Component-resolved diagnosis in egg allergy

The term component-resolved diagnosis has been coined to designate diagnostic tests based on pure allergen molecules which are produced either by recombinant expression of allergen-encoding cDNAs or by purification from natural allergen sources [16]. Measurement of specific IgE antibodies to individual egg white components could be of importance in predicting different disease manifestations in egg-allergic patients.

Allergenic components of egg white

Egg white of the domestic chicken (*Gallus domesticus*) represents the albumin fraction of the egg and contains more allergenic proteins than the yolk. Egg white contains more than 20 different glycoproteins, most of which have been purified. Ovomucoid (OVM) (Gal d 1, 11%), ovalbumin (OVA) (Gal d 2, 54%), ovotransferrin (Gal d 3, 12%) and lysozyme (Gal d 4, 3.4%) [17,18] have been identified as major allergens [19–21] (Table 1). Studies in humans utilizing Radio Allergo Sorbent Test (RAST) reported the order of allergenicity as ovomucoid > ovalbumin > ovotransferrin > lysozyme [22**]. In addition, two new allergen candidates in egg white have been identified recently: egg white cystatin and lipocalin-type prostaglandin D synthase (L-PGDS) [23].

Ovomucoid

Although OVA is the most abundant protein in egg white, OVM has been shown to be the dominant allergen in egg [22**,24–26]. OVM is a highly glycosylated molecule containing 186 amino acid residues and is known to exhibit a trypsin inhibitor activity [27]. In two different studies [18,28**], children with persistent egg allergy had significantly higher specific IgE levels to OVM than children who outgrew their egg allergy. A favorable prognosis was associated with the absence or a decline in OVM-specific IgE titers [18]. The allergenicity of proteins depends mostly, but not exclusively, on their resistance to heat and digestive enzymes [29], reflecting their capacity to stimulate a specific immune response [17]. The importance of OVM in egg allergy may be due to its unique characteristics such as relative stability against heat [30] and digestion with proteinases

Key points

- Although molecular-based technologies are promising to improve the diagnosis of egg allergy, oral food challenge will still be necessary for many patients.
- Measurement of specific IgE to individual egg white components has been shown to predict different clinical patterns of egg allergy.
- Specific IgE to ovomucoid, considered as the immunodominant allergen in hen's egg white, has been shown to be a risk factor for persistent allergy and indicates that neither raw nor heated egg is likely to be tolerated.
- Although specific IgG4 to OVM and OVA has been shown to have no value in the diagnostic of egg allergy, specific ratio IgE/IgG4 might be utilized as a marker in following the development of tolerance and resolution of egg allergy.
- Peptide-based microarray immunoassays are currently under development and epitope profiling of egg white allergens could radically improve the diagnosis of egg allergy.

[22**,31,32], compared with other egg white components. This is possibly related to the presence of strong disulfide bonds that stabilize the protein [26].

An earlier study concluded that IgE binding activity to pepsin-digested OVM was of diagnostic value for distinguishing the food challenge-positive patients from negative patients, and that patients with high IgE-binding activity to pepsin-treated OVM were unlikely to outgrow egg allergy [33]. From another point of view, gastric digestion has been demonstrated to reduce the allergenicity of OVM [34], which can explain why some patients have skin contact reactions to egg, but not ingestion reactions [35]. Significant differences in specific IgE to OVM were found in egg-allergic patients, depending on the reactivity to raw and heated egg, in which low levels of specific IgE to OVM were associated with tolerance to heated egg [22**]. Furthermore, it has been suggested that quantification of specific IgE to OVM could be useful in guiding the physician in the decision

Table 1 Major egg white allergens[a]

Allergen	Common name	Constitute (%)	Mw (kDa)	Carbohydrate (%)	Heat-treated	Digestive enzyme-treated	Allergenic activity	Test code[b] (in-vitro tests)
					IgE binding activity			
Gal d 1	Ovomucoid	11	28	25	Stable	Stable	+++	f233
Gal d 2	Ovalbumin	54	45	3	Unstable	Unstable	++	f232
Gal d 3	Ovotransferrin/conalbumin	12	76.6	2.6	Unstable	Unstable	+	f323
Gal d 4	Lysozyme	3.4	14.3	0	Unstable	Unstable	++	k208

[a] Reproduced with authorization from Benhamou AH, state of the art for egg allergy, Allergy 2010, 65:283–289.
[b] Test code for in-vitro UniCAP system.

whether to perform a challenge or not. Recently published data suggest that a concentration of specific IgE to OVM higher than approximately 11 kU$_A$/l (positive decision point based on 95% clinical specificity) indicates a high risk of reacting to heated egg as well as raw egg. At the same time, a concentration lower than approximately 1 kU$_A$/l (negative decision point, based on 95% clinical sensitivity) means that there is a low risk of reaction to heated egg, even if the patients might well react to raw egg [36**]. Lemon-Mule et al. [37*] investigated immunologic changes associated with ingestion of baked egg in children with egg allergy. Greater levels of specific IgE to OVM were found in children reacting to baked egg (baked with wheat flour in a form of a muffin or a waffle) compared with patients tolerant to baked egg and lightly cooked egg (e.g. French toast). However, in this study, only very high level of specific IgE to OVM (>50 kU$_A$/l) was highly predictive of heated egg reactivity. This might be explained by the so-called matrix effect [38,39], because egg was baked with wheat matrix. Kato et al. [40] previously showed a decreased solubility of OVM when egg was mixed with wheat flour and wheat gluten and heated, suggesting that OVM forms complexes with gluten leading to aggregation and insolubilization, and potentially decreased digestibility. Further studies are required to confirm the utility of specific IgE to OVM in predicting symptomatic egg allergy.

Ovalbumin

Ovalbumin is a phosphorylated glycoprotein with unknown biological function [41]. Its complete sequence of 385 amino acids has been determined [42]. Debate had flourished over the immunodominance of OVA as the major egg allergen; however, it has been shown that the use of contaminated commercial OVA led to an overestimation of its dominance as a major egg allergen in egg-sensitive patients [18]. A recent study [43] using experimental ImmunoCAP test confirmed that commercially available OVA contains a considerable amount of OVM as well as some ovotransferrin. In this study, a very sensitive affinity purification method with monoclonal chimeric antibodies was applied to reduce contamination with other allergens.

Several studies found higher specific IgE to OVA in egg-sensitized and egg-allergic patients compared with non-allergic controls [36**,37*,43]. In contrast to OVM, OVA is heat-labile and undergoes conformational changes to form more stable, and possibly less allergenic, S-ovalbumin upon exposure to elevated temperature [41,44]. This means that the IgE-binding epitopes on OVA might be destroyed after heating, suggesting that children who have specific IgE primarily to OVA are likely to tolerate heated egg [22**,32,45]. A recent study [30] investigated the T-cell immunogenicity of chemically glycated ovalbumin termed advanced glycation end products (AGEs),

produced by the Maillard reaction that occurs between reducing sugars and proteins during thermal processing of foods. The glycation structures of AGEs are suggested to function as pathogenesis-related immune epitopes in food allergy. Interestingly, T-cell immunogenicity of OVA was enhanced by the Maillard reaction, indicating a critical role for thermal processing in allergenicity of OVA.

Ovotransferrin and lysozyme

Ovotransferrin (also called conalbumin) is a nonheme, iron-binding, acute-phase glycoprotein in egg white [27]. As OVA, ovotransferrin is a heat-labile allergen, but it was reported that when coupled to bivalent or trivalent metal ions, it could form heat-stable complexes [46]. However, little scientific evidence is available currently regarding the direct relationships of the heavy metals in egg and egg allergy. Although ovotransferrin is considered to be a major allergen in egg white, the role of specific IgE antibody to ovotransferrin in the diagnosis of egg allergy has not been determined. Lysozyme is a glycosidase commonly used as a food preservative due to its antibacterial properties, in some pharmaceuticals and foods (e.g. eye drops and cheese) [27]. Egg-allergic individuals sensitized to lysozyme may therefore react when exposed to such products [47,48]. Moreover, being widely used in the food and pharmaceutical industry, lysozyme is also considered an important occupational allergen, causing asthma via the inhalation route [49,50].

Allergenic components of egg yolk

The main allergen in egg yolk, chicken serum albumin, also called alpha-livetin (Gal d 5), is thought to be involved in the bird-egg syndrome [51,52]. In this syndrome, the primary sensitization is to airborne bird allergens with the secondary sensitization or cross-reactivity with albumin in egg yolk (Gal d 5). These patients experience respiratory symptoms such as rhinitis and/or asthma with bird exposure and immediate allergic symptoms with egg ingestion [52,53]. Testing-specific IgE to Gal d 5 might therefore confirm the diagnosis of bird egg syndrome. Several other potential allergens have been identified in egg yolk, including vitellenin (apovitellenin I) and apoprotein B (apovitellenin VI), although their roles in egg allergy remain unclear.

