研究成果の刊行に関する一覧表

雑誌

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Torii S, Torii A, Itoh K, Urisu A, Terada A, Fujisawa T, Yamada K, Suzuki H, Ishida Y, Nakamura F, Kanzato H,Sawada D, Nonaka A, Hatanaka M, Fujiwara S	Effects of oral administration of Lactobacillus acidophilus L-92 on the symptoms and serum markers of atopic dermatitis in children.	Int Arch Allergy Immunol.	154	236-245	2010
Sakai S, Adachi R, Akiyama H, Teshima R, Doi H, Shibata H, Urisu A.	Determination of walnut protein in processed foods by enzyme-linked immunosorbent assay: Interlaboratory study.	J AOAC Int.	93	1255-1261	2010
Sakai Y., Ishihata K., Nakano S., Yamada T., Yano T., Uchida K., Nakao Y., Urisu A., Adachi R., Teshima R., Akiyama H.	Specific detection of banana residue in processed foods using polymerase chain reaction,	J Agric Food Chem.	58	8145-8151	2010

Ⅳ．研究成果の刊行物・別冊

ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

A convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression in cultured mast cells

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Keywords
allergy test; food allergy; IgE; luciferase; mast cell.

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Abstract

Background: For the detection of allergen-specific IgE in sera, solid-phase IgE-binding assays like the CAP test are commonly used. Although such immunochemical methods are very sensitive, they frequently produce false positives. Degranulation of the human IgE receptor (FcεRI)-transfected rat mast cell (RBL) lines seems to be a possible indicator for human IgE, but spontaneous mediator release from these cells in the presence of human sera is not negligible.
Methods: The nuclear factor of activated T-cells (NFAT)-responsive luciferase reporter gene was stably transfected into human FcεRI-expressing RBL-SX38 cells. One established clone (RS-ATL8) was sensitized with 1 : 100 dilution of sera from patients with egg white allergy and then stimulated with purified or a crude extract of egg white allergen.
Results: Sensitization with 15 pg/ml IgE was sufficient to detect IgE crosslinking–induced luciferase expression (EXiLE) by anti-IgE stimulation. Allergen-specific EXiLE was elicited by as little as 1 fg/ml of egg white protein without cytotoxicity. There was a good correlation between results with EXiLE and oral food challenge tests on patients with egg allergy (P = 0.001687, Fisher's exact test). The measured values of EXiLE and the CAP test also correlated well (R = 0.9127, Spearman's test).
Conclusion: The EXiLE test using RS-ATL8 cells is a promising *in vitro* IgE test to evaluate the biological activity of the binding between IgE and allergens.

Food allergy occurs in 5–10% of infants and preschool children and 1–2% of school children in Japan (1). Ideally, oral food challenge (OFC) tests would be available for each patient with food allergy to determine his/her responsive allergens. However, it is often the case that OFC tests are avoided because of the patient's age, physical condition or clinical history (2). The *in vitro* allergen-specific IgE test using patients' sera, like ImmunoCAP (CAP test), is widely used for the initial screening purposes for responsive allergens. The CAP test is a highly automated, convenient and very sensitive method (sub ng/ml) for detecting serum IgE binding to allergens (3). However, results of specific IgE binding to allergens cannot always be translated into a clear diagnosis, especially in the cases of food allergy (4, 5). Such clinically irrelevant results in serum IgE tests can be partly explained by cross-reactive carbohydrate determinants (CCDs) (5). The CCD-specific IgE in patients' sera can bind to the carbohydrate residue(s) in the allergen. However, if the carbohydrate determinant has only one site per allergen, such binding between the IgE and allergen would not induce mast cell activation because of failure to crosslink the high-affinity IgE receptor (FcεRI) on the mast cells (6).

High-affinity IgE receptor is a heterotetrameric receptor composed of an α subunit, a β subunit, and a homodimer of γ subunit (7). Among these subunits, only the α subunit has a binding ability to IgE, and expression of only the α subunit is sufficient for high-affinity binding to human IgE (8). So far, there are no useful human mast cell lines that express

Abbreviations
EWP, Egg white proteins; EXiLE, IgE crosslinking–induced luciferase expression; FCS, Fetal calf serum; FcεRI, High-affinity IgE receptor; LDH, Lactate dehydrogenase; NFAT, Nuclear factor of activated T-cell; OFC, oral food challenge; OVA, Ovalbumin; RS-ATL8, RBL-SX38 cell stably transfected with NFAT-luciferase, clone 8.

abundant FcεRI and grow well (9–12). Therefore, human FcεRI-overexpressing rodent mast cell lines may be a useful system for reflecting crosslinking of FcεRI on mast cells triggered by patients' IgE and specific allergens. We and several other groups have transfected a rat basophilic leukemia-derived mast cell line, RBL-2H3, with the α subunit gene or a complete set of α/β/γ subunit genes of the human FcεRI, and analyzed the usefulness of the system (13–17). Among these cell lines, α/β/γ-transfected RBL cells were found to have the potential to be sensitized with diluted patients' sera and degranulate after the addition of specific allergens. In particular, RBL-SX38 cells, generated by Wiegand et al. (14), were found to be the most effective (18). However, human serum was cytotoxic at high concentrations (typically, more than 1 : 10–1 : 20). To avoid cytotoxicity, investigators had to sufficiently dilute serum (16), or remove the cytotoxic factors by adsorbing the sera to wild-type RBL-2H3 cells (15, 17, 18). These treatments could reduce the IgE concentration in diluted sera, or increase experimental uncertainty through increased manipulations. Moreover, the level of degranulation was relatively low after such treatments, so artificial 'accelerators' of degranulation, such as an adenosine analogue (15) or deuterium oxide (D_2O; 12–14), were required to measure meaningful responses. These compounds have been reported to potentiate the degranulation of mast cells (19–22), but the addition of high concentrations of D_2O increased spontaneous mediator release from these cells (18, 20, 21).

