ゲームの目的

ランチメニューを自分で選択することの疑似体験を通して、食物アレルギーについて理解を深めていきます。

食物アレルギー

食べ物を食べたときに、おなかが痛くなったり、吐き気がしたり、皮膚にじんましんができるなどの「アレルギー症状」があらわれることがあります。ショック症状（アナフィラキシーショック）を起こし死に至る場合もあります。このように、食べ物に含まれる「アレルゲン」とよばれるタンパク質にからだが反応したことが原因で起こるアレルギーを「食物アレルギー」といいます。アレルギーを引き起こすアレルゲンは、人によって異なります。小学生100人のうち3人くらいが、食物アレルギーをもっています。また、誰もが食物アレルギーを引き起こす可能性があります。食物アレルギーの人に、その人のアレルゲンが含まれている食べ物を無理やりすすめ、食べさせてはいけません。

ゲームの目標

各プレーヤーが、月曜日から金曜日までの5日間のランチメニューを、同じようなメニュー（種類）に偏らないようにしながら決めていくゲームです。また、プレーヤーは、食物アレルギーの症状を起こす自分のアレルゲンに注意してメニューを選ばないといけません。メニューの中から和食や洋食、中華など、いろいろな種類をランチメニューとして選び、5日間のランチを楽しみましょう。

使用する道具 カードが2種類（「メニューカード」と「アレルゲンカード」）があります。

メニューカード（5色、合計50枚）

表面には、メニュー名（「ハンバーグ」、「オムライス」など）が書かれ、カードの色は、そのメニューの種類（アオ：和食、キイロ：洋食、アカ：中華、ミドリ：エスニック、ピンク：軽食）をあらわしています。

裏面には、そのメニューで使う食材に含まれるアレルゲン（卵、乳、小麦など）とポイント（0～4）が書かれています。

	オモテ	ウラ
アオ：和食		
キイロ：洋食		
アカ：中華		
ミドリ：エスニック		
ピンク：軽食		

アレルゲンカード

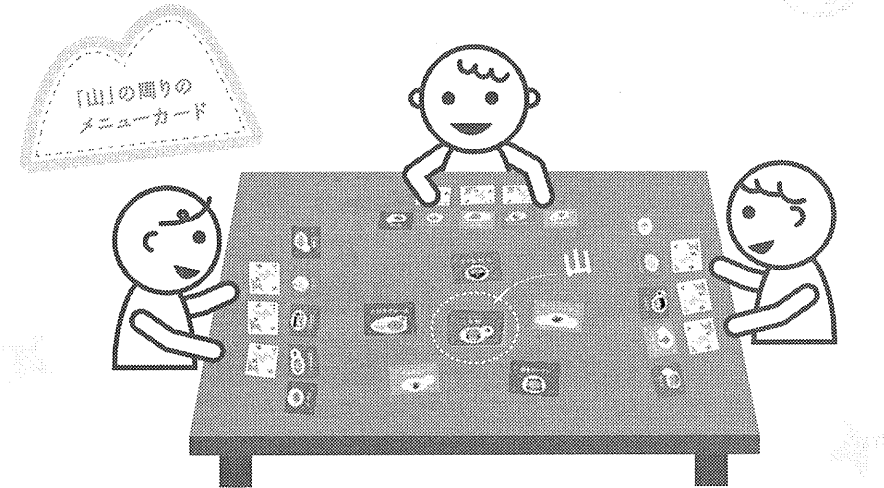
（アレルゲン名7種類各2枚、アレルゲン名記載なし6枚）

裏面にはそのプレーヤーが気を付けなければならないアレルゲン名がひとつ書かれています（「卵」、「乳」、「小麦」「そば」、「落花生」、「えび」「かに」）。また、アレルゲン名が書かれていないカードもあります。

オモテ　ウラ

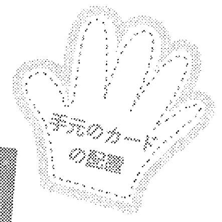
ゲームを始める前に

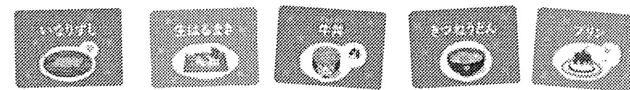
1. メニューカードはよくきって、メニュー名が書いてある表面を上にしてひとつにまとめ、真ん中におきます。これを「山」と呼びます。上から順番に5枚とり、「山」の周りに並べます。