Microarray-based component-resolved diagnosis

Protein microarray has recently become available for measuring specific IgE and commercialized in the form of the ImmunoCAP-ISAC or Immuno Solid phase Allergen Chip (VBC Genomics-Vienna, Austria; Phadia, Uppsala, Sweden) [54,55]. It currently has 103 native/recombinant component allergens from 43 allergen sources and includes nGal d 1, nGal d 2, nGal d 3 and nGal d 5. This technology has two main advantages: it assesses simultaneously specific IgE to different

components and requires small amounts of serum, which is especially relevant in children. Moreover, Immuno-CAP-ISAC can be considered as a cost-efficient approach, as it delivers results for over 100 components. Ott *et al.* [56**] published the first study on the clinical performance of a component-based microarray with respect to the outcome of the OFC in suspected egg allergy. No advantage was found compared with the current diagnostic tests, that is skin prick test and specific IgE to egg white. However, a recent study [57*] suggested that the protein microarray has a good ability to predict the OFC results in egg-allergic children and could be used as a second-level assay, if the Immuno-CAP-specific IgE to egg white is less than 95% clinical decision points. In this study, this led to a decrease in the number of OFCs to be performed, as well as of positive OFCs with a subsequent decrease in severe reaction risk. Discrepancies between these two studies are probably due to difference in patient selection [57*]. Further large-scale studies are warranted before the protein microarray can be introduced into routine management of patients with egg allergy.

Potential role of ovalbumin and ovomucoid-specific IgG4 in the diagnosis of egg allergy

It was recently demonstrated that specific IgG4 does not add additional value to IgE measurement in the diagnostic procedure of egg allergy [58*]. This is in contrast to immunotherapy trials in which increase in specific IgG4 levels is associated with acquisition of tolerance. Protective or blocking functions for this subclass are assumed [59,60]. Because the balance between allergen-specific IgE and IgG4 production may have an impact on whether clinical allergy or tolerance develops, the determination of the ratio of specific IgE/IgG4 antibodies might be superior to the absolute amount of IgG4 for assessing an ongoing status of egg sensitization. Measurements of specific ratio IgE/IgG4 to OVA and/or OVM have been shown to be useful in following the development of oral tolerance and outgrowing egg allergy in the research studies [37*,61]. However, measurement of specific

IgG4 has not been validated sufficiently to be used in clinical practice.

Role of epitope mapping in the diagnosis of egg allergy

Food allergens must at least partially survive digestion and absorption from the gastrointestinal tract to be immunogenic. This fact has led to the hypothesis that individuals who generate IgE antibodies recognizing a greater number or a specific pattern of sequential epitopes (e.g. those not easily destroyed by denaturation and partial digestion) are more likely to have clinical allergy rather than asymptomatic IgE sensitization [62]. There have been a few studies on the IgE-binding epitopes in OVM, and the reported binding sites resemble each other (Table 2) [26,27,28**,63]. Egg white-specific IgE antibodies that recognize sequential or conformational epitopes of OVM and OVA can distinguish different clinical phenotypes of egg allergy. It has been shown that egg-allergic patients with IgE antibodies reacting against sequential epitopes tended to have persistent allergy, whereas those with IgE antibodies primarily reacting against conformational epitopes tended to have transient allergy [26,28**]. In the study by Jarvinen *et al.* [28**], seven patients with persistent egg allergy had IgE that recognized four sequential epitopes of OVM. In contrast, none of the 11 children with transient egg allergy had specific IgE to these epitopes. These observations were supported by a separate study [22**] in which sera obtained from patients with persistent egg allergy had high IgE-binding activity to pepsin-treated OVM.

In the past, epitope mapping was mainly performed using SPOT membrane-based immunoassays [64–66] in which the peptides were synthesized on the nitrocellulose membrane and then incubated with the patient's sera. However, synthesis of large numbers of peptides is relatively error-prone, time-consuming, labor-intensive and expensive, and has limitations because of the specific chemistry of the method. A large volume of serum is

Table 2 Sequential IgE-binding epitopes of ovomucoid (Gal d 1)

	Ref.	Ref. no.	Year	IgE epitope				
Domain 1	Cooke and Sampson	[26]	1997	AA 1-20				AA 49-56
	Jarvinen *et al.*	[28**]	2007	AA 1-10	AA 9-20			AA 47-56
	Holen *et al.*	[63]	2001	AA 1-14	AA 11-24	AA 31-44		AA 51-64
	Mine and Wei Zhang	[67]	2002			AA 32-42	AA 40-50	AA 56-66
Domain 2	Cooke and Sampson	[26]	1997		AA 85-96		AA 115-122	
	Jarvinen *et al.*	[28**]	2007				AA 113-124	
	Holen *et al.*	[63]	2001	AA 61-74		AA 101-114	AA 121-134	
	Mine and Wei Zhang	[67]	2002	AA 71-75	AA 80-90	AA 101-105	AA 121-130	
Domain 3	Cooke and Sampson	[26]	1997		AA 175-186			
	Jarvinen *et al.*	[28**]	2007					
	Holen *et al.*	[63]	2001					
	Mine and Wei Zhang	[67]	2002		AA 159-174	AA 179-186		

AA, amino acid; Ref., reference.

required, and there is also a limitation of the number of targeted peptides. With the development of microarray technology and evolution in peptide synthesis techniques, peptide microarray-based immunoassays for epitope mapping of egg allergens could be the next step. Indeed, analyzing epitope-specific binding with this assay may further increase the positive predictive value of laboratory tests, provide information on the natural history of egg allergy, that is whether the patients may outgrow their allergy, and perhaps provide information on the potential severity of the allergic reaction to egg. Also, characterization of IgE epitopes of egg allergens is of fundamental importance in the design of immunotherapeutics.

Conclusion

Molecular diagnosis technologies will improve diagnosis of IgE-mediated egg allergy. Measurement of specific IgE antibodies to individual egg white components has been shown to predict different clinical patterns of egg allergy. Component-resolved diagnosis based on a microarray platform is especially promising. However, a better purification of individual allergens is required in order to avoid contamination and overestimation of specific IgE level to different egg allergens (components). On the basis of data from other food allergens, peptide microarray-based immunoassay could facilitate determination of egg allergy phenotypes. This test is currently under development. None of these molecular-based tests is ready to be used in clinical practice and an oral food challenge will still be necessary in many patients for the diagnosis of egg allergy.

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Original Paper

Effects of Oral Administration of *Lactobacillus acidophilus* L-92 on the Symptoms and Serum Markers of Atopic Dermatitis in Children

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Abstract
Background: Few studies have investigated the complementary effects of long-term oral administration of *Lactobacillus acidophilus* on traditional medical therapy in the treatment of patients with atopic dermatitis (AD). *Methods:* The Atopic Dermatitis Area and Severity Index was used to evaluate AD severity. Symptom severity was assessed using the symptom score. The effect of medical therapy was evaluated by adding the medication score, calculated as the sum of each product of the amount of steroid ointment used for therapy and its designated strength graded on a 4-point scale, to the symptom score. The complementary effect of long-term oral administration of *L. acidophilus* strain L-92 (L-92) as a probiotic or biogenic strain in patients with AD was evaluated using the symptom-medication score, which was calculated as the sum of the symptom score and medication score. Both a preliminary casuistic study and a double-blind-ed, placebo-controlled study were performed to evaluate the effects of L-92 on the symptoms of AD in children. *Results:* Orally administered L-92 significantly ameliorated the symptoms of AD in Japanese children. L-92 also affected the serum concentrations of thymus and activation-regulated chemokine in a time-dependent manner. *Conclusions:* The results of the preliminary trial and the double-blinded, placebo-controlled study revealed a complementary effect of oral L-92 on the standard medical therapy (topical application of a steroid ointment) in patients with AD that was mediated, at least in part, by alterations in the Th1/Th2 balance.

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Introduction

Factors influencing immune regulation, including intervention with probiotics [1, 2] or biogenics [3] to reduce the microbial burden, have been implicated in the manifestation of allergic diseases. Atopic dermatitis (AD) is a commonly encountered chronic inflammatory disease of the skin that affects 0.3–20% of children worldwide and

is characterized by relapsing pruritic eczema [4]. The prevalence of atopic diseases in children has increased steadily in developed countries, significantly impacting health care resources and triggering extensive research in the field of atopic dermatitis. In addition, the discomfort suffered by patients with AD is significant.

Genetic predispositions to epidermal barrier dysfunction [5] and atopic diathesis [6] are thought to be the main factors involved in the etiology of AD. However, a number of cases show complete healing in the infantile phase, and improvement around puberty is also common [7]; therefore, it has been suggested that environmental factors may also play a critical role in determining the manifestations of AD. Kalliomaki et al. [8] first demonstrated the influence of probiotics isolated from the gut microbiota in reducing the incidence of AD in children. Prophylactic intervention using gut microbiota has been carried out based on the 'hygiene hypothesis' [9], which is based on the observed differences among countries in the prevalence of diseases in populations with similar genetic backgrounds. Such interventions are considered a kind of supplement for the frequent infections that do not occur during development in well-developed countries.