Crosslinking of FcεRI on mast cells will also induce marked gene expression of chemokines, cytokines, and other proteins (23). A number of transcription factors participate in such responses, and we previously demonstrated that nuclear factor of activated T-cells (NFAT) appeared to play one of the most important roles in FcεRI crosslinking–induced gene expression in RBL-2H3 cells (24).

Here, we show that the introduction of a NFAT-responsive luciferase reporter gene into human FcεRI-expressing RBL cells is a convenient method for detecting IgE crosslinking–induced mast cell activation with low-background and high sensitivity. We designated the novel method as the 'EXiLE' test; IgE crosslinking–induced luciferase expression test.

Materials and methods

Cells

RBL-SX38 cells, expressing the human FcεRI α/β/γ-subunits, were a kind gift from Dr Kinet at Beth Israel Deaconess Medical Center (Boston, MA), and were maintained as previously reported (14). The NFAT-regulated luciferase reporter gene plasmid containing hygromycin resistance gene were purchased from Biomyx (San Diego, CA, USA). The plasmid was linearized by *Bgl* I digestion, and was transferred into RBL-SX38 cells using Lipofectamine 2000 (Invitrogen, Rockville, MD, USA) following the manufacturer's protocol. Stable transfectants were selected using 600 μg/ml hygromycin. The RS-ATL8 cell line, the highest luciferase responder subclone after stimulation with 10 nM phorbol myristate acetate and 10 μM ionomycin, was established by limiting dilution and was grown in minimum essential medium (MEM) (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Nichirei Biosciences, Tokyo, Japan), penicillin/streptomycin, GlutaMAX-I, 1.2 mg/ml geneticin, and 200 μg/ml hygromycin (Invitrogen).

Human sera and allergens

Patients with suspected egg allergy referred to our hospital for investigation were enrolled in the study. Most of the patients had atopic dermatitis and asthma. Informed consent was obtained from patients, their parents, or both to collect and investigate serum samples further. The study was approved by the institutional review board of National Institute of Health Sciences and Ethics Committee of Fujita Health University School of Medicine. All sera were collected before food challenge to measure total and allergen-specific IgE levels using ImmunoCAP. Every patient was subjected to double-blind, placebo-controlled food challenges with heated egg and was judged positive if he or she was unable to eat the equivalent of a whole boiled egg, as described previously (25). Pooled sera from healthy donors were purchased from Cosmo Bio (Tokyo, Japan). All sera were stored at −80°C until use. Purified ovalbumin (OVA; grade V, >98%) was from Sigma (St. Louis, MO, USA). To determine the allergen detection limit, a 5 mg/ml egg white-extracted protein (EWP) solution (Greer labs, Lenoir, NC, USA) was used. Egg white extract (100 mg/ml of freeze-dried egg white powder in stock solution) prepared for the scratch diagnostic tests was purchased from Torii Pharmaceutical (Tokyo, Japan) for the experiments to determine the correlations between the allergy tests.

Luciferase assay

RS-ATL8 cells (5×10^4 cells/50 μl/well) were plated onto a clear-bottom white 96-well plate (ViewPlate Perkin Elmer, Waltham, MA, USA), and were incubated for several hours at 37°C in a 5% CO_2 incubator. Then, 5 μl of partly diluted (11 : 100) or serially diluted sera in MEM containing 10% FCS was added to the supernatant (final dilution in the former case, 1 : 100). After overnight incubation, the cells were washed once with sterile PBS and then stimulated for 3 h at 37°C in a 5% CO_2 incubator with allergens diluted in MEM containing 10% FCS (50 μl/well). After stimulation, 50 μl of luciferase substrate solution containing cell lysis reagent (ONE-Glo, Promega Corp., Tokyo, Japan) was added to the cells, and chemiluminescence was measured using an EnVision multilabel plate reader (Perkin Elmer). Luciferase expression levels are represented as the fold increase of light units compared with the background expression, after subtraction of a blank control (without cells).

β-Hexosaminidase assay

Stimulation-induced β-hexosaminidase release from RBL cells was measured as described previously with some

modifications (13). RBL-SX38 cells were plated and sensitized with serially diluted sera as described earlier. After overnight incubation, the cells were washed twice with normal PIPES buffer (140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM glucose, 0.1% BSA and 10 mM PIPES, pH 7.4). OVA (1 μg/ml) or goat anti-human IgE antibody (Bethyl laboratories, Montgomery, TX, USA) diluted in prewarmed PIPES buffer was added to the cells (50 μl/well). After incubation for 30 min at 37°C, supernatants were collected, and residual cells were lysed with 0.2% Triton X-100. Activity of β-Hexosaminidase in the medium and within the cells was determined by a fluorometric assay using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as a substrate (0.1 mM in 100 mM citrate, pH4.5). The reaction was stopped with 0.25 M glycine buffer after 30 min incubation at 37°C. The plate was read on a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA, USA) using 380 nm excitation and 440 nm emission filters. Degranulation levels are represented as follows;

$$R(\%) = (R_{sup} - \text{blank})/(R_{sup} - \text{blank} + R_{ppt} - \text{blank}) \times 100$$

where R is the degranulation level; R_{sup} is released enzyme activity in the supernatant; R_{ppt} is residual enzyme activity within the cell; blank is the buffer control.