2. 各プレーヤーは順番に、「山」から5枚ずつカードを引き、そのまま裏返さずに自分の前に並べます。

3. 次に、アレルゲンカードをよく切って、各プレーヤーに3枚ずつ配ります。裏面に書かれているアレルゲン名が見えないように配ってください。配られたアレルゲンカードは、自分の前に並べているメニューカードの下に並べて置いてください。





4. 各プレーヤーは、自分のアレルゲンカードの裏面を他のプレーヤーにわからないように見て、自分が気をつけなければならないアレルゲン名を確認します。アレルゲン名が何も書かれていないカードもあります。自分のアレルゲンが少ない方がメニューは選びやすくなります。確認が終わったら、全てのアレルゲンカードは、そのまま裏面を伏せたままにしておきます。

5. 次に各プレーヤーは、自分のメニューカードの裏面を他のプレーヤーにわからないように見て、そのメニューの食材に含まれているアレルゲンを確認します。メニューカードに、自分のアレルゲンカードのアレルゲン名が書いてあった場合は、そのメニューは自分の「健康によくない」、「食べられない」ことを意味します。

シナリオ

カードゲーム「アレルギーゲーム」を利用した授業

進め方の例	備考
① 授業の説明（約 2 分） この時間は、カードゲームを使って学習します。まず最初に、簡単なアンケートをします。そのあとゲームをして、説明をしたあと、最後にもう一度アンケートをしてから終わります。	**前提事項として、子どもの前で話すときは常に笑顔で。声を張らない範囲で大きな声で、はっきりと、ゆっくり話す。わかりやすい説明を心がける。**
② 児童第 1 回アンケート実施（約 5 分） アンケートを配布します。アンケートに回答したくないひとは答えなくてもよいです。成績には関係ありません。アンケートに協力してくれる人は、学年・クラス・出席番号・性別を書いて、始めてください。時間は約 5 分です。**アンケートの質問を読み上げる。** 書き終わった人は、答え忘れたところがないか、見直してください。 まだ時間がほしい人はいますか？　いなければ回収します。 表紙を上にして、後ろから集めてください。	・【児童・第 1 回】と書いてある封筒から、アンケートを配布し、余りは封筒に入れる。 ・出席番号の記入を忘れないよう、呼びかける。 ・回答中、教室内の見回りは控える。 ・集めたアンケートは、【児童・第 1 回】と書いてある封筒に戻す。
③ 食物アレルギーについて説明（10 分） さて、みんなは食物アレルギーという言葉を聞いたことがありますか。今日は食物アレルギーについて学習します。 食べ物を食べたときに、おなかがいたくなったり、吐き気がしたり、皮膚にじんましんができたりする、アレルギー症状があらわれることがあります。場合によっては、ショックを起こして死んでしまう場合もあります。食べ物に含まれているアレルゲンとよばれるタンパク質にからだが反応したことが原因でおこるアレルギーを食物アレルギーといいます。**今アレルギーが無い人も後々アレルギーが出てくることもあります。** アレルギーを引き起こすアレルゲンは、人によって違います。 お友達のなかには、食物アレルギーがあるので、給食などでみんなと違うメニューや食材を使ったものを食べているひとがいます。 食物アレルギーがあるときに、どうやってメニューを選ぶのか、それをみなで体験してみます。 ④ ゲーム実施（約 25 分） 1）カードの配布と説明 **それではみなさん、ゲームの説明に入ります。まずはカードを配ります。まだ触らずに待っていてくださいね。** **（配布後）** **まずはこのキラキラが描いてある白いカードを、裏向きのまま、1 人 3 枚ずつ配ってください。配りましたね。前を見てください。私はこの 3 枚です（持っているアレルゲンカードを見せる）。これは、どういうことか説明します。このゲームの中で、僕は卵とえびを食べることができません。この白いカード（無地）は、何も書いていないので関係ありません。みなさんも、自分のカードに何が書いてあるか確認してください。それはみなさんがこのゲームの中で食べることができないアレルゲンです。** **次に、食べ物のカードを絵が書いてある方を上にして、1 人 5 枚ず**	まず、4 人から 5 人のグループをつくっておく。 カードの配布 パネル 1 で説明 **机が班分けされていなければ、班分けさせる。** **卵・えび・無地のカードをあらかじめもっておく。（アレルゲンはなんでも良い）** **各机を見回る人がいる場合、3 枚とも無地の子どもがいないかどうかを確認する。**

