon–glass homogenizer and diluted with 10 mL of homogenizing medium. The nuclear fraction was eliminated by centrifugation at 750 *g* for 10 min and the supernatant was centrifuged at 17 400 *g* for 20 min to obtain the crude synaptosomal pellet. The pellet was suspended in 19 volumes of HEPES buffer (composition in mM (except where stated): 10 HEPES, 150 NaCl, 6.2 KCl, 1.2 Na_2HPO_4, 1.2 $MgSO_4$, 10 glucose, 10 μM pargylin, 0.1% ascorbic acid, pH 7.4). All steps for the tissue preparation were performed at 4°C. The sample of suspension was incubated at 37°C for 15 min and cooled on ice. [^{3}H]Serotonin (10, 20, 30, 50 and 100 nM) was added and the uptake started by incubation at 37°C for 4 min, during

which time the uptake is linearly dependent on time. The reaction was terminated by rapid filtration and radioactivity was determined in a liquid scintillation counter as described above. Nonspecific uptake of [^{3}H]serotonin was determined in the presence of 500 μM serotonin and specific uptake was expressed as fmol min^{-1} (μg protein)$^{-1}$. All assays were conducted in duplicate.

Behavioural test

Mice received St John's wort (100, 300 and 900 mg kg^{-1}), paroxetine (1 mg kg^{-1}) or sertraline (10 mg kg^{-1}) orally and control mice received vehicle. At 1–12 h after the administration, mice were subjected to the forced swimming test or marble-burying test. The forced swimming test was essentially similar to that described previously (Porsolt et al 1978). A glass cylinder (height 25 cm, diameter 10 cm) was utilized and this was filled with water maintained at 23°C (depth 10 cm). Mice were dropped individually into the cylinders, and left there for 6 min. A mouse was judged to be immobile when it floated in an upright position and made only small movements to keep its head above water. The total immobility time(s) of each mouse was expressed as the sum of the immobility periods observed during the 6-min forced swimming test.

For the marble-burying test, an open cubic transparent plastic box (22.5 × 33.8 × 14.0 cm) was used, and 20 clean glass marbles (15 mm diameter) were evenly spaced (5 cm apart) on sawdust (5 cm deep) as previously described (Njung'e & Handley 1991; Ichimaru et al 1995). Mice were placed into the cubic box individually for 30 min and the number of marbles left uncovered was counted. The results of the marble-burying test were expressed as the number of marbles covered with sawdust by mice during 30-min testing period.

The locomotor activity of mice was measured for 30 min by an activity sensor (NS-AS01; Neuroscience Inc., Tokyo, Japan) in the same apparatus utilized for the marble-burying test (without marbles and sawdust) as a separate experiment. The results were represented as activity counts (the number of movement of mice) during the 30-min testing period.

Data analysis

Analysis of binding data was performed as described previously (Yamada et al 1980). The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]paroxetine were estimated by Rosenthal analysis of the saturation data (Rosenthal 1967). Kinetic parameters (K_m and V_{max}) were calculated using Lineweaver–Burk plots. The ability of St John's wort and SSRIs to inhibit specific [^{3}H]paroxetine (0.3 nM) binding and [^{3}H]serotonin (10 nM) uptake in-vitro was estimated by IC50 value, which is the molar concentration of unlabelled drug necessary for displacing 50% of specific binding or uptake (estimated by log probit analysis). The inhibition constant, K_i, was calculated from the equation, $K_i = IC50/(1 + L/K_d)$, where L is the concentration of the radioligand.

Statistical analysis was performed by the non-parametric Kruskal–Wallis test followed by Dunn's post test for multiple comparisons utilizing the Prism 4.0 program (GraphPad Inc., San Diego, CA). The number of determinations (n) was noted in each figure and table, and the level of $P < 0.05$ was considered significant.

Results

In-vitro inhibitory effect of St John's wort and SSRIs on specific [^{3}H]serotonin uptake and [^{3}H]paroxetine binding in mouse brain

The [^{3}H]serotonin (10–100 nM) uptake and [^{3}H]paroxetine (0.1–2.0 nM) binding in mouse brain were saturable with K_m and K_d values of 21.5 and 0.13 nM, respectively, in accordance with previous observations (Habert et al 1985; Singer et al 1999). The total uptake of [^{3}H]serotonin into brain synaptosomes was reduced to a nonspecific level by incubation at 4°C or by the presence of 10 μM fluoxetine instead of 500 μM serotonin. St John's wort (hyperforin equivalent: 30–300 nM) and fluvoxamine (1–100 nM), fluoxetine (1–100 nM), paroxetine (0.1–10 nM) and sertraline (1–100 nM) inhibited synaptosomal uptake of [^{3}H]serotonin in a concentration-dependent manner and the inhibitory effect was in the order: paroxetine > sertraline > fluvoxamine > fluoxetine >> St John's wort. For St John's wort, both the IC50 for [^{3}H]serotonin uptake and the K_i for [^{3}H]paroxetine binding were expressed as the corresponding concentration of hyperforin calculated from the amount (3.2%) of this constituent (Table 1). IC50 values of SSRIs for [^{3}H]serotonin uptake were similar to their K_i values for [^{3}H]paroxetine binding. On the other hand, St John's wort exerted little inhibitory effect on specific [^{3}H]paroxetine binding in-vitro.

Effect of oral administration of St John's wort and SSRIs on specific [^{3}H]serotonin uptake and [^{3}H]paroxetine binding

The effect of oral administration of St John's wort and SSRIs on [^{3}H]serotonin uptake into mouse brain synaptosomes

Table 1 In-vitro inhibition by St John's wort and SSRIs of specific [^{3}H]serotonin uptake and [^{3}H]paroxetine binding in mouse brain

Drug	IC50 (nM) for [^{3}H]serotonin uptake	K_i (nM) for [^{3}H]paroxetine binding
St John's wort (by hyperforin equiv.)	179 ± 8 (4)	> 1000 (4)
Fluvoxamine	6.29 ± 1.40 (4)	5.52 ± 0.82 (3)
Fluoxetine	12.8 ± 1.2 (4)	10.8 ± 1.9 (3)
Paroxetine	0.17 ± 0.05 (4)	0.54 ± 0.04 (3)
Sertraline	3.11 ± 0.18 (4)	3.39 ± 1.25 (3)

Values for St John's wort are expressed as hyperforin content of the 3.2% extract. Values are mean ± s.e. of 3 or 4 mice (no. of replicates given in parentheses).