More recently, according to the revised hygiene hypothesis [10], altering the intestinal colonization pattern during infancy has an impact on the immune system. Manipulation of the intestinal microflora using pro-, pre- or synbiotics, or more directly with biogenics, is an innovative way to prevent or treat AD. However, there is little information on the activity or effectiveness of different genera, species and strains of lactic acid bacteria that are used as probiotics or biogenics. In addition, the effectiveness of this type of intervention also remains controversial [11]. The use of effective probiotics or biogenics is quite beneficial as supportive therapy for both AD patients and their families because of their negligible side effects and potential to cure the disease.

Lactobacilli are the most frequently examined probiotics with efficacy in the management of allergic diseases. It is hypothesized that these probiotics have immunoregulatory properties and induce mucosal tolerance, mediated in part by their immunoregulatory functions. *Lactobacillus acidophilus* strain L-92 (L-92) has been used as a probiotic or biogenic strain in Japan. This strain has been reported to demonstrate antiallergic effects in patients with an allergy to Japanese cedar pollen [12] or perennial allergic rhinitis [13].

While the mechanism underlying the antiallergic effects of this probiotic in these clinical trials remains unknown, the effect of L-92 on the immunologic response has been gradually clarified over time. When administered orally, L-92 lowers the level of allergen-specific immunoglobulin E (IgE) in the blood [14]. In addition, L-92 has been shown to stimulate IL-12 production from dendritic cells (DC) and to induce the generation of T helper type 1 (Th1) cells from naïve T cells [15]. These phenomena suggest that L-92 might exert its effect, at least in part, by suppressing Th2 responses through the activation of Th1 cells. Another proposed mechanism is that L-92 might attenuate CD4+ T cell responses by inducing DC-mediated apoptosis, and this might be beneficial in the treatment of allergic diseases resulting from CD4+ T cell hyperresponsiveness, especially Th2 cells. Furthermore, heat-killed, lyophilized L-92 stimulates Peyer's patch (PP) cells to produce high levels of TGF-β and IgA simultaneously [14]. L-92 has also been suggested to induce regulatory T (Treg) cells in the PP through the possible activation of DC. This might be involved in the attenuation of the excessive activation of CD4+ T cells observed in mice immunized repeatedly with ovalbumin (OVA) [16, 17].

In this study, we examined the safety and beneficial effects of L-92 as a probiotic or biogenic food ingredient in children with AD.

Materials and Methods

Subjects and Study Design
Preliminary Casuistics
The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Kami-iida Dai-ichi General Hospital. The preliminary study was performed from the first week of November 2004 to the end of December 2005 and used a commercialized fermented milk product (FM) produced with viable L-92 (containing approx. 3×10^{10} colony-forming units of the bacterium; Calpis, Kanagawa, Japan); this study was an open trial on 20 children (age 4–15 years) who had been referred to the Department of Pediatrics, Kami-iida Dai-ichi General Hospital, Yamada Clinic or the Department of Pediatrics, Banbuntane Hotokukai Hospital, Fujita Health University for suspected AD that was not complicated by an allergy to cow's milk. These facilities are located in the Aichi prefecture in Japan.

Patients were enrolled by members of a site management organization (Ethic Co. Ltd., Tokyo, Japan) who were not involved in the casuistic study. We explained the aim and protocol of the casuistic study to the patients and their parents, asked if they were willing to participate, and screened the patients' serum to ensure that they did not have cow's milk protein-specific IgE. We checked for skin infections simultaneously. The study protocol was approved by the ethics committees of all participating facilities, and written informed consent for participation in the respective studies was obtained from each child's parents. At the analysis stage, the age of the children in the preliminary study (n = 20) ranged

from 4 to 15 years (mean age 4.47; SD 2.65; male:female ratio 11:9). Twenty-two children were enrolled, and 2 children were excluded from the casuistic study: one child encountered difficulty during blood collection and the other required antibiotics during the experimental period.

The symptoms of the remaining subjects included pruritus, atopic eczema and subjective symptoms reported by the parents (such as itching, scratching and other symptoms related to the general skin condition). The preliminary casuistics were conducted to evaluate the possible complementary effects of supplementation of continued medical therapy with oral L-92 in the control of AD.

After a 4-week run-in period, the subjects received 150 ml of milk fermented with L-92 (containing 3×10^{10} live bacteria) once daily for 8 consecutive weeks. Medical examinations by physicians and collection of blood samples and fecal specimens were conducted at 4-week intervals during the experimental period.

Validity Affirmation Study

The study was performed in a randomized, double-blinded, placebo-controlled manner, conducted in accordance with the principles of the Declaration of Helsinki and approved by the ethics committees of the institutions participating in the trial. The study enrolled 60 children (age 1–12 years) referred to the Department of Allergy, Daido Hospital; the Department of Clinical Research, Mie National Hospital or the Department of Allergy, Aichi Children's Health and Medical Center (in addition to 1 of the 3 institutions mentioned above) for suspected AD without a concomitant cow's milk allergy. All these facilities are located in the Chukyo area in Japan. The symptoms of these children were similar to those of the children enrolled in the preliminary study. Written informed consent for participation in the respective studies was obtained from each child's parents.

The experiment was performed from the second week of January 2007 to the first week of April 2007. Randomization was performed by members of the site management organization who were not involved with the study and who used a computer-generated permuted randomization in each institution. In this study, the institution was considered a stratification factor because of a possible symptom evaluation bias using the Atopic Dermatitis Area and Severity Index (ADASI) scoring system between the facilities. There was no obvious difference between the two groups (table 2). Placebo and heat-inactivated L-92 groups were given either unsupplemented milk components (placebo, 1,000 mg dextrin; n = 30) or 900 mg dextrin supplemented with 100 mg of heat-treated L-92 (Calpis; n = 29). We asked each patient and their parents not to change the patient's lifestyle or skin care regimen during the study period.

Inclusion criteria for the study were: (1) tolerance to cow's milk; (2) no evidence of skin infection, including infectious impetigo or dermatomycosis, at enrollment; (3) no recent history of antibiotic use; (4) clear steroid dependency for maintaining their skin condition; (5) no complication with seasonal allergic rhinitis; (6) no habit of consuming materials that may affect the intestinal microbiota, including medicine for intestinal disorders and fermented foods such as fermented milk. These criteria were fulfilled by all children included in the study population.

Exclusion criteria for the study were: (1) use of antibiotics during the experiment for a skin infection; (2) inadequate skin care; (3) noncompliance with scheduled visits; (4) inadequate intake of

the experimental foods; (5) the intake of fermented foods containing probiotics.

At the analysis stage, the age of the children in the validity affirmation study ranged from 1 to 12 years (placebo group, n = 24: mean age 4.25 years, SD 2.44, male:female ratio 16:8; L-92 group, n = 26: mean age 5.04 years, SD 2.97, male:female ratio 20:6).

Evaluation of the Complementary Effect of the Experimental Food

In both studies, the atopic eczema severity was evaluated by physicians using the ADASI [18]. Briefly, on diagrams showing the body with marker points for the front and back, the involved areas are painted with 1 of 3 different colors (green, blue and red) according to disease severity. Skin areas with only slight erythema are painted green. The more severely affected skin areas with infiltrating erythema and more or less severe scaling are painted blue. Skin areas with severe inflammation, oozing and/or scaling or lichenification are painted red. The area fraction of each of the 3 severity grades of skin changes is calculated by counting the points on each color field and dividing by the total number of points falling on the body diagram.

The ADASI score was calculated using the following formula: ADASI = (1 Ag + 2 Ab + 3 Ar) · (I + 1), where Ag, Ab and Ar are the fractions of the green, blue and red areas, respectively, and I is the itching score, which is assessed on a 0–5 scale by the patients or their parents. ADASI score ranges from 1 to 18.

The primary outcome for both studies was the symptom-medication score (SMS), which is calculated as the sum of the ADASI score and the medication score (MS). The MS is used to correct for the effect of the applied topical corticosteroids and represents the sum of the product of the intensity factor and the amount (expressed in grams) for each steroid ointment used in a 4-week period. The strength factors of steroid ointments are defined as topical corticosteroids classified in weak (category V), mild (category IV), strong (category III) or very strong (category II) ranks designated as 0.05, 0.1, 0.2 or 0.3, respectively. The strength grading of topical corticosteroids has been previously described in the 'Guidelines for management of atopic dermatitis' by the Japanese Dermatological Association [19].

Secondary outcomes included the white blood cell (WBC) count, number of eosinophils, serum C-reactive protein concentration, serum total IgE concentration (both the preliminary casuistics and the validity affirmation study), and serum thymus and activation-regulated cytokine (TARC) concentration (validity affirmation study). The validity affirmation study sample size was determined using instructive information from prior clinical trials for seasonal and perennial allergic rhinitis using L-92 [12, 13].

Probiotic or Biogenic Bacterium Supplementation

In the preliminary study, the daily dose (150 g) of the administered commercial FM contained approximately 3×10^{10} colony-forming units of L-92.