つ配ってください。 それでは自分の前に、5枚並べてみましょう。では、一番右のメニューカードの裏をこっそり見てみてください。その料理に入ってるアレルゲンが書いてあります。みなさん、その料理を食べることができますか？僕は〇〇というメニューカードを食べることができませんでした。卵が入ってるからですね。それでは、5枚のメニューカードをすべて、こっそり見てみましょう。 （待機） 配られたカードの中で、食べれない料理がある人は手を挙げてくださいー（挙手させる、笑顔でうなずきながら、確認）はい、それでは食べれないカードを食べれるカードへと変えていきましょうね。 2）ルールの説明 それではルールを説明します。 まず、メニューカードはよくきって、ひとつにまとめ、真ん中におきます。これを「山」と呼びます。上から順番に5枚とり、「山」の周りに並べます。このとき、メニューの名前とその絵が描いてある表面をみんなで見れるようにしましょう。裏は見てはいけませんよ。 次に、じゃんけんをして順番を決めましょう。 勝った人から時計まわりに順番にゲームをすすめます。 それでは最初の人、よく聞いていてくださいね。今から、自分のメニューと、場に出ているメニューの1枚を交換します。まずは、自分のメニューの中で、食べられないものがあれば、それを場に出しましょう。そして、山の周りの5枚のうち自分の食べられそうなものをとってください。交換しましたか？交換したら、新しいカードの裏をこっそり見てみましょう。自分の食べられないものはありませんでしたか？大丈夫かな？ それでは、時計回りに、1人1回ずつやってください。終わった班から、静かに待っていてね。 （全員1回ずつ終わったところで） はい、それではみなさん。もうひとつ、教えておくことがあります。 もう全部食べられるよ！って言う人、何人かいますよね。自分の目の前にあるメニューが全部食べられるようになったら、今度は、いろんな色を集めましょう。全部で5つの色があります。わかりますか？ （確認させる） もう一度確認しますよ。 ・まずは自分の料理が全部食べられるものであること。 ・そして、そのうえでなるべくたくさんの色があること。 この2つを目指して頑張ってください。 そしてもう一つ。「山」の周りにならんでいるカードのなかに欲しいメニューカードがない場合は、「山」の周りの5枚全部を捨て、「山」から新しくメニューカードを5枚上からめくって並べて入れ替えすることができます。入れ替え後、交換しますが、交換できるのは1枚	パネル2で説明 各グループじゃんけんの終了を確認 パネル3で説明 5枚全部交換ルールは小学生対象なら無くても良い。 黒板に書いておくのも良い。 ルールがわかったか確認。

（か５枚全部）です。 　カードの総入れ替えは**１人１回**だけです。捨てられたカードは、メニューの絵が描いてある表向きのまま、「山」とは別にまとめておきます。（※アレルゲンカードは交換しません） 　わかりましたか。 　**それでは１人後２回ずつやります。２周終わった**グループは、メニューカードにどんなアレルゲンが含まれているのか、おともだちのアレルゲンは何なのか、カードをお互いに見せあいっこしていてください。 3）勝敗の決定 　全員、メニューカードとアレルゲンカードを裏返してください。 　メニューカードのなかに、自分のアレルゲン名が書かれていたら、それは自分の健康によくない、食べられないので５日間のメニューから省きます。 **（グループ単位で勝敗を決める場合）** 　自分の手元のメニューカードが５枚そろっていて、５色になっている人が優勝です。 　５色のひとが２人以上いるとき、勝敗は、メニューカードの右上に書かれているポイント数の合計点が多いひとのほうが勝ちになります。 　５色でない人のポイント数の合計の計算では、同じ色のカードはより健康によいと思うメニューのカード１枚だけを選んで、そのポイントだけを数えます。 　獲得したポイント数によって順位が決まります。 　**（クラスなど全員から優勝者を決める場合）** 　**食べられるメニューカードがまず５枚全部になっている人、手をあげてください。** 　**では次に、その５枚がすべて違う色の人手をあげていてください。** 　**次に、カードの右上にポイントが書いてあります。その５色５枚のポイントの合計を計算してください。そのポイントが●点以上の人、手をあげてください。**	**机を回りながら、パスは無しであることも状況を見て説明する。** 各グループ終了したのを確認する。 **５枚持っている人を挙手させた上で、その人たちの中で 3pt 以上の人～は手を上げ続けてくださいーとやっていき、最後に残った人の名前と持っていたアレルゲンを聞いて拍手する。**
⑤　ふりかえり 　さて、みなさん、アレルゲンカードをみてください。ここには、日本人の食物アレルギーの人に多いアレルゲンが書いてあります。何が書いてありますか。アレルゲンはこれだけではありません。カードにある７つのアレルゲンについては、お菓子やカップめんなどの加工食品に含まれていたら、その表示に必ず書かなければいけないと国が決めているものです。 　メニューカードをみてください。メニューに含まれているアレルゲンは、いろいろです。どのメニューにどんなアレルゲンが含まれているでしょうか。作る人によって、このメニューカードには書かれていないアレルゲンが含まれる場合もあります。 　さて、お友達がメニューカードを選ぶとき、お友達にすすめたりし	ポイント ・日本に代表的なアレルゲン７つ ・加工食品の義務表示の品目となっている。 ・メニューを選ぶときに、友達にすすめても、アレルギーがあったら断ること。無理に食べさせてはならないこと。 ・食物アレルギーがある場合には、食べる前に、自分のアレルゲンが含まれていないか必ず確認すること。 ・今は、アレルギーがなくても、これからアレルギーになる場合があること。