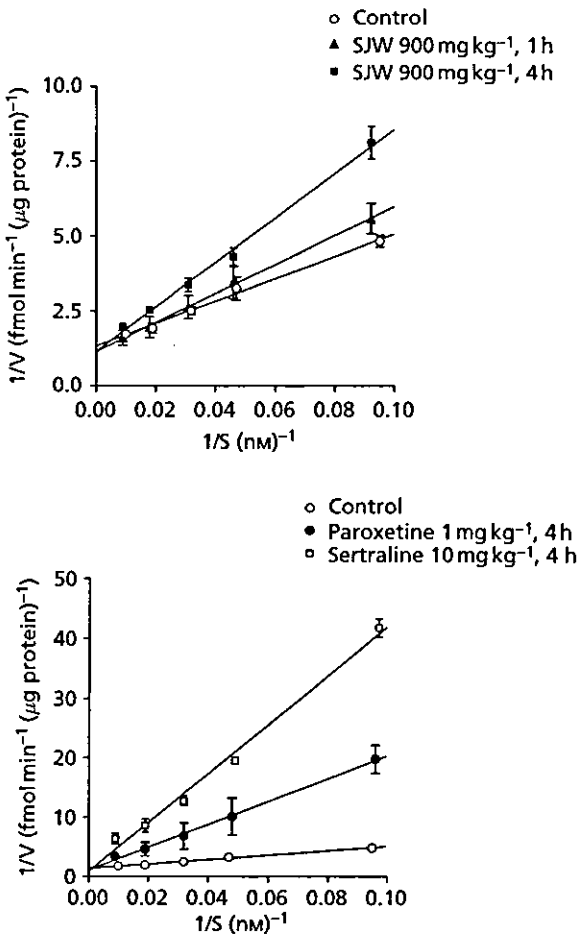

Figure 1 Lineweaver–Burk analysis for synaptosomal [^{3}H]serotonin uptake in mouse brain after oral administration of St John's wort (SJW), paroxetine and sertraline. Mice received St John's wort (900 mg kg^{-1}), paroxetine (1 mg kg^{-1}) or sertraline (10 mg kg^{-1}), and were exsanguinated by taking blood from the descending aorta at 1 or 4 h after the administration. Specific [^{3}H]serotonin (10–100 nM) uptake into brain synaptosomes was measured. Each point represents mean ± s.d. of 4 mice.

Table 2 Effect of oral administration of St John's wort and SSRIs on the K_m and V_{max} values for specific [^{3}H]serotonin uptake into mouse brain synaptosomes

Drug	n	Time (h)	K_m (nM)	V_{max} (fmol min^{-1} (μg protein)$^{-1}$)
Control	4		27.9 ± 1.2	0.75 ± 0.02
St John's wort 300 mg kg^{-1}	4	4	30.1 ± 0.1	0.82 ± 0.02
St John's wort 900 mg kg^{-1}	4	1	35.0 ± 1.0	0.82 ± 0.05
St John's wort 900 mg kg^{-1}	4	4	49.6 ± 4.2*	0.75 ± 0.02
St John's wort 900 mg kg^{-1}	3	12	27.4 ± 4.0	0.75 ± 0.06
Paroxetine 1 mg kg^{-1}	4	4	178 ± 37*	0.92 ± 0.06
Sertraline 10 mg kg^{-1}	4	4	208 ± 34**	0.57 ± 0.01*

Values are means ± s.e. of 3 or 4 mice. *$P < 0.05$, **$P < 0.01$, compared with control values.

Table 3 Effect of oral administration of St John's wort and SSRIs on K_d and B_{max} values for specific [^{3}H]paroxetine binding in mouse brain

Drug	n	Time (h)	K_d (nM)	B_{max} (fmol (mg protein)$^{-1}$)
Control	5		0.13 ± 0.01	292 ± 5
St John's wort 900 mg kg^{-1}	5	4	0.11 ± 0.01	272 ± 9
Paroxetine 1 mg kg^{-1}	4	4	1.96 ± 0.26*	204 ± 20*
Sertraline 10 mg kg^{-1}	4	4	2.07 ± 0.30*	272 ± 43

Values are means ± s.e. of 4 or 5 mice. *$P < 0.05$, compared with control values.

was examined. Synaptosomal [^{3}H]serotonin uptake was little affected by oral administration of St John's wort, at a dose of 300 mg kg^{-1} (Figure 1, Table 2) but was significantly suppressed by a dose of 900 mg kg^{-1}. In fact, the K_m values were enhanced by 25.4 and 77.8%, respectively, at 1 and 4 h after the St John's wort (900 mg kg^{-1}) administration compared with control values, the value at 4 h being statistically significant, with little change in V_{max} values. Similarly, paroxetine (1 mg kg^{-1}) and sertraline (10 mg kg^{-1}) brought about significant increase in K_m values for [^{3}H]serotonin uptake 4 h after their oral administration, the increase being 6.38 and 7.46 fold for paroxetine and sertraline, respectively. A significant (24%) decrease in the V_{max} value was also observed for sertraline.

Oral administration of paroxetine (1 mg kg^{-1}) and sertraline (10 mg kg^{-1}) produced significant (15.1 and 15.9 fold, respectively) increases in K_d values for specific [^{3}H]paroxetine binding in mouse brain at 4 h compared with the control value, and also a significant (30%) decrease in the B_{max} value (paroxetine) (Table 3). On the contrary, St John's wort (900 mg kg^{-1}) had little effect on K_d and B_{max} for brain [^{3}H]paroxetine binding.

