図2　Cytokine production by spleen T cells from OK-432-treated mice at the induction phase—TLR2 ko and wild type mice

Cytokine production by spleen T cells from OK-432 treated or PBS-treated mice at the induction phase. The enriched T cells (5×10⁵ cells) from the spleen of OK-432 treated or PBS-treated mice sensitized with OVA or naive mice were cultured with OVA in the presence of MMC-treated spleen cells (5×10⁵ cells) for 48h at 37℃. All mice were immunized with OVA/ALUM. The proliferation activities of spleen T cells was assessed by incorporation of [³H]TdR. IL-4 and IFN-γ production by the T cells was assessed by ELISA. The datas are representative of four independent experiments using pooled cells from five mice and are shown as mean of triplicate determinations±SD. ＊P＜0.005

制することが期待され，ヒトでの予防的治療としての可能性がある．生後にTh1型のサイトカイン産生Th細胞を誘導する環境因子，さらに介在因子として重要な働きをしているものとして，自然免疫に関与するTLRが注目されている．TLRを介した刺激がTh1型の免疫応答を惹起する機序としては，ウィルス由来のRNAやグラム陽性球菌のPGN，グラム陰性桿菌のLPSなどが，TLR3やTLR4などを介してIL-12の産生を誘導し未分化T細胞からのTh1細胞への分化を誘導すると考えられている⁵⁾⁶⁾．

2. マウススギ花粉症モデルにおけるCry j 1/Cry j 2 遺伝子導入米の経口投与の影響

人間は自らの身体に巧妙に構築された粘膜免疫系のシステムを保有している．近年の粘膜免疫学の進歩により，粘膜面を介した抗原の投与により，感染防御を強化するワクチン療法の治療戦略を展開すると同時に，一方では経口免疫寛容の理論に基づくアレルギー性炎症の制御を目的とした検討が行われるようになった．口から食べる食物ワクチンの概念は，抗原のデリバリーシステムからすると日常的で自然であり，毎日の食生活そのものは超身近な存在である．

農業生物資源研究所の高岩らにより開発されたマウスのCry j 1 およびCry j 2 のT細胞エピトープ遺伝子導入米を用いた経口免疫療法のマウスを用いた研究(図3～5)では，東京大学医科学研究所の清野研究室と著者らの島根大学耳鼻
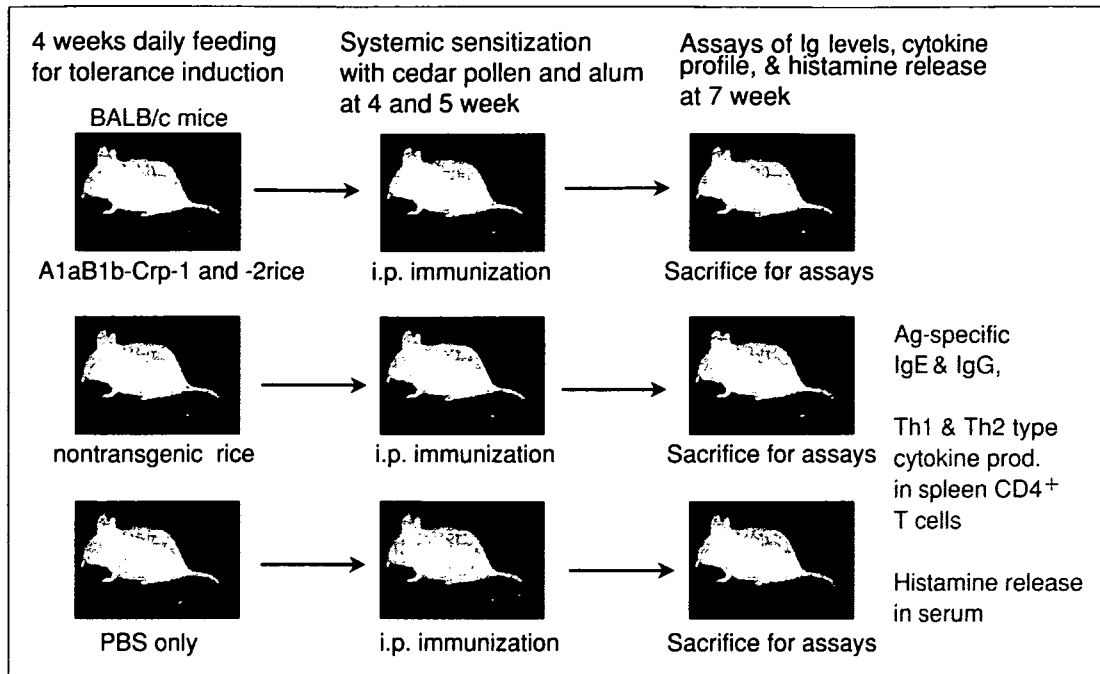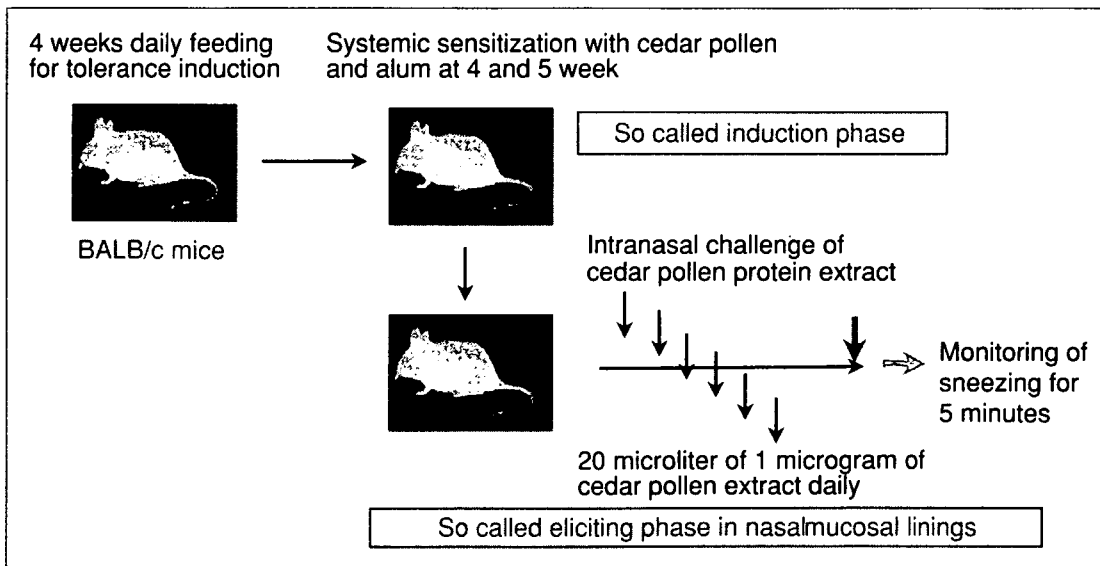
図 3　Experimental protocol（1）

図 4　Experimental protocol（2）to see the nasal symptoms

科との共同研究により，マウススギ花粉症モデ
ルで，血中スギ特異的IgE産生の低下や，脾臓 T
細胞のアレルゲン特異的Th2型サイトカイン産生
の有意な低下が認められ，さらにはアレルゲン
の点鼻感作による反応相での鼻症状の緩和が観
察された[7]．

　この研究成果は，経口免疫寛容の概念を基本
にした画期的な研究成果であり，国際的にも高
い評価を受けている[8]．今後，遺伝子組み換え技
術を応用したスギ花粉症緩和米として，安全性
の確認と同時に，ヒトでの臨床研究の成果が大

いに期待されるところである．

反応相制御についての実験的検討

1．マウスアレルギー性鼻炎モデルにおけるLPS
　の影響

　アレルギー性鼻炎の実効相において中心的な
役割を担う肥満細胞では，in vitroの実験により，
LPS刺激によりTLR4を介してTh2型サイトカイン
産生が誘導されることが証明されているが，in vivo
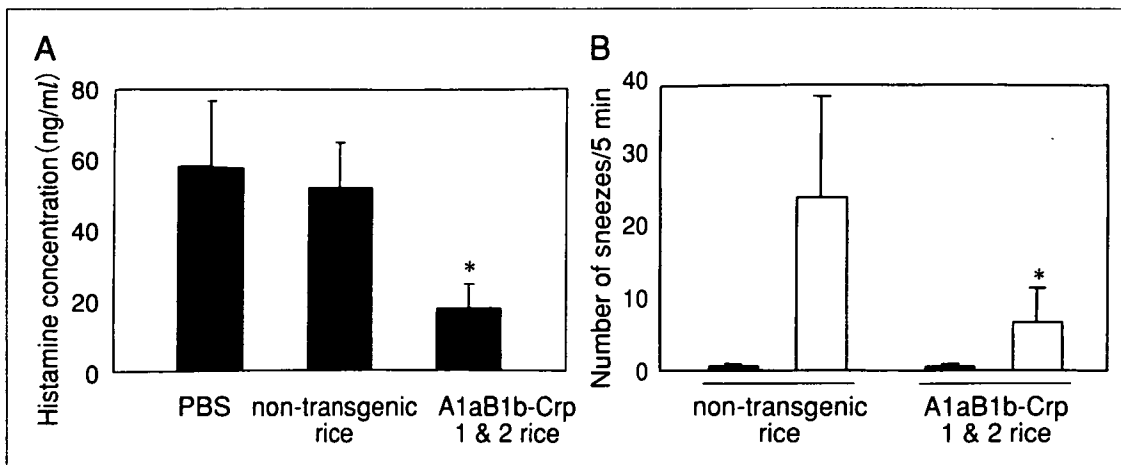でのデータは少ない．われわれは，マウスアレ
ルギー性鼻炎モデルを作製し，反応相における

図5　Serum histamine levels and sneezing rate upon allergen exposure of mice orally fed with A1aB1b-Crp-1 and -2 rice seeds

Serum histamine levels (A) and the number of sneezes (B) were inhibited in the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds. The number of sneezes was counted in the 5 min after the last nasal challenge at week 8 (white bars). Sham-challenged mice were nasally administered with 20ml of PBS in the same manner (black bars). Data are expressed as mean±SD. *P<0.01 for the group of mice fed with A1aB1b-Crp-1 and -2 seeds in comparison with the group of mice fed with nontransgenic rice seeds.
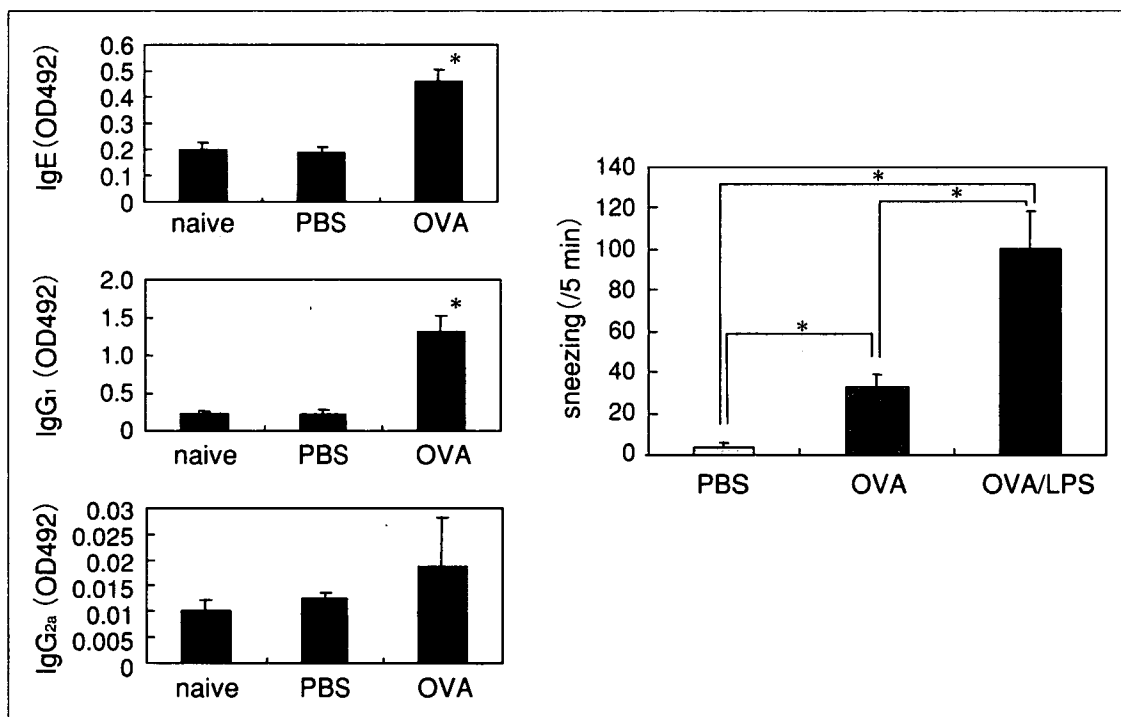

図6　Effect of LPS on nasal symptoms (Balb/c mice) —eliciting phase

LPSの影響について検討した[9][10].