ましましたか。お友達は、自分のアレルゲンが入っていたら、ちゃんとだめだめと断ることができていましたか。食物アレルギーがあるひとに、無理に食べ物をすすめてはいけません。 　食物アレルギーがあったら、食べる前に、そのメニューに自分のアレルゲンが含まれていないか、お店のひとにきいたりして、必ず確認をしましょう。 　いままで食物アレルギーでなくても、これからアレルギーになる場合があります。	
⑥　アンケート（約３分） 　最後に、もう１度アンケートをします。さっきと同じように、協力してくれる人は、学年・クラス・出席番号・性別を書いてから、始めてください。周りの人と相談したりしないで答えてください。 **（質問文を読み上げる）** 　書き終わった人は、答え忘れたところがないか、見直してください。 　　まだ時間がほしい人はいますか？　いなければ回収します。表紙を上にして、後ろから集めてください。	・【児童・第１回】と書いてある封筒から、アンケートを配布し、余りは封筒に入れる。 ・出席番号の記入を忘れないよう、呼びかける。 ・回答中、教室内の見回りは控える。 ・集めたアンケートは、【児童・第１回】と書いてある封筒に戻す。

Molecular diagnosis of egg allergy

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Purpose of review
Allergy to hen's egg is common in infancy and childhood. Oral food challenges are often required to diagnose egg allergy, because of the limitation in the diagnostic accuracy of skin test and specific IgE to egg white. New molecular diagnostic technologies have been recently introduced into allergological research. In this article, we will review the recent literature regarding the potential value of these tests for the clinical management of egg-allergic patients.

Recent findings
Component-resolved diagnosis that can be combined with the microarray technology is promising as measurement of specific IgE antibodies to individual egg white components has been shown to predict different clinical patterns of egg allergy. Specific IgE to ovomucoid has been identified as a risk factor for persistent allergy and could indicate reactivity to heated egg. Ovomucoid and ovalbumin IgE and IgG4-binding epitope profiling could also help distinguish different clinical phenotypes of egg allergy. Particularly, egg-allergic patients with IgE antibodies reacting against sequential epitopes tend to have more persistent allergy.

Summary
Using recombinant allergens, IgE-binding epitopes, and microarrays, molecular-based technologies show promising results. However, none of these tests is ready to be used in clinical practice and oral food challenge remains the standard for the diagnosis of egg allergy.

Keywords
allergy, component, diagnosis, egg, microarray

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Introduction

After cow's milk, hen's egg allergy is the second most common food allergy in infants and young children [1–5]. A recent meta-analysis [6] of the prevalence of food allergy estimated that egg allergy affects 0.5–2.5% of young children. Egg allergy is closely associated with atopic dermatitis and was found to be present in about two-thirds of children with positive oral food challenges (OFCs) performed for allergy evaluation of atopic dermatitis [7]. The risks of sensitization to aeroallergens [8] and asthma [9] are also increased in egg-allergic children. The prognosis of egg allergy in young children is favorable and the majority of cases resolve within first years of life [10,11]. Today, the standard therapy for egg allergy is strict avoidance [12]. However, hen's egg is a versatile ingredient used in the cooking of many cultures, including a wide range of manufactured food products and the dietary avoidance of egg can thus be challenging [13].