LDH release assay

Cytotoxicity of human sera on RS-ATL8 cells was measured by a lactate dehydrogenase (LDH) release assay using the Cytotoxicity Detection Kit^{PLUS} (Roche Diagnostics K.K., Japan) following the manufacturer's protocol, and an EL340 (BioTek Instruments, Winooski, VT, USA).

Statistics

The cytotoxic effect of human serum was analyzed using a 1-way ANOVA followed by Dunnett's test compared with negative controls without human serum. Fisher's exact test was performed to analyze correlations between the OFC and EXiLE or CAP tests. Spearman's rank correlation test was performed to compare the EXiLE and CAP test.

Results

RS-ATL8 cells

In the present study, we transfected an NFAT-responsive luciferase reporter gene into human FcεRI α/β/γ-expressing RBL-SX38 cells. We obtained 16 clones after hygromycin resistance selection, and found that clone number 8 was the highest responder to phorbol ester and ionomycin (data not shown). This clone was designated as RS-ATL8; RBL-SX38 cell stably transfected with NFAT-Luciferase clone 8. The cells were sensitized with serially diluted healthy donor's serum overnight, and then stimulated with 1 μg/ml goat anti-human IgE antibody for 3 h. A dose-dependent increase in EXiLE in the RS-ATL8 cells was observed (Fig. 1). The original serum contained 99 ng/ml (41 IU/ml) of IgE as revealed by ELISA. If an expression level of two-fold background (without serum) was set as a cut-off, EXiLE by anti-IgE crosslinking exceeded the cut-off at 15 pg/ml of human IgE (0.006 IU/ml). In our preliminary results, sensitization and stimulation periods of 16 h (overnight) and 3 h, respectively, resulted in the highest luciferase expression (data not shown).

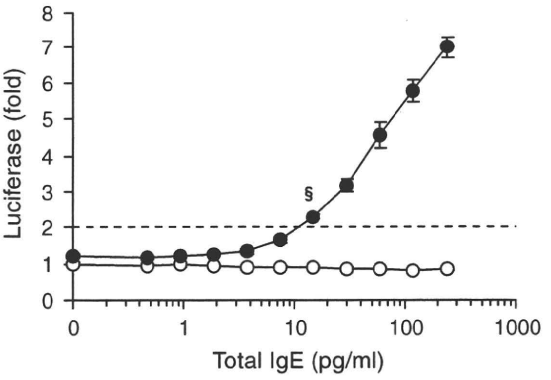

Figure 1 Detection limit of IgE crosslinking-induced luciferase expression. RS-ATL8 cells were sensitized with serial dilutions of healthy donor's serum in 10% Fetal calf serum-containing medium. The total IgE concentration in the original serum was determined by ELISA. After overnight incubation, the cells were stimulated with (closed circles) or without (open circles) 1 μg/ml of anti-human IgE for 3 h. Dashed line, two-fold level of the background (control without serum) luciferase expression. §IgE = 15 pg/ml (0.006 IU/ml). Data are means ± SEM (n = 4).

Comparison of EXiLE to the β-hexosaminidase release method

As previously reported, RBL-SX38 cells are capable of effectively degranulating after sensitization using sera from patients with peanut allergy and the addition of peanut allergen (15, 19). However, human serum (1 : 10) was cytotoxic to the cells, so it had to be cross-adsorbed against wild type RBL-2H3 cells prior to the sensitization of RBL-SX38 cells (15, 19). Moreover, when degranulation assays were performed, it was usual for an 'accelerator' of degranulation, like D_2O or 5′-N-ethylcarboxamide, to be added to the medium (15–18). Here, RBL-SX38 cells or RS-ATL8 cells were similarly sensitized with serially diluted serum from egg allergy patient's (total IgE, 12 700 IU/ml; egg white specific IgE, > 100 U_A/ml), and stimulated with 1 μg/ml of OVA and anti-human IgE for 30 min in PIPES buffer or for 3 h in 10% FCS-containing MEM, respectively (Fig. 2). RBL-SX38 cells degranulated following stimulation with a specific allergen even without a degranulation accelerator (Fig. 2A). However, as serum concentration increased, spontaneous release increased, particularly in cases of more than 0.1% serum. The minimum and maximum spontaneous release was 14.6% and 34.9% of total enzyme, respectively. Meanwhile, the background luciferase expression levels were gradually decreased at > 0.3% serum (Fig. 2B). EXiLE by OVA exceeded 2.0 at 0.1% serum, and was > 2.0 until 3% serum.