Day 0 とday 7 にOVA, alumをBalb/cマウスに腹腔内投与して感作を成立させ, day 14に血清を採取してELISA法によりOVA特異的抗体価を測定した. Day 21〜28まで, OVAおよびLPSの点鼻を行い, マウスアレルギー性鼻炎モデルを作製した.

最終点鼻直後より5分間くしゃみの回数を測定し, 鼻粘膜組織を採取して組織学的検討を行った. 鼻粘膜における Th2型のサイトカインの発現について, 免疫沈降−western blot法により検討した. その結果, くしゃみの回数は, OVA単独点鼻群と比較して, OVAとLPS点鼻群において有意な増加を認めた(図6). 鼻粘膜組織では, OVA単独点鼻群において好酸球浸潤を認めたが, OVAとLPS点鼻群では好酸球浸潤がより顕著となっ
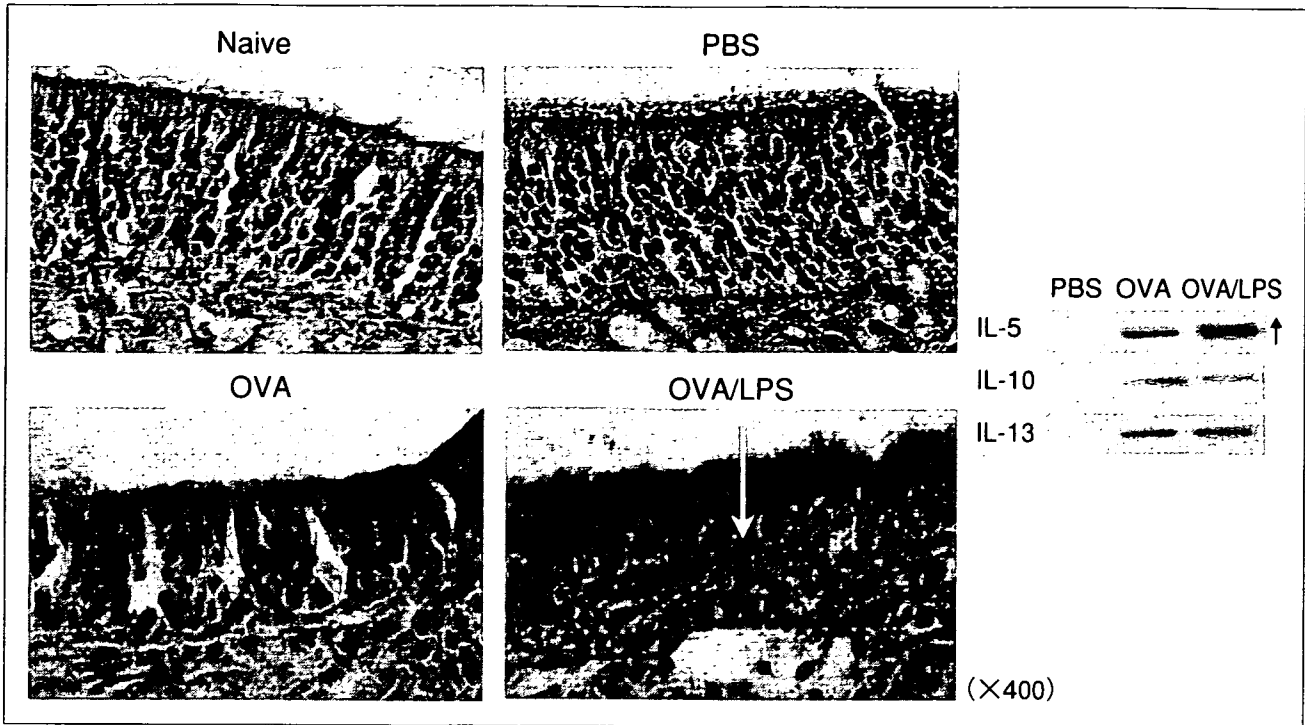
図7　Eosinophil infiltration and Th2 type cytokine production

た(図7). 鼻粘膜のTh2型サイトカイン発現の検討では, IL-5, IL-10, IL-13いずれもOVA単独点鼻群で発現を認めたが, OVAとLPS点鼻群ではOVA単独点鼻群と比較してIL-5の発現の増強を認めた(図7). 続いて, TLR4の遺伝子変異マウスであるC3H/HeJマウスと, 野生型のC3H/HeNマウスとを用いて, LPSの影響について検討した. その結果, TLR4の遺伝子変異マウスであるC3H/HeJマウスでは, 反応相におけるLPSの同時点鼻投与の影響(くしゃみの回数, 好酸球浸潤, Th2型サイトカイン産生)を認めなかった. 上記の結果から, 実効相においてLPSが肥満細胞のTLR4を介しIL-5発現を誘導することによりアレルギー性炎症の増悪因子として作用することが示唆された.

2. マウスアレルギー性鼻炎モデルにおけるIL-15の役割

IL-15は粘膜免疫に関与する細胞群の増殖維持因子として重要な役割を果たしている. IL-15あるいはIL-15Rαの遺伝子欠損マウスでは, 腸管上皮間γδ型T細胞, NK, NKT細胞およびメモリーCD8T細胞が減少しており, さらにIL-15遺伝子導入マウスでは, メモリーCD8T細胞の増加, Tc1反応を介しての気道アレルギー性炎症の抑制が報告されている[11]. さらに, IL-15は肥満細胞の

増殖活性化因子として知られており, 本研究では, 粘膜面でのアレルギー反応におけるIL-15の役割を調べるため, IL-15KOマウスと野生型マウスのマウスアレルギー性鼻炎について比較検討した[12].

その結果, OVA感作後のIL-15KOマウスにおけるOVA特異的IgE量および脾臓におけるTh1/Th2応答は, 野生型マウスと比較して有意差はなかった. 感作マウスにおけるOVA点鼻後の症状は, IL-15KOマウスで増悪しており, 鼻粘膜への好酸球浸潤も亢進していた(図8). 野生型マウス骨髄由来肥満細胞(bone marrow-derived mast cell；BMMC)とIL-15KOマウス由来BMMCでは, FcεRおよびCD117の発現に差は認められなかったが, IL-15KOマウス由来BMMCで脱顆粒率が高く, リコンビナントIL-15を添加することで野生型マウスおよびIL-15KO由来いずれのBMMCでも脱顆粒が抑制された.

さらに, OVAで感作した野生型マウスにOVAとともにリコンビナントIL-15を点鼻投与したところ(図9), 症状および鼻粘膜への好酸球浸潤が抑制された結果(図10)より, IL-15は鼻粘膜局所の実効相におけるTh2反応を抑制することにより, アレルギー反応を制御しているものと考えられた. さらに, IL-15は肥満細胞の脱顆粒を抑
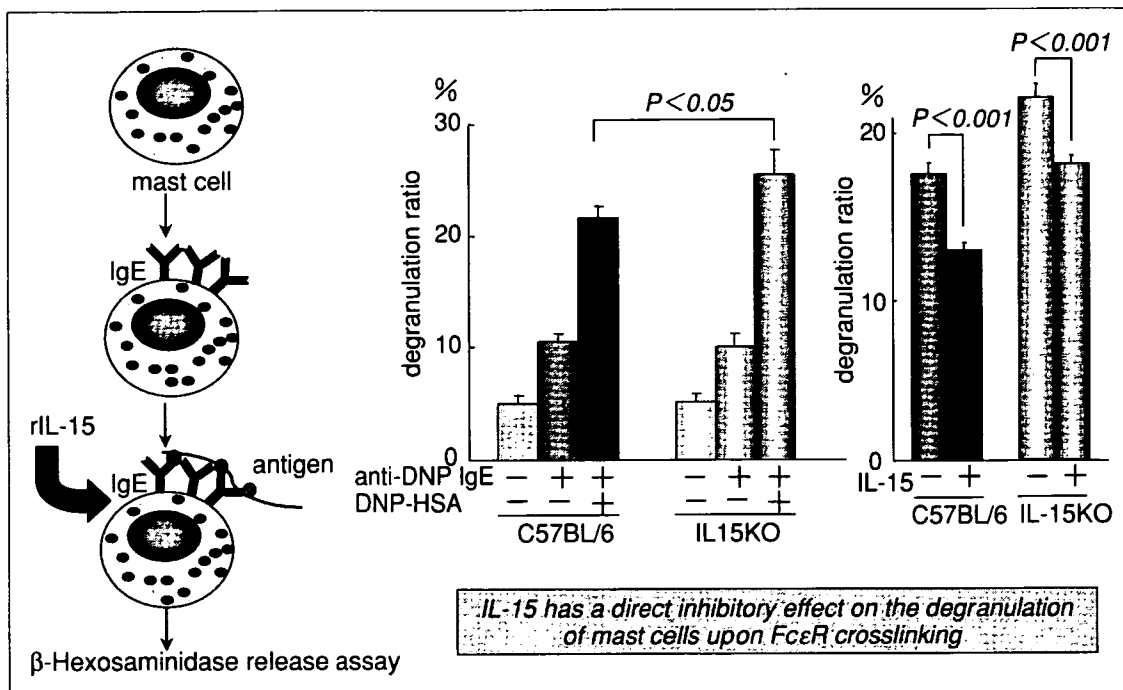
図8 Degranulation of BMMCs derived from IL-15KO mice and C57BL/6 mice stimulated with IgE cross-linking
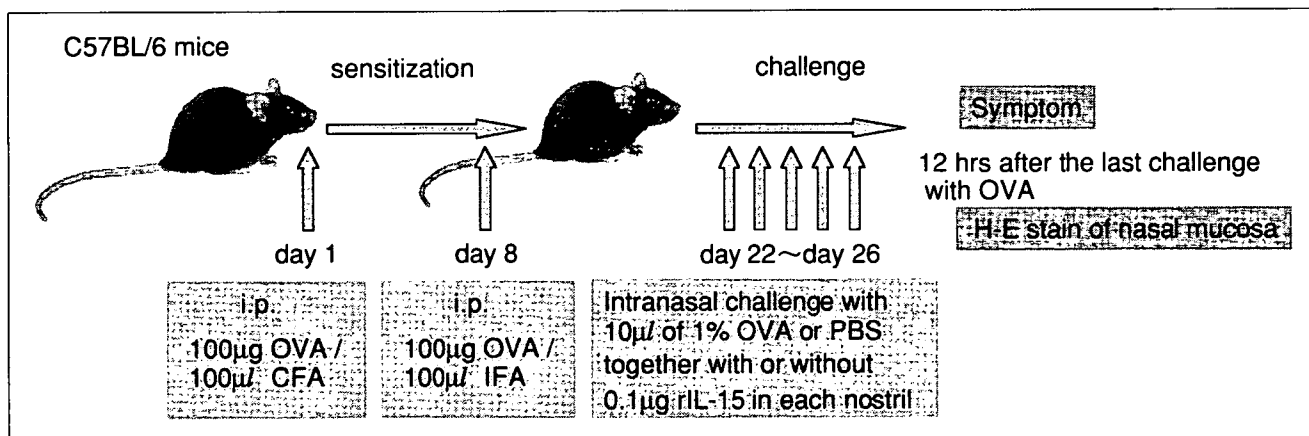
図9 Experimental design