In the validity affirmation study, the active experimental food was supplemented with 100 mg of dried and heat-killed L-92 at a concentration equivalent to a bacterial count of approximately 1.5 $\times$ 10^{11} and 900 mg of dextrin. Heat treatment was performed by an independent microbiologist at Hokkaido Sugar (Tokyo, Japan). The food-quality liquid L-92 concentrate was maintained and heated in a tank approximately 20,000 liters in volume with a
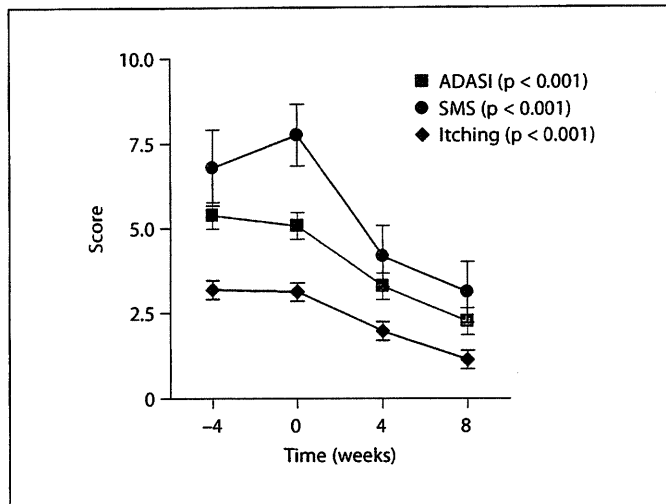
Fig. 1. Time-dependent changes in the ADASI, SMS and itching scores of children with AD with administration of L-92 FM in the preliminary casuistic study. The probabilities shown in parentheses refer to the statistical significances of the time factor from the ANOVA. The data are presented as the mean ± SEM.

Table 1. Study design of preliminary study and scheduled visits

Observation	Ingestion period		
–4 weeks	0 weeks	4 weeks	8 weeks
ADASI	ADASI	ADASI	ADASI
	SMS	SMS	SMS
WBC	WBC	WBC	WBC
Eosinophil	Eosinophil	Eosinophil	Eosinophil
Total IgE	IgE	IgE	IgE
CRP	CRP	CRP	CRP
	Fecal microbiota	Fecal microbiota	Fecal microbiota
Atopy diary (every day) ——————————————————————→			

Patients at entry: 22; analyzed: 20.
IgE = Immunoglobulin E; CRP = C-reactive protein.

steam jacket. When the concentrate reached 85°C, the tank was held at that temperature for a further 15 s, after which it was cooled with chilled water passed through the same jacket to 5°C. The inactivated concentrates were freeze-dried before being added to dextrin. The efficacy of the heat treatment and the bacterial concentration in the formulas were controlled using both a standard plate count method and particle counting with a Multisizer 3 Coulter Counter (Beckman Coulter, Tokyo, Japan).

Blood Examination
Blood samples were collected four times in both experiments. All analyses were conducted by SRL (Tokyo, Japan).

Analysis of Fecal Microbiota
The parents collected fecal samples by scooping up specimens from floating paper sheets placed on the water in the toilet bowl prior to defecation. The specimens were immediately cooled to 6–8°C and delivered to the research laboratories within 24 h. All samples were cultured and analyzed according to the method of Mitsuoka et al. [20–22].

Statistics
In the preliminary experiment, an analysis of variance (ANOVA) and Dunnett's test or Bonferroni's multiple comparison procedure were applied to the obtained time-dependent data. All analyses were performed using SPSS for Windows, version 12 (SPSS Japan, Tokyo, Japan).

In the validity affirmation study, a split-plot ANOVA was used, and sub-analyses were then conducted using a split-plot ANOVA and linear regression analyses for the data from each group. These analyses were carried out using SAS, version 9.1 for Windows (SAS Institute Japan, Tokyo, Japan).

Results

Preliminary Casuistic Study
Clinical Symptoms
Table 1 shows the study design and the visit schedule. Subjects were evaluated by medical examination of the skin and blood. After screening, 22 patients with mild, moderate or severe symptoms were selected. One patient dropped out because of difficulties in blood collection and another dropped out due to antibiotic use for a skin infection that developed during the experimental period. Ultimately, 20 patients (age 4–15 years; initial severity of eczema: 4 mild, 11 moderate, 5 severe) were enrolled in this open trial.

Statistically significant time-dependent changes in the symptom score of the ADASI, which was evaluated as a measure of atopic eczema severity, were observed after L-92-containing FM supplementation ($p < 0.001$, factor of time; fig. 1). Simultaneously, significant time-dependent decreases in the MS were also observed (data not shown). Therefore, highly significant changes in the SMS were detected after daily administration of L-92 FM ($p < 0.001$, factor of time; fig. 1).

The score for itching as a subjective symptom that was recorded in an atopy diary maintained by the patients' parents also decreased after the initiation of L-92 FM supplementation ($p < 0.001$, factor of time; fig. 1).

Blood Examination
No abnormal clinical changes were noted during the assessment period. No changes in serum aspartate ami-

Table 2. Clinical characteristics of intervention and placebo groups

Characteristics	Placebo group	Intervention with L-92	Significance
Patients	24	26	
Age, years	4.25±2.44	5.04±2.97	0.422
Sex, male:female	16:8	20:6	0.424
Initial state of symptoms			
Mild cases	11	11	
Moderate cases	11	14	0.956
Severe cases	2	1	
SMS	4.83±0.57	4.46±0.57	0.552
WBC, count/μl	9,458.8±566.7	9,404.8±572.4	0.947
Eosinophil, count/μl	624.7±77.9	462.5±58.2	0.100
Total IgE, IU/ml	2,010.5±775.2	1,859.5±731.5	0.888
CRP, ng/ml	1,247.5±726.2	1,169.2±397.2	0.923
TARC, pg/ml	3,172.5±1,301.3	1,855.1±890.8	0.650

Data are given as average ± SEM. Figures in parentheses are average ± SD. Patients at entry: 60; analyzed: 50. TARC = Thymus and activation-related chemokine.

Fig. 2. Inconsistent time-dependent changes in the *Bacteroidaceae* and *Lactobacillus* counts in the intestinal microbiota of children with AD with administration of L-92 FM in the preliminary casuistic study. The probability figures in both tracing areas refer to the statistical significances of the time factor from the ANOVA analyzing changes in the number of these bacterial groups. The data are presented as the mean ± SEM.

notransferase, alanine aminotransferase or lactate dehydrogenase were observed during the preliminary experiment. There were no noticeable changes in blood biochemical parameters, including the total plasma IgE concentration, which represents the extent of atopic sensitization; the plasma C-reactive protein and hematological measurements, including red blood cell count, packed cell volume, hemoglobin concentration, mean corpuscular volume or mean corpuscular hemoglobin concentration; neutrophil, lymphocyte or monocyte counts.

In contrast, a decrease in the WBC and absolute eosinophil count was observed during the course of administration of L-92 FM. The initial values of these parameters were abnormally elevated compared with the normal values. The WBC count was significantly decreased after 8 weeks of supplementation with L-92 FM (p = 0.041; data not shown). The eosinophil count showed a trend towards a decrease 8 weeks after the start of oral administration of L-92 FM (p = 0.085; data not shown).

Therefore, the *L. acidophilus* strain L-92 evaluated in this study did not adversely affect the health of the volunteers, which is consistent with its label as a probiotic bacterium (data not shown).

Fecal Microbiota

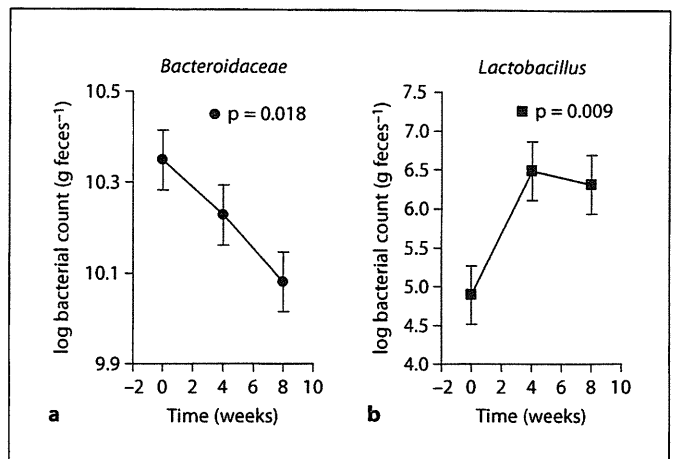As shown in figure 2, a significant decrease in the total fecal count of *Bacteroidaceae* (p = 0.034) and a sig-

nificant increase in the fecal count of *Lactobacillus* (p = 0.007) were observed. No significant changes in the fecal count of other examined microbial groups, families, genera or species, including *Enterobacteriaceae*, *Enterococcaceae*, staphylococci, yeasts, *Bacillus*, *Bifidobacterium*, *Eubacterium*, *Peptococcaceae*, *Clostridium*, or lecithinase-positive *Clostridium* strains, were observed (data not shown).