Effect of St John's wort and SSRIs on marble-burying behaviour and immobility time in the forced swimming test

Oral administration of St John's wort (300, 900 mg kg^{-1}) suppressed marble-burying behaviour in mice in a dose-dependent manner (Figure 2). The decrease in the number of marbles buried by mice at 4 h was 26.2% after a dose of 300 mg kg^{-1}; after 900 mg kg^{-1} the decrease was 46.4, 95.2 and 29.8% at 1, 4 and 12 h, respectively (the decrease at 4 h

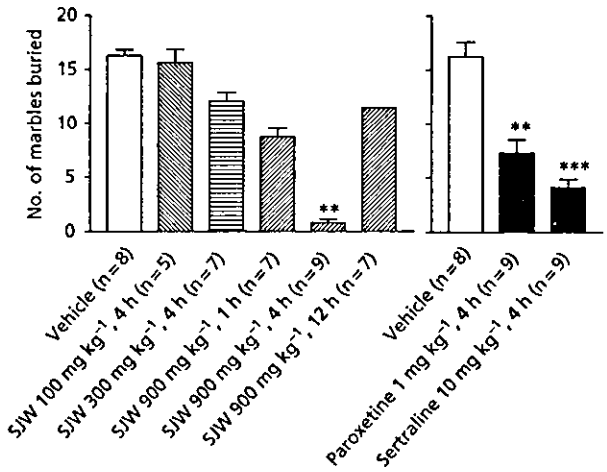

Figure 2 Effect of oral administration of St John's wort (SJW), paroxetine and sertraline on marble-burying behaviour in mice. Mice received St John's wort (100, 300 or 900 mg kg^{-1}), paroxetine (1 mg kg^{-1}) or sertraline (10 mg kg^{-1}) orally, and they were subjected to the marble-burying test 1–12 h later. Mice were placed into the open cubic plastic box (22.5 × 33.8 × 14.0 cm) where 20 clean glass marbles (15 mm diameter) were evenly spaced (5 cm apart) on sawdust (5 cm deep), and the number of marbles buried by mice during the testing period (30 min) was counted. Each column represents the mean ± s.e. of 5–9 mice. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with vehicle control values.

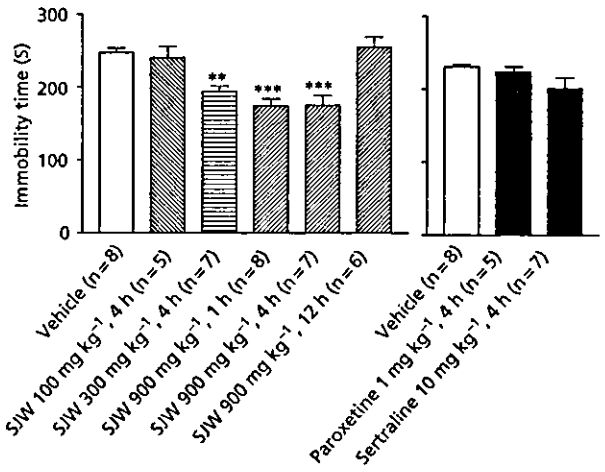

Figure 3 Effect of oral administration of St John's wort (SJW), paroxetine and sertraline on the immobility time of mice in the forced swimming test. Mice received St John's wort (100, 300 or 900 mg kg^{-1}), paroxetine (1 mg kg^{-1}) or sertraline (10 mg kg^{-1}) orally, and they were subjected to the forced swimming test 1–12 h later. Mice were dropped individually into glass cylinders (height: 25 cm, diameter: 10 cm) containing 10 cm of water, and left there for 6 min. A mouse was judged to be immobile when it floated in an upright position, and made only small movements to keep its head above water. The total immobility time of each mouse was expressed in seconds as the sum of immobility periods during the 6-min forced swimming test. Each column represents mean ± s.e. of 5–8 mice. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with vehicle control values.

after 900 mg kg^{-1} was statistically significant). Similarly, paroxetine (1 mg kg^{-1}) and sertraline (10 mg kg^{-1}) significantly attenuated the marble-burying behaviour at 4 h after oral administration (54.6 and 74.5%, respectively).

In the forced swimming test, there was significant decrease in the immobility time of mice after oral administration of St John's wort and the reduction rates were 21.8% (300 mg kg^{-1}, 4 h), 29.6 and 29.2% (900 mg kg^{-1}, 1 and 4 h, respectively) (Figure 3). On the other hand, the immobility time of the mice was unaffected by oral administration of paroxetine and sertraline. Locomotor activity counts during the testing period (30 min) in mice remained unchanged after oral administration of each drug compared with the vehicle control group (Table 4).

Table 4 Effect of oral administration of St John's wort and SSRIs on total activity in mice

Drug	n	Time (h)	Total activity (counts)
Control	7		1160 ± 40
St John's wort 100 mg kg^{-1}	5	4	1080 ± 40
St John's wort 300 mg kg^{-1}	6	4	1210 ± 50
St John's wort 900 mg kg^{-1}	6	1	1100 ± 90
St John's wort 900 mg kg^{-1}	5	4	1090 ± 50
St John's wort 900 mg kg^{-1}	5	12	1220 ± 70
Paroxetine 1 mg kg^{-1}	7	4	1150 ± 50
Sertraline 10 mg kg^{-1}	7	4	1160 ± 40

Values are means ± s.e. of 5–7 mice.

Discussion

The relationship between brain serotonin transporter binding, effects on serotonin uptake and behaviour in mice was investigated after oral administration of St John's wort, in comparison with SSRIs, to elucidate the pharmacological relevance of in-vitro observation. In in-vitro experiments, fluvoxamine, fluoxetine, paroxetine and sertraline inhibited both [^{3}H]serotonin uptake and [^{3}H]paroxetine binding in mouse brain at a nanomolar range. The calculated IC50 values for [^{3}H]serotonin uptake and the K_i values for [^{3}H]paroxetine binding of each SSRI became similar and there was a significant ($r = 0.98$, $P < 0.01$) correlation between both parameters.