制することにより，鼻アレルギー症状を制御している可能性も示唆された．

おわりに

アレルギー性鼻炎の発症予防と発症後の完全治癒をめざした方法論の確立に向けて，上記に紹介したように，多くの研究が推進されている．治癒を求める治療手段としての確立の基本は，アレルゲン特異的なT細胞のTh2 responseへのバイアスをTh1タイプに引き戻すことが肝要であるし，いわゆる調節性T細胞の効率的な誘導も目標となる．さらに，感作が成立し発症してからの治療手段には限界があるように思うが，感

作が成立する前に予防的な免疫療法を行うことにより，発症を回避する方向性をめざす(いわゆる早期介入療法，early intervention)ための研究のさらなる推進や，臨床現場での治療法の開発と環境作りが望まれる．アレルギー性鼻炎も悪性腫瘍と同様，prophylactic medicine(予防医学)への変貌を遂げなければならない時期に来ていることを最後に述べて筆をおく．
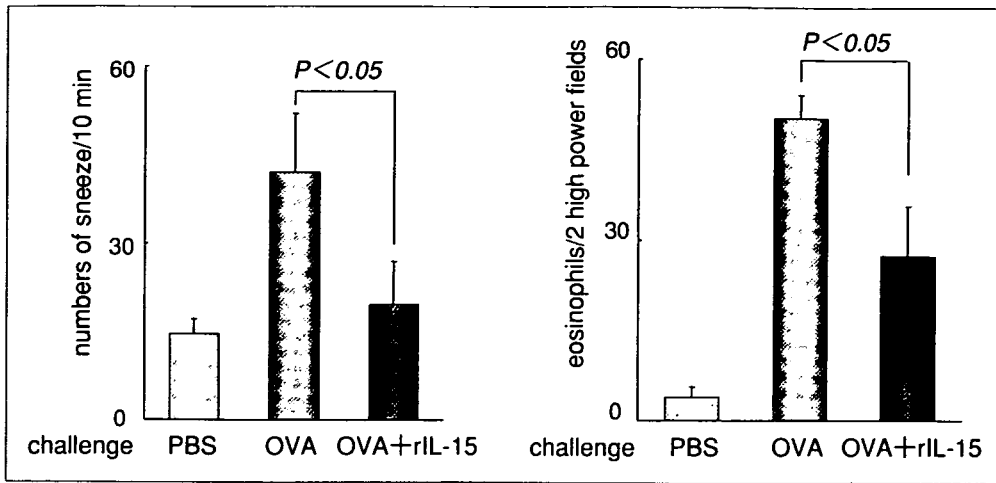
図10 Intranasal administration of rIL-15 reduced allergic symptom and eosinophil infil-
tration into nasal mucosa

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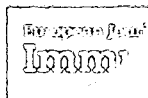
Accumulation of intestinal intraepithelial lymphocytes in association with lack of polymeric immunoglobulin receptor

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Immunoglobulin A (IgA) is transported by the polymeric immunoglobulin receptor (pIgR) through epithelial cells of the gut, the airways, the tear and salivary glands, and the lactating mammary gland, and IgA accumulates in serum and the intestinal lamina propria of pIgR-deficient (pIgR$^{-/-}$) mice. Intraepithelial lymphocytes (IEL) increased in number and Thy-1$^+$CD8$\alpha\beta^+$TCR$\alpha\beta^+$ IEL preferentially expanded in the small intestine (SI) of pIgR$^{-/-}$ mice. Cytotoxic activity of SI-IEL was comparable in pIgR$^{+/+}$ and pIgR$^{-/-}$ mice. Accumulation and cytotoxic activity of SI-IEL was attenuated in germ-free pIgR$^{-/-}$ mice. Furthermore, Thy-1$^+$CD8$\alpha\beta^+$ IEL did not expand in pIgR$^{-/-}$TCR$\beta\delta^{-/-}$ mice compared with TCR$\beta\delta^{-/-}$ mice, and SI-IEL from pIgR$^{-/-}$TCR$\beta\delta^{-/-}$ mice as well as TCR$\beta\delta^{-/-}$ mice expressed perforin and granzyme B mRNA and serine esterase. The proliferative status of SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice was similar, but adoptive transfer experiment showed that SI-IEL from pIgR$^{-/-}$ mice might have a stronger tendency to migrate into the intestinal epithelia than those from pIgR$^{+/+}$ mice. These results demonstrate that the accumulation of Thy-1$^+$CD8$\alpha\beta^+$TCR$\alpha\beta^+$ IEL in pIgR$^{-/-}$ mice triggered by intestinal microorganisms needed the expression of functional TCR and might be caused by lymphocyte migration into the intestinal epithelia.

Introduction

Immunoglobulin A (IgA) is secreted into the gut lumen, and secretory IgA (sIgA) has been considered important for the defense against intestinal infection. Antigen-specific IgA is induced by immunizing with pathogens or toxins and may successfully prevent infection [1–3]. Moreover, indigenous intestinal bacteria augment development of IgA-producing cells, and dendritic cells in the intestine capture *Enterobacter cloacae* (murine indigenous intestinal bacteria), followed by helping B cells to produce IgA [4–6]. Therefore, indigenous intestinal bacteria as well as pathogens play a pivotal role in the development of IgA-producing cells in the intestine.

Novel functions of IgA have recently been clarified. Bacterial cell wall components induce intestinal epithelial cells (IEC) to produce pro-inflammatory cytokines. However, when anti-LPS IgA was given to the basolateral side of an IEC monolayer, production of pro-inflammatory cytokines in response to LPS was suppressed [7].

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Abbreviations: αβ-IEL: TCRαβ$^+$ IEL · **CMFDA:** 5-Chloromethylfluorescein diacetate · **CMTMR:** 5-(and-6)-(((4-Chloromethyl)benzoyl)amino)tetramethylrhodamine · **CV:** Conventional · γδ-IEL: TCRγδ$^+$ IEL · **GF:** Germ-free · **IEC:** Intestinal epithelial cell · **IEL:** Intraepithelial lymphocyte · **LI:** Large intestine · **pIgR:** Polymeric immunoglobulin receptor · **SI:** Small intestine · **sIgA:** Secretory IgA

Mice in which anti-OVA IgA had been induced beforehand could trap OVA in their IEC after OVA was injected intravenously [8]. These results indicate that IgA can maintain the intestinal homeostasis by preventing inflammation and accelerating clearance of wastes.

Besides IgA-producing cells, there are a huge number of T cells in the intestinal mucosa. Oral infection with reovirus induces viral antigen-specific $CD8\alpha\beta^+TCR\alpha\beta^+$ cells in the intestinal epithelia, and systemic infection with lymphocytic choriomeningitis virus or vesicular stomatitis virus causes the accumulation of $CD8\alpha\beta^+TCR\alpha\beta^+$ cells in the intestinal epithelia as well as in peripheral lymphoid tissues [9–11]. These results demonstrate that intraepithelial lymphocytes (IEL) bearing $TCR\alpha\beta$ contribute to the defense against pathogenic microorganisms. Moreover, the proliferative rate of IEC is significantly lower in $TCR\delta^{-/-}$ mice than in wild-type mice, and intraperitoneal injection of anti-$TCR\delta$ mAb induces DNA fragmentation and detachment of IEC [12, 13]. Therefore, $TCR\gamma\delta^+$ IEL may be involved in the intestinal homeostasis by regulating proliferation and apoptosis of IEC.

The basolateral side of IEC expresses polymeric immunoglobulin receptor (pIgR), which captures dimeric IgA produced by plasma cells in the intestinal lamina propria and transports IgA into the gut lumen. In order to clarify the importance of sIgA, others and we established pIgR-deficient ($pIgR^{-/-}$) mice and confirmed that IgA was markedly reduced in the intestinal secretion and accumulated in the serum of $pIgR^{-/-}$ mice [14, 15]. Although $pIgR^{-/-}$ mice had leaky mucous membranes and an excessive uptake of *Escherichia coli* antigens from the gut [14], $pIgR^{-/-}$ mice did not suffer severe infection, even if they were bred under conventional conditions. These observations indicate that the disadvantages due to the absence of sIgA export may be compensated for by other immune functions in the intestine. In this report, we show that IEL remarkably increase and that Thy-$1^+CD8\alpha\beta^+TCR\alpha\beta^+$ cells preferentially accumulate in the intestinal epithelia of $pIgR^{-/-}$ mice. We also analyzed the mechanisms by which Thy-$1^+CD8\alpha\beta^+$ IEL accumulate.

Results

Development of IEL in $pIgR^{-/-}$ mice

To clarify the influence of pIgR deficiency on the intestinal immune system, we isolated IEL from the small intestine (SI) of $pIgR^{+/+}$ and $pIgR^{-/-}$ mice. SI-IEL were more numerous in $pIgR^{-/-}$ mice than in $pIgR^{+/+}$ mice, the difference being mainly due to the increase of $TCR\alpha\beta^+$ cells. Among $TCR\alpha\beta^+$ IEL ($\alpha\beta$-IEL) subsets, Thy-1^+ $\alpha\beta$-IEL and $CD8\alpha\beta^+$ $\alpha\beta$-IEL were greater in number, and the relative proportion of $CD8\alpha\alpha^+$ $\alpha\beta$-IEL was smaller in $pIgR^{-/-}$ mice than in $pIgR^{+/+}$ mice. A slight increase in Thy-1^+ $TCR\gamma\delta^+$ IEL ($\gamma\delta$-IEL) and a small decline of $CD8\alpha\alpha^+$ $\gamma\delta$-IEL due to the pIgR deficiency were also observed. A significant increase of Thy-1^+ $\alpha\beta$-IEL and $CD8\alpha\beta^+$ $\alpha\beta$-IEL was detected in $pIgR^{-/-}TCR\delta^{-/-}$ mice compared with $pIgR^{+/+}TCR\delta^{-/-}$ mice (Table 1). Two-color flow cytometry analysis revealed that $\alpha\beta$-IEL expressing both Thy-1.2 and $CD8\alpha\beta$ preferentially increased in $pIgR^{-/-}$ mice (data not shown).