Correct diagnosis of egg allergy is an absolute prerequisite for appropriate and potentially lifesaving preventive measures. The current tools available for diagnosis of egg allergy include the clinical history, physical examination, prick skin test and specific IgE to egg white. None of these parameters achieved sufficiently high predictive values and thus the majority of children still need to undergo clinician-supervised OFC to determine the clinical relevance of IgE sensitization. However, OFCs are resource-consuming and associated with a risk for severe anaphylaxis [14]. Although it has been shown that sensitivity and specificity of skin prick tests could be optimized using the end point titration approach [15], new testing methodologies are still needed for determining the presence and severity as well as the likelihood of resolution of egg allergy. Molecular diagnostic technologies have been recently introduced into allergological research as promising tools. Instead of measuring the IgE response to complex allergen extracts, specific responses on the level of individual allergens ('component-resolved diagnosis') or the epitopes of those allergens ('epitope mapping or profiling') are evaluated. We will discuss the potential role of these tools in the diagnosis of egg allergy.

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 [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

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.

Table 1 Major egg white allergens[a]

					IgE binding activity			
Allergen	Common name	Constitute (%)	Mw (kDa)	Carbohydrate (%)	Heat-treated	Digestive enzyme-treated	Allergenic activity	Test code[b] (in-vitro tests)
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, ImmunoCAP-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 ImmunoCAP-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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Papers of particular interest, published within the annual period of review, have been highlighted as:

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

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Key Words
Atopic dermatitis · Children · Probiotics · Biogenics · *Lactobacillus acidophilus* L-92

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-blinded, 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

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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 (Thl) 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 Thl 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: $ADASI = (1\,Ag + 2\,Ab + 3\,Ar) \cdot (I + 1)$, 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 1.5×10^{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

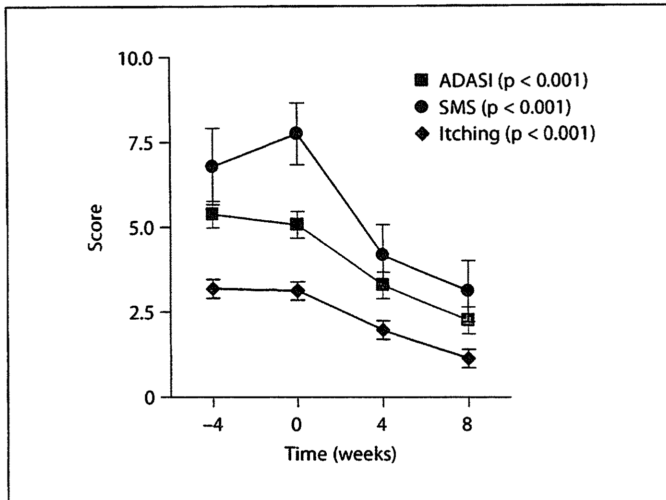

Fig. 1. Time-dependent changes in the ADASI, SMS and itching scores of children with AD with administration of L-92 FM in the preliminary casuistic study. The probabilities shown in parentheses refer to the statistical significances of the time factor from the ANOVA. The data are presented as the mean ± 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	0.956
Moderate cases	11	14	
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.

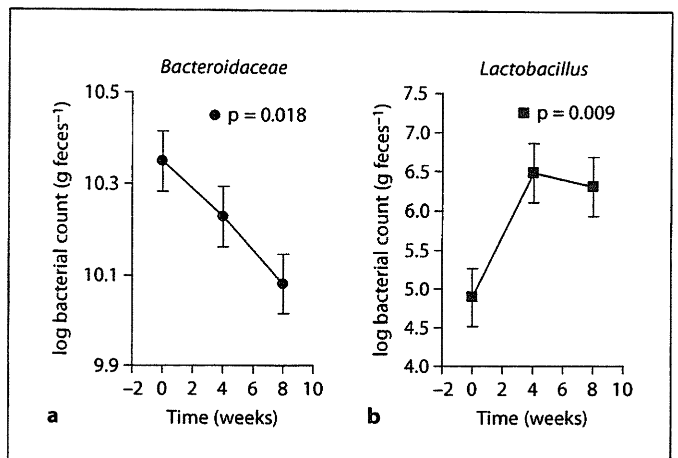

Fig. 2. Inconsistent time-dependent changes in the *Bacteroidaceae* and *Lactobacillus* counts in the intestinal microbiota of children with AD with administration of L-92 FM in the preliminary casuistic study. The probability figures in both tracing areas refer to the statistical significances of the 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 significant 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-