Validity Affirmation Study
Clinical Symptoms
Atopic Eczema. Initially, 60 patients were included in the study. Of these, 10 patients were excluded from the analysis for the following reasons: noncompliance with scheduled visits, antibiotic use for skin infections, antibiotic use for systemic mycoplasma infection and inadequate compliance with the necessary skin care. A total of 26 patients in the L-92 group and 24 patients in the placebo group were included in the final analysis (table 2). Table 3 shows the study design and the visit schedule.

Figure 3 shows the flow of the participants through each stage of the randomization trial.

Figure 4b shows the changes in the SMS of the two groups. As determined using a split-plot ANOVA, the time-dependent changes in the SMS and the rates of decrease of the SMS differed between the two groups (p = 0.0474; interaction of time × group; table 4a). There-

fore, subsequent ANOVA analyses were independently applied to each group. The subanalyses indicated that the administration of the L-92-containing test food significantly decreased the SMS in a time-dependent manner ($p = 0.0127$, term of time; table 4b). Furthermore, a simple regression analysis showed no significant regression in the placebo group, whereas a highly significant negative regression was observed between time and SMS in the L-92 group (slope, −0.35224904; $p = 0.000125$; table 4c).

Blood Examination

No abnormal clinical changes were noted during the assessment period. No noticeable changes in blood biochemical parameters or hematological indices were noted, as described for the preliminary open trial above. Therefore, the heat-killed L-92 powder did not adversely affect the health of the volunteers, which is consistent with its label as a biogenic bacterium (data not shown).

In this study, the trend of the time course of changes in the serum level of TARC, which reflects chemotactic

Fig. 3. Profile flow chart of the validity affirmation study.

Enrollment: 60 subjects randomized

Allocation: 30 assigned to placebo | 30 assigned to L-92

Follow-up:
1 lost at visit 2 (mycoplasma infection) — 29 completed study
1 lost at visit 1 (refusal to participate) — 29 completed study

Analysis:
24 analyzed
5 excluded from analysis
−4 due to antibiotic use
−1 due to visit noncompliance

26 analyzed
3 excluded from analysis
−2 due to antibiotic use
−1 due to inadequate skin care

Fig. 4. Time course of changes in the SMS of children with AD in the placebo and intervention groups in the double-blinded validity affirmation study using heat-killed L-92. The probability figures in the tracing area refer to the statistical significances of the interaction of time × group from the ANOVA analyzing patterns of reduction of these evaluation indices. The data are presented as the mean ± SEM.

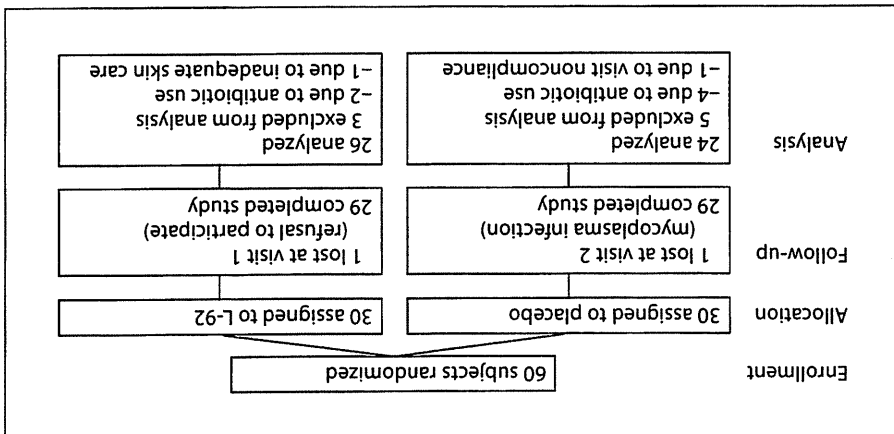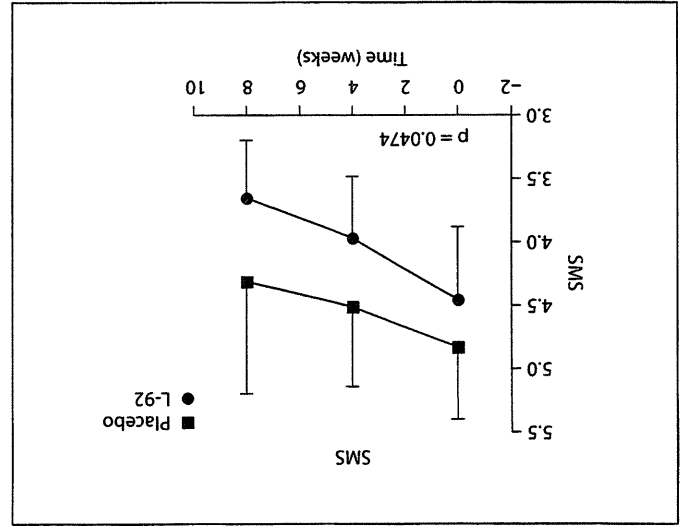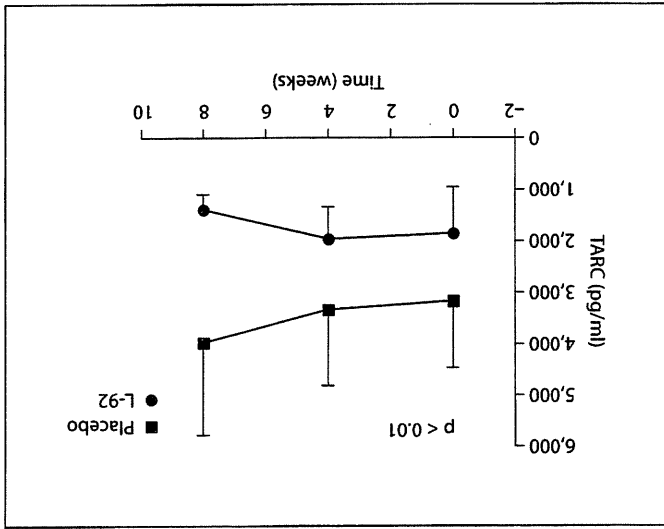SMS graph: SMS (5.5, 5.0, 4.5, 4.0, 3.5, 3.0); $p = 0.0474$; Time (weeks) −2 0 2 4 6 8 10; ● L-92; ■ Placebo

Fig. 5. Changes in the serum TARC concentration in children with AD in the placebo and intervention groups in the double-blinded validity affirmation study conducted using heat-killed L-92. The probability figures in the tracing area refer to the statistical significances of the interaction of time × group from the ANOVA analyzing patterns of changes in these determination values. The data are presented as the mean ± SEM.

TARC graph: TARC (pg/ml) (6,000; 5,000; 4,000; 3,000; 2,000; 1,000; 0); $p < 0.01$; Time (weeks) −2 0 2 4 6 8 10; ● L-92; ■ Placebo

Table 3. Study design of validity affirmation study and scheduled visits

Observation	Ingestion period		
−4 weeks	0 weeks	4 weeks	8 weeks
ADASI	ADASI	ADASI	ADASI
	SMS	SMS	SMS
WBC	WBC	WBC	WBC
Eosinophil	Eosinophil	Eosinophil	Eosinophil
IgE	IgE	IgE	IgE
CRP	CRP	CRP	CRP
TARC	TARC	TARC	TARC
	Test foods ⟶		
Atopy diary (every day) ⟶			

Table 4a. Analysis of variance for repeated measures of SMS

Factor	Probability
Primary	
Group	0.7840
Institution	0.5506
Group × institution	0.8351
Secondary	
Time	0.0835
Time × group	0.0474
Time × institution	0.1674
Time × group × institution	0.1288

Table 4b. Subanalysis by group for repeated measures of SMS

Factor	Group probability	
	placebo	L-92
Primary		
Instutution	0.1024	0.2267
Secondary		
Time	0.6093	0.0127
Time × institution	0.4018	0.7729

Table 4c. Regression analysis (changes in SMS by group)

Group	Factor	Probability	Slope
Placebo	Regression	NS	
L-92		0.000125	−0.35224904

stimulation of Th2, but not Th1, cells, was significantly different between the group receiving the placebo and the group receiving heat-killed and lyophilized L-92 powder (p < 0.01; interaction of time × group; fig. 5).

Discussion

The *L. acidophilus* L-92 strain used in these studies was selected as the probiotic or biogenic bacterium on the basis of a report demonstrating that the oral administration of this strain lead to the suppression of the elevation of the total serum IgE level following repeated immunization with OVA as a model allergen in an animal model [14]. The strain has also been reported to result in improvements in the symptoms of Japanese cedar pollinosis [12] and perennial allergic rhinitis [13] in placebo-controlled clinical studies. Therefore, L-92 is thought to be potentially effective against allergic diseases caused by type-I hypersensitivity reactions.