Next, we analyzed IEL from the large intestine (LI) of $pIgR^{+/+}$ and $pIgR^{-/-}$ mice. In contrast to SI-IEL, the number of $TCR\alpha\beta^+$ cells and $TCR\gamma\delta^+$ cells in LI-IEL and the relative ratio of Thy-1^+ cells in each LI-IEL subset

Table 1. Cellular composition of SI-IEL in $pIgR^{+/+}$ and $pIgR^{-/-}$ mice

Subset	8–9 weeks		18–23 weeks		9–19 weeks	
	$pIgR^{+/+}$	$pIgR^{-/-}$	$pIgR^{+/+}$	$pIgR^{-/-}$	$pIgR^{+/+}\delta^{-/-}$	$pIgR^{-/-}\delta^{-/-}$
	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)
No. of $\alpha\beta$-IEL ($\times10^6$)	2.1±1.0	5.2±0.7**	5.1±2.3	11.0±2.4**	3.6±0.5	6.8±1.1**
% of Thy-1^+ $\alpha\beta$-IEL	30.9±6.6	65.3±9.7**	38.3±20.5	66.8±8.0	38.5±13.3	66.2±10.1**
% of $CD4^+$ $\alpha\beta$-IEL	13.0±5.7	9.0±1.7	14.1±7.5	12.6±10.1	5.3±2.6	4.5±1.4
% of $CD8\alpha\alpha^+$ $\alpha\beta$-IEL	60.5±13.7	31.7±13.1*	48.0±20.1	25.6±14.4	58.3±12.5	37.0±15.0
% of $CD8\alpha\beta^+$ $\alpha\beta$-IEL	17.1±4.7	52.9±12.2**	14.2±5.6	37.7±15.4**	31.8±10.2	51.2±12.8*
No. of $\gamma\delta$-IEL ($\times10^6$)	2.6±0.6	4.0±0.9*	5.0±1.8	10.4±1.8**	Not detected	Not detected
% of Thy-1^+ $\gamma\delta$-IEL	25.3±4.0	34.1±4.5*	25.7±8.9	34.7±6.2		
% of $CD4^-8^-$ $\gamma\delta$-IEL	9.8±2.5	15.3±3.4*	10.2±3.5	9.1±1.8		
% of $CD8\alpha\alpha^+$ $\gamma\delta$-IEL	87.5±2.7	80.7±4.1*	86.2±3.9	85.9±3.1		

*$p<0.05$, **$p<0.01$: significant differences between $pIgR^{+/+}$ and $pIgR^{-/-}$ mice.

Table 2. Cellular composition of LI-IEL in pIgR$^{+/+}$ and pIgR$^{-/-}$ mice

Age	Gender	Genotype	No. of cells ($\times 10^5$)[a]		% of Thy-1$^+$ cells	
			αβ-IEL	γδ-IEL	αβ-IEL	γδ-IEL
9 weeks	male	pIgR$^{+/+}$	1.58	0.27	79.9	57.9
		pIgR$^{-/-}$	1.71	0.54	78.8	66.0
18 weeks	male	pIgR$^{+/+}$	2.06	0.62	81.3	59.9
		pIgR$^{-/-}$	1.88	0.77	83.7	67.1
8 weeks	female	pIgR$^{+/+}$	0.77	0.18	89.9	56.1
		pIgR$^{-/-}$	1.14	0.38	89.3	63.4
21 weeks	female	pIgR$^{+/+}$	3.34	0.69	85.2	55.0
		pIgR$^{-/-}$	2.97	1.08	78.0	55.4

[a] LI-IEL from three mice/group were pooled, and the number of αβ-IEL or γδ-IEL per mouse was calculated according to the following formula: No. of αβ-IEL or γδ-IEL = total cell number × % of αβ-IEL or γδ-IEL /3.

were not different between pIgR$^{+/+}$ and pIgR$^{-/-}$ mice (Table 2).

To clarify whether SI-IEL from pIgR$^{-/-}$ mice are functionally normal, anti-TCR mAb-redirected cytotoxic activity was measured. Cytotoxic activity of αβ-IEL was higher in pIgR$^{-/-}$ mice than in pIgR$^{+/+}$ mice (Fig. 1A). However, taking into consideration that pIgR$^{-/-}$ mice contained a higher proportion of αβ-IEL than pIgR$^{+/+}$ mice (Table 1), cytotoxic activity of αβ-IEL should not be different between pIgR$^{+/+}$ and pIgR$^{-/-}$ mice. γδ-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice showed cytotoxic activity to the same degree (Fig. 1B).

Constitution and cytotoxic activity of SI-IEL in germ-free pIgR$^{-/-}$ mice

We generated germ-free (GF) pIgR$^{-/-}$ mice to determine the involvement of intestinal microorganisms in the development of SI-IEL in pIgR$^{-/-}$ mice. The serum IgA level was remarkably higher in pIgR$^{-/-}$ mice than in pIgR$^{+/+}$ mice under either conventional (CV) or GF conditions. Absolute numbers of αβ-IEL and γδ-IEL decreased in GF pIgR$^{-/-}$ mice compared with CV pIgR$^{-/-}$

mice, and the degree of decline by microbial deprivation was more drastic for αβ-IEL than for γδ-IEL. The relative proportion of Thy-1$^+$ and CD8αβ$^+$ αβ-IEL was greater in pIgR$^{-/-}$ mice under CV conditions, but these αβ-IEL subsets did not accumulate in GF pIgR$^{-/-}$ mice. When GF mice were transferred to conventional conditions and bred for 6 months, a more remarkable increase in the number of Thy-1$^+$ and CD8αβ$^+$ αβ-IEL was induced in pIgR$^{-/-}$ mice than in pIgR$^{+/+}$ mice (Table 3).

Microbial deprivation drastically reduced the cytotoxic activity of αβ-IEL, irrespective of whether mice were pIgR$^{+/+}$ or pIgR$^{-/-}$, resulting in very low cytotoxic activities of αβ-IEL in both GF pIgR$^{+/+}$ and GF pIgR$^{-/-}$ mice. In contrast, γδ-IEL displayed high and similar levels of cytotoxic activity in either GF pIgR$^{+/+}$ or GF pIgR$^{-/-}$ mice (Fig. 2).

Constitution of SI-IEL in pIgR$^{-/-}$ mice defective in TCR expression

To investigate how the accumulation of Thy-1$^+$CD8αβ$^+$ αβ-IEL was induced in pIgR$^{-/-}$ mice, we generated pIgR$^{-/-}$ mice lacking both TCRβ and TCRδ genes (βδ$^{-/-}$).
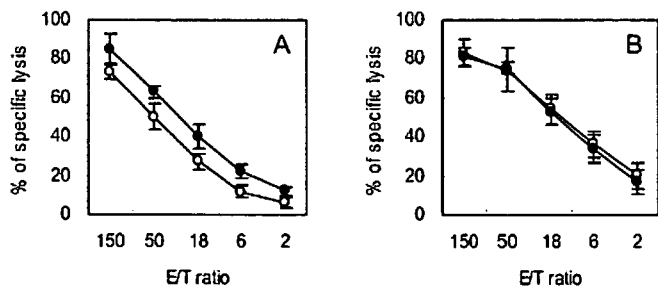
Fig. 1. Cytotoxic activity of SI-IEL from mice bred under conventional conditions. Cytotoxic activity of (A) αβ-IEL and (B) γδ-IEL from pIgR$^{+/+}$ (○) and pIgR$^{-/-}$ mice (●) were analyzed, and means ± SD of data from three mice are shown.

Fig. 2. Cytotoxic activity of SI-IEL from mice bred under germ-free conditions. Cytotoxic activity of (A) αβ-IEL and (B) γδ-IEL from pIgR$^{+/+}$ (○) and pIgR$^{-/-}$ mice (●) were analyzed, and means ± SD of data from three mice are shown.
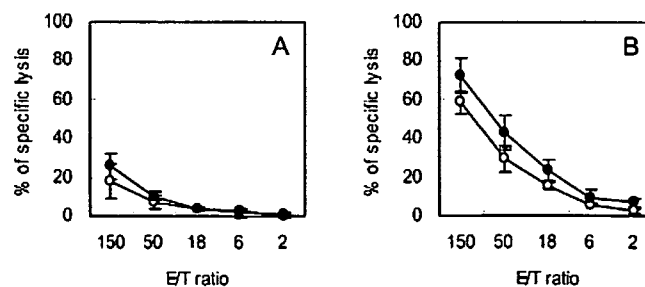
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Table 3. Serum IgA level and cellular composition of SI-IEL in pIgR$^{+/+}$ and pIgR$^{-/-}$ mice bred under conventional (CV), germ-free (GF), and conventionalized (Cvd) conditions

Parameter	CV		GF		Cvd	
	pIgR$^{+/+}$	pIgR$^{-/-}$	pIgR$^{+/+}$	pIgR$^{-/-}$	pIgR$^{+/+}$	pIgR$^{-/-}$
	(n=3)	(n=3)	(n=3)	(n=3)	(n=4)	(n=4)
Serum IgA (μg/ml)	223±106	8,217±1,025	39±13	988±381	359±143	19,602±4,327*
No. of αβ-IEL (×10^6)	4.9±0.9	5.9±1.3	1.7±0.3	2.5±0.7	3.1±0.3	9.5±0.9*
% of Thy-1$^+$ αβ-IEL	25.4±2.9	54.9±10.9	30.8±1.1	24.8±2.5	61.2±0.3	75.7±0.9*
% of CD4$^+$ αβ-IEL	4.8±1.4	4.7±1.5	16.0±2.0	11.5±4.7	11.9±9.0	5.7±0.3*
% of CD8αα$^+$ αβ-IEL	69.0±3.8	50.3±12.2	59.0±3.4	68.8±6.5	35.9±5.7	20.5±1.9*
% of CD8αβ$^+$ αβ-IEL	20.2±3.2	40.4±13.9	17.8±1.6	13.6±2.4	48.0±7.6	69.8±0.7*
No. of γδ-IEL (×10^6)	5.1±1.8	5.3±1.4	3.2±1.4	3.9±0.8	4.0±0.2	6.3±1.4*
% of Thy-1$^+$ γδ-IEL	41.3±8.5	57.5±7.3	21.1±2.7	26.3±4.8	29.9±3.3	30.9±4.3
% of CD4$^-$8$^-$ γδ-IEL	9.5±2.1	11.1±2.0	7.9±2.5	7.4±1.7	11.0±2.3	7.7±0.9
% of CD8αα$^+$ γδ-IEL	86.1±2.1	82.4±3.6	87.1±3.9	90.0±1.3	84.4±2.4	87.3±2.2

*$p<0.05$: significant differences between pIgR$^{+/+}$ and pIgR$^{-/-}$ mice bred under each breeding condition.

Serum IgA levels were lower in βδ$^{-/-}$ mice than in pIgR$^{+/+}$ mice, and IgA accumulated in the serum of pIgR$^{-/-}$βδ$^{-/-}$ mice compared with βδ$^{-/-}$ mice. While SI-IEL were more abundant in pIgR$^{-/-}$ mice than in pIgR$^{+/+}$ mice, the absolute numbers of SI-IEL in pIgR$^{-/-}$βδ$^{-/-}$ and βδ$^{-/-}$ mice were not different. Although Thy-1$^+$B220$^-$ and CD8αβ$^+$ cells accumulated in SI-IEL of pIgR$^{-/-}$ mice compared with pIgR$^{+/+}$ mice, the pool size of Thy-1$^+$B220$^-$ and CD8αβ$^+$ cells in SI-IEL was very small in both βδ$^{-/-}$ and pIgR$^{-/-}$βδ$^{-/-}$ mice (Table 4).