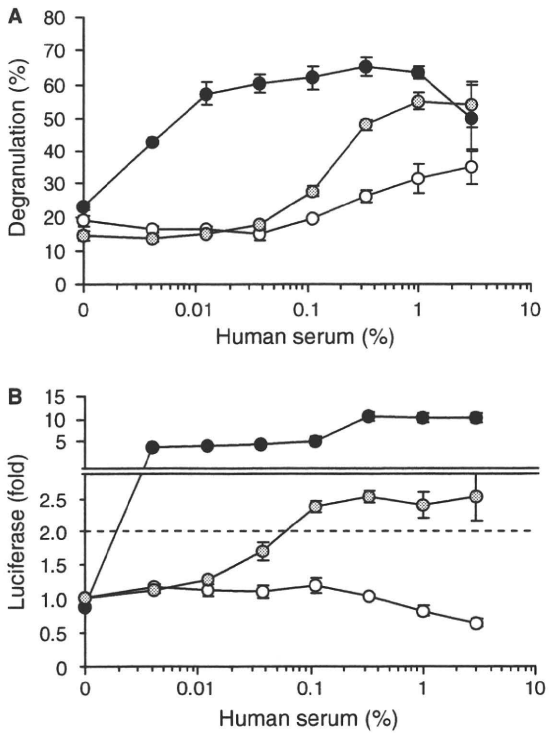

Figure 2 Comparison of degranulation and luciferase expression. RBL-SX38 cells (A) and RS-ATL8 cells (B) were sensitized with serial dilutions of egg white allergy patient's serum (total IgE, 12 700 IU/ml; egg white specific IgE, >100 U_A/ml) overnight. Cells were stimulated with 1 μg/ml ovalbumin (shaded circles), 1 μg/ml anti-human IgE (closed circles), or solvent alone (open circles). Solvent does not include any degranulation accelerator such as D_2O. Degranulation after 30-min stimulation (A), and luciferase expression after 3-h stimulation (B) are shown. Dashed line in B, two-fold level of background (control without serum) luciferase expression. Data are means ± SEM (n = 4 in a, n = 3 in b).

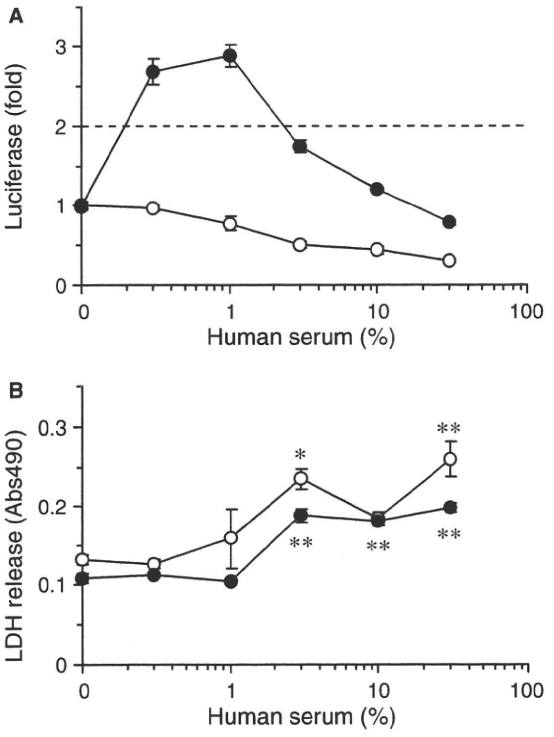

Figure 3 Appropriate serum dilution. RS-ATL8 cells were sensitized with serial dilution of pooled healthy donor's serum overnight. Cells were stimulated with 1 μg/ml anti-human IgE (closed circles), or solvent alone (open circles) for 3 h. (A) IgE crosslinking–induced luciferase expression by anti-human IgE; (B) Cytotoxicity of human serum on the RS-ATL8 cells revealed by lactate dehydrogenase release. Dashed line in a, two-fold level of background (control without serum) luciferase expression. Data are means ± SEM (n = 4). *P < 0.05, **P < 0.01 (Dunnett's test).

Anti-IgE stimulation elicited dramatic increases in luciferase in RS-ATL8 cells throughout the serum concentration range tested (0.004–3%), whereas β-hexosaminidase release by anti-IgE stimulation decreased nearly to the spontaneous release levels at higher serum concentrations (Fig. 2A).

Appropriate serum dilution for EXiLE in RS-ATL8 cells

To determine the appropriate dilution factor for human sera, anti-human IgE-induced luciferase expression and cell viability after sensitization of RS-ATL8 cells with serially diluted healthy donor's serum were measured. LDH is a stable cytosolic enzyme and is released to the medium if the plasma membrane is damaged (26). Anti-IgE stimulation elicited a marked increase in the luciferase expression in RS-ATL8 cells sensitized with 0.3% and 1% serum; however, at higher serum concentrations (≥3–30%), the expression decreased depending on the serum concentration (Fig. 3A). The decrease was also seen in the nonstimulated controls. Such decreases in luciferase expression inversely correlated to LDH release (Fig. 3B), suggesting that human serum has a cytotoxic activity on the RS-ATL8 cells. In preliminary experiments, this effect could be decreased by more than 50% by heating the sera to 56°C for 30 min, suggesting heat-labile factors (maybe complement) cause the cytotoxicity. However, heat-inactivation was not to be performed in our experiments because IgE is also heat-labile (27). Therefore, we concluded that a 1 : 100-dilution was the most appropriate to determine EXiLE without cytotoxicity.