On the contrary, St John's wort inhibited [^{3}H]serotonin uptake into mouse brain synaptosomes in a concentration-dependent manner but had little effect on [^{3}H]paroxetine binding, which is consistent with previous reports (Gobbi et al 1999; Singer et al 1999). The IC50 value expressed as hyperforin equivalent of 3.2% extract became 179 nM and it was similar to the value for hyperforin (205 nM) reported previously (Chatterjee et al 1998). These data suggest that the inhibitory effect of St John's wort on serotonin uptake into brain synaptosomes is not due to direct interaction with [^{3}H]paroxetine binding sites

on serotonin transporters, and hyperforin plays a major role as an active ingredient of this extract in-vitro.

A series of bioactive compounds, such as phenylpropanes, flavonol derivatives, biflavones, proanthocyanidines, xanthones, phloroglucinols, some amino acids, naphthodianthrones and essential oil constituents, have been identified as constituents contained in St John's wort (Nahrstedt & Butterweck 1997) and extensive scientific studies have contributed towards the elucidation of their pharmacological effects and mode of action (Butterweck 2003). However, the effects and the mechanism of action of these constituents are still a matter of debate and the pharmacological effects of St John's wort can not be explained by a single compound such as hyperforin, as previously described (Butterweck 2003; Butterweck et al 2003). Thus, based on recent reports, it is likely that synergistic and antagonistic interaction among the various ingredients is important and multiple bioactive compounds contribute to the antidepressive effects of St John's wort in a complex manner. Therefore, in this study, the effects of systemic administration of St John's wort (whole extract) on brain serotonin transporters, serotonin uptake and behaviour in mice were investigated, since the situation under the influence of pharmacokinetic and pharmacodynamic factors makes it possible to take not only its constituents but also its metabolites into consideration.

Oral administration of St John's wort ($900\,mg\,kg^{-1}$), paroxetine ($1\,mg\,kg^{-1}$) and sertraline ($10\,mg\,kg^{-1}$) produced a significant increase in K_m value for [^{3}H]serotonin uptake into mouse brain synaptosomes after 4 h. Paroxetine and sertraline significantly suppressed specific [^{3}H]paroxetine binding in mouse brain as revealed by a significant increase in K_d, whereas St John's wort had little effect on binding parameters for [^{3}H]paroxetine. Thus, in accordance with in-vitro experiments, our data suggest that SSRIs, but not St John's wort, suppress serotonin uptake into brain synaptosomes through binding to serotonin transporters and that none of the constituents or metabolites have a significant affinity for serotonin transporters in mouse brain after oral administration of St John's wort. Systemic administration of St John's wort ($125–500\,mg\,kg^{-1}$, p.o.) significantly increased serotonin levels in the cortex of rats and noradrenaline and dopamine levels in the diencephalon (Calapai et al 1999), and hyperforin alone ($10\,mg\,kg^{-1}$, i.p.) produced a significant increase in extracellular serotonin, noradrenaline, dopamine and glutamate in the rat locus coeruleus using microdialysis (Kaehler et al 1999). Nonselective inhibition by St John's wort of various neurotransmitter uptake into presynaptic neurons has been reported. According to the in-vitro observation, the mechanism underlying this inhibitory effect might be due to an increase in free intracellular sodium concentration by hyperforin (Singer et al 1999) through interaction with amiloride-sensitive sodium-conductive pathways such as the Na^+ channel and Na^+–H^+ exchanger (Wonnemann et al 2000). It is well known that the sodium gradient is the driving force of all neurotransmitter transporters (Lester et al 1994) and this property would result in nonselective uptake inhibition by St John's wort. Such nonspecific uptake inhibition of St John's wort, which may be one of the possible mechanisms contributing to the antidepressant effect, is supported by our observation that this extract inhibited synaptosomal uptake of serotonin without interacting with serotonin transporters.

The marble-burying test and forced swimming test are utilized to evaluate the therapeutic effects in obsessive–compulsive disorder and depression, respectively (Porsolt et al 1978; Njung'e & Handley 1991; Ichimaru et al 1995) and we investigated the potential behavioural activity of St John's wort in comparison with SSRIs. Oral administration of St John's wort ($900\,mg\,kg^{-1}$) significantly suppressed marble-burying behaviour in mice, with little change in locomotor activity, and the time-course of the suppression of this behaviour approximately paralleled that of [^{3}H]serotonin uptake inhibition. Similarly, significant attenuation of this behaviour was observed after oral treatment with paroxetine ($1\,mg\,kg^{-1}$) and sertraline ($10\,mg\,kg^{-1}$). In accordance with our results, the suppressive effect of St John's wort ($300\,mg\,kg^{-1}$, p.o.) on marble-burying behaviour in mice was reported by Skalisz et al (2004). We have previously found that brain serotonin transporter occupancy by SSRIs significantly correlated well with their suppressive effects on marble-burying behaviour after oral administration in mice (unpublished observation), suggesting that St John's wort exerts its suppressive effect on marble-burying behaviour through the activation of serotonergic neurotransmission. In the forced swimming test, St John's wort, but not SSRIs, significantly decreased the immobility time of mice without changing locomotor activity. The potential antidepressant activity of St John's wort in the forced swimming test is well known (Chatterjee et al 1998; Butterweck et al 2003) and the lack of efficacy of SSRIs on this behavioural model at the doses used here is also consistent with previous reports (Cervo et al 1991; Sánchez & Meier 1997). Systemic treatment with paroxetine ($3\,mg\,kg^{-1}$, s.c.) and sertraline ($10\,mg\,kg^{-1}$, s.c.) selectively increased the brain level of extracellular serotonin but not noradrenaline and dopamine (Bymaster et al 2002), and imipramine and desipramine, which have a high affinity for noradrenaline transporters, are utilized as positive controls in the forced swimming test (Egawa et al 1995; Sánchez & Meier 1997). Since St John's wort significantly suppressed both the marble-burying behaviour and the immobility time in the forced swimming test, it is plausible that St John's wort administered systemically enhances not only serotonergic neurotransmission but also noradrenergic neurotransmission to pharmacologically effective levels. Also, the involvement of other neurotransmitter pathways cannot be excluded (Calapai et al 1999; Kaehler et al 1999).