To examine whether SI-IEL developing in βδ$^{-/-}$ and pIgR$^{-/-}$βδ$^{-/-}$ mice belong to the T cell lineage, we analyzed expression of CD3ε and pre-Tα mRNA in SI-IEL. RT-PCR analysis revealed that SI-IEL from βδ$^{-/-}$ and pIgR$^{-/-}$βδ$^{-/-}$ mice expressed CD3ε and pre-Tα mRNA to the same level as those from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice. In contrast, λ5 mRNA was detected in bone marrow cells, but was below detectable levels in SI-IEL from pIgR$^{+/+}$, pIgR$^{-/-}$, βδ$^{-/-}$, and pIgR$^{-/-}$βδ$^{-/-}$ mice (Fig. 3).

Perforin and granzyme B expression in SI-IEL of pIgR$^{-/-}$ mice defective in TCR expression

We examined the expression of perforin and granzyme B mRNA and serine esterase activity in SI-IEL from pIgR$^{+/+}$, pIgR$^{-/-}$, βδ$^{-/-}$, and pIgR$^{-/-}$βδ$^{-/-}$ mice, in order to see whether functional TCR expression was needed for cytotoxic lymphocytes to populate the intestinal epithelia of pIgR$^{-/-}$ mice. Both perforin and granzyme B mRNA were detectable in SI-IEL from pIgR$^{+/+}$, pIgR$^{-/-}$, βδ$^{-/-}$, and pIgR$^{-/-}$βδ$^{-/-}$ mice. SI-IEL from pIgR$^{+/+}$, pIgR$^{-/-}$, βδ$^{-/-}$, and pIgR$^{-/-}$βδ$^{-/-}$ mice also exhibited serine esterase activity, although the activity in βδ$^{-/-}$ and pIgR$^{-/-}$βδ$^{-/-}$ mice was higher than in pIgR$^{+/+}$ and pIgR$^{-/-}$ mice. In contrast, splenocytes expressed very small amounts of perforin and granzyme B mRNA, irrespective of the genotypes, and showed much lower levels of serine esterase activity (Fig. 4).

Table 4. Serum IgA level and cellular composition of SI-IEL in pIgR$^{+/+}$, pIgR$^{-/-}$, βδ$^{-/-}$, and pIgR$^{-/-}$βδ$^{-/-}$ mice

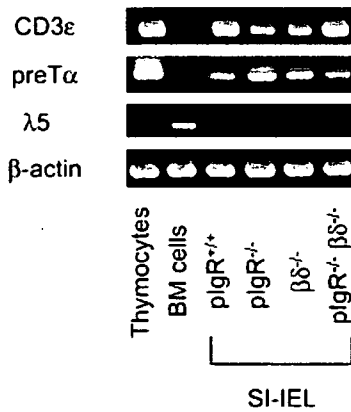| Parameter	pIgR$^{+/+}$	pIgR$^{-/-}$	βδ$^{-/-}$	pIgR$^{-/-}$βδ$^{-/-}$
	(n=3)	(n=3)	(n=3)	(n=3)
Serum IgA (μg/ml)	160±77	13,690±3,531	48±5	1,505±655
No. of SI-IEL (×10^6)	4.4±0.8	10.7±3.2	2.6±1.4	2.9±1.0
% of Thy-1$^+$B220$^-$ IEL	35.3±10.8	55.5±17.3	5.1±1.2	5.2±3.1
% of CD8αα$^+$ IEL	61.0±5.4	58.0±7.6	43.7±12.4	55.1±3.2
% of CD8αβ$^+$ IEL	16.9±4.9	24.5±5.9	2.9±0.5	2.9±0.4

Fig. 3. Expression of CD3ε and pre-Tα mRNA in SI-IEL. Total RNA in thymocytes and bone marrow (BM) cells from C57BL/6 mouse and SI-IEL from pIgR$^{+/+}$, pIgR$^{-/-}$, β$\delta^{-/-}$, and pIgR$^{-/-}$β$\delta^{-/-}$ mice was subjected to RT-PCR analysis.
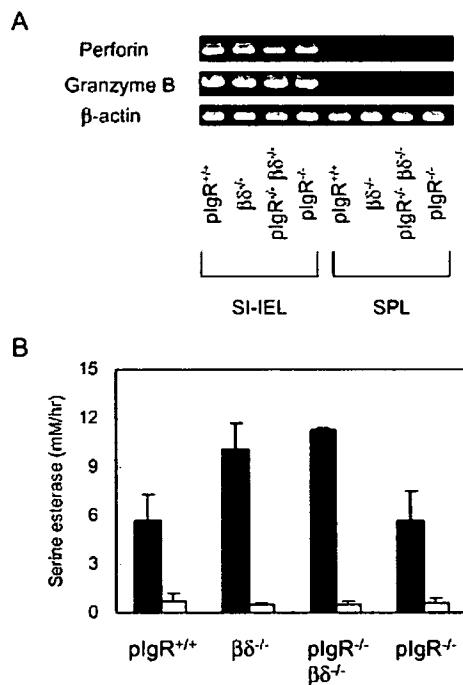

Fig. 4. Cytotoxic machinery in SI-IEL and splenocytes. (A) Expression of perforin and granzyme B mRNA in SI-IEL or splenocytes (SPL) from pIgR$^{+/+}$, β$\delta^{-/-}$, pIgR$^{-/-}$β$\delta^{-/-}$ and pIgR$^{-/-}$ mice. Total RNA in each cell preparation was subjected to RT-PCR analysis. (B) Serine esterase activity in SI-IEL (filled columns) or splenocytes (open columns) from pIgR$^{+/+}$, β$\delta^{-/-}$, pIgR$^{-/-}$β$\delta^{-/-}$ and pIgR$^{-/-}$ mice was measured, and means ± SD of data from three mice of each genotype are shown.

Proliferative capacity of SI-IEL in pIgR$^{-/-}$ mice

The finding that SI-IEL numbers increase in pIgR$^{-/-}$ mice indicates that proliferation of SI-IEL may be accelerated in the absence of sIgA. To examine this possibility, we analyzed the DNA content of SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice. As shown in Fig. 5A, both αβ-IEL and γδ-
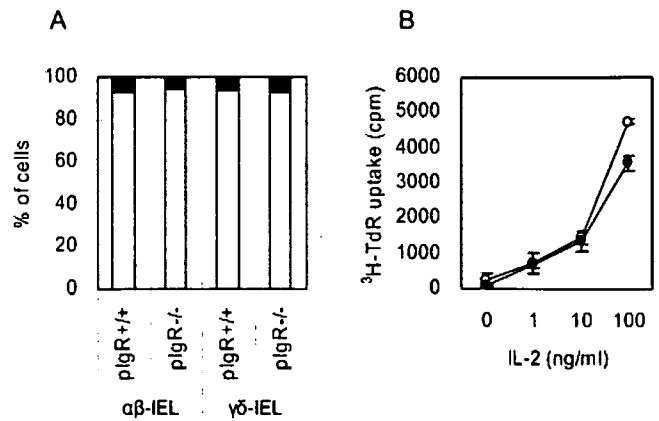

Fig. 5. Proliferative status of SI-IEL. (A) DNA content in SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice was analyzed with flow cytometry. Dotted and filled areas in columns show the percentages of cells containing 2N DNA (G1 stage) and more than 2N DNA (S + G2/M stage). (B) Proliferative response to rIL-2 of SI-IEL from pIgR$^{+/+}$ (○) and pIgR$^{-/-}$ mice (●).

IEL isolated from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice had no differences in their percentages of cells in the S + G2/M stage. Furthermore, when SI-IEL were cultured in medium alone or in the presence of rIL-2, the proliferative response of SI-IEL from pIgR$^{-/-}$ mice was not greater than that of SI-IEL from pIgR$^{+/+}$ mice (Fig. 5B).

Migration of adoptively transferred SI-IEL

Finally, we tried to distinguish whether the accumulation of SI-IEL in pIgR$^{-/-}$ mice was caused by an accelerated entry of lymphocytes into the intraepithelial space. SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice were labeled with fluorescent dye and intravenously transferred to β$\delta^{-/-}$ mice. Transferred cells were detected in the spleen of the recipient mice 1 and 3 days after the injection, but were not detected 4 days after the injection. SI-IEL from pIgR$^{-/-}$ mice did not appear to be superior to those of pIgR$^{+/+}$ mice in their capability to migrate to the spleen (Fig. 6A). In contrast, the number of SI-IEL from pIgR$^{-/-}$ mice detected in the intestinal epithelia of the recipient mice was constantly higher than that of SI-IEL from pIgR$^{+/+}$ mice during days 1–6 after the transfer (Fig. 6B).

Discussion

In this report, we compared IEL of pIgR$^{+/+}$ and pIgR$^{-/-}$ mice to clarify the influence of pIgR deficiency on the intestinal immune system. We found that SI-IEL markedly accumulated, and the pool size of Thy-1$^+$CD8αβ$^+$ αβ-IEL preferentially expanded in the SI of pIgR$^{-/-}$ mice. The accumulation of SI-IEL and the higher
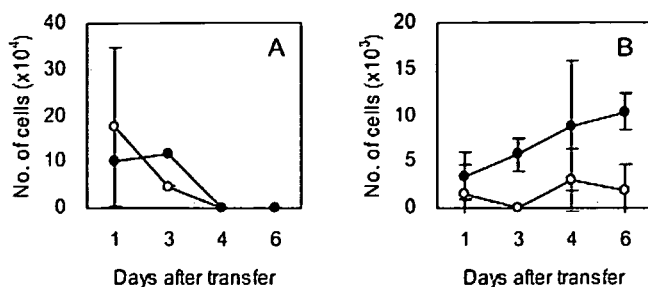
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Fig. 6. Adoptive transfer of SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice. SI-IEL from pIgR$^{+/+}$ (○) and pIgR$^{-/-}$ mice (●) were labeled with CMTMR and CMFDA, respectively, injected intravenously into βδ$^{-/-}$ mice, and transferred cells in (A) splenocytes and (B) SI-IEL of the recipient mice were analyzed with flow cytometry. Each point shows the mean ± SD of data from two to four mice.

proportion of Thy-1$^+$CD8αβ$^+$ αβ-IEL in pIgR$^{-/-}$ mice were abolished under GF conditions, demonstrating that intestinal microorganisms were involved in the increase of SI-IEL caused by the absence of pIgR. Thy-1$^+$CD8αβ$^+$ αβ-IEL expand in response to colonization of intestinal microorganisms in three strains of inbred mice [16]. Therefore, the accumulation of Thy-1$^+$CD8αβ$^+$ αβ-IEL in pIgR$^{-/-}$ mice may be an enlargement of the physiological response occurring in wild-type mice as a result of the adaptation to indigenous intestinal microorganisms.