Detection limit of allergen by means of EXiLE

We next tried to determine the detection limit of egg white allergen using the patient's serum. RS-ATL8 cells were sensitized with 1 : 100-diluted patient serum or healthy donor serum overnight, and were stimulated with EWP serially diluted in MEM containing 10% FCS for 3 h. Figure 4 shows that EXiLE by EWP peaked at 1 ng/ml and exceeded 2.0 at 1 fg/ml. The lot-to-lot reproducibility and effect of cell passage number on the EXiLE test were deter-

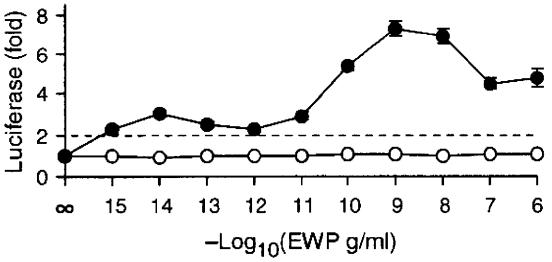

Figure 4 Detection limit of egg white allergen. RS-ATL8 cells were sensitized with 1 : 100-diluted healthy donor's serum (open circles) or egg allergy patient serum (closed circles) overnight. Cells were stimulated with the indicated concentrations of egg white proteins (EWP) diluted in 10% Fetal calf serum-containing medium for 3 h. Dashed line, two-fold level of background (0 g/ml EWP) luciferase expression. Data are means ± SEM (n = 4).

mined; 1 ng/ml EWP induced a 7.3 ± 1.1 (mean ± SEM) fold increase in luciferase expression across six different time points (Fig. S1). It was impossible to perform the same experiment on β-hexosaminidase release from RBL-SX38 cells, because significant spontaneous release (49%) was induced by sensitization with the 1 : 100-diluted serum (data not shown).

Comparison of EXiLE to OFC and CAP tests

Nineteen sera from suspected egg-allergy patients who had been diagnosed by the OFC test (double-blind placebo-controlled food challenge test) with heated egg and also by the egg white–specific serum IgE test (CAP test) were subjected to the EXiLE test for comparison. RS-ATL8 cells were sensitized with 1 : 100-diluted patients' sera, and stimulated with 0, 1, 10, 100, and 1000 ng/ml of EWP, and EXiLE by EWP was calculated by assuming the background expression with no allergen was 1.0, and was judged to be positive if the maximum EXiLE value (Max EXiLE) was ≥2.0. Table 1 summarizes the results; 12 of 19 patients were positive in the OFC test, 18 of 19 were positive in the CAP test (class 2 as a cut-off; specific IgE ≥ 0.70 U_A/ml, a level indicated in the manufacturer's instruction), and 12 of 19 were positive in the EXiLE test. Among 12 OFC positive patients' sera, 11 and 12 were predicted to be positive by EXiLE and CAP tests, respectively, while among 7 OFC negative patients' sera, 6 and 1 were predicted to be negative by EXiLE and CAP tests, respectively. As revealed by Fisher's exact test, the OFC and EXiLE tests correlated well (P = 0.001687), whereas the OFC and CAP tests did not (P = 0.3684).

The EXiLE scores 1, 2, 3, and 4 correspond to the minimum concentrations of responsive EWP; 1000, 100, 10, and 1 ng/ml, respectively, while 0 means a nonresponder. We

Table 1 EXiLE in RS-ATL8 cells sensitized with egg-allergy patients' sera

Patient	Age, Sex	OFC* test	totIgE (IU/ml)	CAP (U_A/ml)	CAP class	CAP* test†	EXiLE (fold) by EWP (ng/ml) 1	10	100	1000	Max EXiLE (fold)	EXiLE score‡	EXiLE* test§	Anti-IgE EXiLE (fold)
#79	6, M	+	1922	**76.8**	5	+	**2.3**	**3.4**	**4.5**	**3.5**	**4.5**	4	+	16.7
#77	13, M	+	1273	**68.6**	5	+	**4.1**	**8.8**	**9.5**	**7.5**	**9.5**	4	+	17.9
#78	9,F	+	844	**48.3**	4	+	**4.8**	**8.3**	**8.4**	**8.6**	**8.6**	4	+	17.6
#80	6,M	+	381	**35.1**	4	+	1.5	**3.0**	**3.9**	**5.0**	**5.0**	3	+	12.0
#74	9,M	+	2663	**29.9**	4	+	**3.9**	**3.3**	**4.5**	**4.8**	**4.8**	4	+	20.9
#90	14, F	+	12323	**25.6**	4	+	1.5	**2.5**	**2.8**	**2.8**	**2.8**	3	+	16.7
#84	5,M	+	331	**13.2**	3	+	1.5	1.7	**2.3**	1.9	**2.3**	2	+	7.84
#83	10, M	+	772	**8.98**	3	+	1.3	1.7	**2.5**	**3.2**	**3.2**	2	+	17.7
#85	8,M	+	619	**5.57**	3	+	1.6	**2.5**	**3.4**	**3.1**	**3.4**	3	+	24.2
#81	10, F	+	795	**4.54**	3	+	1.5	**2.2**	1.8	**2.7**	**2.7**	3	+	19.5
#76	6,M	+	509	**3.91**	3	+	1.4	1.7	**2.0**	**2.2**	**2.2**	2	+	23.0
#94	9,F	+	653	**2.99**	2	+	0.8	1.3	1.3	1.5	1.5	0	−	11.8
#73	9,F	−	7339	**33.4**	4	+	1.8	**2.2**	**2.2**	1.9	**2.2**	3	+	26.9
#67	13, M	−	22600	**4.46**	3	+	1.0	1.2	1.4	1.6	1.6	0	−	14.2
#82	10, M	−	1470	**3.45**	2	+	1.2	1.2	1.5	1.4	1.5	0	−	16.7
#95	5,F	−	578	**2.03**	2	+	1.1	1.2	1.3	1.5	1.5	0	−	10.0
#75	6,M	−	1006	**1.17**	2	+	0.8	0.9	0.9	1.0	1.0	0	−	25.0
#100	9,M	−	5356	**0.89**	2	+	0.9	1.3	1.5	1.4	1.5	0	−	23.0
#99	13, M	−	475	<0.34	0	−	1.1	1.1	1.3	1.5	1.5	0	−	7.13