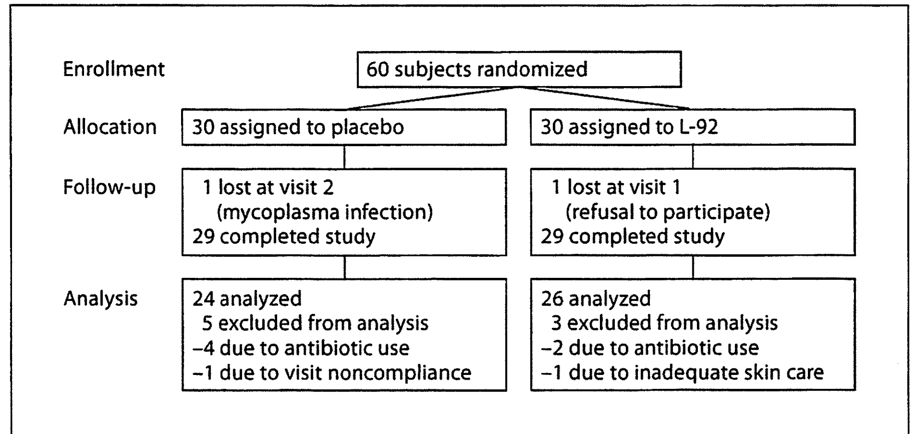

Fig. 3. Profile flow chart of the validity affirmation study.

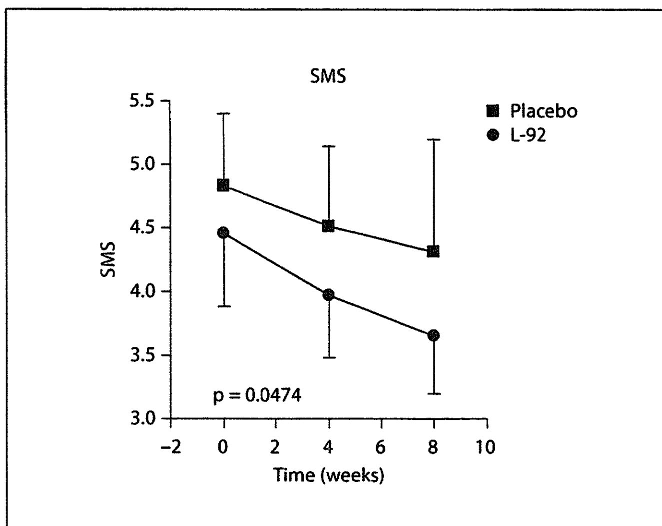

Fig. 4. Time course of changes in the SMS of children with AD in the placebo and intervention groups in the double-blinded validity affirmation study using heat-killed L-92. The probability figures in the tracing area refer to the statistical significances of the interaction of time × group from the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

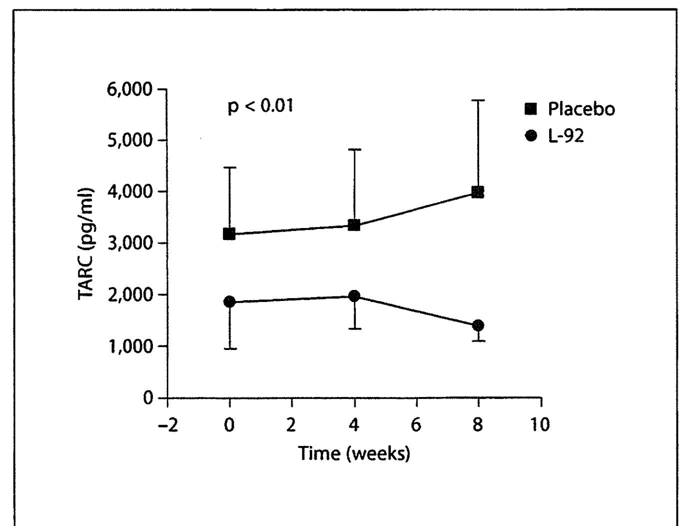

Fig. 5. Changes in the serum TARC concentration in children with AD in the placebo and intervention groups in the double-blinded validity affirmation study conducted using heat-killed L-92. The probability figures in the tracing area refer to the statistical significances of the interaction of time ×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

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

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 suggests 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 anti-allergic 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.

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