AD is a commonly encountered chronic inflammatory skin disease characterized by relapsing pruritic eczema. Genetic predispositions to epidermal barrier dysfunction [23] and atopic diathesis [24] are believed to be the main causes underlying the development of AD. However, various studies have indicated a complex etiology for AD, with activation of multiple immunologic and inflammatory pathways being important [25]. At least two forms of AD have been described: an 'extrinsic' form, associated with IgE-mediated sensitization and accounting for 70–80% of patients, and an 'intrinsic' form, not associated with IgE-mediated sensitization but accounting for 20–30% of patients [26]. Both forms of AD are characterized by eosinophilia. Although a considerable number of AD patients have an allergic constitution [27], the contribution made by the IgE-mediated hypersensitivity reactions to the pathogenesis and clinical severity of AD remains controversial. This disease is not considered a pure type I, so-called IgE-mediated, allergic disorder. Therefore, we conducted this study with the objective of determining if the oral administration of L-92 might effectively ameliorate the symptoms of childhood AD.

We conducted two autonomous clinical studies examining the complementary effect of L-92 in the treatment of AD in children. In the preliminary study, the SMS improved in a time-dependent manner (fig. 1). However, in the casuistic study, the main effect following ingestion of L-92 was confounded by the effect of the time factor. For this reason, changes in the SMS should be considered with caution. However, some objective markers, such as

the WBC and eosinophil counts, also showed changes consistent with the changes in the SMS, although to variable degrees. These results suggest a complementary effect of long-term oral administration of L-92 FM with traditional medical therapy.

It should be noted that the medical treatment prescribed by the subjects' attending physicians continued throughout the experimental period in this study. During the 4-week run-in period, almost no changes in symptoms were observed. Although some placebo effect might be included, the SMS seemed to change just after the start of L-92 FM administration. In addition, no adverse effects were observed in the preliminary study following the administration of live L-92. Therefore, we advanced to the stage II study, in which heat-treated L-92 lyophilized powder was used as the biogenic substance.

In the stage II validity affirmation study, a statistically significant time-dependent decrease in the SMS was observed only in the group administered heat-treated L-92 powder. This finding clearly shows that the long-term oral administration of heat-killed L-92 enhances the efficacy of traditional medical therapy in subjects with AD. This finding suggests that L-92 may be an important food ingredient for AD patients that might reduce their dependence on steroid treatment. There is no clear regulation for evaluating the primary or complementary effects of food on the biological responses in clinical trials at this point. This validity affirmation study was not intended to evaluate the primary effect of L-92 intake; rather, it was designed to extract the complementary effect of food supplementation from the whole effect during treatment with topical corticosteroids, so we think that the per-protocol analysis would give a more complete evaluation.

It could be argued that L-92 not only works as a probiotic in the live state but also in the heat-killed state. It is generally considered that heat-killed L-92 might not significantly affect the composition of the intestinal microbiota of patients. Therefore, the underlying mechanism(s) of the observed complementary effect of L-92 in AD patients can be explained, at least in part, by the direct actions of the bacterial cells or bacterial cell component(s) as biogenic substances. These substances may affect the host immune system via the gastrointestinal tract. The term 'biogenics' has been defined and suggested as a category of functional foods by Mitsuoka [3]. The term refers to physiologically active substances that directly modulate the functions of organisms following oral administration without having any effect on the intestinal bacterial balance.

In this respect, there has been only one exception reported. Terada et al. [28] reported that a heat-killed strain of *Enterococcus faecalis* demonstrated the ability to change the human intestinal bacterial composition. It has also been shown that cell preparations altered digestive flow in an experimental animal model [29]. These findings suggest that heat-killed cells of some lactic acid bacteria may exert beneficial effects on intestinal disorders through possible changes in the composition of the intestinal microbiota. This might also explain the ameliorative effect of L-92 in patients with AD. Changes in composition of the intestinal microbiota may be involved in the antiatopic effect of heat-killed L-92. There is some evidence suggesting that intestinal inflammatory reactions and disruptions in intestinal barrier function are involved in the pathogenesis of AD [30]. In addition, recent studies have suggested that gastroenteropathy might exist in children with AD. Therefore, orally administered L-92 as a probiotic or biogenic bacterium may result in restoration of the intestinal barrier function directly or via modification of the intestinal bacterial composition. In the preliminary casuistic study, during the L-92 FM administration period, the decreased fecal count of *Bacteroidaceae* and increased fecal count of *Lactobacillus* were compatible with each other. Some species of the genus *Bacteroides*, such as *B. fragilis* and *B. vulgatus*, have been implicated in intestinal inflammation and colitis [31, 32], which may indicate the relative health of our patients.

The benefit of L-92 in the live state over the heat-killed organism cannot be excluded from the results of this study. Further detailed studies should be conducted to clarify the mechanisms underlying the antiallergic effects of L-92, especially with regard to the anti-inflammatory actions exerted in the intestine. A precise understanding of the mechanism underlying the improvement of AD symptoms following administration of L-92 is critical to develop more effective management strategies for reducing steroid dependence, especially for children. This is an important role for this category of functional foods. Such foods may benefit patients with allergy without any adverse side effects. No matter how large or small the relief L-92 may provide to AD patients, we think that it is worthwhile because it is important to improve the quality of life of the patients.

The stratified analysis based on the initial severity of the skin symptoms showed that the validity of L-92 was detectable to a greater extent in patients with moderate or severe initial symptoms than in those with mild initial symptoms. The reason for this observation is not entirely clear; however, it could be discussed from the point of

view of the detectional characteristics of the ADASI. The clinical index is calculated based on the point of view that itching is a critical element in the diagnosis of the disease. Itching is sensed more by patients with advanced disease, which may explain the results of the stratified analysis. On the basis of this finding, to obtain a clearer picture of the primary effect of L-92 administration, information about the patient's initial symptoms can be used as a significant covariate. In addition, because there was a good correlation between the severity of the initial symptoms and total serum IgE concentration, the total serum IgE value may also be used as an important covariate. This issue must be addressed in a future study.

The time course of changes in the serum concentration of TARC (fig. 5), which represents a marker of Th2 activation, was significantly different between the placebo and L-92 groups (p < 0.01). This finding suggests that L-92 administration may induce escape from Th2-biased immune responses [33]. The serum TARC level has been correlated with and may be directly reflected by the severity of the AD [34–36]; therefore, we used the serum TARC concentration as one of the secondary outcomes in the validity affirmation study.

We suggest the possibility that orally ingested L-92 may be transported into the intestinal lymphatics, including the PP, and may somehow modulate the Th1/Th2 balance throughout the entire body. Even though little is known about the component(s) of L-92 and the cell populations important in the induction of IL-12, which stimulates the differentiation of Th0 cells to Th1 cells, L-92 has been found to induce the release of cytokines from cultured splenocytes [14, 15]. This may explain our observations.

A second possibility is the induction of apoptosis of Th2 cells by L-92 cells. L-92 induces the apoptosis of differentiated Th2 cells and decreases the secretion of IL-4 from these cells, suggesting that L-92 might regulate the Th1/Th2 balance through this pathway [15]. Moreover, L-92 upregulates the expression of B7-H1 and downregulates the expression of B7-H2 on DC, and DC exposed to L-92 also induce the apoptosis of antigen-stimulated T cells. These findings indicate that L-92 attenuates the CD4+ T cell response by inducing DC-mediated apoptosis and that it might exert beneficial effects in patients with diseases resulting from the hyperresponsiveness of CD4+ T cells.

A third possibility is the induction of Treg cells, which might affect the responsiveness of Th2 cells. Furthermore, cultured PP cells isolated from OVA-immunized mice fed heat-killed, lyophilized L-92 simultaneously produced high levels of TGF-β and IgA compared to cells from control chow-fed mice [14]. This observation sug-gests an essential role of L-92 in the suppression of Th2-induced allergic inflammation [16] and induction of oral tolerance [17]. These data may also indicate that L-92 induces Treg cells in the PP by activating macrophages, which might lead to the attenuation of the excessive activity of the CD4+ T cells in mice repeatedly immunized with OVA. The mechanism of Treg induction has been assessed using human DC [37]. In this report, while some *Lactobacillus* species stimulated monocyte-derived DC and facilitated Treg cell activation, other *Lactobacillus* species had no activity. The former species were recognized by the C-type lectin DC-specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and antibodies against DC-SIGN neutralized the Treg activation activity. These findings strongly suggest that some *Lactobacillus* species stimulate the induction of Treg via a signaling pathway mediated by DC-SIGN. L-92 may be this type of *Lactobacillus* species.

These observations lend support to the observed antiallergic activity of L-92. We have not examined the effect of L-92 on the induction of Th17 cells [38], which produce IL-17 and IL-22. This type of Th cell plays a critical role not only in the inflammatory response in allergic disorders but also in the responses that mediate autoimmune diseases [39]. It has been suggested that the inflammatory reactions induced by Th17 may be regulated by Foxp3+ Treg. This pathway could be involved in the complementary 'ceasefire' effect on the inflammatory responses in AD that we observed with oral administration of L-92. Further studies are needed to elucidate the precise mechanisms underlying the clinical effects of L-92 in patients with allergic diseases.