Conclusions

This study has shown that oral administration of St John's wort inhibits brain serotonin uptake without interacting with the transporter molecule. Furthermore, this extract effectively suppressed both the marble-burying behaviour and the immobility time in the forced swimming test in mice. These results provide the first in-vivo evidence to suggest that the mode of antidepressant action of St John's wort differs from that of SSRIs. Thus, this study may have a significant impact on phytotherapy with St John's wort.

References

Biber, A., Fischer, H., Römer, A., Chatterjee, S. S. (1998) Oral bioavailability of hyperforin from hypericum extracts in rats and human volunteers. *Pharmacopsychiatry* **31**: 36–43

Brenner, R., Azbel, V., Madhusoodanan, S., Pawlowska, M. (2000) Comparison of an extract of hypericum (LI 160) and sertraline in the treatment of depression: a double-blind, randomized pilot study. *Clin. Ther.* **22**: 411–419

Butterweck, V. (2003) Mechanism of action of St John's wort in depression: what is known? *CNS Drugs* **17**: 539–562

Butterweck, V., Christoffel, V., Nahrstedt, A., Petereit, F., Spengler, B., Winterhoff, H. (2003) Step by step removal of hyperforin and hypericin: activity profile of different *Hypericum* preparations in behavioral models. *Life Sci.* **73**: 627–639

Bymaster, F. P., Zhang, W., Carter, P. A., Shaw, J., Chernet, E., Phebus, L., Wong, D. T., Perry, K. W. (2002) Fluoxetine, but not other selective serotonin uptake inhibitors, increases norepinephrine and dopamine extracellular levels in prefrontal cortex. *Psychopharmacology* **160**: 353–361

Calapai, G., Crupi, A., Firenzuoli, F., Costantino, G., Inferrera, G., Campo, G. M., Caputi, A. P. (1999) Effects of *Hypericum perforatum* on levels of 5-hydroxytryptamine, noradrenaline and dopamine in the cortex, diencephalon and brainstem of the rat. *J. Pharm. Pharmacol.* **51**: 723–728

Cervo, L., Grignaschi, G., Rossi, C., Samanin, R. (1991) Role of central serotonergic neurons in the effect of sertraline in rats in the forced swimming test. *Eur. J. Pharmacol.* **196**: 217–222

Cervo, L., Rozio, M., Ekalle-Soppo, C. B., Guiso, G., Morazzoni, P., Caccia, S. (2002) Role of hyperforin in the antidepressant-like activity of *Hypericum perforatum* extracts. *Psychopharmacology* **164**: 423–428

Chatterjee, S. S., Bhattacharya, S. K., Wonnemann, M., Singer, A., Müller, W. E. (1998) Hyperforin as a possible antidepressant component of hypericum extracts. *Life Sci.* **63**: 499–510

Egawa, T., Ichimaru, Y., Imanishi, T., Sawa, A. (1995) Neither the 5-HT_{1A}- nor the 5-HT_2-receptor subtype mediates the effect of fluvoxamine, a selective serotonin reuptake inhibitor, on forced-swimming-induced immobility in mice. *Jpn. J. Pharmacol.* **68**: 71–75

Ernst, E., Rand, J. I., Barnes, J., Stevinson, C. (1998) Adverse effects profile of the herbal antidepressant St. John's wort (Hypericum perforatum L.). *Eur. J. Clin. Pharmacol.* **54**: 589–594

Gobbi, M., Valle, F. D., Ciapparelli, C., Diomede, L., Morazzoni, P., Verotta, L., Caccia, S., Cervo, L., Mennini, T. (1999) *Hypericum perforatum* L. extract does not inhibit 5-HT transporter in rat brain cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **360**: 262–269

Habert, E., Graham, D., Tahraoui, L., Claustre, Y., Langer, S. Z. (1985) Characterization of [^{3}H]paroxetine binding to rat cortical membranes. *Eur. J. Pharmacol.* **118**: 107–114

Ichimaru, Y., Egawa, T., Sawa, A. (1995) 5-HT_{1A}-receptor subtype mediates the effect of fluvoxamine, a selective serotonin reuptake inhibitor, on marble-burying behavior in mice. *Jpn. J. Pharmacol.* **68**: 65–70

Kaehler, S. T., Sinner, C., Chatterjee, S. S., Philippu, A. (1999) Hyperforin enhances the extracellular concentrations of catecholamines, serotonin and glutamate in the rat locus coeruleus. *Neurosci. Lett.* **262**: 199–202

Lester, H. A., Mager, S., Quick, M. W., Corey, J. L. (1994) Permeation properties of neurotransmitter transporters. *Annu. Rev. Pharmacol. Toxicol.* **34**: 219–249

Linde, K., Ramirez, G., Mulrow, C. D., Pauls, A., Weidenhammer, W., Melchart, D. (1996) St John's wort for depression – an overview and meta-analysis of randomized clinical trials. *BMJ* **313**: 253–258

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275

Malagié, I., Deslandes, A., Gardier, A. M. (2000) Effects of acute and chronic tianeptine administration on serotonin outflow in rats: comparison with paroxetine by using *in vivo* microdialysis. *Eur. J. Pharmacol.* **403**: 55–65

Nahrstedt, A., Butterweck, V. (1997) Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. *Pharmacopsychiatry* **30**: 129–134

Njung'e, K., Handley, S. L. (1991) Evaluation of marble-burying behavior as a model of anxiety. *Pharmacol. Biochem. Behav.* **38**: 63–67

Philipp, M., Kohnen, R., Hiller, K. O. (1999) Hypericum extract versus imipramine or placebo in patients with moderate depression: randomised multicentre study of treatment for eight weeks. *BMJ* **319**: 1534–1538

Porsolt, R. D., Anton, G., Blavet, N., Jalfre, M. (1978) Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur. J. Pharmacol.* **47**: 379–391

Rosenthal, H. E. (1967) A graphic method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.* **20**: 525–532

Sánchez, C., Meier, E. (1997) Behavioral profiles of SSRIs in animal models of depression, anxiety and aggression. Are they all alike? *Psychopharmacology* **129**: 197–205

Schrader, E. (2000) Equivalence of St John's wort extract (Ze 117) and fluoxetine: a randomized, controlled study in mild-moderate depression. *Int. Clin. Psychopharmacol.* **15**: 61–68