RS-ATL8 cells were sensitized with 1 : 100-diluted egg-allergy patients' sera overnight, and stimulated with the indicated concentrations of EWP or 1 μg/ml anti-IgE for 3 h. *Correlation between OFC and CAP tests was P = 0.3684, and that between OFC and EXiLE tests was P = 0.001687 (Fisher's exact test). †CAP test was considered positive if the class was ≥2 (i.e. ≥0.70 U_A/ml) ‡EXiLE score varies from 0 to 4. Score 1, 2, 3, and 4 means its EXiLE exceeds 2.0 at EWP concentrations of 1000, 100, 10, and 1 ng/ml, respectively. Score 0 means negative. §EXiLE test was judged to be positive if Max EXiLE was more than the cut-off level (2.0). Values greater than cut-off levels are represented in Bold. EWP, Egg white proteins; EXiLE, IgE crosslinking–induced luciferase expression; OFC, oral food challenge.

analyzed the correlation between the values and scores of the CAP and EXiLE tests by a Spearman's rank test. Figure 5 illustrates that there are very good correlations between the CAP and EXiLE scores (R = 0.9319, P < 0.001), and between the CAP (specific IgE concentration) and Max EXiLE values (R = 0.9127, P < 0.001).

Max EXiLE values and the ratio of specific/total IgE concentrations were also compared and found to correlate well, but the Spearman's correlation coefficient was slightly lower (R = 0.8561, P < 0.001) than that between CAP and Max EXiLE. These results seemed to be consistent with a previous study by Dibbern Jr. et al. (15), looking at the degranulation of RBL-SX38 cells.

Discussion

In the present study, we have established a stable luciferase-reporting humanized RBL clone, RS-ATL8, derived from the previously described RBL-SX38 cells (14, 15). RBL-SX38 cells released β-hexosaminidase on activation following sensitization with serum from patients with egg allergy and stimulation with EWP, without any degranulation accelerators

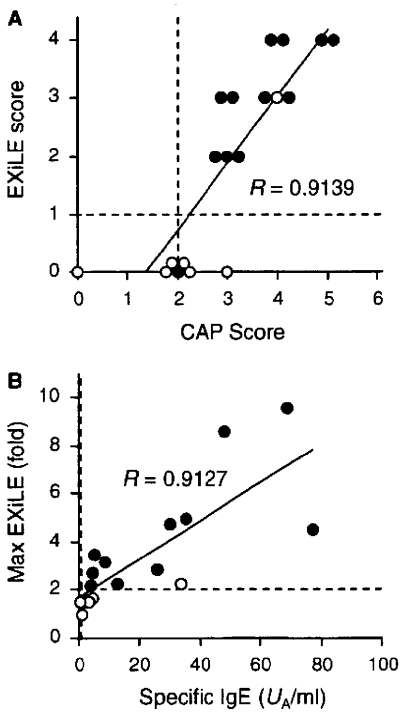

Figure 5 Correlation between CAP test and IgE crosslinking-induced luciferase expression (EXiLE) test. Results in Table 1 are depicted in graphic form. Correlation between CAP classes and EXiLE scores (A), and that of allergen-specific IgE concentration and Max EXiLE (B) are shown. Closed and open circles, subjects from oral food challenge positive and negative, respectively. Vertical and horizontal dashed lines, cut-off levels of the CAP and EXiLE test, respectively. R-values of Spearman's rank correlation test were 0.9139 (P < 0.001) in A, and 0.9127 (P < 0.001) in B.

(Fig. 2A). However, there was significant spontaneous release from serum-sensitized but nonstimulated cells, which varied from patient to patient (data not shown). In a previous report, Ladics et al. described that humanized RBL cells, including RBL-SX38 cells, were not conducive to using a broad range of allergic subjects sera, because of difficulties in generating consistent, robust responses (15). In our system, human sera can be diluted 1 : 100, avoiding serum cytotoxicity (Fig. 3B), and generate consistent responses without any artificial accelerators with very high sensitivity (≥15 pg/ml IgE). In appropriate conditions, our system can detect at least 1 fg/ml of allergen (Fig. 4). It is noteworthy that the medium used here is supplemented with 10% FCS, which includes incredible amounts of nonspecific proteins, ensuring the robustness of the system. Indeed, antigen-specific luciferase expression was observed either by purified allergen (Fig. 2B) or by a mixture of egg white allergens (Fig. 4) with the same patient's serum. So far, nine food allergens (egg white, egg yolk, milk, wheat, buckwheat, peanut, shrimp, crab, and sesame) and three inhaled allergens (mite, cedar pollen, and cat dander) were capable of inducing EXiLE in RS-ATL8 cells sensitized with specific patients' sera. Some of the EXiLE experiments undertaken for the screening of allergens are shown in Fig. S2. As allergens are in the liquid phase in our system, the degree-of-freedom for testing allergens appears to be higher than in a solid phase system like CAP.