In conclusion, our data suggest that L-92 works as a probiotic and a biogenic in patients with AD, even children, and its daily intake is within the practical range of consumption.

Acknowledgments

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A PATIENT WITH SALMON ROE ALLERGY SHOWING TAXONOMY-UNRELATED CROSS-REACTIVITY WITH SEA URCHIN ROE

In Japan, salmon (*Oncorhynchus keta*; phylum Chordata, family Salmonidae) eggs (salmon roe [SR]) are commonly eaten raw in dishes such as sushi, and the number of patients who develop severe allergies after initial SR intake has been increasing in recent years. In Western countries, one patient developed unexpected anaphylaxis after consuming rainbow trout (*Oncorhynchus mykiss*; family Salmonidae) roe,[1] and another adult developed anaphylactic symptoms after initial SR intake.[2] On the basis of the induction of allergy by initial SR intake, there is a possibility that SR allergy develops through sensitization due to cross-reactivity with other foods. However, the source of such SR sensitization remains unclear.[3]

We encountered a patient with allergy to SR who developed a cross-reactive allergic reaction to the eggs of sea urchins (phylum Echinodermata; sea urchin roe [UR]), which are distantly taxonomically related. There have been no previous reports of cross-reactivity between the Chordata and Echinodermata phyla. We confirmed the cross-reactivity between them by inhibition enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

The patient first developed SR allergy at 4 years of age. He had previously tolerated chicken eggs and had also eaten fish meat, including salmon and the roe of other kinds of fish (herring and pollock roe), without any reaction. Immediately after first ingesting an SR egg, he became pale, experienced abdominal pain, vomited, and had diarrhea. His SR specific IgE antibody level was subsequently found to be high (91.9 UA/mL) using the ImmunoCAP test (Phadia AB, Uppsala, Sweden). At the age of 10 years, because he wished to eat UR, which is a popular sushi dish, we planned an oral challenge after skin prick tests. The oral challenge was performed without discontinuing the oral administration of pranlukast for his underlying asthma. Because the skin prick test result was positive (wheal diameters: UR extract, 3 mm; 10 mg/mL histamine, 4 mm; and saline, 0 mm), a very small amount (corresponding to 0.1 mg) of UR was orally administered using the open challenge method after preparing for his admission. He developed abdominal pain 15 minutes after the challenge, excreted soft feces, and experienced itching around his eyes. The oral challenge test result was regarded as positive, and the test was terminated. The above symptoms disappeared without any treatment. We subsequently realized that we could have obtained more objective data if we had measured plasma histamine levels.

To examine the cross-reactivity between SR and UR, we performed an inhibition ELISA study with the patient's sera, as described in a previous report.[3] Extracts from UR, salmon meat, SR,
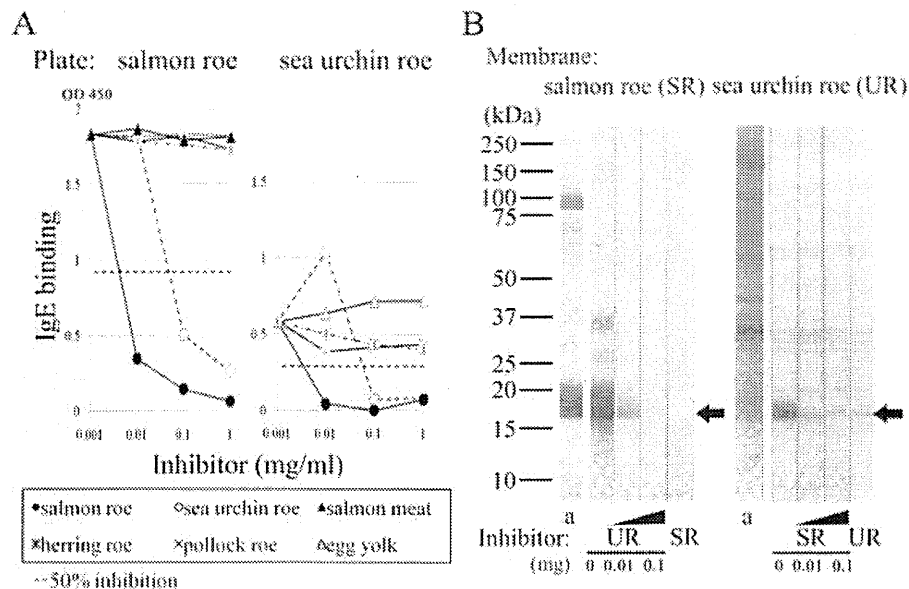
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Figure 1. Inhibition assay and immunoblot results. A, Inhibition enzyme-linked immunosorbent assay between salmon roe (SR) and sea urchin roe (UR). Preincubation of the patient's serum with heterogeneous SR or UR extract induced more than 50% inhibition of the IgE specific to each extract. Other kinds of fish roe and chicken yolk did not affect IgE binding to either extract. B, Inhibition immunoblot between SR and UR. IgE specific to low-molecular-weight proteins (arrows) isolated from the 2 extracts mutually inhibited each other. Lane a: Amido black stained.

herring roe, Alaskan pollock roe, and chicken egg yolk were obtained in the previously reported manner.[3] Specific IgE to SR and UR almost completely inhibited each other's binding. Interestingly, other kinds of fish roe were not only poor inhibitors of IgE specific to UR, to which they were not taxonomically related, but also poor inhibitors of IgE specific to SR, to which they were closely related taxonomically (Figure 1A). This finding suggested that there was no correlation between taxonomy and allergenicity. Then, we attempted to identify the relevant allergen by performing an inhibition immunoblot study. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in a 4% to 12% NuPAGE Novex Bis-Tris Mini Gel (Invitrogen, Carlsbad, California) under reducing conditions. Immunoblotting of the proteins and the detection of bound serum IgE were performed as previously reported.[4] Specific IgE to 2 proteins of approximately 18 kDa that were isolated from UR or SR (1 protein was isolated from each roe) mutually inhibited each other (Figure 1B). We performed amino acid analysis of the N-terminal of the 18-kDa UR protein and identified an 18-kDa egg cortical vesicle protein (NP_999641). A search revealed homology with the complement C1q-like protein 4 precursor (ACM08719) of Atlantic salmon (Salmo salar) with a Basic Local Alignment Search Tool (BLAST) expected value of 2 × 10⁻¹⁰ (−10). Both proteins belong to the C1q family, and a member of this family has been reported to be localized in mature eggs in the prespawning ovary.[5] These findings suggest that the homology of these proteins could cause cross-reactivity.

There have been 3 case reports of UR allergy.[6–8] As allergens, 2 high-molecular-weight proteins (118 kDa[7] and 160 kDa[8]) have been reported, and the latter was identified as major yolk protein. However, these reports did not mention cross-reactivity. In Figure 1B, the patient's IgE did not react with these proteins. Therefore, the 18-kDa protein was considered to be a novel allergen that displays the unique characteristic of cross-reacting with SR.

This is the first study, to our knowledge, to demonstrate unexpected cross-reactivity between the Echinodermata and Chordata phyla. It is important to bear in mind that cross-reactivity can occur in taxonomically unrelated species.

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REVIEW ARTICLE

Japanese Guideline for Food Allergy

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ABSTRACT

Food allergy is defined as "a phenomenon in which adverse reactions (symptoms in skin, mucosal, digestive, respiratory systems, and anaphylactic reactions) are caused in living body through immunological mechanisms after intake of causative food."

Various symptoms of food allergy occur in many organs. Food allergy falls into four general clinical types; 1) neonatal and infantile gastrointestinal allergy, 2) infantile atopic dermatitis associated with food allergy, 3) immediate symptoms (urticaria, anaphylaxis, etc.), and 4) food-dependent exercise-induced anaphylaxis and oral allergy syndrome (i.e., specific forms of immediate-type food allergy).

Therapy for food allergy includes treatments of and prophylactic measures against hypersensitivity like anaphylaxis. A fundamental prophylactic measure is the elimination diet. However, elimination diets should be conducted only if they are inevitable because they places a burden on patients. For this purpose, it is highly important that causative foods are accurately identified. Many means to determine the causative foods are available, including history taking, skin prick test, antigen specific IgE antibodies in blood, basophil histamine release test, elimination diet test, oral food challenge test, etc. Of these, the oral food challenge test is the most reliable. However, it should be conducted under the supervision of experienced physicians because it may cause adverse reactions such as anaphylaxis.

KEY WORDS

elimination diet, food allergy, IgE-mediated type, non-IgE-mediated type, oral food challenge test

1. DEFINITION OF FOOD ALLERGY

The Japanese Pediatric Guideline for Food Allergy 2005,[1,2] published in 2005, defines food allergy as "a phenomenon in which adverse reactions (symptoms in skin, mucosal, digestive, respiratory systems, and anaphylactic reactions) are caused in living body through immunological mechanisms after intake of causative food."