CD8αα$^+$ TCRγδ$^+$ cells constitute 30–50% of total SI-IEL in normal mice, and Thy-1$^+$CD8αβ$^+$ αβ-IEL were relatively enriched in TCRδ$^{-/-}$pIgR$^{-/-}$ mice compared with TCRδ$^{-/-}$pIgR$^{+/+}$ mice. These results demonstrate that accumulation of Thy-1$^+$CD8αβ$^+$ αβ-IEL does occur in the absence of sIgA, independently of γδ-IEL. It has been considered that CD8αβ$^+$ αβ-IEL are thymus derived while CD8αα$^+$ αβ-IEL and CD8αα$^+$ γδ-IEL develop extrathymically [17, 18], but the thymus-independent development of CD8αα$^+$ αβ-IEL and CD8αα$^+$ γδ-IEL is still controversial [19, 20]. The result that Thy-1$^+$CD8αβ$^+$ αβ-IEL accumulate in pIgR$^{-/-}$ mice even in the absence of γδ-IEL is consistent with the notion that αβ-IEL develop independently of γδ-IEL [12].

sIgA and cytotoxic T cells are considered the defense mechanism against intestinal infection of pathogens, and cytotoxic T cells induced in response to pathogenic infection are Thy-1$^+$CD8αβ$^+$TCRαβ$^+$ cells. Therefore, it is possible that blockade of IgA transcytosis due to pIgR deficiency allows intestinal commensal bacteria or pathogens to penetrate into the intestinal mucosa and stimulate Thy-1$^+$CD8αβ$^+$ αβ-IEL. However, cytotoxic activities of the αβ-IEL of pIgR$^{+/+}$ and pIgR$^{-/-}$ mice were almost comparable (Fig. 1), and the abilities of CD8αβ$^+$ αβ-IEL of pIgR$^{+/+}$ and pIgR$^{-/-}$ mice to produce IFN-γ mRNA were similar (Yamazaki, K., unpublished observation). These results suggest that the accumulation

of Thy-1$^+$CD8αβ$^+$ αβ-IEL in pIgR$^{-/-}$ mice may not result from intestinal inflammation due to microbial infection. Molecular analysis of bacterial 16S ribosomal RNA detected no difference between ileal microorganisms of pIgR$^{+/+}$ and pIgR$^{-/-}$ mice [21]. These results indicate that commensal bacteria, but not pathogenic bacteria, could induce the expansion of Thy-1$^+$CD8αβ$^+$ αβ-IEL in pIgR$^{-/-}$ mice, although there may be a difference in intestinal microorganisms between pIgR$^{+/+}$ and pIgR$^{-/-}$ mice that cannot be detected with the above method. On the other hand, abnormalities of the intestinal microflora in the SI of activation-induced cytidine deaminase (AID)-deficient mice have been reported [22]. AID$^{-/-}$ mice are deficient in sIgA due to blocking of the class switch into IgA-producing cells, but they contain a greater number of IgM-producing cells in the intestinal lamina propria. In contrast, pIgR$^{-/-}$ mice are deficient in sIgA due to blockade of IgA transcytosis and contain more IgA-producing cells in the intestinal lamina propria than pIgR$^{+/+}$ mice [23]. These results suggest that IgA accumulating in the intestinal lamina propria can maintain a normal intestinal microflora in pIgR$^{-/-}$ mice, and the combination of sIgA deficiency and dysregulated IgM production in the intestinal lamina propria may allow the expansion of particular intestinal microorganisms in AID$^{-/-}$ mice [24].

Comparative analysis of SI-IEL from pIgR$^{-/-}$ and pIgR$^{-/-}$βδ$^{-/-}$ mice revealed that Thy-1$^+$CD8αβ$^+$ IEL did not accumulate in pIgR$^{-/-}$ mice defective in TCR expression. CD3$^-$CD8αα$^+$ SI-IEL exist in athymic RAG-deficient mice [25], and development of SI-IEL has proceeded to the stage of cells expressing CD3ε and pre-Tα mRNA in βδ$^{-/-}$ and pIgR$^{-/-}$βδ$^{-/-}$ mice. Therefore, TCR expression is necessary for SI-IEL to accumulate in the intestinal epithelia of pIgR$^{-/-}$ mice. However, it is unclear whether TCR-mediated signaling could augment the expansion of Thy-1$^+$CD8αβ$^+$ TCRαβ$^{+/+}$ cells in pIgR$^{-/-}$ mice.

SI-IEL are highly activated lymphocytes exhibiting cytotoxic activity. We found that perforin and granzyme B mRNA and serine esterase activity were detected in SI-IEL, irrespective of pIgR and/or TCR expression. These results demonstrate that synthesis of cellular components necessary for the cytotoxic activity in the intestinal environment does not always require functional TCR expression. SI-IEL and IEC interact with various arrays of surface molecules other than TCR, and these interactions, such as between the activating NK receptor (NKG2D) on IEL and its ligand MICA on IEC, may be involved in the activation of IEL [26].

IgA can be detected in the intestine of βδ$^{-/-}$ mice, and almost all of their IgA is derived from B-1 cells [27]. We observed that IgA transcytosis was blocked in pIgR$^{-/-}$βδ$^{-/-}$ mice, followed by an elevation of the serum IgA level (Table 4). However, the relative

proportion of Thy-1$^+$ cells and CD8αβ$^+$ cells in SI-IEL was not different between βδ$^{-/-}$ and pIgR$^{-/-}$βδ$^{-/-}$ mice. SI-IEL contain a significant number of NK cells in both mice and humans [28, 29]. Therefore, we suppose that NK cells or other non-T cells may be enriched in SI-IEL of pIgR$^{-/-}$βδ$^{-/-}$ mice and that they play an important role for their intestinal homeostasis.

The pathways for Thy-1$^+$CD8αβ$^+$ αβ-IEL to accumulate in pIgR$^{-/-}$ mice remain to be elucidated. In mice reconstituted by OT-II transgenic T cells expressing OVA-specific TCR, proliferation of CD8$^+$ OT-II transgenic T cells was observed in Peyer's patches and mesenteric lymph nodes when OVA was given orally [30]. However, this may not be the case in pIgR$^{-/-}$ mice, because the cell cycle and the in vitro proliferative response of SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice were very similar (Fig. 5). On the other hand, CD8$^+$ T cells triggered by dendritic cells of Peyer's patches tended to migrate to the intestinal mucosa, and CD8αβ$^+$ αβ-IEL from mice infected by Toxoplasma gondii efficiently migrated to the intestinal epithelia of recipient mice after adoptive transfer [31, 32]. After equal numbers of SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice were transferred, more SI-IEL from pIgR$^{-/-}$ mice were detected in the intestinal epithelia of the recipient mice. These results support that SI-IEL from pIgR$^{-/-}$ mice could migrate more efficiently into the intestinal epithelia than SI-IEL from pIgR$^{+/+}$ mice. However, we cannot rule out the possibility that SI-IEL from pIgR$^{-/-}$ mice proliferated more vigorously after having migrated into the intestinal epithelia of the recipient mice.

In contrast to SI-IEL, the relative composition of LI-IEL was not different between pIgR$^{+/+}$ and pIgR$^{-/-}$ mice. It is known that LI-IEL and SI-IEL are markedly different in terms of cell constitution and function [33], but the reason is still unclear. Our results also show that SI-IEL and LI-IEL differently respond to a deficiency in sIgA export.

Intestinal epithelium is always subjected to various kinds of antigenic exposure that may be dangerous to the host, and both sIgA and SI-IEL are indispensable for intestinal homeostasis, by neutralizing pathogens, destroying infected cells, and then suppressing the inflammatory responses. Our results suggest that SI-IEL accumulate in order to compensate for the absence of sIgA. Likewise, a compensatory defense mechanism provided by IEL has been suggested in the gut of immunodeficient humans [34]. We would like to propose that an intimate cooperation of sIgA, SI-IEL, and other humoral and cellular components in the intestinal mucosa may be responsible not only for defense against infection but also for intestinal homeostasis.

Materials and methods

Mice

pIgR$^{-/-}$ mice were backcrossed to C57BL/6 mice ten times [15]. GF pIgR$^{+/+}$ and pIgR$^{-/-}$ mice were generated by caesarian operation and maintained in a vinyl isolator. TCRβ$^{-/-}$ and TCRδ$^{-/-}$ mice of C57BL/6 background were kindly provided by Dr. Susumu Tonegawa (Massachusetts Institute of Technology, Cambridge, MA). To generate mice defective in both TCRβ and TCRδ genes (βδ$^{-/-}$ mice), TCRβ$^{-/-}$ mice and TCRδ$^{-/-}$ mice were crossed. By crossing pIgR$^{-/-}$ mice and βδ$^{-/-}$ mice, mice having mutations in the TCRβ, TCRδ, and/or pIgR genes were obtained.

Cell preparation

Spleen was teased over gauze, and bone marrow in femurs was pushed out using a syringe equipped with a 26-gauge needle. After the cell suspension was centrifuged, hemolytic buffer (0.144 M NH$_4$Cl, 0.017 M Tris-HCl pH 7.65) was added to the cell pellet and the suspension was kept at room temperature for 5 min. Thereafter, cells were washed with HBSS.

SI, from the pyloric sphincter to the ileo-cecal junction, was removed, and the intestinal contents were washed away with HBSS. After opening longitudinally and cutting into segments of 1–2 cm length, the segments were washed in 0.45 mM DTT, 10 mM HEPES pH 7.3 in HBSS. The segments were vigorously shaken in 10 mM HEPES pH 7.3, 5% FCS in RPMI 1640 at 37°C for 45 min (200 strokes/min). Separated cells from the intestinal wall were poured into a glass wool column, and cells passing through the column were centrifuged. Cell pellets were suspended in 30% Percoll, followed by centrifugation at 600×g for 20 min at 20°C. Cells at the bottom were suspended in 44% Percoll and poured onto 70% Percoll. After centrifugation at 600×g for 20 min at 20°C, cells at the interface were collected as SI-IEL. SI-IEL usually contained B220$^+$CD3$^-$ cells by 3–7% and CD4$^+$ cells by 4–6%. LI-IEL were similarly collected from large intestine, covering the ascending colon to the rectum, except for omitting the density gradient centrifugation in 30% Percoll.

Flow cytometric analysis

Cells were stained with antibodies and analyzed with an EPICS Altra flow cytometer. The following antibodies were used at 10–20 μg/ml: PE-conjugated anti-TCRβ (H57–597, hamster IgG2λ), FITC-conjugated anti-TCRδ (GL3, hamster IgG2κ), biotinylated anti-Thy-1.2 (30-H12, rat IgG2bκ), biotinylated anti-CD8α (53–6.7, rat IgG2aκ), PE-conjugated anti-CD4 (RM4–5, rat IgG2aκ), PE-conjugated anti-CD8β (53–5.8, rat IgG1κ), or PE-conjugated anti-B220 (RA3–6B2, rat IgG2aκ) (all from BD PharMingen, San Diego, CA). Biotinylated antibodies were detected by TRI-conjugated streptavidin (Caltag Laboratories, Burlingame, CA). Before analysis, we confirmed that isotype- and concentration-matched irrelevant rat mAb [PE-conjugated rat IgG1 (LODNP1; Immunotech, Marseille, France), biotinylated rat IgG2a (R35–95; BD PharMingen), or biotinylated rat IgG2b (A95–1; BD PharMingen)] did not stain mouse SI-IEL.

Cytotoxic activity assay

P815 cells were labeled by adding 100 μCi $Na_2{}^{51}CrO_4$ and incubating at 37°C for 1 h. SI-IEL were put into a 96-well flat-bottom microtiterplate in the presence of anti-TCRβ (H57–597) or anti-TCRδ (GL-3) mAb at 1 μg/ml (both from BD PharMingen), and then ^{51}Cr-labeled P815 cells were added at 3,000 cells/well. The microtiterplate was incubated at 37°C for 6 h, and radioactivity released into the supernatant was counted. Specific lysis was calculated with the following formula: % of specific lysis = (cpm of experimental release − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release) x 100. Maximal release was defined as the radioactivity released by standing ^{51}Cr-labeled P815 cells in 3.75% Triton X-100.