As shown in Table 1, comparative analysis of the EXiLE test to the OFC test demonstrated that the predicted results with the EXiLE test correlated well to the final diagnosis obtained with the OFC test (P = 0.001687, Fisher's exact test), whereas the results with the CAP test had no significant correlation to the OFC test (P = 0.3684). The low correlation between the CAP and OFC tests here could be partially explained by the high sensitivity of the CAP test. In CAP tests, a class of 2 (≥0.70 U_A/ml) is usually adopted for a cut-off point following the manufacturer's instructions. However, Ando et al. (25) recently reported that the optimal cut-off point of egg white–specific IgE was 2.82 and 7.38 U_A/ml, for the OFC tests with raw and heated egg white, respectively. In addition, Komata et al. (28) also reported that the 95% probability was achieved at 30.0 U_A/ml for patients with egg white allergy who were aged ≥2. Considering these aspects, the class of 2 might be too strict to exclude false positives, at least for the patients in the present study. If a CAP class of 3 (≥3.50 U_A/ml) was set as a cut-off in Table 1, the correlation of the results with the CAP test and OFC test would increase to a significant level (P = 0.009546). Therefore, the CAP test is so sensitive that it seems to be suitable for the first screening of allergen-specific IgE in the sera.

Both of the scores (Fig. 5A) and values (Fig. 5B) from the EXiLE and CAP tests correlated well. These results strongly suggest that an increase in the luciferase expression observed in RS-ATL8 cells reflects crosslinking of the antigen-specific IgE bound to FcεRI on the mast cells. Therefore, these three systems seemed to correlate well, but there were several specific differences in the results between them (Table 1). Compared with the OFC results, the EXiLE test predicted one false-negative (#94) and one false-positive (#73). Reasons for

this are unclear, but the false-negative result might be because of the higher detection limit of the EXiLE system, as serum #94 contained no more than 2.99 U_A/ml specific IgE. Another possibility is that other substances, like chemokines and cytokines, can induce histamine release from human basophils (29, 30). As RS-ATL8 cells only have human FcεRI, the cells would not be activated through IgE-independent mechanisms like these. This might also be an explanation for the false-positive case. If neutralizing IgG antibodies (31) against EWP were present *in vivo*, it would not be strange that oral challenge did not show marked symptoms in the patient, whereas antibodies other than IgE in the patient's serum will be washed away from the medium before the addition of specific antigen to RS-ATL8 cells in the present study. A qualitative change in epitopes by heat and/or digestion could be another possibility.

One noteworthy example is patient #67, whose serum contained 4.46 U_A/ml (class 3) of egg white–specific IgE, but can eat eggs with no allergic symptoms. With this serum, EXiLE levels were gradually increased to 1.6 depending on EWP concentration, but did not exceed the 2.0 cut-off level (Table 1). Considering the substantially high level of total IgE in his serum (22 600 IU/ml), most of the specific IgE seems to be biologically meaningless cross-reactive antibodies, lacking the ability to activate mast cells and basophils (5, 32). It is important to distinguish such patients from the CAP-positive patient group because food avoidance is unnecessary and would lower the quality of life for such patients (33). The EXiLE test could be suitable for this purpose, screening allergen-specific IgE in sera following immunochemical *in vitro* tests like CAP.

This novel *in vitro* technique might be applicable to: (i) second screening of allergen-specific IgE in the serum following CAP tests, (ii) cross-reactivity tests between known allergens and potential allergens (safety assessment of gene-modified crops), (iii) standardization of allergen extracts for clinical usage, (iv) screening of novel allergens and/or epitopes, (v) detection of small amounts of allergens in foods, (vi) epidemiological study of allergens using a serum bank, and (vii) high-throughput screening for the seed compounds of anti-allergic drugs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Lot-to-lot reproducibility and the effect of passage number on the EXiLE test.
Figure S2. Allergen screening using EXiLE test.

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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.

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 (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 1.5×10^{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

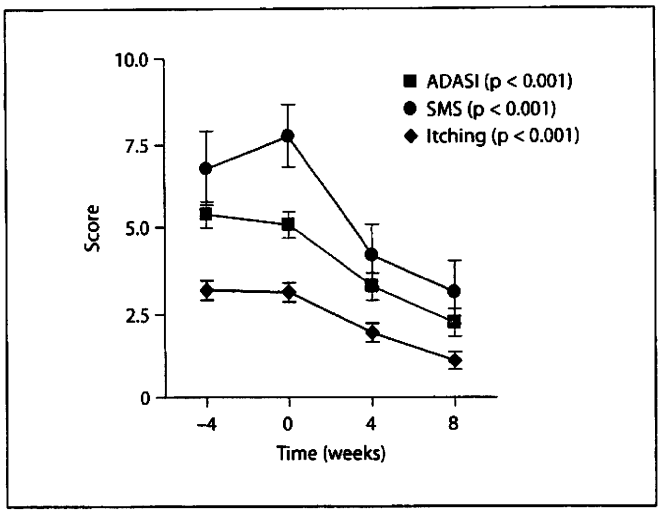

Fig. 1. Time-dependent changes in the ADASI, SMS and itching scores of children with AD with administration of L-92 FM in the preliminary casuistic study. The probabilities shown in parentheses refer to the statistical significances of the time factor from the ANOVA. The data are presented as the mean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

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).

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.

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.