2. EPIDEMIOLOGY OF FOOD ALLERGY

2.1. PREVALENCE OF IMMEDIATE-TYPE FOOD ALLERGY

Food allergy is common among infants aged 0-1 years and decreases with aging, which indicates that tolerance develops with aging. The estimated prevalence in Japan is 5-10% among infants and 1-2% among schoolchildren. The prevalence of food allergy, reported from various countries, is shown in Table 1.

2.2. CAUSATIVE FOODS

Eggs, dairy products, wheat, buckwheat, shrimp and peanuts are the common causative foods of immediate-type food allergy, indicated by the national surveys of food allergy during 1998-1999, conducted by the Review Committee on the Countermeasure for the Food Allergy of the Ministry of Health and Welfare (Fig. 1). As shown in Figure 2, patients aged less than 1 year of age account for 29.3%, and those aged ≤ 8 years account for 80.1%. The number of patients decreases with aging. Patients aged ≥ 20 years account for 9.2%. This is not a small number. Eggs, dairy products and wheat are 3 major allergens among those aged ≤ 6 years, while shrimp, fish, and

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Table 1 Prevalence of food allergy reported from various countries

Year	Reporter	Country	Subject	Number of subjects	Methods	Diagnosis	Preva-lence	Journal
1994	Young E	UK	All ages	7,500 households	Interview + DBPCFC	Food intolerance	1.4-1.8%	Lancet
1994	Jansenn JJ	Nether-lands	Adults	1,483 persons	Questionnaire + DBPCFC	Food allergy, food intolerance	0.8-2.4%	J Allergy Clin Immunol
1999	Kristjansson I	Sweden, Iceland	Children (aged 18 months)	652 persons	Questionnaire + DBPCFC	Food allergy	2.00%	Scand J Prim Health Care
2001	Kanny G	France	All ages	33,110 persons	Questionnaire (two-step survey)	Food allergy	3.52%	J Allergy Clin
2004	Zuberbier T	Germany	All ages	4,093 persons	Questionnaire + DBPCFC	Food allergy	3.60%	Allergy
2005	Imai	Japan	School children	8,035,306 persons	Questionnaire	Food allergy	1.30%	J Jpn Pediatr Soc
2005	Rance F	France	School children	2,716 persons	Questionnaire	Food allergy	4.70%	Clin Exp Allergy
2005	Pereira B	UK	School children (aged 11 years)	757 persons	Questionnaire + Open challenge test	Food allergy	2.30%	J Allergy Clin Immunol
			School children (aged 15 years)	775 persons	Questionnaire + DBPCFC	Food allergy	2.30%	
2005	Osterballe M	Denmark	3 years old	486 persons	Questionnaire + Food challenge test	Food allergy	2.30%	Pediatric Allergy Immunol
			Aged ≥3 years	301 persons	Questionnaire + Food challenge test	Food allergy	1.00%	
			Adults	936 persons	Questionnaire + Food challenge test	Food allergy	3.20%	
2005	Penard-Morand C	France	School children (aged 9-11 years)	6,672 persons	Questionnaire	Food allergy	2.10%	Allergy
2006	Venter C	UK	1-year-old children	969 persons	Questionnaire + Open challenge test	Food allergy	5.50%	J Allergy Clin Immunol
					Questionnaire + DBPCFC	Food allergy	2.20%	
2006	Venter C	UK	6-year-old children	798 persons	Questionnaire + Open challenge test	Food allergy	2.50%	Pediatric Allergy Immunol
					Questionnaire + DBPCFC	Food allergy	1.60%	

fruits are common among those aged >6 years (Table 2).

3. PATHOLOGY, SYMPTOMS AND CLINICAL TYPES OF FOOD ALLERGY

3.1. PATHOLOGY OF FOOD ALLERGY

IgE is often involved in food allergies (IgE-mediated food allergy).[3] In some patients, symptoms develop via immunological mechanisms not involving IgE (non-IgE-mediated food allergy).[4] Both IgE-mediated and non-IgE-mediated reactions may be involved in the development of food allergies (mixed type food allergy).

Food provides essential nutrients for humans. The antigenicity of foods is reduced when they are di-gested into low-molecular substances. However, even in adults with mature digestive functions, the anti-genicity remains to some extent after foods are ab-sorbed into the living body. Orally ingested foods are foreign substances (non-self). If antigenicity remains, they should be immunologically eliminated, but are not eliminated. Healthy individuals have mechanisms for preventing allergic reactions to foreign food anti-gens, including a physicochemical barrier during food digestion and absorption in the digestive tract and an immunological barrier to reduce the antigenic-ity of foods absorbed in the digestive tract. The for-mer includes digestion into low-molecular substances by digestive enzymes (e.g., pepsin) and denaturation by gastric acid. The latter includes the inhibition of
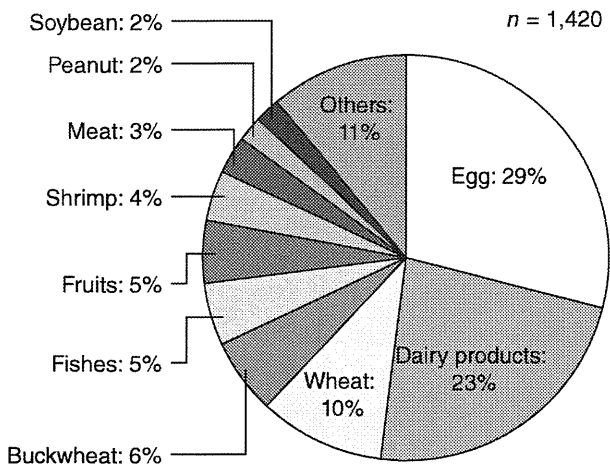
Fig. 1 Causative foods of immediate-type food allergy (national surveys by the Ministry of Health and Welfare during 1998-1999).

absorption of food antigens via secretory IgA and the establishment of oral immunotolerance to suppress allergic reactions to food antigens ingested from the digestive tract.[5]

In patients with food allergy, oral immunotolerance, which is normally established against orally ingested food antigens, may not be established or may be compromised after establishment. However, it is unknown why oral immunotolerance is not established in patients with food allergy.

Food allergy is common in infants because physical, biochemical and immunological barriers are underdeveloped during infancy.

3.2. SYMPTOMS OF FOOD ALLERGY
Symptoms of food allergy include skin, digestive, nasal, ocular, respiratory and systemic symptoms (Table 3).
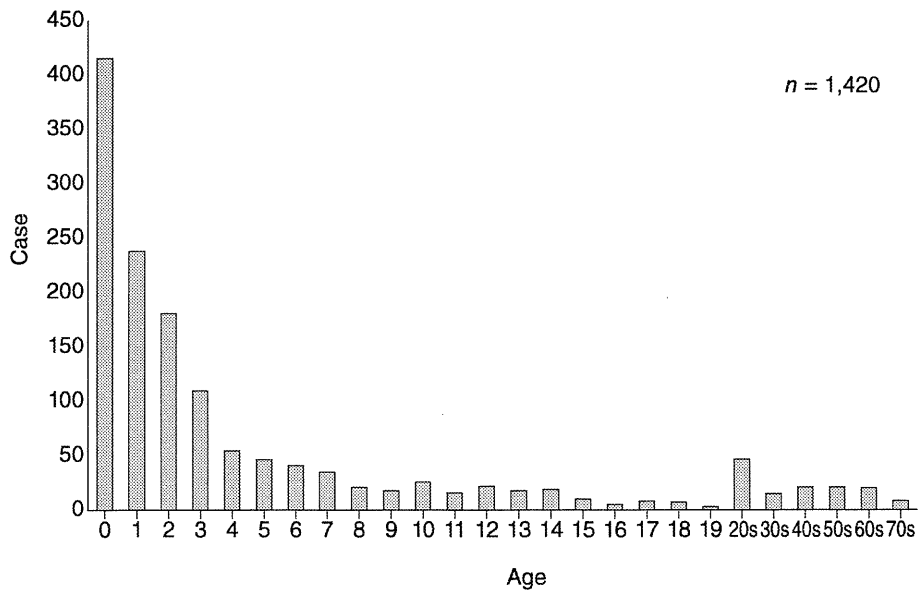

Fig. 2 Age distribution of immediate-type food allergy (national surveys by the Ministry of Health and Welfare during 1998-1999).

Table 2 Causative foods of immediate-type food allergy by age

	0 year (n = 416)	1 year (n = 237)	2-3 years (n = 289)	4-6 years (n = 140)	7-19 years (n = 207)	>20 years (n = 131)
No. 1	Egg 47.4%	Egg 30.4%	Egg 30.8%	Egg 25.0%	Buckwheat 14.0%	Seafood 16.0%
No. 2	Dairy products 30.8%	Dairy products 27.8%	Dairy products 24.2%	Dairy products 24.3%	Shrimp 13.0%	Shrimp 14.5%
No. 3	Wheat 9.6%	Wheat 8.4%	Wheat 12.1%	Wheat 8.6%	Wheat 10.6%	Buckwheat 12.2%
Total	87.8%	66.6%	67.1%	57.9%	37.6%	42.7%

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