Singer, A., Wonnemann, M., Müller, W. E. (1999) Hyperforin, a major antidepressant constituent of St. John's Wort, inhibits serotonin uptake by elevating free intracellular Na^{+1}. *J. Pharmacol. Exp. Ther.* **290**: 1363–1368

Skalisz, L. L., Beijamini, V., Andreatini, R. (2004) Effect of hypericum perforatum on marble-burying by mice. *Phytotherapy Res.* **18**: 399–402

Volz, H. P. (1997) Controlled clinical trials of hypericum extracts in depressed patients—an overview. *Pharmacopsychiatry* **30**: 72–76

Wheatley, D. (1997) LI 160, an extract of St. John's wort, versus amitriptyline in mildly to moderately depressed outpatients – a controlled 6-week clinical trial. *Pharmacopsychiatry* **30**: 77–80

Woelk, H. (2000) Comparison of St John's wort and imipramine for treating depression: randomized controlled trial. *BMJ* **321**: 536–539

Wong, A. H., Smith, M., Boon, H. S. (1998) Herbal remedies in psychiatric practice. *Arch. Gen. Psychiatry* **55**: 1033–1044

Wonnemann, M., Singer, A., Müller, W. E. (2000) Inhibition of synaptosomal uptake of ^{3}H-L-glutamate and ^{3}H-GABA by hyperforin, a major constituent of St. John's Wort: the role of amiloride sensitive sodium conductive pathways. *Neuropsychopharmacology* **23**: 188–197

Yamada, S., Yamamura, H. I., Roeske, W. R. (1980) Characterization of alpha-1 adrenergic receptors in the heart using [^{3}H]WB4101: effect of 6-hydroxydopamine treatment. *J. Pharmacol. Exp. Ther.* **215**: 176–185

Lymphocyte and Plasma Vitamin C Levels in Type 2 Diabetic Patients With and Without Diabetes Complications

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Diabetes has been considered to be associated with oxidative stress. It has been suggested that increased free radicals and decline of antioxidant defense mechanisms induce diabetic micro- and macrovascular complications (1–3). Vitamin C is one of the major antioxidants and is detected in various blood components (4). However, measurements of vitamin C levels have shown inconsistent results, and the interpretation of vitamin C levels in diabetes as an antioxidant biomarker has not been clarified (5–8). In this study, we investigated the lymphocyte and plasma vitamin C levels in type 2 diabetic patients with and without diabetes complications.

RESEARCH DESIGN AND METHODS— Forty-one patients with type 2 diabetes (63 ± 8.9 years [mean ± SD]; 25 men and 16 women) attending the Department of Endocrinology and Metabolism at Shizuoka City Hospital were recruited. Type 2 diabetes was diagnosed according to the American Diabetes Association criteria. The duration of illness was 11 ± 8.3 years, fasting plasma glucose was 137 ± 43 mg/dl, and HbA_{1c} levels were 7.1 ± 1.0%. Twenty-six patients had diabetes complications with neuropathy, retinopathy, or nephropathy, and 15 patients had no complications. Both diabetic groups were matched by age, sex, fasting plasma glucose, and HbA_{1c} level (63 ± 9.7 years, 18 men and 8 women, 137 ± 45 mg/dl, and 7.2 ± 1.0% for diabetic patients with complications compared with 64 ± 7.5 years, 7 men and 8 women, 137 ± 42 mg/dl, and 6.8 ± 0.8% for diabetic patients without complications, respectively). The duration of illness was longer in the diabetic patients with complications than in diabetic patients without complications (13 ± 9.1 vs. 7.7 ± 5.2 years, respectively, $P = 0.051$). For the normal control subjects, 50 age- and sex-matched healthy volunteers (63 ± 5.7 years, 31 men and 19 women) were recruited. The participants taking vitamin supplements were excluded from the study. All participants gave informed consent before entering the study. The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at the hospital.

Blood samples were obtained by vein puncture in the morning while the patients were in the fasting state. Lymphocytes and plasma were prepared by centrifugation and the Ficoll gradients method, then immediately treated with metaphosphoric acid (final 5% wt/wt) to stabilize vitamin C (9,10). These processes were performed within 2 h under cooled conditions on ice to obtain reliable data. The vitamin C samples were stored at −80°C until analyzed, and the vitamin C (ascorbic acid, reduced form) levels were measured by high-performance liquid chromatography with the electrochemical detector method (11). All samples were handled and stored similarly in both diabetic patients and control subjects.

The lymphocyte and plasma vitamin C levels in type 2 diabetic patients were compared with those of the control subjects. The differences between the vitamin C levels in type 2 diabetic patients with and without diabetes complications were also studied. Statistical analysis was performed with the unpaired Student's *t* test to compare the data between diabetic patients and control subjects and between type 2 diabetic patients with and without diabetes complications. A P value <0.05 was considered significant.

RESULTS— The lymphocyte vitamin C level in diabetic patients was significantly lower than in control subjects (18 ± 4.5 vs. 28 ± 7.9 nmol/mg protein, $P < 0.0001$), whereas the plasma vitamin C level was not different (59 ± 19 vs. 53 ± 18 μmol/l, $P = 0.17$) (Fig. 1*A* and *B*). There were no significant linear correlations between the lymphocyte and plasma vitamin C levels in diabetic patients ($r = 0.011$, $P = 0.95$) as well as in control subjects ($r = 0.14$, $P = 0.35$). The lymphocyte vitamin C level in diabetic patients with complications was significantly lower than in those without complications (17 ± 3.3 vs. 21 ± 5.4 nmol/mg protein, $P = 0.011$) (Fig. 1*C*), whereas the plasma vitamin C level was not different (59 ± 18 vs. 59 ± 21 μmol/l, $P = 0.97$).

CONCLUSIONS— Increased oxidative stress in diabetes could contribute to depletion of antioxidants such as vitamin C (2,3). In this report, we demonstrated that the lymphocyte vitamin C level is significantly lower in type 2 diabetic patients, but we could not observe such an association in plasma vitamin C levels. The plasma concentration of vitamin C is considered to be strongly correlated with transient consumption of foods such as fruit, supplements, and vegetables (4).