Serine esterase assay

SI-IEL or splenocytes were suspended in 1% Triton X-100, 10% FCS, 5×10^{-5} M 2-ME in RPMI 1640 and incubated at 37°C for 20 min. After centrifugation at 500×g for 7 min, the supernatant was recovered. Of the cell supernatant or cysteine solution, 50 μl was mixed in 950 μl substrate solution [0.2 mM N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT), 0.22 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 0.01% Triton X-100 in PBS], followed by incubation at 37°C for 30 min. Absorbance at 415 nm of the reaction mixture was measured, and the serine esterase activity was calculated using the regression line between cysteine concentration and the absorbance.

RT-PCR analysis

Total RNA was isolated using ISOGEN (Nippongene, Toyama, Japan), and cDNA was synthesized with the use of a cDNA synthesis kit (QIAGEN, Hilden, Germany). Primers used in PCR and sizes of DNA products were as follows: β-actin, 5′-ATG GAT GAC GAT ATC GCT-3′ and 5′-ATG AGG TAG TCT GTC AGG T-3′, 570 bp [35]; pre-Tα, 5′-GTG TCA GGC TCA ACC ATC AGG-3′ and 5′-GCA GAA GCA GTT TGA AGA GGA-3′, 449 bp [36]; CD3ε, 5′-ATG GCC AAG AGC TGC CT-3′ and 5′-AGA ATA CAG GTC CCG CT-3′, 383 bp [37]; λ5, 5′-CTT GAG GGT CAA TGA AGC TCA GAA GA-3′ and 5′-CTT GGG CTG ACC TAG GAT TG-3′, 337 bp [38]; perforin, 5′-TGC TAC ACT GCC ACT CGG TCA-3′ and 5′-TTG GCT ACC TTG GAG TGG GAG-3′, 401 bp [39]; granzyme B, 5′-CTC CAC GTG CTT TCA CCA AA-3′ and 5′-GGA AAA TAG TAC AGA GAG GCA-3′, 493 bp [39]. DNA products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Cell cycle analysis

SI-IEL were washed with PBS containing 0.1% glucose and stained with FITC-conjugated anti-TCRβ (H57–597; BD PharMingen) or FITC-conjugated anti-TCRδ mAb. After extensive washing, cells were fixed with 70% ethanol at 4°C overnight. Thereafter, cells were centrifuged, and 1 ml of propidium iodide (1 mg/ml), RNase A (1,000 Kunitz units/ml) in PBS/0.1% glucose was added to the pellet. Following incubation at room temperature for 30 min, DNA content was analyzed with an EPICS Altra flow cytometer after gating out TCRαβ$^+$ cells and TCRγδ$^+$ cells.

Proliferation assay

SI-IEL were put of 96-well U-bottom microtiterplates and cultured in medium (10% FCS, 5×10^{-5} M 2-ME in RPMI 1640) alone or together with recombinant human IL-2 (1, 10, 100 ng/ml; Shionogi Pharmaceutical Co., Osaka, Japan) for 3 days. Cells were pulsed with 0.5 μCi [^{3}H]thymidine for 8 h, and radioactivity incorporated into cells was counted.

Adoptive transfer experiment

SI-IEL from pIgR$^{+/+}$ and pIgR$^{-/-}$ mice were incubated with 100 μM 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) and 10 μM 5-chloromethylfluorescein diacetate (CMFDA) (both from Molecular Probes, Eugene, OR), respectively, at 37°C for 30 min and washed with HBSS. Stained cells were adjusted to 1×10^8 cells/ml in HBSS, and an equal volume of each cell suspension was mixed. βδ$^{-/-}$ mice were intravenously given 0.2 ml of the mixed cell suspension and sacrificed 1, 3, 4, and 6 days later. Transferred cells in the spleen and the intestinal epithelia of the recipient mice were detected with an EPICS Altra flow cytometer. The absolute number of transferred cells was calculated by multiplying the total cell number by the ratio of transferred cells.

Statistical analysis

Significance of difference between two groups was evaluated by unpaired Mann-Whitney statistics with two-sided tests.

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Original Paper

Effects of Probiotics on Allergic Rhinitis Induced by Japanese Cedar Pollen: Randomized Double-Blind, Placebo-Controlled Clinical Trial

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Abstract
Background: Lactobacillus casei strain Shirota (LcS) has been found to exert antiallergic effects in animal experiments, but there is little information about its clinical effects in human patients with allergy. *Methods:* We performed a randomized double-blind, placebo-controlled study to investigate the effects of LcS in patients with allergic rhinitis triggered by Japanese cedar pollen (JCP). Participants were asked to drink fermented milk containing LcS (LcS group) or placebo (control group) for 8 weeks. Clinical symptoms and immunological parameters were compared between the two groups. *Results:* Symptom-medication scores (SMS) worsened in accordance with the increase in the amount of scattered JCP. In terms of the nasal and ocular SMS, there was no significant difference between the LcS group and the placebo group during the ingestion period. In the subgroup of patients with moderate-to-severe nasal symptom scores before starting the ingestion of test samples, supplementation with LcS tended to reduce nasal SMS. *Conclusion:* These results indi-

cate that fermented milk containing LcS does not prevent allergic symptoms in patients sensitive to JCP, but may delay the occurrence of allergic symptoms in patients with moderate-to-severe nasal symptom scores.

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Introduction

Recently, patients with allergic rhinitis have been increasing in Japan, and the incidence of allergic rhinitis caused by Japanese cedar pollen (JCP) is estimated to be in the range of 10–15% among Japanese people [1]. This tendency could be a serious problem from the standpoint of socioeconomic aspects, because the allergic symptoms are unpleasant for patients and often cause various disturbances in their ordinary life during the particular season. Moreover, the medical bill for treating allergic symptoms is not negligible.

The general treatment of allergic rhinitis is administration of antihistamines, laser evaporation of the inferior turbinate, Vidian neurectomy and immunotherapy. These modalities are considered efficient but have some problems. Antihistamines sometimes cause side effects

Table 1. Characteristics of the participants in the LcS group and the placebo group

Characteristics	LcS group	Placebo group	Significance
Number of patients (male:female)	55 (22:33)	54 (21:33)	NS
Age, years	39.3 ± 8.0	39.5 ± 10.9	NS
Total IgE, IU/ml	198.9 ± 273.8	160.0 ± 247.0	NS
Anti-JCP IgE, IU/ml	15.6 ± 21.5	14.0 ± 15.7	NS
Nasal SMS	1.33 ± 0.72	1.42 ± 0.88	NS
Ocular SMS	0.94 ± 1.01	0.95 ± 0.97	NS
Swelling of nasal mucosa	1.24 ± 1.00	1.20 ± 0.86	NS
Color of nasal mucosa	1.45 ± 0.90	1.52 ± 0.91	NS
Amount of mucus	0.89 ± 0.76	0.89 ± 0.66	NS
Nature of mucus	1.45 ± 1.32	1.50 ± 1.31	NS

NS = Not significant; SMS = symptom-medication score.

such as sleepiness, thirst or gastrointestinal disturbance, and their dose and timing of administration should be strictly controlled. Laser evaporation of the inferior turbinate, Vidian neurectomy and immunotherapy cause a great burden to the patient, such as hospitalization, or sometimes a long ambulatory treatment period. Under these circumstances, food products to prevent or improve allergic symptoms that are easily available in ordinary life are required. Thus, yogurt, tea and herbs have been shown to potentially relieve the allergic symptoms [2–5].

Supplementation with milk fermented with *Lactobacillus paracasei* 33, *L. acidophilus* L-92 or *Bifidobacterium longum* BB536 has been shown to suppress the subjective symptoms and may modulate immunological parameters in allergic rhinitis patients [6–9]. These findings support the opinion that stabilization of the intestinal microflora by administration of probiotics may prevent the development of allergic rhinitis.

L. casei strain Shirota (LcS) suppresses the IgE production of splenocytes by enhanced interleukin 12 secretion by macrophages in vitro [10], and its administration prevents the elevation of the IgE level and induction of anaphylactic symptoms after sensitization with ovalbumin in animal models [11, 12]. Therefore, it is worthwhile examining whether LcS could improve allergic symptoms in humans. We evaluated the effect of fermented milk containing LcS in patients with allergic rhinitis to JCP in a randomized double-blind, placebo-controlled study. This report shows that ingestion of fermented milk containing LcS did not prevent clinical symptoms or abnormal immunological parameters in patients allergic to JCP, but may delay the occurrence of subjective symptoms in patients with moderate-to-severe nasal symptom scores.

Materials and Methods

Subjects
To carry out this study, we recruited participants enrolled for human studies in the nontreated subject bank of Soiken Inc. (Osaka, Japan), which is an organization for evaluating the functions of foods or food-derived materials in humans. We explained the aim and protocol of this study, asked if they were willing to participate and screened for subjects having specific IgE for JCP by a scratch test using allergen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) and a radioallergosorbent test. Exclusion criteria were as follows: use of antihistamines or antiallergic medication at the time of the screening test; any recent history of acute rhinitis, sinusitis, nasal polyp, hypertrophic rhinitis, septal deformity or asthma; severe disorder of the liver, kidney, heart, respiratory organs, endocrine glands or metabolism; treatment with hyposensitization therapy; frequently drinking dairy products containing lactic acid bacteria, and cow's milk allergy. The study was performed in accordance with the Declaration of Helsinki and approved by the local ethics committee, which is independent of Soiken Inc. Written informed consent was obtained from all the participants.

Study Design
The study was performed in a randomized double-blind, placebo-controlled manner. Experiments were performed from January 22 to April 15, 2005. Randomization was performed by doctors, who were not involved in this study design. All of the enrolled subjects were randomly assigned to the LcS group or the placebo group according to computer-generated permuted-block randomization. There was no obvious difference in the two groups (table 1). The LcS group drank fermented milk containing LcS (4×10^{10} CFU/80 ml) and the placebo group drank unfermented milk. The composition of fermented milk containing LcS and placebo was the same except that the placebo did not contain LcS, and the lactic acid level of placebo was adjusted to the level of fermented milk containing LcS. Before delivery to the participants, we confirmed that the fermented milk contained more than 5×10^8 CFU/ml of LcS, and both fermented milk and placebo had no contamination with other bacteria.
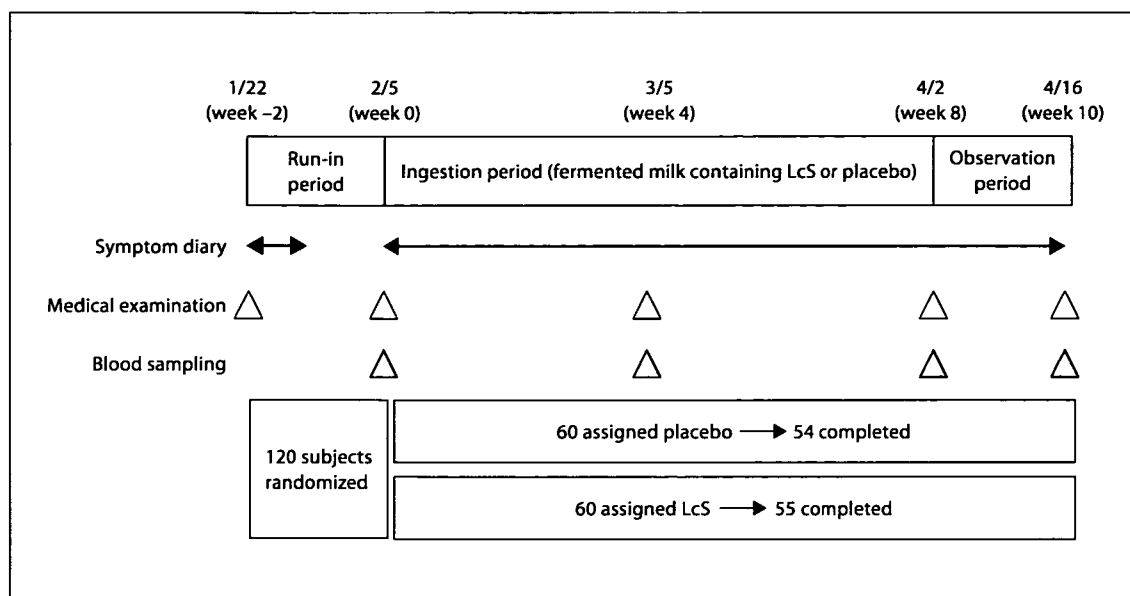
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Fig. 1. Study protocol. The clinical trial was carried out from January 22 to April 15, 2005. Participants were asked to drink fermented milk containing LcS or placebo for 8 weeks, and to record their symptoms in a diary every day. Medical examination was conducted 5 times, and blood samples were taken 4 times during the study.