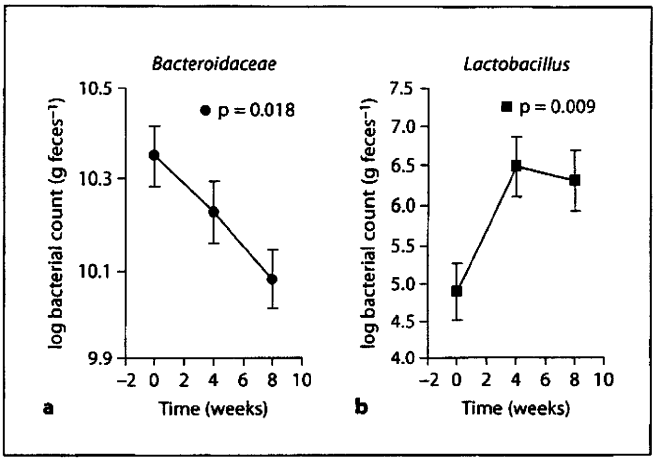

Fig. 2. Inconsistent time-dependent changes in the *Bacteroidaceae* and *Lactobacillus* counts in the intestinal microbiota of children with AD with administration of L-92 FM in the preliminary casuistic study. The probability figures in both tracing areas refer to the statistical significances of the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

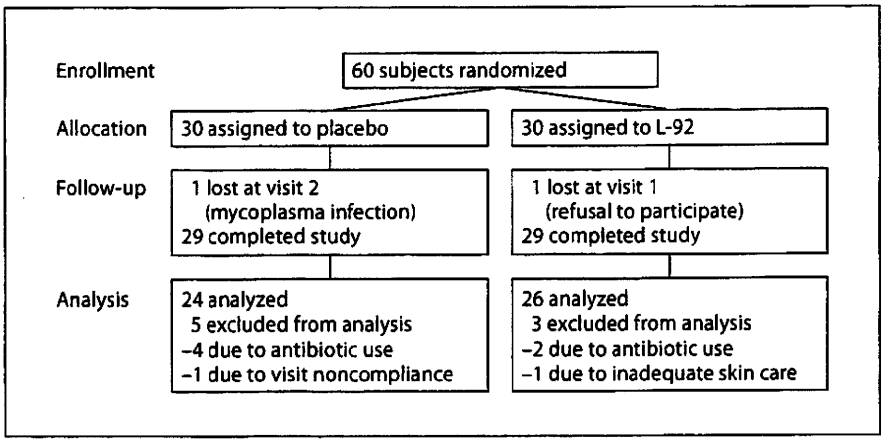

Fig. 3. Profile flow chart of the validity affirmation study.

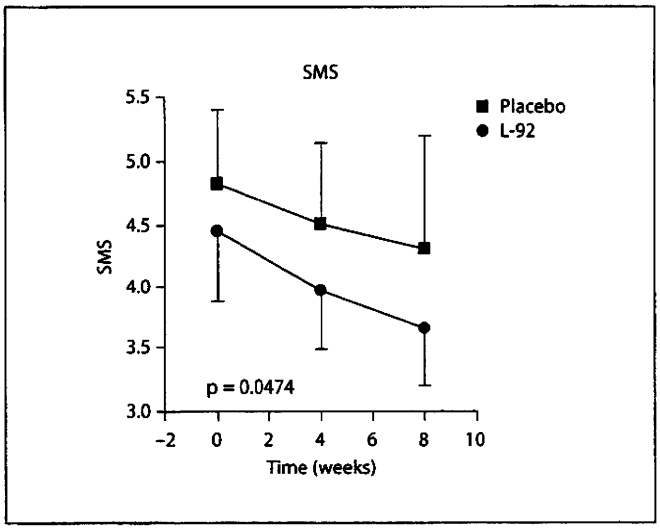

Fig. 4. Time course of changes in the SMS of children with AD in the placebo and intervention groups in the double-blinded validity affirmation study using heat-killed L-92. The probability figures in the tracing area refer to the statistical significances of the interaction of time × group from the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

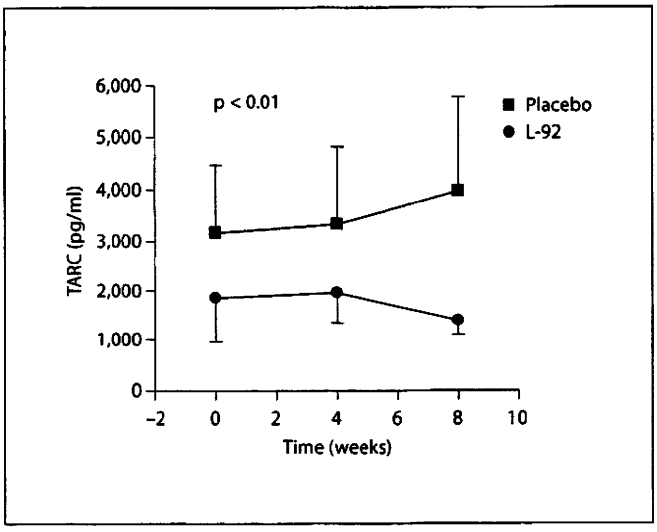

Fig. 5. Changes in the serum TARC concentration in children with AD in the placebo and intervention groups in the double-blinded validity affirmation study conducted using heat-killed L-92. The probability figures in the tracing area refer to the statistical significances of the interaction of time × 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.

Acknowledgments

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