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A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

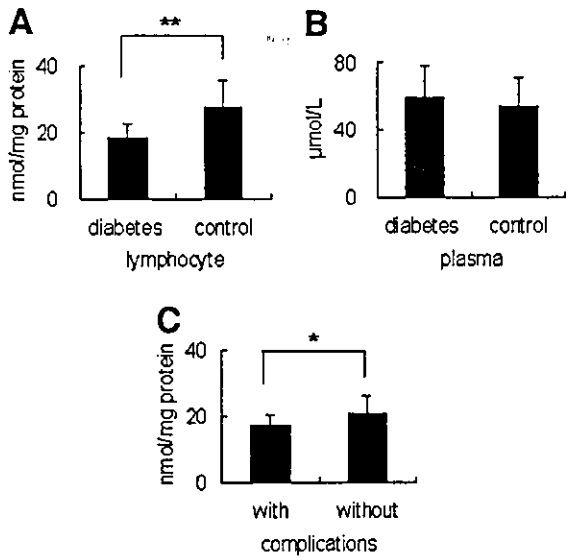

Figure 1—*Lymphocyte and plasma vitamin C levels in type 2 diabetic patients* (n = *41*) *and control subjects* (n = *50*). *A: Lymphocyte vitamin C level in diabetic patients was significantly lower than that in the control subjects* (**P < *0.0001*). B: *Plasma vitamin C level in diabetic patients was not different from that in the control subjects* (P = *0.17*). *C: Lymphocyte vitamin C level in diabetic patients with complications* (n = *26*) *was significantly lower than that in those without complications* (n = *15*) (*P = *0.011*). *The horizontal bars represent the mean ± SD.*

Compared with plasma, lymphocyte has been reported to maintain a vitamin C concentration as large as 80- to 100-fold across the plasma membrane (12,13) and to have cell-membrane transporting mechanisms between vitamin C and glucose (14,15). In diabetes, therefore, the measurement of lymphocyte vitamin C might be expected to be a more reliable antioxidant biomarker than plasma vitamin C level.

It is unclear whether leukocyte vitamin C correlates with diabetes complications. VanderJagt et al. (5) reported that vitamin C levels in mononuclear leukocytes were decreased in the whole group of type 1 diabetic patients compared with control subjects but were not different between patients with and without long-term complications. We showed the significant lower lymphocyte vitamin C levels in patients with type 2 diabetes with complications compared with those without complications. However, the results should be interpreted carefully because of the small sample size and because the differences of lymphocyte vitamin C level among different diabetes complications are not fully clarified. Further studies are required to investigate the precise correlations of lymphocyte vitamin C with duration or severity of diabetes and to establish the clinical usefulness of lymphocyte vitamin C level as a biomarker in developing diabetes complications.

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References

1. Giugliano D, Ceriello A, Paolisso G: Oxidative stress and diabetic vascular complications. *Diabetes Care* 19:257–267, 1996
2. Maritim AC, Sanders RA, Watkins JB 3rd: Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol* 17: 24–38, 2003
3. Hasanain B, Mooradian AD: Antioxidant vitamins and their influence in diabetes mellitus. *Curr Diab Rep* 2:448–456, 2002
4. Omaye ST, Schaus EE, Kutnink MA, Hawkes WC: Measurement of vitamin C in blood components by high-performance liquid chromatography: implication in assessing vitamin C status. *Ann N Y Acad Sci* 498:389–401, 1987
5. VanderJagt DJ, Harrison JM, Ratliff DM, Hunsaker LA, Vander Jagt DL: Oxidative stress indices in IDDM subjects with and without long-term diabetic complications. *Clin Biochem* 34:265–270, 2001
6. Cunningham JJ, Ellis SL, McVeigh KL, Levine RE, Calles-Escandon J: Reduced mononuclear leukocyte ascorbic acid content in adults with insulin-dependent diabetes mellitus consuming adequate dietary vitamin C. *Metabolism* 40:146–149, 1991
7. Sinclair AJ, Taylor PB, Lunec J, Girling AJ, Barnett AH: Low plasma ascorbate levels in patients with type 2 diabetes mellitus consuming adequate dietary vitamin C. *Diabet Med* 11:893–898, 1994
8. Schorah CJ, Bishop N, Wales JK, Hansbro PM, Habibzadeh N: Blood vitamin C concentrations in patients with diabetes mellitus. *Int J Vitam Nutr Res* 58:312–318, 1988
9. Margolis SA, Davis TP: Stabilization of ascorbic acid in human plasma, and its liquid-chromatographic measurement. *Clin Chem* 34:2217–2223, 1988
10. Umegaki K, Yoshimura M, Nishimuta M, Esashi T: A practical method for determination of vitamin C in plasma by high-performance liquid chromatography with an electrochemical detector. *J Jpn Soc Nutr Food Sci* 2:107–111, 1999
11. Washko PW, Hartzell WO, Levine M: Ascorbic acid analysis using high-performance liquid chromatography with coulometric electrochemical detection. *Anal Biochem* 181:276–282, 1989
12. Bergsten P, Amitai G, Kehrl J, Dhariwal KR, Klein HG, Levine M: Millimolar concentrations of ascorbic acid in purified human mononuclear leukocytes: depletion and reaccumulation. *J Biol Chem* 265: 2584–2587, 1990
13. Evans RM, Currie L, Campbell A: The distribution of ascorbic acid between various cellular components of blood, in normal individuals, and its relation to the plasma concentration. *Br J Nutr* 47:473–482, 1982
14. Bergsten P, Yu R, Kehrl J, Levine M: Ascorbic acid transport and distribution in human B lymphocytes. *Arch Biochem Biophys* 317:208–214, 1995
15. Ngkeekwong FC, Ng LL: Two distinct uptake mechanisms for ascorbate and dehydroascorbate in human lymphoblasts and their interaction with glucose. *Biochem J* 324:225–230, 1997