To assure the viability of LcS, we prepared fermented milk every week during the study period. Furthermore, we checked the acidity and sugar level in LcS-containing fermented milk and confirmed that these values were within standardized levels. We did not check the intestinal microflora in the volunteers in this study. However, it has already been verified that LcS can be detected in feces after supplementation of LcS-containing fermented milk [13–15]. We asked all the participants not to change their ordinary lifestyle during the study. The participants drank 80 ml of placebo or fermented milk containing LcS daily for 8 weeks. The schedule of the study is shown in figure 1. Participants were asked to record their nasal and ocular symptoms and medication in a diary during the study period. Moreover, they underwent medical examination by an otolaryngologist 5 times during the study.

Evaluation of Symptoms and Medical Examination of Subjects

The scores of nasal and ocular symptoms have been defined by the Japanese Society of Allergology [1, 16]. Briefly, sneezing, runny nose, stuffy nose, itchy eyes and watery eyes were each scored from 0 to 4 according to the severity of symptoms by the participants, and the medication score was estimated based on the efficacy of medicines (table 2). The medication score, which was described in the guidelines of the Japanese Society of Allergology, is determined by medication usage (table 2). The clinical condition of the nasal cavity (swelling and color of nasal mucosa, amount and nature of mucus) was scored from 0 to 3 for each feature according to the severity by the otolaryngologist (table 3). The symptom-medication score (SMS) was calculated by summing the symptom score and the medication score.

Table 2. Symptom score and medication score for evaluating the subjective symptoms

a Symptom score

Score	Sneez-ing[a]	Runny nose[b]	Stuffy nose	Itchy eyes	Watery eyes
0	0	0	none	none	none
1	1–5	1–5	mild	mild	mild
2	6–10	6–10	moderate	moderate	moderate
3	11–20	11–20	severe	severe	severe
4	>21	>21	violent	violent	violent

b Medication score

Score	Medicine
1	oral antihistamine, oral histamine release inhibitor, nose or eye drops (without steroids)
2	local administration of steroids
3	oral antihistamine plus local administration of steroids

Sneezing = Average number of sneezing attacks in a day; runny nose = average number of times patient blew nose in a day.

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Table 3. Standard for doctors of otolaryngology to assess the nasal cavity

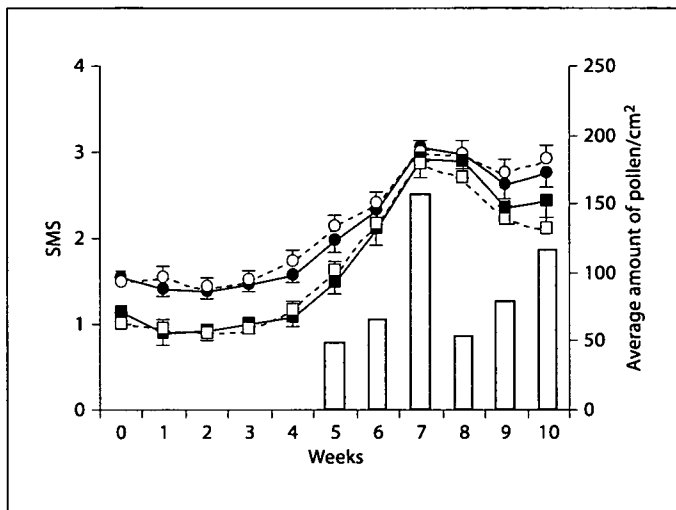| Score	Swelling of mucosa	Color of mucosa	Amount of mucus	Nature of mucus
0	none	normal	none	none
1	middle concha observable	light red	adhesion level	purulent
2	between 1 and 3	red	between 1 and 3	viscous
3	middle concha unobservable	bluish	filled	aqueous

Fig. 2. Nasal and ocular SMS throughout the study period. Nasal SMS in the LcS group (●) and placebo group (○) and ocular SMS in the LcS group (■) and placebo group (□) are shown as means ± SE. Filled columns show the average amount of pollen (JCP and hinoki pollen) scattered in the area where the studies were carried out.

Blood Examination

Blood samples were collected 4 times during the study, and anti-JCP IgE, eosinophil number, eosinophil cationic protein (ECP) and the balance of Th1 cells to Th2 cells (Th1/Th2 ratio) were determined. The anti-JCP IgE level was evaluated by the radioallergosorbent test. The ECP level was measured by radioimmunoassay (Unicap system; Roche Diagnostics KK, Sweden). Peripheral blood mononuclear cells were stimulated with phorbol myristate acetate plus ionomycin for 4 h in the presence of brefeldin A, and stained with anti-CD4 antibody. After cells had been fixed and permeabilized, the accumulated γ-interferon and interleukin 4 in CD4+ T cells were stained and measured by a flow cytometer. The ratio of γ-interferon+ CD4+ T cells to interleukin-4+ CD4+ T cells was expressed as Th1/Th2 balance.

Statistical Analysis

SMS were averaged each week. Differences in SMS and description of the nasal mucosa between the groups were evaluated by the Mann-Whitney U test. Differences in immunological pa-

rameters were assessed by the unpaired Student's t test. Data were analyzed using SPSS software (version 11.5, SPSS Inc., Chicago, Ill., USA).

Results

Scattering of JCP

The Osaka Prefectural Institute of Public Health and the Japan Weather Association reported that much JCP was scattered in the spring of 2005, and the level of JCP reached 10–30 pollen/cm² on the first 10 days of March (4–5 weeks after the start of ingestion), being maximal (approx. 600 pollen/cm²) in the last 10 days of March (7 weeks). Thereafter, the amount of scattered JCP gradually declined, although it was still detected at more than 10–30 pollen/cm² until the beginning of April (9 weeks). Furthermore, hinoki pollen started to scatter from the beginning of April, causing a biphasic change in the average amount of total pollen in the spring of 2005 (fig. 2).

Study Population

One hundred and twenty subjects were enrolled, but 11 subjects declined to take part in the study for personal reasons. As a result, 109 subjects (54 subjects in the placebo group and 55 in the LcS group) completed the study and their data were analyzed (fig. 1). There was no difference between the groups in terms of age, total and anti-JCP IgE level, and severity of allergic symptoms before the study (table 1).

Subjective Symptoms

Nasal and ocular SMS began to rise during the first 10 days of March in 2005 (4 weeks after the start of ingestion) and increased in association with the increase in scattered JCP. Deterioration of nasal SMS was delayed 1 week in the LcS group compared with the placebo group, but the difference in nasal SMS between the groups was not significant during the study (fig. 2). However, when the patients were divided into two categories ('mild' and
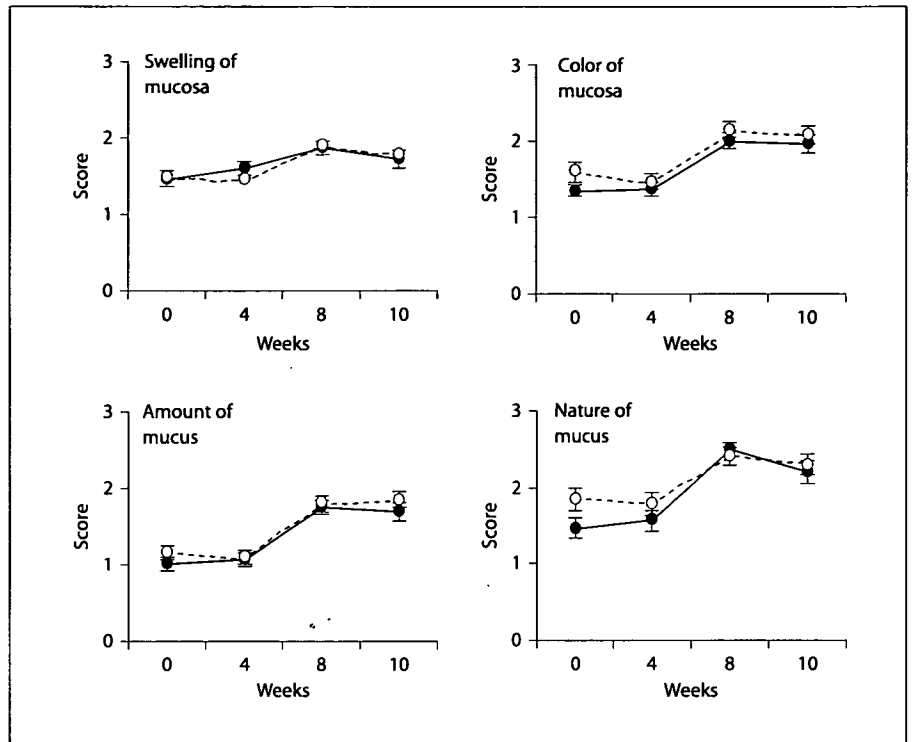
Fig. 3. Score of condition of nasal mucosa throughout the study. Data are presented as means ± SE. ● = LcS group; ○ = placebo group.

'moderate-to-severe') based on the nasal symptom score before the study, the nasal SMS in moderate-to-severe cases in the LcS group was lower at 4 and 5 weeks than in the placebo group (LcS group n = 13; placebo group n = 11; data not shown).

Medical Examination of Nasal Cavity

Scores of swelling and redness of the nasal mucosa, and the amount and nature of mucus deteriorated in association with the increase in scattered JCP. However, none of these scores differed between the groups (fig. 3).

Blood Examination

Immunological parameters associated with allergic symptoms increased in response to the amount of scattered JCP. Compared with the value before ingestion, the ECP level rose by 4 weeks and the anti-JCP IgE level and eosinophil number increased by 8 weeks. No difference was detected in immunological parameters between the two groups during the study (fig. 4). We divided the participants into mild cases and moderate-to-severe cases based on the nasal symptom score before the study and performed the statistical analysis, but there was no significant difference between the two groups (data not shown).

Side Effects

Ten subjects suffered from a cold, 3 subjects developed diarrhea, and 1 subject vomited during the study. All of these disorders were transient and there was no concern that they were related to the ingestion of fermented milk containing LcS (data not shown).

Discussion

We investigated the effect of fermented milk containing LcS on allergic symptoms triggered by JCP in a randomized double-blind, placebo-controlled study. The results showed that nasal and ocular SMS worsened in accordance with the increase in scattered JCP, confirming that the subjective symptoms of allergic rhinitis appear swiftly in response to scattered JCP. We have also performed a similar study in the spring of 2004 when a very small amount of JCP was scattered. Most patients did not experience any problem related to allergic symptoms, and nasal and ocular SMS in the participants did not rise in the spring of 2004 (data not shown). Taken together, these observations demonstrate that subjective symptoms are good parameters to assess allergic rhinitis during the JCP season.

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