日常診療からみた食の安全性

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要　旨

最近、食品の疾病予防や治療的な効果が注目され、機能性食品として保健機能食品および“いわゆる健康食品”が一般に急速に広まりつつある。それに伴い日常診療においても補完代替医療の一つとして、食の安全性や有効性に関する情報を充分に吟味して診療にあたる必要性に迫られることが多くなってきている。食の安全性に関しては食品自体による有害反応や医薬品との相互作用が問題となるが、食品は医薬品と異なり、法に基づいた監視規制が弱く、かつ情報収集体制の整備も遅れている。また個人の体験談や専門家の意見などが主流を占めているのが現状であり、より科学的で信頼性ある確かなエビデンスとして提供されているものが極めて少ない。現在、国立健康・栄養研究所が主体となり、健康食品等の安全性と有効性に関する科学的なデータベースの作成が進んでおり、医療従事者のみならず一般消費者にとっても貴重な情報提供源となっていくものと期待される。

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<Summary>

Recently functional foods have been spreading out rapidly to the public in Japan because of their anticipated effects on the disease prevention as complementary medicine. Therefore, it is essential for medical practitioners to examine closely enough the information on the safety and effectiveness of functional foods. Food safety should be considered as an adverse reaction by food itself or an interaction with co-administered drugs. However the national regulations against food safety are less strict compared to those of the registered drugs and the organizing system of collecting safety information has not been satisfactory achieved yet. As a result, there has been extremely few information available as reliable clinical evidences, and individual experiences or an opinion of a clinical specialist influence the decision of taking functional foods in the public. The National Institute of Health and Nutrition has constructed recently the scientific database system on the safety and effectiveness of functional foods and the database is expected to become a valuable information source for citizen as well as medical practitioners.

Food Safety from a Viewpoint of Clinical Practice

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1. はじめに

医師という職業柄、日常診療で患者と接していると、健康食品に関する患者からの訴えが最近非常に増えていることに気づく。その中には、「糖尿病にカテキンが効くって本当ですか？」といった効能的な質問から、「がんになった知人が通信販売でアガリスクを買って服用しているが、副作用が出ないかと心配している」とか、「スリムになりたくて痩せ薬を中国から個人輸入してみたが、飲んでも大丈夫だろうか？」といった安全性に関する疑問も多く聞かれる。診療現場でこのような状況が多く発生するようになってきた背景には、昨今の食品の疾病予防や治療的な効果に対する期待から、機能性食品として保健機能食品および“いわゆる健康食品”が一般に急速に広まってきたことが多大に影響していると思われる。それに伴い、従来は健康食品の使用に対してあまり関心を示さなかった医師においても、補完代替医療の一つとして、食の安全性や有効性に関する情報を充分に吟味して診療にあたる必要性に迫られるようになってきている。本稿では臨床医の立場から、臨床研究のエビデンスを踏まえ、最近話題になっている健康食品等の安全性情報を提示しつつ、食の安全性について考えてみたい。

2. 臨床研究とクリニカル・エビデンス

クリニカル・エビデンスに基づいた臨床研究と臨床試験の位置づけを略図で示す（図1）。臨床研究は人を対象とした研究全てを包含しており、その中には臨床試験以外に、症例報告や疫学的な観察研究、コホート研究など

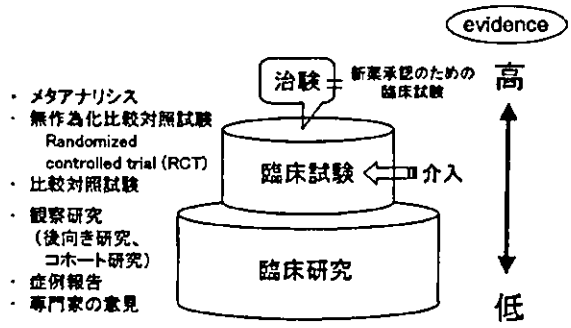

図1 臨床研究とエビデンス・レベル
Figure 1 Clinical research and the level of evidences

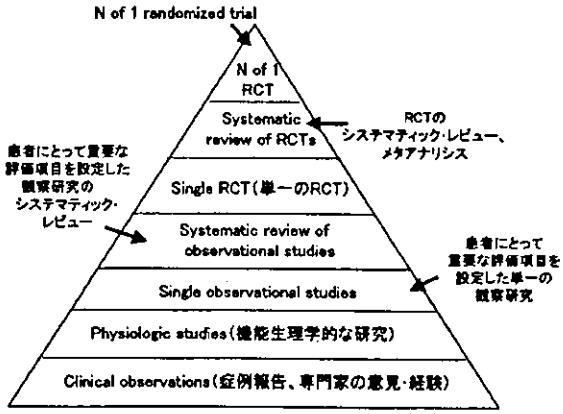

図2 エビデンスの強さによるヒエラルキー（文献1より改変）
Figure 2 A hierarchy of strength of evidence

が含まれる。臨床試験は、人為的な介入を行い前向きに評価項目を設定して行う臨床研究であり、専門家の意見や症例報告よりも科学的なエビデンスとしては上位に位置づけられる[1]。“治験”は新薬承認のために行う臨床試験である。臨床試験の中でさらにエビデンスが高いのが、介入群と非介入（対照）群への割付けを無作為に行う無作為化比較対照試験（randomized controlled trial, RCT）であり、またRCTをシステマティック・レビューしたメタ・アナリシスである（図2）。

健康食品等の安全性や有効性に関するエビデンスのレベルをみてみると、その多くは個人の体験談や専門家の意見などが主流を占めているのが現状であり、より科学的で信頼性ある確かなエビデンスとして提供されているものは極めて少ない。保健機能食品の中で機能性食品として国が個別に認めている特定保健用食品（いわゆるトクホ）は最もエビデンスを重視しているが、その認可基準は医薬品と比較し非常に緩やかであり、長期投与の安全性や医薬品との相互作用に関するデータは要求されていない[2,3]。今後、食の安全性を更に重視する観点からも、エビデンスの高い臨床試験により検証していく必要がある。

3. 健康食品等による有害反応

健康食品等の有害反応の具体例について、その発生原因に基づき、医薬品成分の違法添加、健康食品の成分自体によるもの、さらに併用医薬品との相互作用に分類して提示する（図3）。